JCI The Journal of Clinical Investigation

Dual Ca2+ requirement for optimal lipid peroxidation of low density lipoprotein by activated human monocytes.

Q Li, A Tallant, M K Cathcart

Research Article

The oxidative modification of LDL seems a key event in atherogenesis and may participate in inflammatory tissue injury. Our previous studies suggested that the process of LDL oxidation by activated human monocytes/macrophages required O_2^- and activity of intracellular lipoxygenase. Herein, we studied the mechanisms involved in this oxidative modification of LDL. In this study, we used the human monocytoid cell line U937 to examine the role of Ca^{2+} in U937 cell-mediated lipid peroxidation of LDL. U937 cells were activated by opsonized zymosan. Removal of Ca^{2+} from cell culture medium by EGTA inhibited U937 cell-mediated peroxidation of LDL lipids. Therefore, Ca^{2+} influx and mobilization were examined for their influence on U937 cell-mediated LDL lipid peroxidation. Ca^{2+} channel blockers nifedipine and verapamil blocked both Ca^{2+} influx and LDL lipid peroxidation by activated U937 cells. The inhibitory effects of nifedipine and verapamil were dose dependent. TMB-8 and ryanodine, agents known to prevent Ca^{2+} release from intracellular stores, also caused a dose-dependent inhibition of LDL lipid peroxidation by activated U937 cells while exhibiting no effect on Ca^{2+} influx. Thus, both Ca^{2+} influx through functional calcium channels and Ca^{2+} mobilization from intracellular stores participate in the oxidative modification of LDL by activated U937 cells. $^{45}Ca^{2+}$ uptake experiments revealed profound Ca^{2+} influx during the early stages of U937 cell activation, however, the Ca^{2+} ionophore 4-bromo A23187 [...]

Find the latest version:

https://jci.me/116355/pdf

Dual Ca^{2+} Requirement for Optimal Lipid Peroxidation of Low Density Lipoprotein by Activated Human Monocytes

Qing Li,*‡ Ann Tallant,* and Martha K. Cathcart*‡
*Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195; and ‡Department of
Regulatory Biology, Cleveland State University, Cleveland, Ohio 44115

Abstract

The oxidative modification of LDL seems a key event in athero-genesis and may participate in inflammatory tissue injury. Our previous studies suggested that the process of LDL oxidation by activated human monocytes/macrophages required O_2^- and activity of intracellular lipoxygenase. Herein, we studied the mechanisms involved in this oxidative modification of LDL. In this study, we used the human monocytoid cell line U937 to examine the role of Ca^{2+} in U937 cell-mediated lipid peroxidation of LDL. U937 cells were activated by opsonized zymosan. Removal of Ca^{2+} from cell culture medium by EGTA inhibited U937 cell–mediated peroxidation of LDL lipids. Therefore, Ca^{2+} influx and mobilization were examined for their influence on U937 cell–mediated LDL lipid peroxidation. Ca^{2+} channel blockers nifedipine and verapamil blocked both Ca^{2+} influx and LDL lipid peroxidation by activated U937 cells. The inhibitory effects of nifedipine and verapamil were dose dependent. TMB-8 and ryanodine, agents known to prevent Ca^{2+} release from intracellular stores, also caused a dose-dependent inhibition of LDL lipid peroxidation by activated U937 cells while exhibiting no effect on Ca^{2+} influx. Thus, both Ca^{2+} influx through functional calcium channels and Ca^{2+} mobilization from intracellular stores participate in the oxidative modification of LDL by activated U937 cells. ^{45}Ca^{2+} uptake experiments revealed profound Ca^{2+} influx during the early stages of U937 cell activation, however, the Ca^{2+} ionophore 4-bromo A23187 was unable to induce activation of U937 cells and peroxidation of LDL lipids. Release of intracellular Ca^{2+} by thapsigargin only caused a suboptimal peroxidation of LDL lipids. Our results indicate that although increases in intracellular Ca^{2+} levels provided by both influx and intracellular Ca^{2+} mobilization are required, other intracellular signals may be involved for optimal peroxidation of LDL lipids by activated human monocytes. (*J. Clin. Invest.* 1993. 91:1499–1506.) Key words: human macrophages • calcium influx • calcium mobilization • signal transduction • low density lipoprotein peroxidation

Introduction

Activated human monocytes/macrophages can oxidize normal human LDL and transform it to a cytotoxin (1). The process of LDL lipid peroxidation by activated human monocytes has been shown to require superoxide anion (O_2^-) released from activated monocytes/macrophages, since scavenging of O_2^- by superoxide dismutase prevents peroxidation of LDL lipids (2). Metal ion chelators and general antioxidants can also inhibit monocyte-mediated peroxidation of LDL lipids (1, 2). Our previous studies suggested that there was an initiation of oxidation by O_2^- and propagation of oxidation mediated by other free radicals, possibly lipid radicals (2). It was also found that intracellular lipoxygenase was essential for oxidation of LDL lipids (3). Cellular lipoxygenases catalyze the oxidation of polyunsaturated fatty acids to fatty acid hydroperoxides. Fatty acid hydroperoxides may propagate oxidation by forming reactive lipid peroxyl radicals.

Oxidized LDL has been shown to be toxic to proliferating target cells in vitro, such as fibroblasts and endothelial cells (1, 4, 5). If activated monocytes/macrophages were able to oxidize LDL in vivo, the resultant toxic lipoproteins could mediate tissue injury in a variety of inflammatory responses. This may likely occur in the developing atherosclerotic lesion where lipid-laden foam cells of monocyte origin are surrounded by LDL accumulating in high concentration in the vascular interstitial space (5, 6). Thus, it appears that phagocyte-derived free radicals, both superoxide anion and lipid peroxyl radicals, oxidize LDL and that the products of lipid peroxidation carried by oxidized LDL can effect more extensive or more distant tissue injury than that directly mediated by monocyte-derived free radicals.

Although both the release of reactive oxygen species by activated human monocytes/macrophages and the requirement for cellular lipoxygenases have been correlated with oxidation of LDL lipids, the precise mechanisms involved in the oxidation are not entirely understood. In particular, little is known regarding the intracellular signaling required for activated monocytes to mediate oxidation of LDL. Studies on the required intracellular signaling can help elucidate the specific mechanisms of activated monocyte-mediated oxidation of LDL and conversion of LDL to cytotoxin as well as suggest optimal means for intervening and preventing this process.

For the majority of our studies opsonized zymosan (ZOP)^1 has been used as the activation stimulus for human monocytes. ZOP resembles a type of stimulus that might be encountered in vivo. Since ZOP can activate the monocyte through Fc recep-

---

1. *Abbreviations used in this paper:* IC$_{50}$, inhibitory concentration; LPO, lipid peroxide; MDA, malondialdehyde; PLC, phospholipase C; TBA, thiobarbituric acid; TMB, 8-(*N,N*-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride; ZOP, opsonized zymosan.

tors, complement receptors, and as a stimulator of phagocytosis (7), it is likely that several secondary messenger pathways of signal transduction are put into motion. Our hypothesis regarding activation signals and their transduction to intracellular signaling systems is that upon activation of monocytes/macrophages by ZOP, several intracellular signal transduction systems are involved and that some of these may be required for cell-mediated LDL oxidation whereas others may not. Several secondary messenger–generating pathways, such as the activation of lipoxygenases, phospholipases, or protein kinases form networks of coregulation. In a number of these signal transduction pathways, Ca^{2+} is a critical mediator. Thus, in this study we evaluated the importance of Ca^{2+} influx from the extracellular environment and Ca^{2+} mobilization from intracellular stores in the peroxidation of LDL lipids by activated macrophages.

It has been reported that Ca^{2+} channel blockers may prevent atherogenesis (8); however, the precise mechanisms remain to be elucidated. In this paper, classic slow Ca^{2+} channel blockers known to block Ca^{2+} entry through selective Ca^{2+} channels in the plasma membrane were used to block the entry of extracellular Ca^{2+} from cell culture medium. Agents known to prevent release of intracellular Ca^{2+} from internal membrane stores were also tested. Data presented here indicate that both extracellular and intracellular Ca^{2+} contribute to the process of peroxidation of LDL lipids induced by activated human macrophages. Prevention of Ca^{2+} entry or Ca^{2+} mobilization substantially interferes with the conversion of normal LDL to an oxidized and toxic entity. Our data also suggest that neither of these Ca^{2+} resources alone is sufficient for fully triggering monocyte-mediated LDL lipid peroxidation.

Methods

Cell culture

U937 cells obtained from the American Type Culture Collection (Rockville, MD) were cultured in 150-cm² flasks (Corning Glass Inc., Corning, NY) in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with 10% bovine calf serum (Hyclone Laboratories Inc., Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco Laboratories, Grand Island, NY) at 37°C in a humidified atmosphere of 90% air/10% CO_2. U937 cells were maintained in log phase and were not allowed to exceed 1×10^6/ml. For experiments U937 cells were washed twice with RPMI 1640 without serum and 5×10^5 cells/ml were plated into 12-well tissue culture plates (Costar Corp., Cambridge, MA). U937 cells were cocultured with 0.5 mg LDL cholesterol/ml. Various reagents were included during the 24-h incubation of U937 cells with LDL as noted to test for their effects on U937 cell-mediated lipid peroxidation of LDL. The total volume per well was 1.0 ml.

Lipoprotein preparation

LDL was prepared according to previously described methods that minimize oxidation and exposure to endotoxin (9). Each batch of LDL was assayed for endotoxin contamination by the Limulus amebocyte lysate assay. Final endotoxin contamination was < 0.015 U/ml. LDL was stored in 0.5 mM EDTA and adjusted to 10 mg cholesterol/ml. Immediately before use, LDL was dialyzed at 4°C against phosphate-buffered saline without calcium or magnesium (Gibco Laboratories). LDL was used at a final concentration of 0.5 mg cholesterol/ml.

Measurement of lipid peroxidation

The peroxidation of LDL lipids was measured by both the thiobarbituric acid (TBA) assay and lipid peroxide (LPO) assay.

TBA assay. The presence of lipid oxidation products on LDL was determined by a modification of the assay described by Schuh et al. (10), which detects malondialdehyde (MDA) and MDA-like compounds reacting with TBA. Compounds that react with TBA are referred to as TBA-reactive substances (TBARS). Briefly, 0.75 ml 25% trichloroacetic acid was added to 0.3-ml samples, followed by 0.75 ml of 1% TBA. The samples were then vortexed and incubated for 45 min at 95°C; after which they were centrifuged (1,000 g) for 15 min. Supernatant TBARS were detected by fluorescence at 515 nm excitation and 553 nm emission. Malondialdehyde bis (dimethyl acetal), i.e., 1,1,3,3, tetramethoxypropane (Aldrich Chemical Co., Milwaukee, WI) was used as the standard at concentrations of 0–10 nmol MDA/ml. Samples were assayed in duplicate. Data represent the mean±SEM of experimental results obtained in three similar experiments and are expressed in terms of MDA equivalents (nmol MDA/ml of sample).

LPO assay. To detect lipid peroxides on LDL we performed the LPO assay of El-Saadani et al. (11). The principle of this assay is based on the oxidative capacity of lipid peroxides to convert iodide to iodine, which can be measured spectrophotometrically at 365 nm. A stoichiometric relationship is observed between the amount of organic peroxides assayed and the concentration of iodine produced (11). Thus, the amount of lipid peroxides can be calculated using an extinction coefficient. For this assay, 100 μl of sample were added to 1 ml of working reagent (200 mM monobasic potassium phosphate, pH 6.2, 120 mM potassium iodide, 0.15 mM sodium azide, 2.0 g/liter Triton X-100, 0.1 g/liter benzalkonium chloride, 0.01 mM ammonium molybdate, 0.02 mM butylated hydroxy toluene, and 0.024 mM EGTA) and then incubated in the dark at room temperature for 30 min. After incubation, the samples were read using a spectrophotometer (model DU-64; Beckman Instruments, Inc., Fullerton, CA) at wavelength 365 nm. The quantity of lipid peroxides in samples of oxidized LDL was calculated according to the equation: nmol lipid peroxide/ml = 447.15 × OD_{365}.

$^{45}Ca^{2+}$ uptake assay

U937 cells at 2×10^6 cells/ml in HBSS (Gibco Laboratories) were preincubated at 37°C with test agents for 30 min. At zero time, LDL and ZOP were added together with 2 μCi $^{45}Ca^{2+}$/ml. At given time intervals, 100 μl of cell suspension was loaded onto 500 μl Ficoll-Paque (Pharmacia Inc., Piscataway, NJ) and centrifuged for 2 min in an Eppendorf centrifuge 5413. The cells were rapidly filtered under vacuum on GF/C glass-fiber filters (Whatman International Ltd., Maidstone, England) that were previously rinsed with 2 ml HBSS. The filters were further rinsed with 3 ml HBSS, air dried, and placed in 10 ml of scintillation liquid (ICN Biomedicals, Inc., Irvine, CA). Radioactivity was determined with a β-scintillation counter. All experiments were performed in triplicate.

Detection of toxicity of test agents

The toxicity of test agents to U937 cells was determined by both the [¹⁴C]adenine and the [⁵¹Cr]sodium chromate release assays. These assays gave very similar results when U937 cells were injured with increasing doses of hydrogen peroxide in support of previously published comparisons of these sensitive assays for cell injury/toxicity (12).

[¹⁴C]adenine release assay (13). Briefly, 1×10^7 U937 cells in 20 ml RPMI 1640 were labeled overnight by incubating with 10 μCi [¹⁴C]-adenine (ICN Radiochemicals, Irvine, CA). For experiments, U937 cells were washed twice with RPMI 1640, and 5×10^5 cells/ml, ZOP, and LDL were plated into 12-well tissue culture plates in the presence or absence of test agents. After a 24-h incubation, the amount of [¹⁴C]-adenine release by U937 cells was detected by counting 100 μl of the supernatant fluid using a β-counter (model LS-3801; Beckman Instruments, Inc.). [¹⁴C]adenine release from ZOP-activated U937 cells in the absence of test agents was defined as background and 0% release and interpreted as a lack of toxicity. [¹⁴C]adenine release by 0.2% SDS was defined as 100% release and interpreted as maximum toxicity. The data are expressed as: % release = [(sample release − background)/(maximum release − background)] × 100%.

[^{51}Cr]*Sodium chromate release assay (14)*. 1×10^7 U937 cells in 20 ml RPMI 1640 were labeled overnight by incubating with 100 μCi of sodium chromate-51 (Du Point/New England Nuclear, Boston, MA). After labeling, the cells were washed twice with RPMI 1640, and 5 $\times 10^5$ cells/ml, ZOP, and LDL were plated into 12-well tissue culture plates in the presence or absence of test agents. After 24 h, 100 μl of the supernatants containing released isotope were removed and placed in glass tubes to be counted on a gamma counter. Controls consisted of U937 cells incubated with ZOP (0% release) or 0.2% SDS (100% release). The data are calculated according to above equation.

Intracellular Ca²⁺ measurement

Intracellular Ca²⁺ was measured using the fluorescent indicator Fura 2 (15). In U937 cells, at 1×10^7/ml of Krebs–Ringer solution with the following composition (mM): (125 NaCl, 5 KCl, 1.2 $MgSO_4$, 6 glucose, 1 $CaCl_2$, and 25 Hepes, pH 7.5) containing 0.2% BSA, were incubated for 20 min at 37°C in 4 μM Fura 2-AM (Molecular Probes, Inc., Eugene, OR). The cells were subsequently diluted fourfold and incubated an additional 20 min to completely hydrolyze the entrapped ester. The cells were pelleted and resuspended at 1.33×10^6/ml Krebs–Ringer solution containing BSA for measurements. Fura 2 measurements were conducted in an air-driven dual-wavelength spectrofluorometer using excitation wavelengths of 340 and 380 nm and emission at 500 nm. Maximal fluorescence of the Ca²⁺-saturated dye was measured in the presence of 0.1% Triton X-100 and minimal fluorescence was measured in the presence of 5 mM EGTA and 32 mM Tris, pH 8.3. Autofluorescence was corrected by measurement in the presence of excess $MnCl_2$. The level of cytosolic Ca²⁺ was calculated according to the procedure of Grynkiewicz et al. (15).

Chemicals

8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8), nifedipine, and verapamil were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Ethylene glycol-bis(β-aminoethyl ether) tetracetic acid (EGTA), lanthanum chloride, and 4-bromo A23187 were purchased from Sigma Chemical Co. (St. Louis, MO). Ryanodine was purchased from Progressive Agri Systems, Inc. (Wind Gap, PA). Thapsigargin was purchased from Cal Biochem (San Diego, CA). Zymosan, purchased from ICN Nutritional Biochemicals (Cleveland, OH), was opsonized (16) and used at a concentration of 2 mg/ml to activate U937 cells. TMB-8, nifedipine, verapamil, ryanodine, 4-bromo A23187, and thapsigargin were solubilized in DMSO as 100-fold stock solutions. EGTA and lanthanum chloride were solubilized in H_2O as 100-fold stock solutions. Opsonized zymosan was suspended in RPMI 1640 as 20-fold stock solutions. For experiments these reagents were diluted to designated concentrations in RPMI 1640 as indicated.

Results

The TBA assay is a widely used method to detect MDA and MDA-like compounds derived from lipid oxidation products (10); and the LPO assay is used to detect the presence of lipid peroxides (11). In our studies both of these methods were used to detect the lipid oxidation products on LDL mediated by activated human monocytes. U937 cells, ZOP, and LDL were incubated together for 24 h. Results of three experiments are summarized in Fig. 1. LDL was significantly oxidized by ZOP-activated U937 cells as detected by both the TBA assay (Fig. 1 A) and the LPO assay (Fig. 1 B). Little if any lipid peroxidation was observed in cocultures of LDL and unactivated U937 cells. Removal of Ca²⁺ from the cell culture medium (there is 0.4 mM Ca²⁺ in RPMI 1640) by adding the Ca²⁺-chelating agent EGTA resulted in inhibition of LDL lipid peroxidation by activated U937 cells. In the presence of EGTA the peroxidation of LDL lipids by ZOP-activated U937 cells was decreased in a
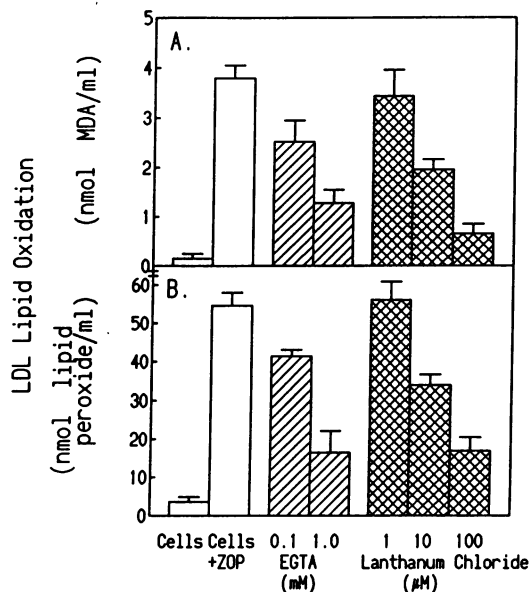

Figure 1. LDL lipid peroxidation by activated U937 cell is inhibited by EGTA and lanthanum chloride. A total 5×10^5 cells were incubated with LDL and ZOP (2 mg/ml) in the presence (*hatched bars*) or absence (*open bar*) of EGTA or lanthanum chloride at 37°C for 24 h in a humidified incubator with 10% CO_2. Different concentrations of test reagents were added to ZOP-activated U937 cells as indicated: EGTA 0.1 mM and 1 mM or lanthanum chloride 1, 10, and 100 μM. After incubation, LDL lipid peroxidation was assessed by both the TBA assay and the LPO assay as described in Methods. The data are depicted as the mean±SEM of data obtained in three similar experiments. (A) Oxidation of LDL lipids was measured by the TBA assay and expressed as nmol MDA/ml. (B) The degree of lipid peroxidation was measured by the LPO assay and expressed as nmol lipid peroxide/ml.

dose-dependent fashion. Lanthanum chloride (1, 10, and 100 μM) also showed dose-dependent inhibition of LDL lipid peroxidation by ZOP-activated U937 cells, thus indicating an important role for Ca²⁺.

Since there are two sources of Ca²⁺ that are available to the cell, namely influx from the extracellular medium and release from intracellular stores, we first examined the role of Ca²⁺ influx in the monocyte-mediated peroxidation of LDL lipids. We evaluated the effect of two structurally different Ca²⁺ channel blockers at various concentrations. U937 cells, ZOP, LDL, and various Ca²⁺ channel blockers were incubated together. In the absence of Ca²⁺ channel blockers, the LDL lipid peroxidation mediated by activated U937 cells was 3.78 nmol MDA/ml and 55.45 nmol lipid peroxide/ml, respectively. The results of three experiments are shown in Fig. 2. Both verapamil (Fig. 2 A) and nifedipine (Fig. 2 B) significantly inhibited peroxidation of lipids on LDL in a concentration-dependent manner. Our results show that both Ca²⁺ channel blockers inhibited LDL lipid peroxidation by activated U937 cells. The influx of extracellular Ca²⁺ was thus required for oxidation of LDL lipids by ZOP-activated U937 cells.

The participation of Ca²⁺ released from intracellular stores was also examined. Data shown in Fig. 3 indicate that prevention of release of Ca²⁺ from intracellular stores by adding either ryanodine (Fig. 3 A) or TMB-8 (Fig. 3 B) resulted in a dose-dependent inhibition of LDL lipid peroxidation by activated U937 cells as detected by both the TBA assay and the LPO
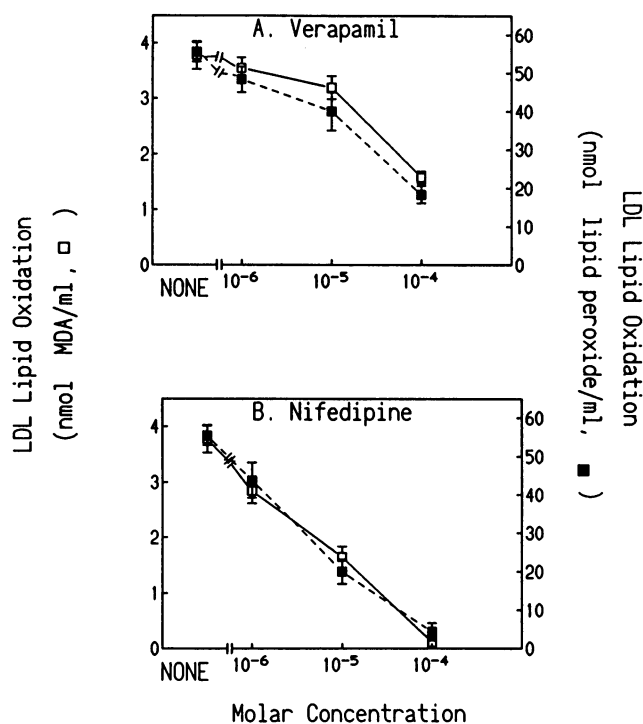
Figure 2. Ca^{2+} channel blockers, nifedipine and verapamil, inhibit LDL lipid peroxidation by activated U937 cells. U937 cells (5×10^5 cells/ml) were incubated with LDL and ZOP (2 mg/ml) in the presence of Ca^{2+} channel blockers. The incubation was at 37°C for 24 h in a humidified incubator with 10% CO_2. After incubation, peroxidation of LDL lipids was determined by both the TBA assay (*left ordinate, open squares and solid lines*) and the LPO assay (*right ordinate, closed squares and dashed lines*) as described in Methods. The data are depicted as the mean±SEM in nmol MDA/ml or nmol lipid peroxide/ml of three similar experiments. (*A*) Activated U937 cells were incubated with LDL and 1, 10, or 100 μM verapamil or without verapamil. (*B*) Activated U937 cells were incubated with LDL and 1, 10, or 100 μM nifedipine or without nifedipine.

assay. These results suggest that either blockade of Ca^{2+} entry or prevention of Ca^{2+} mobilization from internal stores inhibited monocyte-mediated peroxidation of LDL lipids by activated U937 cells.

By using $^{45}Ca^{2+}$ labeling, we found that peak Ca^{2+} influx in ZOP-activated U937 cells occurred during the first hour of activation (data not shown). In these experiments we also examined the effects of nifedipine, verapamil, lanthanum chloride, TMB-8, and ryanodine on $^{45}Ca^{2+}$ uptake after activation of U937 cells. To obtain sufficient uptake of $^{45}Ca^{2+}$ in these experiments, the concentration of U937 cells was fourfold higher than in the LDL lipid peroxidation experiments; accordingly we increased the concentrations of these hydrophobic test agents by fourfold as well to keep the same ratio of test agents to cells. U937 cells (2×10^6 cells/ml) were preincubated with test agents for 30 min. At zero time, LDL, ZOP, and $^{45}Ca^{2+}$ were added together. After 1 h of incubation, 100 μl of the cell suspension was treated to remove the ZOP and uptake of $^{45}Ca^{2+}$ was determined. The results of a representative experiment are demonstrated in Fig. 4. There was an eightfold increase in $^{45}Ca^{2+}$ uptake in ZOP-activated U937 cells. Significant blockade of $^{45}Ca^{2+}$ influx was caused by 40 and 400 μM nifedipine, 40 and 400 μM lanthanum chloride, or by 400 μM verapamil
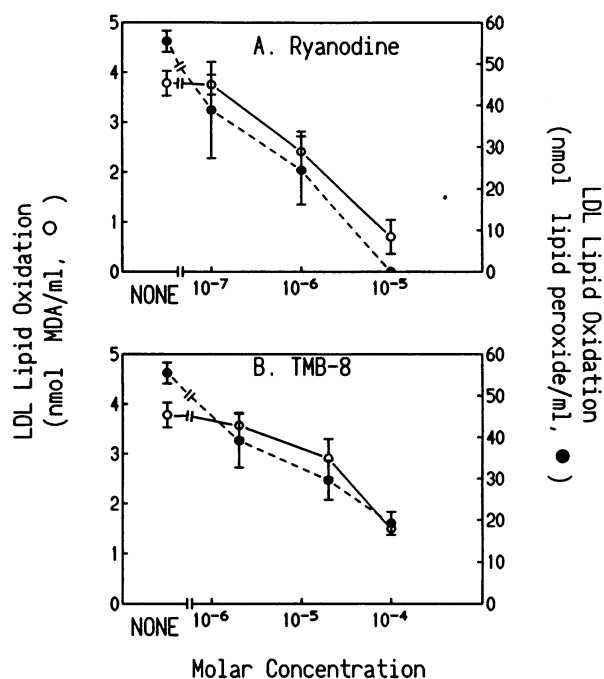

Figure 3. Agents that prevent release of Ca^{2+} from intracellular stores inhibit LDL lipid peroxidation by activated U937 cells. U937 cells (5×10^5 cells/ml) were incubated with LDL and TMB-8 or ryanodine for 24 h. The incubation conditions were the same as indicated in Fig. 2. After 24 h incubation LDL lipid peroxidation was determined by both the TBA assay (*left ordinate, open circles and solid lines*) and the LPO assay (*right ordinate, closed circles and dashed lines*) as described in Methods. The data are depicted as the mean±SEM of three similar experiments. (*A*) Activated U937 cells were incubated with LDL and 0.1, 1, or 10 μM ryanodine or without ryanodine. (*B*) Activated U937 cells were incubated with LDL and 2, 20, or 100 μM TMB-8 or without TMB-8.

($P < 0.05$). As expected, neither TMB-8 nor ryanodine blocked $^{45}Ca^{2+}$ influx by ZOP-activated U937 cells.

To determine whether Ca^{2+} release from intracellular stores and influx of Ca^{2+} from extracellular sources regulated different aspects of monocyte-mediated LDL lipid peroxidation, we investigated whether the inhibitory effects of these agents were additive. We found that the inhibition of monocyte-mediated LDL lipid peroxidation was significantly greater with ryanodine and nifedipine or verapamil together than that caused by any of these agents alone. The results of experiments are displayed in Fig. 5. Ryanodine (1 μM) significantly increased the inhibitory effects of verapamil (1, 10, or 100 μM) and nifedipine (1 or 10 μM) on LDL lipid peroxidation by activated U937 cells ($P < 0.05$). A similar statistically significant additive effect was observed with TMB-8 (20 or 100 μM) plus nifedipine (1 or 10 μM) (data not shown).

We also calculated the IC_{50} for nifedipine, TMB-8, and ryanodine. These were, respectively, 9.71, 99.77, and 1.63 μM for the production of TBA-reactive material and 11.3, 110.2, and 1.2 μM for the LPO assay. Since the relationship between the concentration of verapamil and the inhibitory effect were not linear, we did not calculate the IC_{50} of verapamil. These concentrations are very similar for the two measures of lipid peroxidation and are consistent with those shown to inhibit Ca^{2+} channels or Ca^{2+} release in other cell systems (17–19).
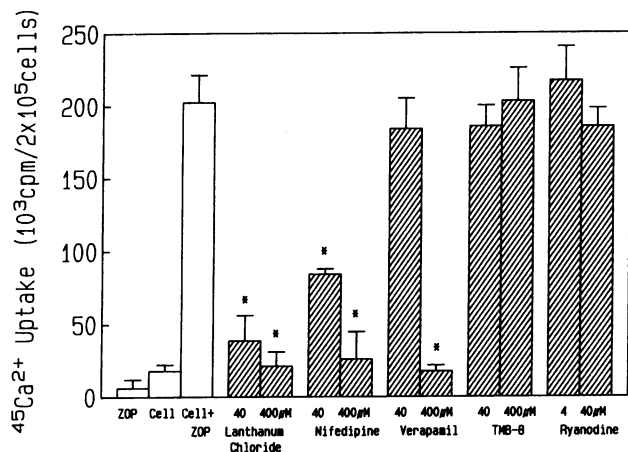
Figure 4. $^{45}Ca^{2+}$ uptake by ZOP-activated U937 cells. U937 cells (2 × 10^6 cells/ml) in HBSS were preincubated with test agents at 37°C for 30 min. At zero time, LDL and ZOP were added together with 2 µCi $^{45}Ca^{2+}$/ml. At given time intervals, 2 × 10^5 cells were separated, filtered and counted with a β-scintillation counter as described in Methods. All experiments were performed in triplicate. The results are presented as mean±SD of data obtained in one of two similar experiments. Open bars show experimental results without test agents. Hatched bars show experimental results with different test agents as indicated. 40 and 400 µM nifedipine, 40 and 400 µM lanthanum chloride significantly inhibits $^{45}Ca^{2+}$ influx (*P < 0.05). 400 µM verapamil also significantly inhibits $^{45}Ca^{2+}$ influx (*P < 0.05). The significance of inhibition was determined by Student's *t* test.

To examine the Ca^{2+} involvement in activating U937 cells to oxidize LDL, a Ca^{2+} ionophore was tested for its ability to induce LDL lipid peroxidation by U937 cells. In these experiments, U937 cells (5 × 10^5 cells/ml), LDL, and 4-bromo A23187 (0.1 and 1 µM) were incubated together. After 24 h of
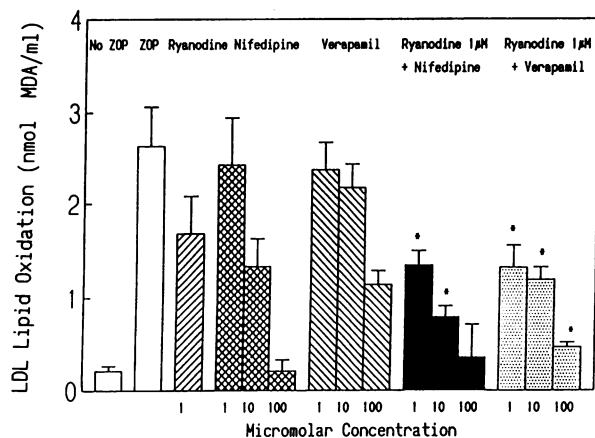

Figure 5. Ryanodine plus verapamil or nifedipine causes greater inhibition of LDL lipid peroxidation by activated U937 cells than any of these drugs alone. U937 cells (5 × 10^5 cells/ml) were incubated in the presence (*filled bars*) or absence (*open bars*) of test agents as indicated. The incubation conditions were the same as indicated in Fig. 2. After the 24-h incubation, LDL lipid peroxidation was determined as described in Methods. The data are depicted as the mean±SEM of three similar experiments. The significance of the inhibition by both drugs together as compared with either drug separately was determined by Student's *t* test (*P < 0.05).

incubation, LDL lipid peroxidation was measured. Data of representative experiments are shown in Fig. 6. 4-bromo A23187 failed to induce U937 cell-mediated LDL lipid peroxidation. We also tested whether triggering Ca^{2+} release from intracellular stores could induce LDL lipid peroxidation by U937 cells. Thapsigargin, which is known to induce the release of intracellular Ca^{2+} from internal membrane stores (20), was used. Data are shown in Fig. 6. 1 µM thapsigargin did not induce peroxidation of LDL lipids by U937 cells whereas 10 µM thapsigargin (the maximal nontoxic dosage as determined by [^{14}C]adenine release, data not shown) induced LDL lipid peroxidation to 35% of that mediated by ZOP-activated U937 cells. We also examined thapsigargin-induced intracellular Ca^{2+} release by using Fura 2–loaded cells. As expected, thapsigargin caused release of intracellular Ca^{2+} from a basal level of 70 nM to 1.3 µM (Fig. 6, *inset*) without causing a change in influx of extracellular Ca^{2+} as indicated by $^{45}Ca^{2+}$ uptake (data not shown).

To evaluate the cytotoxicity of the agents used in these studies, we tested the toxicity of Ca^{2+} channel blockers and inhibitors of intracellular Ca^{2+} mobilization by measuring [^{14}C]-adenine release and chromium-51 release by drug-treated U937 cells. In these experiments, [^{14}C]adenine- or chromium-51-labeled U937 cells were incubated with test reagents as described in Methods. The experimental results are summarized in Table I. Our data indicate that none of the concentrations of test reagents used for the studies reported here showed any toxic effects on U937 cells as measured by either assay. We also examined the drug combinations used to generate the data pre-
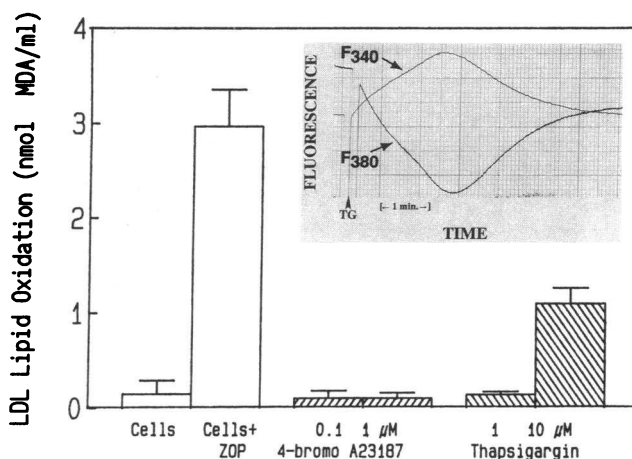

Figure 6. Effects of 4-bromo A23187 and thapsigargin on LDL lipid peroxidation by U937 cells. U937 cells (5 × 10^5 cells/ml) were incubated with LDL in the presence (*hatched bars*) or absence (*open bars*) of 4-bromo A23187 or thapsigargin at 37°C for 24 h in a humidified incubator with 10% CO_2. Different concentrations of 4-bromo A23187 or thapsigargin were added to U937 cells as indicated: 4-bromo A23187 0.1 or 1 µM, thapsigargin 1 or 10 µM. After the 24-h incubation, LDL lipid peroxidation was determined as described in Methods. The data are depicted as the mean±SEM of three similar experiments. LDL lipid peroxidation induced by ZOP was 2.97 nmol MDA/ml. 4-bromo A23187 did not induce LDL lipid peroxidation by U937 cells. 10 µM thapsigargin-induced LDL lipid peroxidation was 35% of that mediated by ZOP-activated U937 cells. (*Inset*) Intact U937 cells loaded with Fura 2 were challenged with 10 µM thapsigargin. Fura 2 measurements were conducted as described in Methods. The 340/380 nm tracings are representative of at least three different experiments.

Table I. Cytotoxic Effects of Ca^{2+} Channel Blockers and Inhibitors of Intracellular Ca^{2+} Mobilization on U937 Cells

Incubation additions*	Adenine release		Chromium release	
	10^3 cpm‡	Percent release§	10^3 cpm‡	Percent release§
Unactivated cells				
+ LDL	11.66±0.33	—	4.25±0.04	—
Activated cells				
+ LDL +				
DMSO (solvent control)	19.18±0.83	0	3.95±0.10	0
0.2% SDS (total release)	43.29±0.48	100	24.42±1.00	100
Nifedipine				
10 µM	16.05±0.37	0	3.29±0.03	0
100 µM	17.97±1.13	0	4.07±0.15	0.6
Verapamil				
10 µM	18.83±0.33	0	3.56±0.01	0
100 µM	19.52±0.11	1.4	4.67±0.03	3.5
La³⁺				
10 µM	13.07±0.30	0	2.88±0.07	0
100 µM	13.47±0.10	0	3.52±0.08	0
TMB-8				
10 µM	16.88±0.34	0	ND	0
20 µM	ND‖		3.87±0.11	0
100 µM	16.58±0.44	0	4.51±0.10	2.7
Ryanodine				
0.1 µM	11.85±0.31	0	ND	
1 µM	15.19±0.41	0	3.84±0.10	0
10 µM	16.04±0.23	0	3.93±0.22	0

* U937 cells (5×10^5/ml) were radiolabeled with [^{14}C]adenine or chromium-51 and then incubated together with LDL (0.5 mg/ml) and a variety of test agents as indicated in Methods. ‡ Release of radioactivity was determined as described in Methods. All experiments were performed in triplicate. The results are expressed as mean±SD of data obtained in one of two similar experiments. § Percent release of [^{14}C]adenine or chromium-51 was determined by the equation described in Methods. ‖ ND, not done.

sented in Fig. 5 and found that none of these drug combinations was toxic for U937 cells (data not shown). Additionally, in a pilot study, we examined whether the effect of nifedipine could be reversed by removing it after pretreating U937 cells, to determine whether it irreversibly injured the cells. We found that after removing nifedipine (100 µM) U937 cells were capable of oxidizing LDL lipids to a similar degree as untreated cells; thus providing further support that an agent that inhibits calcium influx can profoundly inhibit LDL lipid peroxidation without injuring the monocytes.

Finally, we tested the antioxidant activity of the Ca^{2+} channel blockers and agents preventing Ca^{2+} release from intracellular stores that were used in the above studies to rule out a nonspecific inhibitory effect on U937 cells. The antioxidant activity was determined by measuring the effect of these test agents on copper-induced LDL lipid peroxidation. (Briefly, CuSO$_4$ [5 µM] and LDL [0.5 mg cholesterol/ml] were incubated together in the presence or absence of test agents at 37°C for 24 h. After incubation the degree of lipid peroxidation on LDL was determined by the TBA assay.) Nifedipine, verap-

amil, lanthanum chloride, or TMB-8 had no antioxidant activity at the concentrations used in these experiments. Ryanodine at 0.1 and 1 µM had no antioxidant activity whereas, at a concentration of 10 µM, it caused a 68% inhibition of copper-induced LDL lipid peroxidation (data not shown).

Discussion

The oxidation of LDL by monocytes requires monocyte activation by certain stimulating agents such as ZOP or bacterial LPS (1, 2). Zymosan is a particulate stimulus comprised of yeast cell walls and is opsonized with human serum before activating monocytes. Therefore, ZOP is coated with immunoglobulin and complement and likely interacts with monocytes through Fc and complement receptors as well as triggering phagocytosis. Our data show that the LDL lipid peroxidation by ZOP-activated U937 cells requires both the influx of extracellular Ca^{2+} and the release of sequestered intracellular Ca^{2+}, since either blockade of Ca^{2+} influx by Ca^{2+} channel blockers or prevention of Ca^{2+} mobilization by TMB-8 and ryanodine abolished the LDL lipid peroxidation. Thus, our findings indicate that Ca^{2+} functions as an important mediator in the process of LDL lipid peroxidation by ZOP-activated U937 cells.

Increases in intracellular Ca^{2+} can be achieved either by release of Ca^{2+} from internal stores or by influx of extracellular Ca^{2+} from medium. First, we examined the Ca^{2+} influx through Ca^{2+} channels in the cell plasma membrane. Both slow Ca^{2+} channel blockers nifedipine (dihydropyridine) and verapamil (phenylakylamine) have been shown to bind with Ca^{2+} channels on the plasma membrane in a variety of cells and tissues (21). The blockade of Ca^{2+} channels by nifedipine or verapamil affects a wide range of cell functions, such as reducing contraction of vascular smooth muscle and cardiac muscle (21), inhibiting proliferation and activation of T lymphocytes (17), and interfering with differentiation of U937 cells (22). Our data show that in the presence of nifedipine or verapamil the peroxidation of LDL lipids by activated monocytes is significantly decreased (Fig. 2) as is ^{45}Ca^{2+} influx (Fig. 4). Similarly, removal of extracellular Ca^{2+} by EGTA or by addition of lanthanum chloride, which competes with Ca^{2+} for Ca^{2+} channels (23) (or other Ca^{2+}-binding sites), has the same inhibitory effect on LDL lipid peroxidation by activated U937 cells (Fig. 1). Our results thus indicate that blocking influx of extracellular Ca^{2+} inhibits LDL lipid peroxidation by activated U937 cells.

Our data also show differential effects of nifedipine and verapamil. Nifedipine caused 92% inhibition of monocyte-mediated LDL lipid peroxidation at a concentration of 100 µM whereas verapamil showed only 55% inhibition. Unfortunately, we were unable to measure intracellular Ca^{2+} concentrations using Fura 2 fluorescence because of the fact that ZOP is an extremely turbid particulate suspension. ^{45}Ca^{2+} uptake studies showed that there was an eightfold increase in intracellular calcium because of influx of Ca^{2+} upon monocyte activation by ZOP. Although both nifedipine and verapamil blocked the influx of extracellular Ca^{2+} at high concentrations, at lower concentrations only nifedipine blocked the influx of extracellular Ca^{2+} (see Fig. 4). These data suggest that U937 cells may contain multiregulated or multiple types of receptor-operated Ca^{2+} channels. Existence of multiple types of Ca^{2+} channels in the same tissue has been reported by Ratz and Flaim (24), who found that two types of receptor-operated Ca^{2+} channels ex-

isted in smooth muscle of bovine coronary arteries, only one of which was sensitive to verapamil. Alternatively, nifedipine and verapamil may have differing binding sites and affinity for the same Ca^{2+} channel (25, 26), since their molecular structures are not the same. Furthermore, since U937 cells do not have the classic voltage-sensitive L type channel, it is likely that nifedipine and verapamil operate differently in these cells than in smooth muscle cells. Precedence for the use of nifedipine and verapamil as inhibitors of Ca^{2+} influx has been established in U937 cells (22) and other nonexcitable cells such as T cells (17) and neutrophils (27). Finally, it was reported that in neutrophils (27) and skeletal muscle (28) nifedipine can also bind to intracellular plasma membrane such as sarcoplasmic reticulum (28) to prevent Ca^{2+} release. Thus, at high concentrations nifedipine may block both Ca^{2+} influx from extracellular medium and release from intracellular stores in U937 cells and thus prevent Ca^{2+}-triggered intracellular signal transductions. Besides blockade of Ca^{2+} influx, it has been reported that nifedipine and verapamil have other potentially antiatherosclerotic effects, since both nifedipine and verapamil inhibit intracellular cholesteryl esterification in macrophages (29). The Ca^{2+} dependence of this latter observation remains to be determined.

We also examined the contribution of Ca^{2+} mobilization from internal stores to monocyte-mediated peroxidation of LDL lipids. Two agents reported to prevent Ca^{2+} release from internal membrane stores, TMB-8 and ryanodine, were used. TMB-8 has been reported to prevent mobilization of Ca^{2+} from intracellular stores without altering Ca^{2+} influx into the cell (18, 30, 31). Ryanodine is believed to bind to the Ca^{2+} release channel in the intracellular Ca^{2+} stores (32). Our data demonstrated that both TMB-8 and ryanodine had dose-dependent inhibitory effects on LDL lipid peroxidation by activated U937 cells (Fig. 3); an effect that was independent of direct antioxidant effects of these agents. Furthermore, neither agent inhibited $^{45}Ca^{2+}$ influx (Fig. 4). These results indicate that intracellular Ca^{2+} mobilization is required in the process of LDL lipid peroxidation.

Additionally, our data also show that Ca^{2+} channel blockers and agents known to prevent release of intracellular Ca^{2+} can have additive effects (Fig. 5). The inhibitory effects of these two types of reagents in combination were greater than either alone. These findings support the concept that increases in intracellular Ca^{2+} from intracellular and extracellular sources are not functionally equivalent. Similar observations have been made by Haverstick et al. (33) in T lymphocytes. Our findings indicate that intracellular Ca^{2+} concentrations regulated by both influx from the extracellular medium and release from intracellular stores are critical elements of the activation of monocytes/macrophages.

We also investigated whether direct stimulation of an increase in intracellular Ca^{2+} was sufficient to induce the peroxidation of LDL lipids observed with ZOP-activated U937 cells. U937 cells and LDL were incubated together with the Ca^{2+} ionophore 4-bromo A23187 or thapsigargin. This Ca^{2+} ionophore did not induce LDL lipid peroxidation with 5×10^5 U937 cells/ml (Fig. 6). Furthermore, the release of intracellular Ca^{2+} by thapsigargin only induced a suboptimal level of LDL lipid peroxidation (Fig. 6). This level of LDL lipid peroxidation was not enhanced by incubation with both thapsigargin and 4-bromo A23187 (data not shown). Therefore, the increases in intracellular Ca^{2+} levels are critical for optimal per-

oxidation of LDL lipids by activated U937 cells to occur. Both influx and mobilization of Ca^{2+} are required but are not sufficient for stimulating LDL lipid peroxidation equivalent to that induced by ZOP.

The functional role of Ca^{2+} in the cellular response to exogenous stimulus likely involves the induction of several different intracellular signal transduction pathways. As is well recognized, monocytes and other phagocytes, if stimulated by ZOP, exhibit receptor-mediated induction of multiple intracellular signal transduction pathways (7, 34, 35). The effect of ZOP has been suggested to induce the activation of phospholipase C (PLC), which catalyzes hydrolysis of phosphatidylinositides (34) and generates the intracellular second messengers inositol phosphates (35) and diacylglycerol. Inositol phosphates induce the mobilization of intracellular Ca^{2+} from internal stores. Thus, the increase in intracellular Ca^{2+} levels with ZOP could be mediated by inositol phosphates generated by activation of PLC. Recently, Van De Winkel et al. (36) showed that crosslinking of IgG Fcγ receptor by monoclonal antibodies induced an increase in intracellular Ca^{2+} levels in U937 cells. Phospholipase A_2 has been shown to be a Ca^{2+}-dependent signaling mechanism as well (37). In other studies, we have found that inhibition, protein kinase C, phospholipase A_2, or lipoxygenase pathways by using selective inhibitors destroys the ability of ZOP-activated U937 cells to oxidize LDL (reference 3 and unpublished data).

Taken together, our results indicate that increases in intracellular Ca^{2+} are required for peroxidation of LDL lipids by activated monocytes. Although increased intracellular Ca^{2+} levels are necessary for LDL lipid peroxidation, several other intracellular signal transduction pathways are likely involved, since neither stimulation of Ca^{2+} influx from extracellular medium nor Ca^{2+} release from intracellular stores could individually or together induce LDL lipid peroxidation by monocytes equivalent to that induced by ZOP. ZOP must activate other signal transduction pathways that act in concert with increased intracellular Ca^{2+} to stimulate the peroxidation of LDL lipids by U937 cells.

Acknowledgments

References

1. Cathcart, M. K., D. W. Morel, and G. M. Chisolm. 1985. Monocytes and neutrophils oxidize low density lipoprotein making it cytotoxic. *J. Leukocyte Biol.* 38:341–350.
2. Cathcart, M. K., A. K. McNally, D. W. Morel, and G. M. Chisolm. 1989. Superoxide anion participation in human monocyte-mediated oxidation of low-

density lipoprotein and conversion of low-density lipoprotein to a cytotoxin. *J. Immunol.* 142:1963–1969.

3. Cathcart, M. K., A. K. McNally, and G. M. Chisolm. 1991. Lipoxygenase-mediated transformation of human low density lipoprotein to an oxidized and cytotoxic complex. *J. Lipid Res.* 32:63–70.

4. Hessler, J. R., D. W. Morel, L. J. Lewis, and G. M. Chisolm. 1983. Lipoprotein oxidation and lipoprotein-induced cytotoxicity. *Arteriosclerosis.* 3:215–222.

5. Jurgens, G., H. F. Hoff, G. M. Chisolm, and H. Esterbauer. 1987. Modification of human serum low density lipoprotein by oxidation-characterization and pathophysiological implications. *Chem. Phys. Lipids.* 45:315–336.

6. 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. *N. Engl. J. Med.* 320:915–924.

7. Czop, J. K., and K. F. Austen. 1980. Functional discrimination by human monocytes between their C3b receptors and their recognition units for particulate activators of the alternative complement pathway. *J. Immunol.* 125:124–128.

8. Schmitz, G., J. Hankowitz, and E. M. Kovacs. 1991. Cellular processes in atherogenesis: potential targets of Ca^{2+} channel blockers. *Atherosclerosis.* 88:109–132.

9. Cathcart, M. K., G. M. Chisolm, A. K. McNally, and D. W. Morel. 1988. Oxidative modification of low density lipoprotein (LDL) by activated human monocytes and the cell lines U937 and HL60. *In Vitro Cell. Dev. Biol.* 24:1001–1008.

10. Schuh, J., G. F. Fairclough, and R. H. Haschemeyer. 1978. Oxygen-mediated heterogeneity of apo-low-density lipoprotein. *Proc. Natl. Acad. Sci. USA.* 75:3173–3177.

11. El-Saadani, M., H. Esterbauer, M. El-Sayed, M. Goher, A. Y. Nassar, and G. Jurgens. 1989. A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagents. *J. Lipid Res.* 30:627–630.

12. Warren, J. B., and U. S. Ryan. 1989. Endothelial injury assessed by isotope release: ^{3}H-adenine compared with ^{51}Cr. *In Vitro Cell. Dev. Biol.* 25:334–335.

13. Shirhatti, V., and G. Krishna. 1985. A simple and sensitive method for monitoring drug-induced cell injury in cultured cells. *Anal. Biochem.* 147:410–418.

14. Varani, J., S. H. Phan, D. F. Gibbs, U. S. Ryan, and P. A. Ward. 1990. H_2O_2-mediated cytotoxicity of rat pulmonary endothelial cells. Changes in adenosine triphosphate and purine products and effects of protective interventions. *Lab. Invest.* 63:683–689.

15. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

16. Johnston, R. B. 1981. Secretion of superoxide anion. *In* Methods for Studying Mononuclear Phagocytes. D. O. Adams, P. J. Edelson, and H. Koren, editors. Academic Press, New York. 489–497.

17. Birx, D. L., M. Berger, and T. A. Fleisher. 1984. The interference of T cell activation by calcium channel blocking agents. *J. Immunol.* 133:2904–2909.

18. Donowitz, M., S. Cusolito, and G. W. G. Sharp. 1986. Effects of calcium antagonist TMB-8 on active Na and Cl transport in rabbit ileum. *Am. J. Physiol.* 250:G691–G697.

19. Zimanyi, I., and I. N. Pessah. 1991. Comparison of [^{3}H]ryanodine receptors and Ca^{2+} release from rat cardiac and rabbit skeletal muscle sarcoplasmic reticulum. *J. Pharmacol. Exp. Ther.* 256:938–946.

20. Thastrup, O., P. J. Cullen, B. K. Drobak, M. R. Hanley, and A. P. Dawson. 1990. Thapsigargin, a tumor promter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. USA.* 87:2466–2470.

21. Hosey, M. M., and M. Lazdunski. 1988. Calcium channels: molecular pharmacology, structure and regulation. *J. Membr. Biol.* 104:81–105.

22. Pedrinaci, S., F. Ruiz-Cabello, O. Gomez, A. Collado, and F. Garrido. 1990. Protein kinase C-mediated regulation of the expression of CD14 and CD11/CD18 in U937 cells. *Int. J. Cancer.* 45:294–298.

23. Gould, R. J., K. M. M. Murphy, and S. H. Snyder. 1982. [^{3}H]-Nitrendipine-labeled calcium channels discriminate inorganic calcium agonists and antagonists. *Proc. Natl. Acad. Sci. USA.* 79:3656–3660.

24. Ratz, P. H., and S. F. Flaim. 1985. Acetylcholine- and 5-hydroxytryptamine-stimulated contraction and calcium uptake in bovine coronary arteries: evidence for two populations of receptor-operated calcium channels. *J. Pharmacol. Exp. Ther.* 234:641–647.

25. Catterall, W. A. 1988. Structure and function of voltage-sensitive ion channels. *Science (Wash. DC).* 242:50–61.

26. Zobrist, R. H., K. M. Giacomini, W. L. Nelson, and J. C. Giacomini. 1986. The interaction of phenylalkylamine calcium channel blockers with 1,4-dihydropyridine binding site. *J. Mol. Cell. Cardiol.* 18:963–974.

27. Rosales, C., and E. J. Brown. 1992. Calcium channel blockers nifedipine and diltiazem inhibit Ca^{2+} release from intracellular stores in neutrophils. *J. Biol. Chem.* 267:1443–1448.

28. Rios, E., and G. Brum. 1987. Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. *Nature (Lond.).* 325:717–720.

29. Daugherty, A., D. L. Rateri, G. Schonfeld, and B. E. Sobel. 1987. Inhibition of cholesteryl ester deposition in macrophages by calcium entry blockers: an effect dissociable from calcium entry blockade. *Br. J. Pharmacol.* 91:113–118.

30. Chiou, C. Y., and M. H. Malagodi. 1975. Studies on the mechanism of action of a new Ca^{2+} antagonist, 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscles. *Br. J. Pharmacol.* 53:279–285.

31. Mix, L. L., R. J. Dinerstein, and M. L. Villereal. 1984. Mitogens and melittin stimulate an increase in intracellular free calcium concentration in human fibroblasts. *Biochem. Biophys. Res. Commun.* 119:69–75.

32. Fill, M., and R. Coronado. 1988. Ryanodine receptor channel of sarcoplasmic reticulum. *Trends Neurosci.* 11:453–457.

33. Haverstick, D. M., V. H. Engelhard, and L. S. Gray. 1991. Three intracellular signals for cytotoxic T lymphocyte-mediated killing. Independent roles for protein kinase C, Ca^{2+} influx, and Ca^{2+} release from internal stores. *J. Immunol.* 146:3306–3313.

34. Leslie, C. C., and D. M. Detty. 1986. Arachidonic acid turnover in response to lipopolysaccharide and opsonized zymosan in human monocyte-derived macrophages. *Biochem. J.* 236:251–259.

35. Hoffman, T., C. Brando, E. F. Lizzio, Y. L. Lee, M. Hansen, A. K. Tripathi, M. Taplits, J. Puri, E. Bonvini, T. G. Abrahamsen, et al. 1991. Calcium-dependent eicosanoid metabolism by concanavalin A-stimulated human monocytes *in vitro*. Synergism with phorbol ester indicates separate regulation of leukotriene B_4 synthesis and release. *J. Immunol.* 146:692–700.

36. Van De Winkel, J. G. J., W. J. M. Tax, C. W. M. Jacobs, T. W. J. Huizinga, and P. H. G. M. Willems. 1990. Cross-linking of both types of IgG Fc receptor, FcγRI and FcγRII, enhances intracellular free Ca^{2+} in the monocytic cell line U937. *Scand. J. Immunol.* 31:315–325.

37. Chang, J., J. H. Musser, and H. McGregor. 1987. Phospholipase A_2: function and pharmacological regulation. *Biochem. Pharmacol.* 36:2429–2436.