Chronic Exposure of Smooth Muscle Cells to Minimally Oxidized LDL Results in Depressed Inositol 1,4,5-Trisphosphate Receptor Density and Ca$^{2+}$ Transients

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Abstract—Oxidized LDL (oxLDL) (0.1 mg/mL) increased [Ca$^{2+}$]$_i$ in vascular smooth muscle cells (VSMCs) within 5 to 10 seconds of incubation. This increase was mediated via an inositol 1,4,5-trisphosphate (IP$_3$)-dependent release of Ca$^{2+}$ from the sarcoplasmic reticulum. However, atherosclerosis is a gradual process in which VSMCs are more likely exposed to low concentrations of oxLDL over extended periods 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$^{2+}$]$_i$. VSMCs were incubated with 4- to 100-fold lower [oxLDL] for up to 6 days. The conditions were not cytotoxic. Basal [Ca$^{2+}$]$_i$ was not altered. Surprisingly, however, after chronic exposure to oxLDL, a brief addition of oxLDL (0.1 mg/mL) or norepinephrine failed to elicit the expected rise in Ca$^{2+}$$_i$. Because the acute effects of oxLDL on control cells were mediated through an IP$_3$-dependent pathway, we investigated the integrity of the VSMC IP$_3$ receptors. Immunocytochemical analysis and Western blots revealed a depression in the density of IP$_3$ receptors after chronic exposure of VSMCs to oxLDL. These changes in IP$_3$ receptors have significance under atherosclerotic conditions as well. Immunocytochemical analysis revealed a decrease in IP$_3$ receptor density in the medial layer under atherosclerotic plaques in situ. Our data, therefore, demonstrate a striking difference between the acute and chronic effects of oxLDL on VSMC calcium. Whereas acute exposure to oxLDL stimulates [Ca$^{2+}$]$_i$, chronic exposure results in depressed Ca$^{2+}$ transients, apparently through a decrease in IP$_3$ receptor density. These changes have functional implications for the atherosclerotic vessel in vivo, and our data implicates oxLDL in this process. (Circ Res. 1999;85:515-523.)

Key Words: oxidized LDL • vascular smooth muscle cell • atherosclerosis • Ca$^{2+}$ • sarcoplasmic reticulum

The initiation and development of atherosclerotic lesions are correlated with circulating cholesterol and LDL levels.$^1$ Oxidative modification of LDL (oxLDL) enhances its atherosclerotic properties and may represent the first step in the initiation of atherosclerosis.$^1,2$ OxLDL can induce a massive deposition of cholesterol esters in vascular smooth muscle cells (VSMCs) and macrophages to transform them into foam cells within the atherosclerotic lesion.$^3,4$ However, the precise mechanism whereby oxLDL initiates and propagates the atherosclerotic lesion is still not known.

The current concept regarding the development of atherosclerosis is dominated by the assumption that the accumulation of lipids is the primary risk factor. Less research attention has been directed toward the role of calcium in this process. However, a significant increase in cellular calcium has been identified in atherosclerotic vessels.$^5$ The increase in cell calcium is thought to be involved in several important processes associated with atherosclerosis. These include abnormalities in VSMC contraction and proliferation, excessive secretion of extracellular matrix proteins (such as collagen, elastin, and proteoglycans), secretion of chemoattractants and growth factors, and platelet aggregation.$^6,7$

Recently, several studies have demonstrated that an acute administration of oxLDL generates a Ca$^{2+}$ transient in different cell types (for review, see Reference 8). The ability for a single exposure of oxLDL to generate a rapid Ca$^{2+}$ transient was suggested to be important in atherosclerosis. However, this stimulatory effect of an acute exposure of oxLDL on VSMC [Ca$^{2+}$]$_i$ appears to be in conflict with many studies that have shown arterial smooth muscle to be unresponsive to a variety of vasoactive agents under atherosclerotic conditions.$^9-12$ The discrepancy may be due to the time of exposure of VSMCs to oxLDL. Atherosclerosis is a relatively slow, gradual process. It may be more relevant to this disease state to selectively examine the cellular effects of oxLDL, an important atherosclerotic factor, over a period of time. The purpose of this study, therefore, was to determine the effect of oxLDL on [Ca$^{2+}$]$_i$ in VSMCs that have been chronically

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incubated with low [oxLDL]. Surprisingly, our results demonstrate that cells become nonresponsive to oxLDL after they are incubated for extended periods of time with oxLDL. This is accompanied by a striking change in inositol 1,4,5-trisphosphate (IP3) receptor density in VSMCs. This change is also observed in situ in the atherosclerotic plaques obtained from cholesterol-fed rabbits.

**Materials and Methods**

**LDL Isolation and Oxidation**
LDL (density 1.019 to 1.063 g/mL) was prepared by sequential ultracentrifugation. EDTA (0.1 mmol/L) was added throughout the isolation to prevent oxidation of LDL. The LDL fraction was extensively dialyzed against 0.15 mol/L NaCl and 0.1 mmol/L EDTA (pH 7.4), sterile-filtered, and stored at 4°C. The protein content and cholesterol content (free and esterified) of LDL were measured as described.

The absence of LDL oxidation during isolation or before its use in experiments was confirmed by an absence of malondialdehyde reactive products and oxidized cholesterol.

The EDTA concentration in native LDL was reduced before LDL oxidation. Native LDL was diluted 10-fold in 150 mmol/L NaCl (pH 7.4) and oxidized by incubation with a solution of 50 μmol/L FeCl3, and 0.25 mmol/L ADP for 3 hours at 37°C. The extent of LDL oxidation was evaluated by (1) measurement of thiobarbituric acid reactive substances, (2) electrophoretic mobility on agarose gels (using the Chiron Diagnostic Lipoprotein System), and (3) measurement of α-tocopherol content by high-performance liquid chromatography.

**Vascular Smooth Muscle Cells**
An explant technique was used to generate primary cultures of VSMCs from normal rabbit thoracic aorta. The aorta from a male New Zealand White rabbit (2.5 to 3 kg body weight) was isolated and cleaned of excess fat and connective tissue. The aorta was cut into 2- to 3-mm sections and transferred to a culture dish with growth medium (20% FBS in DMEM) and 5% antibiotic antymycotic (Gibco BRL). The explants were incubated in a humidified incubator equilibrated with 5% CO2 and maintained at 37°C. Initial migration of VSMCs was observed within the first 6 days. After 7 days of migration, these explants were transferred to a new culture dish for further migration. VSMCs from the second phase of migration were used in our experiments. To induce differentiation, VSMCs were placed (for 5 to 6 days) in a serum-free medium as described.

**Chronic Treatment of VSMCs With oxLDL**
VSMCs used in our experimental protocol were from either the first or second passage. The cells were serum-starved for 6 days before exposure to oxLDL. In chronic experiments, VSMCs were exposed for a period of 6 days to different concentrations of freshly prepared oxLDL (0.001 to 0.025 mg cholesterol/mL LDL). The medium containing freshly oxidized LDL was added daily to the cultured cells. The control cells were also kept in culture for the same length of time as the treated cells. The cytotoxicity of different concentrations of oxLDL was assessed by two tests: (1) lactate dehydrogenase (LDH) released into the culture medium and (2) ethidium homodimer staining of cell nuclei (Viability/Cytotoxicity Assay Kit, Molecular Probes).

**IP3 Content**
VSMCs with and without treatment with oxLDL were washed with ice-cold PBS, then scraped down and homogenized. This homogenate was used to measure d-myo-inositol 1,4,5-trisphosphate (IP3) content in VSMCs with a radioisotopic assay kit (Amersham).

**Immunocytochemistry**
VSMC phenotype was identified using monoclonal antibodies against smooth muscle α-actin, myosin, and caldesmon (Sigma-Aldrich). For the identification of IP3 receptors within VSMCs, quiescent cells were fixed and incubated with monoclonal anti-IP3 receptor antibodies that recognize all 3 types of IP3 receptors (ie, type I, II, and III) (Calbiochem). These cells were further incubated with a secondary antibody conjugated to FITC. The fluorescent images were obtained with a Bio-Rad MRC-600 confocal system.

**Immunohistochemistry**
The aortas from both control and 0.5% cholesterol-fed (12- to 14-week diet) male New Zealand White rabbits were cut into 5-mm sections and placed in a mold covered with O.C.T. embedding compound (Sakura Finetek, Torrance, Calif). These molds were frozen and cut into 7-μm sections using a Leitz 1720 Cryostat. Before use, these slides were fixed in 1:1 solution of acetone and methanol (−20°C) for 15 minutes. These sections were then blocked with 5% skim milk and incubated with monoclonal anti-IP3 receptor antibody (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). Rabbits were obtained from local suppliers and maintained in the Animal Care Facility at St. Boniface Hospital Research Centre under the guidelines of the Canadian Council on Animal Care.

**Western Blotting**
After treatment of VSMCs with oxLDL, cells were lysed with lysis buffer (1% SDS, 100 mmol/L NaCl, 62.5 mmol/L Tris-HCl [pH 7.6], 1 mmol/L PMSF, and 10 μg/mL leupeptin). Cell extracts were denatured with 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 minutes. The samples were separated on a 6% SDS polyacrylamide gel and transferred electrophoretically onto a polyvinylidene difluoride membrane before incubating with a polyclonal anti-IP3 receptor antibody (Chemicon International Inc.). The blots were incubated with HRP-conjugated goat anti-rabbit IgG. The IP3 receptor was detected with Pierce Super Signal detection system.

**Calcium Measurements**
Measurement of [Ca2+]i in a single cell was carried out using a Spex spectrophotofluorometer, as described in detail previously. Cultured VSMCs were loaded with 2 μmol/L fura-2 for 20 minutes at 22°C in a Krebs-Henseleit buffer (in mmol/L: NaCl 120, NaHCO3 25, KCl 4.8, KH2PO4, 1.2, MgSO4 1.25, CaCl2, 1.8, and dextrose 8.6). The fluorescent intensities of fura-2, which reflects bound and unbound calcium, were determined by exciting the cell at 340 and 380 nm and recording emission at 505 nm. The maximum and minimum fluorescence signals were obtained by adding 10 μmol/L 4-Bromo-A23187 and 5 mmol/L EGTA, respectively, at the end of the experiment to calibrate the signal with the [Ca2+]i.

**Statistical Analysis**
Data were expressed as mean±SE. 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 at P<0.05.

**Results**

**Acute Exposure of VSMCs to oxLDL**

*What Is the Mechanism Responsible for the Enhanced Ca2+ Transient After Acute Exposure of VSMCs to oxLDL?*

LDL was oxidized with an Fe-ADP free radical generating system. This LDL exhibited characteristics consistent with limited oxidation. The Fe-ADP-treated LDL exhibited a modest increase in electrophoretic mobility in comparison to native LDL, a limited 33% increase in malondialdehyde...
products indicative of lipid peroxidation and a 21% decrease in LDL vitamin E content (from 12.7 mg/L before oxidation to 10.1 mg/L after exposure to the Fe-ADP). These characteristics are consistent with the generation of a minimally modified oxLDL.

We first examined the effects of a concentration of oxLDL frequently used in other studies for the cellular effects of oxLDL.8 As shown in Figure 1, 0.1 mg/mL oxLDL caused a rapid increase in Ca²⁺. The peak increase in Ca²⁺ was observed within 5 to 10 seconds of exposure to oxLDL. The Fe-ADP free radical generating system did not have any effect on Ca²⁺ by itself. Native LDL elicited either no change or a modest increase in the Ca²⁺ transient in VSMCs (data not shown). Removing oxLDL from the solution bathing the VSMCs (Figure 1A) could eliminate the effects of oxLDL on Ca²⁺. The increase in [Ca²⁺]i induced by 0.1 mg/mL oxLDL was also observed in a calcium-free solution (Figure 1B). Therefore, the increase in Ca²⁺ induced by oxLDL likely resulted from a release of Ca²⁺ from the sarcoplasmic reticulum (SR). This increase in [Ca²⁺]i in response to oxLDL occurred in all cells irrespective of the basal [Ca²⁺].

Ca²⁺ release from the SR can occur via an IP₃ signaling mechanism.22 Therefore, oxLDL was tested for its capacity to increase VSMC IP₃ levels. The basal IP₃ concentration was ~20 pmol/mg protein. The IP₃ levels in VSMCs acutely exposed to 0.1 mg/mL oxLDL for 20 seconds increased by ~5 fold (Figure 2). To further identify the potential signaling pathway through which oxLDL acted to cause a release of calcium from the SR, we used NCDC, which disrupts the phosphatidylinositol cascade in the cell via an inhibition of phospholipase C.23 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 3). NCDC-treated cells were completely unresponsive in 3 other cases. Incubation of VSMCs with 50 μmol/mL NCDC blocked IP₃ formation even after stimulation with 0.1 mg/mL oxLDL (Figure 2).

**Chronic Exposure of VSMCs to oxLDL**

What Effect Does Chronic Exposure of VSMCs to oxLDL Have on Cell Integrity?

Although VSMCs were acutely responsive to oxLDL, these cells may have a different response after extended exposure to oxLDL in culture. VSMCs 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

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Representative results demonstrating the acute effect of oxLDL on Ca²⁺ in a single VSMC. A, OxLDL (0.1 mg cholesterol/mL) induced a rapid increase in Ca²⁺. The resting calcium level was reestablished by removing oxLDL from the solution bathing the cell. B, OxLDL (0.1 mg cholesterol/mL) induced a rapid increase in Ca²⁺ in a calcium-free solution.

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** Effect of chronic exposure to oxLDL on intracellular IP₃. VSMCs were treated with ±0.1 mg/mL oxLDL and in the presence or absence of 50 μmol/mL NCDC (phospholipase C inhibitor), and IP₃ concentration was quantified 20 seconds 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 IP₃ was quantitated as above. The IP₃ values were adjusted to milligrams of protein content. The values presented are mean±SE of 3 to 6 different experiments. **P<0.001 vs control.

![Figure 3](http://circres.ahajournals.org/)

**Figure 3.** Blunting of the effect of oxLDL on VSMC Ca²⁺ by NCDC. A, Effect of 0.1 mg cholesterol/mL oxLDL on a single cell. B, VSMCs were pretreated with 50 μmol/L NCDC for 30 minutes, then stimulated with 0.1 mg cholesterol/mL oxLDL. This is a representative recording from one cell.
changed daily. This medium contained freshly prepared oxLDL. Control cells were maintained for the same amount of time in an identical medium except for the addition of oxLDL.

OxLDL has a cytotoxic effect on cultured cells. Therefore, it was important to assess the effect of these concentrations of oxLDL on the viability of VSMCs. The viability of VSMCs was tested using the Live/Dead assay and also examined with LDH release from the cells. Incubation of cells with 0.025 mg/mL oxLDL over a 6-day period did not induce a cytotoxic effect as determined by the Live/Dead assay, nor was there a significant release of LDH into the medium in comparison to control untreated cells (data not shown). Subsequent experiments, therefore, were undertaken at [oxLDL] ≤ 0.025 mg/mL.

**Will Chronic Exposure of VSMCs to oxLDL Alter Basal [Ca\(^{2+}\)]?**

A small proportion of the VSMCs 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 that is typical of VSMCs. 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 among the different experimental groups (P > 0.05). [Ca\(^{2+}\)], was 182 ± 22 nmol/L (n = 24), 154 ± 14 nmol/L (n = 32), and 219 ± 19 nmol/L (n = 22), for cells treated with 0, 0.005, and 0.025 mg/mL oxLDL, respectively.

**Will Chronic Exposure to oxLDL Alter Cellular Ca\(^{2+}\) Transients in Response to Subsequent Stimulation by oxLDL?**

VSMCs were incubated with oxLDL for 6 days, then washed and examined for their ability to respond with an increase in [Ca\(^{2+}\)], to an acute application of 0.1 mg/mL oxLDL. The extended treatment of VSMCs with different [oxLDL] had a pronounced effect on Ca\(^{2+}\), regulation. Some, but not all, of the VSMCs responded to oxLDL with an increase in Ca\(^{2+}\). By examining the number of cells that responded to oxLDL with a rise in [Ca\(^{2+}\)], in comparison to the total number of cells tested in each experimental group, a responder ratio was obtained (Figure 4). Less than 10% of VSMCs treated with higher [oxLDL] (0.01 and 0.025 mg/mL) for 6 days subsequently responded to 0.1 mg/mL oxLDL with a change in Ca\(^{2+}\). A relatively low [oxLDL] (0.005 mg/mL) led to a 40% decrease in responder ratio compared with control. There was no difference in responder ratio between control VSMCs and the cells chronically treated with 0.001 mg/mL oxLDL.

The Ca\(^{2+}\) transient was altered even in the cells that did respond to oxLDL. As shown in representative recordings from single cells (Figure 5), 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. VSMCs 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). The chronic incubation of VSMCs 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\(^{2+}\), transients. However, it is also interesting to note that the lowest [oxLDL] tested (0.001 mg/mL) induced a Ca\(^{2+}\) transient with a half-time that was approximately twice as long as the control cells (Table).

To test whether oxLDL is responsible for the alteration in the Ca\(^{2+}\) transient, we used native LDL in the same chronic setting. VSMCs 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 cells responded to oxLDL with a Ca\(^{2+}\) transient (data not shown).

To test the responsiveness of chronically treated cells to other agonists that target SR calcium release, we used norepinephrine (α-adrenergic agonist). Binding of norepinephrine to its α-adrenergic receptor will result in activation of phospholipase C, formation of IP\(_3\), and [Ca\(^{2+}\)], release from the SR. As shown in Figure 6, control VSMCs responded to norepinephrine with a rapid increase in [Ca\(^{2+}\)], whereas VSMCs treated with 0.025 mg/mL oxLDL for 6 days failed to show an increase in [Ca\(^{2+}\)], after exposure to norepinephrine.
Percentage of Change in \([\text{Ca}^{2+}]_i\), From Basal Level in Smooth Muscle Cells Chronically Exposed to oxLDL for 6 Days

<table>
<thead>
<tr>
<th>[oxLDL], mg/mL</th>
<th>0</th>
<th>10</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.04±0.05</td>
<td>69.48±11.1</td>
<td>62.8±12.5</td>
<td>45.5±9.0</td>
<td>28.6±5.2</td>
<td>20.7±3.1</td>
<td>17.1±2.8</td>
</tr>
<tr>
<td>0.001</td>
<td>0.02±0.02</td>
<td>57.7±17.6*</td>
<td>71.1±22.4*</td>
<td>69.0±19.6*</td>
<td>54.5±11.6*</td>
<td>44.1±10.6*</td>
<td>37.8±11.1*</td>
</tr>
<tr>
<td>0.005</td>
<td>0.10±0.06</td>
<td>13.0±4.3*</td>
<td>12.2±3.7*</td>
<td>10.7±2.7*</td>
<td>10.1±1.9*</td>
<td>10.0±2.6*</td>
<td>8.1±2.9</td>
</tr>
<tr>
<td>0.0075</td>
<td>0.08±0.07</td>
<td>8.6±6.3*</td>
<td>9.1±6.8*</td>
<td>7.7±4.8*</td>
<td>8.6±3.2*</td>
<td>7.5±3.0*</td>
<td>8.0±3.1</td>
</tr>
<tr>
<td>0.01</td>
<td>0.03±0.08</td>
<td>0.26±0.2*</td>
<td>0.9±0.4*</td>
<td>0.3±0.4*</td>
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<tr>
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<td>0.07±0.05</td>
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<td>2.4±1.3*</td>
<td>2.4±1.3*</td>
<td>1.5±1.3*</td>
</tr>
</tbody>
</table>

Values represent mean±SE of 4 to 20 separate experiments. *P<0.05.

**What Is the Mechanism Responsible for the Chronic Effects of oxLDL on VSMC Ca^{2+}?**

VSMCs may have lost their capacity to respond to oxLDL with an increase in \([\text{Ca}^{2+}]_i\), because of (1) a decrease in oxLDL binding to the cell surface or (2) an alteration in the intracellular signaling, which effected the increase in \([\text{Ca}^{2+}]_i\). Because 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. However, we could address the second possibility. As demonstrated previously (Figure 2), the acute effects of oxLDL on \([\text{Ca}^{2+}]_i\), were likely due to an IP_{3}-mediated release of \([\text{Ca}^{2+}]_i\) from the SR. Therefore, it was reasonable to hypothesize that an extensive incubation of VSMCs with oxLDL resulted in a change in the IP_{3} signaling pathway. VSMCs were treated with 0.025 mg/mL oxLDL for 3 or 6 days. These cells were then fixed and treated with a monoclonal antibody against the IP_{3} receptor. This anti-IP_{3} antibody recognizes all 3 known isoforms of the IP_{3} receptor (type I, II, and III). However, because VSMCs contain 73% of type I IP_{3} receptors, the signal likely emanates mostly from type I IP_{3} receptors.

Figure 7a through 7c 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.

VSMCs that were treated with oxLDL for 3 days (Figure 7d through 7f) 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 7g through 7i). This was quantitated by Western immunoblots. As shown in Figure 8, incubation of VSMCs 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 2). Thus, chronic exposure of VSMCs to oxLDL leads to a significant depression in IP_{3} signaling in the cells due to an attenuated generation of IP_{3} and a decrease in IP_{3} receptor density.

**Are the Changes in IP_{3} Receptor Density Found in Atherosclerotic Tissue In Situ?**

The possibility exists that the cell culture conditions used may not mimic the in vivo situation in atherosclerosis. It is unknown whether vascular IP_{3} receptors are affected by atherosclerosis. Aortic tissue was removed from rabbits fed a 0.5% cholesterol-supplemented diet for 12 to 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 9). IP_{3} receptors stained strongly in the medial layer of control aorta (Figure 9A), whereas this staining was reduced in the medial section of atherosclerotic tissue (Figure 9B). This difference in IP_{3} receptor density was also observed in aortic sections that contained both plaque and an unaffected area (Figure 9C). As shown in Figure 9C, IP_{3} receptor density was higher in the medial layer of an area with no plaque, whereas the same medial layer below the plaque region exhibited a reduction in fluorescence intensity.

**Discussion**

**Acute Effects of oxLDL**

Our data demonstrate that the transient exposure of VSMCs to oxLDL results in an immediate rise in \([\text{Ca}^{2+}]_i\). OxLDL induced the \([\text{Ca}^{2+}]_i\) transient through a phosphoinositide-mediated release of \([\text{Ca}^{2+}]_i\) 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. Second, a blocker of phospholipase C activity, NCDC, blunted the rise in intracellular IP_{3} and...
Third, the effects of oxLDL on VSMC Ca$^{2+}$ could be observed even in the absence of [Ca$^{2+}$], and finally, the inactivation of SR function by prior treatment with ryanodine blocked the effects of oxLDL on VSMC Ca$^{2+}$ (data not shown). 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 used at the same concentration had no effect on [Ca$^{2+}$] on its own.

**Chronic Effects of oxLDL**

The effects of oxLDL under acute exposure conditions contrast sharply with the effects of oxLDL after VSMCs have been chronically exposed to low [oxLDL]. Chronic exposure of VSMCs to oxLDL resulted in a loss in the capacity of oxLDL to initiate a Ca$^{2+}$ transient. This alteration in Ca$^{2+}$ transient was due to the oxidation of LDL, because native LDL in a similar chronic setting did not show any effect on the Ca$^{2+}$ transient. The oxLDL preparation used in the present study was minimally modified, and the concentrations used were 10- to 100-fold below those used by many other investigations that have tested the effects of oxLDL on cell function.8,27,28 This reinforces the potency that small quantities of oxLDL have on cell function and emphasizes the potential pathological significance of the effects observed.

Many studies have implicated oxygen-derived free radicals and oxLDL in the atherosclerotic process.1-4 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. Further, the autoantibody titers correlate with the severity of the plaque formation. These findings would suggest that the oxLDL must be elevated chronically. It is also likely that this oxLDL is trapped in the interstitial space and cells would be chronically exposed to it. Several other studies have demonstrated that oxLDL or its products increase within the plaque as the plaque advances in severity. These data do not prove that VSMCs 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

Figure 9. IP3 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. Sections (7 μm each) were prepared and incubated with anti-IP3 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 IP3 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. D, Immunofluorescence exhibited by a section stained with preimmune sera. IEL indicates internal elastic lamina; M, medial layer or tunica media; and A, adventitial layer or tunica adventitia.
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 than 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. 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 VSMCs. Interestingly, however, foam cells also failed to elicit a Ca$^{2+}$ transient in response to any agonist tested (data not shown). This would suggest that oxLDL may induce a transitional change in VSMC function that precedes significant morphological changes.

We have identified one factor that may contribute to the inability of oxLDL to induce a Ca$^{2+}$ transient after VSMCs have been chronically exposed to oxLDL. We have focused our work on 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 VSMCs involves a lesion in IP$_3$ signaling. Because oxLDL appeared to induce a release of Ca$^{2+}$ from SR via an IP$_3$-mediated mechanism under acute exposure conditions (Figures 1 through 3), 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 VSMCs exposed to oxLDL over time. We cannot rule out an alternative possibility that oxLDL binding to the VSMCs is depressed after chronic incubation periods. However, cellular lipid accumulation continues even in the presence of elevated [oxLDL].$^1$ This would suggest that the cells have a limited capacity to downregulate oxLDL receptors and thereby control oxLDL interactions with the cell.$^1$ Furthermore, the observation that another agonist (norepinephrine) that binds to a different surface receptor than oxLDL but initiates the same IP$_3$ signaling pathway as oxLDL also is incapable of generating a Ca$^{2+}$ transient argues strongly that the primary defect is the IP$_3$ signaling.

The mechanism responsible for the downregulation in IP$_3$ receptors is unclear. However, Wojcikiewicz et al.$^34$ demonstrated a downregulation in IP$_3$ receptor type I as a result of prolonged exposure of human neuroblastoma to carbachol (a muscarinic agonist). They suggested that persistent elevation in [Ca$^{2+}$], may be the mechanism leading to downregulation of these receptors. A similar downregulation of IP$_3$ receptors and desensitization of Ca$^{2+}$ release were also observed in rat A7r5 aortic smooth muscle cells as the result of chronic stimulation with vasopressin.$^{35}$ Their data and other studies suggested that this downregulation was due to proteasomal degradation and accelerated proteolysis.$^{35,36}$ Ultimately, IP$_3$ generation was also depressed in VSMCs chronically treated with oxLDL (Figure 2). This would argue against an association of elevated [IP$_3$], with a downregulation of IP$_3$ receptors.$^{36}$ The depression in IP$_3$ generation also suggests an inhibition of phospholipase C activity by oxLDL over extended periods of time. Definitive proof of this hypothesis awaits experimental testing.

Our study also provides important evidence that IP$_3$ receptor density is decreased in vivo in the atherosclerotic plaque. Because the IP$_3$ pathway represents an important mechanism for controlling tension within VSMCs (via its effects on Ca$^{2+}$), these defects will have important effects on vasoactivity. Our findings agree well with observations that atherosclerosis leaves arterial smooth muscle unresponsive to vasoactive agents.$^{9–12}$ The present data demonstrate that cells that are in contact with oxLDL over extended periods of time exhibit an inability to elevate [Ca$^{2+}$], to a level that would support normal contractile activity. The lesions identified in IP$_3$ signaling and Ca$^{2+}$, 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.

[Ca$^{2+}$] also has been suggested to play an important role in the development of atherosclerotic plaques. Excessive secretions of extracellular matrix proteins, cytokines, and chemotactants have been associated with alterations in Ca$^{2+}$.$^6,7,37$ Just as important, changes in gene expression, cell growth, and proliferation have been suggested to occur through changes in [Ca$^{2+}$]. Our initial studies have indicated that oxLDL is capable of inducing a release of Ca$^{2+}$ from stores in proximity to the nucleus. This would suggest that oxLDL may be capable of inducing a Ca$^{2+}$-mediated signal that 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 VSMCs to respond to oxLDL may represent an initial signaling response of these cells to the atherogenic environment. This may change dramatically as these cells advance in severity through the various stages of atherosclerosis, ultimately rendering the vessel hyporesponsive to a variety of vasoactive agents. OxLDL may play a key role in changing the Ca$^{2+}$ regulatory mechanisms in VSMCs during this process.

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References


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