SPHINGOSINE KINASE REGULATES OXIDIZED LOW DENSITY LIPOPROTEIN MEDIATED CALCIUM OSCILLATIONS AND MACROPHAGE SURVIVAL

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Content Summary: This study shows that oxLDL induces activation of sphingosine kinase in murine macrophages. Sphingosine kinase activation increases sphingosine-1-phosphate levels which leads to mobilization of calcium from intracellular stores and the generation of calcium oscillations. These calcium oscillations, as previously reported, lead to an inhibition of macrophage apoptosis.

Abbreviations: BMDM, bone marrow derived macrophage; [Ca^{2+}], intracellular calcium; eEF2, eukaryotic elongation factor-2; IP_3R, inositol-1,4,5-triphosphate receptor; oxLDL, oxidized low density lipoprotein; RyR, ryanodine receptor; S1P, sphingosine-1-phosphate; SERCA, sarco-endoplasmic reticulum ATPase; SK, sphingosine kinase.
ABSTRACT

We recently reported that oxidized low density lipoprotein (oxLDL) induces an oscillatory increase in intracellular calcium ([Ca$^{2+}$]$_i$) levels in macrophages. Furthermore, we have shown that these [Ca$^{2+}$]$_i$ oscillations mediate oxLDL's ability to inhibit macrophage apoptosis in response to growth factor deprivation. However, the signal transduction pathways by which oxLDL induces [Ca$^{2+}$]$_i$ oscillations have not been elucidated. In this study, we show that these oscillations are mediated in part by intracellular mechanisms, as depleting extracellular Ca$^{2+}$ did not completely abolish the effect. Inhibiting sarco-endoplasmic reticulum ATPase (SERCA) completely blocked [Ca$^{2+}$]$_i$ oscillations, suggesting a role for Ca$^{2+}$ re-uptake by the ER. The addition of oxLDL resulted in an almost immediate activation of sphingosine kinase (SK), which can increase sphingosine-1-phosphate (S1P) levels by phosphorylating sphingosine. Moreover, S1P was shown to be as effective as oxLDL in blocking macrophage apoptosis and producing [Ca$^{2+}$]$_i$ oscillations. This suggests that the mechanism in which oxLDL generates [Ca$^{2+}$]$_i$ oscillations may be (1) activation of SK, (2) SK-mediated increase in S1P levels (3) S1P-mediated Ca$^{2+}$ release from intracellular stores, and (4) SERCA-mediated Ca$^{2+}$ re-uptake back into the ER.
INTRODUCTION

Atherosclerosis is a chronic inflammatory disease of large and medium-sized arteries and macrophages play a central role in its initiation and progression (1, 2). The accumulation of macrophages in lesions is due in part to recruitment of monocytes from the bloodstream (2), and also to proliferation of macrophages within atherosclerotic lesions (3-6). Oxidized low-density lipoprotein (oxLDL) plays an important role in atherogenesis, in part because of its effects on macrophage recruitment and retention (7, 8). Initial oxidation of LDL and formation of what is often referred to as “minimally modified” LDL stimulates adjacent endothelial and smooth muscle cells to release monocyte chemotactic protein-1 (MCP-1), which facilitates the recruitment of monocytes into the arterial wall. OxLDL itself is chemotactic for monocytes by virtue of its lysophosphatidylcholine content.

At high concentrations, oxLDL can be toxic to cultured macrophages and other cells, but at lower concentrations it has been clearly shown to promote macrophage proliferation and inhibit apoptosis (9-15). Our group has recently reported that oxLDL inhibits macrophage apoptosis by activating eukaryotic elongation factor-2 (eEF2) kinase (also known as Ca²⁺/calmodulin dependent kinase III) (16). eEF2 kinase activation and inhibition of macrophage apoptosis is mediated an oscillatory increase in [Ca²⁺]. However, the signal transduction pathways involved in oxLDL mediated [Ca²⁺] oscillations have not been elucidated.

Ca²⁺ is a ubiquitous intracellular signal responsible for controlling numerous cellular processes. These processes range from muscle contraction to synaptic transmission, and from cellular proliferation to apoptosis (17). Ca²⁺ can relay specificity in signalling through its high degree of spatial and temporal diversity (18). Ca²⁺ released into the cytoplasm can function as a second messenger that can mediate cell survival or induce apoptosis (19). Within the same cell
Ca\(^{2+}\) signals can have dual roles in response to the same stimulus, depending on the temporal pattern of the Ca\(^{2+}\) elevations. For example, distinct temporal patterns of Ca\(^{2+}\) elevation are associated with positive versus negative selection of developing T cells in the thymus (20, 21). Weak TCR activation induces Ca\(^{2+}\) oscillations, whereas strong TCR activation induces sustained Ca\(^{2+}\) elevation. The former activates nuclear factor of activated T cells (NFAT) optimally and thereby up-regulates expression of the pro-survival cytokine IL-2, whereas the latter up-regulates the pro-apoptotic BH3-only protein Bim.

Spingosine-1-phosphate (S1P) plays an important role in many cellular processes including regulation of Ca\(^{2+}\) signals (22-26) and cell survival and proliferation (27-35). Intracellular levels of S1P are tightly regulated by the equilibrium between its formation, which is catalyzed by sphingosine kinase (SK), and its degradation, which is catalyzed by S1P lyase and S1P phosphatases (36). S1P produced in response to agonists has the ability to function intracellularly as a second messenger or after secretion in an autocrine/paracrine fashion to activate S1P receptors (formerly known as EDG receptors) on the cell surface (37). Although S1P is thought to mobilize [Ca\(^{2+}\)]\(_i\) via interaction with its surface receptors, increasing evidence suggests an important intracellular role for S1P in mediating Ca\(^{2+}\) increases within the cell (38, 39). However, the exact mechanism in which S1P mediates Ca\(^{2+}\) mobilization is still uncertain.

In the present study, we describe a mechanism in which oxLDL generates [Ca\(^{2+}\)]\(_i\) oscillations by (1) oxLDL-mediated activation of SK, (2) SK-mediated increase in S1P levels (3) S1P-mediated Ca\(^{2+}\) release from intracellular stores, and (4) SERCA-mediated Ca\(^{2+}\) re-uptake back into the ER.
MATERIALS AND METHODS

Materials. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), Dulbecco’s phosphate buffered saline (DPBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Fluo-4-AM, pluronic acid, and propidium iodide (PI) were purchased from Invitrogen (Burlington, ON, Canada). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was from Promega (Madison, WI). Phenazine methosulfate (PMS), phosphatidylcholine (PC), lys phosphatidylcholine (lysoPC), and sphingomyelin were obtained from Sigma-Aldrich (St. Louis, MO). The sphingosine kinase inhibitor 2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole (SKI), U-73122, and hydrated 1-(((5-(4-nitrophenyl)-2-furanyl)-methylene)-amino)-2,4-imidazolidinedione sodium salt (dantrolene) were purchased from Calbiochem (San Diego, CA). Protein A Sepharose beads, L-[35S]methionine, and L-[4,5-3H]leucine were from Amersham Biosciences (Piscataway, NJ). [32P]ATP was from Perkin Elmer (Waltham, MA). BCA protein assay reagents, BSA standards, and SuperSignal Femto Substrate were purchased from Pierce (Milwaukee, WI). Goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was from DAKO Diagnostics (Mississauga, ON, Canada). G60 silica gel plates were from Whatman (Piscataway, NJ). SDS-PAGE molecular weight standards and polyvinylidene difluoride (PVDF) membranes were provided by Bio-Rad (Hercules, CA). BioMax MR film was from Kodak (Rochester, NY).

Lipoprotein isolation and oxidation. Low density lipoprotein (d = 1.019-1.063 g/ml) was isolated from EDTA-anticoagulated whole blood of fasting healthy normolipidemic donors(15).
Copper oxidation was performed by incubating LDL (200 µg/ml) with 5 µmol/L CuSO₄ in DPBS containing 0.90 mmol/L CaCl₂ and 0.49 mmol/L MgCl₂ for 24 hours at 37°C. The reaction was stopped by addition of 40 µmol/L butylated hydroxytoluene (BHT) and 300 µmol/L EDTA. The oxidized LDL was then washed and concentrated to approximately 1.5 mg/ml using Amicon Centricon Plus-20 ultrafilters (Millipore, Bedford, MA). After a 0.45 micron filtration, protein concentration of oxidized LDL was determined using BCA protein assay.

Relative electrophoretic mobility (Rf) of modified lipoproteins was assessed using a Ciba-Corning (East Walpole, MA) electrophoresis apparatus and Titan agarose gels (Beaumont, TX) in 50 mmol/L barbital buffer, pH 8.6 according to the manufacturer’s instructions. BSA was added to lipoprotein samples to ensure reproducible migration distances. Lipoprotein bands were visualized by staining with Fat Red 7B. All oxLDL used in this study was extensively modified with an Rf value greater than or equal to 3 when compared with nLDL.

**Cell culture.** L929 cells (kindly provided by Dr. J.W. Schrader, Biomedical Research Centre, BC, Canada) were seeded in TufRol™ roller bottles (BD Falcon, San Jose, CA) at a density of 1.5 x 10⁴ cells per cm² and cultured in media (DMEM, 10% FBS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 50 U/ml penicillin, 50 µg/ml streptomycin) containing 20 mmol/L HEPES at 37°C in a 5% CO₂ atmosphere. After 15 days, the media were harvested and centrifuged at 800 x g for 10 minutes. The supernatant was filter sterilized through a 0.22 micron filter. This L929-cell conditioned media (LCM) contains approximately 10,000 U/ml of M-CSF (40).

Bone marrow cells were obtained from the femurs of 6-8 week old female CD1 mice (Charles River Laboratories, Wilmington, MA) as previously described (15). Cells were
cultured in media containing 10% LCM for 18 hours at 37°C in a 95% humidity atmosphere containing 5% CO₂. After 18 hours, non-adherent cells were isolated and differentiated into macrophages by culturing them in medium containing 10% LCM until 80% confluence was reached (5-6 days). Cells were washed to remove non-adherent cells and harvested using a rubber cell scraper (Sarstedt, Montreal, QC, Canada).

**Calcium imaging.** BMDM were seeded in 6-well plates at 5.0 x 10⁴ cells per cm² and grown for 24 hours. Cells were then washed with Ca²⁺ free DPBS and incubated in Ca²⁺ free, HEPES buffered DPBS containing 2 µmol/L Fluo-4-AM for 30 minutes at room temperature. Fluo-4-AM was dissolved with 20% pluronic acid in DMSO to make a 2 mmol/L stock solution. Cells were washed again with DPBS and incubated in HEPES buffered medium with inhibitors where indicated for 10 minutes at room temperature to allow for de-esterification of the acetoxymethyl group. Medium was then removed and fresh media containing test compounds and inhibitors where indicated were added. Fluorescence was measured every 0.6 seconds for 2 minutes using an inverted Leica TCS SP2 AOBS laser scanning confocal microscope with a 10X objective. Image analysis was performed using Leica LCS software and fluorescence of every cell in each field was measured. On average, 68.2 +/- 11.1 cells were separately analyzed per condition in each experiment. Cells exhibiting an increase of fluorescence at least 2 times that of background, followed by a decrease in fluorescence and another increase in fluorescence were scored as positive for calcium oscillations. Each condition was performed in duplicate within the experiment and data shown are representative of at least 3 independent experiments.
Cell viability assay. BMDM were seeded in 96-well plates at 5.0 x 10^4 cells per cm^2 and grown for 24 hours. Cells were washed and incubated with medium with or without compounds as indicated for 24 hours. MTS/PMS solution was then added to each well to a final concentration of 333 µg/ml MTS and 25 µmol/L PMS. After incubation for 2 hours at 37 °C, the absorbance at 490 nm was recorded using a Molecular Devices VersaMax microplate reader. Correlation between macrophage number and formation of formazan product has been previously established (11). Each condition was performed in triplicate within the experiment and data is representative of at least 3 independent experiments.

Apoptosis Assay. BMDM were seeded in 6-well plates at 5.0 x 10^4 cells per cm^2 and grown for 24 hours. Cells were then washed and incubated with medium containing compounds as indicated for 24 hours. Cells were harvested using a rubber cell scraper and fixed in 70% cold ethanol for 30 minutes. Cells were then washed with DPBS and stained with 3 µmol/L PI in DPBS containing 0.1% Triton X-100 and 0.73 µmol/L RNase A. DNA content was analyzed by flow cytometry on the FL-3 channel with appropriate gating used to exclude debris and cellular aggregates. Ten thousand events were counted for analysis. Each condition was performed in triplicate within the experiment and representative of at least 3 independent experiments.

Sphingosine kinase activity assay. BMDM were seeded in 60 mm plates at 5.0 x 10^4 cells per cm^2 and grown for 24 hours. Cells were then washed and incubated with medium in the absence of M-CSF for 4 hours. OxLDL (25 µg/ml) was then added for the time points indicated. Afterwards, cells were washed with DPBS and lysed with ice-cold solubilization buffer (50 mmol/L Tris-Cl pH 8.0, 150 mmol/L NaCl, 1% Nonidet P-40 (IGEPAL CA-630), 10% glycerol,
154 nmol/L aprotinin, 2.90 µmol/L bestatin, 2.34 µmol/L leupeptin, 1.46 µmol/L pepstatin, 2.80 µmol/L trans-epoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64), 1 mmol/L sodium fluoride). Lysates were centrifuged at 20,000 x g for 10 minutes, and the protein content of supernatants quantified by BCA protein assay. Equal amounts of protein were then incubated with 50 µmol/L sphingosine in 0.4% fatty acid-free BSA, 10 µCi of [³²P]ATP and 100 mmol/L MgCl₂. The reaction was carried out for 30 minutes at 37 °C and stopped by the addition of 20 µl of 1 N HCl and 800 µl of chloroform/methanol/HCl (100:200:1, vol/vol/vol). After 10 minutes, 240 µl of chloroform and 240 µl of 2 mol/L KCl were added, and the samples were centrifuged at 3000 x g for 5 minutes. The aqueous layer was aspirated, and 250 µl of the organic layer were transferred to new glass tubes. The samples were evaporated under nitrogen gas and then resuspended in chloroform/methanol/HCl (100:200:1, vol/vol/vol). Labelled S1P was resolved by thin layer chromatography on G60 silica gel plates with 1-buthanol/methanol/acetic acid/water (80:20:10:20, vol/vol/vol). Labelled S1P was then imaged and quantified using a Bio-Rad FX phosphor-imager.

Statistical analyses: Categorical data (proportion of cells showing Ca²⁺ oscillations) were assessed with both Student's t-test and chi-square test. Continuous data were assessed with Student's t-test. Comparisons showing p values less than 0.05 were deemed significant.
RESULTS

Lysophosphatidylcholine (lysoPC) in oxLDL is not responsible for the generation of $[\text{Ca}^{2+}]_i$ oscillations. OxLDL and one of its components, lysoPC, have both been shown to induce an increase in $[\text{Ca}^{2+}]_i$ in macrophages (41-44). While 10 μmol/L lysoPC was able to elicit $[\text{Ca}^{2+}]_i$ oscillations in BMDMs to some extent, a considerably lower percentage of cells were positive for $[\text{Ca}^{2+}]_i$ oscillations compared to cells treated with oxLDL (the 25μg/ml of oxLDL used contains approximately 6 μmol/L lysoPC(45)) (Figure 1). Furthermore, phosphatidylcholine (PC) treatment elicited a response similar to that of lysoPC (Figure 1). Hence even though PC is converted to lysoPC during the LDL oxidation reaction (45), it is unlikely that the lysoPC content in oxLDL is responsible for the observed $[\text{Ca}^{2+}]_i$ oscillations.

Extracellular Ca$^{2+}$ plays a partial role in the generation of $[\text{Ca}^{2+}]_i$ oscillations. An increase in $[\text{Ca}^{2+}]_i$ can be mediated by an influx of Ca$^{2+}$ from the extracellular environment or from intracellular Ca$^{2+}$ stores. To assess the contribution of extracellular Ca$^{2+}$, BMDM were incubated in medium lacking Ca$^{2+}$. Under these conditions, the percentage of cells showing Ca$^{2+}$ oscillations in response to oxLDL was reduced to less than half the level observed in cells incubated in media containing Ca$^{2+}$ (Figure 2). This indicates that while the presence of extracellular Ca$^{2+}$ is required for the full effect of oxLDL, release from intracellular stores accounts for much of the observed $[\text{Ca}^{2+}]_i$ oscillations.

Thapsigargin blocks oxLDL generated $[\text{Ca}^{2+}]_i$ oscillations. During the course of a Ca$^{2+}$ transient, the release of calcium from stores is followed by re-uptake via a number of pumps and exchangers that remove Ca$^{2+}$ from the cytoplasm. Sarco-endoplasmic reticulum ATPase
(SERCA) is one of the pumps that returns Ca\(^{2+}\) from the cytoplasm to the ER (46).

Thapsigargin, an epoxide derivative that selectively prevents Ca\(^{2+}\) binding to SERCA (47-49), completely blocked oxLDL-generated [Ca\(^{2+}\)]\(_i\) oscillations (Figure 3). This suggests that SERCA is responsible for Ca\(^{2+}\) re-uptake to produce [Ca\(^{2+}\)]\(_i\) oscillations.

**Inhibition of phospholipase C or ryanodine receptor (RyR) does not block oxLDL mediated macrophage survival.** Two well-studied mechanisms of Ca\(^{2+}\) release from intracellular stores involve inositol-1,4,5-triphosphate receptors (IP\(_3\)Rs) and RyRs. Activation of phospholipase C (PLC) results in the conversion of phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)) to diacylglycerol (DAG) and IP\(_3\). The IP\(_3\) stimulates IP\(_3\)R-mediated Ca\(^{2+}\) release from the ER. U-73122 is a selective inhibitor of PLC in this pathway (IC\(_{50}\) ~ 3 µmol/L) (50). Inhibition of PLC by U-73122 did not selectively inhibit oxLDL’s pro-survival effect (Figure 4A) in that it also decreased survival in control cells incubated without oxLDL. Dantrolene inhibits Ca\(^{2+}\) release from RyR channels (51). Inhibition of RyR-mediated Ca\(^{2+}\) release with this drug also did not block oxLDL’s pro-survival effect (Figure 4B).

**S1P generates [Ca\(^{2+}\)]\(_i\) oscillations and promotes macrophage survival.** S1P can act as a second messenger to induce Ca\(^{2+}\) mobilization within the cell (38, 39). We demonstrated that S1P can generate [Ca\(^{2+}\)]\(_i\) oscillations in BMDMs within the same time frame and as effectively as oxLDL (Figure 5). Furthermore, the same concentration of S1P promoted BMDM survival (Figure 6A) and blocked apoptosis (Figure 6B) to the same extent as oxLDL. S1P arises from the phosphorylation of sphingosine by SK (36). It has been previously reported that oxLDL can
activate SK in SMCs (52, 53) suggesting that the induction of \([\text{Ca}^{2+}]_i\) oscillations by oxLDL in BMDM might be mediated by its ability to increase phosphorylation of sphingosine.

*SK is activated in response to oxLDL.* To determine if oxLDL can activate SK in BMDMs we used an *in vitro* kinase assay to measure the ability of cell lysates to phosphorylate sphingosine. There was a 1.5-fold increase in SK activity almost immediately after the addition of oxLDL (Figure 7). This rapid activation of SK lends plausibility to the suggestion it could be a mechanism for mediating the \([\text{Ca}^{2+}]_i\) oscillations observed in response to oxLDL.

*Inhibition of SK blocks oxLDL mediated \([\text{Ca}^{2+}]_i\), oscillation and macrophage survival.* To test if SK activation is required for the ability of oxLDL to induce \([\text{Ca}^{2+}]_i\), oscillation, we used a selective inhibitor of SK (54). Figure 7 shows that this compound effectively blocks SK activation by oxLDL. It also completely blocked oxLDL-generated \([\text{Ca}^{2+}]_i\), oscillations (Figure 8) and oxLDL-mediated macrophage survival (Figure 9). These results strongly suggest that oxLDL induced Ca\(^{2+}\) mobilization is mediated by increased generation of S1P via SK activation.
DISCUSSION

A number of groups have reported that oxLDL induces an increase in $[\text{Ca}^{2+}]_i$ (41-44), and our studies extend this observation by demonstrating that at least in macrophages, this is an oscillatory increase. These oscillations involved $\text{Ca}^{2+}$ release from intracellular stores, and required SERCA to return cytosolic $\text{Ca}^{2+}$ to the ER. S1P is known to induce intracellular calcium release in other cells (37) and we found it to be as effective as oxLDL at inducing $[\text{Ca}^{2+}]_i$ oscillations in BMDM. The addition of oxLDL resulted in an almost immediate activation of SK, which is the major cellular pathway to production of S1P.

Inhibition of SK activation blocked not only oxLDL-generated $[\text{Ca}^{2+}]_i$ oscillations, but also oxLDL-mediated macrophage survival. This links $\text{Ca}^{2+}$ signaling with the pro-survival effects of oxLDL. Delivery of S1P by oxLDL itself is unlikely because S1P is lost during the oxidation process (55). Furthermore, native LDL, which contains significant amounts of S1P (55), does not elicit a $\text{Ca}^{2+}$ response similar to S1P, suggesting that endogenous production, perhaps in the plasma membrane, may be required to induce calcium oscillation (56). Our results do not exclude a role for other components of oxLDL such as oxysterols in stimulating intracellular $[\text{Ca}^{2+}]_i$ oscillations. However, the fact that the effect of oxLDL was mimicked by exogenous S1P and inhibited by an SK inhibitor suggests that S1P plays a major role. In addition, a recent study in U937 macrophages found that increased intracellular $\text{Ca}^{2+}$ in response to 7β-hydroxycholesterol was mediated by influx of extracellular $\text{Ca}^{2+}$ and was non-oscillatory (57). Both of these features are different from our findings with oxLDL-induced $\text{Ca}^{2+}$ signaling in BMDM.

The exact mechanism in which S1P mediates $\text{Ca}^{2+}$ mobilization is still uncertain. $\text{Ca}^{2+}$ release mediated by S1P occurs independently of IP$_3$Rs and RyRs (58). One possible candidate
is SCaMPER (sphingolipid Ca\(^{2+}\) release mediating protein of the endoplasmic reticulum) (58). SCaMPER is a 181 amino acid protein that was first shown to mediate sphingolipid-gated Ca\(^{2+}\) release from intracellular stores in Xenopus oocytes. More recently, antisense knockdown of SCaMPER mRNA was shown to substantially reduce sphingolipid-induced calcium release in human and rat cardiomyocytes (59). However, SCaMPER shares no similarity to any other known [Ca\(^{2+}\)]\(_{i}\) channels and is a small protein with only one transmembrane domain (58). Thus, it is unlikely to itself be a Ca\(^{2+}\) channel. Furthermore, a study showed that there is little correlation between its intracellular location and that of known [Ca\(^{2+}\)]\(_{i}\) stores (60).

We recently reported that oxLDL mediated [Ca\(^{2+}\)]\(_{i}\) oscillations lead to activation of the Ca\(^{2+}\)/calmodulin dependent kinase, eEF2 kinase (16). Both the increase in [Ca\(^{2+}\)]\(_{i}\) oscillations and the activation of eEF2 kinase were blocked by BAPTA-AM, an intracellular Ca\(^{2+}\) chelator. Addition of oxLDL also resulted in the phosphorylation of eEF2, the only known substrate of eEF2 kinase. The eEF2 kinase selective inhibitors TS-4 and TX-1918 blocked the ability of oxLDL to promote survival of BMDMs. Withdrawal of M-CSF resulted in the activation of p38 MAPK, an effect that is blocked with the addition of oxLDL, and eEF2 kinase can be negatively regulated by p38 MAPK. Together, these results indicated that oxLDL can positively regulate eEF2 kinase activity by both (1) generating an oscillatory increase in [Ca\(^{2+}\)]\(_{i}\), and (2) inhibiting its negative regulation by p38 MAPK. The only known substrate of eEF2K is eEF2, a monomeric GTPase that regulates peptide chain elongation. Phosphorylation of eEF2 inhibits its activity thereby reducing the rate of protein synthesis. In keeping with its ability to activate eEF2K, addition of oxLDL results in a decrease in overall protein synthesis in BMDMs (16).

Paradoxically, this effect of eEF2K activation has been shown to result in increased viability of cells under conditions of metabolic stress (e.g., growth factor withdrawal).
The results described herein extend our previous observations, and suggest a model in which oxLDL activates SK, triggering S1P-mediated oscillatory Ca$^{2+}$ release from intracellular stores, which in turn leads to activation of eEF2K and energy conservation via inhibition of protein synthesis, culminating in increased macrophage survival.
References


**FIGURE LEGENDS**

*Figure 1. LysoPC in oxLDL is not responsible for the generation of [Ca^{2+}]_{i} oscillations.*

Calcium mobilization was visualized using fluo-4-AM and confocal fluorescence microscopy. After incubation with fluo-4-AM, BMDM were washed and media containing either nLDL (25 µg/ml), oxLDL (25 µg/ml), PC (10 µmol/L), or lysoPC (10 µmol/L) was added at time 0. Fluorescence values as a function of time were measured for every cell in the field and cells positive for [Ca^{2+}]_{i} oscillations are expressed as a fraction of total cells. **p < 0.01 compared to cells treated with oxLDL.

*Figure 2. Extracellular Ca^{2+} plays a partial role in the generation of [Ca^{2+}]_{i} oscillations.*

Calcium mobilization was visualized using fluo-4-AM and confocal fluorescence microscopy. After preincubation with fluo-4-AM, BMDM were washed and nLDL or oxLDL (both 25 µg/ml) in media containing Ca^{2+}, or oxLDL (25 µg/ml) in Ca^{2+}-free media was added at time 0. Fluorescence values as a function of time were measured for every cell in the field and cells positive for [Ca^{2+}]_{i} oscillations are expressed as a fraction of total cells. *p < 0.05 compared to cells treated with nLDL.

*Figure 3. Thapsigargin blocks oxLDL generated [Ca^{2+}]_{i} oscillations.* Calcium mobilization was visualized using fluo-4-AM and confocal fluorescence microscopy. After incubation with fluo-4-AM, BMDM were washed and media containing 25 µg/ml nLDL, oxLDL, or oxLDL + thapsigargin (1 µmol/L) was added at time 0. Fluorescence values as a function of time were measured for every cell in the field and cells positive for [Ca^{2+}]_{i} oscillations are expressed as a fraction of total cells. **p < 0.01 compared to cells treated with oxLDL alone.
Figure 4. Inhibition of phospholipase C or RyR does not block oxLDL mediated macrophage survival. (a) BMDM were washed and incubated with media alone or oxLDL (25 µg/ml), in the presence U-73122 or dantrolene at the concentrations indicated for 24 hours. Viability was measured by the bioreduction of MTS and expressed as a ratio normalized to absorbance values of cells cultured in 10% M-CSF conditioned media.

Figure 5. S1P generates [Ca$^{2+}$]$_i$ oscillations. Calcium mobilization was visualized using fluo-4-AM and confocal fluorescence microscopy. After incubation with fluo-4-AM, BMDM were washed and media containing either nLDL (25 µg/ml), oxLDL (25 µg/ml), or S1P (30 µmol/L) was added at time 0. Fluorescence values as a function of time were measured for every cell in the field and cells positive for [Ca$^{2+}$]$_i$ oscillations are expressed as a fraction of total cells. ** p < 0.01 compared to cells treated with nLDL.

Figure 6. S1P promotes macrophage survival. BMDM were washed and incubated with media alone, oxLDL (25 µg/ml), or S1P (30 µmol/L) for 24 hours. (a) Viability was measured by the bioreduction of MTS and expressed as a ratio normalized to absorbance values of cells cultured in 10% M-CSF conditioned media. (b) Apoptosis was assessed by measuring the percentage of cells with subdiploid DNA. Data are expressed as a ratio normalized to fluorescence values of cells cultured in the absence of M-CSF. ** p < 0.01 compared to cells treated with media alone.

Figure 7. SK is activated in response to oxLDL. BMDM were washed and incubated in medium without M-CSF for 4 hours. OxLDL (25 µg/ml in medium without M-CSF) or oxLDL + SKI
(30 µmol/L) was then added for the time periods indicated. SK activity was assessed by measuring the ability of lysates to phosphorylate sphingosine with an in vitro kinase assay. Data are normalized to values of cells at time 0. * p < 0.05 compared to cells at time 0.

**Figure 8. Inhibition of SK blocks oxLDL generated [Ca²⁺]ᵢ oscillations.** Calcium mobilization was visualized using fluo-4-AM and confocal fluorescence microscopy. After incubation with fluo-4-AM, BMDM were washed and media containing nLDL (25 µg/ml), oxLDL (25 µg/ml), or oxLDL (25 µg/ml) + SKI (30 µmol/L) was added at time 0. Fluorescence values as a function of time were measured for every cell in the field and cells positive for [Ca²⁺]ᵢ oscillations are expressed as a fraction of total cells. ** p < 0.01 compared to cells treated with oxLDL alone.

**Figure 9. Inhibition of SK Blocks oxLDL Mediated Macrophage Survival.** BMDM were washed and incubated with media alone or oxLDL (25 µg/ml), in the presence or absence of SKI (30 µmol/L) for 24 hours. (a) Viability was measured by the bioreduction of MTS and expressed as a ratio normalized to absorbance values of cells cultured in 10% M-CSF conditioned media. (b) Apoptosis was assessed by measuring the percentage of cells with subdiploid DNA. Data are expressed as a ratio normalized to fluorescence values of cells cultured in the absence of M-CSF. ** p < 0.01 compared to cells treated with oxLDL alone.
Figure 1

![Graph showing the comparison of nLDL, oxLDL, PC, and lysoPC](image-url)
Figure 3
by guest, on October 22, 2017

www.jlr.org Downloaded from
Relative SK Activity

Time (minutes)

oxLDL
oxLDL + SKI

*
Figure 9

- oxLDL
- + oxLDL

Viability

Control  SKI

Apoptosis

Media Alone  oxLDL  oxLDL + SKI

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