Oxidized LDL–Mediated Macrophage Survival Involves Elongation Factor-2 Kinase

Johnny H. Chen, Maziar Riazy, Ewan M. Smith, Christopher G. Proud, Urs P. Steinbrecher, Vincent Duronio

Objective—Macrophage survival and proliferation is believed to be a contributing factor in the development of early atherosclerotic lesions. Oxidized low density lipoprotein (oxLDL), a key mediator in the pathogenesis of this disease, has been shown to block apoptosis in macrophages deprived of growth factor. In this report, we investigate the mechanism of oxLDL-mediated macrophage survival.

Methods and Results—OxLDL, but not native LDL (nLDL), induces an immediate and oscillatory increase in intracellular calcium ([Ca\(^{2+}\)]). We also show that the calcium/calmodulin dependent kinase, eukaryotic elongation factor-2 kinase (eEF2 kinase), is activated in response to oxLDL, an effect that can be blocked by inhibiting calcium mobilization. Furthermore, selective inhibition of eEF2 kinase reverses the prosurvival effect of oxLDL and results in cellular apoptosis. p38 MAP kinase, a negative regulator of eEF2 kinase, is activated on growth factor withdrawal, a response that can be inhibited by oxLDL. Finally, we show that oxLDL, by activating eEF2 kinase, phosphorylates and therefore inhibits eEF2, resulting in an overall decrease in protein synthesis.

Conclusion—These results indicate a novel signaling pathway in which oxLDL can block macrophage apoptosis by mobilizing calcium and activating eEF2 kinase. (Arterioscler Thromb Vasc Biol. 2009;29:92-98.)

Key Words: oxidized LDL ■ eEF2 kinase ■ macrophage ■ apoptosis ■ calcium
In this study, we report that oxLDL can activate eEF2 kinase in bone marrow–derived macrophages (BMDM), resulting in the inhibition of protein synthesis and inhibition of apoptosis during growth factor deprivation.

**Materials and Methods**

An expanded Materials and Methods section can be found in the supplemental materials (available online at http://atvb.ahajournals.org). In brief, density ultracentrifugation isolation of LDL and its oxidation by copper sulfate was carried out as previously described. Bone marrow–derived macrophages were isolated from the femurs of 6- to 8-week-old female CD-1 mice. Calcium mobilization was assessed by confocal fluorescence microscopy. Human eEF2 was purified from HL-60 cell homogenates with antihuman eEF2. Activity of eEF2 kinase was quantified by monitoring the formation of phospho-eEF2 with an anti-human phospho-eEF2 antibody. Cell viability was assessed by measuring the rate of reduction of a formazan dye. Apoptosis was assessed by flow cytometry of cells stained with propidium iodide. Protein synthesis was assessed by measuring rates of incorporation of L-[35S]methionine and L-[4,5-3H]leucine.

**Results**

**OxLDL Induces an Increase in [Ca\textsuperscript{2+}].** OxLDL has been previously shown to mobilize calcium in peritoneal macrophages and macrophage-like cell lines. Using Fluo-4-AM and fluorescence confocal microscopy, we were able to visualize calcium fluxes in real-time in response to oxLDL treatment. Fluorescence intensity of each cell was continually measured over a 2-minute period immediately after addition of compounds. On average, 69 ± 15 cells were separately analyzed per condition in each experiment. Cells showing a doubling of fluorescence intensity over the baseline were scored as positive for an increase in [Ca\textsuperscript{2+}]. OxLDL induced an increase in [Ca\textsuperscript{2+}] in 66.4% of cells, whereas with native LDL (nLDL) only 26.4% of cells were positive (Figure 1D). Because fluorescence intensity of each cell was independently measured over time, we were able to make the novel observation that the increase in [Ca\textsuperscript{2+}], induced by oxLDL, but not nLDL, actually involves calcium oscillations (Figure 1A). In contrast, this specific effect was almost completely absent in nLDL-treated cells. Additionally, the magnitude of the [Ca\textsuperscript{2+}] increase was, on average, lower in cells treated with nLDL in comparison with cells treated with oxLDL (Figure 1B). As expected, preincubation with the cell permeable calcium chelator, BAPTA-AM, completely ablated calcium mobilization (Figure 1C and 1D).
OxLDL mobilizes calcium into the cytosol of murine macrophages within 15 minutes (Figure 2a).

Figure 2. eEF2 kinase activation and regulation. Activity of eEF2 kinase was measured in BMDM lysates after the incubations described below using immunoblot analysis for phosphorylated eEF2. A, BMDM were incubated in medium without M-CSF for 4 hours, and 25 μg/mL oxLDL was then added for the times indicated. B, BMDM were incubated in media without M-CSF for 4 hours. Then, the p38 MAPK inhibitor SB202190 (15 μM), the PKC inhibitor Ro 32-0432 (10 μM), the Ca2+/calmodulin inhibitor BAPTA-AM (20 μM), the Hsp90 inhibitor geldanamycin (12 nM), or the eEF2 kinase inhibitors TS-4 (3.5 μM) or TX-1918 (8 μM) were added to cells for 10 minutes. OxLDL (25 μg/mL) was then added for a further 30 minutes.

**eEF2 Kinase Is Activated in Response to oxLDL**

EEF2 is the only known substrate for eEF2 kinase, which it phosphorylates at Thr56. Activity of eEF2 kinase was assessed by incubating BMDM lysates with eEF2 and measuring levels of phosphorylated eEF2 by immunoblotting. An increase in eEF2 kinase activity was detectable as early as 15 minutes after the addition of oxLDL and was maximal by 30 minutes (Figure 2a).

EEF2 kinase is a calcium/calmodulin-dependent kinase that can be activated when [Ca2+]i levels increase. Our results demonstrate that oxLDL mobilizes calcium into the intracellular space almost immediately after its addition, an effect that can be blocked by 20 μM BAPTA-AM (Figure 1C and 1D). At the same concentration, BAPTA-AM also blocks oxLDL-mediated eEF2 phosphorylation (Figure 2B). This suggests that eEF2 kinase is activated in response to oxLDL via an increase in [Ca2+]i.

Previous reports have suggested that oxLDL-mediated macrophage proliferation is dependent on calcium mediated activation of protein kinase C (PKC). Of the PKC isoforms that are activated in response to Ca2+ (α, β, γ), only PKCα and PKCβ are expressed in macrophages. Depletion of PKC by incubation (for 72 hours) with phorbol myristate acetate (PMA) or incubation with the PKCα/β selective inhibitor Ro 32-0432 did not alter the viability of macrophages, or their survival response to oxLDL treatment (supplemental Figure I).

**eEF2 Kinase Activity Is Required for oxLDL-Mediated Macrophage Survival**

The eEF2 kinase selective inhibitors TS-4 and TX-1918 effectively blocked the phosphorylation of eEF2 by oxLDL (Figure 2B). At corresponding concentrations, both TS-4 and TX-1918 lowered the viability of cells treated with oxLDL to levels similar to that of cytokine-starved cells (Figure 3A and 3B). These inhibitors had no effect on the viability of macrophages not treated with oxLDL, indicating that they act specifically on the antiapoptotic effect of oxLDL and do not simply decrease macrophage viability. Moreover, both TS-4 and TX-1918 induced cellular apoptosis in oxLDL-treated BMDM to levels similar to that of cytokine-starved cells (Figure 3F).

During purification, eEF2 kinase has been found to be tightly associated with Hsp90, and disruption of the eEF2 kinase/Hsp90 complex by geldanamycin inhibited the clonogenicity of glioblastoma cell lines. To determine whether the prosurvival effect of MPO-LDL is similar to that of copper-oxidized LDL, macrophage viability was measured in response to treatment with MPO-LDL. As seen in supplemental Figure II, 50 μg/mL MPO-LDL was as effective as 25 μg/mL copper oxLDL in promoting macrophage survival, and the addition of the eEF2 kinase selective inhibitor, TX-1918, blocked this effect.

Recently, an eEF2 kinase-dead transgenic mouse line was developed. Our preliminary experiments with these mice shows that eEF2 kinase activity plays a positive role in oxLDL-mediated macrophage survival (supplemental Figure III). Future studies will further evaluate the effect of the lack of eEF2 kinase activity on oxLDL-dependent macrophage survival in vivo as well as the effect on atherosclerosis progression in a mouse model.

Taken together, these results indicate that the ability of oxLDL to block macrophage apoptosis is dependent on its ability to activate eEF2 kinase.

**p38 MAPK Negatively Regulates eEF2 Kinase Activity and Is Phosphorylated upon Growth Factor Withdrawal**

Knebel et al reported that p38 MAPK phosphorylates eEF2 kinase at Ser359 and Ser396, and inhibits its activity by
Other p38 MAPK isoforms are of lesser importance because p38\(\alpha\) (which phosphorylates eEF2 kinase at Ser377) did not affect its activity and p38\(\beta\) (which phosphorylates eEF2 kinase at Ser396) caused only a modest decrease in activity. To assess the role of p38 MAPK isoforms in the antiapoptotic effect of oxLDL, we incubated BMDM with anisomycin, a pyrrolidine antibiotic that activates p38 MAPK. At 250 nmol/L, anisomycin blocked activation of eEF2 kinase by oxLDL (Figure 2B). At the same concentration, anisomycin also decreased macrophage viability (Figure 3D) and induced apoptosis (Figure 3F). SB202190, a selective inhibitor of p38\(\alpha\) and p38\(\beta\) but not p38\(\gamma\), was not able to rescue macrophages from apoptosis because of macrophage colony stimulating factor (M-CSF) withdrawal (Figure 3E), in agreement with the limited role of p38\(\alpha\) and p38\(\beta\) in regulating eEF2 kinase activity as discussed above.

P38 MAPK phosphorylation was observed as early as 15 minutes after M-CSF withdrawal (Figure 4A). Of interest, p38 MAPK phosphorylation was inhibited by the addition of oxLDL (Figure 4B). Thus, oxLDL can not only activate eEF2 kinase by mobilizing \([Ca^{2+}]\), but can also inhibit its negative regulation by blocking p38 MAPK activation. Conversely, as would be predicted based on the ability of anisomycin to activate p38 MAPK, the addition of anisomycin negated the inhibition of p38 MAPK phosphorylation by oxLDL (Figure 4B).

Ceramide Activates p38 MAPK and Negatively Regulates eEF2 Kinase Activity

Our group has previously shown that incubation of BMDM in the absence of M-CSF results in activation of acid sphingomyelinase (ASMase) and an increase in ceramide levels. OxLDL blocked both of these effects, whereas the addition of C2-ceramide blocked the ability of oxLDL to induce survival. In the present study, we found that C2-ceramide can also block activation of eEF2 kinase by oxLDL (Figure 2B). As well, C2-ceramide induced phosphorylation of p38 MAPK (Figure 4C), in agreement with previous reports in other macrophage cell types. At present, we cannot be certain whether the effect of ceramide on eEF2 kinase is mediated entirely by its activation of p38 MAPK or whether there is another mechanism as well.

Protein Synthesis Is Reduced in Response to oxLDL

eEF2 is a monomeric GTPase that facilitates translocation of peptidyl t-RNA from the ribosomal A site to P site. Phosphorylation of eEF2 by eEF2 kinase leads to an inhibition of eEF2 and therefore of protein synthesis. To determine whether the oxLDL-mediated phosphorylation of eEF2 has the expected effect on protein synthesis, the in vivo incorporation rates of \(L-[{35}S]\)methionine and \(L-[{4,5}\text{-}^3H]\)leucine in response to oxLDL were measured. Within 30 minutes of oxLDL treatment, the rate of incorporation of both \(L-[{35}S]\)methionine and \(L-[{4,5}\text{-}^3H]\)leucine was reduced.
5). This correlated well to the timing of eEF2 phosphorylation in response to oxLDL (Figure 2A), suggesting that oxLDL mediates a reduction in protein synthesis via the activation of eEF2 kinase.

**Discussion**

Calcium is a universal second messenger that regulates a number of diverse cellular processes including cell proliferation, development, motility, and secretion. In all eukaryotic cells, Ca$^{2+}$ is required in both the extracellular environment and intracellular stores for cell growth and division. Recent studies have proposed that Ca$^{2+}$ may be an important factor in the early development and progression of atherosclerotic lesions. The use of calcium antagonists in clinical trials has been reported to retard the progression of the disease. Furthermore, alterations in Ca$^{2+}$ gradients by oxLDL have been implicated in the formation of macrophage-derived foam cells.

Ca$^{2+}$ can relay specificity in signaling through its temporal diversity. In response to some types of stimuli, there may be repetitive Ca$^{2+}$ spikes (Ca$^{2+}$ oscillations). Whereas a transient or sustained increase in [Ca$^{2+}$], is generally associated with apoptosis, calcium oscillations favor cell survival by enhancing mitochondrial bioenergetics. Cells respond to calcium oscillations using highly sophisticated mechanisms, including the ability to interpret changes in its frequency. One family of molecular machines that can interpret such frequency changes is the calcium/calmodulin-dependent kinases.

eEF2 kinase, or calcium/calmodulin-dependent kinase III, is a highly regulated enzyme. To date, there are 7 known phosphorylation sites. Phosphorylation at 6 of these sites through the p38 MAPK, mTor, Erk, AMPK or PKA pathways regulate its activity either positively or negatively. Specific agonists and inhibitors targeting mTOR, Erk, and PKA all failed to alter macrophage viability in response to oxLDL (data not shown).

In this article, we identified a novel mechanism by which oxLDL prevents macrophage apoptosis in response to growth factor withdrawal. OxLDL initiates an almost immediate oscillatory increase in [Ca$^{2+}$]. This is followed by the activation of the Ca$^{2+}$ sensitive eEF2 kinase. Specific inhibition of eEF2 kinase activity completely blocks the antiapoptotic effects of oxLDL. Whereas a previous study suggested a role of PKC in calcium-induced proliferation of peritoneal macrophages in response to oxLDL, our results suggest that PKC is not required for the antiapoptotic effect of oxLDL in BMDM.

Upon growth factor withdrawal p38 MAPK phosphorylation was detected, an effect that can be blocked by the addition of oxLDL. Phosphorylation of eEF2 kinase by p38...
MAPK negatively regulates its activity. Activation of p38 MAPK by anisomycin was shown to block oxLDL activation of eEF2 kinase and induce apoptosis. Taken together, these observations suggest that oxLDL positively regulates eEF2 kinase not only by increasing [Ca\textsuperscript{2+}], but through inhibiting its negative regulation by blocking p38 MAPK activation. A summary of the signaling pathways involved in oxLDL-mediated macrophage survival can be found in the supplemental Figure IV.

Activation of eEF2 kinase results in the phosphorylation and inhibition of its only known substrate, eEF2. eEF2 is a monomeric GTPase and serves as an elongation factor that facilitates translocation of peptidyl t-RNA from the ribosomal A site to P site. The addition of oxLDL resulted in the reduction of protein synthesis in a time frame that correlates well with our observation of eEF2 phosphorylation by oxLDL.

Recent studies have implicated the important role of eEF2 kinase in promoting cells during times of stress, such as hypoxia or nutrient deprivation. A possible mechanism is that eEF2 phosphorylation can inhibit the synthesis of proteins that promote apoptosis, as has been suggested in studies involving postischemic neuronal cells. Additionally, Terai et al have shown that the activation of eEF2 kinase protects cardiomyocytes against ER stress–induced apoptosis during hypoxia, via inhibition of ER stress–induced production of misfolded proteins. Wu et al, however, link the protective role of eEF2 kinase with the conservation and replenishment of cellular energy during nutrient deprivation, through an eEF2 kinase-dependent activation of autophagy.

The precise nature of how the activation of eEF2 kinase leads to macrophage survival is still unclear. One hypothesis is that the activation of eEF2 kinase, via oxLDL-mediated increase in [Ca\textsuperscript{2+}], during times of nutrient deprivation or withdrawal of M-CSF, switches the cells from an apoptotic to an autophagic cell survival state. Our study has shown that oxLDL-mediated cell survival requires eEF2 kinase activation, which leads to phosphorylation and inhibition of eEF2 and a reduction in protein synthesis. This suggests that oxLDL may promote cell survival during times of nutrient deprivation by lowering cellular energy consumption via a reduction in protein translation.

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Disclosures
None.

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SUPPLEMENT MATERIAL.

SUPPLEMENTAL MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle’s Medium (DMEM), RPMI-1640, fetal bovine serum (FBS), Dulbecco’s phosphate buffered saline (DPBS), HEPES, BAPTA-AM, Fluo-4-AM, pluronic acid, and propidium iodide (PI) were purchased from Invitrogen (Burlington, ON, Canada). L929 cells were provided by Dr. J.W. Schrader (Biomedical Research Centre, BC, Canada) and L929 cell-conditioned media (LCCM) was used as a crude source of M-CSF as previously described \(^1\). [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was from Promega (Madison, WI). Myeloperoxidase, glucose oxidase, catalase, phenazine methosulfate (PMS), anisomycin, and geldanamycin were obtained from Sigma-Aldrich (St. Louis, MO). SB202190, rottlerin and TX-1918 were provided by Calbiochem (San Diego, CA). Protein A Sepharose beads, L-[\(^{35}\)S]methionine, and L-[4,5-\(^{3}\)H]leucine were from Amersham Biosciences (Piscataway, NJ). TS-4 was a kind gift from Dr. Y. Uehara (National Institute of Infectious Diseases, Tokyo, Japan). BCA protein assay reagents, BSA standards, and SuperSignal Femto Substrate were purchased from Pierce (Milwaukee, WI). Ro 32-0432, and PKCd antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to eEF2, phospho-eEF2 (Thr56), p38, and phospho-p38 (Thr180/Tyr182) were obtained from Cell Signaling Technology (Beverly, MA). Goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was from DAKO Diagnostics (Mississauga, ON, Canada). 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**

Procedures for LDL isolation and copper oxidation are as previously reported by our group ². Myeloperoxidase oxidized LDL (MPO-LDL) was prepared by incubating LDL (0.2 mg/mL) at 37°C in 50 mmol/L sodium phosphate, pH 7.0 and 10 µmol/L EDTA in the presence of 30 nmol/L myeloperoxidase, 500 µmol/L glucose, 125 nmol/L glucose oxidase, and 0.5 mmol/L NaNO₂ for 8 or 24 hours. The oxidized LDL was then washed and concentrated to 1.5 mg/ml using Amicon Centrifilus 20 ultrafilters (Millipore, Bedford, MA). After a 0.45 micron filtration, exact protein concentrations of modified LDL were then determined using BCA protein assay. Extent of oxidation was evaluated using agarose gel electrophoresis and thin-layer chromatography. All copper-oxidized LDL preparations were heavily oxidized, with electrophoretic mobility in agarose gels of about 4 times that of native LDL. When oxidized for 24 hours, MPO-LDL had similar electrophoretic mobility and extent of lipid oxidation as copper oxLDL. MPO-LDL oxidized for 8 hours, had electrophoretic mobility of approximately 2.7 times that of native LDL, but showed no pro-survival activity (data not shown).

**Cell Culture**

Bone marrow cells were obtained from the femurs of 6-8 week old female CD-1 mice, or from eEF2 kinase inactive transgenic mice (and litter mate controls), and macrophages were isolated from these as previously described ².
Measurement of $[\text{Ca}^{2+}]_i$ Mobilization

BMDM were seeded in 6-well plates at $5.0 \times 10^4$ cells per cm$^2$ and grown for 24 hours. Cells were then washed with calcium free DPBS and incubated for 30 minutes at room temperature in calcium free DPBS with 2 µmol/L Fluo-4-AM (prepared as a 2mmol/L solution, dissolved in 20% pluronic acid in DMSO). Cells were then washed with DPBS and incubated in HEPES buffered medium for 10 minutes at room temperature to allow for de-esterification of the acetoxymethyl group. Medium was then replaced with fresh medium containing test compounds. Fluorescence was measured in real-time using an inverted Leica TCS SP2 AOBS laser scanning confocal microscope using a 10X objective. Image analysis was performed using Leica LCS software and fluorescence of every cell in each field was measured. Each condition was performed in duplicate within the experiment and data shown are representative of at least 3 independent experiments. Statistical significance was calculated by unpaired Student’s t-Test.

Cell Viability Assay

BMDM were seeded in 96-well plates at $5.0 \times 10^4$ cells per cm$^2$ and grown for 24 hours. Cells were then washed and incubated with media containing test compounds 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 $^3$. Each condition was performed in triplicate within the experiment and data is representative of at least 3 independent experiments. Statistical significance was calculated by unpaired Student’s t-Test.
Flow Cytometry

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 media containing oxLDL to a concentration of 25 µg/ml and/or other compounds 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 gating to exclude debris and cellular aggregates. Ten thousand events were counted for analysis. Each condition was performed in triplicate within the experiment and data are representative of at least 3 independent experiments. Statistical significance was calculated by unpaired Student’s t-Test.

eEF2 Kinase Activity Assay

To prepare human eEF2 substrate, 3.0 x 10^7 HL-60 cells were washed with DPBS and lysed with 1 ml 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 aprotin, 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), and 1 mmol/L sodium fluoride). Lysates were centrifuged at 20,000 x g for 10 minutes, and supernatants were incubated with 3 µg of anti-eEF2 antibody and rotated at 4°C for 2 hours. Lysates were further incubated for 1 hour with 30 µl of Protein A Sepharose beads. The beads were then washed four times with solubilization buffer and used as a substrate in the assay.
BMDM were seeded in 100 mm dishes 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 followed by treatments as indicated. Afterwards, cells were washed with DPBS and lysed with ice-cold solubilization buffer. Lysates were centrifuged at 20,000 x g for 10 minutes, and the protein content of supernatants was quantified using a BCA protein assay. 300 µl of lysate containing 300 µg total protein was then transferred to the substrate beads and incubated at 37°C for 1 hour. Beads were then spun down and washed three times with ice-cold solubilization buffer. Beads were mixed with sample buffer (100 mmol/L tris-Cl pH 6.8, 70 mmol/L SDS, 10% glycerol, 1.5 mmol/L bromophenol blue, 150 mmol/L β-mercaptoethanol) and heated to 70°C for 10 minutes. The contents were then analyzed by immunoblotting with an antibody to phosphorylated eEF2.

**Immunoblotting**

Cells were washed with DPBS and lysed with ice-cold solubilization buffer. Lysates were centrifuged at 20,000 x g for 10 minutes, and the protein content of supernatants was quantified using BCA protein assay. Sample buffer was added to the lysates and heated to 70°C for 10 minutes. 50 µg of protein from each sample was loaded onto a SDS-PAGE gel. Gels were calibrated using pre-stained SDS-PAGE low molecular weight standards. Proteins were then transferred electrophoretically to PVDF membranes and then incubated with 1 µg/ml primary antibody in Tris-buffered saline (TBS) containing 3 mmol/L sodium azide, and either 1% skim milk or 1.5 mmol/L BSA at room temperature for 2 hours. After three washes with TBS containing 0.1% Tween 20 (TBS-T), membranes were incubated with horseradish peroxidase-conjugated secondary antibody at 1:10,000 dilution in TBS containing 3 mmol/L sodium azide,
and either 1% skim milk or 1.5 mmol/L BSA at room temperature for 30 minutes. After six washes with TBS-T, membranes were treated with SuperSignal Femto Substrate and chemiluminescence was visualized by exposure to BioMax MR film. Results shown are representative of at least 3 independent experiments.

**Protein Synthesis Assay**

BMDM were seeded in 6-well plates at 5.0 x 10⁴ cells per cm² and grown for 24 hours. Cells were washed and incubated with media in the absence of M-CSF for 3 hours. Cells were then washed and incubated with media containing 1/10th the normal concentration of either methionine (0.0201 mmol/L) or leucine (0.0802 mmol/L) in the absence of M-CSF for 1 hour. OxLDL at a concentration of 25 µg/ml was then added for the times indicated. 5µCi of either L-[³⁵S]methionine or L-[4,5-³H]leucine was added 10 minutes prior to harvesting of cells. Cells were then washed with DPBS and lysed with ice-cold solubilization buffer. Protein was precipitated by adding trichloroacetic acid (TCA) to a final concentration of 0.6 mmol/L. The precipitate was washed three times with 0.6 mmol/L TCA and finally with 95% ethanol. Radioactivity was then measured using a scintillation counter. Each condition was performed in triplicate within the experiment and data are representative of at least 3 independent experiments. Statistical significance was calculated by unpaired Student’s t-Test.
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Supplemental Videos. OxLDL increases \([\text{Ca}^{2+}]\). 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 (a) oxLDL (25 µg/ml), (b) nLDL (25 µg/ml), or (c) oxLDL (25 µg/ml) + BAPTA-AM (20 µmol/L) were added at time 0.

Supplemental Figure I. PKC is not involved in macrophage survival. Viability was measured by the bioreduction of MTS and expressed as a fraction normalized to absorbance values of cells incubated with M-CSF. PKCδ protein levels were assessed by immunoblotting. (A) BMDM were washed and incubated with media alone or with oxLDL (25 µg/ml) in the presence or absence of Ro 32-0432 (1 µmol/L) for 24 hours. (B, C) BMDM were grown for 48 hours in the presence or absence of PMA (10 nmol/L). Cells were then washed and incubated with media alone or with oxLDL (25 µg/ml) in the presence or absence of PMA (10 nmol/L) for 24 hours.

Supplemental Figure II. MPO-LDL promotes macrophage survival. Viability was measured by the bioreduction of MTS and expressed as a fraction normalized to absorbance values of cells incubated with M-CSF. BMDM were washed and incubated with compounds as indicated for 24 hours. ** p<0.01 for MPO-LDL (50µg/ml) compared to No M-CSF, and for MPO-LDL (50µg/ml) + TX-1918 (8 µmol/L) compared to MPO-LDL (50 µg/ml).

Supplemental Figure III. eEF2 kinase activity contributes to the pro-survival effect of oxLDL. BMDM from wild type mice or transgenic mice expressing catalytically inactive eEF2 kinase
were cultured for 24 hours in the presence of either 10% M-CSF conditioned media or oxLDL (25 µg/ml). (A) Viability was measured by the bioreduction of MTS and normalized to values for cells incubated with M-CSF. (B) Apoptosis was quantified using flow cytometry to measure the percentage of cells with subdiploid DNA * p<0.05, ** p<0.01 compared to wild type from a single experiment performed in triplicate.

Supplemental Figure IV. OxLDL mediated macrophage survival: a working model. Abbreviations used: ASMase - acid sphingomyelinase, eEF2K - eukaryotic elongation factor-2 kinase, cytoC - cytochrome C, Hsp90 - heat shock protein 90, PKB - protein kinase B, PI-3K - phosphatidylinositol-3 kinase.
Supplementary Figure I

A

Viability

Control  Ro 32-0432

- oxDLDL + oxDLDL

B

PKC

PMA  +  +  -  -

oxLDL  -  +  -  +

C

Viability

Control  PMA

- oxDLDL + oxDLDL
Supplemental Figure II

![Bar chart showing viability results for different conditions.](image)

- NoM-CSF
- 25 μg/ml Copper oxLDL
- 12 μg/ml MPO oxLDL
- 25 μg/ml MPO oxLDL
- 50 μg/ml MPO oxLDL
- 50 μg/ml MPO oxLDL + 6 μmol/L TX-1918

**Legend:**
- Bars represent viability levels.
- Significant differences are indicated by "**".