ROLE OF OXIDIZED LOW DENSITY LIPOPROTEIN IN CALCIUM HOMEOSTASIS IN VASCULAR SMOOTH MUSCLE CELLS

BY

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ABSTRACT

Low density lipoprotein (LDL) is an important risk factor for atherosclerotic disease. LDL may be oxidized (oxLDL) by free radicals in a lipid and an aqueous environment. OxLDL plays an important role in atherosclerosis, possibly by altering Ca^{2+} within vascular smooth muscle cells (VSMC). Acute exposure of VSMC to oxLDL immediately increased $[Ca^{2+}]_i$ through an IP_3 mediated pathway. However, atherosclerosis is a gradual process in which VSMCs are more likely exposed to low concentrations of oxLDL over extended periods of time rather than acute exposures. It is very possible, therefore, that lower [oxLDL] and longer exposure times may induce a very different response with regard to regulation of $[Ca^{2+}]_i$. VSMC incubated with oxLDL (0.001-0.025 mg/ml) for up to 6 days significantly decreased $[Ca^{2+}]_i$ transients in response to a variety of inotropic agents. OxLDL did not have a cytotoxic effect. Therefore, we hypothesized that a disruption in the IP_3 or ryanodine dependent release of SR Ca^{2+} may be the mechanism responsible for this effect. As detected by immunocytochemical analysis chronic exposure of VSMC to oxLDL induced a depression in the density of both IP_3 and ryanodine receptors. This trend was also observed in aortic sections from rabbits maintained on a high cholesterol diet. Similar treatment conditions significantly increased the total SERCA2 ATPase content. At higher [oxLDL], we observed a significant loss of myosin and actin as the cell phenotype changed. In addition, these proteins formed giant aggregates which appeared to be in the process of being expelled from the cell. Our data demonstrate, therefore, that the change in $Ca^{2+}{}_i$ is very different depending upon prior exposure of VSMC to oxLDL. The two effects of oxLDL on $Ca^{2+}{}_i$ may play very different but equally important pathogenic roles in atherosclerosis. The acute effects may play an important pathogenic role in the initial atherosclerotic process, the chronic effects of oxLDL may provide a mechanism for the altered vascular tone during atherosclerosis. Furthermore, the process of transforming VSMC into foam cell morphology involves an unusual extrusion mechanism for contractile proteins which was dependent upon oxidation products within the LDL.

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1. General Anatomy of the Vascular Wall:

Formation of the vascular system occurs at the early stages of gestational period. At this stage, different precursor cells such as angioblasts will differentiate to generate the vasculature. In humans, vascular buds originating from the epicardium have been established to be the origin of coronary vessels (115, 120). Differentiation and development of endothelial cells from epi- and endocardium will result in the formation of a complex vascular structure (arteries, capillaries, and veins). The process of formation of a new vascular bed is called *vasculogenesis*, while the budding and branching of vessels to form a new vessel is called *angiogenesis* (190, 212).

In general, arteries are very elastic. This elasticity will allow them to contract and expand with pulsatile flow of blood. Arteries consist of three distinct layers or tunics. The inner most layer is called the tunica intima. It consists of a single endothelial layer (under no pathological condition). The muscular middle layer is called the tunica media which consists mainly of smooth muscle cells. A layer of elastic ring known as the internal elastic lamina separates the tunica intima from the tunica media. The main function of the tunica media is contraction and relaxation therefore controlling lumen size. An external elastic lamina defines the boundary between tunica media and the last

layer in the arterial wall. The tunica adventicia is the last external layer. This layer provides strength and consists mostly of connective tissue and fibroblasts.

Veins have the same layers as the arterial system but they are structurally different. The tunica intima in the venous system has developed valves to prevent a back flow of blood. The tunica media consists of a thin layer of smooth muscle cells while the tunica adventicia is very thin due to the very low venous blood pressure.

a. Vascular Smooth Muscle Cells:

Smooth muscle cells are the main cellular constituents of the tunica media of any vascular blood vessel. These cells are arranged in a helical and longitudinal pattern. The contraction of smooth muscle cells will dictate the extent of change in luminal diameter and blood flow.

b. Structure of Smooth Muscle Cells:

Smooth muscle cells are long, spindle shaped cells. Their size ranges from 5-10 μm in diameter and 100-400 μm in length, due to the presence of smooth muscle cells sub-type in the vascular tree.

The contractile apparatus in smooth muscle cells consists of the thick filament myosin (15 nm in diameter) which is surrounded by numerous long and thin actin filaments (6 nm diameter) and intermediate filaments like desmin (10 nm in diameter) and vimentin. The actin filaments are anchored into dense

bodies in the cytoplasm or attached to the plasma membrane. These dense bodies are composed of several proteins such as vinculin, metavinculin, talin and α-actinin (29, 211). Dense bodies have a similar function as the Z-disk in skeletal and cardiac myocytes (29). The spatial organization of these myofilaments (thin, thick and intermediate) within smooth muscle cells provides the characteristic elongated and spindle shape in the cells.

2. Mechanism of Smooth Muscle Cells Contraction:

a. Excitation-Contraction Coupling:

Excitation-contraction coupling (E-C coupling) refers to a chain of cellular events that is triggered by the stimulation of smooth muscle cells by external signals that lead to an increase in intracellular calcium and contraction. Stimulation of the cell membrane by depolarization (which is referred to as electromechanical coupling) will result in the generation of an action potential and an increase in intracellular calcium. Depolarization of smooth muscle cells will result in opening of voltage-operated-calcium channels (VOC) on the plasma membrane that results in influx of Ca^{2+} ions into the cell (258). Influx of Ca^{2+} through Ca^{2+} channels will depolarize the membrane and trigger Ca^{2+}-induced-Ca^{2+} release (259).

Hormonal or pharmacological stimulation of the smooth muscle cells that is independent of membrane potential is called pharmacomechanical coupling (51, 257, 260, 261). As the result of hormonal stimulation Ca^{2+} enters into the

cytoplasm through two pathways: receptor-operated-calcium channels (ROC) on the plasma membrane or Ca^{2+} channels in the sarcoplasmic reticulum (11, 70, 169, 259).

During E-C coupling intracellular Ca^{2+} elevates from 120-140 nM in the resting smooth muscle cell to 500-700 nM in the contracted smooth muscle cell (305). This elevated Ca^{2+} binds to calmodulin in a 4:1 ratio. The Ca^{2+}-calmodulin complex will then bind to and activate myosin light chain kinase (MLCK) (59) which result in phosphorylation of myosin at serine-19. Phosphorylated myosin will trigger cross-bridge formation with actin filaments and the initiation of smooth muscle contraction (296).

b. The Involvement of Ion Channels in E-C Coupling in Smooth Muscle:

The contraction and relaxation of vascular smooth muscle cells during electromechanical coupling depends on the conductance of several ions including K^+, Cl^-, and Ca^{2+}.

i. K^+ channels:

Smooth muscle cells hold their resting membrane potential from –70 to –50 mV. This is due to an outward K^+ efflux through K^+ channels (21, 245). Outward movement of K^+ along its concentration gradient will result in a net negative charge inside the cells. The intracellular K^+ concentration is maintained primarily by the Na^+-K^+ ATPase (Na^+ pump) (21, 245).

There are different types of K^+ channels present in smooth muscle cells. These include Ca^{2+} activated K^+ channels (K_{Ca}), ATP-dependent K^+ channels (K_{ATP}), inward rectifier K^+ channels, and delayed rectifier K^+ channels. K_{Ca} conductance is sensitive to the intracellular calcium concentration. K_{Ca} conductance will result in hyperpolarization of smooth muscle cells. Conversely, K_{ATP} channel conductance increases when intracellular ATP levels decrease and there is an accumulation of ADP and H^+ within the smooth muscle cells (21, 182, 245). K_{ATP} plays an important role during ischemia. Accumulation of H^+ and the depletion of ATP will result in activation of K_{ATP} leading to its hyperpolarization and dilatation. Inward K^+ rectifier conductance is active even at a resting potential. Its main function is to maintain resting potential. Finally, the delayed K^+ rectifier channel is not calcium dependent and opens upon depolarization to restore repolarization (21, 112, 182, 245).

ii. Chloride channels:

The presence of this channel has been reported in different types of smooth muscle cells, such as rabbit portal vein and ear artery, and guinea pig ileum (101, 227). This channel is activated by α-adrenergic receptor stimulation with norepinephrine and requires calcium for activation (140).

iii. Ca^{2+} channels:

There are two main types of VOC in smooth muscle cells: L-type and T-type calcium channels. L-type calcium channels play a greater role in the E-C coupling process in smooth muscle cells (169, 315). The T-type calcium current activates and inactivates at a more negative membrane potential than the L-type calcium current. The inactivation of the L-type calcium current is both calcium and voltage dependent, whereas inactivation of T-type calcium channels is mainly voltage dependent. The L-type calcium current has a high threshold and is long lasting. This induces an inward current that is necessary for the action potential. In contrast, the T-type calcium channels have a low threshold and a transient opening time (21).

The receptor operated calcium channels (ROC) are insensitive to membrane potential. These channels activate as a result of binding of a specific agonist (such as ATP, norepinephrine, angiotensin II, vasopressin) to their receptor. This results in pharmacomechanical coupling and contraction. These channels are permeable to both Ca^{2+} and Na^{+} ions (11).

c. Regulation of Intracellular Calcium:

Regulation of intracellular free [Ca^{2+}] depends upon a balance between the influx of calcium into the cytoplasm from extracellular space or intracellular

release site (like the sarcoplasmic reticulum) and the efflux of calcium out of the cytoplasm.

i. Calcium influx:

The influx of calcium can occur as a result of stimulation of VOC or ROC (as described above). This will lead to an increased influx of extracellular calcium into the cytoplasm. The initial increase in intracellular calcium will induce a further release of calcium from the SR. The Ca^{2+}-induced Ca^{2+} release mechanism was first demonstrated in skinned skeletal and cardiac muscle cells (74, 75). Since the amount of calcium entering the smooth muscle cells during the action potential is too low to initiate any contraction, the Ca^{2+}-induced Ca^{2+} release mechanism was also proposed to occur in smooth muscle cells (288). Saida in 1978 demonstrated the presence of Ca^{2+}-induced Ca^{2+} release in saponin skinned and SR intact smooth muscle cells (242, 243). Two main SR Ca^{2+} release channels have been identified on vascular smooth muscle cells. These channels are ryanodine receptor sensitive and inositol 1,4,5-triphosphate (IP_3) receptor sensitive calcium release channels. During Ca^{2+}-induced Ca^{2+} release, the initial increase in intracellular calcium level will activate the ryanodine receptor. Activation of this receptor will result in opening of the calcium channel associated with the ryanodine receptor. The ryanodine receptor is also sensitive to adenine nucleotides, caffeine, Mg^{2+} and intracellular pH (113, 122).

In contrast to skeletal and cardiac muscle cells in which their contractility depends on presence of extracellular Ca^{2+}, smooth muscle cells could be induced to contract under experimental conditions in the absence of extracellular Ca^{2+} (285, 286). Deth and coworker in 1977 demonstrated that a number of agonists such as norepinephrine, vasopressin, histamine, and angiotensin could release intracellular calcium from the SR calcium pool (67, 68). The above agonists will bind to their plasma membrane receptors to cause Ca^{2+} release and pharmacomechanical coupling. The major pathway for this calcium release is the activation of a phospholipase C (PLC) and formation of second messengers. Activation of PLC by any cell membrane agonist will result in a cascade of events leading to formation of IP_3 and 1,2-diacylglycerol (DAG). IP_3 and DAG act as second messengers and play an important role in many cellular processes (17, 142). IP_3 and DAG can be generated either through G protein coupled receptors and PLC-β isoforms or through tyrosine kinase receptors and PLC-γ isoforms (15, 16). The IP_3 which is rapidly released into the cytoplasm activates the IP_3 receptor on the SR leading to a rapid release of Ca^{2+} (18, 30).

At least three subtypes of the IP_3 receptor exist due to alternative splicing of IP_3 receptor genes. The full length cDNA of type 1(IP_3R1), type 2 (IP_3R2) and type 3 (IP_3R3) IP_3 receptors have been cloned (23, 87, 277). All of the three subtypes are closely related with approximately 70% homology. IP_3R1 is expressed in many cell types (86), IP_3R2 is expressed in brain and heart (207), whereas IP_3R3 is expressed in most of the tissues except nervous system (23). In

vascular smooth muscle cells, IP_3R1 and IP_3R3 mRNA has been detected (64). IP_3R1 has been localized to the SR (189). Affinity binding assays demonstrated different binding affinities for IP_3 to different IP_3R subtypes. Newton and coworkers (1994) showed a higher affinity of IP_3R2 for binding to IP_3 than IP_3R1 (183). IP_3R3 had the lowest affinity for binding to IP_3 (303). In smooth muscle cells, binding of IP_3 to IP_3R1 is 10 fold higher than its binding to IP_3R3 (183).

The activation and opening of the IP_3 receptor is calcium dependent. Cytoplasmic calcium has a biphasic role on calcium release through the SR IP_3 receptor (123). Increasing the intracellular calcium to approximately 300 nM will result in a stimulation of IP_3 mediated calcium release (71, 123, 160, 170). A further increase in intracellular calcium concentration will inhibit any response to IP_3 mediated calcium release through IP_3 receptors (160, 168). The role that calcium plays in the activation of IP_3 receptors is very similar to Ca^{2+} induced Ca^{2+} release through the ryanodine receptor. However, the IP_3 receptor cannot be activated by calcium alone and the presence of IP_3 is essential for this activation (45, 71, 118, 123, 160).

ii. Calcium efflux:

There are several proteins involved in lowering elevated intracellular $[Ca^{2+}]$ in smooth muscle cells. These include the plasma membrane or sarcolemmal Ca-ATPase, SR Ca-ATPase (SERCA2) and Na^{+}-Ca^{2+} exchanger.

These proteins play an important role in the relaxation and the reduction vascular tone.

Plasma membrane calcium pump activity depends on hydrolysis of ATP for extrusion of Ca^{2+} into extracellular space. The activity of the SL Ca-ATPase is stimulated by cAMP dependent protein kinase, cGMP dependent protein kinases and protein kinase C (PKC) (169, 310).

Both endoplasmic reticulum and sarcoplasmic reticulum play a major role in regulating the uptake of excess cytosolic calcium via an ATPase pump. There are many sarcoplasmic reticulum and endoplasmic reticulum Ca^{2+} ATPase isoform. These pumps are called SERCA ATPase pumps and are derived from three genes called SERCA1, SERCA2, and SERCA3. However, alternative splicing of SERCA1 and SERCA2 generates additional isoforms of the SERCA ATPase pump (i.e. SERCA1a, 1b and SERCA2a, and 2b). The SERCA2 ATPase is more predominant in vascular smooth muscle cells whereas the SERCA1 ATPase is more predominant in fast skeletal muscle cells and the SERCA3 ATPase is mostly present in non-muscle cells (5, 34, 44, 191, 213).

In smooth muscle cells phospholamban plays an important role in controlling SERCA2 ATPase activity (191, 213). Dephosphorylated phospholamban inhibits SERCA2 ATPase pump activity whereas phosphorylation of this protein by cAMP and cGMP dependent protein kinases or PKC will activate the calcium pump (191).

The Na^+-Ca^{2+} exchanger also plays a role in the regulation of intracellular [Ca^{2+}]. This exchanger does not directly require ATP for its function. The inward drive of Na^+ along its concentration gradient will result in efflux of calcium from cytoplasm (172, 217, 218). Na^+-Ca^{2+} exchanger is thought to be an important factor in modulating VSMC relaxation via this significant role in Ca^{2+} efflux from the cell (172, 217, 218).

II. Pathology of Vascular Wall:

1. Atherosclerosis: Definition and Risk Factors:

Atherosclerosis is a chronic disease process in the intima of the vessel which gradually leads to vascular obstruction. Atherosclerosis is the major cause of coronary heart disease, cerebrovascular disease, and peripheral vascular disease. Atherosclerosis is a chronic disease that starts in youth and progresses until death. There are several risk factors associated with the disease. These risk factors could be labeled into two groups: constitutional factors and controllable factors. Examples of constitutional factors include age, sex, and genetic predisposition and for the latter are life style, diet, activity levels and cigarette smoking.

a. Risk Factors:

i. Age:

Epidemiological studies support the age dependent increase in the incidence of coronary heart disease in different populations (125, 138). The onset of this disease starts at childhood (304). The initiation and progression depends largely on nutritional factors (12). Moczar and coworkers demonstrated that an attenuation of matrix macromolecule biosynthesis in an age dependent fashion in rabbits (171). This is due to an age dependent elastase activity in the lesion-free portion of the aorta (224, 225).

ii. Sex:

Gender plays an important role in atherosclerosis. Men tend to suffer from coronary heart disease at a younger age than women do. The average life span of a woman is 8-10 years more than a man (98). However, this gap lessens with aging. This is due to a rapid increase in coronary heart disease in post-menopausal women. In average, pre-menopausal women have higher serum high density lipoprotein (HDL) and lower low density lipoprotein (LDL) levels. This alteration in serum lipid profile is due to estrogen which protects against the early development of coronary heart disease (98).

iii. Behavioral factors:

Personality type has been associated with a propensity for coronary heart disease. For instance, people with type A personality have a higher chance of

developing coronary heart disease. These people show an exaggerated sense of time and are extremely competitive (98).

iv. Hypercholesterolemia:

Elevation of serum lipids plays the major role in the atherosclerotic process and constitutes one of the main risk factors for coronary heart disease. Many animal species such as primates (76, 77, 163), rabbits (229, 230), swine (91, 92), and hamsters (186) develop atherosclerotic lesions when fed a high cholesterol diet. Attenuation of serum cholesterol using diet or drugs in human clinical trials demonstrated a decrease in fatal and nonfatal coronary artery disease in men and women.

v. Hypertension:

High blood pressure is one of the major risk factors in coronary heart disease. Hypertensive animal models show medial smooth muscle hypertrophy and hyperplasia (195). Women with high blood pressure are more prone to cardiovascular complications than normotensive women (6).

vi. Diabetes:

Diabetes has a major impact on cardiovascular diseases. It has been shown that platelets from diabetic patients release more growth factors in the form of PDGF and IGF which result in proliferation of smooth muscle cells into the

intima (104). The combination of insulin with IGF *in vitro* can lead to an increase in LDL uptake by smooth muscle cells (147). Diabetic patients can accumulate substances called advance glycolysation endproducts (AGE). These substances are chemotactic for human monocytes in vitro, and therefore, can play an important role in atherosclerosis (42).

vii. Cigarette Smoking:

Smoking is a potent risk factor in coronary heart disease. The level of coronary heart disease is dependent upon the number of cigarettes smoked (320,321). The number of cigarettes smoked and inhalation of its toxic material appears to be correlated with the development of coronary and peripheral vascular disease (320,321). Cigarette smoke contains mutagenic substances and it may result in the formation of free radicals in the plasma which further results in oxidation of lipoprotein and initiation of atherosclerotic lesions. Furthermore, cigarette smokers have lower HDL levels which is very important in reverse transport of cholesterol (232).

viii. Immune Injury:

There are many studies that suggest an involvement of the immune system in the process of atherosclerosis (144, 251, 272). Immunoglobulins and T lymphocytes, B lymphocytes, immunocomponents and macrophages have been identified in atherosclerotic plaques(109, 144). Endothelial cells in cholesterol

fed rabbits express attachment molecules that can bind and attract monocytes and lymphocytes (58).

2. Role of Intima in Atherosclerosis:

Atherosclerosis is an intimal disease. The normal artery consists of a thin layer of endothelial cells, very few smooth muscle cells, and macrophages. In contrast to the atherosclerotic intima, the normal intima lacks any accumulation of lipids. The intima can be divided into a proteoglycan layer (adjacent to the endothelial layer) and a deeper musculoelastic layer (adjacent to medial layer) (264). The initial lipid accumulation occurs in the deeper area of the intima (265).

3. Initiation and Progression:

The above risk factors will lead to the initiation and progression of atherosclerotic lesions. The lesions have been hypothesized to occur at the site of injury in endothelial cells. This response to injury hypothesis was first introduced by von Rokitansky (294) and Virchow (293). French in 1966 and Ross in 1973 further modified this hypothesis (85, 234, 235). This hypothesis proposes that injury to endothelial cells by mechanical, chemical, viral or immunological agents will result in platelet stimulation, adhesion and aggregation. The aggregated platelets will release PDGF (platelet derived growth factor), which could induce the smooth muscle cell proliferation and migration. However, the

process of lesion formation is much more complicated than initially hypothesized. There are many other factors involved in this process. In contrast to denudation or necrosis of the endothelial layer that was originally proposed to occur, a very subtle change in endothelial function and structure can also lead to lesion formation (61). Furthermore , it has been shown that release of PDGF from platelets is not the only mitogen causing smooth muscle cell migration and proliferation. Other growth factors released from endothelial cells and macrophages play an important role in smooth muscle cells proliferation as well. In addition, free radicals and oxidized byproducts of oxidized LDL have been shown to have mitogenic properties (53, 65, 127, 135, 179).

a. Role of Lipoprotein:

Four major groups of plasma lipoproteins have been identified. These lipoproteins were grouped according to their density and electrophoretic mobility on an agarose gel. The density of pure lipid is less than water and when lipids are incorporated with protein their density increases. Therefore, as the ratio of protein to lipid increases the density of a lipoprotein increases too. This characteristic of lipoprotein enable us to separate lipoproteins into: 1) chylomicrons (0.95 mg/ml), 2)very low density lipoproteins (VLDL) (0.95-1.006 mg/ml), 3) low density lipoproteins (LDL) (1.019-1.1063 mg/ml), and 4) high density lipoproteins (HDL) (1.063-1.21 mg/ml). Upon electrophoresis on an agarose gel, these lipoproteins migrate differently. Chylomicrons show no

migration, followed by LDL (β-lipoprotein), VLDL (pre-β lipoprotein) and HDL (α-lipoprotein).

Among the above lipoproteins, LDL and VLDL have been considered the most atherogenic lipoproteins. VLDL contains mainly triglyceride (56% of total lipids), but also has significant amounts of cholesterol (23% of total lipids). However, LDL contains mostly cholesterol (58% of total lipid) and only 13% triglycerides. The main function of LDL is to transport cholesterol from the liver to the peripheral tissues, where it is used as a precursor for steroid hormone production and also for the repair and control of the fluidity of the cell membrane. HDL has been associated with an attenuation in atherosclerotic lesions via a reverse transport of cholesterol from the peripheral tissue to the liver (97). HDL contains primarily cholesterol (41% of total lipids) and very little triglycerides (13% of total lipids).

Since LDL and VLDL are the most atherogenic lipoprotein, therefore, it is important to discuss their structure and function in more detail with emphasis on LDL. The main apolipoprotein of LDL is apolipoprotein B-100. This apo B-100 also is found in VLDL and considered one of the longest single polypeptide chains known. It consists of 4563 amino acids. VLDL contains other apolipoproteins such as apo C-I, C-II, CIII and apo E. These apolipoprotein are small polypeptides that could be freely transferred between different lipoproteins. There are many functions associated with these lipoproteins such as receptor recognition site (apo B-100 to classical LDL receptor), enzyme cofactors (e.g.

lipoprotein lipase and lecithin: cholesterol acyltransferase), and lipid transport proteins.

VLDL is assembled in liver and secreted into the circulation. It has a very short half-life and its main function is to provide triglyceride to different tissues (e.g. adipose tissue and skeletal muscle). LDL has a much longer half-life. It can last in circulation for up to 2 days. LDL carries 60% of total plasma cholesterol. Its concentration is estimated to be approximately 3 mg/ml of plasma (177). LDL is a enormous molecule with molecular weights between 1.8 to 2.8 million dalton (177). Each molecule of LDL contains neutral lipids in its core. There are approximately 1600 molecules of cholesterol ester and 170 molecules of triglyceride in this region. This is surrounded by a single layer of phospholipids (~ 700 molecules) and free cholesterol (~ 600 molecules) (177). Each of the phospholipids are arranged in a way that the polar head groups are facing the surface of LDL and the hydrophobic tail is located toward the core of LDL. This arrangement of phospholipid ensures the solubility of LDL in plasma. The apolipoprotein B-100 is also embedded in the phospholipid surface and 8-10% of this protein is glycosylated.

LDL plays an important role in the process of lesion formation. First, LDL is the major carrier of cholesterol among other lipoproteins. Accumulation of cholesterol in the form of cholesterol esters has been identified in atherosclerotic lesions. Furthermore, an increase in native LDL concentration also has been documented in early lesions (116, 117). Secondly, patients with homozygous

familial hypercholesterolemia (HFH) and Watanabe heritable hyperlipidemic (WHHL) rabbits, which both lack any functional LDL receptor develop massive atherosclerotic lesions (39, 95). The familial hypercholesterolemic patients die within the second decade of their life from coronary heart disease (83). Finally, animal models on a high cholesterol diet demonstrated atherosclerotic lesion formation (292, 306).

Many cells in the vessel wall such as smooth muscle cells, macrophages, and endothelial cells have receptors that can recognize and internalize LDL. This receptor is referred to as the classical LDL receptor (40, 93). Although LDL is considered to be atherogenic, its uptake via the classical LDL receptor is down-regulated when the cellular need for cholesterol is met (40, 93). Ylä-Herttuala and coworkers (1991) studied the LDL receptor in atherosclerotic lesions using *in situ* hybridization (314). They demonstrated the absence of classical LDL receptor expression on macrophages in these lesions (314). Furthermore, Goldstein and Brown (1977) showed that macrophages failed *in vitro* to accumulate cholesterol ester even when exposed to high concentration of LDL (94). These observations in combination with the data obtained from patients with familial homozygous hypercholesterolemia suggest that receptors other than the classical LDL receptor must be involved in the process of lesion formation. This has lead to the proposal for the existence of a new receptor type that can recognize and take up modified LDL without any down-regulation in its

expression. This receptor is called the scavenger receptor (41, 94). It is present on most of the cells in the atherosclerotic lesions (41, 94).

i. LDL Oxidation & its Properties:

There are many studies in support of the role of oxidation of LDL in initiation of events leading to formation of atherosclerotic lesions. Many studies *in vitro* demonstrated that all the major cells types (macrophages, endothelial cells, smooth muscle cells and lymphocytes) can oxidatively modify LDL (110, 111, 139, 202). Among the above cells, macrophages seem to be the most active in modifying LDL (110, 202). The mechanism of oxidation of LDL is believed to be caused by free radicals. Free radicals are molecules with unpaired electrons that are highly unstable and could cause chain reaction oxidation. In addition to oxidation by cells, LDL can also be oxidized with free radicals generated by transitional metals. This further supports the involvement of free radicals in the formation of oxLDL. Alternatively, Sparrow and coworkers also demonstrated oxidation of LDL by incubation with a crude extract of soybean lipoxygenase (262). The involvement of lipoxygenase in the oxidation of LDL has been demonstrated in cell mediated oxidation of LDL (203, 215). It has been proposed that cellular lipoxygenase (especially 15-lipoxygenase) can generate and secrete superoxide anion into the medium causing lipid peroxidation in LDL (203, 215).

The above were examples of *in vitro* oxidation of LDL, but does oxidation of LDL occurs *in vivo*? There are several line of evidence that strongly suggest

that oxidation of LDL occurs *in vivo*. The first *in vivo* evidence came from immunocytochemical studies on WHHL rabbits. Haberland and coworkers used a monoclonal antibody against malondialdehyde modified LDL to demonstrate the presence of oxidatively modified LDL in the atherosclerotic lesion of these rabbits (106). Malondialdehyde (MDA) is an end oxidation product of lipids that can bind to LDL's apolipoprotein (Apo B-100) leading to uptake of modified LDL via the scavenger receptor (79, 105). Other antibodies to oxLDL have also been used confirming the presence of oxLDL in the atherosclerotic lesion of WHHL rabbits (200, 228). The second piece of evidence suggesting involvement of oxLDL *in vivo* is LDL extracted from atherosclerotic lesions from WHHL rabbits. These LDL particles were characterized by an increase in thiobarbituric acid reactive substances, an increase in electrophoretic mobility, a decreased particle size, an increase in macrophage uptake, an increase in free cholesterol, and a fragmentation of apolipoprotein B (60). These changes in LDL isolated from atherosclerotic lesions were also confirmed in cholesterol fed rabbits and humans (198, 313). In addition, autoantibodies to oxLDL have been isolated from both human and rabbit plasma (72, 197, 198, 204, 244). This indicates the presence of antigenic material to oxLDL in atherosclerotic lesions. Finally, experimental animals (rabbits, mice, hamster and non-human primates) on a high cholesterol diet showed a significant decrease in lesion formation when antioxidant compounds (probucol, butylated hydroxytoluene, and vitamin E) were administrated to the animals (267).

OxLDL has many other properties that are not shared with native LDL besides stimulating accumulation of lipid. The biological properties of oxidized LDL depend upon the extent of its oxidation. More extensive oxidized LDL is cytotoxic to a variety of cells in culture (114, 133, 174, 283). The cytotoxic effect of oxLDL *in vivo* will damage endothelial cells and initiate lesion formation. LDL oxidation can affect gene expression. Fox and coworkers reported that oxLDL can inhibit the expression of platelet drived growth factor (PDGF) in cultured bovine aortic endothelial cells (81, 82) and monocyte derived macrophages (158). In contrast, minimally oxidized LDL stimulate the expression and secretion of colony-stimulating factor and monocyte chemotactic protein 1 (MCP-1) by endothelial cells and smooth muscle cells *in vitro* (57, 214). Furthermore, injection of minimally oxidized LDL into mice caused a significant elevation in serum macrophage colony-stimulating factor (143). These chemotactic properties of oxLDL will result in monocyte adhesion onto endothelial cells and penetration of these cells into the sub-endothelial space where they undergo morphological changes to form macrophages. Macrophage motility is also inhibited by oxLDL leading to entrapment of these cells in the intima. Several groups have also reported that oxLDL is immunogenic (200), mitogenic (53, 219), stimulates platelet aggregations (7, 236), and promotes pro-coagulant activity (248). Finally, many studies have been demonstrated that oxLDL can also affect vascular contractility. Bossaller and coworkers showed that in the atherosclerotic lesion, endothelial cells lose their ability to generate

endothelial dependent relaxing factor (EDRF) (31, 32). Therefore, oxLDL can inhibit EDRF mediated vasodilatation. The mechanism of action is not clear. Kugiyama and coworkers suggested that an alteration in the endothelial membrane may result in a disruption in receptors specific for vasodilatation (134). Other studies suggest more direct inhibition in EDRF release or a direct effect on smooth muscle cells (89, 90).

ii. Protection of LDL against Oxidation:

If oxidized LDL is important in cardiovascular disease processes, then agents which can retard or prevent the oxidation process should prove to be beneficial therapeutic tools. The protective effect of vitamin E or α-tocopherol against cell mediated oxidation of LDL have been demonstrated. Steinbrecher and coworkers demonstrated that addition of α-tocopherol in the media of cultured endothelial cells inhibited cell mediated LDL oxidation (271). Similar results with α-tocopherol was also demonstrated with smooth muscle cells and macrophages *in vitro* (52, 173). The protective effect of different antioxidants such as probucol, α-tocopherol, and butylated hydroxytoluene have been demonstrated in WHHL rabbits and cholesterol fed rabbits and hamsters (268). Probucol has been used as a lipid-lowering drug. It has a very potent antioxidant effect with structural similarity to butylated hydroxytoluene (19, 216). In animal models, probucol not only played an important role as an antioxidant but it also

reduced plasma cholesterol levels. Therefore, the protective effect of probucol is not only due to its antioxidant effect but it has other biological properties. Probucol can inhibit the release of interlukin-1 by monocytes and increase expression of cholesterol ester transfer protein (269). Out of 23 studies, 16 studies showed beneficial effects of antioxidants, 2 studies were borderline, and 5 studies were negative (269).

4. Stages of Plaque Formation:

The atherosclerotic lesion can be grouped into at least three lesion types: the fatty streak, the intermediate lesion, the atheroma, and the fibroatheroma or complicated lesion (264).

The fatty steaks are characterized microscopically by a presence of lipid droplets within the cells resident in the intima. The two main types of cells that have been identified to accumulate large amounts of lipid in the form of cholesterol ester to transform into foam cells are predominantly macrophages and smooth muscle cells (264, 266). Most of lipid accumulation in the fatty streak is intracellular. However, there is some lipid accumulation that is associated with extracellular matrix proteins. Furthermore, not all the fatty streaks progress into the next stage of atherosclerotic lesion. This regression may happen as a result of the reduction in plasma LDL levels, a decrease in oxidative modification of LDL in the intima by antioxidants and/or an elevation in reverse cholesterol transport by HDL (48, 249). However, if the pro-atherogenic factors persist, the fatty

streak will develop into an intermediate lesion. The intermediate lesion has been classified morphologically to be somewhere between a fatty streak and a complicated lesion. This lesion is characterized by the formation of a pool-like aggregate of extracellular lipid droplets. There is a more active recruitment and transformation of monocytes to macrophages and a proliferation of smooth muscle cells from the medial region of the vessel. There is a greater synthesis of collagen, elastin and proteoglycans. This will result in an increase in the size of the lesion. Abnormal formation of connective tissue will lead to the development of a fibrous cap that covers the lipid core of the lesion.

The atheroma is characterized by the presence of dense extracellular lipids in a well defined area of the intima (264). This dense area is called the lipid core. This lipid core is developed from the confluence of smaller lipid aggregates present in the intermediate lesions (264). Macrophages were not present in the lipid core but were identified at the periphery of the lipid core in the shoulder area of the lesion (264). The smooth muscle cells were also scattered and contained lipid droplets. There is no further significant increase in collagen and proteoglycan deposit. However, progression of atheroma leads to formation and deposition of newly synthesized fibrous connective tissue which result in formation characteristic fibroatheroma lesions. This stage of atherosclerosis is characterized by presence of fibrous cap on the top of lipid core. There are substantial increase in synthetic smooth muscle cells and collagen deposits which result in obstruction of vessel lumen.

5. Association of Calcium with the Atherosclerotic Plaque:

Calcification of the vessel wall has been considered to be a hallmark of the atherosclerotic lesion. Deposition of calcium was initially thought to be the result of atherosclerosis rather than the cause of this disease. However, Blumenthal and coworkers demonstrated that medial calcification precedes intimal calcification (24). In addition, Fleckenstein and coworkers demonstrated a 13-fold increase in calcium deposits in the early stages of a fatty streak (78). As the lesion progressed into intermediate lesions and complicated lesions, they observed a 25 and 80-fold increase in calcium deposits, respectively. These observations were based on the total calcium deposited in the arterial wall. However, Strickberger and coworkers showed that aorta from cholesterol-fed rabbits have approximately 5-fold higher intracellular calcium than control rabbit aorta (273).

a. Role of Calcium in Atherosclerosis:

Calcium may play a very important role in the initiation and progression of the atherosclerotic lesion. Many cellular processes that are involved in lesion formation are regulated by changes in the intracellular [Ca^{2+}]. These include the migration and proliferation of smooth muscle cells, release of growth factors, secretion of extracellular matrix protein, activation of platelets, cholesterol

esterification, recruitment of monocytes, and endothelial permeation (193, 208, 231).

b. Evidence for the Action of LDL and Oxidized LDL on Cell Calcium

i. Indirect evidence that LDL alters calcium pools:

Cytosolic calcium plays an essential role in regulating a variety of activities within different cells. Elevation of intracellular calcium can take place via either depolarization of excitable cells or ligand-receptor binding and activation of second messenger systems. These changes mediate contraction of muscle cells, cell proliferation, secretion of different biological products, platelet aggregation, and activation of blood cells. Observation of any of the above changes in cell function, therefore, can be taken as indirect evidence of an elevation in intracellular calcium.

For example, although controversial (8, 89), recent data has shown that LDL induces a dose dependent increase in force of contraction in rat aortic rings (238, 241). The force of contraction was attenuated in the absence of extracellular calcium (238, 241). This would suggest that LDL may stimulate calcium influx from the extracellular space into the cell. LDL has also been shown to stimulate proliferation of smooth muscle cells in culture (145, 250). The mitogenic action of LDL was linked to an elevation of intracellular calcium.

Finally, an increase in the activity of platelets is frequently observed during hypercholesterolemia (50, 188) and atherosclerotic plaque formation

(278). Recently, LDL has been shown to induce aggregation of platelets in the presence of other agonists such as epinephrine (4, 7, 28). Because an increase in the intracellular calcium concentration plays a central role in activation of platelets (223), the process of platelet aggregation can also be used as an indirect indication of an elevation in intracellular calcium.

ii. Direct evidence for the action of LDL on cell calcium

Our understanding about the role that calcium plays in cellular processes has been greatly enhanced by the use of calcium ion indicators (such as fura-2, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid; quin-2, 2-[[2-bis-(carboxymethyl)-amino-5-methylphenoxy]-methyl]-6-methoxy-8-bis (carboxymethyl)-aminoquinoline; and indo-1, 1-[2-amino-5-(6-carboxyindol-2-yl)-phenoxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid) (103). These fluorescent probes can be used to measure the changes in intracellular calcium. Using quin-2, Block et. al. (1988) and Knorr et. al. (1988) were the first to demonstrate that the cellular activation induced by LDL was mediated by an elevation of intracellular calcium (22, 129). With but one exception, this elevation in calcium was also observed by many other groups using a variety of different cell types (Tables 1 & 2).

The time required for LDL to significantly increase intracellular calcium varied depending upon the cell type. In vascular smooth muscle cells, LDL had its maximum

Table 1 - Effect of native LDL on vascular smooth muscle cells.

Cell Type	Effects of LDL on		Time for Maximum Change	Proposed Mechanism				Ref.
	$[Ca^{2+}]_i$	Force		Classic LDL Receptor	Phosphotidylinositol Turnover	Extracellular Ca^{2+}	Ca^{2+} Channel	
r-VSMC	↑	--	15 sec.	--	Maybe	Yes	Yes	(238)
aortic ring	--	↑	--	--	--	--	--	
r-VSMC	↑	--	15 sec.	--	Maybe	Yes	Yes	(241)
aortic ring	--	↑	--	--	--	--	--	
r-VSMC	↑	--	15 sec.	No	No	--	--	(239)
r-VSMC	↑	--	10 sec.	No	No	Yes	--	(308)
r-VSMC	↑	--	20 sec.	--	--	--	--	(237)
r-VSMC	↑	--	30 sec.	--	--	--	--	(302)
r-VSMC	↑	--	-	--	--	--	--	(297,301)
r-VSMC	↑	--	20 sec.	No	--	Yes	No	(299)
r-VSMC	↑	--	30 sec.	--	Yes	--	--	(22)
r-VSMC WKY & SHR	↑	--	5 min.	--	--	--	No	(194)
rab-Femoral artery	--	↔	--	--	--	--	--	(89)
r-VSMC	↑	--	30 sec.	No	Yes	Yes	Maybe	(175)
r-VSMC WKY & SHR	↑	--	--	--	Yes	--	--	(220)
r-VSMC	↑	--	30 sec.	--	--	--	--	(27)
rab-VSMC	↑	--	--	--	--	--	--	(137)
h-VSMC	↑	--	few sec.	--	Yes	Yes	Yes	(25)

Abbreviation : r = Rat; VSMC= Vascular smooth muscle cells; WKY= normotensive Wistar Kyoto rats; SHR= Spontaneous hypertensive rats; rab= Rabbit; h= Human

Table 2 - Effect of native LDL on calcium in various cells.

Cell Type	Effect of LDL on $[Ca^{2+}]_i$	Time for Maximum Change	Proposed Mechanism				Ref.
			Classic LDL Receptor	Phosphotidylinositol Turnover	Extracellular Ca^{2+}	Ca^{2+} Channel	
h-Platelets	↑	30 sec.	Yes	Yes	--	--	(28)
h-Platelets HFH	↑	30 sec.	No	Yes	--	--	(26)
h-Platelets	↑	30 sec.	Yes	Yes	Yes	--	(129)
h-Platelets	↑	15 sec.	Yes	Yes	--	--	(22)
h-Fibroblasts	↑	1.5 min.	--	Yes	--	--	
h-Platelets	↑	30 sec.	Yes	Yes	--	--	(43)
h-Platelets	↑	30 sec.	--	--	--	--	(263)
h-Platelets	↑	--	--	--	Yes	--	(317)
h-Platelets	↑	30 sec.	--	Yes	--	Yes	(256)
h-Endothelial	↑	--	--	Yes	--	--	
h-Fibroblast	↑	15 sec.	No	No	Yes	--	(239)
r-Alveolar type II	↑	60 sec.	Yes	Yes	Yes	--	(295)
Endothelial cell line	↑	20 sec.	Yes	--	Yes	Yes	(247)
b-Aortic endothelial	↔	--	--	--	--	--	(180)
h-lymphocyte	--	1 min.	--	Yes	--	Yes	(153)
rab-Cardiomyocyte	↑	30 min.	No	--	Yes	Yes	(150)

Abbreviations: r= rat; h= Human; b= Bovine; rab= Rabbit; HFH= Homozygous familial hypercholesterolemia.

effect on intracellular calcium from 10-30 seconds (Table 1). The only exception to this relatively rapid elevation of calcium was observed by Orlov et. al. (1993) in which they reported a period of 5 minutes was needed for LDL to exert its maximum action on ^{45}Ca uptake (194). In other cell types, the time required for the maximum change to occur in cellular calcium varied from 15-30 seconds in platelets to 30 minutes in cardiomyocytes (Table 2). This relatively rapid effect is noteworthy and it is not an isolated finding. Many studies have demonstrated LDL effects on cellular calcium within as little as 10-30 seconds (22, 22, 26-28, 237, 238, 240, 241, 247, 256, 298, 302). This is unusual considering the process of receptor-mediated LDL internalization normally takes at least several minutes (9, 35, 40, 255). Distribution of the internalized LDL and transfer of the free cholesterol to the cell membrane would extend the time even further (35). In cardiomyocytes, the effects of LDL on intracellular calcium transients required about 30 minutes to be observed (150). This coincided temporally with a measured increase in cell cholesterol (150). These data agree well with LDL transport studies which demonstrate a half-time of about 32-42 minutes for the process of LDL internalization followed by cholesterol movement to the plasma membrane (35, 84). Thus, the extremely fast effects of LDL reported by the majority of the studies (15-30 seconds) are difficult to explain. Alternatively, lipid transfer from the LDL to the cell may occur through non-receptor mediated pathways like pinocytotic internalization, a receptor-ligand complex that is not internalized (205), or equilibrium diffusion through the aqueous space (128, 255). However, these mechanisms act at approximately the same rate as the receptor-mediated transfer of lipids or slower (128,

252). Several of these studies have attempted to experimentally address this issue. Presently, there is no convincing evidence that LDL acts directly through classical LDL receptors on the smooth muscle cells. Morita et. al. (1989) demonstrated that apolipoprotein B-100 alone can also induce an increase in intracellular calcium (175). In contrast, Sachinidis et. al. (1991) demonstrated that neither pretreatment of smooth muscle cells with apolipoprotein B-100 or monoclonal antibodies against the LDL receptor had any effect on the LDL induced calcium release (239). The involvement of the LDL receptor was also examined in other cell types (247, 295). In platelets, the involvement of the LDL receptor is controversial. One laboratory has reported that the action of LDL was receptor mediated since modification of LDL with cyclohexanedione (which reduces LDL binding to the classic LDL receptor) attenuated the action of LDL on platelets (22, 26, 28). Conversely, another group has observed that platelets from patients with genetic defects in the expression of the apo B/E receptor (i.e. homozygous familial hypercholesterolemia) were sensitive to calcium mobilization by LDL (26). Similar results were also observed in fibroblasts isolated from patients with homozygous familial hypercholesterolemia (239). This would suggest that LDL may act through other membrane bound receptors or lipids in the LDL may diffuse across the aqueous space to the cells.

Another way to address the receptor-ligand interaction issue is to determine the lipoprotein specificity of the LDL-induced effects on intracellular calcium. In cardiomyocytes, HDL does not elicit a change in intracellular calcium transients (239). However, both HDL and LDL induced calcium

mobilization in vascular smooth muscle cells (27). Both LDL and VLDL can induce alterations in vascular smooth muscle cell calcium exchange kinetics when incubated chronically but VLDL appeared to be less potent than LDL (137). Again, the data do not rule out the possibility that cholesterol or other lipid moieties within the lipoprotein may diffuse across the aqueous space and thereby induce their effects on the cell.

The precise pathway of calcium transport that the LDL is affecting is also a matter of critical interest. Calcium plays a central role in the regulation of muscular tone and other physiological functions in muscle cells. The rise in cytoplasmic calcium in cardiomyocytes and vascular smooth muscle cells can be mediated through calcium entry from the extracellular space via the L-type calcium channels, the Na^{+}-Ca^{2+} exchanger, and ligand operated calcium channels (for review see 157). Calcium release from intracellular stores can also be induced via ryanodine-sensitive calcium channels (i.e. calcium induced calcium release) on the sarcoplasmic reticulum (for review see (287, 288). Binding of hormones or ligands to their receptors on the plasma membrane will also lead to activation of phosphatidylinositol pathways via stimulation of phospholipase C. Phospholipase C catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol 1,4,5-triphosphate and diacylglycerol (DAG). In smooth muscle cells, IP_3 can induce an initial release of calcium from the sarcoplasmic reticulum (1) with a second phase of calcium influx across the sarcolemma. The increase in cell calcium induced by LDL, therefore, may be a result of an action on one or more of these ion transport pathways.

Cultured smooth muscle cells incubated for 1-3 days with high levels of LDL (LDL was replaced daily) responded with significantly faster calcium exchange kinetics in both the intracellular and extracellular compartments (137). These calcium exchange alterations would probably favour an accumulation of calcium within the smooth muscle cell similar to that observed in atherosclerotic vessels (137, 208). This work identified in a general manner the cellular membrane system which may have been modified by LDL (sarcolemma) but not the precise pathway within that membrane system which was affected (calcium channel vs. sodium/calcium exchange) nor the mechanism which caused the alteration (direct effects of cholesterol vs. a second messenger pathway). Other work using acute exposure conditions has provided more information in this regard. For example, exposing isolated smooth muscle cells to LDL caused a transient decrease in PIP_2 and a rise in IP_3 (22). This stimulation of IP_3 turnover and subsequent increase in intracellular calcium has been confirmed by other groups (25, 175, 220) with one exception (238-241). The effect of LDL on phosphatidylinositol turnover was also observed in other cell types (Table 2) ((22,152,256,295). The initial elevation of IP_3 is relatively rapid (5 seconds) in rat vascular smooth muscle cell and reached its maximum level at 30 seconds. The intracellular calcium was increased in a phasic fashion (parallel to the elevation of IP_3) and remained elevated for a period of 10 minutes in a tonic fashion (175). Similar phasic increases in calcium were also observed in human vascular smooth muscle cells (25). Bochkov et. al. (1992) demonstrated that the initial increase in intracellular calcium was due to its mobilization from intracellular sources, whereas the second tonic

phase of calcium was mainly due to calcium entry from the extracellular space (25). Others have also demonstrated a dependence of LDL's effect upon extracellular calcium (Table 1). In accord with these data, calcium channel blockers attenuated the action of LDL on vascular smooth muscle (145, 238, 241). However, there is some controversy concerning the involvement of calcium channels (194, 298).

The elevation in intracellular calcium induced by LDL was also observed in other cell types (Table 2). This increase in intracellular calcium in many different cell types was dependent upon extracellular calcium (26, 239, 247, 295) and calcium channel blockers further reduced any increase in cytoplasmic calcium level (150, 152, 247, 256). However, we would caution against interpreting results which were dependent upon the actions of drugs in a cholesterol-loaded cell preparation. These cells appear to exhibit a general sub-sensitivity to drug administration (161). This may occur because of the significant effects cholesterol has on membrane microviscosity (161). Rigidification of the membranes after cholesterol incorporation may make the transport of drugs through these membranes more difficult (312). The data may have important implications for the clinical setting where hypercholesterolemic patients are frequent targets for drug therapy.

iii. Evidence for the action of oxidized LDL on cell calcium

Although less work has been carried out on oxidized LDL, both indirect and direct evidence supporting the capacity of oxidized LDL to alter cellular

calcium homeostasis has been reported. Like native LDL, oxidized LDL can also stimulate contraction in denuded vascular smooth muscle (89). Oxidized LDL increased the response of vascular smooth muscle cells to threshold concentrations of other vasoconstrictive agonists as well (such as norepinephrine, serotonin, or high potassium) (8, 89). This action of oxidized LDL was suppressed in the presence of calcium channel blockers (8, 89) suggesting a role for extracellular calcium in this process.

More direct evidence defining the capacity of oxidized LDL to alter intracellular calcium movements is available. LDL oxidized by a variety of free radical generating systems is capable of inducing an increase in intracellular calcium (Table 3). This has been demonstrated in smooth muscle cells, cardiomyocytes (149), lymphoid and endothelial cells. The time required to elicit the changes in cell calcium varied considerably from 30 seconds to 20 hours (Table 3). The reason for such a large variation is unclear. Smooth muscle and endothelial cells possess the scavenger receptor for the oxidized LDL moiety (38) but cardiomyocytes probably do not (150). Diffusion of the oxidized lipid products through the aqueous space separating the cell from the oxidized LDL would represent a likely mechanism for lipid exchange.

There is considerably less information identifying the precise calcium transport pathway with which oxidized LDL interacts. Extracellular calcium was critical for the stimulatory effects of oxidized LDL on calcium transients in cardiomyocytes (150). Consistent with this finding, the majority of

Table 3- Effect of oxidized LDL on various cells.

Cell Type	FRGS	Effect of ox-LDL		Time for	Proposed Mechanism				Ref.
		$[Ca^{2+}]_i$	Force	Maximum Change	Scavenger Receptor	Phosphotidylinositol Turnover	Extracellular Ca^{2+}	Ca^{2+} Channel	
rVSMC & aortic ring	Cu^{+}	↑	↑	--	--	--	--	Maybe	(301)
rVSMC	Cu^{+}	↑	--	30 sec.	--	--	--	--	(302)
r-VSMC	*	↑	--	--	--	--	--	--	(297)
hVSMC	Cu^{+}	N.D.	--	--	Yes	Yes	--	Yes	(221)
r-Aortic ring	Cu^{+}	--	↑	--	--	--	--	--	(300)
rab-Femoral artery	Cu^{+}	--	↑	--	--	--	--	Yes	(89)
p-Coronary artery	Cu^{+}	--	↑	--	--	--	--	--	(253)
Endothelial cell line	Cu+ & **	↑	--	--	Yes	--	Yes	Yes	(247)
b-Endothelium	UV-C	↑	--	20 hr.	--	--	--	--	(300)
rab-Cardiomyocyte	Fe-DHF	↑	--	16 min.	No	--	Yes	Yes	(149)
Lymphoid cell	UV-C	↑	--	16 hr.	--	--	--	--	(181)

Abbreviations: r= Rat; h= Human; rab= Rabbit; p= Porcine; b= Bovine; FRGS= Free Radical Generating System; UV= Ultra violet; Fe= Ferrous; DHF= Dihydroxyfumaric acid; oxLDL= Oxidatively Modified LDL.

* LDL isolated from atherosclerotic patients

** Acetylated LDL

studies have shown in a variety of cell types that oxidized LDL appears to enhance calcium flux through the slow calcium channels (89, 149, 221, 247).This would agree well with the observation that free radicals on their own can alter calcium channel function (28). The mechanisms whereby oxidized LDL induces its effects is less clear. Data from our laboratory shows that oxidized LDL is more effective in altering the intracellular calcium concentration in cardiomyocytes than native LDL (149, 150). Whereas it took 1 mg/ml native LDL 30 minutes to elicit a significant increase in cytosolic calcium, a ten-fold lower concentration and half this incubation time was required for oxidized LDL to produce a similar effect (149, 150). Free radical attack of LDL can result in oxidation of the cholesterol, the apolipoprotein and peroxidation of the fatty acyl chains in the phospholipid, triglyceride and cholesteryl ester fractions. Thus, any of these fractions may be responsible for the changes. It is clear that the presence of oxidized cholesterol in cardiac sarcolemmal membrane can seriously disturb calcium transport characteristics (136). However, it appears unlikely that oxidized cholesterol within the LDL is responsible for the effects of oxidized LDL on the cardiomyocyte (136). Instead, peroxidized lipid in the oxidized LDL may be associated with the changes. Consistent with this conclusion are the data of Henry and co-workers who found that lysolecithin generated within the LDL by a free radical generating system was capable of dose-dependent increases in smooth muscle contraction and cell calcium levels (159). Mechanistically, it

may be possible for oxidized LDL to induce a stimulation of phosphatidylinositol turnover (221) which may ultimately mobilize intracellular calcium.

B. MATERIALS

I. Chemicals:

Chemicals	*Source*
β-mercaptoethanol	Sigma-Aldrich, Oakville, Canada
α-Tocopherol (Vitamin E)	Sigma-Aldrich, Oakville, Canada
1,1,3,3-tetra-methoxypropane (MDA)	Sigma-Aldrich, Oakville, Canada
2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT)	Sigma-Aldrich, Oakville, Canada
2,2'-Azobis(2,4-dimethylvaleronitrile) (AMVN)	Polysciences Inc., Warrington, PA
2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH)	Polysciences Inc., Warrington, PA
2-nitro-4-carboxyphenyl N, N-diphenylcarbamate (NCDC)	Sigma-Aldrich, Oakville, Canada
4-Bromo-A23187	Sigma-Aldrich, Oakville, Canada
Acetone	Sigma-Aldrich, Oakville, Canada
Adenosine	Sigma-Aldrich, Oakville, Canada
Adenosine diphosphate (ADP)	Sigma-Aldrich, Oakville, Canada
Adenosine triphosphate (ATP)	Sigma-Aldrich, Oakville, Canada
Ascorbate	Sigma-Aldrich, Oakville, Canada
bis(dimethyl acetal 1,1,3,3-tetramethoxypropane)	Sigma-Aldrich, Oakville, Canada
Bovine serum albumin	Sigma-Aldrich, Oakville, Canada
Bromophenol blue	Sigma-Aldrich, Oakville, Canada
Cholesterol esterase	Sigma-Aldrich, Oakville, Canada
Cholesterol oxidase	Sigma-Aldrich, Oakville, Canada
Cholesterol supplemented diet	Purina Test Diets, Richmond, IN, USA

Chemicals	*Source*
Disodium ethylenediamine tetraacetic acid (EDTA)	Mallinckrodt Inc., Kentuky, U.S.A
Dithiobisnitrobenzoic acid (DTNB)	Sigma-Aldrich, Oakville, Canada
Dulbecco's Modified Eagle Medium (DMEM)	Gibco BRL, Canada
Embedding compound (OCT)	Sakura Finetek, Torrance, CA, USA
Endothelin	Sigma-Aldrich, Oakville, Canada
Epinephrine	Sigma-Aldrich, Oakville, Canada
Ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetate (EGTA)	Mallinckrodt Inc., Kentuky, U.S.A
Ferrous chloride ($FeCl_3$)	Sigma-Aldrich, Oakville, Canada
Fetal bovine serum (FBS)	Gibco BRL, Canada
Fluo-3	Molecular Probes, Eugene, USA
Fura-2	Molecular Probes, Eugene, USA
Glutaraldehyde	Sigma-Aldrich, Oakville, Canada
Glycerol	Sigma-Aldrich, Oakville, Canada
Glycine	Gibco BRL, Canada
Heparin	Leo Laboratories Canada LTD. Ajax, Canada
Histamine	Sigma-Aldrich, Oakville, Canada
Indo-1	Molecular Probes, Eugene, USA
Insulin	Sigma-Aldrich, Oakville, Canada
Ionomyosin	Sigma-Aldrich, Oakville, Canada
Lactate dehydrogenase (LDH)	Sigma-Aldrich, Oakville, Canada
Lazaroid (U74500A)	The Upjohn Co., Kalamazoo, MI
Leupeptin	Sigma-Aldrich, Oakville, Canada
Methanol	Sigma-Aldrich, Oakville, Canada
Nicotinamide-adenine dinucleotide (NAD)	Sigma-Aldrich, Oakville, Canada

Chemicals	*Source*
Norepinephrine	Sigma-Aldrich, Oakville, Canada
Paraformaldehyde	Sigma-Aldrich, Oakville, Canada
Penicillin-streptomycin	Gibco BRL, Canada
Phenazine methosulphate (PMS)	Sigma-Aldrich, Oakville, Canada
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich, Oakville, Canada
Ryanodine	Sigma-Aldrich, Oakville, Canada
Selenium	Sigma-Aldrich, Oakville, Canada
Sequi-Blot PVDF membrane	Bio-Rad Laboratories, Canada
Skim milk	Local shops
Sodium dodecyl sulfate (SDS)	Bio-Rad Laboratories, Canada
Tannic acid	Electron Microscopy Sciences, Washington, USA
Taurine	Sigma-Aldrich, Oakville, Canada
Thapsigargin	Sigma-Aldrich, Oakville, Canada
Thimerosal	Sigma-Aldrich, Oakville, Canada
Thiobarbituric acid (TBA)	Sigma-Aldrich, Oakville, Canada
Transferrin	Sigma-Aldrich, Oakville, Canada
Trichloroacetic acid (TCA)	Sigma-Aldrich, Oakville, Canada
Tris-HCl	Sigma-Aldrich, Oakville, Canada
Trolox	Aldrich Chem. Co., Milwaukee, USA
Trypsin-EDTA	Gibco BRL, Canada
Vasopressin	Sigma-Aldrich, Oakville, Canada

II. Antibodies:

Primary Antibody	Type	Host	Source
Anti-SERCA2 ATPase MA3-919 & MA3-910	Monoclonal	Mouse	Affinity BioReagent, Inc. Golden, CO, USA
Anti-IP_3 Receptor AB1622	Polyclonal	Rabbit	Chemicon International, Inc.
Anti-IP_3 Receptor MAB3078	Monoclonal	Mouse	Chemicon International, Inc.
Anti-IP_3 Receptor 407140	Monoclonal	Mouse	Calbiochem-Novabiochem Corp. San Diego, CA, USA
Anti-α-Smooth Muscle Actin	Monoclonal	Mouse	Sigma Biosciences, St. Louis, MO, USA
Anti-Smooth Muscle Myosin	Monoclonal	Mouse	Sigma Biosciences, St. Louis, MO, USA
Anti-Smooth Muscle Caldesmon	Monoclonal	Mouse	Sigma Biosciences, St. Louis, MO, USA

Secondary Antibody	Type	Host	Conjugate	Source
Anti-Mouse Ig Biotinylated	Monoclonal	Goat	--	Amersham Life Sciences
Streptavidin-Texas Red	--	--	Texas Red	Amersham Life Sciences
Anti-Mouse IgG	Monoclonal	Sheep	FITC	Sigma Biosciences, St. Louis, MO, USA
Anti-Mouse Horseradish Peroxidase	Monoclonal	Goat	HRP	Bio-Rad
Anti-Rabbit Horseradish Peroxidase	Monoclonal	Goat	HRP	Sigma Biosciences, St. Louis, MO, USA
Anti-Mouse Cy3	Monoclonal	Goat	Cy3	Sigma Biosciences, St. Louis, MO, USA

C. METHODS

I. Animal Protocol:

Male albino New Zealand rabbits were used in all of our experiments. These rabbits were divided into two groups. The first group of rabbits was on normal rabbit chow diet. These animals were used for the generation of primary smooth muscle cells in culture and freshly isolated smooth muscle cells. The second group of rabbits was on a cholesterol diet. These rabbits were fed 0.5% (wt/wt) cholesterol diet (Purina Test Diets, Richmond, USA) mixed with their normal chow diet. These rabbits were used for lipoprotein isolation and to obtain their atherosclerotic vessels.

The animals were sacrificed with a dose of 1 ml/kg body weight of 10:1 (*vol:vol*) ratio of ketamine (100 mg/ml) to xylazine (100 mg/ml). In order to prevent blood coagulation, 0.2 ml of sodium heparin stock (i.e 1000 i.u./mL) was added to the euthanasia cocktail. The rabbits received 3 ml of the euthanasia cocktail through the marginal ear vein.

II. Lipoprotein Isolation:

Blood from male albino New Zealand White rabbits fed a 0.5% cholesterol-supplemented diet was collected in 5 ml vacutainers containing 7.5 mg EDTA (Disodium ethylenediamine tetraacetic acid). The plasma was further separated from blood cells by centrifugation at 3000 rpm for 20 minutes at 4°C. In order to protect plasma during isolation, dithiobisnitrobenzoic acid (1.5

mmol/L), phenylmethylsulfonyl fluoride (2 mmol/L), and thimerosal (0.08 mg/ml) were added to the plasma. Dithiobisnitrobenzoic acid inhibits lecithin:cholesterol acyltransferase activity, phenylmethylsulfonyl fluoride inhibits proteolysis, and thimerosal protects from any bacterial growth. Plasma lipoproteins were isolated by a serial ultracentrifugation technique (166). Plasma was centrifuged at 38,000 rpm in a Ti70 rotor (Beckman, Canada) at 4°C. After 24 hours of centrifugation the top layer was removed which consisted of chylomicrons (density < 0.996 g/ml) and very low density lipoprotein (VLDL) (density < 1.0063 g/ml). The density of the remaining plasma was adjusted to ~ 1.019 g/ml with an addition of NaCl according to the following equation (NaCl g = Plasma Volume X 0.11698). Low density lipoprotein (LDL) (density 1.019-1.063 g/ml) was then isolated by centrifugation at 43,000 rpm for another 24 hours. EDTA (0.1 mmol/L) was added throughout the isolation to prevent oxidation of LDL. The VLDL and LDL fractions were extensively dialyzed in the dark against 0.15 mol/L NaCl, 0.1 mmol/L EDTA (pH 7.4), sterile filtered (0.2 μm pore size) and stored in small aliquots at 4°C.

The protein content of LDL and VLDL were determined by Lowry's method, and cholesterol (free and esterified) was measured enzymatically as described (156, 192). The absence of LDL and VLDL oxidation during isolation or prior to its use in experiments was confirmed by an absence of malondialdehyde (MDA) reactive products and oxidized cholesterol (79, 151).

III. Lipoprotein Modification:

The EDTA concentration in native LDL was reduced prior to LDL oxidation. Native LDL was diluted 10 fold in 150 mmol/L NaCl (pH 7.4) solution and oxidized by incubation with a solution of 50 μM $FeCl_3$ and 0.25 mmol/L ADP for period of 3 hours at 37°C. The extent of LDL oxidation was evaluated by i) measurement of thiobarbituric acid reactive substances (TBARS) (79, 107), (ii) electrophoretic mobility on agarose gels (using the Chiron Diagnostic Lipoprotein System), and iii) measurement of α-tocopherol content by HPLC (167).

1. TBARS Measurement Assay:

The MDA content of VLDL or LDL was determined by the thiobarbituric acid-reactive substances method (73, 107). Briefly, 1 ml aliquot of 0.375% thiobarbituric acid, 15% trichloroacetic acid, and 0.25 N HCl was added to the lipoprotein and heated for 15 minute at 100°C. This results in the development of a pink chromogen and its absorbance was measured at 535 nm. Freshly diluted malondialdehyde bis(dimethyl acetal 1,1,3,3-tetramethoxypropane) was used as a reference standard and the thiobarbituric acid reactive substances were expressed as nmoles MDA/mg protein.

2. Electrophoretic Mobility Assay:

Oxidation of LDL will result in a negatively charged molecule which can be distinguished from native LDL. The electrophoretic mobility of native LDL and oxidatively/chemically modified LDL was compared on a Chiron Diagnostic Lipoprotein System. LDL samples (1-5 μg cholesterol/ml) were applied onto a precast Universal Electrophoresis agarose gel. The gel was then run in Universal Buffer (43 mmol/L Sodium barbital and 7 mmol/L barbital) at 90 volts for 60 minutes. After electrophoresis, the gel was dried for 20 minutes in an oven at 55°C. For detection of lipoprotein bands the gel was stained with Fat Red 7B stain (180 mg/L stock solution) for 4 minutes, destained in a 70% methanol solution, and dried in an oven at 55°C.

3. Determination of α-tocopherol by HPLC:

Extraction of plasma, native LDL and oxLDL were performed in the dark in order to minimize light-induced α-tocopherol degradation. 250 μl of plasma from normal and cholesterol-fed rabbits was added to an equal volume of tocopherol acetate. This mixture was vortexed for one minute before 500 μl of ethyl acetate:hexane (3:2 vol/vol) was added and vortexed for another minute. This mixture was then centrifuged at 13000 g for 10 minutes, and the supernatant was collected and dried in a speed-vac (Savant Instruments, Inc., Holbrook, NY, USA). The samples were resuspended in 50 μl ethanol and 200 μl acetonitrile, and were filtered using a 22 μm filter. Aliquots (25 μl) were then injected into

the high performance liquid chromatography (HPLC) system for quantification. For LDL and oxLDL (~ 2 mg cholesterol/ml), a 50 μl aliquot of LDL was used for extraction and only ethanol was used in the resuspension of LDL extracts. α-tocopherol quantification was performed using a modified reverse-phase HPLC method (167). Prodigy 5 micron ODS (2) columns were used. The analytical column was 150 mm in length and the internal diameter was 4.0 mm. A guard column of 30 mm in length and 4.0 mm internal diameter was also used. The mobile phase was acetonitrile and methanol in a ratio of 75:25, respectively. Absorption was measured at 280 nm using a Waters 484 Tunable detector. Data was collected using a Waters Baseline 810 program. Tocopherol acetate was used as an internal standard to measure the efficiency of the extraction procedure.

IV. Vascular Smooth Muscle cells:

1. Cells in Culture:

An explant technique was used to generate primary cultures from normal rabbit thoracic aorta (20, 102). The thoracic aorta from a male New Zealand White rabbit (2.5 to 3 kg body weight) was isolated and gently cleaned from connective tissue, excess fat and adherent blood cells from the adventitial surface using a dissecting microscope. The endothelial layer was scraped off. The aorta was cut into 2-3 mm sections and transferred to a culture dish with growth media (20% fetal bovine serum in Dulbecco's Modified Eagle Medium, DMEM) and

5% antibiotic-antimycotic. The explants were incubated in a humidified incubator equilibrated with 5% CO_2 and maintained at 37°C. The first migration of cells was observed between days 6-8. After 7 days of migration, these explants were transferred to a new culture dish for further migration. The cells from the second phase of migration were used in our experiments. In order to induce differentiation, the smooth muscle cells were starved (for 5-6 days) in a serum free media supplemented with transferrin (5 μg/ml), selenium (1 nM), ascorbate (200 μM), and insulin (10 nM). This differentiation period was crucial for full development of contractile proteins in the cultured vascular smooth muscle cells (146).

2. Freshly isolated Cells:

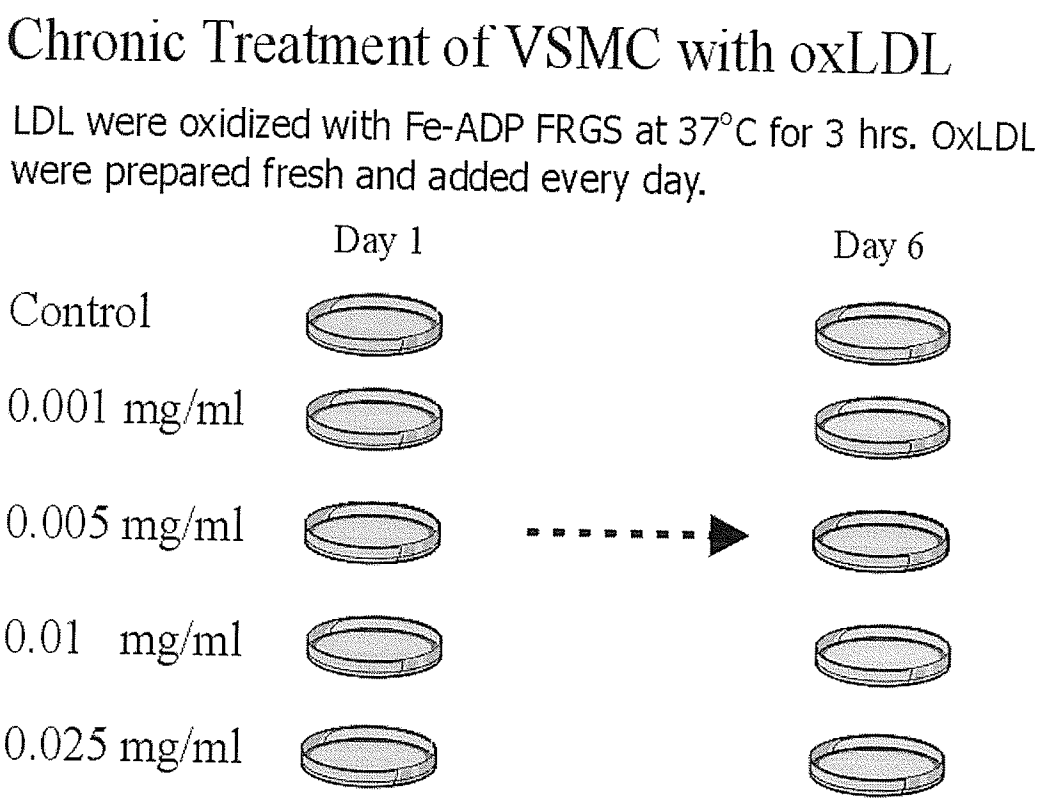
A male New Zealand White rabbit (2.5 to 3 kg body weight) was sacrificed as described above. The portal vein was removed to a physiological solution consisting of 120 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 4.2 mmol/L KCl, 0.6 mmol/L KH_2PO_4, 1.2 mmol/L $MgCl_2$, 11 mmol/L dextrose, 25 mmol/L taurine, 0.02 mmol/L adenosine, and 0.01 mmol/L $CaCl_2$ with pH of 7.4. This solution was constantly bubbled with O_2/CO_2 gas. After removing the adipose tissue and excess connective tissue, the portal vein was cut open longitudinally and the endothelial side was placed down on a dissecting dish and pinned flat onto the dish. All of the remaining fat and adventitial layer was dissected out using fine scissors and forceps under a dissecting microscope. The portal vein

was then cut into small strips (1 by 4 mm) and transferred into a clean test tube containing a digestion solution. The digestion solution consisted of the following: 1 mg trypsin inhibitor, 6 mg collagenase, 0.3 mg protease, and 1% BSA in 5 ml of physiological buffer. The portal vein was allowed to digest for approximately 20 minutes at 37°C under constant bubbling with O_2/CO_2 gas. The pieces of portal vein were removed from digestion solution and washed a few times in a fresh physiological solution containing 3% BSA. The digested portal vein was then triturated gently using a pasteur pipette to free the cells from the tissue. The freed cells were then placed in new dish. The extracellular calcium level was then increased gently in a stepwise fashion to a final concentration of 1 mmol/L. Calcium tolerant, spindly, elongated smooth muscle cells were used in our experimental protocol.

V. Chronic treatment of VSMC with oxLDL:

The smooth muscle cells used in our experimental protocol were from either first or second passage. The cells were serum starved in a defined media for 6 days prior to exposure to oxLDL. In chronic experiments, VSMC were exposed for a period of 6 days to different concentrations of freshly prepared oxLDL (0.001 to 0.1 mg cholesterol/ml LDL). The media containing freshly oxidized LDL was added daily to the cultured cells. The control cells were also kept in culture for the same period of time as the treated cells. The cytotoxicity of different concentrations of oxLDL were assessed by two tests: i) lactate

dehydrogenase (LDH) released into the culture media; ii) Ethidium homodimer staining of cell nuclei (LIVE/DEAD EukoLight Viability/Cytotoxicity Assay Kit, Molecular Probes, Inc.).



VI. Cell Viability Assay:

1. LDH Assay:

For the LDH assays, the VSMC were passaged and seeded in 12-well tissue culture plates. These cells were then starved, as described above, in 1 ml of phenol-red free Dulbecco's Modified Eagle Medium. A 500 µl aliquot of media was collected from each well every day for the LDH assay. This sample was stored at - 20°C. Once thawed, a 100 µl aliquot of Tris buffer (1 M, pH 8.5 stock solution) was mixed with 50 µl of collected media and 25 µl of substrate solution

(0.1 M L-lactate stock solution) in a test tube. After 5 minutes incubation in 37°C water bath, 100 µl of colour reagent (7.5 mmol/L nicotinamide-adenine dinucleotide (NAD), 4.0 mmol/L 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT), and 1.6 mmol/L phenazine methosulphate (PMS) stock solution) were added. These test tubes were further mixed and after 5 minutes incubation in a water bath at 37°C, 2.5 ml of 0.1 N hydrochloric acid was mixed into each test tube. Optical density was measured at 500 nm within 20 minutes against a blank. The blank contained the entire reagents except the substrate solution which was replaced by blank solution (11 mmol/L oxalate and 5.4 mmol/L EDTA) (13).

2. Live/Dead assay:

Vascular smooth muscle cells were passaged and seeded on the glass coverslips. These cells were serum deprived and treated with different concentration of oxLDL. In order to assess the viability of vascular smooth muscle cells chronically treated with oxLDL, we used the LIVE/DEAD EukoLight™ viability/cytotoxicity assay kit (Molecular Probes, Inc., Eugene, OR, USA). This assay kit is a two-colour fluorescent based detection kit. Live cells are distinguished from dead ones by the presence of intracellular esterase activity. This enzyme will cleave the ester conjugate of the non-fluorescent cell permeant calcein-AM to yield highly fluorescent calcein dye that is distinguished

by a very intense green fluorescence. However, the dead cells are distinguished by ethidium homodimer staining of cell nuclei. Ethidium homodimer enters the damaged cells and binds to nucleic acids and will result in generation of red fluorescence in the cell nucleus.

In a typical experiment, smooth muscle cells were treated ± different concentrations of oxLDL. These cells were then washed twice with sterile PBS and incubated in the PBS working solution containing 2 μM calcein-AM and 4 μM ethidium homodimer for 30 minutes at room temperature. These cells were then examined under epifluorescent microscope and the results was recorded on Fuji Provia 400 slide film.

VII. Cellular IP_3 Content Assay:

D-myo-inositol 1,4,5-trisphosphate (IP_3) content in VSMC was measured with a radioisotopic assay kit (Amersham, Canada). Vascular smooth muscle cells were grown in 6 well dishes to confluence. These cells were incubated with defined differentiation media for 6 days prior to the start of experiments. Culture plates containing VSMC were incubated with Krebs-Henseleit solution (120 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 4.8 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.25 mmol/L $MgSO_4$, 1.8 mmol/L $CaCl_2$, and 8.6 mmol/L dextrose) and then were treated with 0.1 mg cholesterol/ml oxLDL for 20 seconds. OxLDL was then removed and all the dishes were washed with ice cold PBS 3 times. These cells

were then frozen with liquid nitrogen. After the liquid N_2 was evaporated, 0.5 ml PBS was added to each dish and cells were scraped off the plates with a policeman scraper. In order to dissociate IP_3 from the cellular contents, scraped cells were homogenized with a Wheaton Teflon homogenizer 20 times. This homogenate was then spun down in a Beckman table top ultracentrifuge (TL 100) at 87,000 rpm for 25 minutes. The supernatant was used to measure IP_3 content by a commercially available radioisotopic method (Amersham, Canada). The protein concentration was determined before and after centrifugation (as described before).

VIII. Lipid Deposition Measurement:

Vascular smooth muscle cells were treated chronically ± different concentrations of oxLDL. After treatment, these cells were washed with PBS 3 times and stained with 1μg/ml Nile Red in acetone for 15 minutes (80, 99, 100). These cells were then washed and mounted on a glass slide. The fluorescent intensity was visualized with a Bio-Rad confocal microscope.

IX. Immunocytochemistry:

Vascular smooth muscle cells were passaged and seeded (~ 300,000 cells per 100 mm dish). These cells were serum starved as described above for 6 days and treated ± oxLDL for different time points. After treatment, smooth muscle

cells were washed twice with PBS and fixed with appropriate fixative for the different antibodies.

1. Actin, Myosin and Caldesmon:

Smooth muscle cells were fixed with 1% paraformaldehyde and permeabilized with 0.1% Triton-X 100. These cells were then treated with monoclonal antibodies for α-smooth muscle actin (1:200), smooth muscle myosin (1:250), and caldesmon (1:500). These cells were extensively washed with PBS and incubated with a secondary antibody conjugated to FITC. Nuclear staining was done with 50 μg/ml Hoescht No. 33258 in PBS. This dual labeling distinguishes between muscle and non-muscle cells. The fluorescent images of the fluorescein-5 isothiocyanate (FITC) and Hoescht No. 33258 were obtained with a Bio-Rad MRC-600 UV-confocal system connected to a Nikon Diaphot 300 epifluorescence microscope. This system is equipped with an argon ion laser able to excite different fluorophores at UV (351 and 363 nm) and visible (488 and 514 nm) wavelengths. The FITC fluorescence was obtained by exciting the cells with a 488 nm laser line and the emission was collected at 520 nm. The Hoescht No. 33258 fluorescence was obtained by exciting the nuclei with a 351 nm laser line and the emission was collected at 450 nm. All of the images were obtained through a Nikon Fluor 40X (N.A.= 1.3) oil immersion lens.

2. IP_3, Ryanodine, and SERCA2 ATPase:

For detection of IP_3 receptors within smooth muscle cells, quiescent cells were fixed with a solution of ice cold acetone and methanol (1:1). These cells were then incubated with a monoclonal anti-IP_3 antibody (1:100) overnight at 4°C. This antibody recognizes all three types of IP_3 receptors (i.e. type I, II, and III) (Calbiochem, San Diego, USA). For ryanodine channels, smooth muscle cells were fixed with ice cold acetone and methanol solution (1:1) and further incubated with monoclonal anti-ryanodine antibodies (1 μg/ml) (Affinity Bioreagents Inc, Golden, CO, USA). Finally, for SERCA2 ATPase, a monoclonal antibody reacting to SERCA2 ATPase was used (1:500) (Affinity Bioreagents Inc, Golden, CO, USA). These cells were further incubated with a secondary antibody conjugated to FITC. The fluorescent images were obtained with a Bio-Rad confocal system as described above.

X. Immunohistochemistry:

Both control and 0.5% cholesterol fed rabbits (12-14 weeks on diet) were sacrificed as described above. The thoracic aortas from both groups were removed and cleaned from any excess tissue and attached blood cells. These aortas were then cut into 5 mm sections and placed in a mold covered with O.C.T. embedding compound (Sakura Finetek, Torrance, USA). These molds were frozen in a mixture of dry ice and ethanol and stored at -70°C until used. The embedded aortas were then cut into 7 microns sections using a Leitz 1720

Cryostat. These sections were mounted on top of superfrost glass slides. Before use, these slides were fixed in a cold 1:1 solution of acetone and methanol (-20°C) for 15 min.

1. Actin and Myosin:

The aortic section were blocked with 5% skim milk. These sections were then incubated with smooth muscle cell specific anti-α-actin (1:400) or anti-myosin (1:500) antibodies. Nuclear staining was done with 50 μg/ml Hoescht No. 33258 in PBS.

2. IP_3, Ryanodine, and SERCA2 ATPase:

These sections were then blocked with 5% skim milk and incubated with monoclonal anti-IP_3, ryanodine, SERCA2 ATPase, or phospholipase C antibodies (1:100) overnight at 4°C. The aortic sections were then incubated with anti-mouse IgG biotinylated whole antibody (from goat) (1:20) followed by streptavidin conjugated to Texas red (1:20) (Amersham Life Science Inc, Oakville, Canada). Nuclear staining was done with 50 μg/ml Hoescht No. 33258 in PBS. The photographs of cell fluorescence were registered on Fuji Provia 400 film with an Olympus BH-2 or a Nikon epifluorescence microscope.

XI. Western Blotting:

Vascular smooth muscle cells were grown on 6 well dishes or 100 mm cell culture dishes (when higher total protein was required). These cells were serum starved and incubated with a defined medium necessary for their differentiation for 6 days. After this period of time, cells were treated ± different concentrations of LDL or oxLDL. After treatment, smooth muscle cells were lysed with lysis buffer (1% SDS, 100 mmol/L NaCl, 62.5 mmol/L Tris-HCl pH 7.6, 1mmol/L PMSF, and 10 μg/ml leupeptin). The cells were then scraped off and transferred into a microfuge tube to be kept on ice. The protein concentration was measured by the modified Lowry assay (156). Cell extracts were denatured with 2X sample buffer (62.5 mmol/L Tris-HCl, 1% SDS, 10% glycerol, 0.01% bromophenol blue, and 20 μg/ml β-mercaptoethanol) at 100°C for 5 min.

1. Actin and Myosin:

The samples (15 μg/lane) were separated on a 3-15% gradient SDS polyacrylamide gel with a 4% stacking gel in the running buffer (0.025 mol/L Tris-HCl, 0.192 mol/L glycine, and 0.1% SDS). The proteins were transferred electrophoretically onto nitrocellulose membrane (Gibco BRL) in transfer buffer (25 mmol/L Tris-HCl, 192 mmol/L glycine, and 20 % methanol). The blots were blocked with 10% skim milk in PBST (i.e. PBS and 0.05% Tween) for 30 minutes at room temperature on a multi-mixer. The membranes were then incubated with smooth muscle specific monoclonal antibodies against α-actin or

myosin (Sigma-Aldrich, Canada). These antibodies were diluted (1:10,000) in 1% skim milk and PBST and incubated at room temperature for 1 hour on a multi-mixer. The blots were further incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) diluted in 1% skim milk and PBST (1:20,000) for 1 hour at room temperature on a multi-mixer. The actin and myosin were detected with the Pierce Super Signal detection system and the blots were exposed to Kodak X-OMAT film.

2. IP_3 Receptors:

The samples (25 μg/lane) were separated on 6% SDS polyacrylamide gel with a 4% stacking gel in the running buffer (0.025 mol/L Tris-HCl, 0.192 mol/L glycine, and 0.1% SDS). The proteins were transferred electrophoretically into Sequi-Blot PVDF membrane (Bio-Rad Laboratories, Canada) in transfer buffer (25 mmol/L Tris-HCl, 192 mmol/L glycine, 0.05% SDS, no methanol). The blots were blocked with 10% skim milk in PBST (i.e. PBS and 0.05% Tween) overnight at 4°C on a multi-mixer. The membranes were then incubated with a polyclonal anti-IP_3-receptor antibody (Chemicon International Inc., USA). The antibody was diluted (1:200) in 1% skim milk and PBST and incubated at 4°C overnight on a multi-mixer. The blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Canada) and diluted in 1% skim milk and PBST (1:3000) overnight at 4°C on multi-mixer.

The IP_3-receptor was detected with the Pierce Super Signal detection system (Rockford, USA).

3. SERCA2 ATPase:

The samples (25 μg/lane) were separated on a 10% SDS gel with a 4% stacking gel in the running buffer (0.025 mol/L Tris-HCl, 0.192 mol/L glycine, and 0.1% SDS). The proteins were transferred electrophoretically into Sequi-Blot PVDF membrane (Bio-Rad Laboratories, Canada) in transfer buffer (25 mmol/L Tris-HCl, 192 mmol/L glycine, and 20 % methanol). The blots were blocked with 10% skim milk in PBST (i.e. PBS and 0.05% Tween) for 1hour at room temperature on a multi-mixer. The membranes were then incubated with a monoclonal anti-SERCA2 ATPase antibody overnight at 4°C on a multi-mixer. The blots were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Canada). The SERCA2 ATPase was detected with the Pierce Super Signal detection system (Rockford, USA).

XII. Calcium Measurements:

Measurement of intracellular calcium in single smooth muscle cells was carried out using two different classes of calcium indicators: single wavelength intensity modulating dye (e.g. fluo-3) and dual wavelength ratiometric dyes (e.g. fura-2 and indo-1). For loading purposes, acetoxymethyl ester (AM) forms of these dyes were used. The AM form can easily penetrate through the plasma

membrane and is entrapped within the cell after cleavage of the ester group by cytoplasmic esterase enzymes.

1. Fura-2:

Measurement of the fura-2 signal was carried out using a SPEX Fluorolog spectrofluorometer (SPEX Industries, Edison,NJ). The SPEX system is connected to a Nikon Diaphot epifluorescent microscope with a set of 100X,40X oil CF Epi-Fluorescence Fluor, and 20X PL2 objective lenses. The microscope stage is equipped with a temperature regulated device that can hold cover slips (Leiden cover slip dish, Medical System Corp., Greenvale, NY).

Vascular smooth muscle cells were washed twice and then loaded with 2 μM fura-2-AM (1 mmol/L stock in dry dimethyl sulfoxide, DMSO) for 20 minutes at 22°C in a Krebs-Henseleit buffer (120 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 4.8 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.25 mmol/L $MgSO_4$, 1.8 mmol/L $CaCl_2$, and 8.6 mmol/L dextrose). After the fura-2 loading, the cells on the cover slips were washed 3 times with Krebs-Henseleit buffer and placed in a Leiden chamber. This Leiden chamber was then placed in an experimental chamber mounted on the microscope and temperature was adjusted to 37°C.

The fluorescent intensities of fura-2 which reflects bound and unbound calcium was determined by alternately exciting a single cell at 340 and 380 nm and recording emission at 505 nm. The maximum and minimum fluorescence

signals were obtained by adding 10 μM 4-Bromo-A23187 and 5 mmol/L EGTA, respectively at the end of the experiment in order to calibrate the signal with the intracellular calcium concentration (103).

2. Fluo-3:

Intracellular calcium concentration was determined using fluo-3-AM. The dye was loaded into the smooth muscle cells by adding 2 μM fluo-3-AM (1 mmol/L stock solution in DMSO) for 20 minutes at 22°C in a Krebs-Henseleit buffer. After loading, the smooth muscle cells were washed three times with Krebs-Henseleit buffer and placed in a Leiden chamber as described above. The fluorescence images of intracellular calcium were obtained with a Bio-Rad MRC-600 UV-confocal microscope. The cells were excited with 488 nm wavelength laser beam through a VHS (high sensitivity violet excitation set) filter block. The emitted fluorescence was measured through a 515 nm low-cut filter.

3. Indo-1:

For ratiometric determination of intracellular calcium, indo-1-AM was used. This dye was loaded into smooth muscle cells by incubating these cells with 2 μM indo-1-AM (1 mmol/L stock solution in DMSO) for 20 minutes at 22°C in a Krebs-Henseleit buffer. After loading, the smooth muscle cells were

washed three times with Krebs-Henseleit buffer and placed in a Leiden chamber as described above. The cells were excited with 351 UV laser line and the emission was recorded at 405 nm (Ca^{2+} bound) and 480 nm (Ca^{2+} free). Filter block IN1 and IN2 were used for indo-1 calcium measurement. The IN1 filter block contains a 380 nm dichroic reflector and IN2 has 440 nm dichroic reflector plus 460 (channel 1) and 405 (channel 2) emission filters.

XIII. Confocal microscopy:

All the confocal images were obtained by using a Bio-Rad MRC-600 UV-laser scanning microscope. This microscope is equipped with a water-cooled argon ion laser (Enterprise, Coherent, USA). This laser can be used to excite a specimen at UV (351 and 363 nm) and visible (488 and 514 nm) wavelength. This confocal is connected to a Nikon Diaphot-300 inverted microscope with a set of 100X and 40X oil CF Epi-Fluorescence Fluor objective lenses. The microscope stage is equipped with a temperature regulated device that can hold cover slips (Leiden cover slip dish, Medical System Corp., Greenvale, NY).

XIX. Statistical Analysis:

Data were expressed as the mean ± standard error (S.E.) The statistical comparisons were made using one-way analysis of variance, followed by the Student-Newman-Keuls test for multiple comparison. Differences between

means were considered significant when $p < 0.05$.

D. RESULTS

I. Modification and Protection of LDL against Oxidation:

Low density lipoprotein can be oxidatively modified both *in vivo* or *in vitro* by free radicals generated by different sources as described in the Review of Literature. The nature and extent of LDL oxidation depends firstly on the damaging free radical species and its site of action and secondly on the anti-oxidative defense mechanism. In this study, we used different free radical generating systems to produce and oxidize LDL at different site. The oxidative modification of LDL was challenged with different antioxidants. We compared the protective effects of two lipophilic antioxidants (α-tocopherol and lazaroid) with two hydrophilic antioxidants (trolox and vitamin C) in the presence of several different free radical generating systems. The efficacy of these antioxidants was tested against a lipophilic or hydrophilic free radical generating system.

LDL was oxidatively modified by a Fe-ADP free radical generating system. This system generated a variety of free radical species such as superoxide anions, hydrogen peroxide and hydroxyl radicals (108). These oxygen derived free radicals can react with unsaturated lipids to form peroxyl radicals (108). The protective effects of α-tocopherol and lazaroid, two lipophilic free radical scavengers, were tested. α-Tocopherol at concentration of up to 2.5 μmol/L did not show any protection against lipid peroxidation in the LDL

(Figure 1). However, lazaroid significantly reduced lipid peroxidation at concentrations as low as 1 μmol/L (Figure 1). Inhibition of thiobarbituric acid reactive substances (TBARS) were significantly reduced at α-tocopherol concentration of 5 μmol/L and this protective effect continued until complete inhibition 10 μmol/L. The protective action of lazaroid against lipid peroxidation remained relatively stable at concentrations ≥7.5 μmol/L. α-Tocopherol and lazaroid showed IC_{50} values of 5.9 and 5.0 μmol/L, respectively (Table 4).

Vitamin C and trolox, two hydrophilic antioxidants tested, exhibited both peroxidative and protective characteristics when a Fe-ADP free radical generating system was used. Trolox induced a significant increase in TBARS production at concentrations up to 0.75 mmol/L (Figure 2), before it significantly reduced the TBARS production at concentrations ≥1.3 mmol/L. The peroxidative effect of vitamin C was observed at concentrations up to 2 mmol/L (Figure 2), whereas concentrations ≥4 mmol/L significantly decreased the TBARS production. Vitamin C had a significantly higher IC_{50} value (5.17 mmol/L) than trolox (1.23 mmol/L) (Table 4).

In order to examine the effect of preincubation of lipophilic or hydrophilic antioxidants on TBARS generation in lipoproteins, VLDL was preincubated with 2.5 μM vitamin E or 0.5 mmol/L trolox for 60 minutes at 37°C prior to oxidation by Fe-ADP. These concentrations were chosen because they exhibited no capacity to inhibit TBARS formation in VLDL without previous pre-incubation

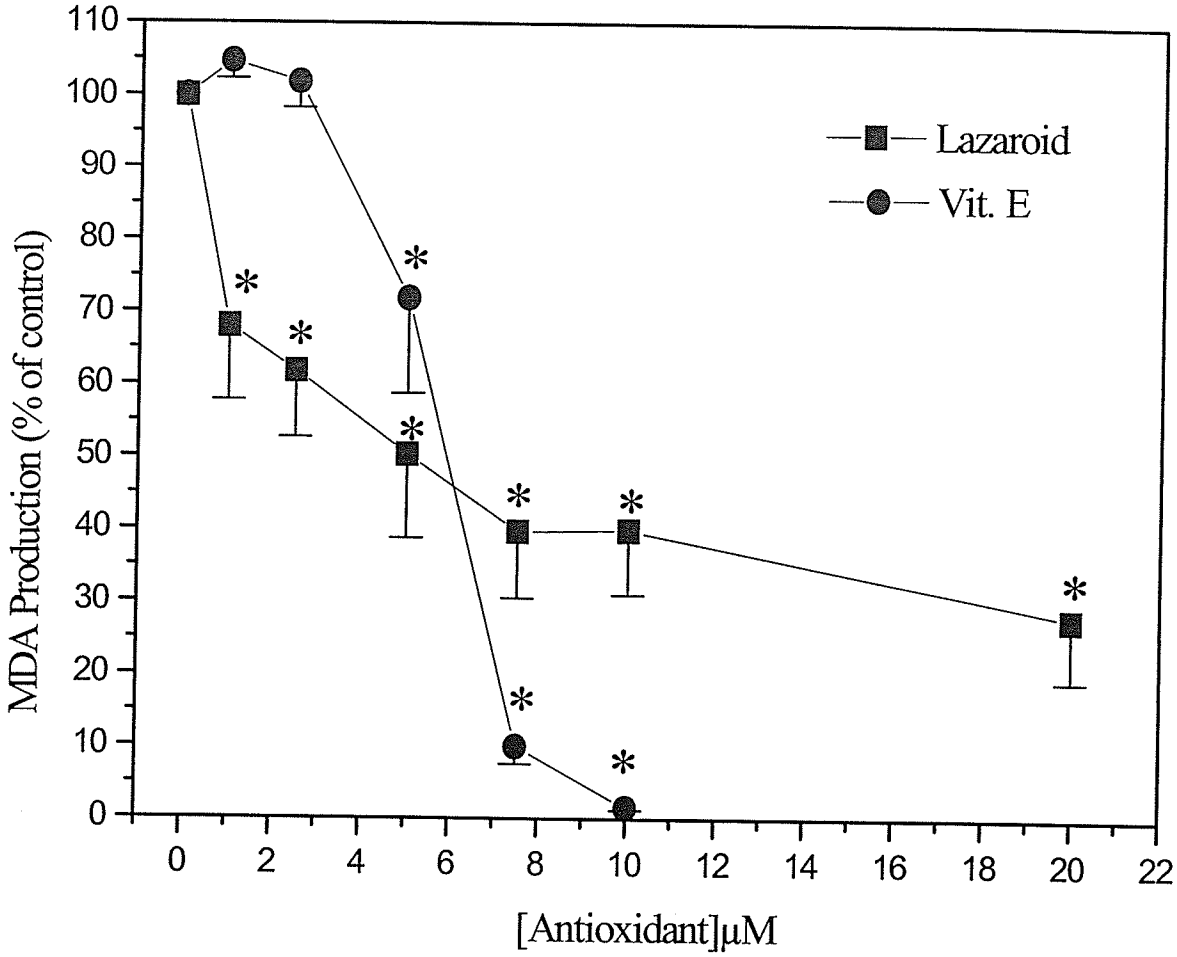


Figure 1. A comparison of the effect of two lipophilic antioxidants, vitamin E and lazaroid on free radical induced MDA formation in LDL. LDL (1 mg cholesterol/ml) was incubated for 60 minutes at 37°C with a Fe-ADP free radical generating system ± different concentrations of the antioxidant. Absolute values for MDA content of oxLDL in the absence of antioxidants (i.e. control) were 36.5 ± 2.3 nmoles/mg protein. Values are means ± S.E. of 5-6 different experiments (*P <0.05).

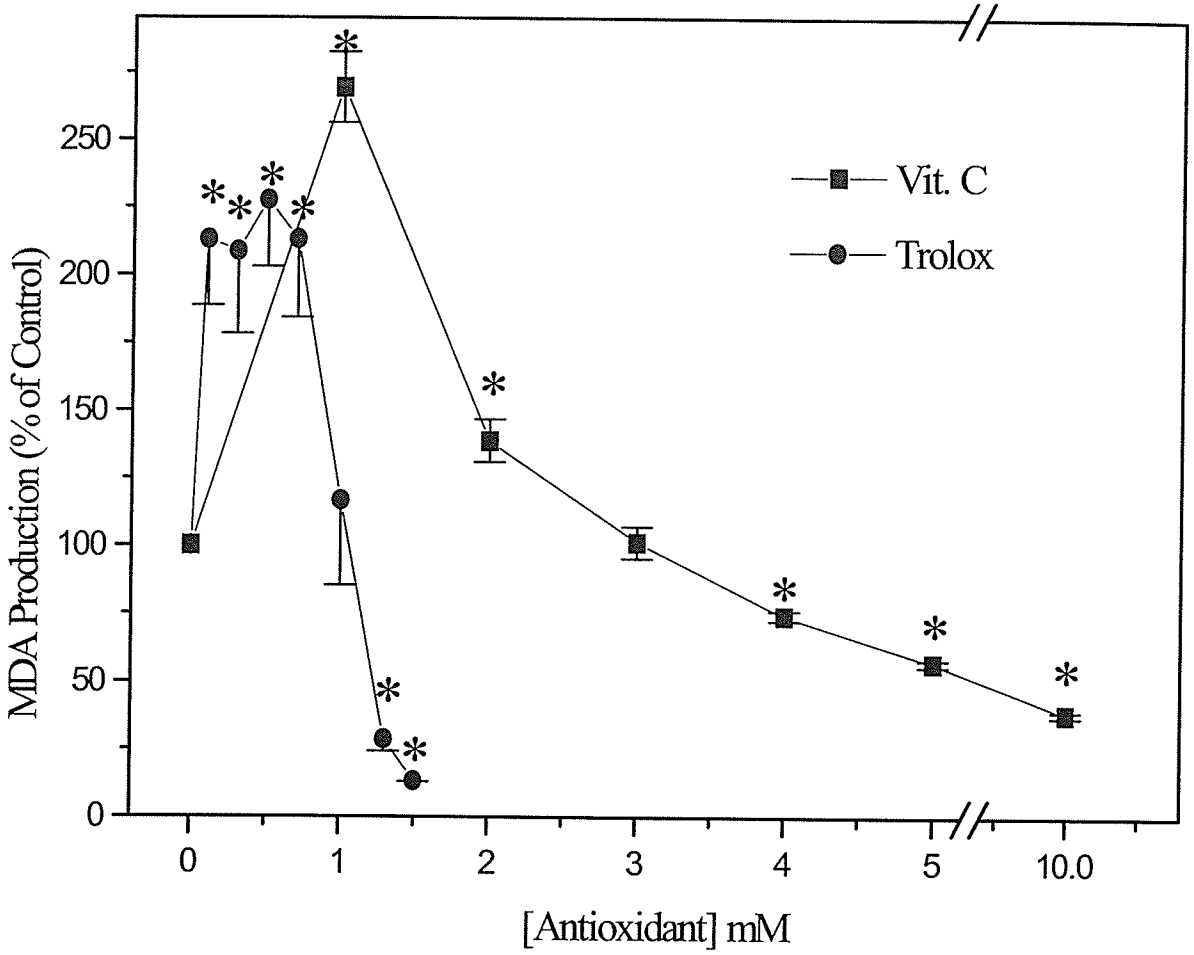


Figure 2. A comparison of the effect of two hydrophilic antioxidants, trolox and vitamin C, on free radical induced MDA formation in LDL. LDL (1 mg cholesterol/ml) was incubated with Fe-ADP for 60 minutes at 37°C ± different concentrations of the antioxidants, vitamin C or trolox. Absolute values for MDA content of oxLDL in the absence of antioxidants (i.e. control) were 44.8 ± 0.2 nmoles/mg protein. Values represent the mean ± S.E. of 5-11 different experiments (*P <0.05).

Table 4. IC_{50} of four antioxidants against LDL lipid peroxidation induced by three different free radical generating systems.

	Free Radical Generating System		
Antioxidant	**Fe-ADP**	**AAPH**	**AMVN**
α-Tocopherol	5.90	0.25	20.00
Lazaroid	5.00	0.22	6.20
Trolox	1.20×10^3	0.08×10^3	0.96×10^3
Vitamin C	5.20×10^3	ND	ND

Values were obtained from data presented in the Figures. Values are in μM. ND; not determined.

of the antioxidant with the VLDL. As shown in Figure 3A, preincubation of VLDL with trolox failed to reduce the TBARS production significantly. Conversely, preincubation of VLDL with α-tocopherol significantly protected against lipid peroxidation (Figure 3B).

In order to more effectively compare the protective effects of lipid soluble versus water soluble antioxidants, it was thought to be useful to examine their effects against two systems which generate the free radicals in either a lipophilic or hydrophilic medium. Therefore, the azo-compounds AMVN and AAPH were used to generate peroxyl radicals within LDL or in the aqueous region surrounding LDL, respectively. These azo-compounds undergo thermal decomposition to generate peroxyl free radicals at a constant rate (185). The TBARS production in LDL was examined first as a function of varying concentrations of AMVN or AAPH (Figure 4). The lipid soluble peroxyl radical generating system (AMVN) induced greater TBARS production in LDL than the water soluble system (AAPH). For the subsequent comparative work, therefore, it was essential to use a concentration of each azo-compound which generated a similar TBARS level in the LDL. Ten mmol/L AMVN and 40 mmol/L AAPH produced approximately the same amount of TBARS (Figure 4). Therefore, these concentrations were used in subsequent work which tested the effects of different free radical scavengers against lipid peroxidation. Lazaroid (≤1 μmol/L) significantly inhibited (80%) TBARS production when the radicals were

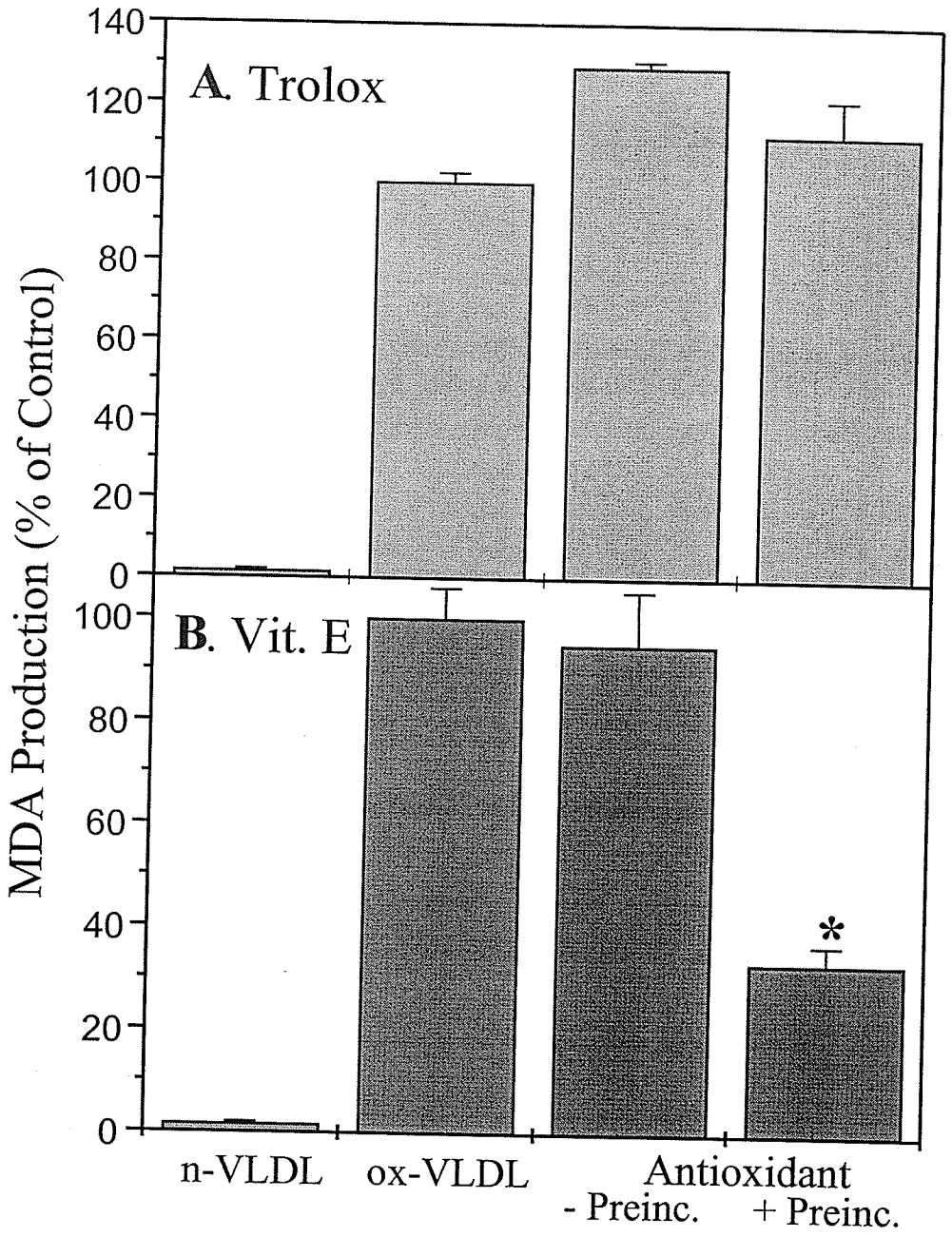


Figure 3. Effect of preincubation of VLDL with trolox or vitamin E on free radical induced MDA formation. LDL (1 mg cholesterol/ml) was pre-incubated in same cases with 0.5 mM trolox or 2.5 μM vitamin E for a period of 60 minutes at 37°C. The LDL was then oxidized with the Fe-ADP free radical generating system (see methods). Values are means ± S.E. of 3-10 different experiments (* $P<0.05$ versus no pre-incubation).

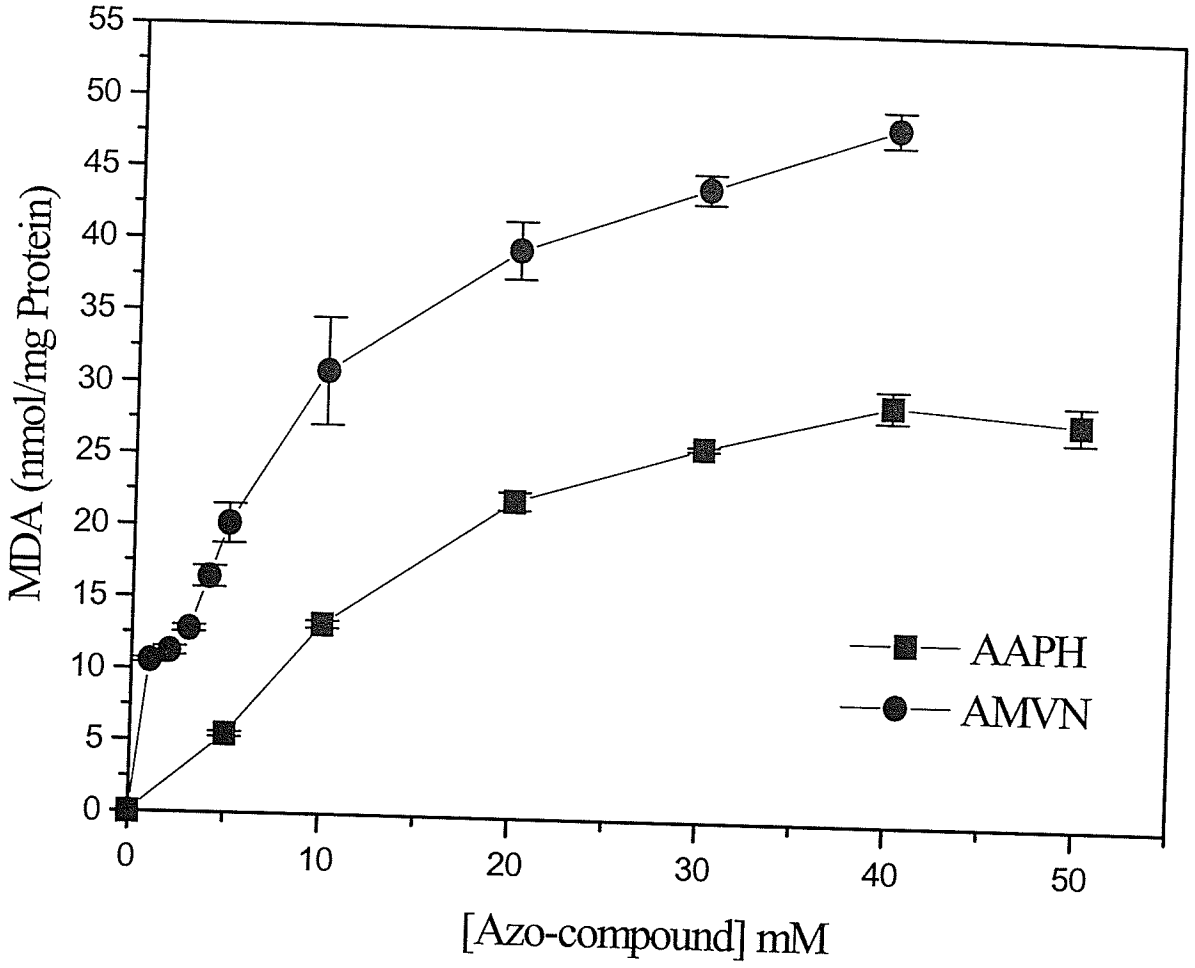


Figure 4. MDA formation as a function of incubation of LDL with varying concentrations of two different azo-compounds. LDL (1 mg cholesterol/ml) was incubated with different concentrations of AAPH and AMVN for 60 minutes at 37°C. Values are means ± S.E. of 3 different experiments (* $P<0.05$).

generated by AAPH (Figure 5). Lazaroid was less effective against peroxyl radicals generated by AMVN. One μmol/L lazaroid protected only 30% against AMVN-induced TBARS formation, and it never achieved more than ~50% inhibition (Figure 5). The IC_{50} values for lazaroid against AAPH and AMVN are 0.22 and 6.2 μM, respectively (Table 4).

In the presence of the water soluble peroxyl free radical generating system AAPH, α-tocopherol exhibited a similar protective effect as lazaroid (Figure 6). As low as 1 μmol/L α-tocopherol significantly reduced TBARS production. However, this action of α-tocopherol was stabilized at concentrations ≥ 7.5 μmol/L (Figure 6). Furthermore, α-tocopherol was far less potent when the free radicals were generated by AMVN. The IC_{50} values for α-tocopherol against AAPH and AMVN were 0.25 and 20 μmol/L, respectively (Table 4). In view of our previous findings (Figure 3), it was hypothesized the α-tocopherol may be more protective versus lipid peroxidation by AMVN if the antioxidant were pre-incubated with the LDL. However, although preincubation of the LDL with 5 or 10 μmol/L α-tocopherol for 24 hours prior to oxidation by AMVN did significantly decrease lipid peroxidation (Figure 6), the pre-incubation step still did not make α-tocopherol as potent against AMVN as it was against AAPH. The IC_{50} value for α-tocopherol after pre-incubation was reduced to 10.5 μmol/L.

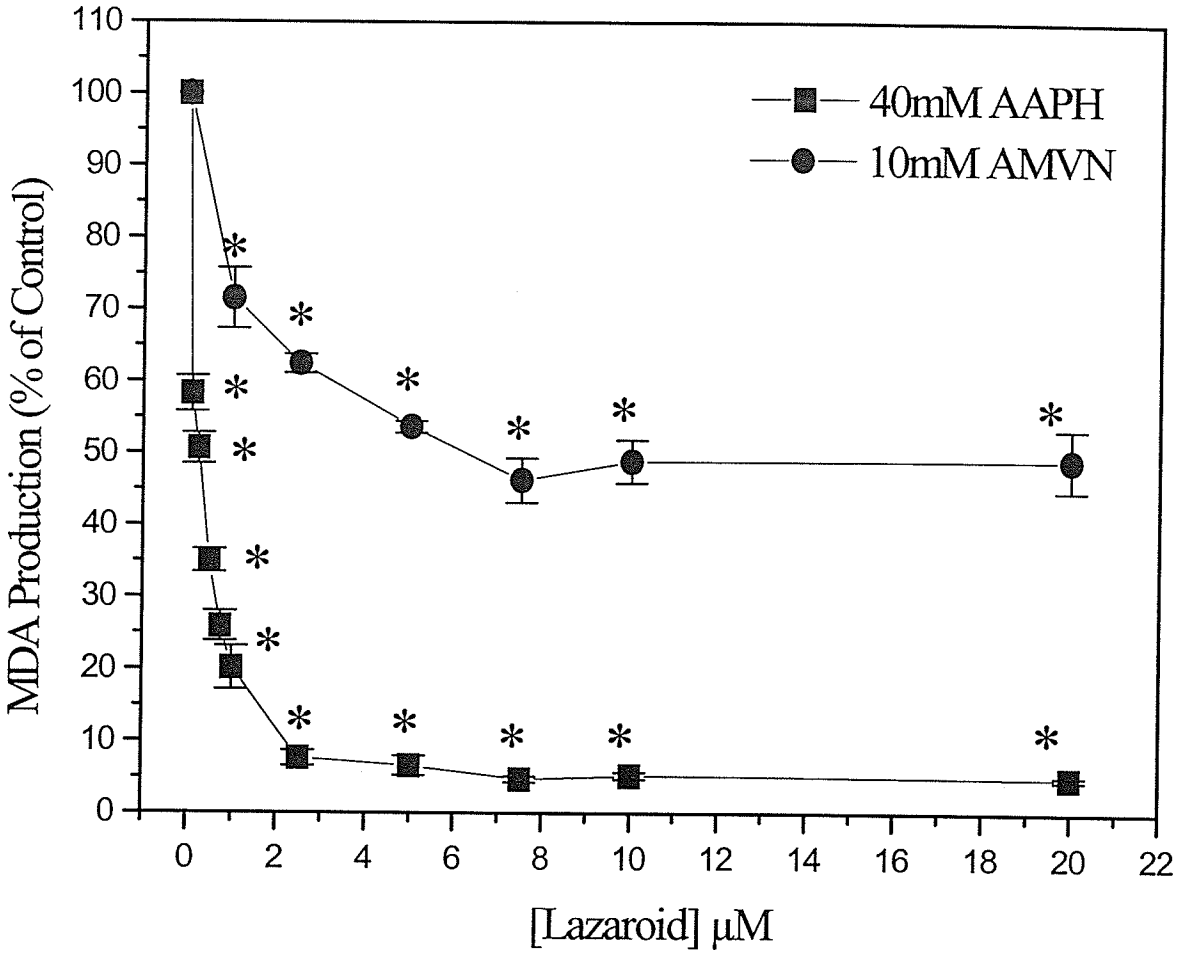


Figure 5. A comparison of the effect of lazaroid on AAPH or AMVN induced MDA production in LDL. LDL (1 mg cholesterol/ml) was incubated with different concentrations of peroxyl radical generating systems, 40 mM AAPH or 10 mM AMVN ± lazaroid for 60 minutes at 37°C. Absolute values for MDA content of oxLDL in the absence of lazaroid (i.e. control) were 42.9 ± 0.2 and 61.1 ± 0.2 nmoles/mg protein for AAPH and AMVN, respectively. Values are means ± S.E. of 3-7 different experiments (* $P<0.05$).

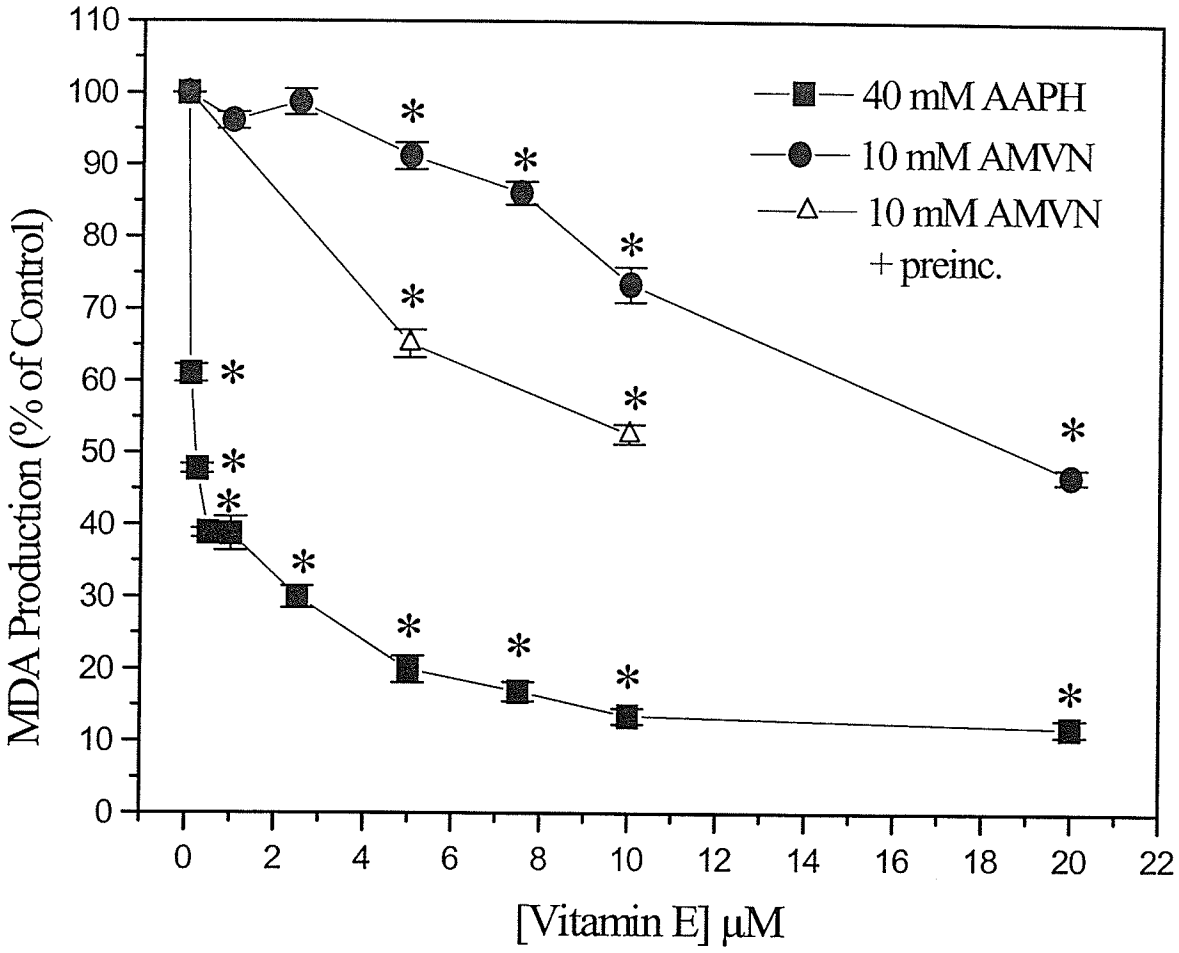


Figure 6. A comparison of the effect of vitamin E on AAPH or AMVN induced MDA production in LDL. LDL (1 mg cholesterol/ml) was incubated with 40 mM AAPH or 10 mM AMVN, for 60 minutes at 37°C ± different concentrations of vitamin E . In one case (Δ) LDL was pre-incubated for 24 hours with 5 or 10 μM vitamin E prior to oxidation by 10 mM AMVN. Absolute values for MDA content of oxLDL in the absence of vitamin E (i.e. control) were 44.4 ± 2.2 and 64.8 ± 1.1 nmoles/mg protein for AAPH and AMVN, respectively. Values are means ± S.E. of 3-7 different experiments (* $P<0.05$).

The protective effect of the water soluble antioxidant trolox was also examined against both AAPH or AMVN. As shown in Figure 7, trolox concentrations of up to 0.1 mmol/L significantly reduced MDA production against both AMVN and AAPH. However, the protective effect of trolox against the AMVN effects remained stable at concentrations ≥ 0.3 mmol/L. Conversely, increasing the trolox concentration to 0.3 mmol/L resulted in a further significant increase in its inhibitory capacity against AAPH induced peroxyl radical effects (Figure 7).

II. Characterization of Primary Smooth Muscle Cells Culture:

We used an explant technique to generate primary smooth muscle cells in culture from rabbit aorta. Smooth muscle cells started to migrate out of the explant within approximately 7 days (Figure 8). The first migration of cells from the explants contains more fibroblasts than smooth muscle cells (246). Therefore, the first migrations of cells from the explant were discarded. The cells from the second phase of migration were used in our experiments. Vascular smooth muscle cells obtained by this technique were homogeneous. These cultures are characterized by spindle shaped cells which form hills and valleys at a confluent stage as previously described (54). In order for smooth muscle cells to proliferate and migrate from the medial region of aorta to the culture dish, they undergo phenotypic modification. These cells change from contractile to synthetic cells. In order to cause differentiation, these cells were deprived from serum and

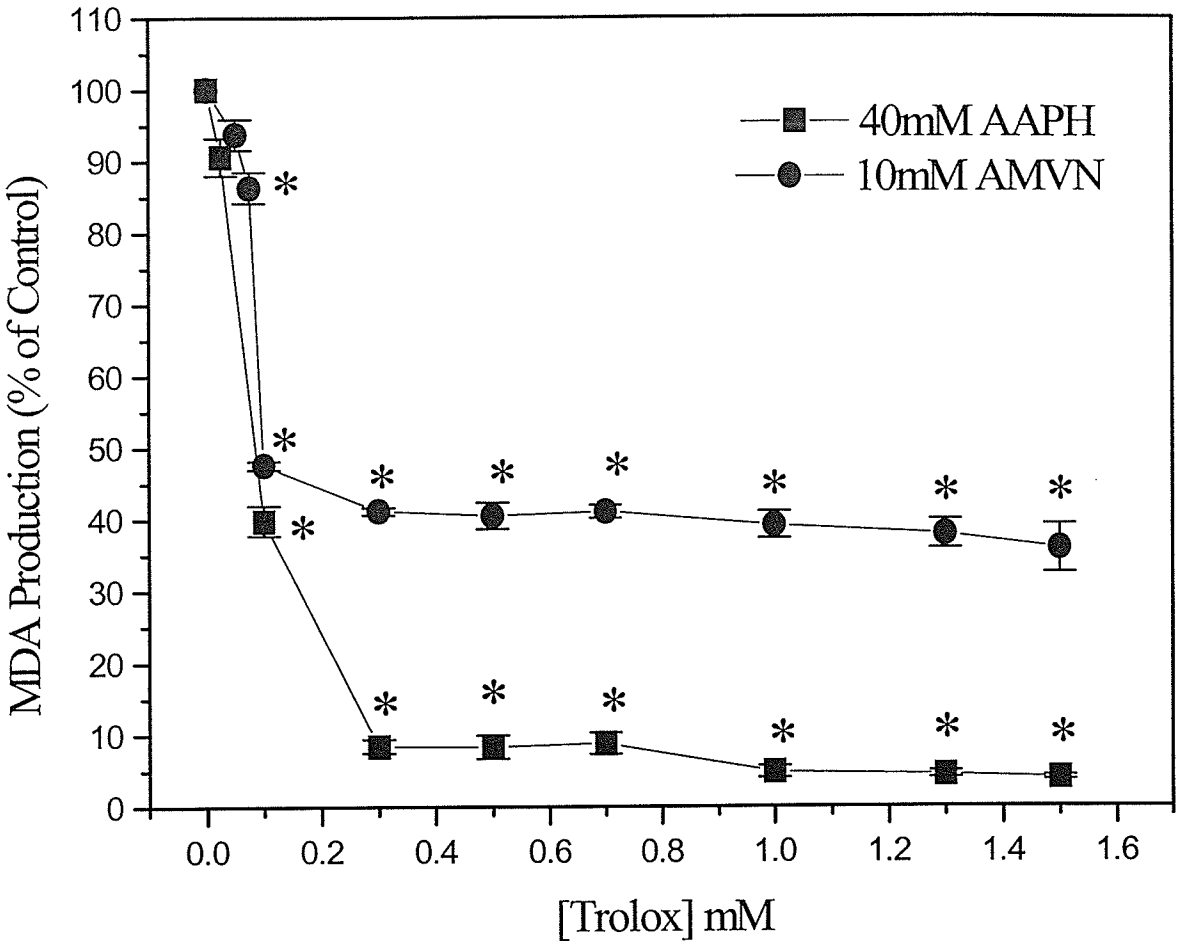


Figure 7. A comparison of the effect of trolox on AAPH or AMVN radical induced MDA production. LDL (1 mg cholesterol/ml) was incubated with different concentrations of trolox and a peroxyl radical generating system, 40 mM AAPH or 10 mM AMVN, for 60 minutes at 37°C. Absolute values for MDA content of oxLDL in the absence of trolox (i.e. control) were 40 ± 7.2 and 46.3 ± 4.4 nmole/mg protein for AAPH and AMVN, respectively. Values are means ± S.E. of 4-5 different experiments (* $P<0.05$).

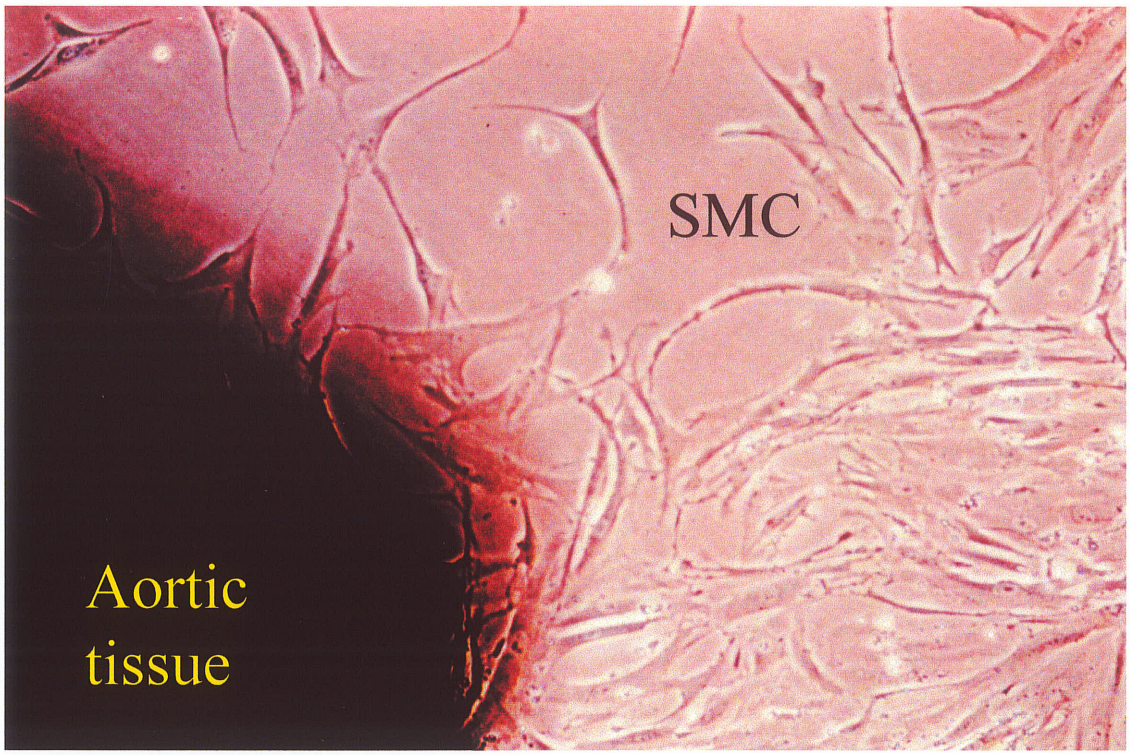


Figure 8. Proliferation and migration of VSMC out of aortic explant tissue. The thoracic aorta from a male New Zealand White rabbit was isolated and gently cleaned from connective tissue, excess fat and adherent blood cells from the adventitial surface. The aorta was cut into 2-3 mm sections and transferred to a culture dish with growth media (as described in the Methods). The first migration of cells was observed between days 6-8.

incubated in defined media necessary for phenotypic modification to a contractile state. Differentiated smooth muscle cells can be identified by using specific markers for cytoskeletal and cytocontractile proteins (88, 132).

Smooth muscle cells that were serum-starved for 6 days reacted strongly with smooth muscle specific α-actin, SM-myosin and caldesmon monoclonal antibodies (Figure 9). When these cells were also stained for their DNA content with Hoescht No. 33258, it is evident that all of the cells exhibited a positive reaction to both stains. This demonstrates the purity of the smooth muscle cell population employed in this study.

Functionally these smooth muscle cells responded to variety of agonists that stimulate cell surface receptors and lead to a release of intracellular calcium. These cells responded to norepinephrine (α-adrenergic receptor), ATP (purinergic receptor), histamine (H_1 receptor), endothelin-1 (ET-1 receptor), vasopressin, BayK 8644 (Ca^{2+} channel opener), methoxamine (α1 agonist), and ouabain (Na pump inhibitor).

III. Acute Exposure of VSMC to OxLDL

1. What is the mechanism responsible for the enhanced Ca^{2+} transient after acute exposure of VSMC to oxLDL?

LDL was oxidized with a 50 μmol/L $FeCl_3$ and 0.25 mmol/L ADP free radical generating system.(149, 316) Under our experimental conditions, LDL

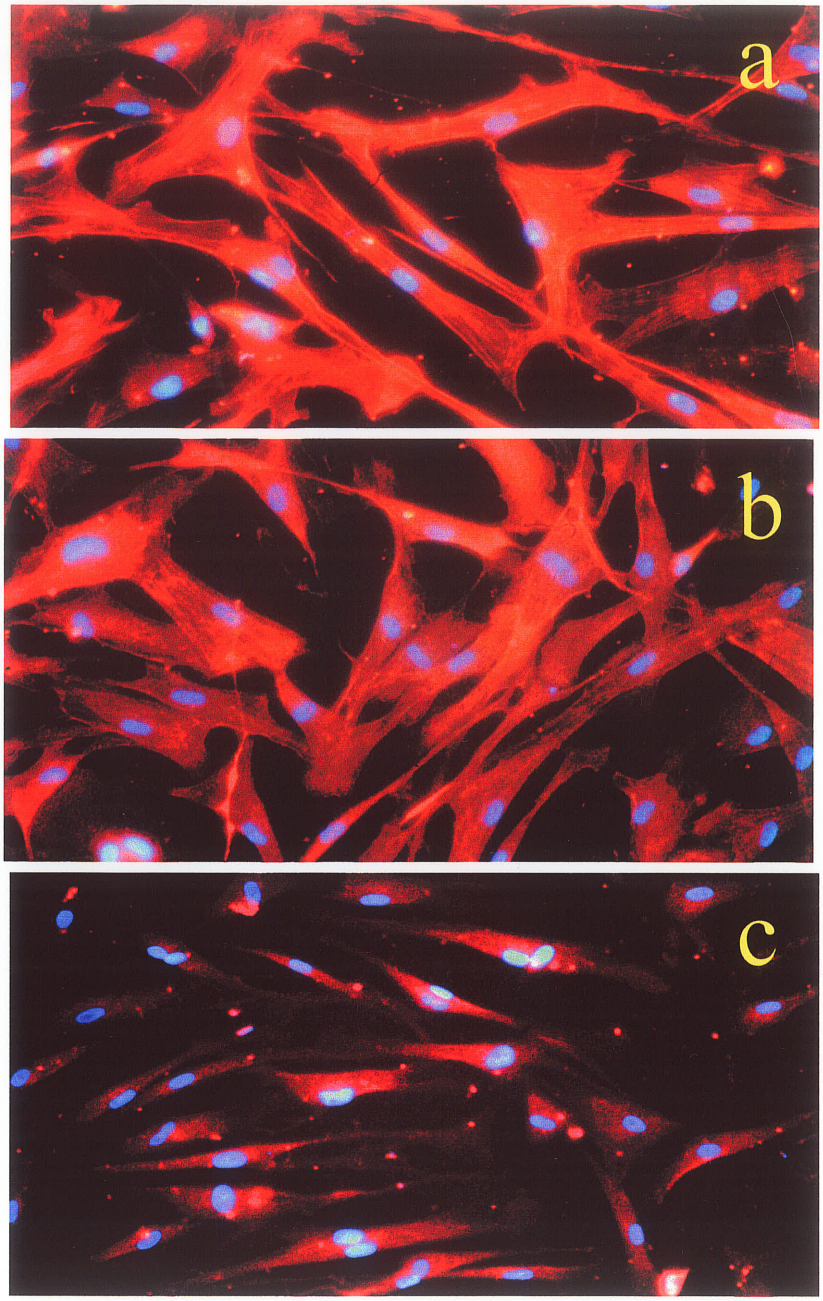


Figure 9. Immunocytochemical analysis of cell phenotype. Cells generated by explant technique were tested for smooth muscle cell homogeneity. Smooth muscle cells were identified by immunostaining for smooth muscle specific α-actin (a), myosin (b) and caldesmon (c). All the primary antibodies were monoclonal and Cy3-anti-mouse secondary was used as the secondary antibody. The nuclei were stained with Hoescht No. 33258.

was minimally oxidized. As shown in Figure 10, the migration of oxLDL on agarose gels was modest compared to native LDL or acetylated LDL. The migration of oxLDL depends on the extent of its oxidation. α-Tocopherol content was also depleted in oxLDL by a modest but significant amount (Figure 10). The oxidation reaction also induced a significant increase in TBARS production which is indicative of lipid peroxidation (Figure 10). However, the amount of TBARS production (8 nmol/mg) was far below values reported by others for oxidative modification of LDL with Cu^{2+} (~50 nmol/mg) (135).

We first examined the effects of a concentration of oxLDL frequently employed in other studies that have investigated the effects of oxLDL on cell Ca^{2+}(162). As shown in Figure 11, 0.1 mg/mL oxLDL caused a rapid increase in the intracellular calcium level. The peak increase in the cell calcium was observed within 5-10 seconds of exposure to oxLDL. This increase was due to oxidative modification of LDL and not due to a direct effect by the free radical generating system used. The Fe-ADP free radical generating system did not have any effect on intracellular calcium by itself. Native LDL elicited either no change or a modest increase in the Ca^{2+} transient in VSMC (data not shown). The effects of oxLDL on intracellular calcium could be eliminated by removing oxLDL from the solution bathing the smooth muscle cell (Figure 11A). The increase in $[Ca^{2+}]_i$ due to acute exposure to 0.1 mg/mL oxLDL was also observed in a calcium-free solution (Figure 11B). Therefore, the increase in intracellular

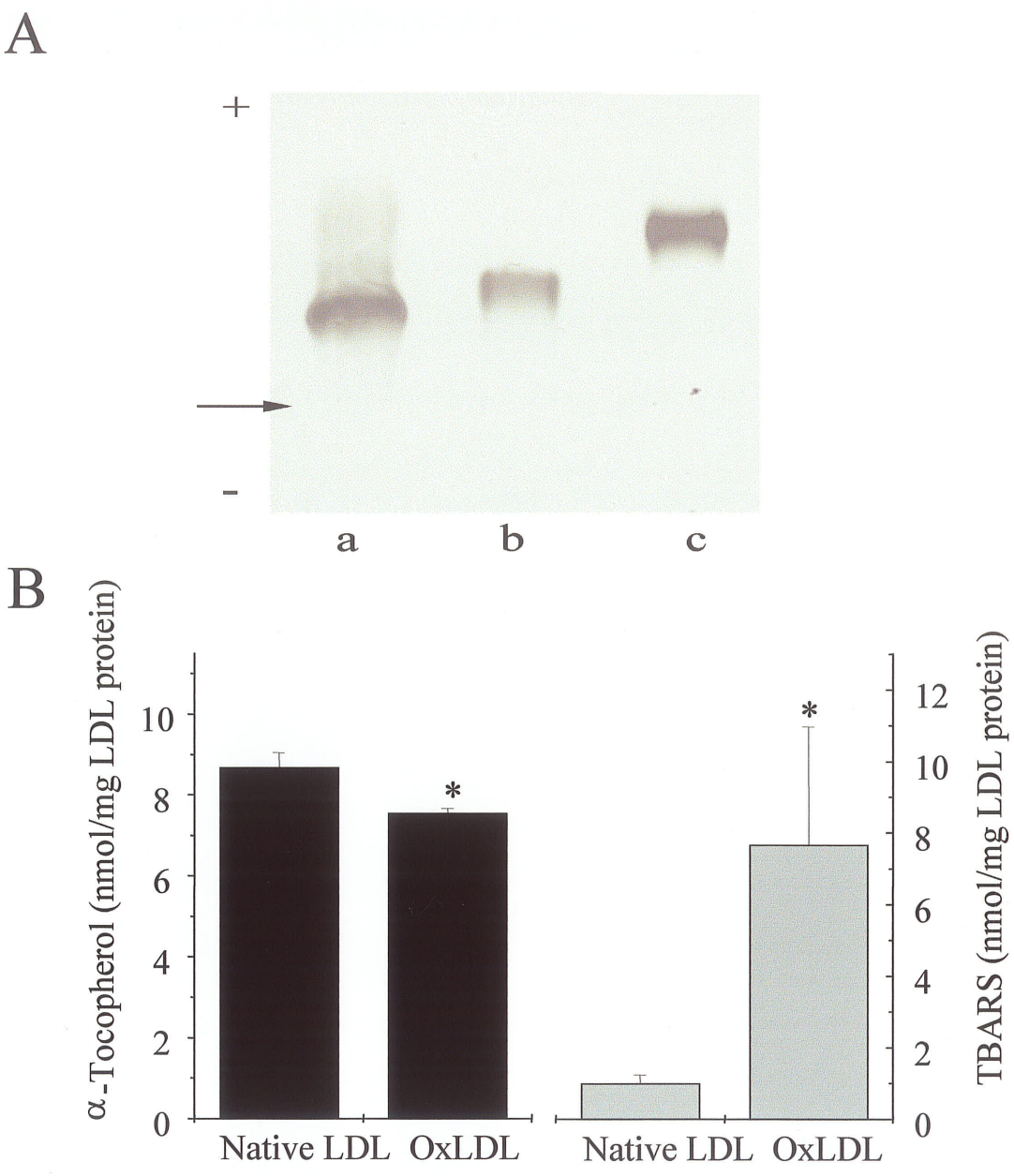


Figure 10. LDL oxidation by Fe-ADP. LDL was oxidized with a solution of 50 μM $FeCl_3$ and 0.25 mM ADP for 3 hours at 37°C. A) Electrophoretic mobility of oxidized LDL on agarose gel, a) native LDL, b) oxLDL, and c) acetylated LDL (arrow indicates the origin of gel). B) Alteration in α-tocopherol level (n = 4) and TBARS production (n = 8). * $P < 0.05$ versus control.

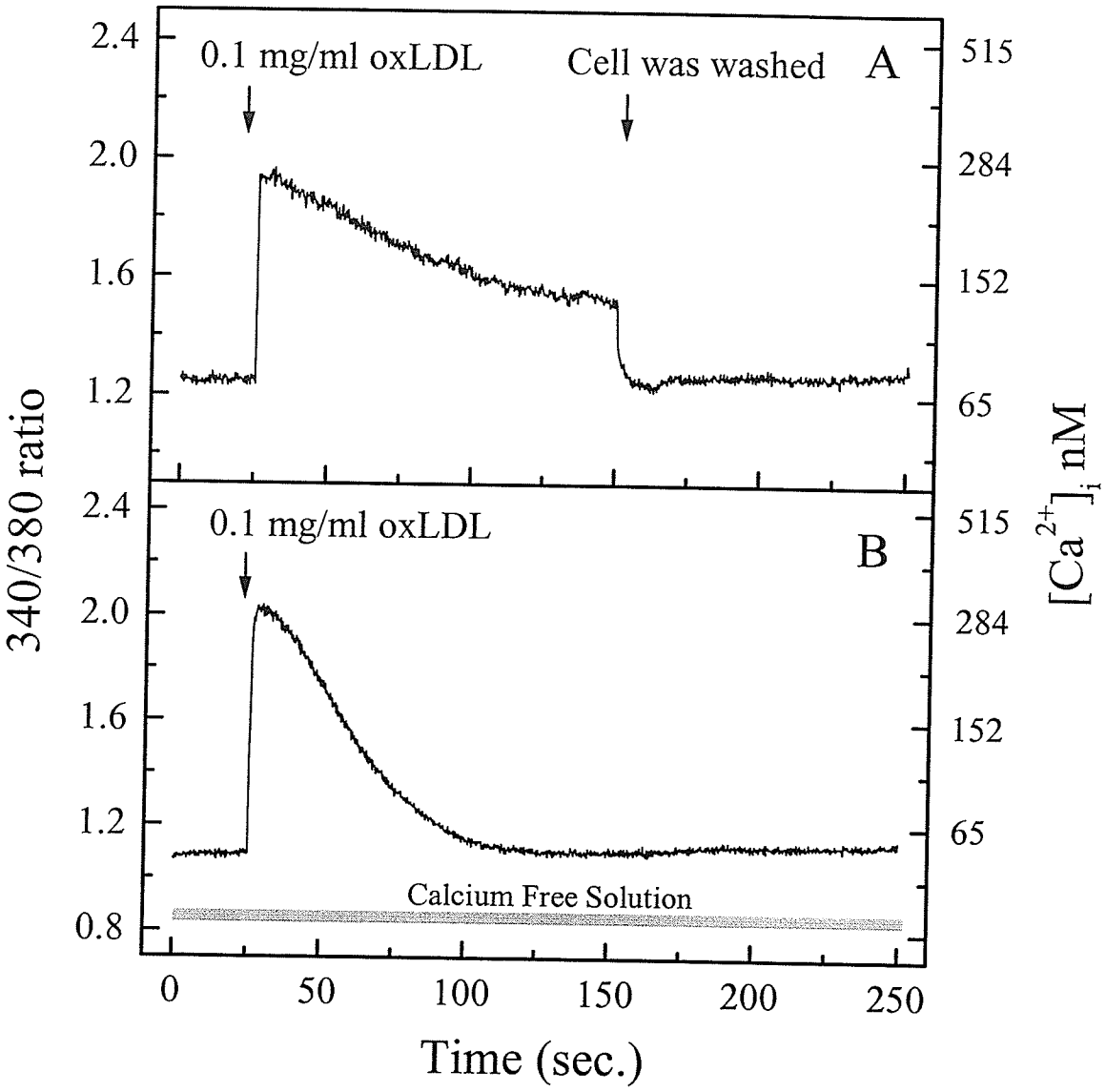


Figure 11. Representative results demonstrating the acute effect of oxLDL on Ca^{2+}_i in a single VSMC. A) OxLDL (0.1 mg cholesterol/mL) induced a rapid increase in Ca^{2+}_i. The resting calcium level was re-established by removing oxLDL from the solution bathing the cell. B) OxLDL (0.1 mg cholesterol/mL) induced a rapid increase in Ca^{2+}_i in a calcium free solution.

calcium induced by oxLDL likely resulted from a release of Ca^{2+} from the sarcoplasmic reticulum (SR).

Calcium release from the SR can occur via an IP_3 signaling mechanism (10). Therefore, oxLDL was tested for its capacity to increase VSMC IP_3 levels. The basal IP_3 concentration was approximately 20 pmol/mg protein. The IP_3 levels in VSMC acutely exposed to 0.1 mg/mL oxLDL for 20 seconds increased by approximately 5-fold (Figure 12). To further identify the potential signaling pathway through which oxLDL acted to cause a release of calcium from the SR, we used 2-nitro-4-carboxyphenyl N, N-diphenylcarbamate (NCDC) which disrupts the phosphatidylinositol cascade in the cell via an inhibition of phospholipase C (281). In 5 separate cell experiments, two cells exhibited a small, delayed response to oxLDL in the presence of NCDC (as shown in one representative experiment in Figure 13). NCDC-treated cells were completely unresponsive in 3 other cases. Incubation of VSMC with 50 μmol/mL NCDC blocked IP_3 formation even after stimulation with 0.1 mg/mL oxLDL (Figure 12).

2. What is the effect of oxLDL on nuclear calcium distribution?

We used confocal microscopy to observe spatial changes in intracellular and intranuclear calcium concentrations. The change in fluorescent intensity was observed in vascular smooth muscle cells using fluo-3 loaded cells (as described above). These cells were stimulated acutely with 0.1 mg cholesterol/mL oxLDL

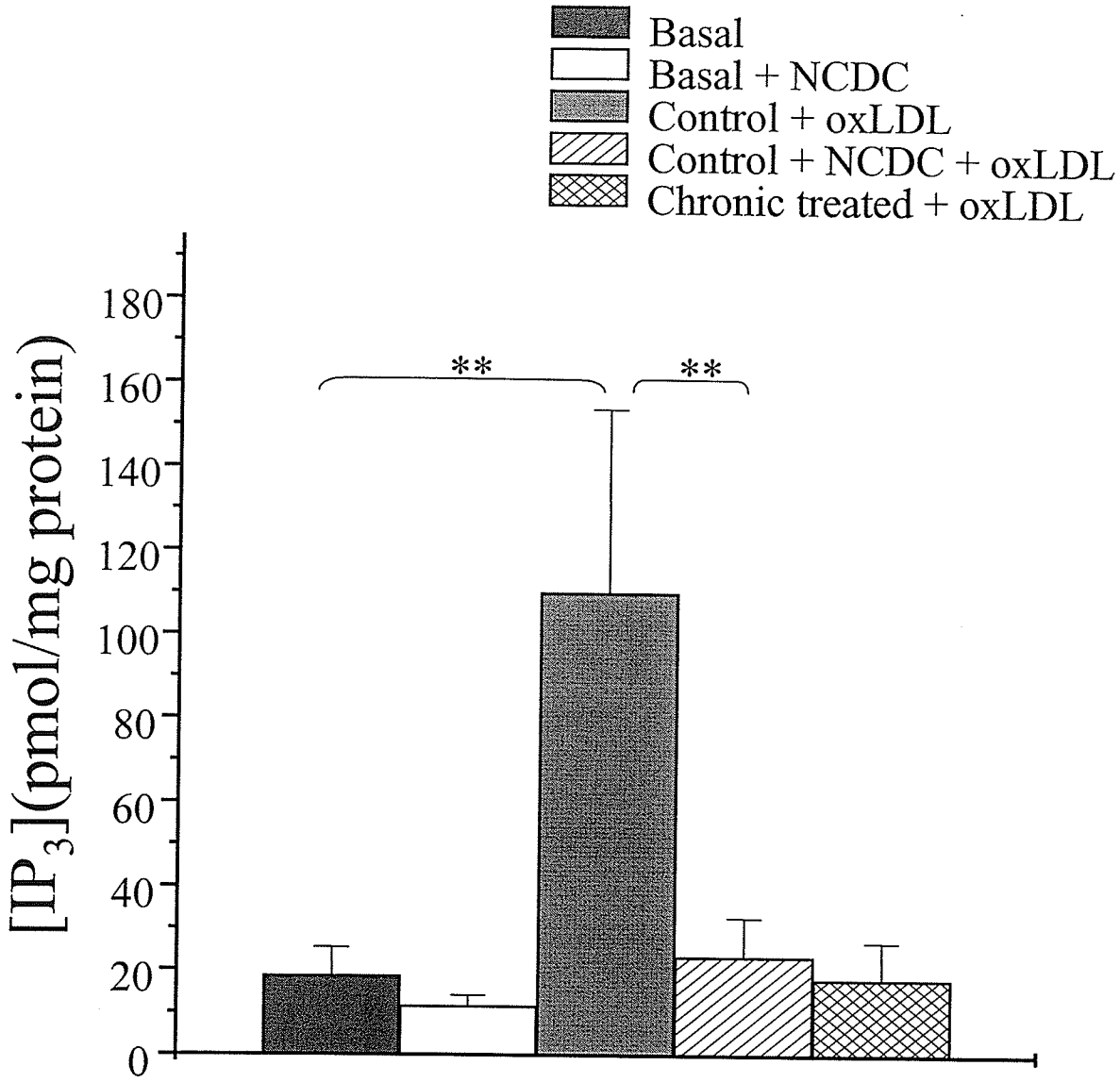


Figure 12. Effect of chronic exposure to oxLDL on intracellular IP_3. VSMC were treated ± 0.1 mg/mL oxLDL and in presence or absence of 50 μmol/mL NCDC (phospholipase C inhibitor) and IP_3 concentration was quantified 20 second after exposure. Cells that were chronically treated with oxLDL (0.025 mg/mL for 6 days) were then exposed to 0.1 mg/mL oxLDL and the IP_3 quantitated as above. All the IP_3 values were adjusted to mg protein content. The values presented are the mean ± S.E. of 3-6 different experiments. * $p < 0.05$ and ** $p < 0.001$ versus control.

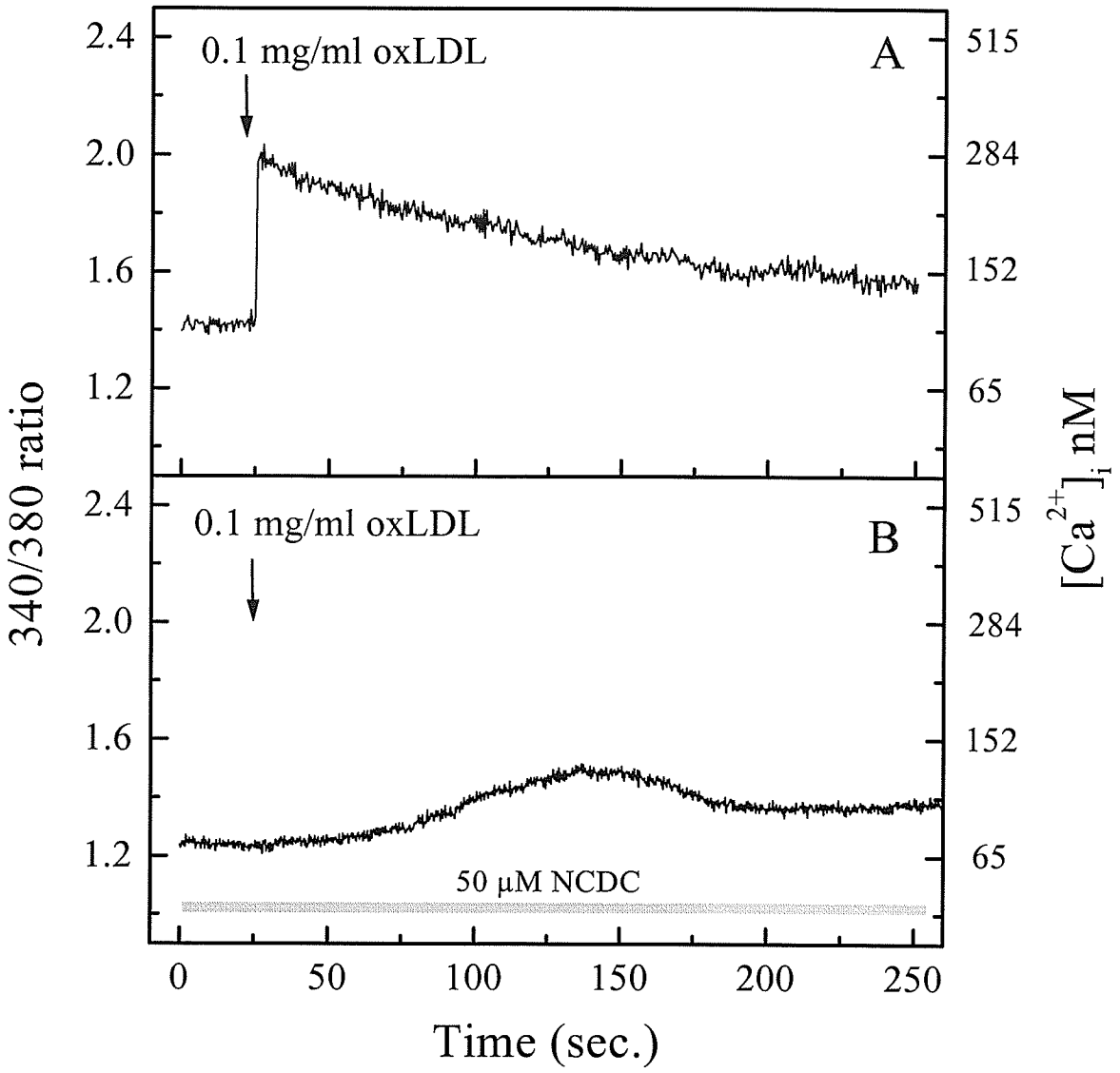


Figure 13. Blunting of the effect of oxLDL on VSMC Ca^{2+}_i by NCDC. A) Effect of 0.1 mg cholesterol/mL oxLDL on a single cell. B) VSMC were pre-treated with 50 μM NCDC for 30 minutes, then stimulated with 0.1 mg cholesterol/mL oxLDL. This is a representative recording from one cell.

and any change in intracellular fluo-3 intensity was registered (Figure 14). After stimulation with oxLDL, there was a rapid increase in the intracellular calcium level. There was a more pronounced change in intranuclear calcium in comparison to cytoplasmic calcium (Figure 14). Some investigators have suggested that the calcium indicator fluo-3 is not accurate for a comparison of cytoplasmic to nuclear calcium changes (206). Under similar calcium concentrations, pH, temperature, and dye concentration, fluo-3 exhibited higher nuclear to cytoplasmic fluorescent intensity in their experiments. The fluorescent intensity of fluo-3 in nuclei was altered independently of pH, temperature, and dye concentration (206). Therefore, we used indo-1 which demonstrated no significant change in nuclear to cytoplasmic fluorescent intensity under similar conditions (206). Indo-1 is a ratiometric calcium indicator that can be excited at 351 nm and its emission can be recorded at 405 and 480 nm. Thus, it is less susceptible to artifactual changes in fluorescence than a non-ratiometric dye like fluo-3. Vascular smooth muscle cells were loaded with indo-1 as described in the Methods, and stimulated with the addition of 0.1 mg cholesterol/mL oxLDL. Figure 15a, shows emission registered at 405 and 480 nm before and after exposure to 0.1 mg/mL oxLDL. Figure 15f, shows the 405/480 ratio values. At rest (Figure 15a and f), there is a very low free calcium level in the cytoplasm. Therefore, the fluorescence intensity at 405 nm is very low and at 480 nm is high. Upon stimulation with oxLDL, there was a rapid elevation in intracellular $[Ca^{2+}]$ which led to a shift in the emission intensity from 480 nm to

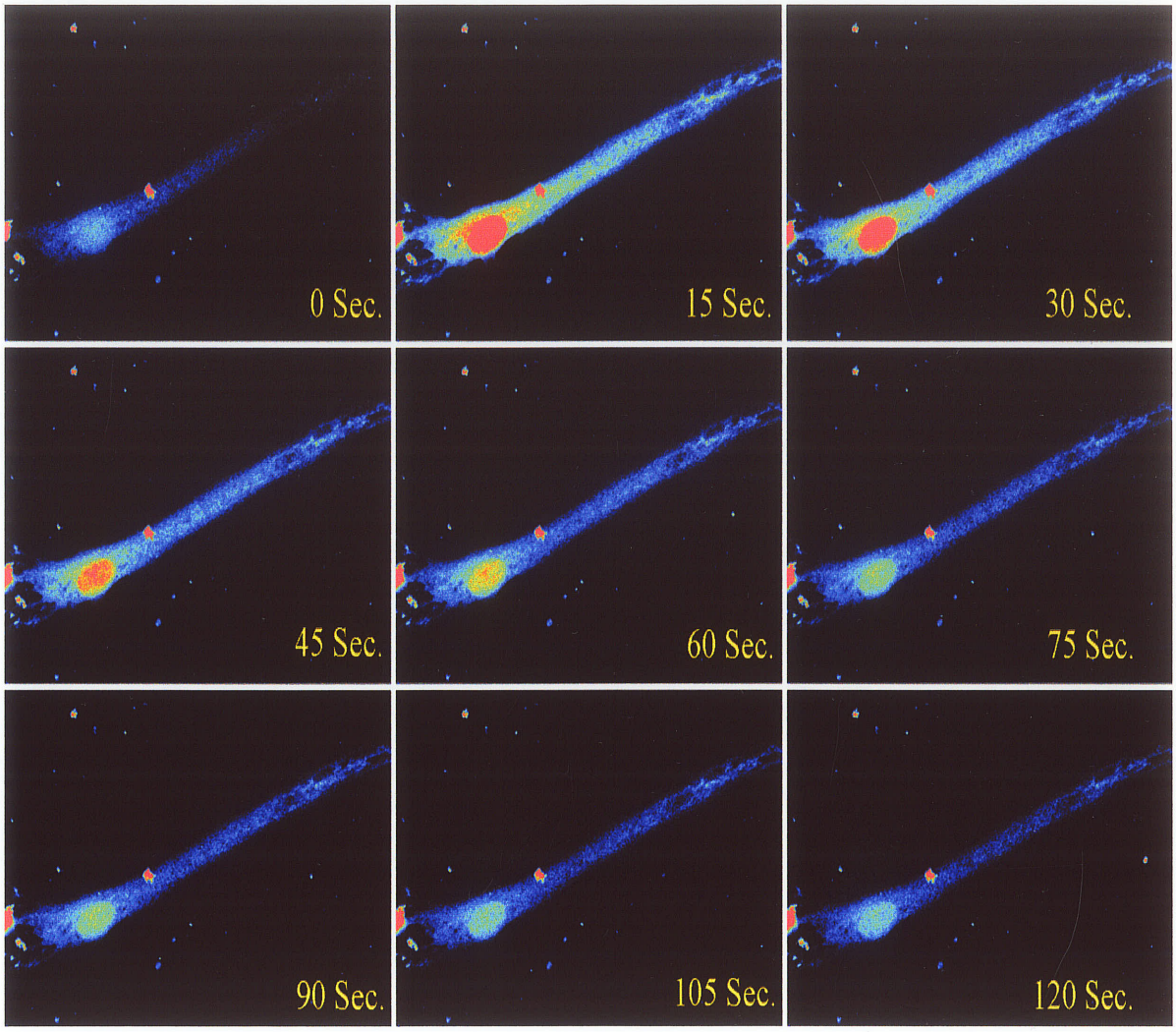


Figure 14. Effect of 0.1 mg/ml oxLDL on the intracellular calcium in fluo-3 loaded VSMC. Vascular smooth muscle cells were loaded with fluo-3 calcium indicator as described in the Methods. Rapid increase in intracellular Ca^{2+} in response to 0.1 mg/ml oxLDL is localized primarily to the nucleus. This is a representative data from 6 different experiments.

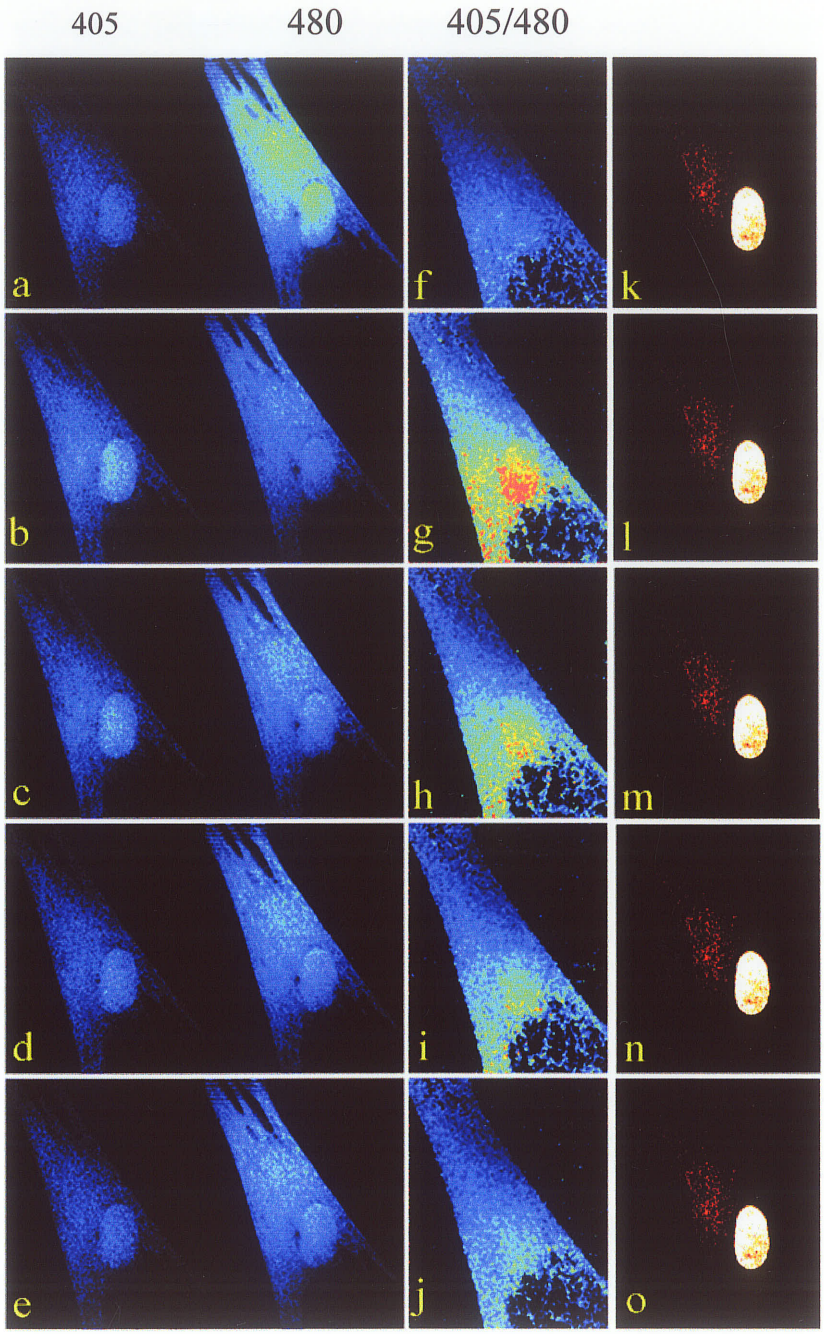


Figure 15. Effect of 0.1 mg/ml oxLDL on the intracellular calcium in indo-1 loaded VSMC. Vascular smooth muscle cells were loaded with indo-1 calcium indicator as described the Methods. VSMC at rest (a and f) and treated with 0.1 mg/ml oxLDL for 15 seconds (b and g), 45 seconds (c and h), 75 seconds (d and i) and 105 seconds (e and j). The nuclei were stained with 50 μg/ml Hoescht No. 33258 (k-o).

405 nm (Figure 15b). This resulted in an increase in fluorescence intensity of the 405/480 ratio (Figure 15g). There was a more pronounced increase in intranuclear calcium in comparison to cytoplasmic (Figure 15g). To identify the nuclear boundary, we used Hoescht No. 33258 to stain DNA. The extent of the increase in cell calcium is also demonstrated in a 3 dimensional calcium map (Figure 16). Again, the large rise in Ca^{2+} that is primarily localized to the nuclear region.

In order to show that oxLDL has the ability to release Ca^{2+} from non-SR calcium stores (i.e. nuclear calcium stores), SR Ca^{2+} was depleted in the smooth muscle cells after treatment with 1 μmol/L thapsigargin (Figure 17). Thapsigargin increases $[Ca^{2+}]_i$ by inhibiting the sequestration of Ca^{2+} through the SR Ca^{2+} pump (126, 155, 282). As shown in Figure 17, after the intracellular Ca^{2+} level plateaued, the cell was further stimulated with 0.1 mg cholesterol/mL oxLDL. OxLDL caused a further increase in intracellular $[Ca^{2+}]$ above that induced by thapsigargin (Figure 17). This indicates that oxLDL can induce the release of Ca^{2+} from other intracellular calcium stores in smooth muscle cells beside the SR.

3. Effect of oxLDL on intracellular calcium in freshly isolated VSMC:

The changes presented above represented the effects of oxLDL on Ca^{2+} homeostasis in cultured cells. To investigate if similar changes in intracellular calcium also appears in freshly isolated cells as a result of exposure to oxLDL,

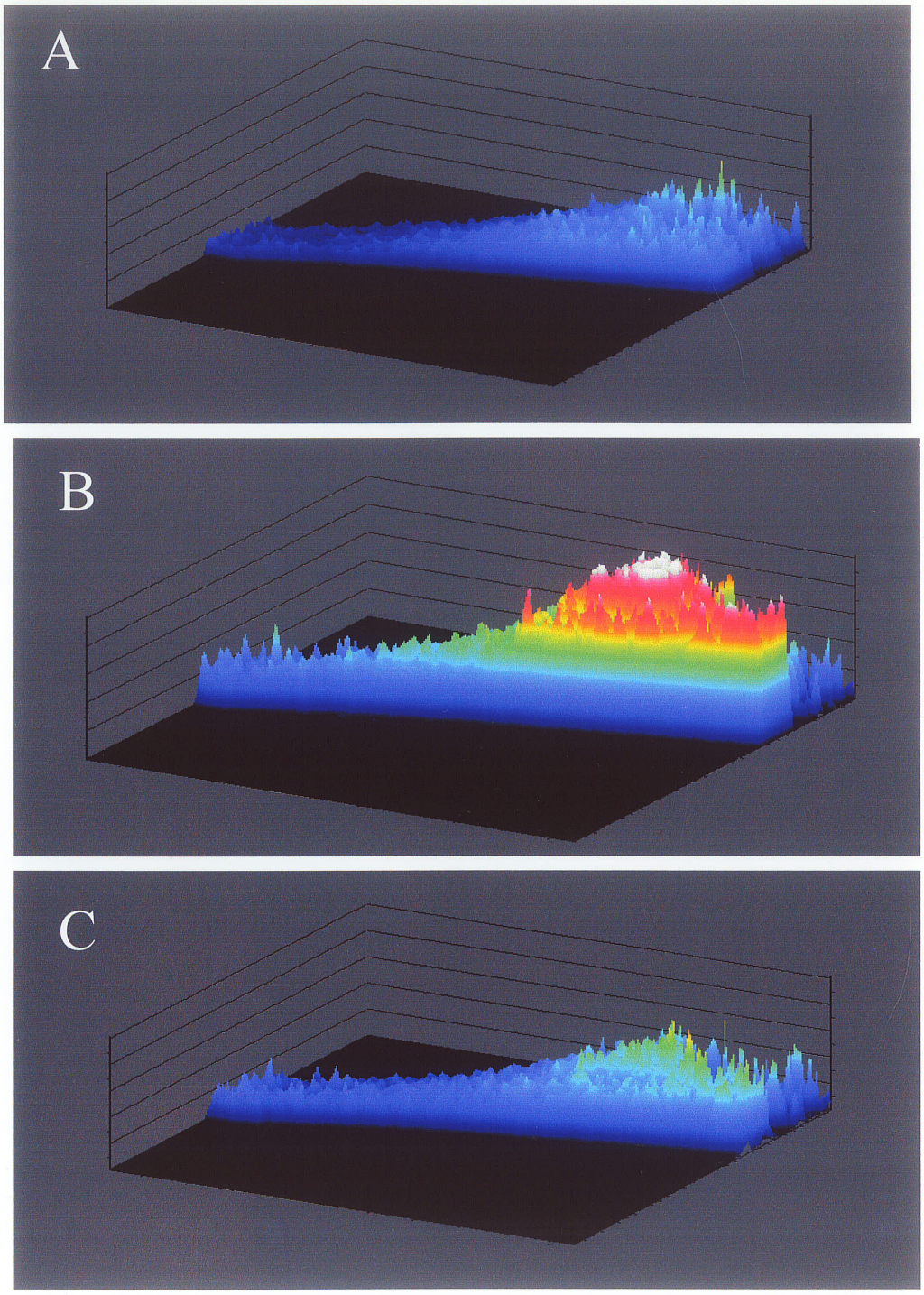


Figure 16. Effect of 0.1 mg cholesterol/ml oxLDL on the intracellular calcium distribution. OxLDL induced a rapid increase in intracellular free calcium. The cell was loaded with indo-1. A) at 0 time, B) 15 sec. after stimulation, and C) 2 min. after stimulation.

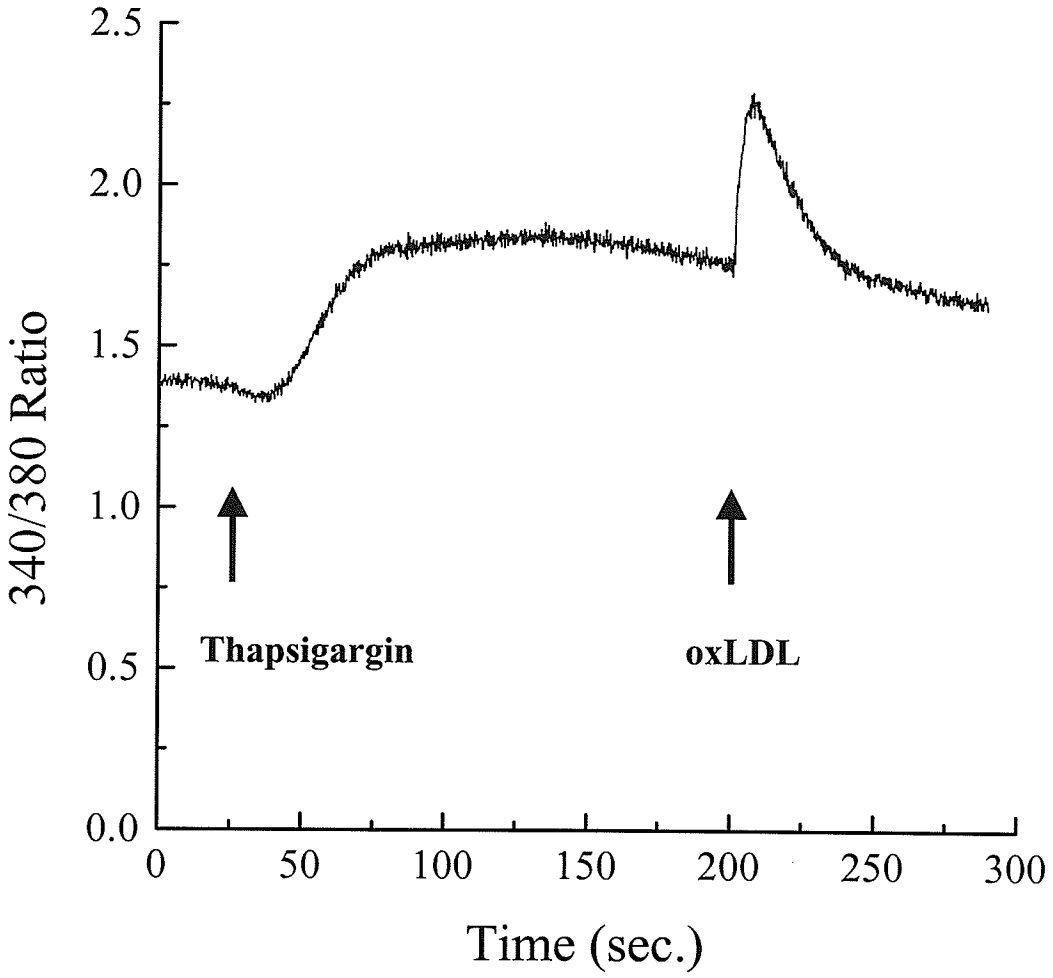


Figure 17. Effect of oxLDL on intracellular calcium after depletion of SR calcium store with thapsigargin. Vascular smooth muscle cells were loaded with fura-2 as described in the Methods. 1μmol/L thapsigargin was added to the cells as indicated by the arrow. After an immediate plateau in intracellular calcium was achived, 0.1 mg/ml oxLDL was added to the cell as indicated by the second arrow.

smooth muscle cells were isolated from portal vein. The isolated cells are long, spindle shaped and calcium tolerant (Figure 18). As shown in Figure 19, addition of 0.1 mg cholesterol/mL oxLDL to a freshly isolated cell resulted in no change in fluorescent ratio (340/380) of fura-2 (Figure 19). However, addition of 100 mmol/L KCl which depolarizes smooth muscle cells caused an immediate increase in intracellular [Ca^{2+}] (Figure 19). There was no change in the distribution of intracellular calcium as a function of exposure to oxLDL as observed by confocal microscope in the cells loaded with fluo-3. Addition of KCl, however, again caused a uniform increase in intracellular calcium and shortening of smooth muscle cells (Figure 20).

IV. Chronic exposure of VSMC to oxLDL

1. Lipid deposits in VSMC chronically treated with oxLDL:

Incubation of smooth muscle cells with oxLDL will result in uptake and accumulation of lipids in these cells. Uptake of oxLDL through scavenger receptors will result in formation of foam cells. Scavenger receptors are present on smooth muscle cells (66, 209, 210). In contrast to receptors for native LDL, the expression of scavenger receptors is not down-regulated (94).

As shown in Figure 21, there is an increase in fluorescent intensity in the smooth muscle cells chronically treated with 0.025 mg/mL oxLDL for 6 days. Nile red will stain both neutral (cholesterol ester) and charged lipids (phospholipids). Charged lipids will exhibit a red fluorescence whereas neutral

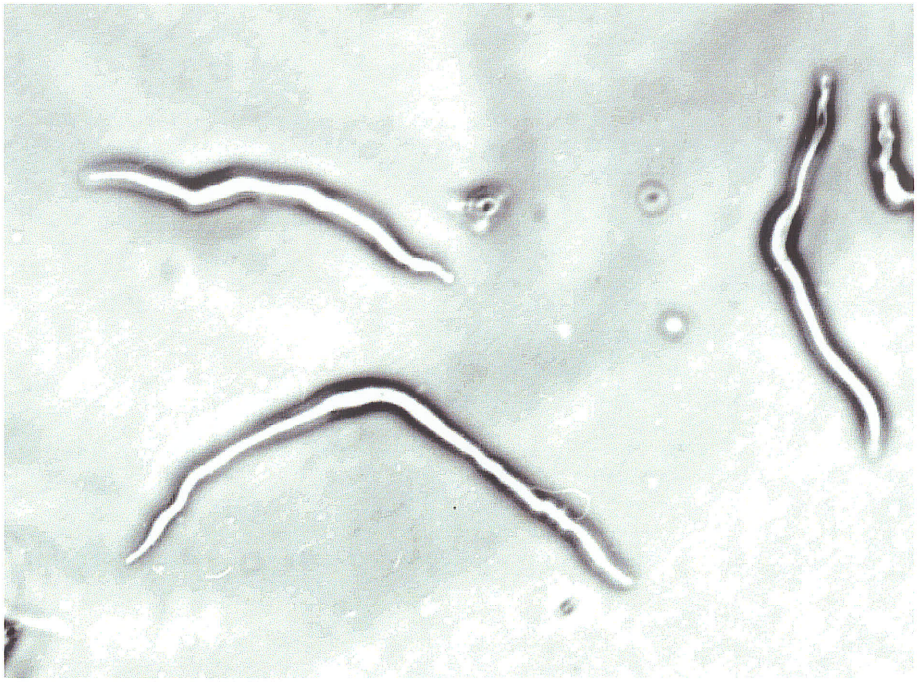


Figure 18. Photograph of freshly isolated portal vein smooth muscle cells. The freshly isolated calcium-tolerant cells were obtained by enzymatic digestion as describe in the Methods.

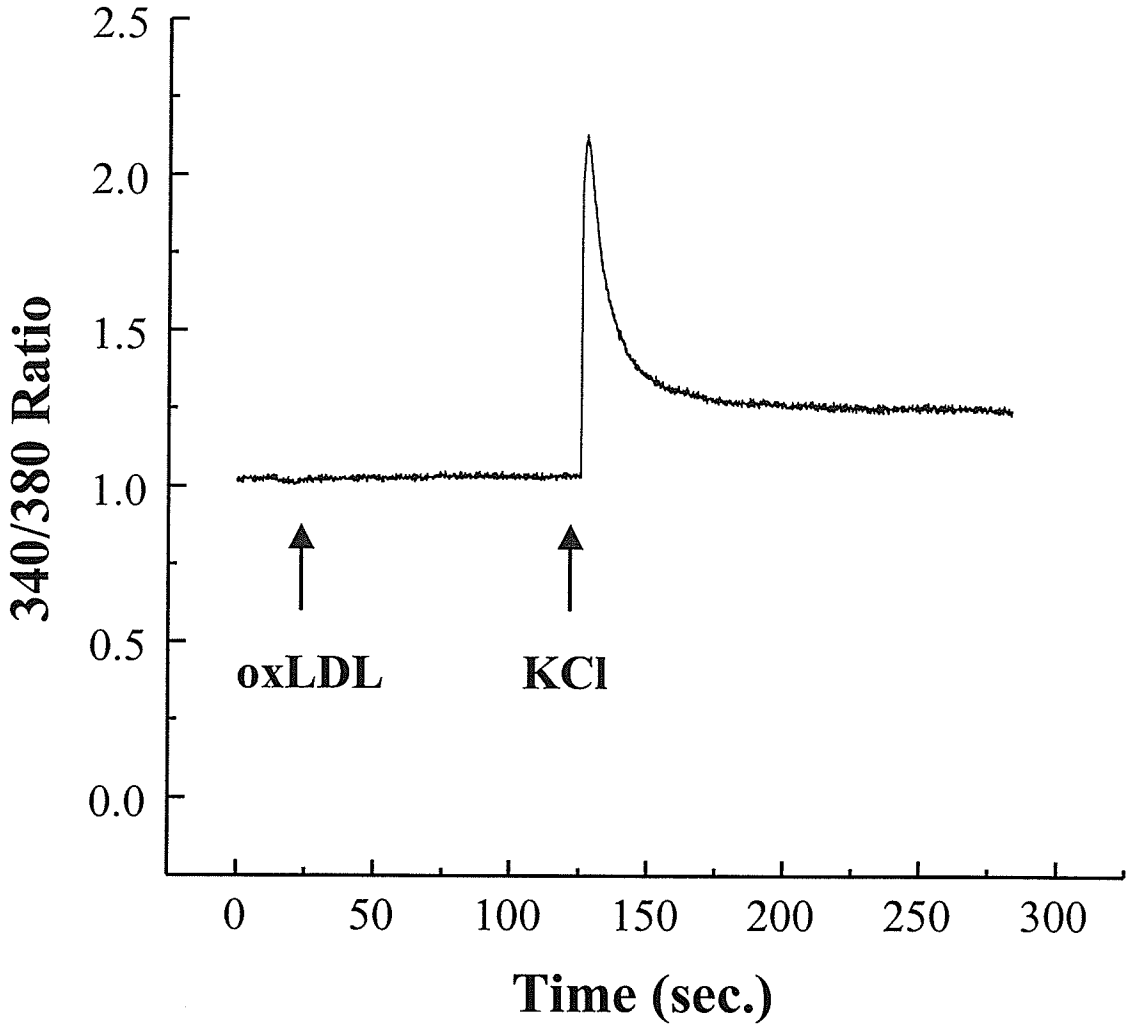


Figure 19. Effect of oxLDL on intracellular calcium in freshly isolated portal vein smooth muscle cells. Portal vein smooth muscle cells were isolated as described in the Methods. These cells were loaded with fura-2 and stimulated with 0.1 mg/ml oxLDL as indicated by the arrow. The cell were further stimulated with 50 mmol/L KCl as indicated by the second arrow.

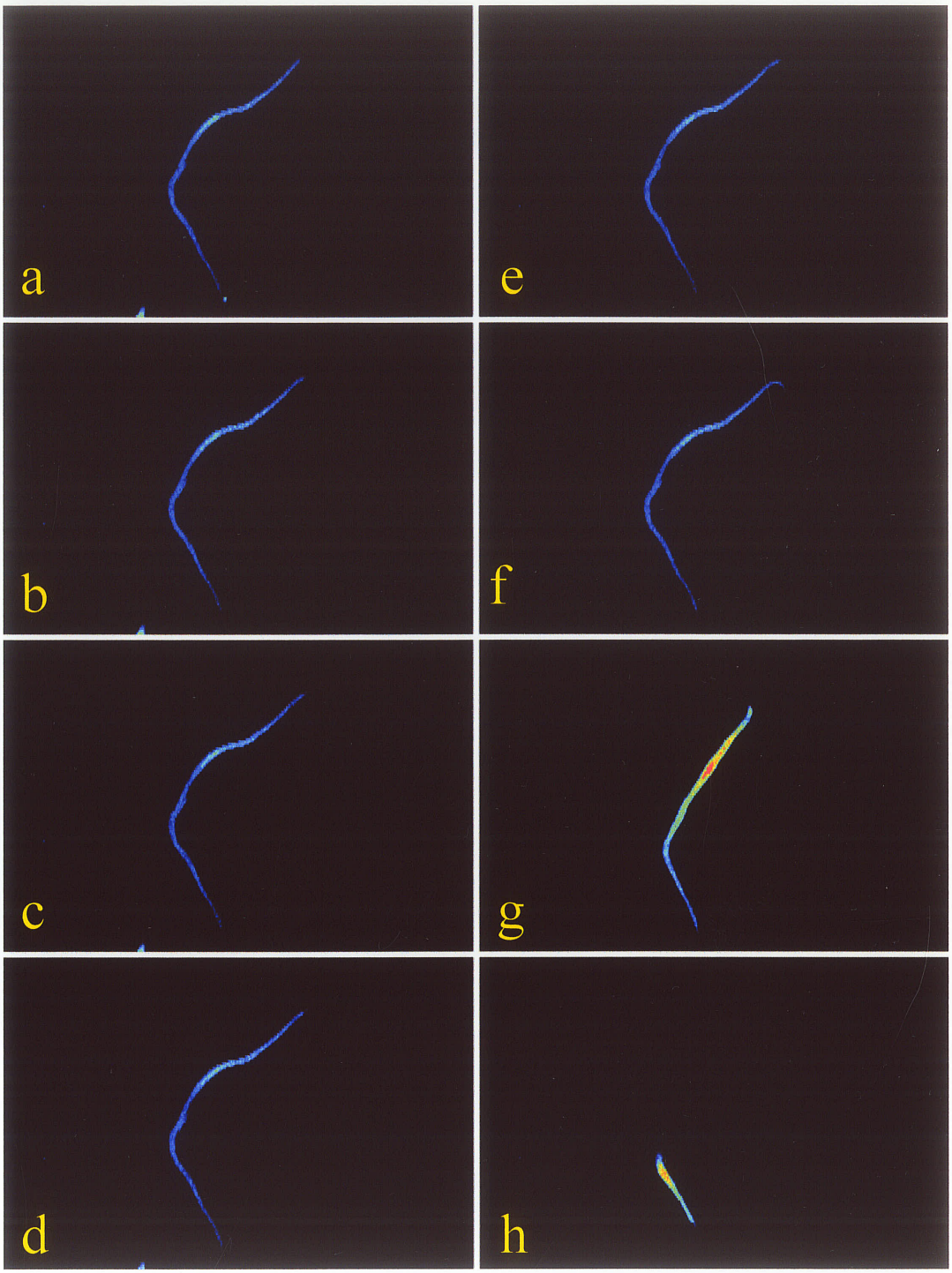


Figure 20. Effect of oxLDL on intracellular calcium distribution in freshly isolated cells. Portal vein smooth muscle cells were isolated and loaded with fluo-3 as described in the Methods. All the images are recorded in 15 second intervals. a) smooth muscle cells at rest, b to e) treatment with 0.1 mg/ml oxLDL, f-h) treatment with 50 mmol/L KCl. This series of images is representative of 4 different experiments.

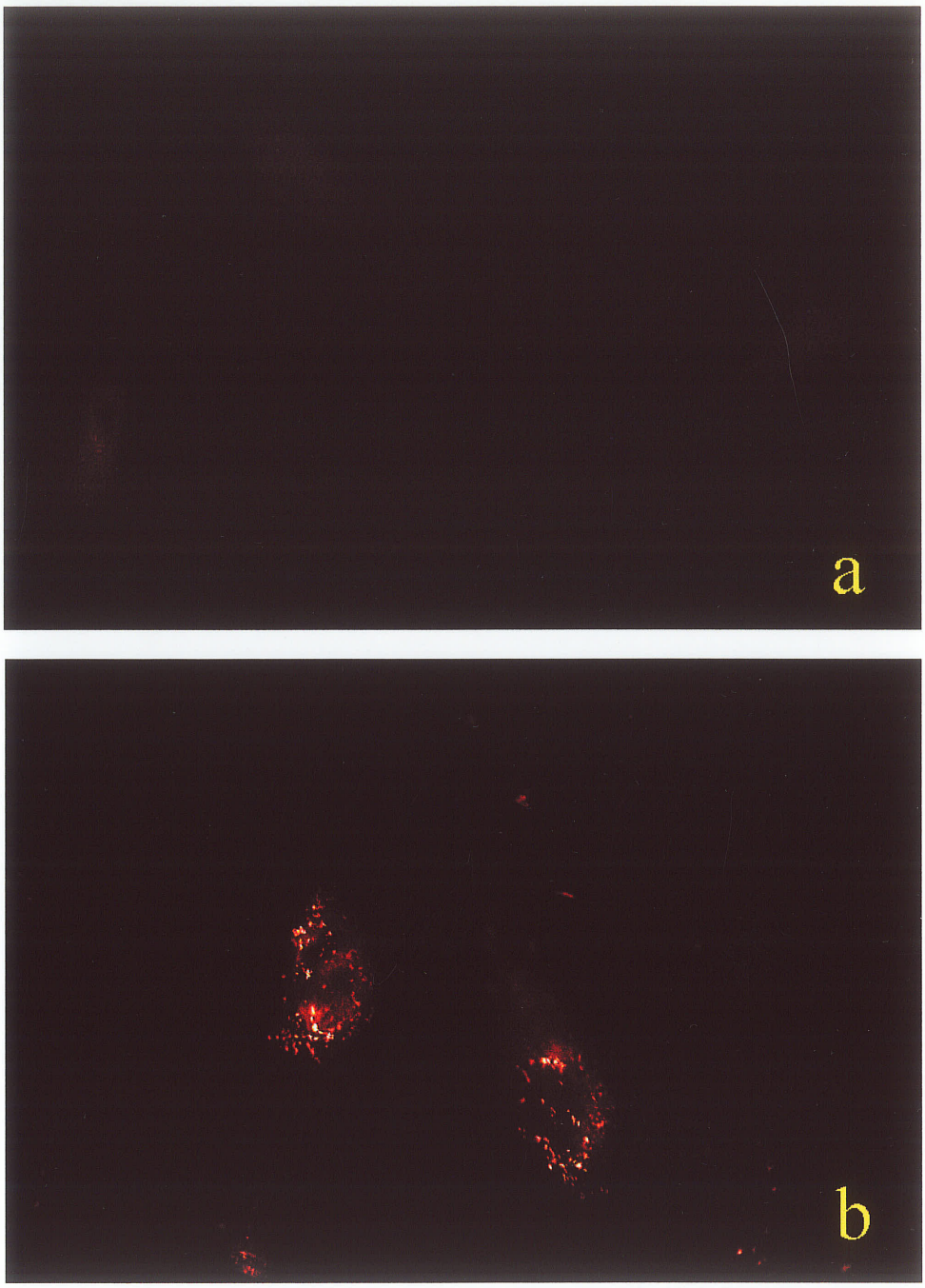


Figure 21. Nile red staining of lipid droplets in cells chronically treated with oxLDL. Vascular smooth muscle cells were treated ± 0.025 mg/ml oxLDL for 6 days. These cells were stained with Nile red as described in the Methods. These images were obtained with confocal microscopy and show an intense accumulation of lipid droplets in chronically treated cells.

lipids fluoresce with a yellow/gold tone (80, 100). In control cells there was a very faint staining of phospholipids in a uniform manner throughout the cell. However, in foam cells there was a strong staining of lipid droplets in the cytoplasm. Formation of extensive lipid droplets in cells in culture after extended exposure to oxLDL was also demonstrated in electron micrographs (Figure 22).

2. What effect does chronic exposure of VSMC to oxLDL have on cell integrity?

Although cultured vascular smooth muscle cells were acutely responsive to oxLDL, these cells may have a different response after extended exposure to oxLDL in culture. Vascular smooth muscle cells were treated for up to 6 days with concentrations that were up to 100 fold lower than those previously tested in the acute experiments. The medium was changed daily. This media contained freshly prepared oxLDL. Control cells were maintained for the same amount of time in an identical media except for the addition of oxLDL.

OxLDL has a cytotoxic effect on cultured cells (174). Therefore, it was important to assess the effect of the concentrations of oxLDL that were used in our experiments on the viability of smooth muscle cells. The viability of vascular smooth muscle cells was tested using the Live/Dead assay. In this test, esterase activity in live cells cleaves the ester group from calcein-AM to generate a green fluorescence. Cells with compromised membrane integrity will allow infiltration of the ethidium homodimer to stain the nuclear contents with a bright red

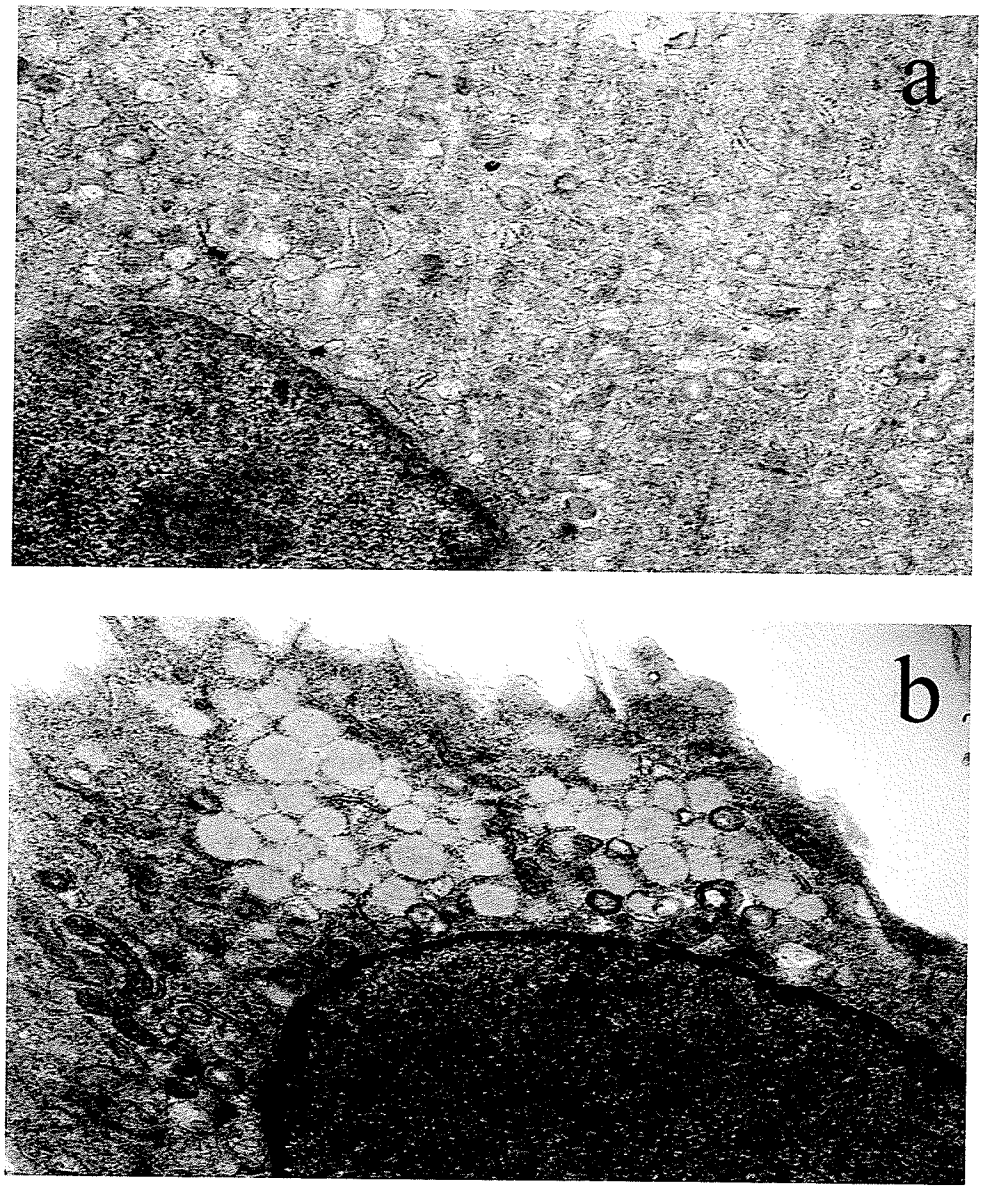


Figure 22. Electromicrograph of control and cells chronically treated with oxLDL. Vascular smooth muscle cells were treated ± 0.025 mg/ml oxLDL for 6 days. These cells were fixed with tannic acid and sectioned horizontally to the bottom of the dish. a) control cells and b) cells treated with 0.025 oxLDL for 6 days.

fluorescence. As shown in Figure 23, the cells treated with 0.005 and 0.025 mg/mL oxLDL for 6 days did not exhibit a red nuclear stain with the ethidium homodimer. This supports the absence of a cytotoxic effect in the oxLDL concentration range used in this study. In order to confirm this observation, we also examined lactate dehydrogenase (LDH) release from the cells. Incubation of cells with 0.025 mg/mL oxLDL over a 6 day period did not induce a significant release of LDH into the media in comparison to control untreated cells (Figure 24). Subsequent experiments, therefore, were undertaken at oxLDL concentrations $\leq$ 0.025 mg/mL.

In order to study the effects of oxLDL on cytoskeletal proteins we used higher concentrations of oxLDL since lower concentrations of oxLDL had very minor effects. The control cells were 99.8 $\pm$ 0.1 % viable (n=1357 cells counted) (Figure 25). Smooth muscle cells treated chronically for 3 days with 0.05 and 0.1 mg cholesterol/mL oxLDL were 96 $\pm$ 1 % (1321 cells counted) and 95 $\pm$ 1 % alive (2828 cells counted) (Figure 25). These data were not affected by necrotic cells lifting free from the culture surface. Cell numbers were unchanged as a function of treatment with 0.05 mg/mL oxLDL over three days (56.5 $\pm$ 6.8 cells were counted per field in untreated preparations as compared to 55.0 $\pm$ 4.0 cells in the experimental group (n>1300 cells counted in total in each group).

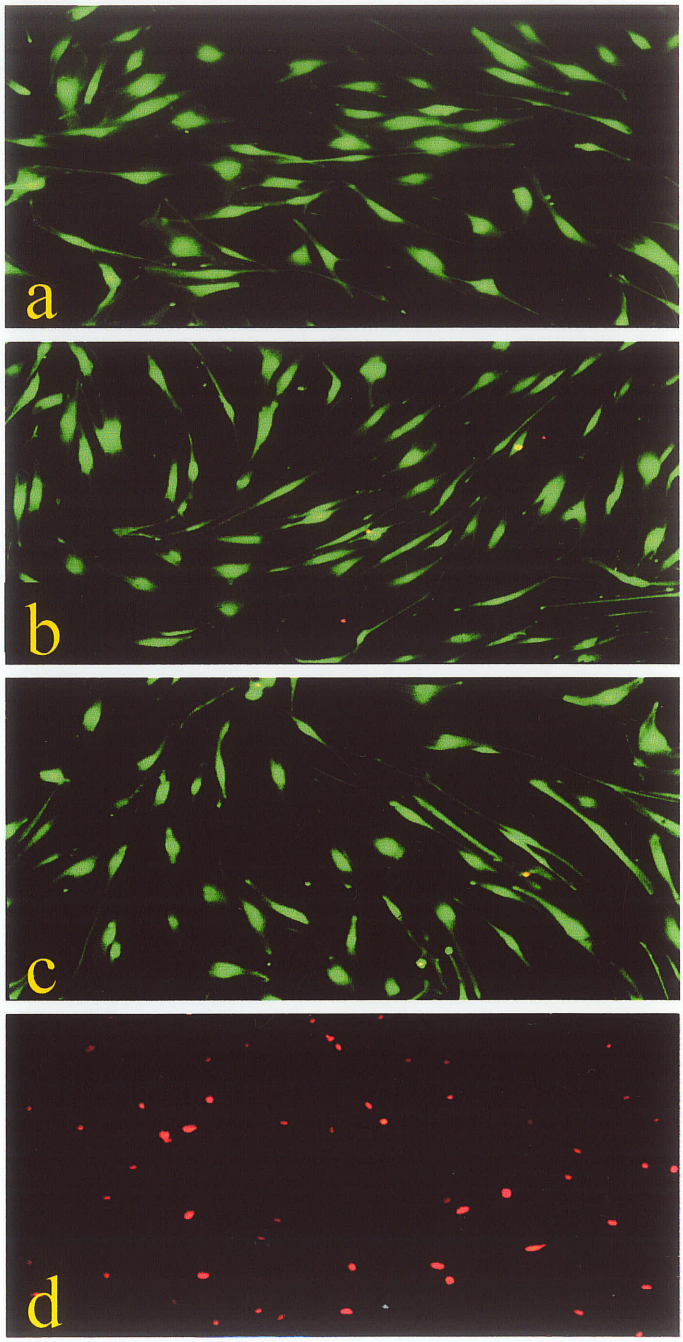


Figure 23 Effect of chronic exposure to oxLDL on viability of the VSMC. Vascular smooth muscle cells stained for live and dead cells after 6 days treatment with oxLDL. a) Control Cells, the green fluorescence is due to cleavage of ester in calcine-AM indicating healthy cells. b) Cells treated with 0.005 mg/ml oxLDL, c) cells treated with 0.025 mg/ml oxLDL, and d) dead cells, membrane was permeablized by digitonin. Note, the red nuclei fluorescence indicate cytotoxic effect of oxLDL.

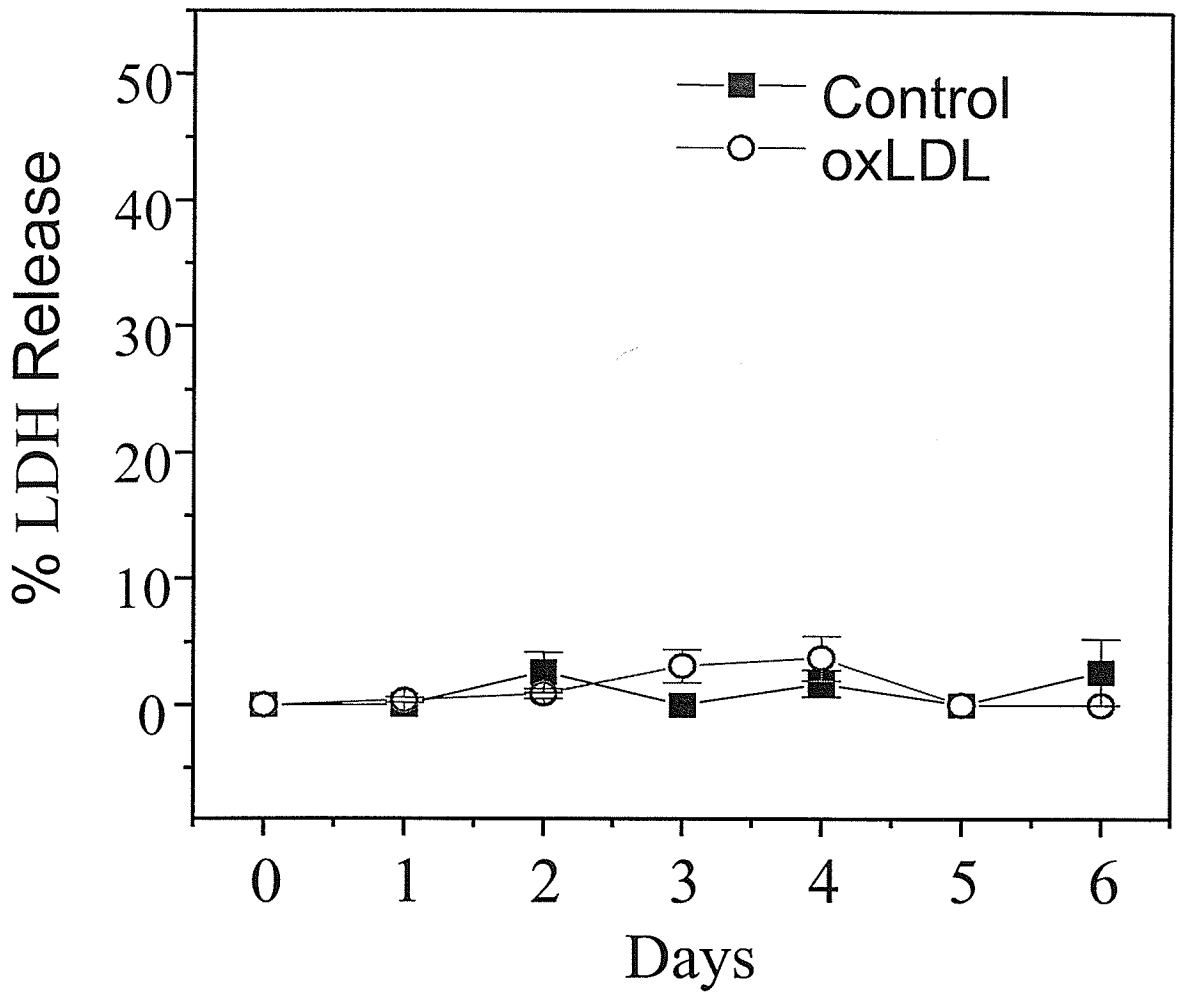


Figure 24. Effect of chronic exposure of VSMC to oxLDL on cellular lactate dehydrogenase (LDH) release. Smooth muscle cells were incubated with no oxLDL (■), 0.025 mg/ml oxLDL (○) for 6 days. The data depict the LDH released as a percent of total LDH present in the cells. The data presented are a mean ± S.E. of 5 separate experiments.

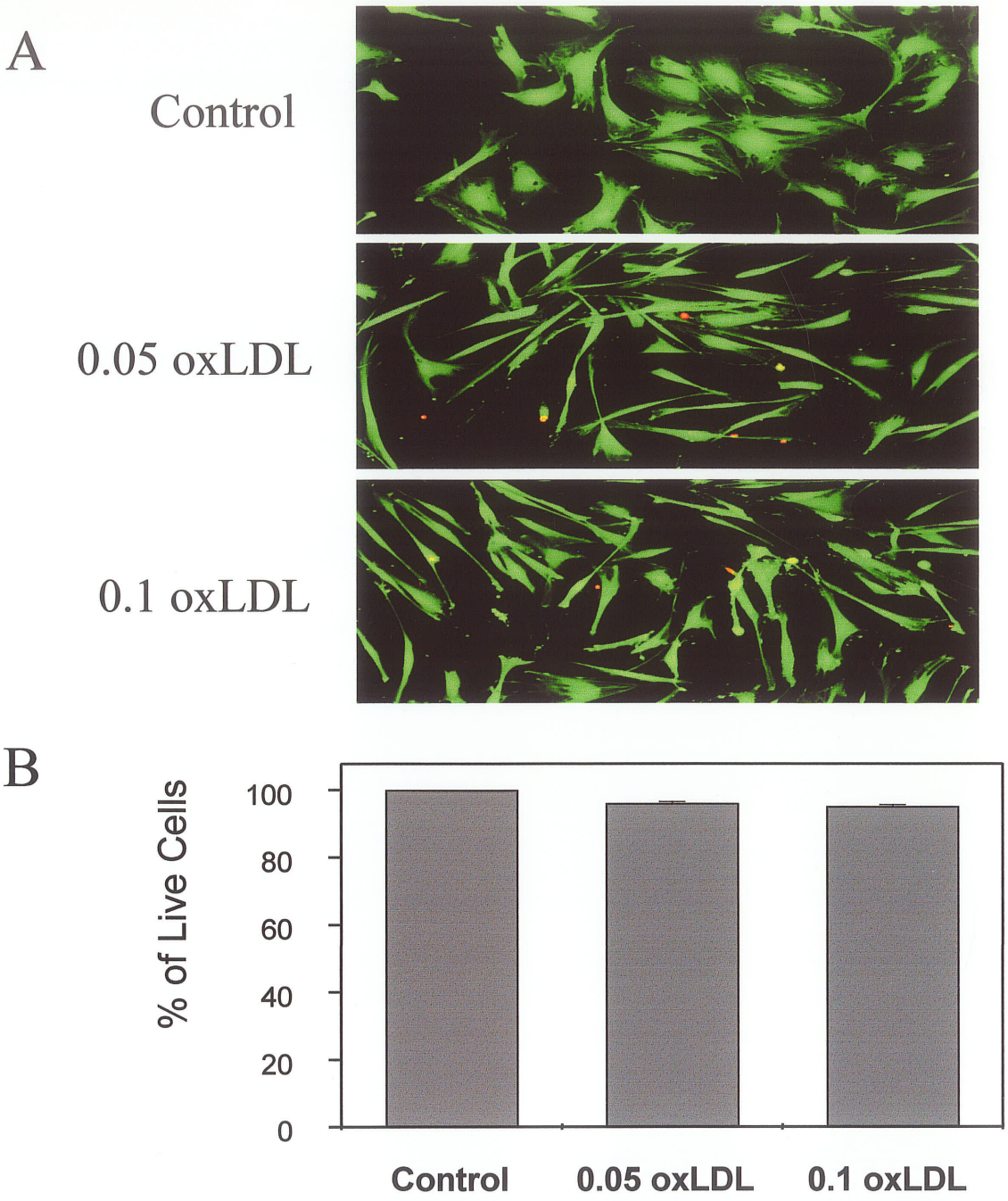


Figure 25. Effect of chronic treatment with oxLDL on vascular smooth muscle cell integrity. VSMC were exposed for 3 days to 0, 0.05, and 0.1 mg cholesterol/ml oxLDL and stained with the ethidium homodimer for cell integrity as described in the Methods. A) Representative results: Live cells stained yellow/green whereas dead cells are identified by staining of nuclei with ethidium homodimer (red fluorescence). B) The number of cells was quantified in several separate experiments (1300 to 2800 cells were counted in each group from 3 separate experiments).

3. Will chronic exposure of VSMC to oxLDL alter basal [Ca^{2+}]?

A small proportion of the vascular smooth muscle cells incubated chronically with oxLDL exhibited a change in cell morphology to foam cells. These cells were not the focus of our study. Instead, we chose to investigate [Ca^{2+}] in cells that maintained the long, spindle shape typical of VSMC. Cells were incubated with 0.001 to 0.025 mg cholesterol/mL for up to 6 days. The basal [Ca^{2+}] was not significantly changed amongst the different experimental groups ($p > 0.05$) (Table 5).

4. Will chronic exposure to oxLDL alter cellular Ca^{2+} transients in response to subsequent stimulation by oxLDL?

VSMC were incubated with oxLDL for 6 days, then washed and examined for their ability to respond with an increase in $[Ca^{2+}]_i$ to an acute application of 0.1 mg/mL oxLDL. The extended treatment of VSMC with different [oxLDL] had a pronounced effect on intracellular Ca^{2+} regulation. Some but not all of the VSMC responded to oxLDL with an increase in intracellular Ca^{2+}. By examining the number of cells that responded to oxLDL with a rise in $[Ca^{2+}]_i$ in comparison to the total number of cells tested in each experimental group, a responder ratio was obtained (Figure 26). Less than 10% of VSMC treated with higher concentration of oxLDL (0.01 and 0.025 mg/mL) for 6 days subsequently responded to 0.1 mg/mL oxLDL with a change in intracellular Ca^{2+}. A relatively low concentration of oxLDL (0.005 mg/mL) led to a 40%

Table 5. Effects of chronic exposure of vascular smooth muscle cells to varying concentrations of oxLDL for 6 days on basal $[Ca^{2+}]_i$.

Chronic Groups [oxLDL] mg/ml	$[Ca^{2+}]_i$ nM	N
Control	182 ± 22	24
0.001	145 ± 23	7
0.005	154 ± 14	32
0.0075	196 ± 29	15
0.01	199 ± 17	29
0.025	219 ± 19	22

Values represent mean ± S.E. N=number of cells examined. There were no significant differences amongst the groups ($p > 0.05$)

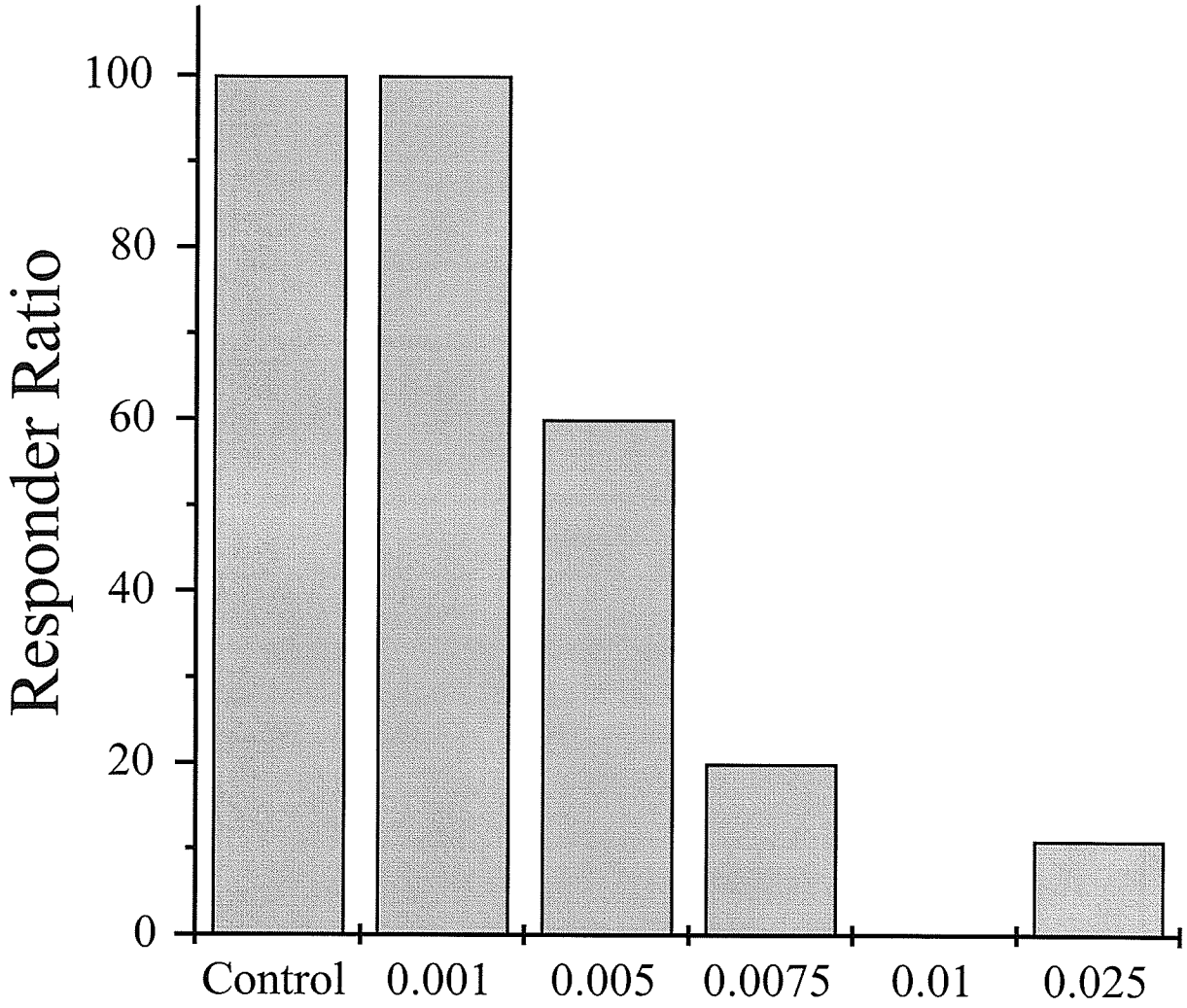


Figure 26. Responder ratio to oxLDL stimulation in VSMC chronically exposed to oxLDL. VSMC were exposed to different concentrations oxLDL (0.001-0.025 mg/mL) for a period of 6 days. These cells were then stimulated with 0.1 mg cholesterol/mL oxLDL. The percentage of cells that responded to oxLDL by an increase in $[Ca^{2+}]_i$ within 1 minute after the application of oxLDL are shown. The values represent 4 to 15 different experiments for each group.

decrease in responder ratio compared to control. There was no difference in responder ratio between control VSMC and the cells chronically treated with 0.001 mg/mL oxLDL.

The calcium transient was altered even in the cells that did respond to oxLDL. As shown in representative recordings from single cells (Figure 27), cells responded immediately as a result of acute stimulation with 0.1 mg/mL oxLDL, however, the peak response was reduced as the concentration of oxLDL used in the chronic incubation period increased. VSMC that were chronically incubated with higher [oxLDL] (from 0.01 to 0.025 mg/mL) failed to respond to the calcium mobilizing effect of oxLDL. Data from a large number of separate experiments were compiled together for analysis (Table 6). The chronic incubation of VSMC for 6 days at concentrations of oxLDL as low as 0.005 mg/mL resulted in a significant blunting of the subsequent effect of oxLDL on $Ca^{2+}{}_i$ transients. However, it is also interesting to note that the lowest [oxLDL] tested (0.001mg/mL) induced a Ca^{2+} transient with a ½ time that was approximately twice as long as the control cells (Table 6).

In order to test if oxLDL is responsible for the alteration in the calcium transient, we used native LDL in the same chronic setting. VSMC were treated for 6 days with different concentrations of native LDL (0.001 to 0.025 mg cholesterol/mL). These cells were then stimulated with 0.1 mg/mL oxLDL. All the cells responded to oxLDL with a Ca^{2+} transient (data not shown).

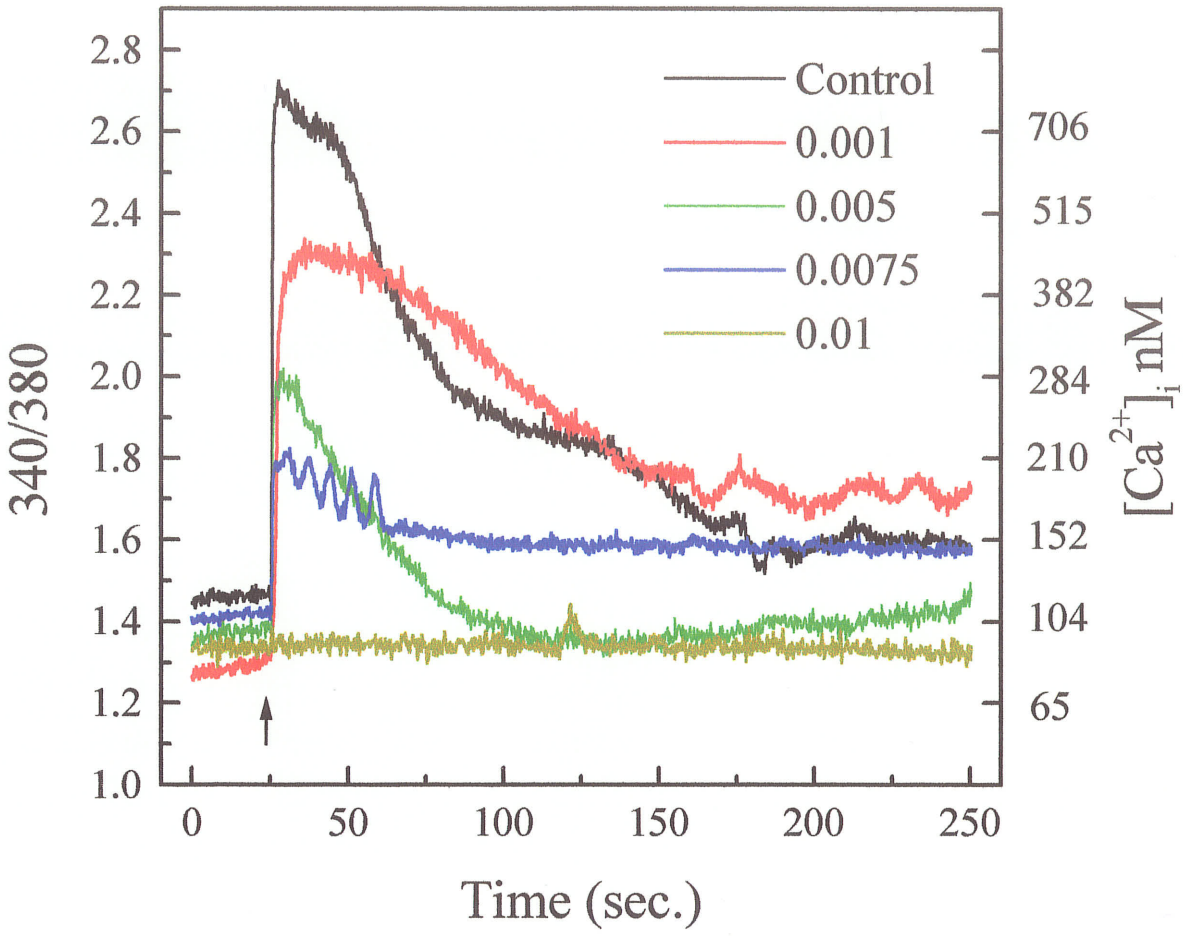


Figure 27. Effect of oxLDL on the intracellular calcium transient in VSMC chronically incubated with oxLDL. Vascular smooth muscle cells were exposed to different concentrations of oxLDL as indicated for 6 days. The tracings are taken from representative cells showing the effect of 0.1 mg/ml oxLDL on the intracellular free calcium. Arrow indicates the addition of oxLDL.

Table 6. Percentage change in intracellular calcium from basal level in smooth muscle cells chronically exposed to oxLDL for 6 days.

[OxLDL]	Time (seconds)						
mg/ml	0	10	15	30	60	90	120
Control	0.04 ± 0.05	69.48 ±11.1	62.8 ± 12.5	45.5 ± 9.0	28.6 ± 5.2	20.7 ± 3.1	17.1 ± 2.8
0.001	0.02 ± 0.02	57.7 ±17.6 *	71.1 ± 22.4 *	69.0 ± 19.6 *	54.5 ± 11.6 *	44.1 ± 10.6 *	37.8 ± 11.1 *
0.005	0.10 ± 0.06	13.0 ± 4.3 *	12.2 ± 3.7 *	10.7 ± 2.7 *	10.1 ± 1.9 *	10.0 ± 2.6 *	8.1 ± 2.9
0.0075	0.08 ± 0.07	8.6 ±6.3 *	9.1 ± 6.8 *	7.7 ± 4.6 *	8.6 ± 3.2 *	7.5 ± 3.0 *	8.0 ± 3.1
0.01	0.03 ± 0.08	0.26 ±0.2 *	0.9 ± 0.4 *	0.3 ± 0.4 *	0.6 ± 0.5 *	0.74 ± 0.8 *	0.10 ± 1.1 *
0.025	0.07 ± 0.05	5.0 ±4.5 *	4.9 ± 3.3 *	3.3 ± 2.0 *	2.4 ± 1.3 *	2.4 ± 1.3 *	1.5 ± 1.3 *

Values represent mean ± S.E. of 4 to 20 separate experiments. * $p < 0.05$.

5. What is the mechanism responsible for the chronic effects of oxLDL on VSMC Ca^{2+}?

Vascular smooth muscle cells may have reduced capacity to respond to oxLDL with an increase in intracellular Ca^{2+} because: i) a decrease in oxLDL binding to the cell surface, or, ii) an alteration in the intracellular signaling which effected the increase in Ca^{2+}_i. The receptors that regulate oxLDL binding to the cell have not been fully characterized and because oxLDL may gain access to the cell through several different receptors, it is impossible to directly assess this first possibility (148, 154). However, we attempted to address the question by using another compound that elevates cellular Ca^{2+} via a similar intracellular pathway but uses a different cell surface receptor. ATP binds to purinergic receptors which stimulates phospholipase C to form IP_3 which stimulates Ca^{2+} release from the sarcoplasmic reticulum (SR) via the IP_3 pathway (62, 275, 280). If ATP did elicit a Ca^{2+} transient in the cells chronically treated with oxLDL (when oxLDL could not), this would identify the site of the lesion as the oxLDL receptor. Conversely, if the cells chronically treated with oxLDL did not respond to ATP with an increase in cellular Ca^{2+}, this control support the argument that the defect resided within the cell or that both receptor types (oxLDL and purinergic) were affected by the chronic incubation with oxLDL. As shown in Figure 28, VSMC which had been incubated with 0.001 to 0.025 mg cholesterol/mL for 6 days exhibited a depressed capacity to respond to ATP. This was qualitatively similar to the effects of oxLDL shown in Figure 26. The

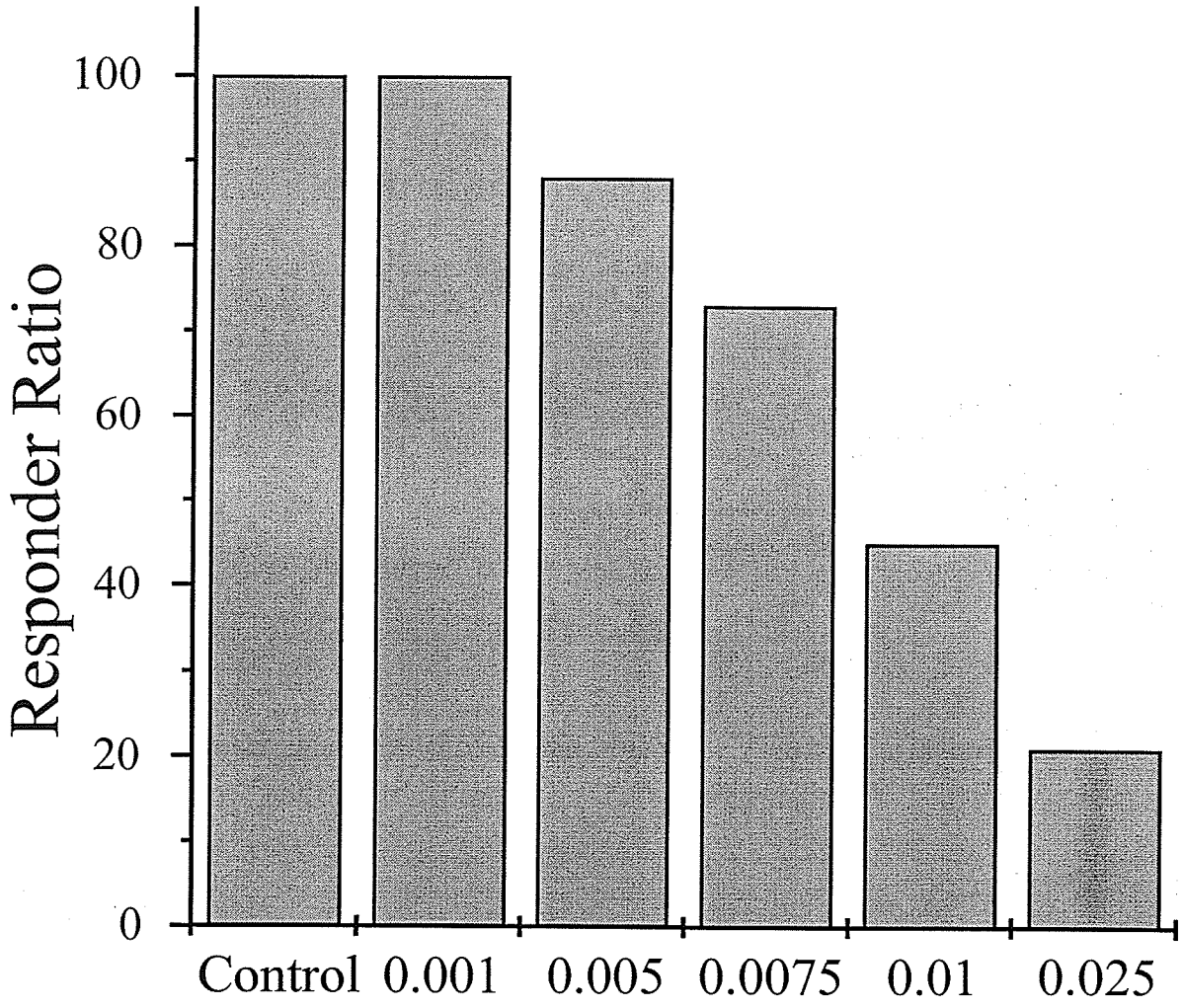


Figure 28. Responder ratio to ATP stimulation in VSMC chronically exposed to oxLDL. Vascular smooth muscle cells were exposed to different concentrations of oxLDL (0.001, 0.005, 0.0075, 0.01, and 0.025 mg/ml) for a period of 6 days. These cells were then stimulated with 100 μM ATP. The percentage of cells that responded to ATP by an increase in $[Ca^{2+}]_i$ are shown. The values represent 9 to 14 different experiments for each group.

majority of cells incubated with oxLDL at concentrations >0.01 mg/mL were no longer responsive to ATP. Furthermore, cells which did respond to ATP exhibited a depressed peak Ca^{2+} transient (Figure 29). This response was again qualitatively similar to that observed with oxLDL in Figure 27. This effect was dependent upon the dose of oxLDL to which the cells were exposed. The results from a number of cells was tabulated (Table 7). A concentration dependent dampening of the Ca^{2+} transient induced by ATP was observed for oxLDL treated cells.

In order to test the responsiveness of chronically treated cells to other agonists that target SR calcium release, we used norepinephrine (α- adrenergic agonist) and endothelin-1 (ET-1 receptor agonist). Binding of norepinephrine to its α-adrenergic receptor or endothelin-1 to its ET-1 receptor will result in activation of phospholipase C, formation of IP_3 and intracellular calcium release from the SR. As shown in Figure 30 and 31, control VSMC responded to norepinephrine or endothelin-1 with a rapid increase in $[Ca^{2+}]_i$, whereas VSMC treated with 0.025 mg/mL oxLDL for 6 days failed to show an increase in $[Ca^{2+}]_i$ after of exposure to norepinephrine or endothelin-1.Chronically treated cells responded in a delayed fashion (~100 second after treatment) with a very small increase in $[Ca^{2+}]_i$ (Figure 30 and 31).

As presented previously, the acute effect of oxLDL on intracellular calcium was likely due to an IP_3 mediated release of calcium from the SR. Therefore, it was reasonable to determine if extensive incubation of VSMC with

Table 7. Percentage change in Ca^{2+}_i from basal level after stimulation with ATP in control VSMC or VSMC chronically treated with oxLDL.

	Time (seconds)						
OxLDL (mg/ml)	0	15	30	60	90	120	N
Control	101.7 ± 0.54	169.5 ± 9.5	160.1 ± 9.7	144.7 ± 5.9	134.7 ± 4.9	132.4 ± 4.1	12
0.001	103.1 ± 0.65	114.4 ± 9.2 *	126.3 ± 1.9	127.2 ± 3.9	123.2 ± 3.7	120.0 ± 3.3	3
0.005	100.6 ± 0.6	122.8 ± 5.2 *	121.5 ± 4.3*	116.2 ± 3.7 *	113.2 ± 3.0 *	112.3 ± 3.0 *	17
0.0075	101.0 ± 0.4	121.4 ± 6.1 *	120.3 ± 5.1*	117.6 ± 4.4 *	113.3 ± 3.6 *	112.2 ± 3.5 *	11
0.01	100.9 ± 0.5	106.0 ± 2.2 *	105.4 ± 2.0 *	104.7 ± 1.7 *	104.2 ± 1.6 *	104.4 ± 1.8 *	20
0.025	101.7 ± 0.6	112.4 ± 5.6 *	111.7 ± 5.1 *	110.2 ± 4.4 *	109.4 ± 4.1 *	109.4 ± 4.0 *	14

Values represent mean values ± S.E. *$p < 0.05$ vs. respective control values.

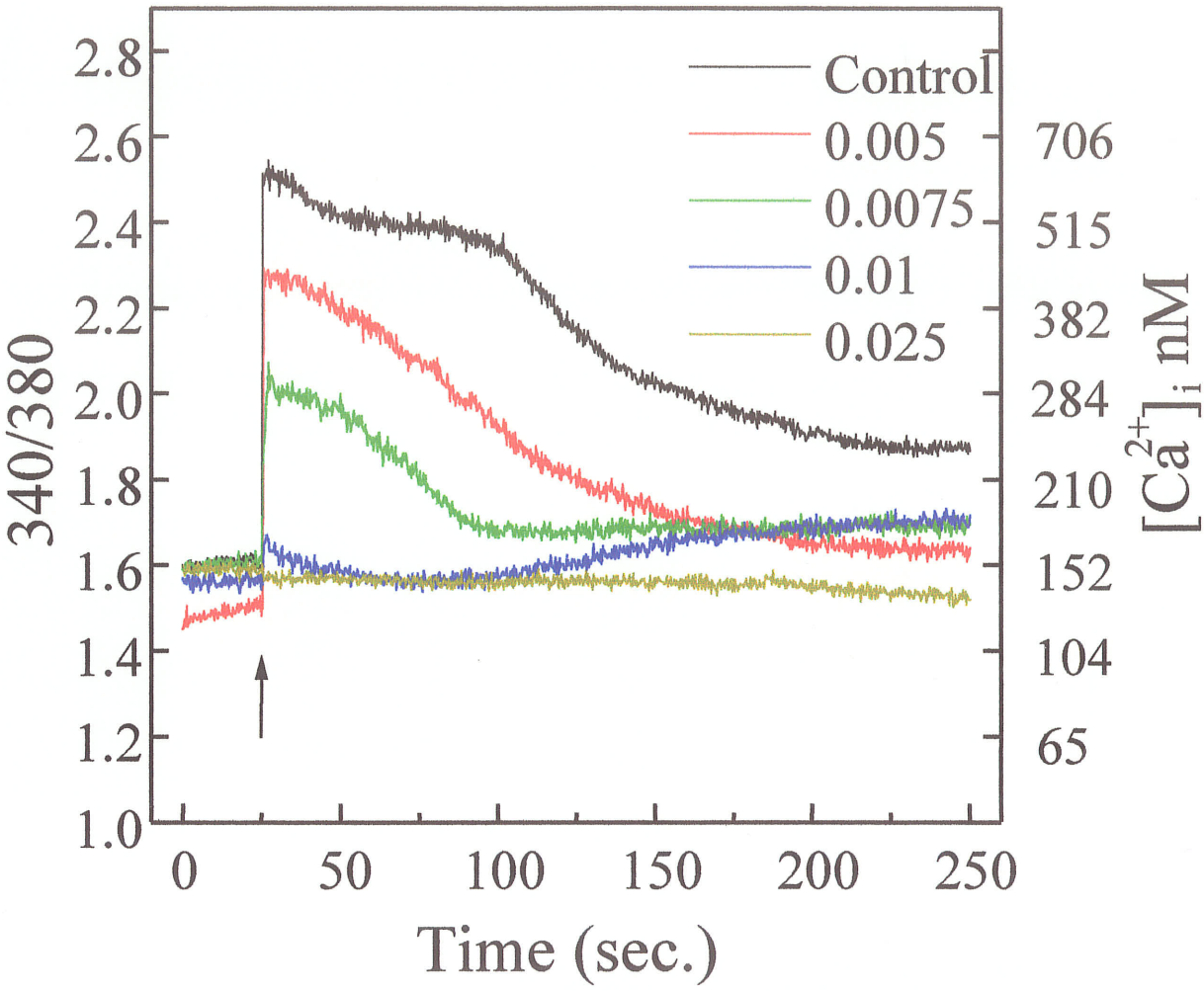


Figure 29. Effect of ATP on the intracellular calcium transients in VSMC chronically incubated with oxLDL. Vascular smooth muscle cells were exposed to different concentrations of oxLDL as indicated for 6 days. The tracings are from representative experiments showing the effect of 100 μM ATP on the intracellular free calcium. Arrow indicates the addition of ATP.

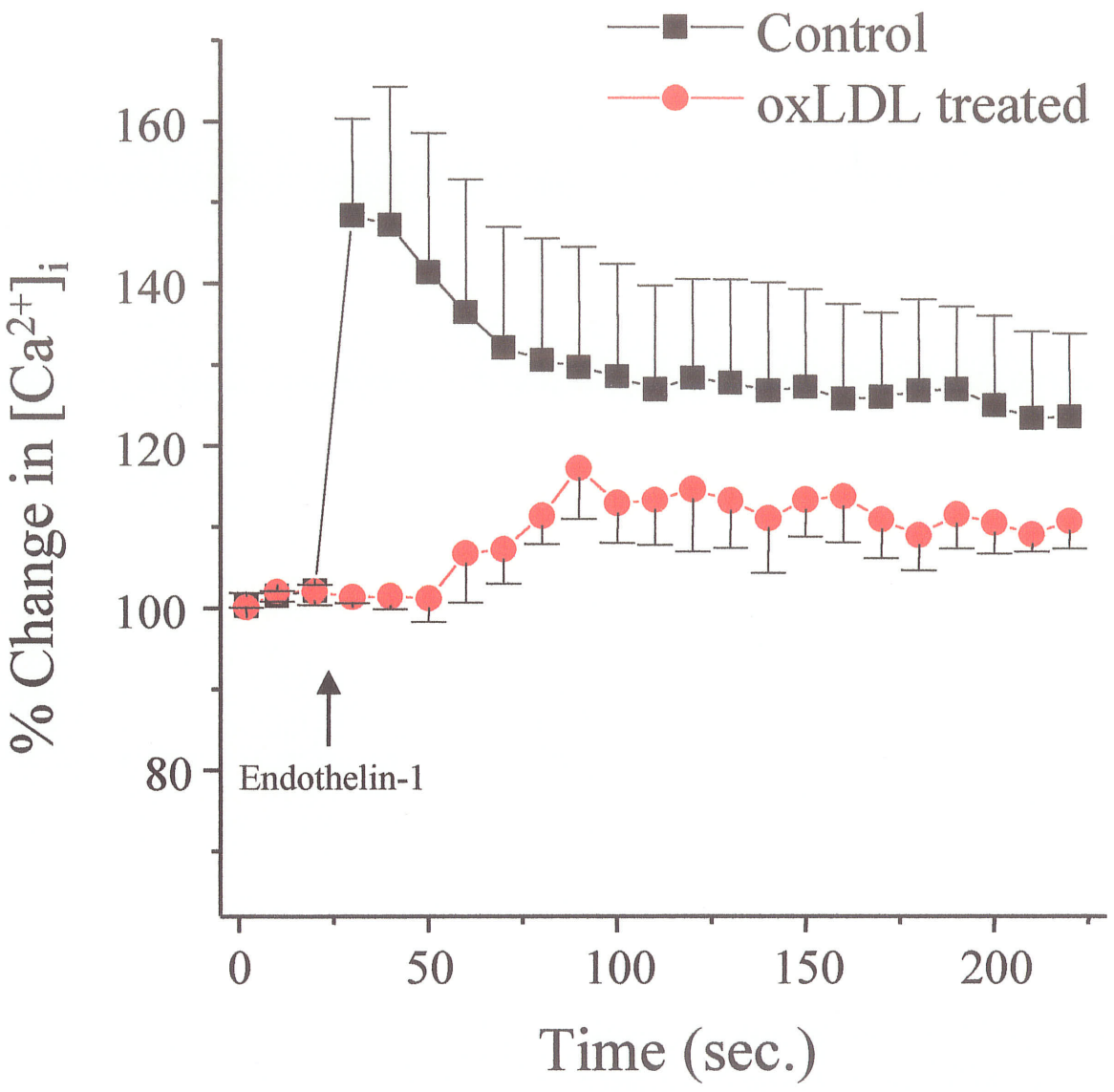


Figure 31. Effect of endothelin-1 on $[Ca^{2+}]_i$ in the VSMC chronically treated with oxLDL. Vascular smooth muscle cells were treated ± 0.025 mg/ml oxLDL for 6 days. These cells were loaded with calcium indicator fura-2 and then stimulated with 5 µmol/ml endothelin-1(as shown by arrow). The values are the mean ± S.E. from 3 different experiments.

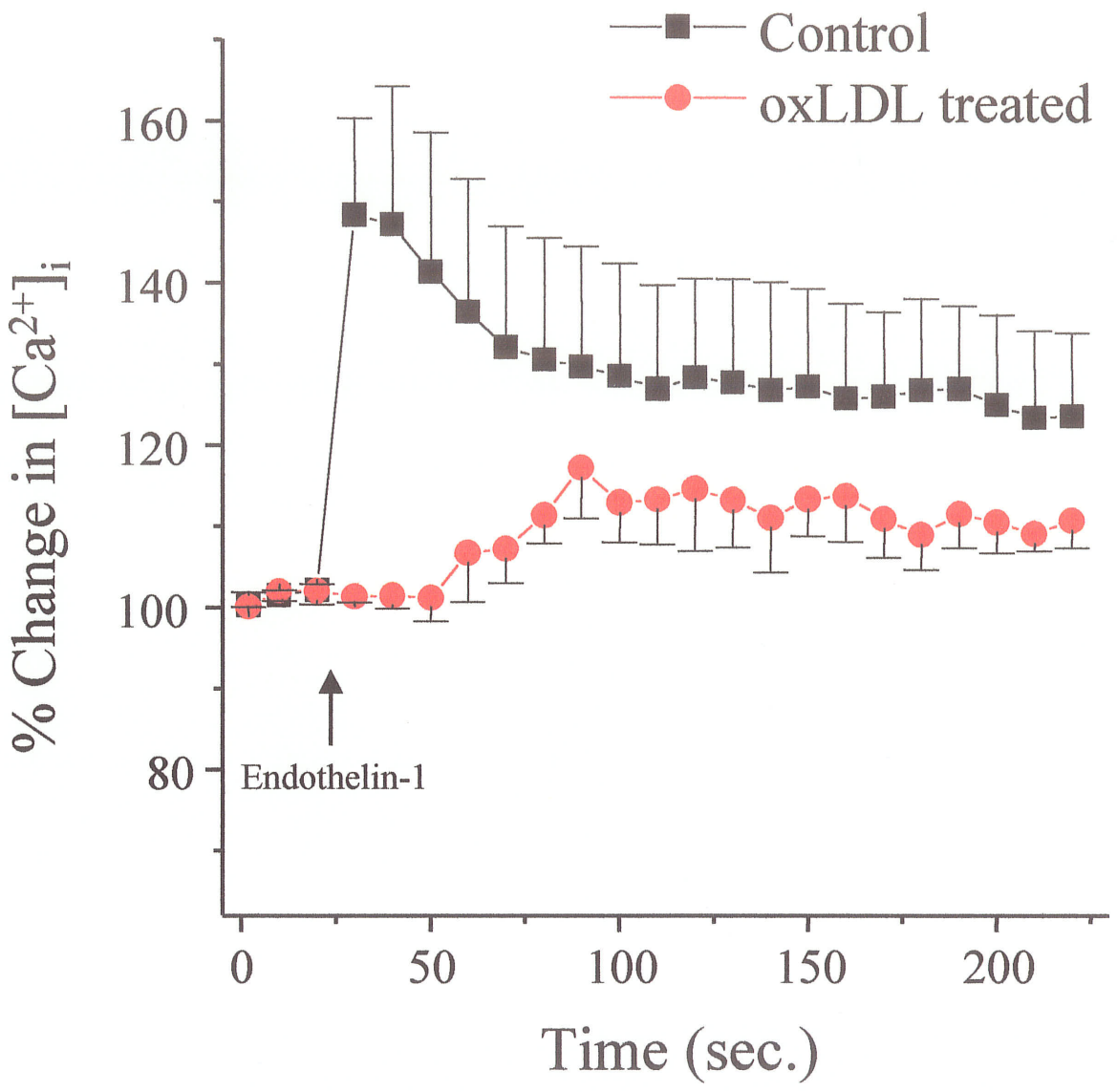


Figure 31. Effect of endothelin-1 on $[Ca^{2+}]_i$ in the VSMC chronically treated with oxLDL. Vascular smooth muscle cells were treated ± 0.025 mg/ml oxLDL for 6 days. These cells were loaded with calcium indicator fura-2 and then stimulated with 5 µmol/ml endothelin-1(as shown by arrow). The values are the mean ± S.E. from 3 different experiments.

oxLDL resulted in a change in the IP_3 signaling pathway. VSMC were treated with 0.025 mg/mL oxLDL for a 3 or 6 day period. These cells were then fixed and treated with a monoclonal antibody against the IP_3 receptor. This anti- IP_3 antibody recognizes all three known isoforms of the IP_3 receptor (type I, II, and III). However, because VSMC contain 73% type I IP_3 receptors, the signal likely emanates mostly from type I IP_3 receptors (63). Figure 32a-c represents results from control cells. These cells show a high expression of IP_3 receptors in the periphery of the nuclei and throughout the cytoplasm. VSMC which were treated with oxLDL for 3 days (Figure 32d-f) exhibited a small decrease in the density of IP_3 receptors from control. However, a longer treatment period (6 days) led to a striking reduction in IP_3 receptor density throughout cells (Figure 32g-i). This was quantitated by Western immunoblots. As shown in Figure 33, incubation of VSMC for 6 days with 0.025 mg/mL oxLDL resulted in a significant decrease in IP_3 receptor density. This defect in IP_3 receptor density was accompanied by a depressed capacity to generate IP_3 in cells chronically exposed to oxLDL (Figure 12). Thus, chronic exposure of VSMC to oxLDL leads to a significant depression in IP_3 signalling in the cells due to an attenuated generation of IP_3 and a decrease in IP_3 receptor density.

This defect in SR regulation of intracellular Ca^{2+} may be associated not only with IP_3 receptor density, but also with a change in ryanodine channel density. Ryanodine channels were detected via immunocytochemical staining. In some cases, ryanodine receptors were expressed in a striking tendril fashion

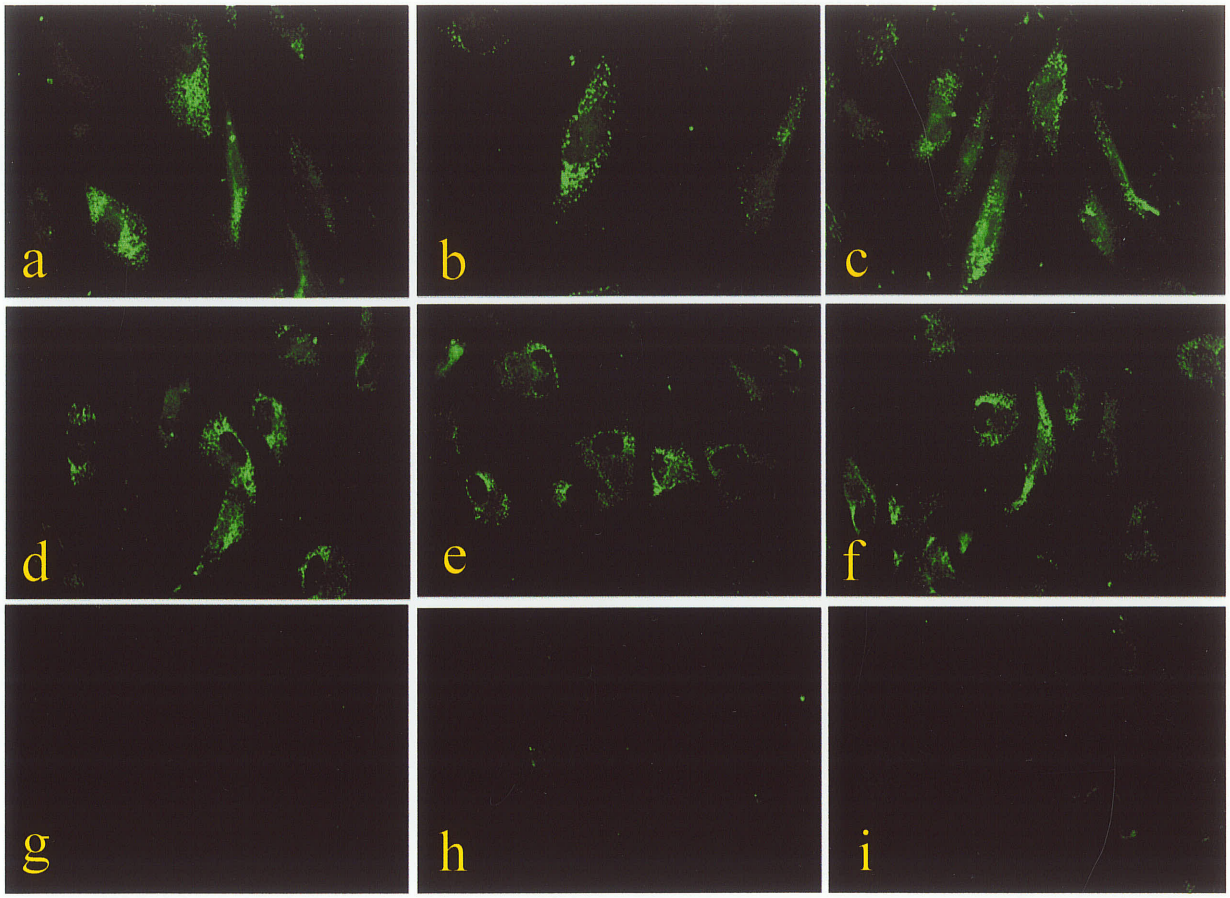


Figure 32. Effect of extended exposure to oxLDL on IP_3 receptor distribution in VSMC. VSMC were treated with 0.025 mg cholesterol/mL oxLDL for 0 (a-c), 3 (d-f), and 6 (g-i) days. These cells were then immunocytochemically stained for IP_3 receptors. Images were collected using a Bio-Rad confocal microscope. All settings were kept constant for comparative reasons. The images were enhanced in size 1.4-1.6 fold.

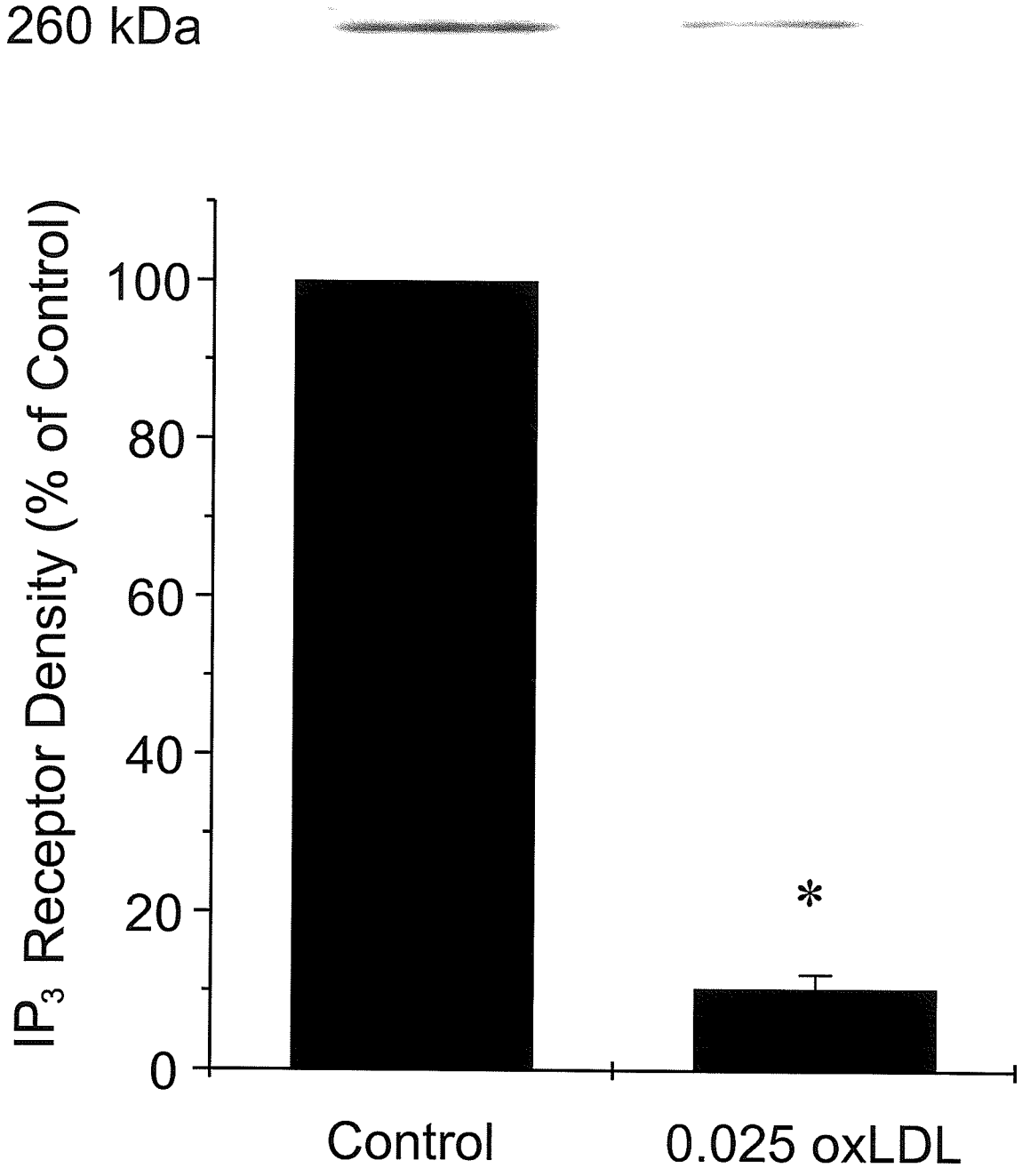


Figure 33. Effect of chronic exposure to oxLDL on IP_3 receptor density in VSMC as determined by western blotting. VSMC were treated with 0.025 mg cholesterol/mL oxLDL for 6 days. A) The polyclonal anti-IP_3 receptor antibody recognizes a 260 Kd protein. B) Values from 4 different experiments were plotted as percentage change from control. * $p < 0.05$ versus control (n=4).

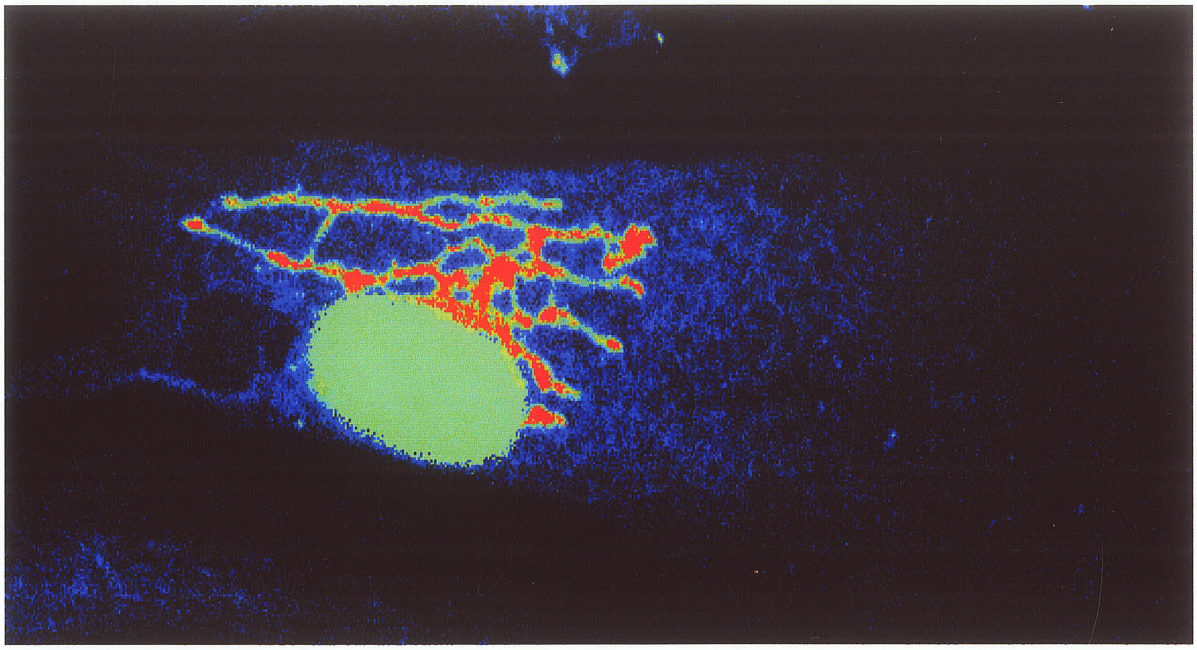


Figure 34. Ryanodine distribution in VSMC stained with monoclonal anti-ryanodine receptor antibody. In some cases we can detect a tendril-like staining pattern of receptor distribution through the smooth muscle cells. The cell has been expanded in size by 3.4 fold to illustrate this receptor distribution. The nuclei were stained with Hoescht No. 33258.

through the cytoplasm in control cells (Figure 34). However, after chronic exposure to 0.025 mg/mL oxLDL, smooth muscle cells showed a gradual loss in ryanodine receptors by 3 days until there was no detectable staining for ryanodine receptors by day 6 (Figure 35).

Since ATP and oxLDL mobilize calcium from the SR, the depressed Ca^{2+} transient in cells chronically treated with oxLDL may be due to a defect in the capacity of the SR to regulate intracellular Ca^{2+}. In order to test the functional integrity of the SR Ca^{2+} release channels, ryanodine was administrated on control VSMC and on cells after chronic exposure to oxLDL. Exposure of control VSMC to ryanodine resulted in a significant rise in $[Ca^{2+}]_i$ (Figure 36). Chronic exposure of vascular smooth muscle cells to 0.025 mg/mL oxLDL for 6 days significantly attenuated the peak Ca^{2+} level in response to ryanodine (Figure 36).

6. Are the changes in IP_3 and ryanodine receptor density found in atherosclerotic tissue in situ?

The possibility exists that the cell culture conditions employed may not mimic the *in vivo* situation in atherosclerosis. It is unknown if vascular IP_3 and ryanodine receptors are affected by atherosclerosis.

Aortic tissue was removed from rabbits fed a 0.5% cholesterol-supplemented diet for 12-14 weeks. This tissue exhibited gross atherosclerotic plaques. Sections from these atherosclerotic aorta and control aorta were examined with immunohistochemical staining for changes in IP_3 receptor density

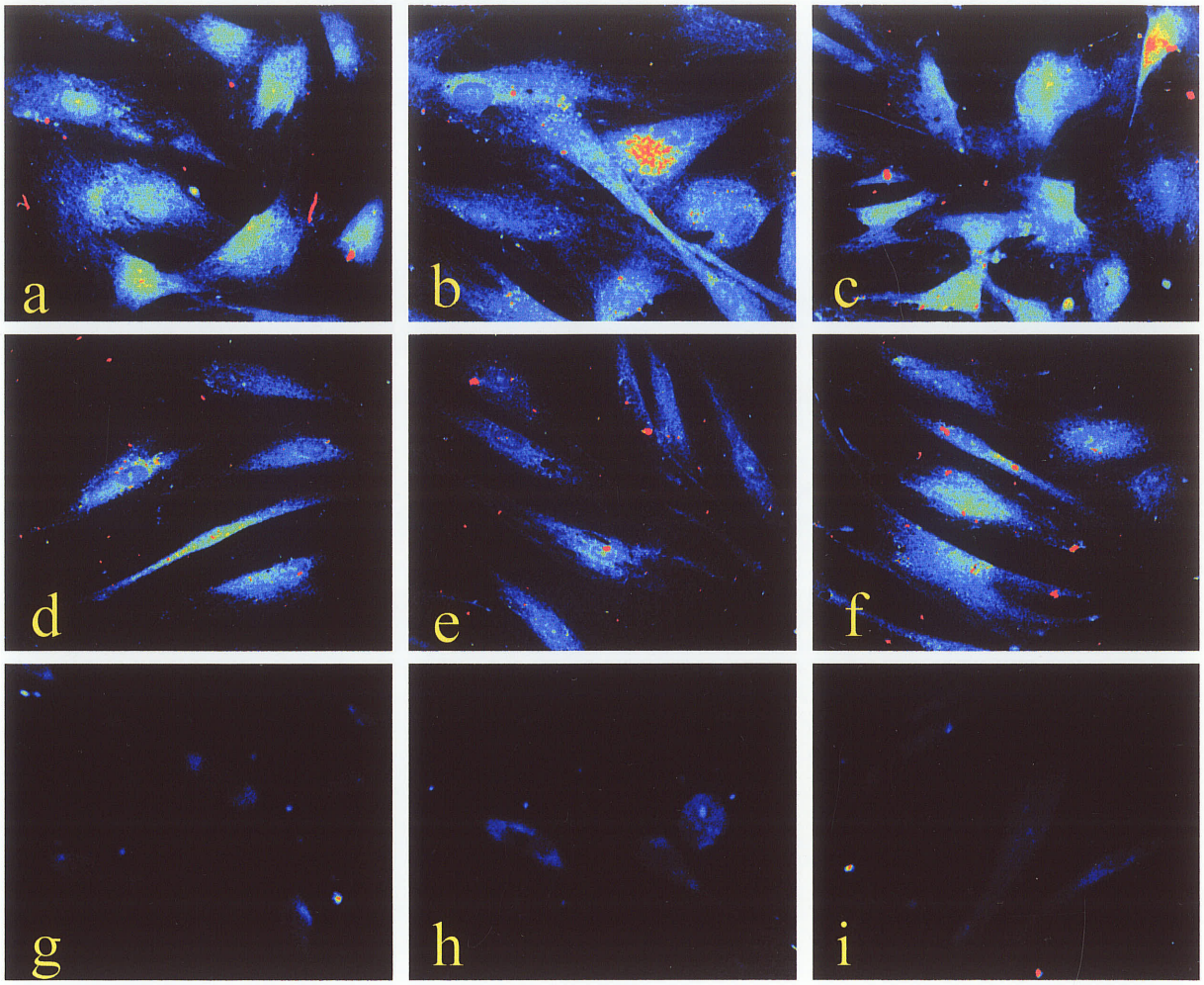


Figure 35. Ryanodine receptor distribution after chronic exposure of VSMC to oxLDL. Smooth muscle cells were treated with 0.025 mg/ml oxLDL for a-c) control; d-f) 3 days; and g-i) 6 days. These cells were stained with monoclonal anti-ryanodine receptor antibody.

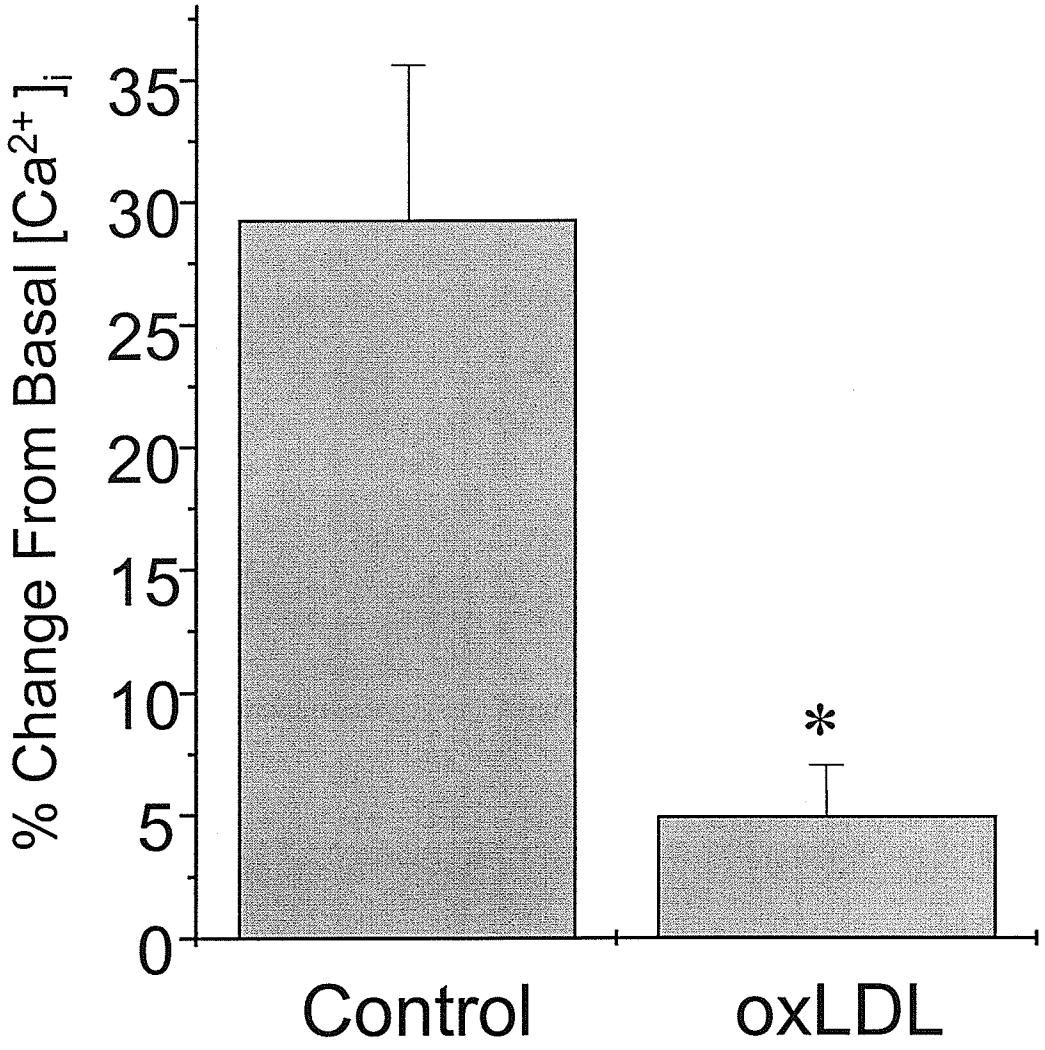


Figure 36. Effect of 1 μM ryanodine on Ca^{2+}_i in control VSMC and VSMC treated with 0.025 mg/ml oxLDL. These cells were stimulated with 1 μM ryanodine and the percentage change from basal level was calculated. Control cells exhibited a significant increase $[Ca^{2+}]_i$ over basal levels in response to ryanodine ($p < 0.05$). Values represent the mean ± S.E. of 4-8 separate experiments. * $p < 0.05$ versus control.

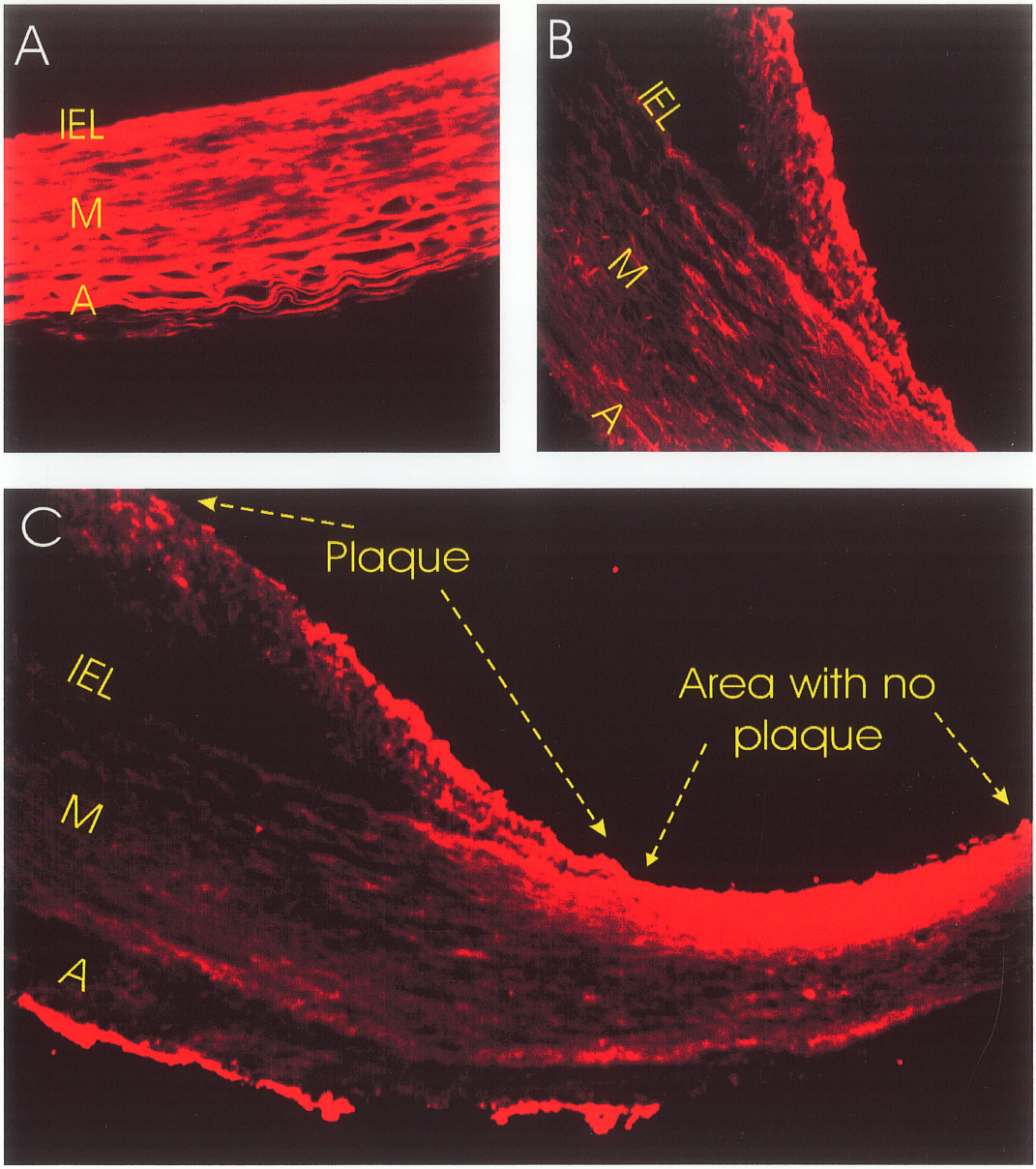


Figure 37. IP_3 receptor density in the medial region of the aorta. Aortic tissue isolated from rabbits fed a control diet or a 0.5% cholesterol supplemented diet. Seven micron sections were prepared and incubated with anti-IP_3 receptor antibody. A) Control aortic section. B) Atherosclerotic region of aorta from cholesterol fed rabbit. C) Aorta from cholesterol fed rabbit showing both plaque region and area with no plaque. Note that the strong IP_3 receptor staining in the area devoid of plaque formation contrasts strongly with the poor receptor density in the plaque region even within the same section. *IEL*= internal elastic lamina, *M* = medial layer or tunica media , and *A* = adventitial layer or tunica adventitia.

(Figure 37). IP_3 receptors stained strongly in the medial section of control aorta (Figure 37A), whereas this staining was reduced in the medial section of atherosclerotic tissue (Figure 37B). This difference in IP_3 receptor density was also observed in aortic sections that contained both plaque and an unaffected area (Figure 37C). As shown in Figure 37C, IP_3 receptor density was higher in the medial layer of an area with no plaque, while the same medial layer below the plaque region exhibited a reduction in fluorescence intensity.

Sections from control and cholesterol-fed rabbits were also examined with immunohistochemistry for ryanodine receptors (Figure 38). There was a striking decrease in immunoreactivity for the ryanodine receptor in the medial region under the plaque compared to control aorta (Figure 38). This change in ryanodine receptor staining intensity was also observed in aortic sections from both the area with a plaque and the area of the vessel that did not contain a plaque. As shown in Figure 38, ryanodine receptor density was more intense in the medial region of these vessels without a plaque, while the same medial layer under the plaque showed a decrease in fluorescent intensity.

This pattern of staining for IP_3 and ryanodine was not due to loss of the cells from medial region under the plaque. Using the nuclear stain Hoescht No. 33258, demonstrate that there is no loss of cells from area under the plaque compared to area with no plaque. Furthermore, staining aortic sections that were adjacent to the section we used for IP_3 and ryanodine staining, we observed uniform staining with both smooth muscle specific α-actin and myosin (Figure

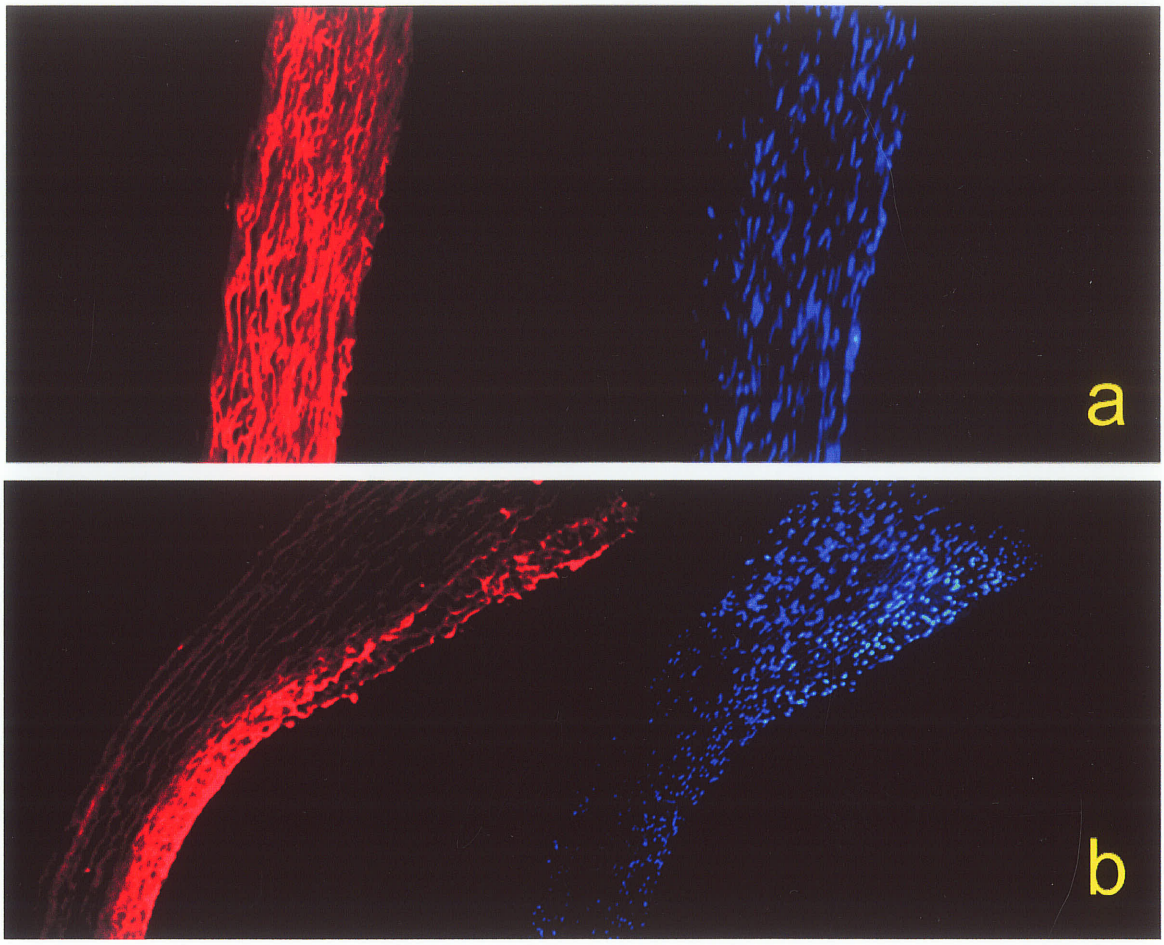


Figure 38. Ryanodine receptor density in control and atherosclerotic aortic sections. Aorta from both control (a) and 0.5% cholesterol fed New Zealand rabbits (b) were sectioned and stained with anti-ryanodine receptor antibody. The same sections were also stained with Hoescht No. 33258 to identify nuclei. Note the presence of cell nuclei throughout the vessel and the plaque.

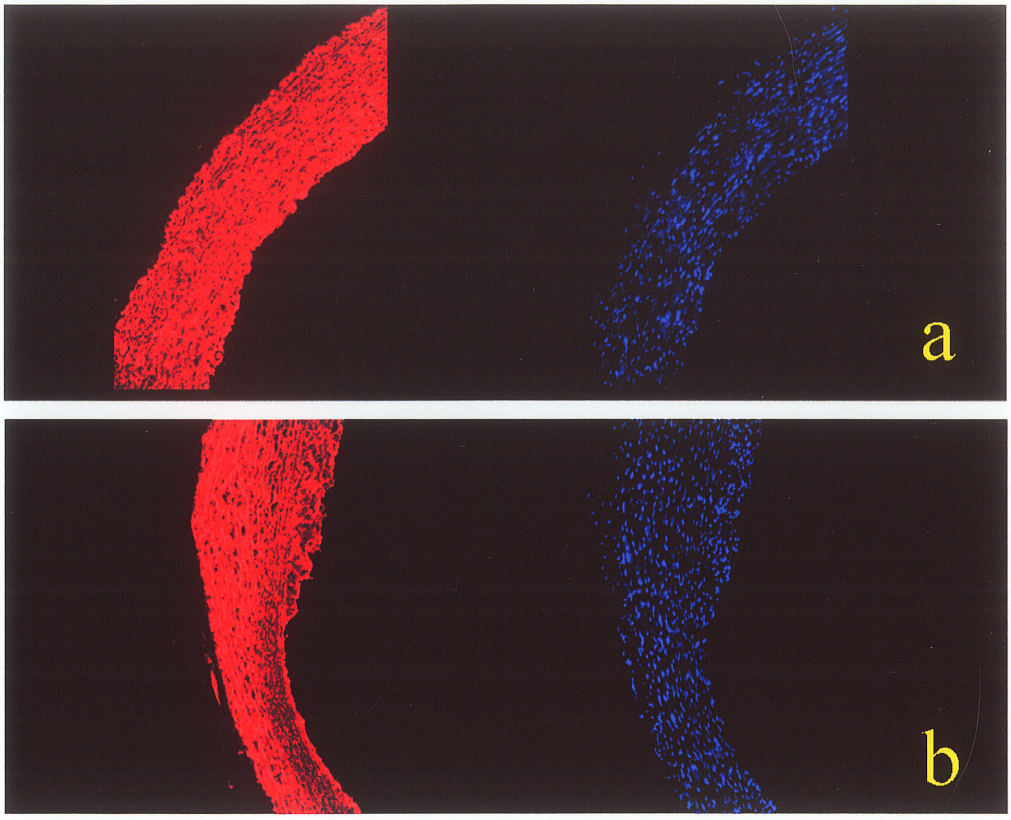


Figure 39. Myosin distribution in control and atherolsclerotic aortic section. Adjacent aortic section from both control (a) and cholesterol fed rabbits (b) were stained with smooth muscle specific monoclonal anti-myosin antibody as positive control. The same sections were also stained with Hoescht No. 33258 to identify nuclei. Note the presence of cell nuclei throughout the vessel and the plaque.

39 showing myosin staining).

7. What is the effect of chronic exposure to oxLDL on SR calcium uptake?

Sarcoplasmic reticulum is a calcium reservoir that plays an important role in calcium release and calcium uptake. Uptake of calcium from the cytoplasm after stimulation occurs through the SR Ca^{2+} ATPase pump or SERCA2 ATPase.

In order to investigate the effect of chronic exposure to oxLDL on SERCA2-ATPase density, vascular smooth muscle cells were treated ± 0.025 mg/mL oxLDL for 3 and 6 days. These cells were fixed and treated with monoclonal anti-SERCA2 ATPase antibody (Figure 40). Control smooth muscle cells showed very diffuse staining for SERCA2 in an area around the nuclei as detected by confocal microscopy (Figure 40a & b). Cells chronically treated for 3 days with 0.025 mg/mL oxLDL showed a slight increase in fluorescence intensity (Figure 40c & d). However, prolonged (6 days) chronic exposure of smooth muscle cells to 0.025 mg/mL oxLDL resulted in a significant increase in fluorescence intensity (Figure 40e & f).

To quantify this increase in SERCA2 ATPase, cells were treated with different concentrations of oxLDL (0.005-0.1 mg/mL) for a 6 day period. These cells were then lysed and scraped from the culture dish and an equal amount of protein was loaded onto a 10% SDS gel. As shown in Figure 41, all the cells treated with different concentrations of oxLDL showed a significant increase in SERCA2 content. This increase in SERCA2 density was also observed in cells treated with a very low concentration of oxLDL (0.005 mg/mL). This

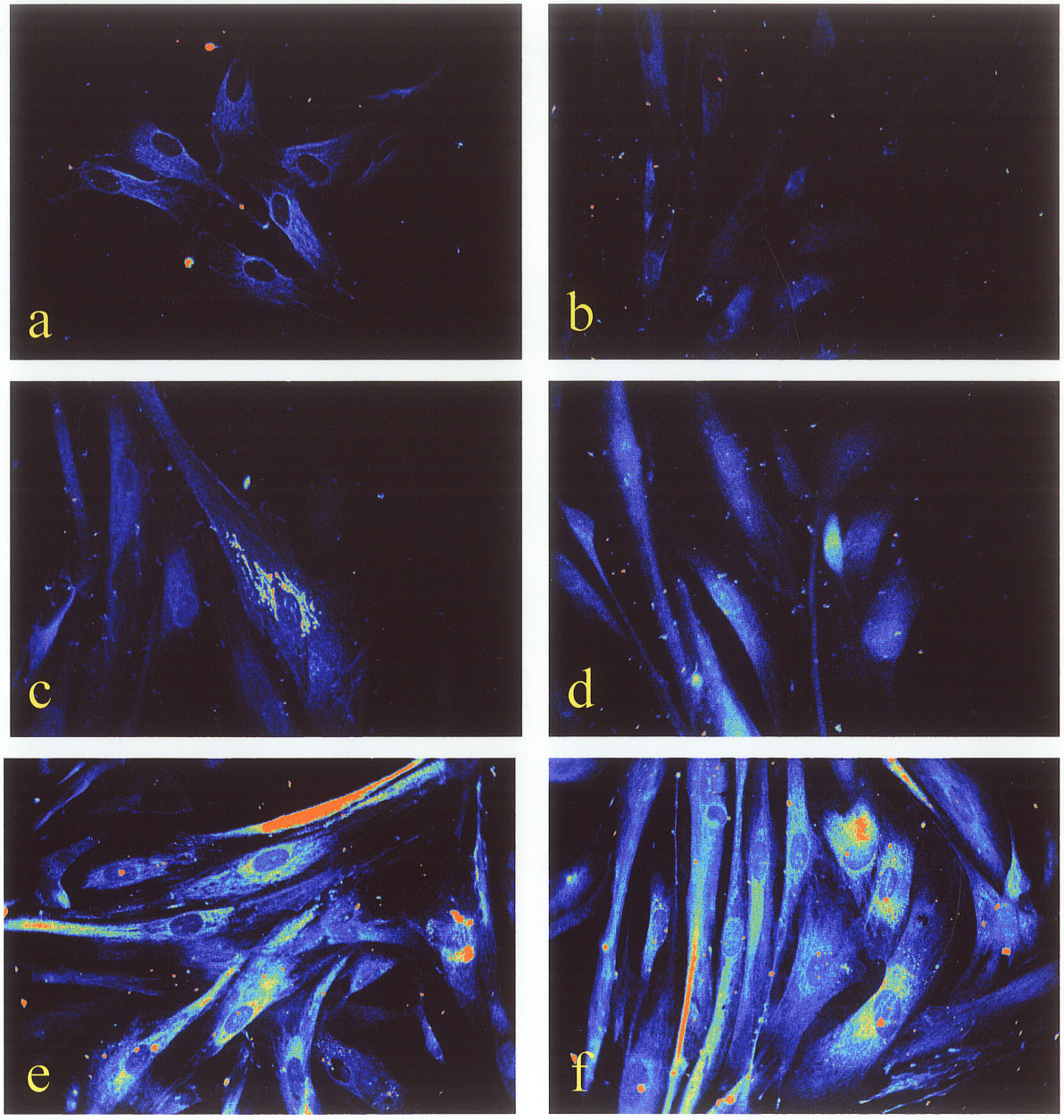


Figure 40. Effects of chronic exposure to oxLDL on SERCA2 ATPase distribution. Smooth muscle cells were treated ± 0.025 mg/ml oxLDL for 3 and 6 days. These cells were fixed and immunostained with monoclonal anti-SERCA2 ATPase antibody for visualization of protein localization. a-b) control cells, c-d) cells treated for 3 days, and e-f) cells treated for 6 days with oxLDL. Data shown are representative of 3-4 experiments.

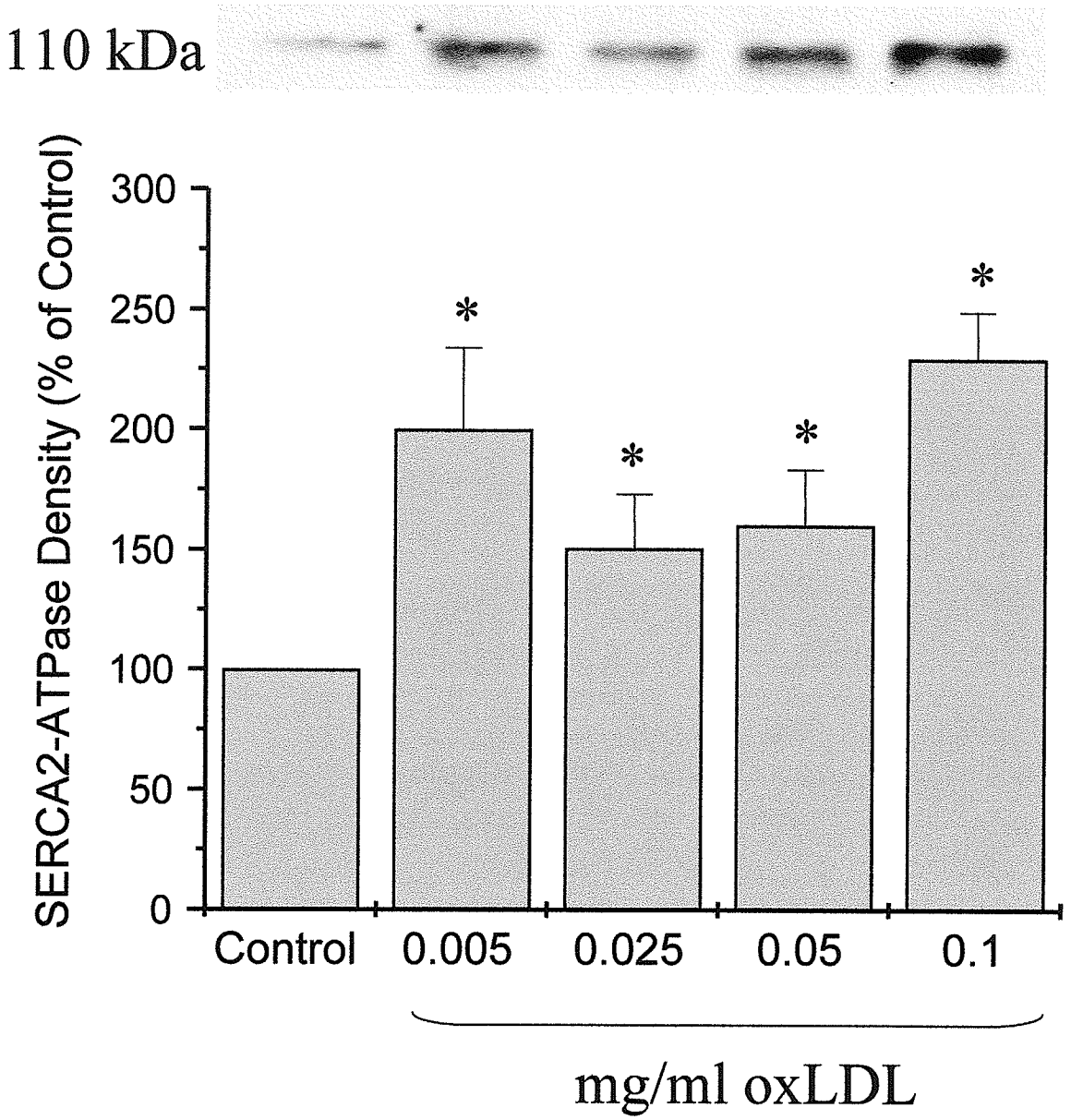


Figure 41. Effects of chronic exposure to oxLDL on SERCA2 ATPase density as determined by Western blotting. Smooth muscle cells were treated with different concentrations of oxLDL (0.005-0.1 mg/ml oxLDL) for 6 days. The total cellular protein was extracted and equal amounts of protein were loaded onto a 10% SDS gel. Values from 3-4 different experiments were plotted as a percentage change from control. * $p < 0.05$ versus control.

concentration is approximately 100 fold lower than that used by most other investigators (162).

In contrast to the increase in SERCA2 ATPase density in treated cells, there was a significant decrease in calcium release from SR. Smooth muscle cells chronically treated with 0.025 mg cholesterol/mL oxLDL for 6 days demonstrated an attenuated calcium response when they were treated with thapsigargin (Figure 42). Thapsigargin is a SERCA ATPase pump inhibitor which results in a depletion of calcium from the SR calcium store.

8. What effect does chronic exposure of VSMC to oxLDL have on VSMC contractile proteins?

During the process of atherosclerosis, smooth muscle cells proliferate and migrate into the intima of vessels, lose their contractile apparatus and modify into a synthetic phenotype. It is unclear what factor within the atherogenic environment is responsible for inducing the loss in smooth muscle myofilaments. As shown by Western blots in Figure 43, the total content of both actin and myosin were altered in smooth muscle cells chronically treated with oxLDL. Smooth muscle cells treated for a period of 6 days with oxLDL showed a significant and consistent decrease in both actin and myosin content in these cells (Figure 43). Increasing concentrations of oxLDL (0.01 to 0.05 mg cholesterol/mL) induced a pattern for the reduction in both actin and myosin, although this was statistically significant for actin only at the highest [oxLDL].

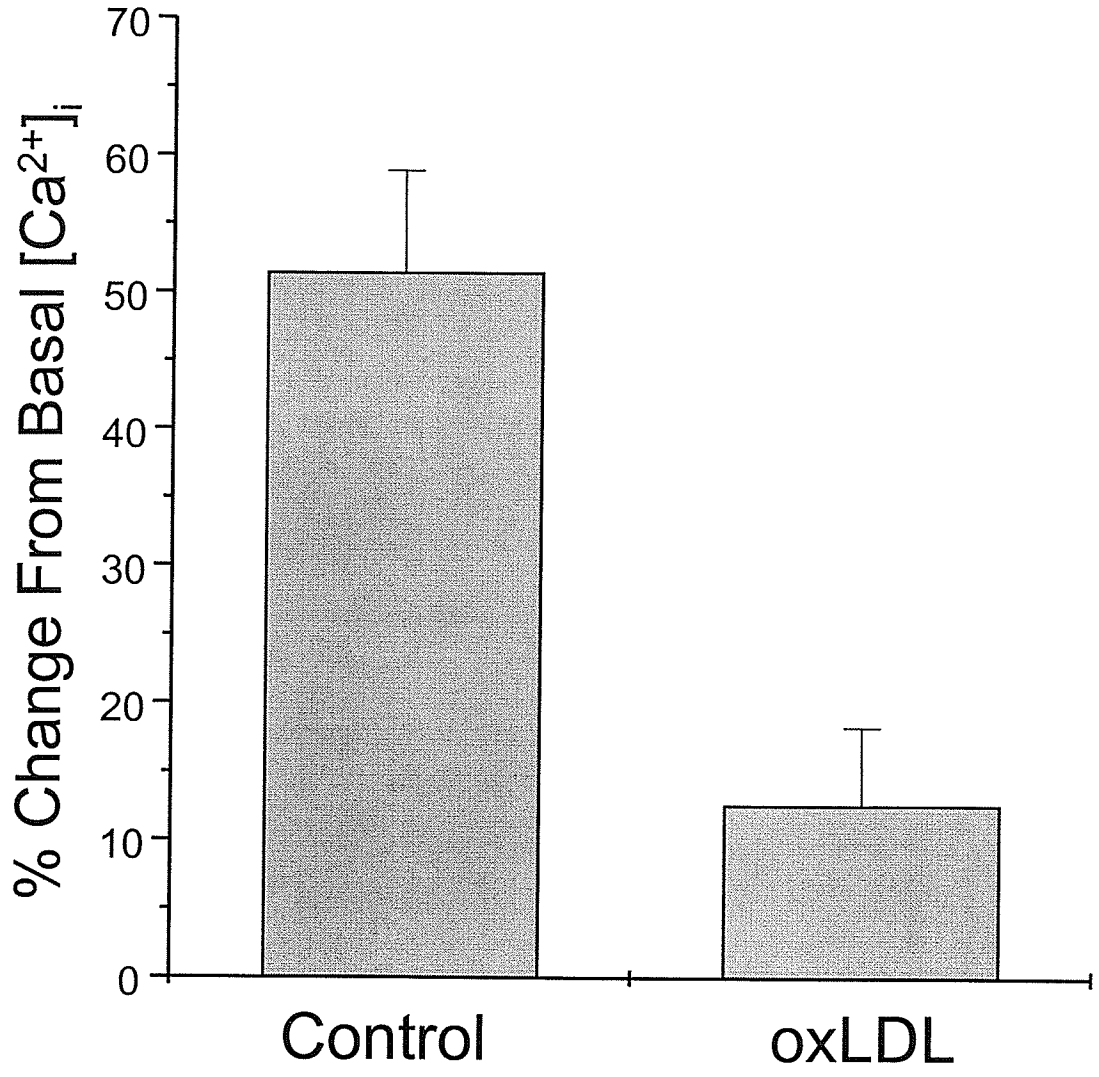


Figure 42. Effect of 1 μM thapsigargin on Ca^{2+}_i in control VSMC and VSMC treated with 0.025 mg/ml oxLDL. These cells were stimulated with 1 μM thapsigargin and the percentage change from basal level was calculated. Control cells exhibited a significant increase $[Ca^{2+}]_i$ over basal levels in response to thapsigargin ($p < 0.05$). Values represent the mean ± S.E. of 4-8 separate experiments. * $p < 0.05$ versus control.

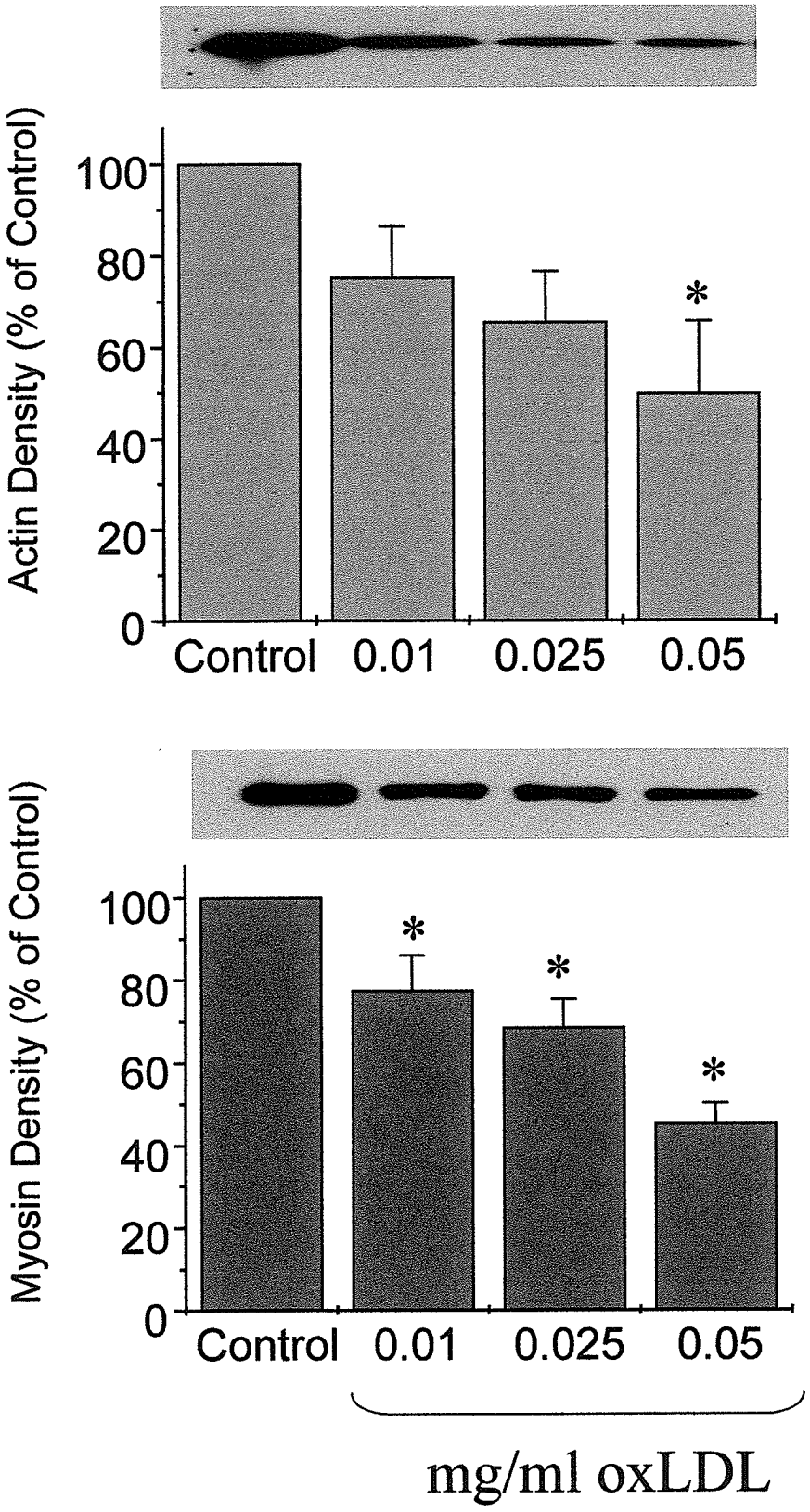


Figure 43. Effect of chronic treatment with oxLDL on α-actin and myosin content in VSMC. Cells were treated with a range of oxLDL (0.01 to 0.05 mg/ml) for 6 days and then probed by Western blot for densities of smooth muscle specific α-actin and myosin. Data are the mean ± S.E. of 6 separate experiments. * ***P < 0.05*** versus control.

This effect of oxLDL on actin and myosin content was also time dependent. Again, a trend to decrease both actin and myosin were observed in smooth muscle cells treated for 3 and 6 days with 0.05 mg/mL oxLDL but this was statistically significant only at the 6 day time point (Figure 44).

These changes were not only restricted to the content of these myofilaments within the smooth muscle cell. Surprisingly, oxLDL also induced a striking disorganization in these myofilaments within the smooth muscle cell. As shown in Figure 45a, using confocal microscopy and immunocytochemical staining, myosin was observed as long, uninterrupted filamentous structures in control cells. However, exposure of smooth muscle cells to 0.1 mg cholesterol/mL oxLDL for a period of 3 days induced a striking disorganization of the myosin filaments (Figure 45b-d). Interestingly, the myosin filaments were furled into large aggregates (as shown in Figure 45b, c, and d). Smooth muscle cells that exhibited visual evidence of a change in cytoskeletal organization were counted to determine the frequency of the abnormality. Fifteen, 24, or 87% of the cells treated for three days with 0.025, 0.05 or 0.1 mg/mL oxidized LDL, respectively, exhibited evidence of some cytoskeletal disorganization similar to that depicted in Figure 45. Because we were forced to employ different techniques (Westerns versus immunocytochemistry), it is difficult to conclusively determine if the myosin content decreased prior to the disorganization of cytoskeletal elements or visa versa. However, since 24% of cells exhibited cytoskeletal disorganization after 3 days of treatment with 0.05

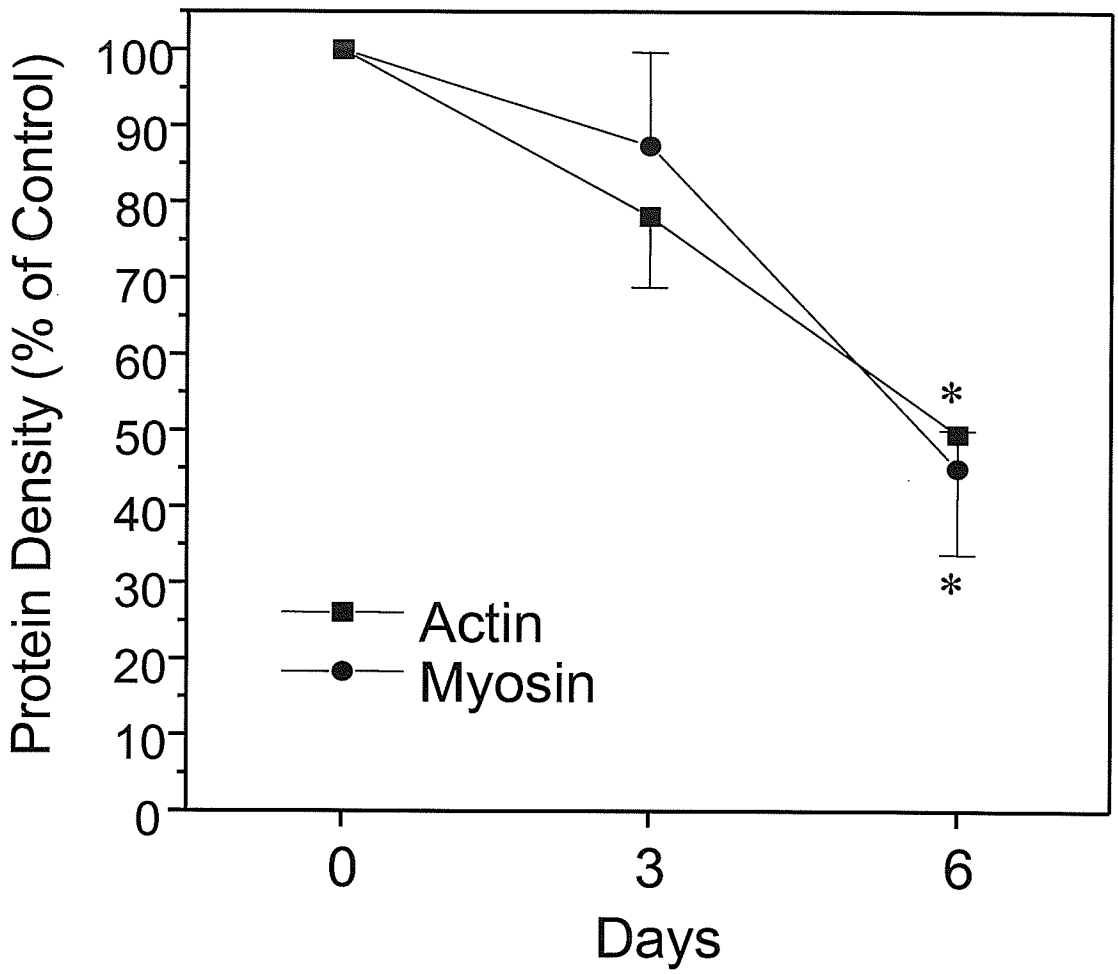


Figure 44. Time dependent effect of oxLDL on α-actin and myosin densities. VSMC were treated with 0.05 mg/ml oxLDL (for 0, 3, and 6 days) and cell extracts were analyzed by Western blots. Data are the mean ± S.E. of 6 separate experiments. * $P < 0.05$ versus control.

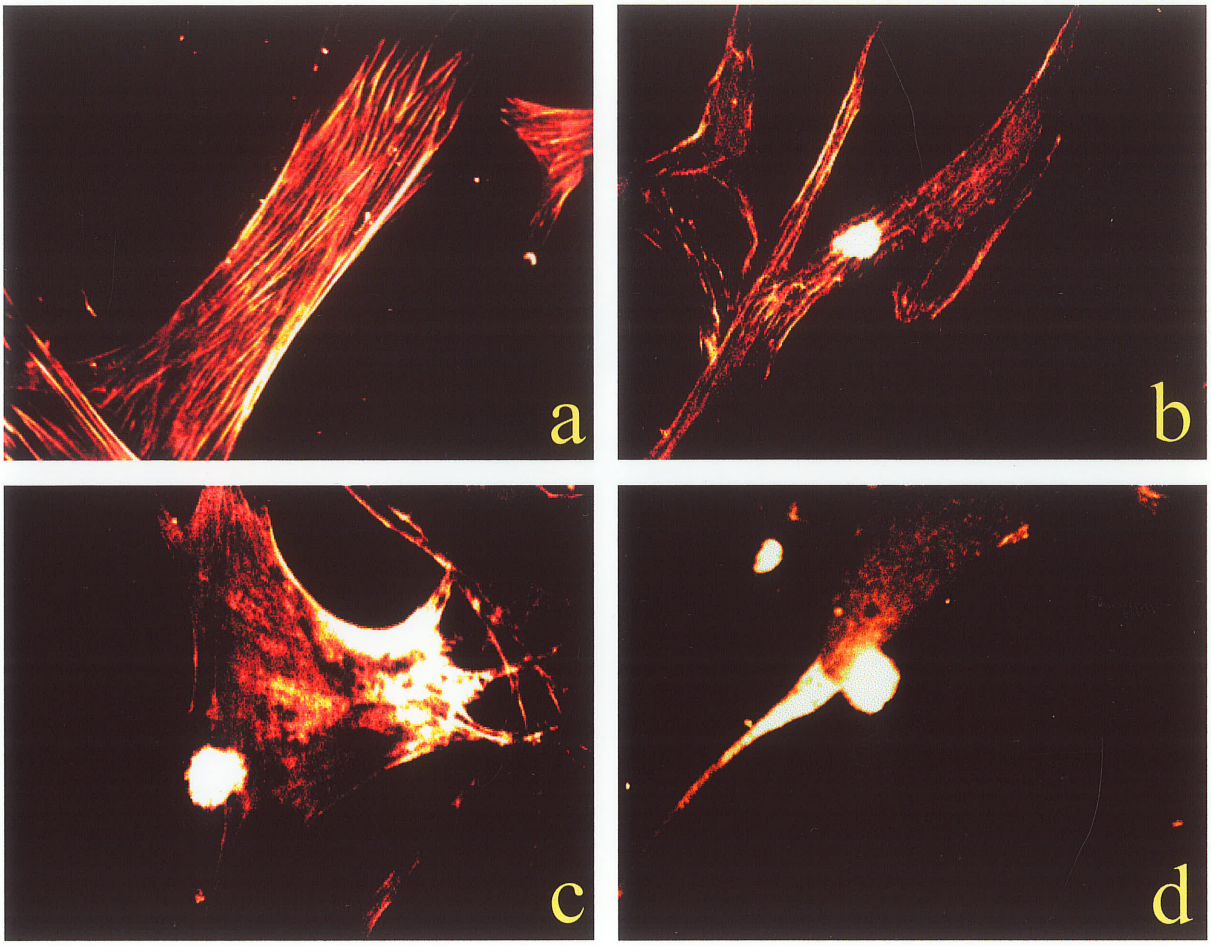


Figure 45. Effect of chronic treatment of VSMC with oxLDL on myosin organization and distribution. VSMC were treated with oxLDL for 3 days and immunostained with smooth muscle specific monoclonal anti-myosin antibody and FITC-conjugated secondary. These images were obtained with a Bio-Rad confocal microscope. a) control smooth muscle cell, b-d) cells treated with 0.1 mg/ml oxLDL.

mg/mL oxLDL and there was no significant effect of this treatment on cytoskeletal protein content (Figure 44), it is possible to suggest that the myofibrillar disorganization may have preceded the reduction in content.

Chronic exposure of cells for 0, 1, 3, and 5 days to a similar concentration of native LDL (0.1 mg cholesterol/mL) failed to induce myosin aggregation (Figure 46a, b, c, d). Cells treated with native LDL (0.1 mg cholesterol/mL) for one day showed no change from control with respect to myosin organization. At day 5, these cells began to show what may be interpreted as contracture bands but clearly provided no evidence of the striking aggregation observed in oxLDL-treated cells. The effect of oxLDL (0.1 mg cholesterol/mL) on myosin was time dependent. Smooth muscle cells were treated with oxLDL for 0, 1, 3, and 5 days (Figure 46e, f, g, h). The cells treated with oxLDL demonstrated early signs of myosin disorganization after only one day of exposure. These alterations were more pronounced at day 3. At this time, myosin filaments began to clearly form into aggregates (Figure 46g). These aggregations of myosin were more pronounced after 5 days. In some cases, large aggregates could be observed in close proximity to the plasma membrane. These appear to be in the process of being expelled from the cell (Figure 47).

Using the confocal microscope, the myosin filaments were optically sectioned in both control and oxLDL-treated smooth muscle cells. This allowed us to study the three dimensional distribution of myosin filaments in these cells. As shown in Figure 48a-f, the myosin filaments were intact and aligned

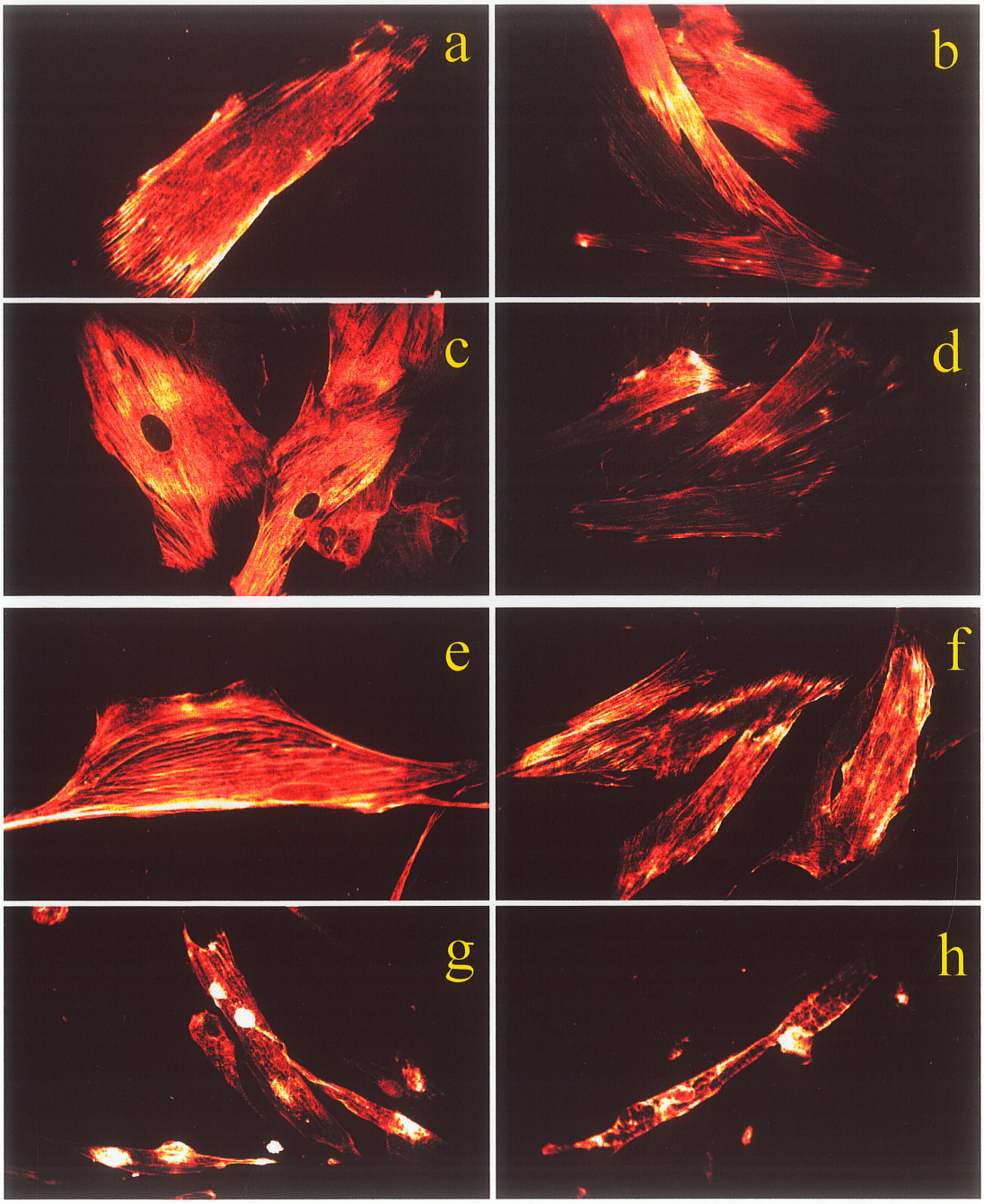


Figure 46. Time dependent effects of native and oxidized LDL on myosin organization in VSMC. VSMC were treated with native LDL (0.1 mg cholesterol/ml) (top panel) or oxidized LDL (bottom panel) and then immunostained with anti-myosin antibody. Cells were treated for different periods of time with native LDL [a) control cell, b) 1 day, c) 3 days, d) 5 days] and oxLDL [e) control, f) 1 day, g) 3 days, h) 5 days].

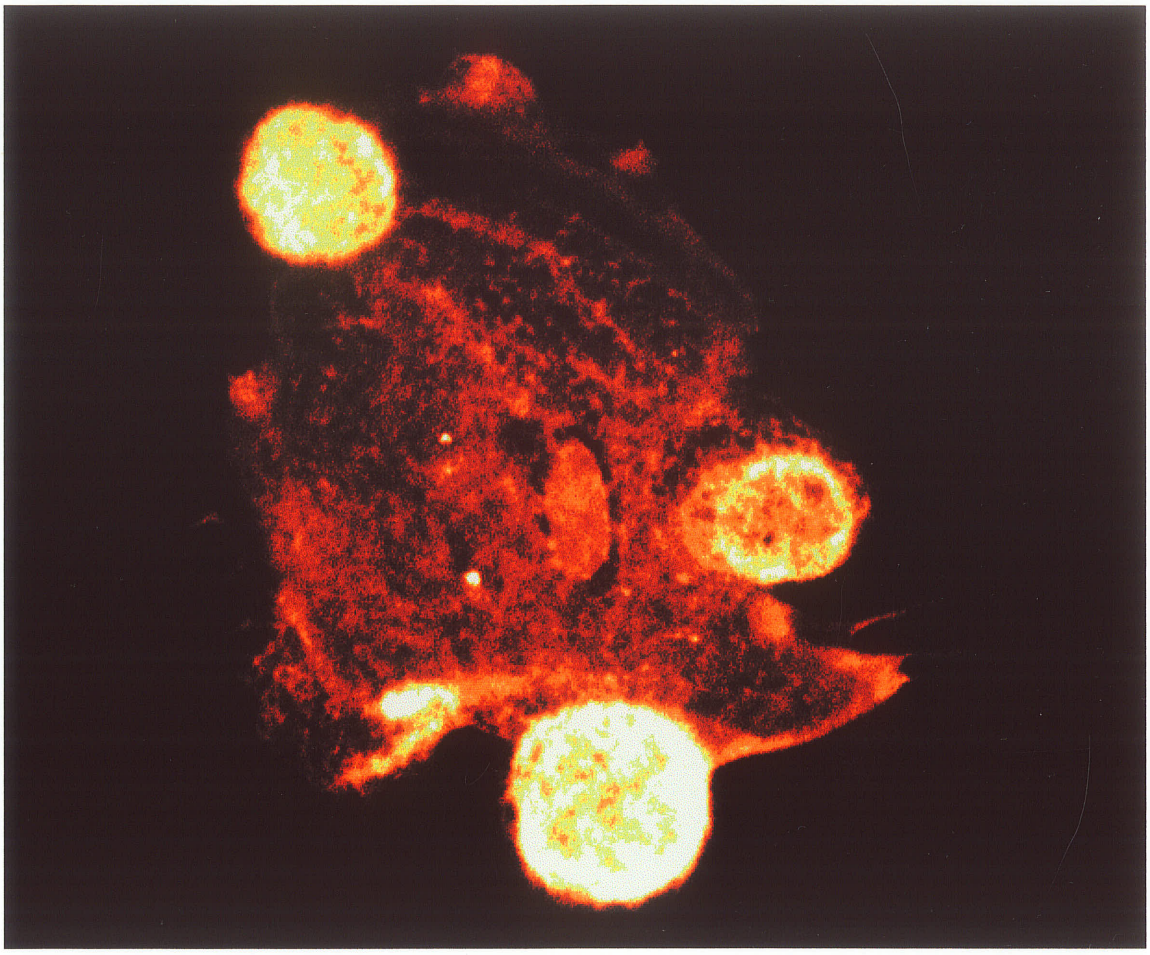


Figure 47. Aggregation of myosin filaments in oxLDL treated cells. Smooth muscle cells were treated with 0.1 mg/ml oxLDL for 3 days. Giant aggregates of myosin seem to be in process of being expelled from the cell.

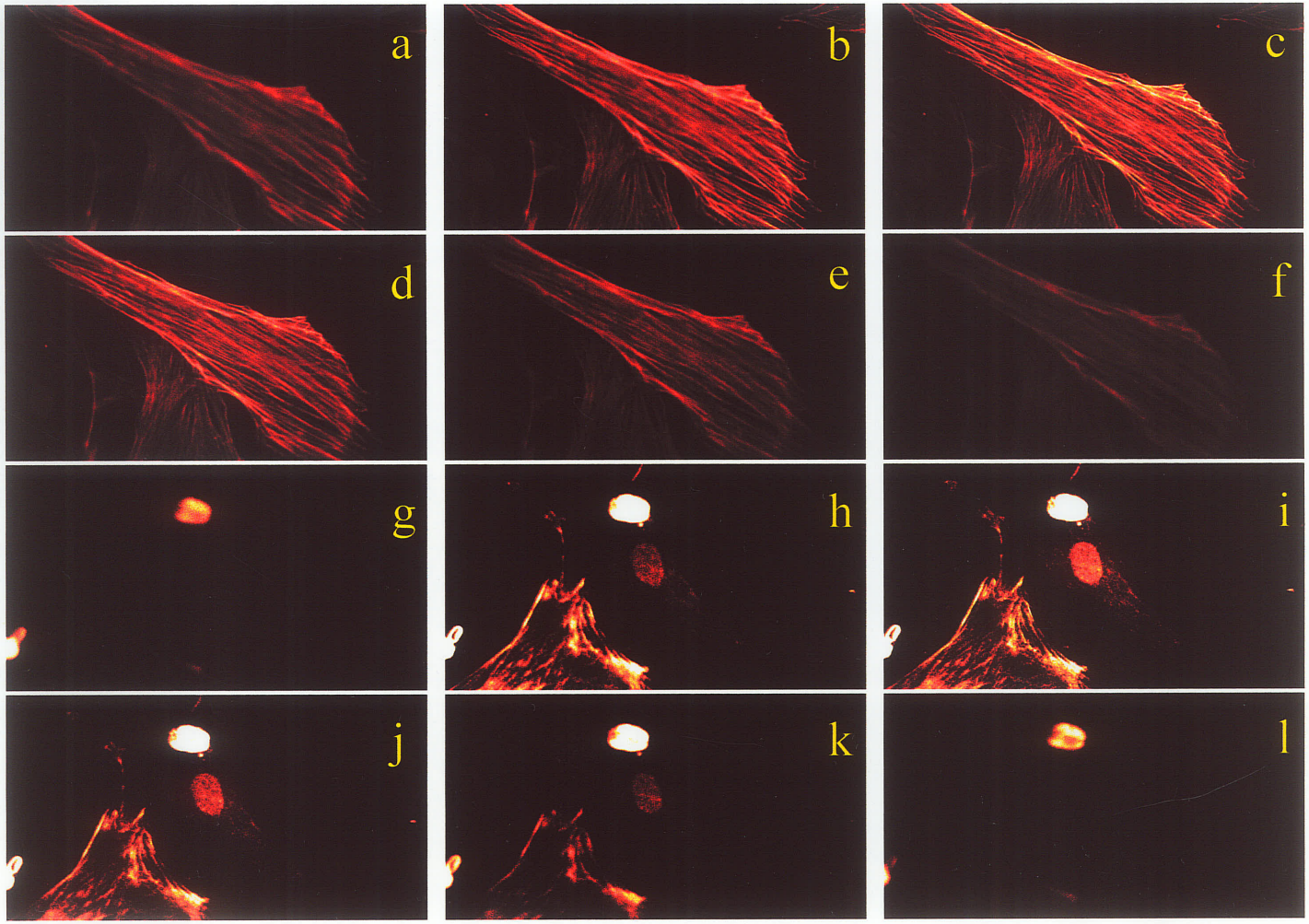


Figure 48. Optical sectioning with confocal microscope of control and treated cells. Cells were immunostained with anti-myosin and optically sectioned with a Bio-Rad confocal microscope. These sections are ~ 0.3 μm apart. Control (a, b, c, d, e, f) and oxLDL treated (g, h, i, j, k, l) cells.

throughout the control cells, whereas these filaments were disassembled and aggregated into a three dimensional ball in the smooth muscle cells treated with oxLDL (Figure 48g-l).

The effects of oxLDL were not limited to just myosin. Actin distribution was affected by oxLDL (0.1 mg cholesterol/mL) in a similar manner (Figure 49e-h). Whereas native LDL had little effect on actin distribution and aggregation within the cell (Figure 49a-d), oxLDL had a time dependent effect on actin organization and aggregation within the smooth muscle cells (Figure 49a-d).

It is possible that the effects on cytoskeletal proteins were induced by a proliferative action of the oxidized LDL on the smooth muscle cells. The ability of oxLDL to stimulate smooth muscle cells to grow and proliferate under our experimental condition was tested by examining for the induction of proliferating cell nuclear antigen (PCNA). Vascular smooth muscle cells were treated ± oxLDL (0.05 and 0.1 mg/mL) for 3 days (Figure 50). There was no statistically significant increase in the expression of PCNA by oxidized LDL under our experimental conditions. This would suggest that oxidized LDL did not induce a proliferative response. The lack of change in cell numbers after oxLDL treatment reported earlier in the result section of this manuscript would further support this conclusion.

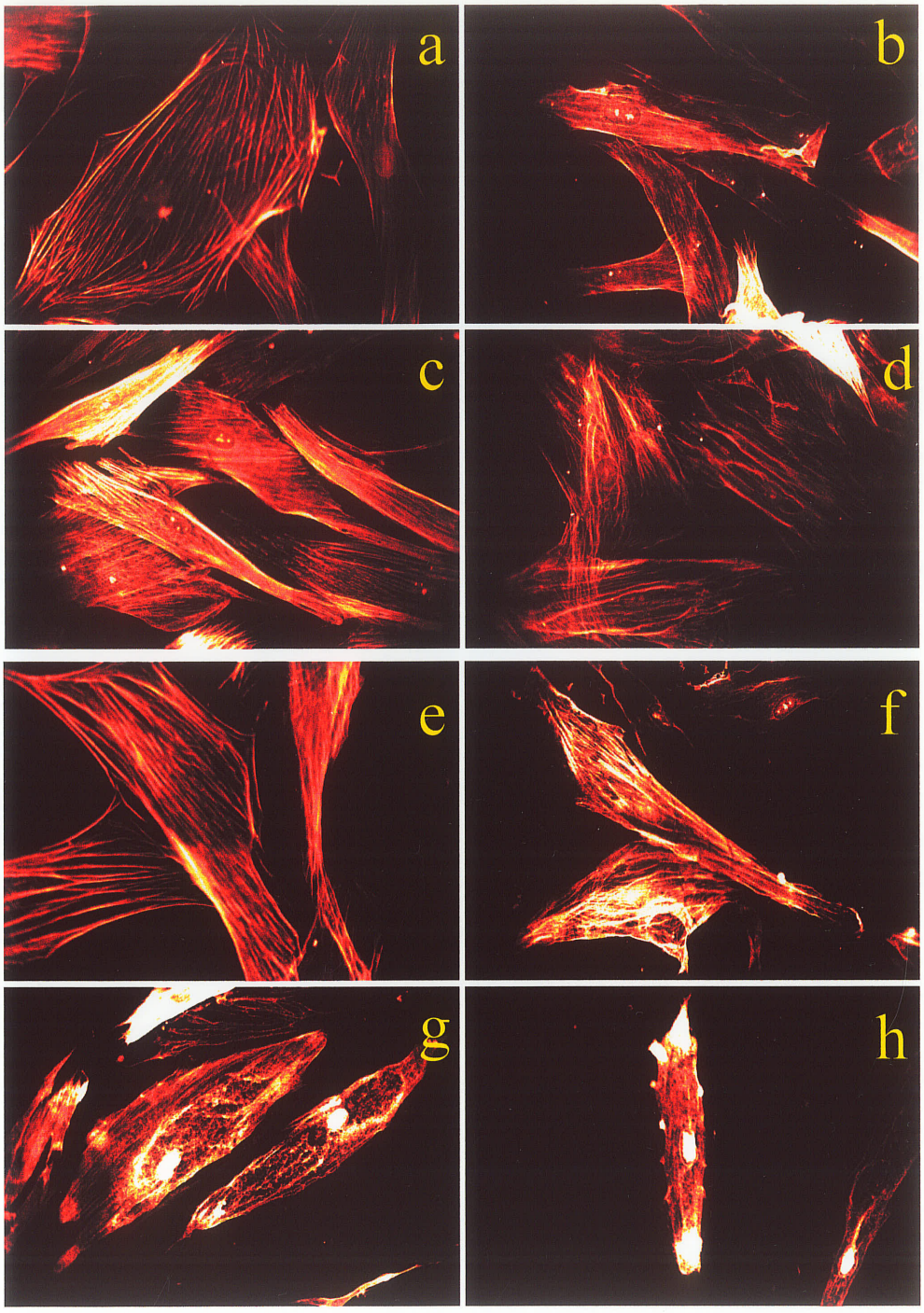
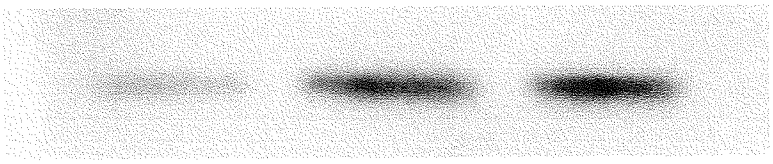


Figure 49. Time dependent effects of native and oxidized LDL on α-actin organization. VSMC were treated with native LDL (0.1 mg cholesterol/ml) (top panel) or oxidized LDL (bottom panel) and then immunostained with smooth muscle specific anti-α-actin antibody. Cells were treated for different periods of time with native LDL [a) control cell, b) 1 day, c) 3 days, d) 5 days] and oxLDL [e) control, f) 1 day, g) 3 days, h) 5 days].



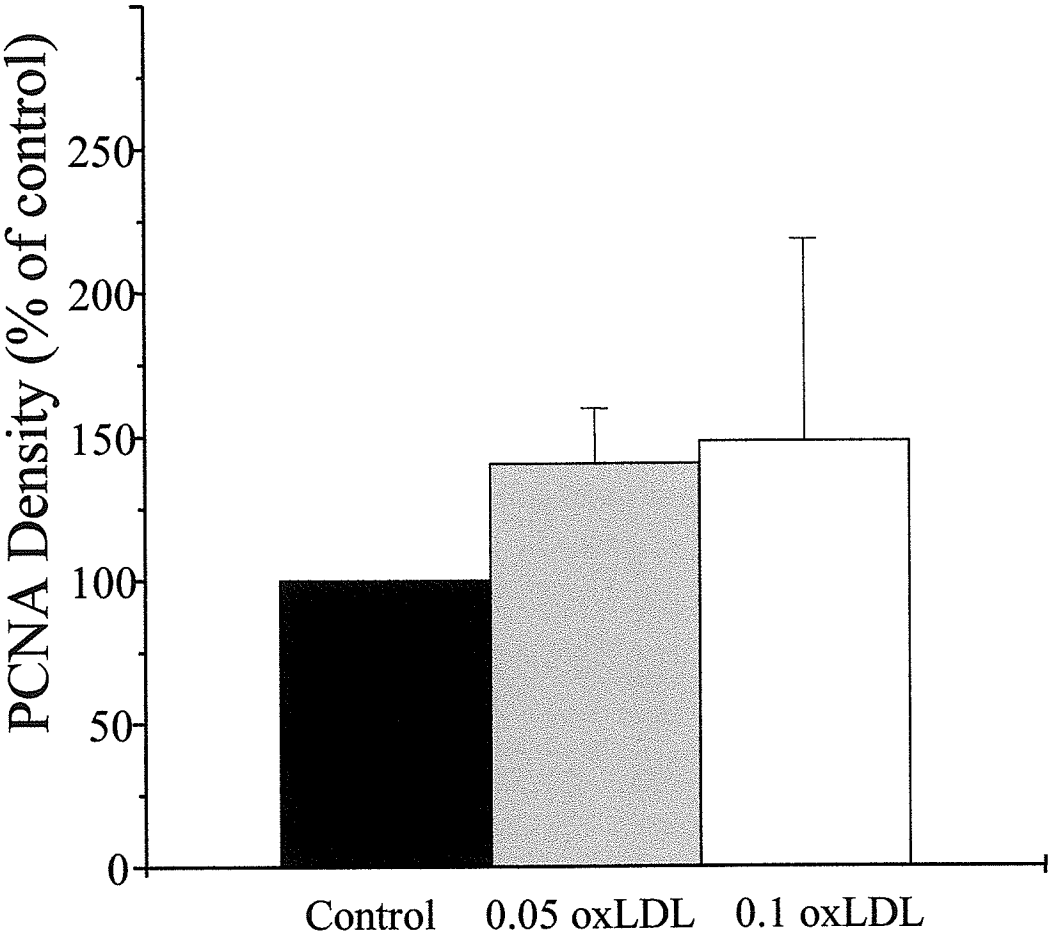


Figure 50. The effect of oxLDL on VSMC proliferation. Smooth muscle cells were treated ± oxLDL (0.05 or 0.1 mg/ml) for 3 days. The total protein was then extracted from these cells and analyzed by Western blots for proliferating cell nuclear antigen (PCNA). Data are the mean ± S.E. of 4 separate experiments.

E. DISCUSSION

I. Protective role of antioxidants against LDL oxidation:

These results demonstrate that lipophilic antioxidants (α-tocopherol and lazaroid) are more effective in protecting LDL from oxidation against free radicals generated by a Fe-ADP system than hydrophilic antioxidants (vitamin C and trolox). The protective effects of α-tocopherol and lazaroid occurred in a dose dependent fashion, whereas vitamin C and trolox had both oxidative and protective effects (Figure 1 and 2). Our data also showed that all of the antioxidants were more protective against water soluble free radicals generated by AAPH than lipid soluble free radicals generated by AMVN (Figure 5-7).

Lipid peroxidation was used in the present study as an index of LDL oxidation. LDL oxidized by free radicals exhibits other products of the oxidation process beside peroxides (107). Therefore, our data are limited to only this oxidation species. However, lipid peroxide products were chosen because of their pathological relevance. They play an important role in atherosclerosis (307). The TBARS assay was used to detect lipid peroxidation products in LDL. There are problems with protein interference inherent in using this method. However, because the LDL molecule is largely composed of lipid, this problem is minimized in our studies. Furthermore, this procedure remains the simplest, most widely used method for identifying the presence of lipid peroxide products and is reliable when used in isolated lipid fractions (107).

Our data demonstrated that α-tocopherol significantly protected lipoproteins against free radicals generated in an aqueous phase but not as well against free radicals generated within the LDL (Figure 6). The orientation of α-tocopherol within the LDL may explain these results. α-Tocopherol, a chain breaking antioxidant, consists of a chroman head group and a phytyl side chain. It is oriented with the chroman head group toward the surface and the hydrophobic phytyl side chain buried within the hydrocarbon region bilayer (284). The head group is responsible for scavenging free radicals, whereas the function of the side chain is believed to retain the α-tocopherol molecule in the membrane (284). Thus, free radicals generated by AMVN in the lipid moiety are in contact with the phytyl chain and further away from the α-tocopherol head group which is essential in the scavenging process. Conversely, the free radical scavenging head group at the surface would be optimal for protecting against free radicals generated in the aqueous phase. Consistent with this, α-tocopherol scavenging properties were significantly better when the free radicals were generated in the aqueous phase by AAPH (IC_{50} = 0.25 μM) as opposed to those generated in the lipid phase by AMVN (IC_{50} = 20.0 μM) (Table 1).

Pre-incubation of α-tocopherol with the LDL also increased its potency. This was likely due to the lipophilicity of α-tocopherol because the pre-incubation protocol did not affect the potency of trolox (which is not lipophilic). It is likely that the pre-incubation period allowed for a greater amount of the lipid

soluble α-tocopherol to be retained within the LDL. This would increase its potency to protect LDL. Distribution of α-tocopherol within the membrane has been shown to alter its antioxidant potency (279). Clearly then, lipophilicity is an important property in the antioxidant as well as the free radical generating system.

The present results also demonstrated that lazaroid (U74500A) can also protect LDL from oxidation by different free radical generating systems. Lazaroid has a steroid structure which can easily incorporate into the lipid bilayer. It has the ability to bind with iron and may act as a bilayer localized chelator of iron (36,37). It has been thought that this iron chelating action is critical in its capacity to act as an antioxidant (36, 37). Our data extend these findings with regard to the mechanism whereby lazaroid functions as an antioxidant. The present results demonstrate that lazaroid can act as a potent antioxidant even in the absence of an iron chelating effect. This conclusion is based upon two observations. First, lazaroid protected LDL from oxidation with a similar potency when the free radicals were generated either by Fe-ADP or AMVN (a free radical generating system which does not rely on iron). Second, when the peroxyl radicals were generated in the aqueous phase by AAPH (another free radical system which is not iron based), lazaroid almost completely inhibited peroxide formation.

When the free radicals were generated by Fe-ADP, both trolox and vitamin C showed a biphasic pro-oxidant and antioxidant effect (Figure 2).

Precisely how these compounds can function in both of these ways is unclear. Vitamin C can reduce ferric ions to ferrous ions which can generate free radicals more rapidly (184). Vitamin C can also be oxidized by oxygen to generate ascorbate radical anion and hydrogen peroxide (184). Trolox demonstrated a biphasic effect only when the free radicals were generated by the Fe-ADP system and not in the presence of AAPH and AMVN. A similar biphasic effect of trolox in an iron environment has also been reported by Laughton et al. (1991) (141). The mechanism whereby low concentrations of trolox promote oxidation is unclear.

In summary, our data demonstrate that the lipid solubility of both antioxidants and free radicals are very important in determining the extent of oxidative damage to LDL. Generally, lipophilic antioxidants demonstrated more potent scavenging properties than hydrophilic antioxidants. This may have important significance in atherosclerotic coronary artery disease. Oxidized LDL appears to play an important causative role in this disease. Antioxidants like α-tocopherol have been shown to offer significant protection against atherosclerotic heart disease (69, 222, 226, 289). These data are certain to stimulate research into the discovery of novel and more potent antioxidant agents. Our data demonstrate that the lipophilicity of an antioxidant is a very important characteristic to be considered during the development of more effective antioxidant agents in the future.

II. The acute effects of oxLDL on intracellular [Ca^{2+}]:

Our data demonstrate that the transient exposure of VSMC to oxLDL results in an immediate rise in $[Ca^{2+}]_i$. OxLDL induced the Ca^{2+} transient through a phosphoinositide-mediated release of Ca^{2+} from intracellular stores located in the SR. This conclusion is supported by several observations. First, oxLDL was capable of generating an increase in intracellular IP_3 levels (Figure 12). Secondly, a blocker of phospholipase C activity, NCDC, blunted the rise in intracellular IP_3 and $[Ca^{2+}]_i$ (Figure 13) Finally, the effects of oxLDL on VSMC Ca^{2+} could be observed even in the absence of extracellular Ca^{2+}(Figure 11). The effects of oxLDL are related to the oxidized products generated within the LDL. Native LDL (when applied to the cells soon after isolation) induced either no change or extremely small increases in VSMC $[Ca^{2+}]$ (data not shown). Fe-ADP at the same concentration employed had no effect on $[Ca^{2+}]_i$ on its own.

In contrast to cultured cells, freshly isolated portal vein smooth muscle cells did not respond to oxLDL with an increase in intracellular calcium over a short exposure time (Figure 19 and 20). These freshly isolated cells are elongated, calcium tolerant cells that respond to depolarization by KCl with an appropriate elevation in intracellular $[Ca^{2+}]$ and contraction (Figure 19 and 20). This lack of response to oxLDL could be due to an absence of oxLDL receptors. The expression of scavenger receptor is more predominant in cells stimulated with growth factors that lead to migrating and proliferating smooth muscle cells (124, 164, 165). Indeed it has been shown that smooth muscle cells lack

scavenger receptors *in vivo*. Pitas and coworkers have demonstrated, however, that scavenger receptors can be expressed in smooth muscle cells as a result of stimulation of these cells with phorbol ester (96, 164, 209). Smooth muscle cells maintained in culture have been proposed to be in a "pre-atherosclerotic" condition (54). This expression of oxLDL receptors and the subsequent development of an intracellular Ca^{2+} responsiveness in culture VSMC may be an important pathological process associated with the atherosclerotic condition *in vivo*. However, we have been unable to isolate cells by enzymatic digestion from an atherosclerotic plaque. Until this can be achieved, we can not directly address the validity of this contention.

Intracellular calcium has been suggested to play an important role in the development of atherosclerotic plaques. Excessive secretions of extracellular matrix proteins, cytokines and chemoattractants have all been associated with alterations in $Ca^{2+}{}_i$ (176, 208, 256). Just as importantly, changes in gene expression, cell growth and proliferation have been suggested to occur through changes in $[Ca^{2+}]_i$. Our studies have indicated that oxLDL is capable of inducing a release of Ca^{2+} from stores in proximity to the nucleus (Figure 14-16). This would suggest that oxLDL may be capable of inducing a Ca^{2+}-mediated signal which could participate in nuclear function. Loss of such signaling may have important implications. However, this remains to be studied in detail in the future. The present data, therefore, suggest that the acute capacity of the VSMC to respond to oxLDL may represent an initial signaling response of the cell to the

atherogenic environment. This may change dramatically as the cell advances in severity through the various stages of atherosclerosis to ultimately leave the vessel hyporesponsive to a variety of vasoactive agents. OxLDL may play a key role in changing the $Ca^{2+}{}_i$ regulatory mechanisms in VSMC during this process.

III. The chronic effects of oxLDL on intracellular Ca^{2+}:

The effects of oxLDL under acute exposure conditions contrast sharply with the effects of oxLDL after VSMC have been chronically exposed to low oxLDL concentrations. Chronic exposure of VSMC to oxLDL resulted in a loss in the capacity of oxLDL to initiate a Ca^{2+} transient (Figure 26 and 27). This alteration in calcium transient was due to the oxidation of LDL, since native LDL in a similar chronic setting did not show any effect on the calcium transient. The oxLDL preparation used in this study was minimally modified and the concentrations employed were 10-100 fold below those used by many other investigations which have tested the effects of oxLDL on cell function (Figure 10) (55, 162, 253). This points to the potency that small quantities of oxLDL have on cell function and emphasize the potential pathological significance of the effects observed.

Many studies have implicated oxygen-derived free radicals and oxLDL in the atherosclerotic process (233, 270, 271, 308). Are cells in the area of a developing plaque exposed to oxLDL for extended periods of time? No direct evidence is available, however, indirect evidence would support this contention.

For example, the generation of autoantibodies to oxLDL strongly suggests that the oxLDL is present for extended periods of time in the body (199, 290). Further, the autoantibody titers correlate with the severity of the plaque formation (199). These findings would suggest that the oxLDL must be elevated chronically. Further, it is likely that this oxLDL is trapped in the interstitial space where cells would be chronically exposed to it (270). Several other studies have demonstrated that [oxLDL] or its products increase within the plaque as the plaque advances in severity (49,276). These data do not prove that VSMC or other vascular cells are in contact with oxLDL over extended periods of time but it is a contention that would be entirely consistent with the general hypothesis of a role for oxLDL in the atherogenic process. Indeed, if one is to assign a role for oxLDL in atherosclerosis, it is far more difficult to argue that cells would be in contact with oxLDL for several minutes in vivo than the contention that cells in a developing atherogenic region are exposed chronically to oxLDL. Our data demonstrate that such conditions have important implications for VSMC function and that these effects are strikingly different that those previously reported under acute exposure periods.

Several factors may be eliminated in the present study as contributory factors to the effects of oxLDL. For example, this was not due to a cytotoxic effect of the oxLDL (Figure 23 and 24). Furthermore, this was not due to a change in VSMC morphology to that of a foam cell. We purposefully examined cells that maintained the spindle shape morphology that is typical of VSMC.

Interestingly, however, foam cells also failed to elicit a Ca^{2+} transient in response to any agonist tested. This would suggest that oxLDL may induce a transitional change in VSMC function that precedes significant morphological changes.

We have identified a few factors that may contribute to the inability of oxLDL to induce a Ca^{2+} transient after VSMC have been chronically exposed to oxLDL. We have focussed our work upon the effects that oxLDL had on intracellular signaling. The present data strongly suggest that the mechanism responsible for the oxLDL-induced change in Ca^{2+} responsiveness within the VSMC involves a lesion in IP_3 signaling. Since oxLDL appeared to induce a release of Ca^{2+} from SR via an IP_3-mediated mechanism under acute exposure conditions (Figures 12 and 13), it is reasonable to argue that the loss in IP_3 receptors under chronic exposure conditions is at least partly responsible for the altered Ca^{2+} transients. Consistent with this hypothesis, we demonstrate for the first time that IP_3 receptor density is strongly depressed in VSMC's exposed to oxLDL over time (Figure 32 and 33). We cannot rule out an alternate possibility that oxLDL binding to the VSMC is depressed after chronic incubation periods. However, cellular lipid accumulation continues even in the presence of elevated [oxLDL] (270). This would suggest that the cells have a limited capacity to down-regulate oxLDL receptors and thereby control oxLDL interactions with the cell (40, 270).

Furthermore, cells were tested for the responsiveness to other agonists that work through similar intracellular mechanisms but different cell surface

receptors. We used physiological agonists such as ATP (purinergic receptor), norepinephrine (α-adrenergic receptor) and endothelin-1 (endothelin receptor) (Figure 28-31). All of these agonists bind to their specific receptor leading to activation of G protein and PLC and formation of IP_3. This ultimately results in an increase of intracellular calcium. The response of the cells to ATP, a known activator of SR Ca^{2+} release (275, 280), was depressed in cells after chronic exposure to oxLDL. This is in striking contrast to the acute effects of ATP on control cells where $[Ca^{2+}]_i$ rises immediately (196, 274). A similar depression in calcium signalling was also observed with epinephrine and endothelin-1. These data strongly suggest that an intracellular mechanism is most likely involved in the loss of Ca^{2+} activation within VSMC chronically-treated with oxLDL.

IP_3 mediated calcium release is not the only channel responsible for the elevation of intracellular calcium as a result of external stimuli. Ryanodine sensitive Ca^{2+} release channels are critical proteins involved in the regulation of $[Ca^{2+}]_i$ in smooth muscle. The density and organization of ryanodine channels were significantly altered as the result of chronic exposure to oxLDL (Figure 35). More direct evidence of the effects of oxLDL on SR Ca^{2+} release was observed with the use of ryanodine. Ryanodine can induce an increase in intracellular Ca^{2+} concentration by opening Ca^{2+} release channels (178). Cells incubated chronically with oxLDL and then stimulated with ryanodine exhibited an attenuated Ca^{2+} transient (Figure 36). This is consistent with a lesion in ryanodine channel function and density.

The mechanism responsible for the down-regulation in IP_3 receptors and ryanodine channels is unclear. However, Wojcikiewicz and coworkers demonstrated a down-regulation in IP_3 receptor type I as a result of prolonged exposure of human neuroblastoma to carbachol (a muscarinig agonist). They suggested that persistent elevation in $[Ca^{2+}]_i$ maybe the mechanism leading to down-regulation of this receptors (309). A similar down-regulation of IP_3 receptors and desensitization of calcium release were also observed in rat A7r5 aortic smooth muscle cells as the result of chronic stimulation with vasopressin (254). Their data and other studies suggested that this down regulation was due to proteasomal degradation and accelerated proteolysis (254, 319)

Our study also provides important evidence that both IP_3 receptor and ryanodine channel density are decreased *in vivo* in the atherosclerotic plaque (Figure 37-39). Because the calcium release from IP_3 and ryanodine channels represents an important mechanism for controlling tension within the VSMC, these defects will have important effects on vasoactivity. Our finding agree well with observations that atherosclerosis leaves arterial smooth muscle unresponsive to vasoactive agents (14, 121, 291, 311). The present data demonstrate that cells that are in contact with oxLDL over extended periods of time exhibit an inability to elevate $[Ca^{2+}]_i$ to a level that would support normal contractile activity. The lesions identified in IP_3 signaling and $Ca^{2+}{}_i$ regulation in the present study would explain the inability of atherosclerotic vessels to exhibit contractile activity in response to vasoactive agents. Our data also demonstrate that these changes in

VSMC function precede any transformation in cellular morphology to the foam cell type that is typical of an atherosclerotic plaque.

In contrast to calcium release channels, we observed a significant increase in the density of SERCA2 ATPase calcium pump in the cells treated with 0.025 mg/ml oxLDL using immunocytochemistry (Figure 40 and 41). Cells treated with a very low concentration of oxLDL also demonstrated a significant increase in total SERCA2 ATPase pump (Figure 41). The mechanism involved in increase in SERC2 ATPase expression by oxLDL is not clear. It is also possible that chronic exposure to oxLDL will lead to a constant stimulation of smooth muscle cells and a transient alteration in $Ca^{2+}{}_i$. Smooth muscle cells appears to undergo an adaptive change in the SERCA2 pump to control $[Ca^{2+}]_i$ at normal levels. This occurs at the same time as these cells downregulate their IP_3 receptors and ryanodine sensitive Ca^{2+} release channels. The results demonstrate the complex action of oxLDL on intracellular Ca^{2+} regulation and protein expression in vascular smooth muscle cells.

IV. Chronic effect of oxLDL on cytoskeletal organization:

Our data demonstrate a significant effect of oxLDL on actin and myosin densities, organization and distribution. Chronic exposure of vascular smooth muscle cells to oxLDL resulted in a decrease in the total cellular protein content of both actin and myosin. It is well recognized that smooth muscle cells lose their contractile proteins in atherosclerotic lesions in vivo (46, 47, 130, 131).

Conversely, smooth muscle cells will also re-express contractile protein in the atheroma under conditions of lipid lowering therapy (2). Our findings, therefore, identify oxLDL as one component within the atherogenic milieu that has the ability to decrease the contractile protein content as the smooth muscle cell phenotype changes.

The concentration of oxLDL used in our work was many fold lower than those used in other studies of oxLDL (3, 33, 55, 253). We employed lower concentrations of oxLDL over longer incubation times. The extended duration of exposure of the cells to oxLDL in the present study was used to mimic more closely the in vivo conditions. Atherosclerosis is a slow, gradual disease. It is unlikely that oxLDL is present only for a limited period of time in relatively high concentrations. Instead, it is more reasonable to propose that small amounts of oxLDL may be in proximity to cells in the sub-endothelial space for extended periods of time. It is important to emphasize that the oxLDL preparation used was minimally modified as well. Our data, therefore, re-emphasize the potential importance of relatively low concentrations of oxLDL to induce phenotypic changes in myofilament composition within the atherogenic smooth muscle cell.

A novel and surprising finding in the present study was the identification of a striking change in the organization of the myofilaments in the smooth muscle cells. Smooth muscle cells chronically treated with oxLDL exhibited a disorganization of actin and myosin into large, ball-shaped aggregates. These findings are not without precedent in another cell type. For example, Colangelo

and co-workers recently showed that f-actin organization was altered in rabbits on a high cholesterol diet (56). Further, cultured human endothelial cells treated with ~200 μg cholesterol/ml oxLDL resulted in alterations in f-actin organization (318). However, the changes in f-actin organization were relatively minor in comparison to the large, striking aggregations of these filaments observed in the present study within vascular smooth muscle cells chronically treated with oxLDL. The higher content of contractile proteins and their linear alignment in smooth muscle cells probably accentuates the disorganizing effects of oxLDL as opposed to those observed in endothelial cells.

The mechanism whereby oxLDL induces the changes in myofilament organization is unclear. However, several possibilities exist based upon evidence obtained here and previously. Native LDL was unable to induce myofilament disorganization in the present study, suggesting that an oxidized component within the oxLDL was responsible for this action. OxLDL contains oxidized species of cholesterol that have been shown to induce myofilament disorganization in endothelial cells (201). This may occur through a MAP kinase mediated effect. MAP kinase activity has been associated with actin organization in endothelial cells (119). OxLDL has been shown to stimulate MAP kinase activity in smooth muscle cells (135). This stimulation occurred through a lipid soluble component within the oxLDL. It is possible, therefore, that oxLDL may stimulate MAP kinase activity in the smooth muscle cells to disorganize contractile proteins under our conditions. It is unlikely that intracellular Ca^{2+} is

involved as a mechanistic agent as proposed elsewhere (318). OxLDL cannot induce changes in intracellular Ca^{2+} under our incubation conditions due to alterations in sarcoplasmic reticulum function and structure. Thus, myofilament changes due to oxLDL were unlikely to be a result of changes in intracellular Ca^{2+} levels.

In summary, our data suggest that oxLDL is capable of decreasing myofilament content within smooth muscle cells in culture. Surprisingly, this effect was preceded by a striking disorganization within the cells that ultimately resulted in the formation of giant myosin and actin aggregates. In some dramatic cases in the present study, these aggregates appeared to be in the process of being expelled from these cells. This is interesting and may have both mechanistic and clinical implications. Mechanistically, this process may explain in part the decrease in myofilament proteins observed in Western blots in the present study. It is tempting to speculate that such a process may be the cause for the presence of autoantibodies to myofilaments in the plasma of patients with coronary artery disease (187). From a clinical standpoint, it is well known that smooth muscle cells are present in the cap of an atherosclerotic plaque and the loss of myofilaments from these cells may de-stabilize the plaque and contribute to rupture and thrombosis (2).

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