Free Rad. Res., Vol. 34, pp. 9-16
Reprints available directly from the publisher
Photocopying permitted by license only

Increase in Nuclear Calcium in Smooth Muscle Cells Exposed to Oxidized Low Density Lipoprotein

HAMID MASSAELI, CECILIA HURTADO, J. ALEJANDRO AUSTRIA and GRANT N. PIERCE*

Division of Stroke & Vascular Disease, St. Boniface General Hospital Research Centre, and the Department of Physiology, University of Manitoba, Winnipeg, Canada

Accepted for publication by Prof. B. Halliwell

(Received March 17, 2000; Revised May 05, 2000)

Vascular smooth muscle cells respond with an increase in intracellular Ca^{2+} within seconds after exposure to oxidized low density lipoprotein (oxLDL). This has been suggested to represent a signaling response that may have implications for gene expression. If so, oxLDL may induce increases in nuclear Ca^{2+} in smooth muscle cells in response to oxLDL. Aortic smooth muscle cells were exposed to 100 μg/ml oxLDL. Large, rapid increases in $[Ca^{2+}]_i$ were observed using fluo-3 as an indicator dye to detect intracellular Ca^{2+}on the stage of a confocal microscope. This was also confirmed using ratiometric imaging of indo signals. These elevations appeared to be localized to the nuclear region of the cell. DNA staining of the cells confirmed its localization to the nuclear/perinuclear region of the cell. Our data demonstrate that oxLDL induces a nuclear localized elevation in Ca^{2+}i that may have important implications for nuclear function.

Keywords: nucleus, atherosclerosis, inositol trisphosphate, gene expression, free radicals

INTRODUCTION

Free radical-induced oxidation of low density lipoprotein (oxLDL) has been identified as a potentially important atherogenic process[1,2]. OxLDL is chemotactic, cytotoxic, has effects on cell proliferation, and can induce foam cell formation[1,2]. All of these processes are thought to be important in generating an atherosclerotic plaque[1,2]. OxLDL can also induce a Ca^{2+} transient in a variety of cells[3–7]. This usually occurs within seconds[4–6]. Many of the processes associated with atherosclerosis (and identified above) are known to be affected by changes in $[Ca^{2+}]_i$[4]. Thus, the effects of oxLDL on $Ca^{2+}{}_i$ have been proposed to function as a signaling response within the cell, possibly to alter gene expression during atherosclerotic conditions[4,7]. If oxLDL-induced changes in $Ca^{2+}{}_i$ are important in an aspect of nuclear function like gene expression, then one might expect changes in nuclear Ca^{2+} levels as a function of exposure to oxLDL. Nuclear Ca^{2+} has been suggested to be involved in cell proliferation, gene expression, excitation-contraction coupling and cell death[8–12]. Many of these processes are central to atherosclerosis and restenosis. The purpose of the present study, therefore, was to determine if oxLDL can induce changes in nuclear Ca^{2+} in aortic smooth muscle cells.

* Address for correspondence: Dr. Grant N. Pierce, Director, Division of Stroke & Vascular Disease, St. Boniface General Hospital Research Centre, 351 Tache Ave., Winnipeg, Manitoba, Canada R2H 2A6. Telephone: 204–235–3414 Fax: 204–231–1151 E-mail: gpierce@sbrc.umanitoba.ca

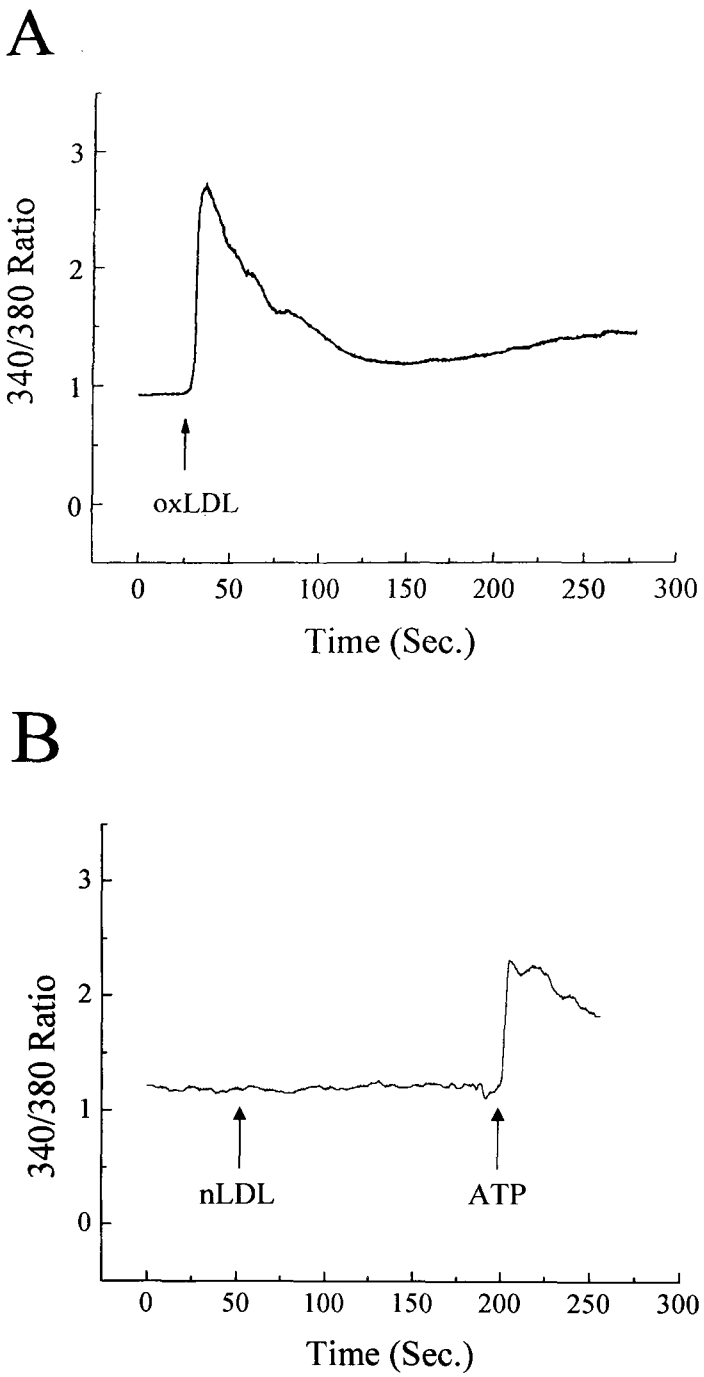


FIGURE 1 Acute effect of oxLDL (0.1 mg cholesterol/ml) on $Ca^{2+}{}_i$ in a single vascular smooth muscle cell. VSMC were loaded with fura-2 and then stimulated with 0.1 mg/ml oxLDL (A) as indicated by an arrow. An increase in intracellular [Ca^{2+}] is reflected by a rise in the 340/380 wavelength ratio. These results are representative of many experiments (n=10). In a separate experiment (B), VSMC were exposed to native LDL (nLDL) (0.1 mg/ml) without generating any change in $Ca^{2+}{}_i$. The addition of 100 μM ATP, however, did induce a rise in $Ca^{2+}{}_i$. This result was representative of many experiments (n=4)

MATERIALS AND METHODS

Low density lipoprotein isolation and oxidation

LDL was isolated from plasma as described previously[3,5,6]. The LDL fraction was extensively dialyzed with 0.15 mM NaCl, 0.1 mM EDTA (pH 7.4), sterile filtered and stored at 4°C in dark. The EDTA concentration in native LDL was reduced prior to LDL oxidation. Native LDL was diluted 10 fold in 0.15 mM NaCl (pH 7.4) and oxidized with 50 μM $FeCl_3$ and 0.25 mM ADP for 3 hours at 37°C, as described[13]. The extent of LDL oxidation was minimal as evaluated by a modest increase of thiobarbituric acid reactive substances (TBARS), a minimal increase in electrophoretic mobility on agarose gels, and a ~20% depletion of α-tocopherol content[3,5,6,13].

Treatment of vascular smooth muscle cells (VSMC) with oxLDL

Primary cultures of aortic smooth muscle cells were generated using an explant technique, as described in detail elsewhere[5,6]. VSMC from the first or second passage were used in all of our experiments. In order to induce differentiation, VSMCs were incubated (for 5–6 days) in serum free media prior to exposure to oxLDL (0.1 mg cholesterol/ml LDL). LDL was always freshly oxidized prior to addition to the cells.

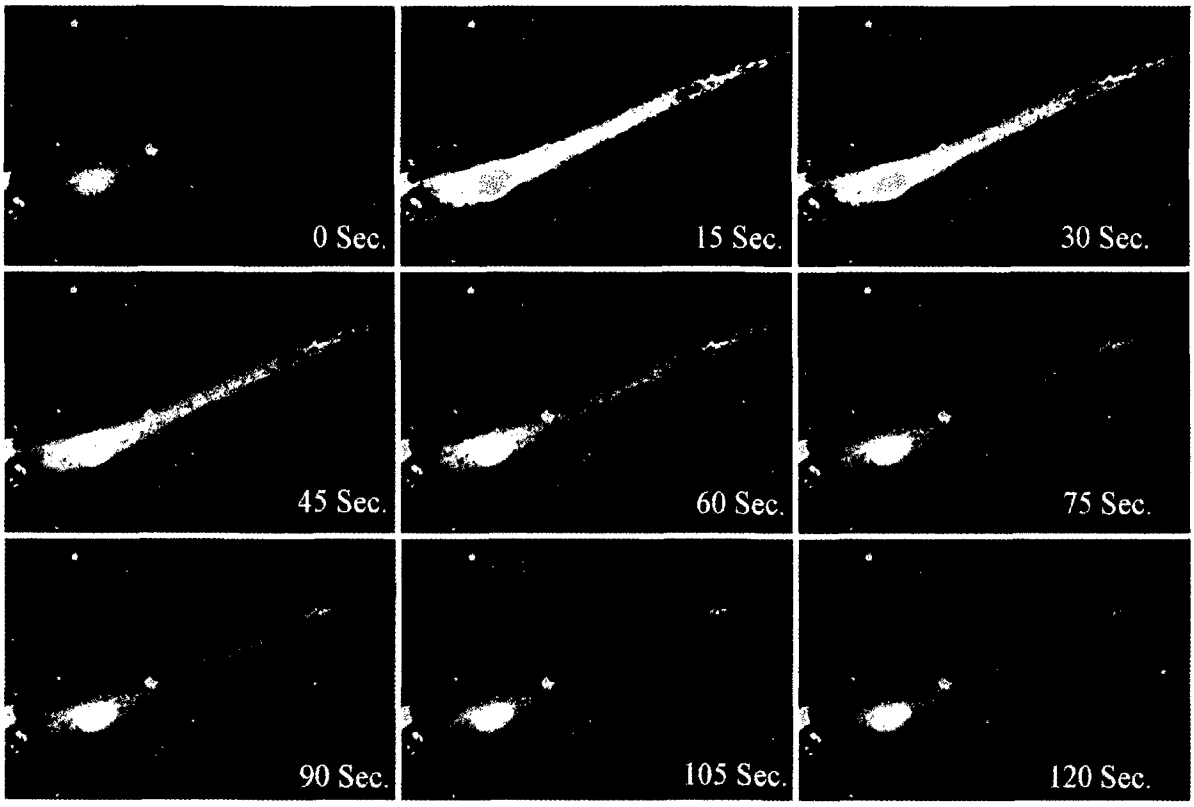
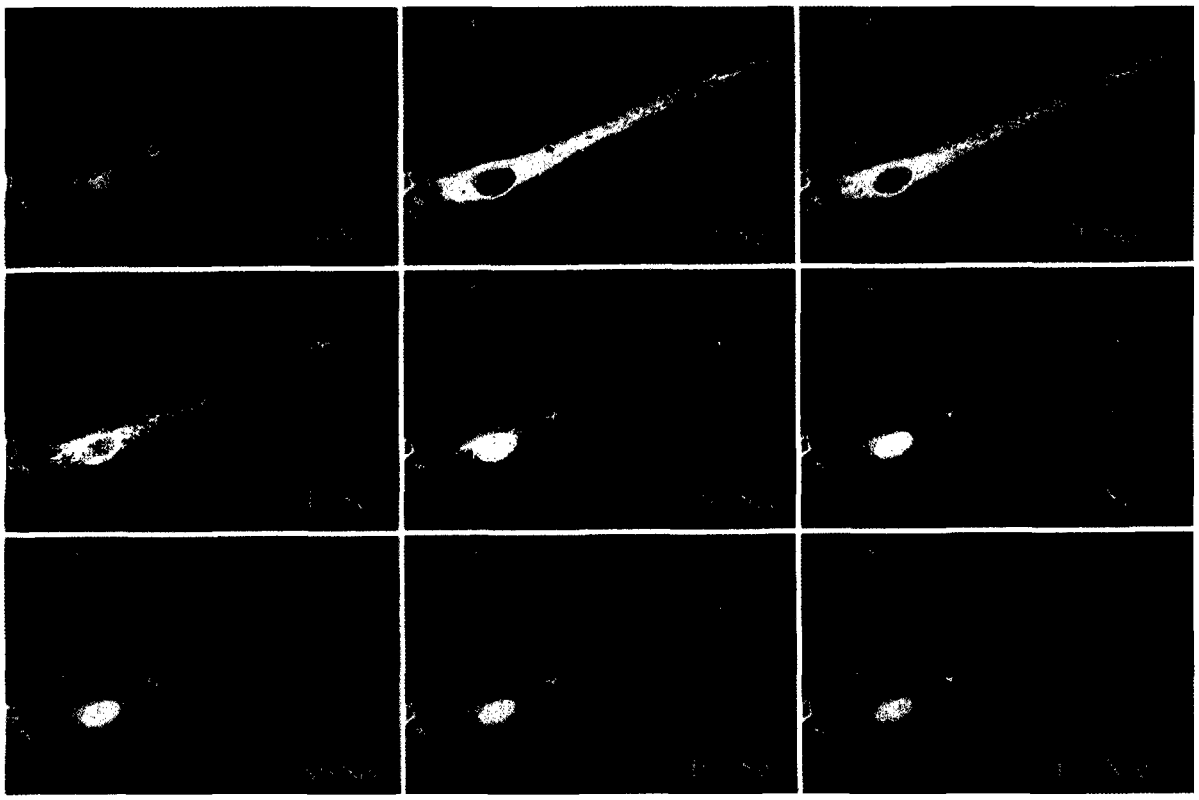


FIGURE 2 Time dependent effect of 0.1 mg cholesterol/ml oxLDL on $Ca^{2+}{}_i$ in a fluo-3 loaded smooth muscle cell. Cells were loaded with fluo-3 as described in the Methods. Rapid increase in $Ca^{2+}{}_i$ in response to oxLDL is found in the cytoplasm and is particularly localized to the nucleus. This result was repeated several times (n = 4) (See Color Plate I at the back of this issue)



Color Plate I (See page 11, Figure 2) Time dependent effect of 0.1 mg cholesterol/ml oxLDL on $Ca^{2+}{}_i$ in a fluo-3 loaded smooth muscle cell. Cells were loaded with fluo-3 as described in the Methods. Rapid increase in $Ca^{2+}{}_i$ in response to oxLDL is found in the cytoplasm and is particularly localized to the nucleus. This result was repeated several times (n = 4)

Calcium measurement

Measurement of $[Ca]_i$ in single smooth muscle cells was carried out as described[5,6]using two different classes of calcium indicators: a single wavelength intensity modifying dye (fluo-3) and two different dual wavelength ratiometric dyes (indo-1 and fura-2). For loading purposes, acetoxymethyl ester (AM) forms of the dyes were used. Cultured VSMCs were incubated with 2 μM fluo-3 for 20 minutes at 22°C in a Krebs-Henseleit buffer (120 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.25 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 8.6 mM dextrose). The cells were then washed three times with the Krebs-Henseleit buffer before being placed in a Leiden chamber. The Leiden chamber was then placed in an experimental chamber that was mounted on the microscope stage. The temperature of the cells was maintained at 37°C. The fluorescent images of intracellular calcium were obtained with a Bio-Rad MRC-600 UV laser scanning confocal microscope. This microscope was fitted with a water-cooled argon ion laser (Enterprise, Coherent, USA). The confocal was connected to a Nikon Diaphot-300 inverted microscope with a 100x or 40x oil CF Epi-Fluorescence Fluor objective lens. The cells were excited with a 488 nm wavelength laser beam through a VHS filter block and the emitted fluorescence was measured through a 515 nm low-cut filter. Indo-1 AM was loaded into cells by incubating them with 2 μM indo-1 for 20 minutes at 22°C in a Krebs-Henseleit buffer, washing them and placing them in a Leiden chamber as above. The cells were excited with a 351 nm UV laser line and emission was recorded at 405 nm (for the bound calcium) and 480 nm (free Ca^{2+}). Filter blocks IN1 and IN2 were used to capture the appropriate signal. The IN1 filter block contains a 380 nm dichroic reflector and IN2 has a 440 nm dichroic reflector plus 460 and 405 nm emission filters in separate channels. The maximum and minimum fluorescence signals were obtained by adding 10 μM 4-Bromo-A23187 and 5 mM EGTA, respectively at the end of the experiment in order to calibrate the signal with the intracellular calcium concentration. Fura-2 AM was loaded into cells in an identical manner as that used for indo-1. Details concerning its use are found elsewhere[5,6].

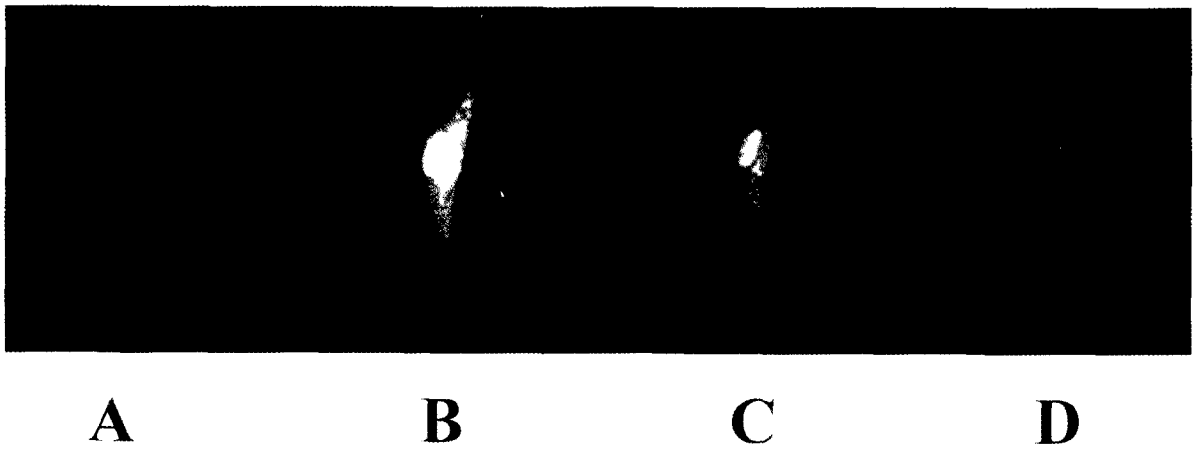


FIGURE 3 Indo-1 fluorescence images from a single smooth muscle cell before (A,B) and 30 seconds after exposure to 0.1 mg/ml oxLDL (C,D). The fluorescence recordings were collected at 405 nm (A,C) and 480 nm (B,D) wavelengths. Note the increase in the signal in a nuclear region at 405 nm in response to oxLDL and the corresponding decrease in this signal at 480 nm. Similar results were obtained from many independent experiments (n = 5) (See Color Plate II at the back of this issue)

Color Plate II (See page 12, Figure 3) Indo-1 fluorescence images from a single smooth muscle cell before (A,B) and 30 seconds after exposure to 0.1 mg/ml oxLDL (C,D). The fluorescence recordings were collected at 405 nm (A,C) and 480 nm (B,D) wavelengths. Note the increase in the signal in a nuclear region at 405 nm in response to oxLDL and the corresponding decrease in this signal at 480 nm. Similar results were obtained from many independent experiments (n = 5)

RESULTS

We first examined the effects of a concentration of oxLDL frequently employed to elicit a Ca^{2+} transient. As shown previously[5,6], oxLDL induced a very rapid increase in intracellular Ca^{2+} (within seconds) (Figure 1A). This increase in Ca^{2+} dissipated with time. Small amounts of Fe-ADP accompanied the oxLDL and may have influenced the Ca^{2+} transient on its own. However, when a similar final concentration of Fe-ADP (5 μM $FeCl_3$, 25 μM ADP) was incubated with the cells in the absence of oxLDL, no Ca^{2+} transient was observed (data not shown). Thus, the generation of the Ca^{2+}transient in the VSMC was clearly due to the oxLDL. Native LDL had no effect on intracellular Ca^{2+} (Figure 1B). This was not due to a defect in the ability of the cell to generate a Ca^{2+} transient because the addition of ATP to the same cell induced a large rise in $Ca^{2+}{}_i$. In four separate experiments, none of the cells responded to native LDL with a rise in $Ca^{2+}{}_i$ but all responded to ATP. In another series of experiments, all cells tested (n=10) had an increase in $Ca^{2+}{}_i$ after exposure to oxLDL.

We used confocal microscopy to observe spatial changes in intracellular distribution throughout the cell in response to oxLDL. Cells were loaded with fluo-3 and acutely stimulated with 0.1 mg/ml oxLDL. After exposure to oxLDL, there was a rapid increase in intracellular Ca^{2+} (Figure 2). Although this can be observed throughout the cell, it was most pronounced in an area that appeared to be the nucleus.

Fluo-3 has been criticized as an indicator dye to detect nuclear pools of Ca^{2+} [14]. Indo-1 is a ratiometric Ca^{2+} indicator dye that is less susceptible to artifactual changes in fluorescence. Vascular smooth muscle cells were loaded with indo-1 and exposed to 0.1 mg/ml oxLDL as above. The 405 nm and 480 nm signals were collected separately and are shown in Figure 3. Note the decrease in the 480 nm signal in the cell nucleus after exposure to oxLDL. The strongest changes occur in a nuclear region in comparison to the cellular cytoplasm. The 405 nm signal changes as well after exposure to oxLDL but this signal increases instead of decreases as in the 480 nm signal. Once again, this change in signal intensity occurred primarily in the nuclear region. These data are consistent with a rise in Ca^{2+} specifically in the nuclear region and argue against a signal artifact. In another experiment, the Ca^{2+} signal from indo-1 was presented in a three dimensional plot (Figure 4). Once again, the data strongly suggest that oxLDL induces a heterogenous, localized increase in $[Ca^{2+}]$ in the nuclear region of the cell.

The data suggest a change in nuclear $[Ca^{2+}]$ in response to oxLDL. To absolutely identify this pool of Ca^{2+} as being nuclear in origin, cells were treated with oxLDL, measured for Ca^{2+} distribution with indo-1, then the same cells were counter-stained with Hoescht 33258 to identify nuclear DNA. As shown in Figure 5, Hoescht staining of the nuclear contents clearly identifies the predominant Ca^{2+} pool as localized to the nuclear/perinuclear space.

DISCUSSION

Our results demonstrate that oxLDL induces a rapid increase in intracellular Ca^{2+}. The heterogeneous nature of the distribution of Ca^{2+} within the cells suggested a nuclear compartment was affected by oxLDL. The co-localization of the Ca^{2+} increase with DNA staining confirmed this hypothesis. The detection of the Ca^{2+} signal in the nuclear region was not an artifact associated with the measurement of the dye's signal. The same qualitative response was observed using two different Ca^{2+} indicator dyes. Furthermore, although artifacts have been associated with non-ratiometric Ca^{2+} indicator dyes, the results were confirmed with the ratiometric indicator dye indo-1. Our results indicate that the oxLDL-induced changes in signal exhibited by both dyes reflect a real change in nuclear/perinuclear Ca^{2+} concentration.

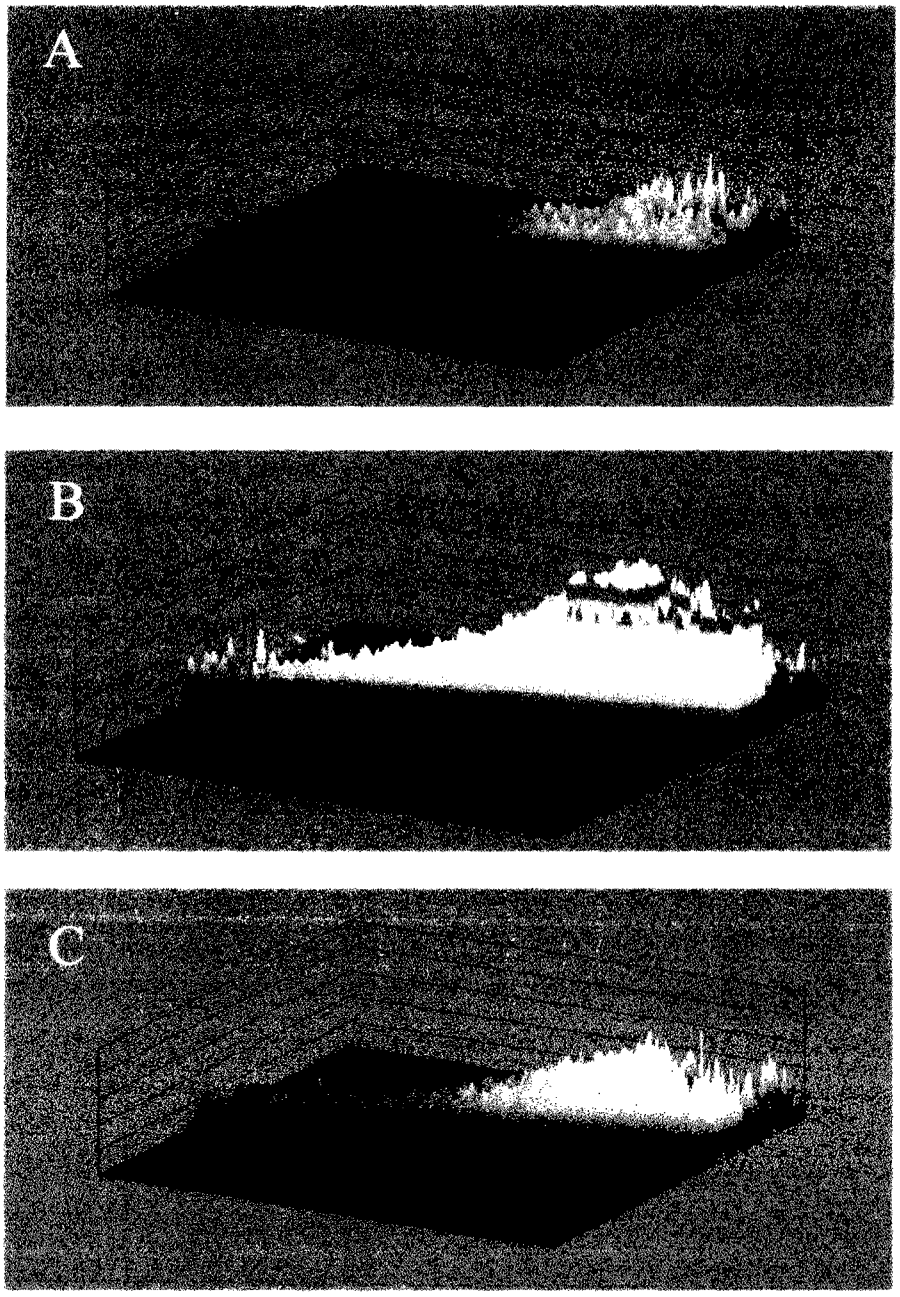
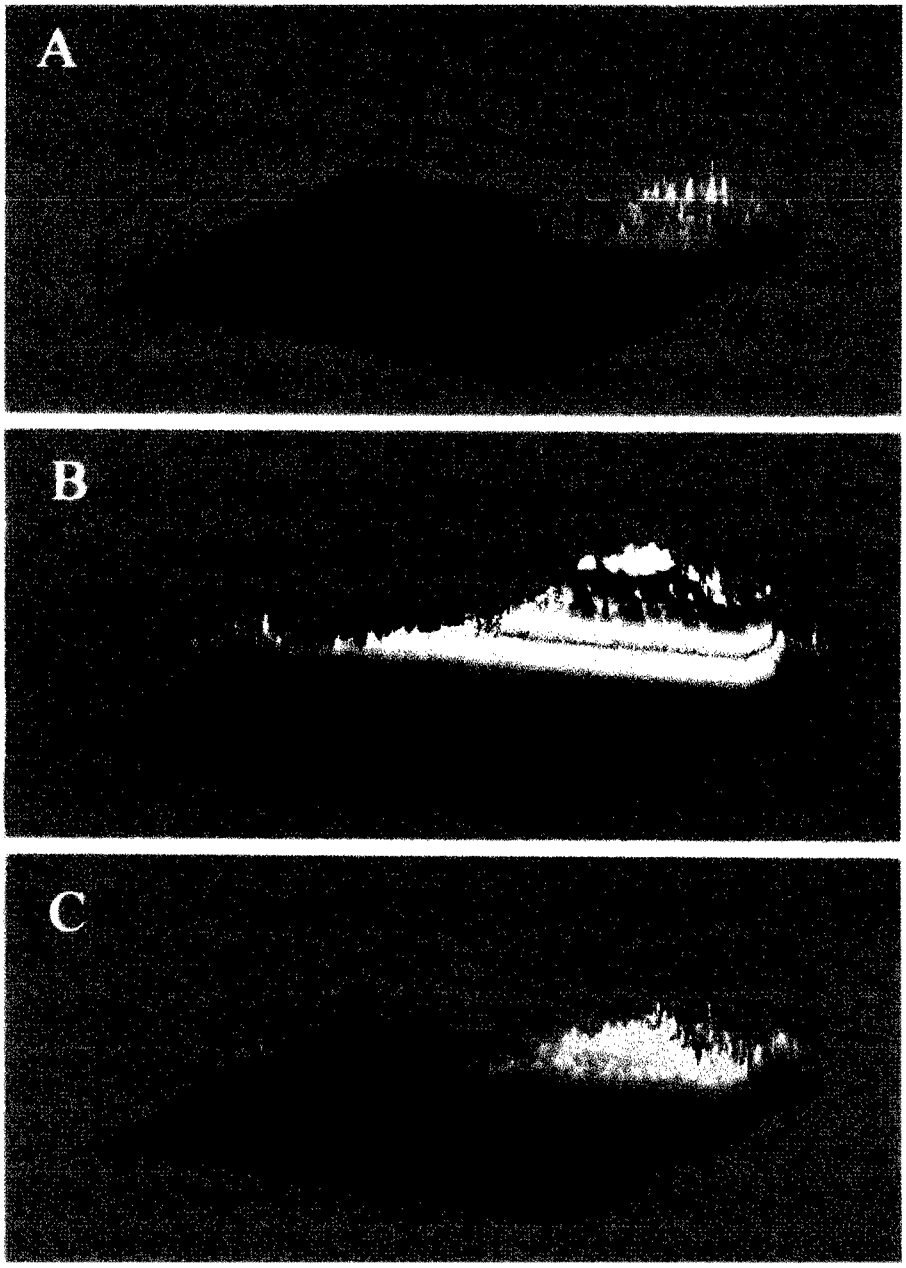


FIGURE 4 Three dimensional plot of Ca^{2+} distribution within a smooth muscle cell exposed to 0.1 mg/ml oxLDL. Cells were loaded with indo-1 and measurements represent the ratio of the 405/480 signals. A: at 0 time; B: 15 seconds after stimulation with oxLDL; C: 2 minutes after stimulation with oxLDL (See Color Plate III at the back of this issue)

Others and we have shown previously that the mechanism whereby oxLDL induces a change in intracellular Ca^{2+} is through an IP_3-induced release of Ca^{2+} from internal stores, likely at the



Color Plate III (See page 14, Figure 4) Three dimensional plot of Ca^{2+} distribution within a smooth muscle cell exposed to 0.1 mg/ml oxLDL. Cells were loaded with indo-1 and measurements represent the ratio of the 405/480 signals. A: at 0 time; B: 15 seconds after stimulation with *oxLDL;* C: 2 minutes after stimulation with oxLDL

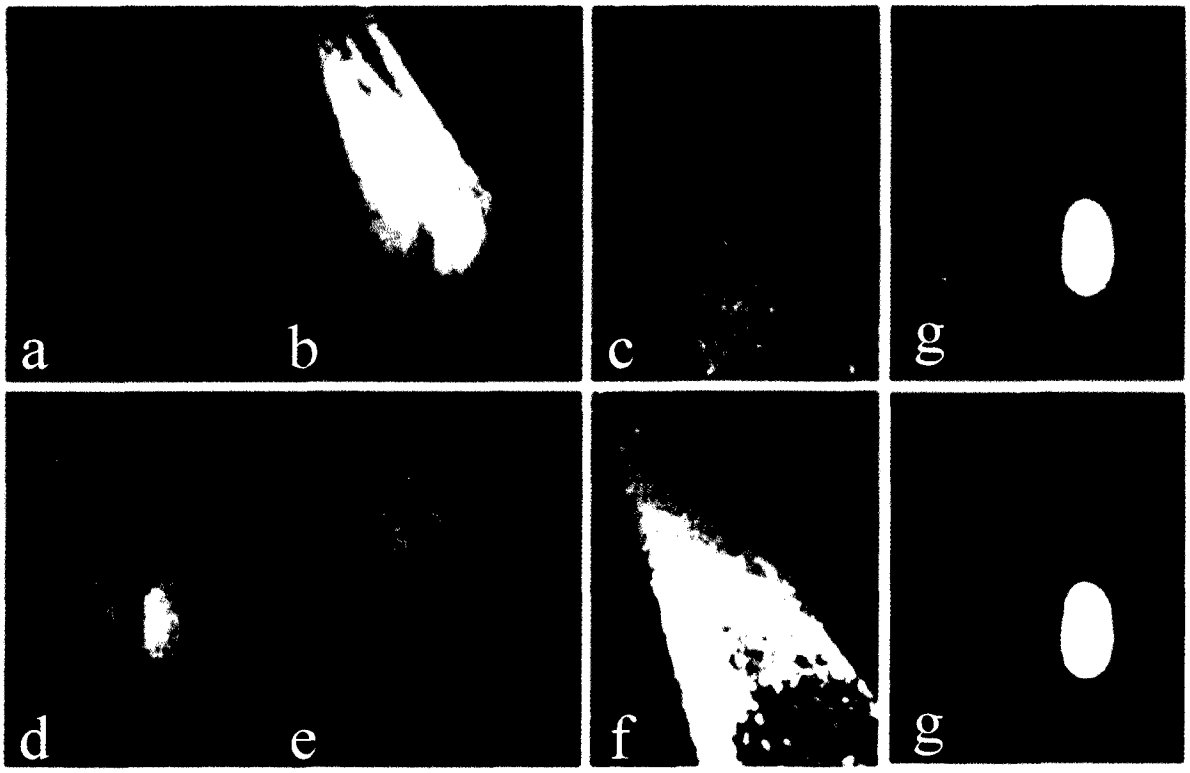
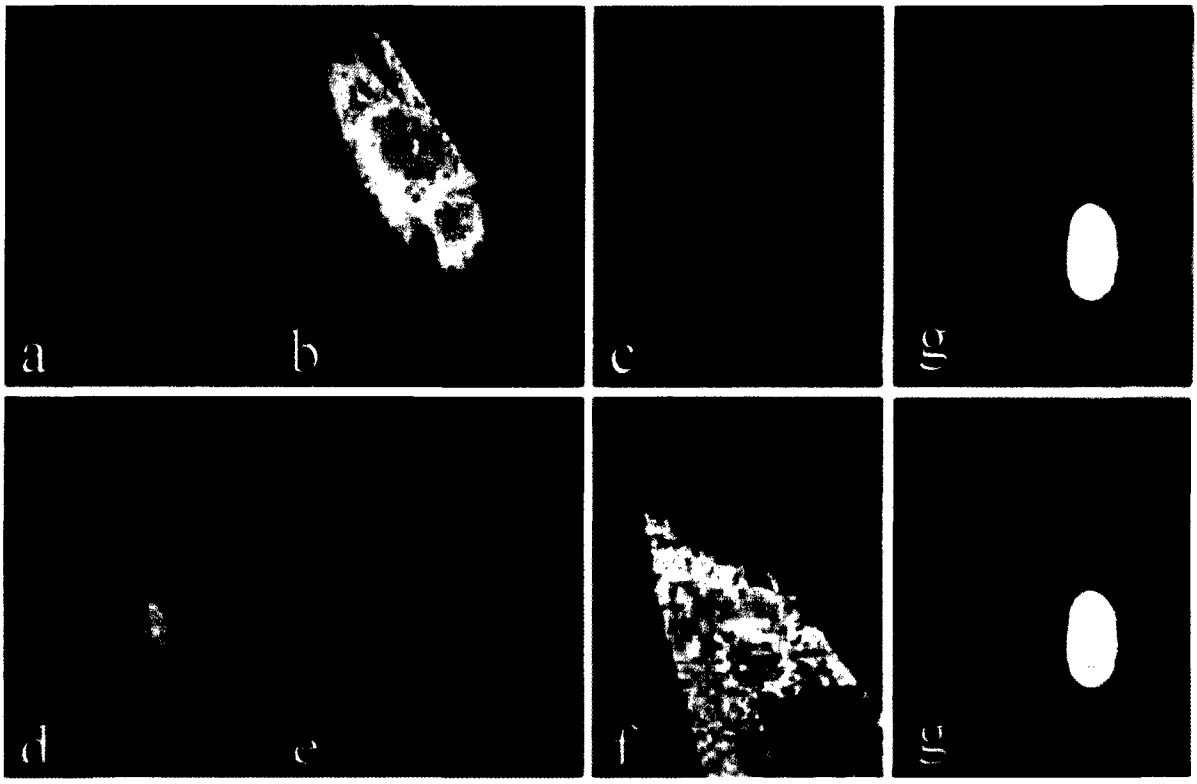


FIGURE 5 Localization of nuclei and Ca^{2+} within a smooth muscle cell before and after incubation with 0.1 mg/ml oxLDL. Cells were loaded with indo-1 and fluorescence was measured at 405 nm (a,d) and 480 nm (b,e) wavelengths, then these signals were ratioed (405/480) (c,f) before (a-c) and 15 seconds after exposure to oxLDL (d-f). The same cell was stained for DNA with 50 ug/ml Hoeschst no. 33258 (g). This image was placed beside both conditions to allow for visual alignment of the Hoeschst staining with the indo-1 fluorescence (See Color Plate IV at the back of this issue)

sarcoplasmic reticulum[4–6]. Since the SR membrane is contiguous with the outer nuclear membrane[9], it is not surprising to find a Ca^{2+} pool at or near the nucleus. Furthermore, it is well known that there are numerous IP_3 release sites in the nuclear membrane[15] that are capable of extensive release of Ca^{2+} both into the nucleoplasm and the space within the two nuclear membranes[16]. Using a confocal microscope, we can conclude that our signal clearly reflects the former nuclear compartment but whether the oxLDL is inducing a release of Ca^{2+} from the near membrane pool is more difficult to conclusively establish. The increase in $[Ca^{2+}]$ within the nucleus declines with time, returning to control levels within a few minutes. This likely reflects a passive movement of Ca^{2+} out of the nuclei and a re-sequestration process into membraneous compartments within the cytoplasm and nucleus. This may occur through ATP-dependent uptake or IP_3 sensitive processes[17].

Nuclear Ca^{2+} is thought to have important functional significance. Intranuclear Ca^{2+} has been suggested to play a role in gene transcription[9,11,12], DNA repair[12], nucleocytoplasmic trafficking[10], cell proliferation[11], excitation-contraction coupling[8], apoptosis[11,12] and cell death[12]. Many of these same functions have relevance for atherosclerosis. For example, oxLDL has been shown to induce cell



Color Plate IV (See page 15, Figure 5) Localization of nuclei and Ca^{2+} within a smooth muscle cell before and after incubation with 0.1 mg/ml oxLDL. Cells were loaded with indo-1 and fluorescence was measured at 405 nm (a,d) and 480 nm (b,e) wavelengths, then these signals were ratioed (405/480) (c,f) before (a-c) and 15 seconds after exposure to oxLDL (d-f). The same cell was stained for DNA with 50 ug/ml Hoeschst no. 33258 (g). This image was placed beside both conditions to allow for visual alignment of the Hoeschst staining with the indo-1 fluorescence

proliferation[18], apoptosis[19], and cellular necrosis[18]. OxLDL also induces dramatic changes in gene expression during the transformation of macrophages and smooth muscle cells into foam cells. It is tempting to speculate that the increase in intranuclear Ca^{2+} induced by oxLDL in the present study represents an initial signaling response that is important in one or more of these processes that are critical in atherogenesis and in restenotic vascular growth.

Acknowledgements

This work was supported by a grant from the Medical Research Council of Canada. H. Massaeli was a Trainee of the Heart and Stroke Foundation of Canada. G.N. Pierce is a Senior Scientist of the Medical Research Council of Canada.

References

1. Steinberg, D., S. Parthasarathy, T.E. Carew, J.C. Khoo, and J.L. Witztum (1989). Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *New England Journal of Medicine* 320, 915–924.
2. Witztum J.L. and D. Steinberg (1991). Role of oxidized low density lipoprotein in atherogenesis. *Journal of Clinical Investigation* 88, 1785–1792.
3. Liu K., H. Massaeli, and G.N. Pierce (1993). The action of oxidized low density lipoprotein on calcium transients in isolated rabbit cardiomyocytes. *Journal of Biological Chemistry* 268, 4145–4151.
4. Massaeli H. and G.N. Pierce (1995). Involvement of lipoproteins, free radicals, and calcium in cardiovascular disease processes. *Cardiovascular Research* 29, 597–603.
5. Massaeli, H., J.A. Austria and G.N. Pierce (1999). Chronic exposure of smooth muscle cells to minimally oxidized LDL results in depressed inositol 1,4,5-trisphosphate receptor density and Ca^{2+} transients. *Circulation Research* 85, 515–523.
6. Massaeli, H., J.A. Austria and G.N. Pierce (2000). Lesions in ryanodine channels in smooth muscle cells exposed to oxidized low density lipoprotein. *Arteriosclerosis, Thrombosis and Vascular Biology* 20, 328–334.
7. Weisser, B., R. Locher, T. Mengden and W. Vetter (1992). Oxidation of low density lipoprotein enhances its potential to increase intracellular free calcium concentration in vascular smooth muscle cells. *Arteriosclerosis, Thrombosis and Vascular Biology* 12, 231–236.
8. Bkaily, G., N. Gros-Louis, R. Naik, D. Jaalouk and P. Pothier (1996). Implication of the nucleus in excitation contraction coupling of heart cells. *Molecular and Cellular Biochemistry* 154, 113–121.
9. Gilchrist, J.S.C., M.P. Czubryt and G.N. Pierce (1994). Calcium and calcium-binding proteins in the nucleus. *Molecular and Cellular Biochemistry* 135, 79–88.
10. Greber, U.F. and L. Gerace (1995). Depletion of calcium from the lumen of endoplasmic reticulum reversibly inhibits passive diffusion and signal-mediated transport into the nucleus. *Journal of Cell Biology* 128, 5–14.
11. Malviya, A.N. and P.J. Rogue (1998). "Tell me where is calcium bred": Clarifying the roles of nuclear calcium. *Cell* 92, 17–23.
12. Santella, L. and E. Carafoli (1997). Calcium signaling in the nucleus. *FASEB J.* 11, 1091–1109.
13. Massaeli, H., S. Sobrattee and G.N. Pierce (1999). The importance of lipid solubility in antioxidants and free radical generating systems for determining lipid peroxidation. *Free Radicals in Biology & Medicine*, 26, 1524–1530.
14. Perez-Terzic, C., L. Stehno-Bittel and D.E. Clapham (1997). Nucleoplasmic and cytoplasmic differences in the fluorescence properties of the calcium indicator Fluo-3. *Cell Calcium* 21, 275–282.
15. Mak, D.D. and J.K. Foskett (1994). Single channel inositol 1,4,5-trisphosphate receptor currents revealed by patch clamp of isolated Xenopus oocyte nuclei. *Journal of Biological Chemistry* 269, 29375–29378.
16. Nicotera, P., S. Orrenius, T. Nilsson and P-O. Berggren (1990). An inositol 1,4,5-trisphosphate-sensitive Ca2+ pool in liver nuclei. *Proceedings of the National Academy of Science USA* 87, 6858–6862.
17. Gerasimenko, O.V., J.V. Gerasimenko, A.V. Tepikin and O.H. Petersen (1995). ATP-dependent accumulation and inositol trisphosphate- or cyclic ADP-ribose-mediated release of Ca2+ from the nuclear envelope. *Cell* 80, 439–444.
18. Auge, N., M-T. Pieraggi, J-C. Thiers, A. Negre-Salvayre, and R. Salvayre (1995). Proliferative and cytotoxic effects of mildly oxidized low-density lipoproteins on vascular smooth muscle cells. *Biochemical Journal* 309, 1015–1020.
19. Reid, V.C., S.J. Hardwick, and M.J. Mitchinson (1993). Fragmentation of DNA in P388D1 macrophages exposed to oxidised low-density lipoprotein. *FEBS Letters* 332, 218–220.