CMLS, Cell. Mol. Life Sci. 61 (2004) 1664–1674
1420-682X/04/131664-11
DOI 10.1007/s00018-004-4080-5

CMLS Cellular and Molecular Life Sciences

Research Article

Accumulation of oxidized lipid-protein complexes alters phagosome maturation in retinal pigment epithelium

G. Hoppe[a, b, *], J. O'Neil[a], H. F. Hoff[a] and J. Sears[a, b]

[a] Department of Cell Biology, Lerner Research Institute, Cleveland, Ohio (USA)
[b] Department of Ophthalmic Research, Cole Eye Institute, mail Stop I-31, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195 (USA), Fax +1 216 445 3670; e-mail: hoppeg@ccf.org

Received 24 February 2004; received after revision 12 April 2004; accepted 4 May 2004

Abstract. Lipid peroxidation has been implicated in many age-associated disorders including macular degeneration of the retina. We sought to elucidate the mechanism by which accumulation of oxidized LDL (oxLDL) reduces the ability of retinal pigment epithelium (RPE) to process photoreceptor outer segments (OS) as a model of peroxidation-induced disruption of phagocytosis. OxLDL did not reduce the lysosomal hydrolytic capacity of the RPE, but efficiently inhibited processing of various internalized proteins. OxLDL caused a delay in the acquisition of late lysosomal markers by newly formed phagosomes. At the same time, an excessive accumulation of markers of early phagosomal compartments was also observed. The activity of phosphatidylinositol 3-kinase (PI3K) was reduced in phagosomes of the RPE treated with oxLDL. These results suggest that accumulation of oxidized lipid-protein complexes in the RPE impedes phagosome maturation by blocking PI3K recruitment to the phagosomal membrane, leading to delayed processing of internalized OS.

Key words. Lipid peroxidation; lysosome; membrane transport; oxidized low-density lipoprotein; phagocytosis; pigment epithelium of retina.

Superior phagocytic capacity of the retinal pigment epithelium (RPE) plays an essential role in the renewal of photoreceptor outer segment membranes (OS) [1]. In mammals, a single RPE cell can process as many as 300 OS tips containing approximately 30,000 photoreceptor disks daily [2]. Selective disruption of this process compromises the viability of photoreceptor cells and quickly leads to retinal degeneration [3–6]. The molecular mechanism of RPE phagocytosis remains largely unclear, but appears to be OS specific, at least at the level of the initial events of recognition, binding, and internalization. Numerous studies in RCS rats have suggested that the RPE utilizes distinct mechanisms for uptake of OS and latex microspheres [7–10]. The c-mer receptor tyrosine kinase dubbed MERTK, which is mutated in the RCS rat, is required for triggering OS internalization, whereas uptake of latex beads is independent of MERTK [11]. On the other hand, OS uptake by RPE appears to share some principal steps with removal of apoptotic cells by macrophages [12]. Both cases involve specific members of the integrin family of receptors, as well as downstream signaling via PKC [13].

In addition to the RCS rat phenotype [4, 14], mutation of MERTK has also been shown to cause retinitis pigmentosa [15], demonstrating the importance of the OS removal function of RPE for degenerative diseases of the retina. Many studies have focused on identifying molecules that govern OS recognition and binding [11, 16–20]. Despite significant progress in deciphering the initial phagocytic steps, the molecular mechanisms un-

* Corresponding author.

derlying advanced stages of OS processing by RPE are unclear, yet may prove critical for our understanding of the pathogenesis of retinal degenerations. In Stargardt macular dystrophy, although mutated ABCR is expressed exclusively in photoreceptors [21], the resulting retinal atrophy is believed to be due to pathological overload of the phago-lysosomal apparatus of RPE by lipofuscin derived from a buildup of the retinoid intermediates [22]. Several lines of evidence link the phagocytic function of RPE to age-related macular degeneration (AMD), a disease which is histochemically characterized in part by RPE overloaded with autofluorescent inclusion bodies, termed lipofuscin [23]. These organelles are reported to originate from lysosomes that accumulate end products of irreversible covalent cross-linking between RPE proteins and the reactive products of lipid peroxidation [24, 25]. Transgenic mice overexpressing functionally compromised cathepsin D, the major lysosomal protease in RPE [26], develop abnormal features in their retina that are typical for AMD, i.e., geographic atrophy, as well as basal laminar deposits [5]. Overall, deficiencies of phagolysosome formation and elimination may be a relevant hypothesis to explain intracellular buildup of poorly degradable lipid-protein cross-links impeding RPE-mediated OS recycling [25].

We have previously developed an experimental model of cultured RPE cells with deficient processing of OS caused by prior uptake of copper-oxidized low-density lipoprotein (oxLDL) [27]. In this model, initial steps of OS interaction with RPE (binding and internalization) are not affected, but proteolytic degradation of OS as well as other macromolecules is significantly impaired. OxLDL-derived mixtures of oxidatively damaged lipids and proteins mimic A2E, a major component of lipofuscin, in several key ways that are pertinent to this study, e.g., lysosomotropy, insolubility, indigestibility, and cytotoxicity at high doses [28–32]. Initially, we established that oxLDL universally suppressed RPE degradation of a variety of internalized particles, regardless of their size, physical state, and molecular composition. We rationalized that the mechanism by which oxLDL blocks degradation may be upstream of actual lysosomal digestion and, therefore, latex beads can substitute for OS, drastically improving the purity of phagosome isolations. Therefore, we sought to determine whether oxLDL causes alterations in phagosomal composition that may explain inefficient proteolysis of internalized molecules by RPE. First, we ruled out direct inactivation of the lysosomal proteases as a cause of the impaired OS processing. Second, we noticed that oxLDL induced an abnormal distribution of Rab5 and EEA1, markers of the early phagosome, consistent with the impeded phagosomal maturation. Finally, we found that, at least in the case of latex bead uptake, oxLDL alters the activity of phagosome-associated phosphatidylinositol-3-kinase (PI3K).

Materials and methods

Reagents

Cell culture media and culture media additives were purchased from Invitrogen (Carlsbad, Calif.). Heat-inactivated CELLect Gold fetal bovine serum (FBS) was purchased from ICN Pharmaceuticals (Irvine, Calif.). All protein gel electrophoresis reagents and instruments were from Bio-Rad (Hercules, Calif.) and Invitrogen. All chemicals were from Sigma (St. Louis, Mo.) unless specified otherwise.

RPE cell culture

Primary cultures of human RPE cells (hRPE) were a gift from Dr. J. Burke (Medical College of Wisconsin, Wis.), and were obtained as confluent cell monolayers at early passages ranging from 3 to 8. Cultures of RPE cells from three different donors, ages 55–80, were used in the study. Cells were maintained and passaged as described previously with biweekly feedings of minimum essential medium (MEM) containing 10% FBS, 500 U/ml penicillin, 500 μg/ml streptomycin, 125 ng/ml fungizone, and 50 μg/ml gentamicin [33].

Lipoproteins

LDL was isolated from fresh plasma obtained from the Cleveland Clinic Blood Bank by sequential ultracentrifugation as a $1.019 < d < 1.063$ g/ml fraction using the procedure of Hatch and Lees [34]. The LDL was dialyzed against 0.15 M NaCl containing 0.5 mM Na_2EDTA, pH 8.5, filter-sterilized and stored at 4 °C. Oxidation of LDL was performed in the presence of 10 μM $CuSO_4$ in 0.15 M NaCl at a protein concentration of 500 μg/ml for 24 h at 20 °C [30, 35]. The concentration of EDTA in LDL preparations was reduced prior to oxidation by overnight dialysis against 0.15 M NaCl. Oxidation was terminated by dialyzing samples into 0.15 M NaCl, 0.3 mM Na_2EDTA, and 20 mM Na phosphate pH 7.4 (PBS), for 24 h. Butylated hydroxytoluene (BHT) dissolved in ethanol was added at a final concentration of 40 μM to prevent oxidation. Acetylated LDL (acLDL) was prepared by treatment of LDL with acetic anhydride as described elsewhere [36].

Photoreceptor OS

Photoreceptor OS were isolated from freshly obtained bovine eyes using a sucrose density gradient as described previously [37, 38]. OS were adjusted to a concentration of 10^8/ml and stored in PBS containing 1 mM $MgCl_2$, 0.1 mM Na_2EDTA, 1 mM DTT, and 10% glycerol. Oxidized OS (oxOS) were prepared by incubating 1 mg/ml OS at 37 °C for 6 h in the presence of 1 mM tert-butyl hydroperoxide and 30 μM $FeSO_4$ [39]. Oxidation was terminated by extensive dialysis of samples against PBS containing 0.3 mM Na_2EDTA and 40 μM BHT. Oxidation

of OS in the presence of 5 μM $CuSO_4$ for 24 h, as described previously [27], did not result in any distinguishable differences in biological activities between copper- and hydroperoxide-oxidized OS.

Uptake and degradation assays

Bovine serum albumin (BSA) was modified by maleic anhydride as described by Brown et al. [36]. Maleylated BSA (mBSA), LDL, and OS were labeled with $Na^{125}I$ using the iodine monochloride procedure as described by Bilheimer et al. [40]. After appropriate pre-treatments as specified in the figure legends, hRPE cells were washed and incubated with ^{125}I-labeled ligands for another 5 h. The media were then removed and assayed for trichloroacetic acid (TCA)-soluble, noniodine degradation products according to the procedure described previously [30, 35]. Cells were washed three times with PBS, dissolved in 0.25 N NaOH, and assayed for cell-associated ^{125}I radioactivity and protein content. All determinations are reported as the mean ± SD of triplicate determinations. To assess proteolysis in hRPE cell extracts, cells were first pre-incubated with LDL or oxLDL as described in the figure legends, followed by preparation of cell homogenates in 250 mM sucrose, 1 mM Na_2EDTA, 0.1% ethanol, pH 6.8 at 4 °C. The homogenates were spun again at 20,000 g to remove particulate matter, and supernatant fractions, defined in this study as cell extracts, were collected, aliquoted, frozen at –70 °C, and subsequently used in experiments over a period of 2 months. Five micrograms of cell extract protein was mixed with 10 μg ^{125}I-LDL or 100 μg ^{125}I-OS in a final volume of 100 μl of 100 mM sodium acetate, 1 mM Na_2EDTA, 1 mM dithiothreitol (DTT), pH 4.5, and incubated for the indicated time periods at 37 °C. The degradation of the radioactive substrate was measured as TCA-soluble, noniodine radioactivity [35]. Cathepsin B activity of hRPE extracts was assayed as follows: 0.5 μg of extract was mixed with 500 μl of acetate buffer, pH 4.0, containing Nα-CBZ-L-lysine p-nitrophenyl ester (CLN) at a final concentration of 2×10^{-4} M and absorbance at 326 nm monitored over a 30-min time interval [35]. To determine the proteolytic activity of enzymes stimulated by the addition of DTT, we conducted all experiments in the presence and absence of DTT. DTT-dependent proteolysis was determined as total degradation (DTT present) minus DTT-independent degradation (DTT omitted).

Phagosome isolation

hRPE cells were plated onto 100-mm Petri dishes and grown for 1 week to confluency. After appropriate pretreatments of the hRPE performed in fresh culture media and specified in the figure legends, phagocytosis was initiated by adding 200 μl of a 2% suspension of carboxylated paramagnetic latex beads (2 μm in diameter; Polysciences, Warrington, Pa.), which were coated with FBS and washed prior to the addition to cell cultures [41]. Cells were then incubated for the indicated time periods at 37 °C in a CO_2 incubator, and experiments was stopped by placing dishes on ice, gentle scraping and pelleting of cells at 200 g. Cells were then resuspended in the homogenization buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM HEPES, pH 7) containing a cocktail of protease inhibitors (Complete; Roche, Indianapolis, Ind.). Cells were homogenized by passing a cell suspension five times through a 26-gauge needle. Phagosomes containing latex beads were isolated using a magnet (Dynal, Oslo, Norway). Phagosomal fractions were resuspended in the homogenization buffer and magnetic isolation of phagosomal as well as nonphagosomal fractions was repeated twice to ensure purity of these subcellular fractions.

Western blotting assay

Laemmli buffer (2×; Invitrogen) supplemented with a 2× cocktail of protease inhibitors (Complete) but without reducing agents, was used to elute hRPE proteins associated with latex beads, as well as to solubilize proteins of nonphagosomal fractions or total cell homogenates. Protein concentrations in extracts were determined using BCA reagent (Pierce, Rockford, Ill.). Equal amounts of protein were loaded and resolved by SDS-PAGE using 4–20% gradient precast minigels (Invitrogen). Proteins separated by SDS-PAGE were electro-transferred onto a PVFD membrane and probed with one of the following antibodies: mouse anti-human LAMP-1, clone H4A3 (Development Studies Hybridoma Bank, Iowa City, Iowa); goat anti-cathepsin D, clone C-20 (Santa Cruz, Calif.); rabbit anti-Rab5, clone S-19 (Santa Cruz); goat anti-EEA1, clone N-19 (Santa Cruz), rabbit anti-p85α, clone Z-8 (Santa Cruz); and mouse anti-phospho-tyrosine, clone PY20 (Santa Cruz). Immunoreactivity was detected with a corresponding second anti-IgG antibody conjugated with HRP (Jackson ImmunoResearch, West Grove, Pa.), followed by the Renaissance reagent (PerkinElmer, Boston, Mass.) as a chemiluminescent substrate.

Cytotoxicity assay

Toxicity of oxLDL to the hRPE was determined by measuring the release of [^{14}C]adenine into culture medium as described by Coffey et al. [29].

PI3K activity assay

Latex bead-containing phagosomal fractions isolated from the hRPE that were pre-incubated with various lipoproteins and inhibitors were washed with 100 mM Tris, pH 7.5, containing 500 mM LiCl, followed by a wash in 20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM Na_2EDTA. The activity of PI3K in phagosomes of the hRPE cells was measured essentially as described elsewhere [42]. Briefly, beads were resuspended in the kinase

buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EGTA) containing 200 μg/ml phosphatidylinositol 4,5-bisphosphate and 50 μCi of γ[^{32}P]ATP. After a 10-min incubation at room temperature, phospholipids were extracted, resolved by thin layer chromatography (TLC), and the incorporation of ^{32}P into phosphatidylinositol 3,4,5-triphosphate (PIP_3) was measured by autoradiography.

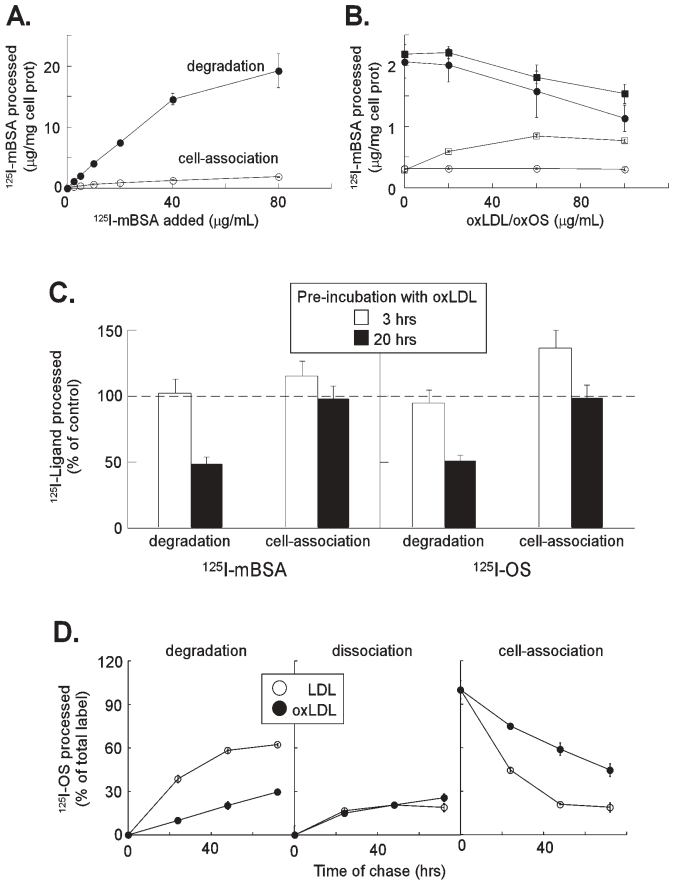

Figure 1. OxLDL blocks intracellular processing of molecules internalized by RPE. (*A*) Dose-dependent degradation and cell-association of ^{125}I-mBSA by hRPE cells. (*B*) Monolayers of hRPE were pre-incubated overnight with indicated concentrations of either oxLDL (●, ○) or oxOS (■, □), followed by the assay for degradation (●, ■) and cell-association (○, □) of ^{125}I-mBSA added for 5 h at a concentration of 5 μg/ml. (*C*) Degradation and cell-association of 5 μg/ml ^{125}I-mBSA or 100 μg/ml ^{125}I-OS was measured for 5 h following a pre-treatment with medium alone or 100 μg/ml oxLDL for either 3 or 20 h. Results are presented as the percent ^{125}I-protein degraded or associated in cells treated with oxLDL of that in cells treated with medium alone. (*D*) The hRPE was pulsed with 1 mg/ml ^{125}I-OS for 3 h followed by washing and addition of fresh medium containing either 100 μg/ml oxLDL or no lipoprotein. At indicated periods of time the distribution of ^{125}I label was measured as described in Material and methods. Results are presented as the percent ^{125}I-OS degraded (TCA-soluble ^{125}I label in medium), dissociated (TCA-insoluble ^{125}I-label in medium), or cell associated of the total ^{125}I radioactivity (media + cells) recovered from the corresponding wells. Each data point indicates the mean ± SD for triplicate samples.

Results

To determine whether oxLDL can induce a deficiency in the processing of targets other than OS [27], we studied the effect of oxLDL on the uptake and catabolism by the hRPE of various ^{125}I-labeled molecules. First, we established that the hRPE exhibits an avid degradation of ^{125}I-mBSA mediated by a saturable and efficient uptake mechanism (fig. 1A). Other tested ^{125}I-labeled ligands, i.e., LDL, methylated LDL, acetylated LDL, copper-oxidized LDL, and asialorosomucoid, demonstrated 10 to 100 times lower rates of uptake and degradation by the hRPE then mBSA (not shown) and, therefore, were excluded from subsequent study. BSA modified by maleic anhydrate is recognized by a number of scavenger receptors expressed on the surface of macrophages, including CD36, which has been reported to be present on RPE cells [19, 43]. We then tested whether oxLDL had any effect on processing of ^{125}I-mBSA by the hRPE. OxLDL binds to CD36 and to other scavenger receptors with high affinity [44]. To avoid the direct competition with ^{125}I-mBSA, we first pre-treated hRPE cells overnight with oxLDL, followed by extensive washing and subsequent incubation with ^{125}I-mBSA. The pre-incubation protocol has been successfully used by us in the past, and leads here to a significant inhibition of mBSA proteolysis, but not cell association by the hRPE (fig. 1B). The distinct lack of an effect of oxLDL on cell association, which is a combination of surface-bound and internalized label, rules out direct competition between mBSA and oxLDL for cell surface binding sites. Degradation of ^{125}I-mBSA was also inhibited by pre-incubation of the hRPE with oxidized OS (oxOS), whereas ^{125}I-mBSA cell association was significantly elevated at the same time (fig. 1B). This latter result was similar to the data reported by Finnemann et al. [43], who noted a modest stimulation of OS uptake by RPE cells when coincubated with oxLDL, presumably due to the cross-linking and activation of CD36. However, we did not observe any stimulation of internalization in response to oxLDL. To resolve this contradiction, we examined the effects of different pre-incubation times with oxLDL on the ability of the hRPE to process ^{125}I-labeled OS or mBSA. Figure 1C shows that a 3-h pretreatment with oxLDL resulted in a small, but statistically significant increase in cell association values for both ^{125}I-OS and ^{125}I-mBSA. Using this approach, we cannot differentiate between surface-bound and internalized label, but one can infer from the data presented by Finnemann et al. [43] that oxLDL increased RPE internalization rates. Finally, to rule out any potential influence of oxLDL on the stages of ligand binding and internalization, we asked whether oxLDL could affect the degradation of ^{125}I-OS internalized prior to the treatment of hRPE cells with the former. We tested this by doing the reverse of the standard pre-incubation protocol, i.e., first pulsing

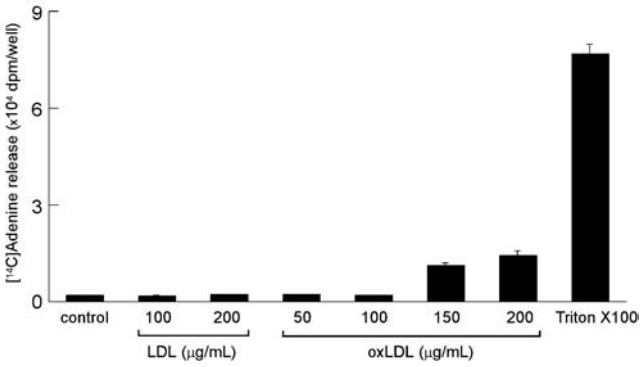

Figure 2. OxLDL is not toxic to hRPE in doses that cause a defect in degradation. hRPE cells were labeled with [^{14}C]adenine for 48 h followed by washing and addition of the indicated concentrations of LDL, oxLDL, 0.5% Triton X-100, or medium alone (control). After a 24-h incubation at 37°C, aliquots of culture medium were removed to measure the release of ^{14}C-radioactivity from the cells. The data are presented as the mean ± SD for triplicate samples.

the hRPE with ^{125}I-OS, allowing internalization of the label during a 4-h chase, and then challenging the hRPE with oxLDL. Figure 1D demonstrates a dose-dependent and efficient inhibition of ^{125}I-OS proteolysis by oxLDL, resulting in a concomitant delay of ^{125}I-OS clearance from RPE cells. Together, these data suggest that oxLDL is capable of inducing comparable degrees of RPE deficiency in degrading molecules of different composition without apparent effect on their rates of internalization.

To rule out a general toxicity of oxLDL as a cause of the altered intracellular proteolysis, we measured membrane integrity of hRPE cells treated with various doses of oxLDL. Concentrations of oxLDL up to 100 μg/ml did not cause any elevation over control values (treatment with LDL) of [^{14}C]adenine release from hRPE cells monolayers pre-loaded with the label, while higher doses of oxLDL resulted in mild increase in permeability of RPE membranes (fig. 2). Thus, the documented deficiency in degradation in hRPE is induced by nontoxic concentrations of oxLDL.

To further investigate the mechanism of altered OS processing in the RPE, we asked whether oxLDL could inactivate lysosomal enzymes responsible for OS breakdown. Cathepsin D, an aspartic protease, is reported to account for the majority of lysosomal proteolytic activity in RPE cells [26], whereas, a thiol protease plays an important regulatory role as well [45]. We have previously suggested that oxLDL-induced inhibition of degradation in macrophages can be explained by the severe suppression of lysosomal thiol protease activity [35]. Therefore, we sought to determine whether lysosomal enzyme activity is perturbed by oxLDL. Total amounts of immunoreactive cathepsin D and cathepsin B, an abundant lysosomal thiol protease, were unchanged in hRPE cells pre-treated with oxLDL (fig. 3A). Since Western blot

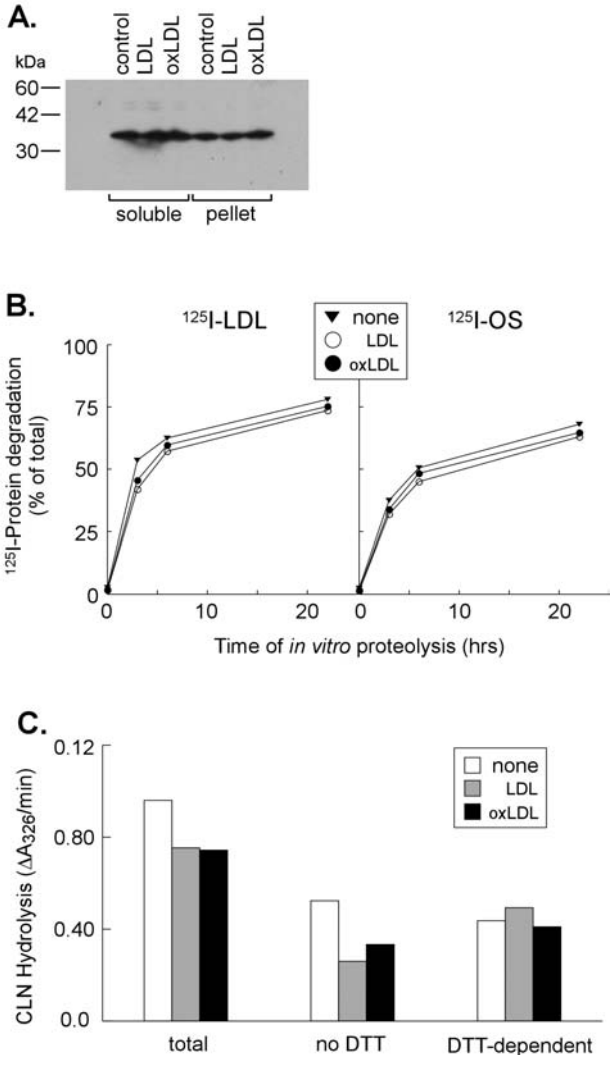

Figure 3. OxLDL does not affect overall proteolytic capacity of hRPE. Cultured hRPE cells were treated overnight with medium alone (control), 100 μg/ml LDL, or 100 μg/ml oxLDL, followed by cell homogenization, separation of RPE cell homogenates into soluble and insoluble fractions by 30-min centrifugation at 20,000 g. (*A*) RPE proteins from each fraction were then resolved by SDS-PAGE and analyzed by Western blotting using anti-cathepsin D polyclonal antibody. (*B*) Soluble fractions of RPE homogenates were adjusted to pH 4.5 and mixed with aliquots of either ^{125}I-LDL or ^{125}I-OS for indicated periods of time, followed by assessing percentage the of ^{125}I-protein converted into TCA-soluble radioactivity. (*C*) Soluble fractions of RPE homogenates were adjusted to pH 4.5 and cathepsin B was measured as hydrolysis of CLN in the presence or absence of 2 mM DTT. DTT-dependent values (latent cathepsin B activity) were obtained by subtracting absorbance measured in the absence of DTT from that in the presence of DTT. Each experiment was performed three to five times with similar results.

analysis cannot differentiate between inactivated and still-active protease, we next tested the proteolytic activity towards various substrates in crude extracts of hRPE cells adjusted to the lysosomal pH of 4.5. Figure 3B shows that pre-treatment of the hRPE with oxLDL did not affect the capacity of their total cell extracts to cleave

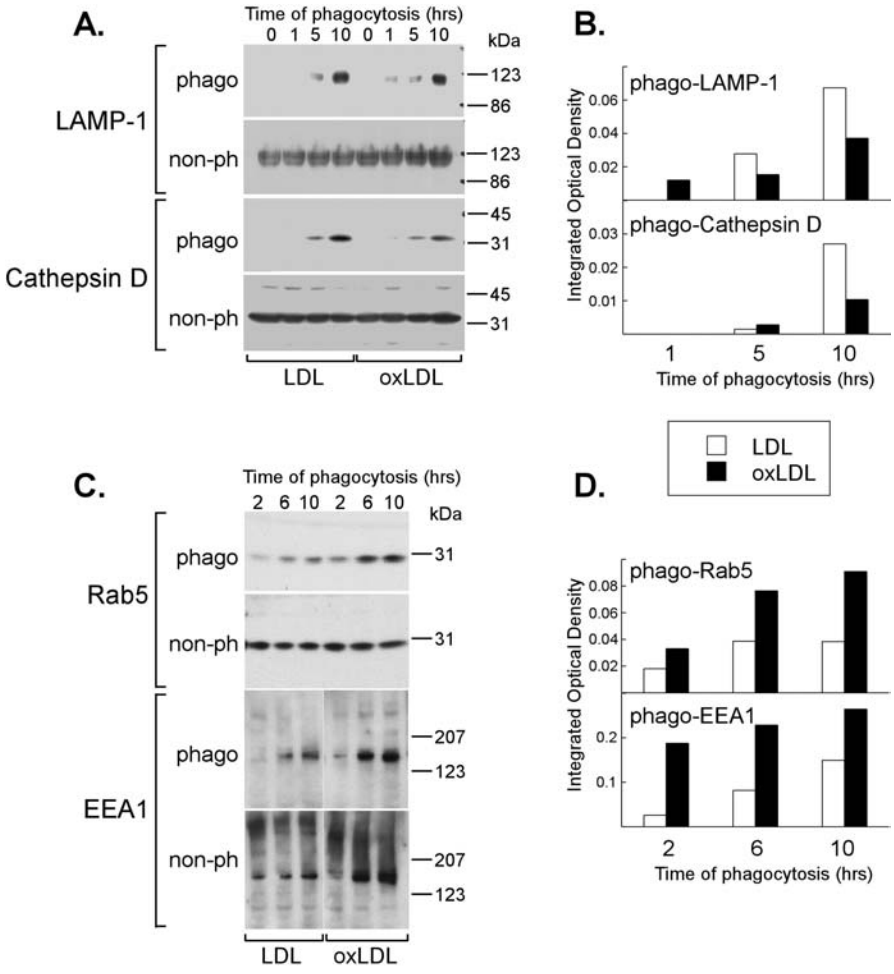

Figure 4. OxLDL augments association of early phagosomal markers with latex-containing phagosomes. Cultures of the hRPE were pretreated for 20 h with either100 μg/ml LDL or 100 μg/ml oxLDL, followed by the removal of lipoprotein-containing medium and extensive washings. FBS-coated magnetic latex beads were then added for the indicated periods of time, after which cell homogenates were prepared and separated into phagosomal and nonphagosomal fractions using the magnet. RPE proteins were then subjected to SDS-PAGE and Western blot analysis using specific antibodies recognizing LAMP-1, cathepsin D, Rab5, or EEA1. (*A*) Late phagosome markers LAMP-1 and cathepsin D. (*C*) Early phagosome markers Rab5 and EEA1. (*B*, *D*) Integrated optical density of the immunoreactive bands was analyzed by densitometry using Scion Image v4.0.2 software. Results are presented as relative optical density of phagosome-associated markers normalized to that of total (phagosomal + nonphagosomal) intracellular levels of a respective marker.

^{125}I-LDL or ^{125}I-OS into small TCA-soluble oligopeptides. To test the thiol protease activity of the RPE extracts, we used hydrolysis of CLN, which has a higher specificity for cathepsin B, and can also be cleaved by other thiol-dependent cathepsins, e.g., L, S, and K [46]. When assayed in the absence or presence of the reducing agent DTT, which activates latent thiol proteases, CLN hydrolysis in cell extracts obtained from oxLDL-treated hRPE cells did not differ from that of LDL-treated cells (fig. 3C). Together, these results rule out global lysosomal enzyme deficiency as a reason for the failure of oxLDL-loaded hRPE to process OS.

Since oxLDL reduced neither hRPE internalization nor proteolytic capacity, we reasoned that it may impede the delivery of lysosomal enzymes to the engulfed OS via blockage of the vesicular transport from the Golgi to newly formed phagosomes. To determine whether oxLDL-treated RPE cells experienced any altered phagosome maturation, we studied the acquisition by hRPE-internalized latex beads of molecules characteristic for early and late stages of phagocytosis. Specifically, lysosome-associated membrane protein 1 (LAMP-1), an abundant lysosomal glycoprotein, and cathepsin D served as markers of late phagosomal compartments, whereas Rab5, a small GTPase, and early endosome antigen 1 (EEA-1), a phosphatidylinositol 3-phosphate-binding protein, are found in early phagosomal compartments [47]. Following accumulation of oxLDL by the hRPE, association of LAMP-1 and cathepsin D with latex-containing phagosomes was diminished by approximately 40–50% (fig. 4A, B), indicating a reduced fusion of lysosomal constituents with newly internalized targets. At the

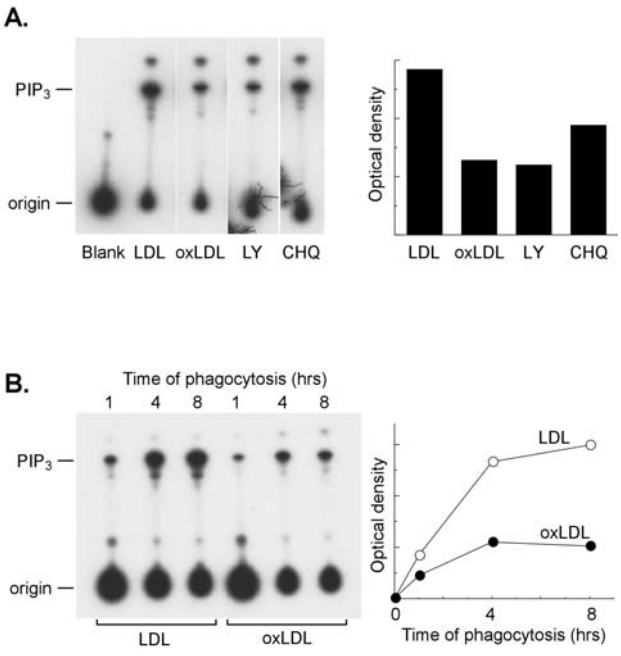

Figure 5. Reduced activity of PI3K in phagosomes of hRPE pretreated with oxLDL. (*A*) Cultures of the hRPE were challenged with FBS-coated magnetic latex beads in the presence of the following reagents: 100 μg/ml LDL, 100 μg/ml oxLDL, 100 μM LY294002 (LY), or 100 μM chloroquine (CHQ). After 4 h incubation, latex-containing phagosomes were isolated from cell homogenates and assayed for PI3K activity. Products of the in vitro kinase reaction were resolved by TLC and phosphorylated PIP_3 was detected by autoradiography. (*B*) Human RPE cells were first pretreated with either 100 μg/ml LDL or 100 μg/ml oxLDL overnight followed by the removal of lipoprotein-containing medium and extensive washings. FBS-coated magnetic latex beads were then added for indicated periods of time, after which cells were homogenated and the isolated phagosomal fraction was tested for PI3K activity as above.

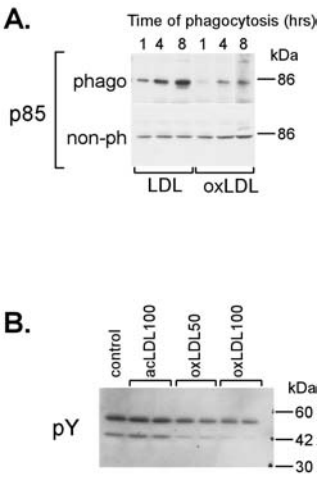

Figure 6. OxLDL causes deficient recruitment of the p85 subunit of PI3K to phagosomes in hRPE. (*A*) Cultures of hRPE were pretreated for 20 h with either100 μg/ml LDL or 100 μg/ml oxLDL, followed by the removal of lipoprotein-containing medium and extensive washings. FBS-coated magnetic latex beads were then added for the indicated periods of time, after which cell homogenates were prepared and separated into phagosomal and nonphagosomal fractions using the magnet. RPE proteins were then subjected to SDS-PAGE and Western blot analysis using specific antibodies recognizing the p85 subunit of PI3K. (*B*) Human RPE cells were incubated overnight with either medium alone (control), or in the presence of 100 μg/ml acLDL, 50 μg/ml oxLDL, or 100 μg/ml oxLDL. At the end of the incubation, cellular proteins were solubilized directly in Laemmli sample buffer, resolved by SDS-PAGE, and probed by Western blotting using an anti-phosphotyrosine antibody (pY).

same time, greater amounts Rab5 and EEA1 were recruited to the latex beads ingested by cells pre-incubated with oxLDL (fig. 4C, D). Since LAMP-1 and cathepsin D are always vesicle-associated, whereas Rab5 and EEA1 can exist in a membrane-bound or cytosolic form, these data suggest that in oxLDL-treated RPE cells, vesicular transport is impaired, but not the acquisition of cytosolic trafficking molecules by phagosomes. When taken together with other effects of oxLDL, e.g., blockade of cathepsin D maturation and its targeting to the cell surface [48], and alteration of the actin cytoskeleton [49], these features resemble the effects of PI3K inhibitors.

Activities of class I and III PI3K are essential for signal transduction and membrane fusion events during phagocytosis [50]. We asked whether the observed blockade in phagosome maturation is associated with any change in class I PI3K activity on the phagosomal membranes. Phagosomal membranes isolated from hRPE cells pretreated with oxLDL demonstrated a sustained decrease in PI3K activity compared to cells treated with LDL (fig. 5). OxLDL was as effective as the highest nontoxic dose of LY294002, a known inhibitor of all PI3K (fig. 5A). To further investigate the mechanism of oxLDL-induced inhibition of PI3K activity, we studied the presence of the PI3K molecule on the phagosomal membrane. The cellular localization and activity of the catalytic subunit of the class I PI3K, p110, is controlled by the regulatory subunit, p85 [51]. The p85 subunit itself is recruited to the membranes via its SH2 domain that binds phosphorylated tyrosines (pY) present on either membrane receptors or adapter proteins [51]. Acquisition of p85 was significantly impaired by phagosomes of the hRPE following the treatment with oxLDL compared to LDL treatment (fig. 6A), suggesting the underlying mechanism for the reduced PI3K activity. We have independently confirmed this result by studying the colocalization of latex beads and p85 in mouse peritoneal macrophages using confocal immunofluorescence microscopy [G. Hoppe, unpublished observation]. Unfortunately, confluent hRPE, unlike macrophages, forms very tightly packed cellular monolayers, making it very difficult to accurately demonstrate overlapping of an abundant membrane protein with phagosomes in the cultured RPE. Since tyrosine phosphorylation is a signal for p85 association with the membranes, we also asked whether oxLDL affected the overall levels of pY. Figure 6B shows

a Western blot analysis with a pY-specific antibody of the total cell lysates obtained from the hRPE that received various forms of modified LDL. Uptake of oxLDL but not acLDL resulted in a global reduction of the pY content in hRPE cells, suggesting that oxLDL may be capable of suppressing phosphotyrosine signaling. Using mouse peritoneal macrophages we also confirmed this result immunohistochemically by observing that oxLDL reduced the phagosome-associated pY immunoreactivity [G. Hoppe, unpublished observation]. Thus, our results indicate that oxLDL inhibits class I PI3K recruitment to phagosomes formed by hRPE internalization of latex beads.

Discussion

Due to the highly pro-oxidative environment of the outer retina secondary to photo-illumination, high oxygen tension, metabolic by-products, and abundant polyunsaturated fatty acids, the RPE is likely to experience elevated oxidative stress [24, 52]. Oxidative breakdown of lipids results in the formation of a number of toxic intermediates, including fatty acid hydroperoxides and reactive aldehydes, most of which are chemically characterized as electrophiles, i.e., biologically active molecules capable of abstracting electrons and thereby forming covalent links with side chains of cysteinyls, lysyls, arginyls, and histidyls [53]. Therefore, the biological effects of oxidized lipids are often attributed to the toxicity associated with the products of lipid peroxidation, including the ability of 'artificial lipofuscin' (UV-oxidized OS) to perturb RPE phagocytosis [54]. Our results demonstrate that accumulation of oxidized products in the RPE leads to the inhibition of intracellular OS processing via a mechanism that cannot be explained by the reduced OS uptake, direct inactivation of lysosomal enzymes or compromised plasma membrane integrity of RPE. Sequential challenge of the hRPE with oxLDL and then with OS created an accumulation of oxLDL and OS in different cellular compartments [G. Hoppe, unpublished observation], and was the key to achieving nontoxic effects on OS processing, which persist even after an additional 24-h recovery period between oxLDL removal and OS addition [27, 48]. Although under our experimental conditions, oxLDL is likely to be chased into a terminal compartment, which has a limited capacity to fuse with newly internalized phagosomes [55], it affects newly internalized molecules, possibly via cytosolic transport or through membrane lateral diffusion of polar lipids [56]. We have recently shown that a relatively polar lipid fraction in oxLDL can account for the majority of its biological activity [41, 57].

The unexpected failure of oxLDL to cause direct inactivation of lysosomal enzymes in the hPRP is partially inconsistent with our previous studies on macrophages, where oxLDL effectively suppressed the activity of the thiol protease, cathepsin B, but not the aspartic protease, cathepsin D [35]. This discrepancy is likely due to the significant differences in endo-phagosomal pathways between macrophages and RPE cells. For example, primary cultures of mouse peritoneal macrophages exhibit far greater rates of uptake and catabolism of various macromolecules, including faster cleavage of rhodopsin and degradation of OS [G. Hoppe, unpublished observations]. The proteolytic repertoires of these cell types are apparently also distinct, with a greater contribution of thiol protease activity to macrophage lysosomal proteolysis. The lack of direct enzyme inactivation by oxLDL is, on the other hand, consistent with the reports that intracellular accumulation of lipofuscin or its major component, A2E, leads to lysosomal dysfunction [58, 59] and inhibition of phagocytosis [54], without apparent effects on any of 23 hydrolytic lysosomal enzymes [60].

In this study, we provide evidence that oxLDL, as a prototype of a lipid-protein cross-link, is capable of arresting phago-lysosome formation in the RPE by selective exclusion of lysosomal markers and PI3K, while inducing an excessive association of the early trafficking markers with the phagosomal membranes. Thus, our results suggest that the poor processing of OS by oxLDL-loaded RPE cells is a result of impaired phagosome maturation, which is associated with deficient PI3K signaling. The role of the class III PI3K in membrane trafficking including phagosome maturation is well known for a variety of phagocytic processes, e.g., autophagy, host defense, and surfactant turnover [50, 61, 62]. On the other hand, the class I PI3K is traditionally implicated in various steps of signal transduction during phagocytosis, e.g., actin remodeling [63]. However, evidence also exists that the activity of this class of PI3K is required for some specific vesicle transport events [64–66]. The product of PI3K activity, PI3P, is thought to contribute to the formation of a fusion-competent multimeric complex on the cytosolic side of the vesicle, in which Rab5 and EEA1 interact with PI3P directly [67]. Recruitment of EEA1 to the membranes in normal conditions has been shown to be PI3P dependent [68], which seemingly contradicts our data on excessive accumulation of EEA1 on the phagosomes with reduced PI3K activity. However, since EEA1 can also be recruited to the membranes via its Rab5-binding domain [69], the following scenario of fusion blockade by oxLDL is possible. OxLDL-mediated reduction in PI3K activity may lead to membrane depletion of PI3P, followed by an assembly of incomplete and fusion-incompetent vesicle docking complexes [70]. Cells in turn overcompensate for the lack of vesicle maturation by excessive recruitment of Rab5 to the fusion-deficient membranes, which triggers abnormal accumulation of EEA1 that still cannot be activated in the absence of PI3P [71]. This proposed mechanism fits remarkably well with the

recently reported data on paradoxical enhancement of Rab5 recruitment to latex-containing phagosomes in macrophages treated with wortmannin, a PI3K inhibitor [72].

Accumulation of lipofuscin within post-mitotic tissues is a common feature of many age-associated disorders including macular degeneration of the retina [23, 73]. We found that accumulation of oxLDL, a prototype for oxidatively damaged undegradable supramolecular complexes, causes phagolysosomal dysfunction in RPE cells through perturbation of intracellular vesicle trafficking without appreciable inactivation of lysosomal enzymes. Lysosomotropic properties of A2E [58], and the ability of lipofuscin to alter lysosome-mediated processes [54, 58, 59] via an apparently nontoxic mechanism [60], reinforces the relevance of our findings.

Acknowledgements. The authors are indebted to Dr. J. Burke and C. Skumatz for a gift of primary human RPE, and on their valuable advice on culturing these cells. We thank Dr N. Sizemore for her help and advice with the PI3K assay. We are grateful to Dr. A. Marmorstein for stimulating discussion of our results. This work was supported in part by the AHA Scientist Development Grant (to G. H.), by NIH Grant HL53315 (to H. F. H.), by funds from the Foundation Fighting Blindness (to G. H. and H. F. H.), and the CCF Clinical Investigator Development Award (CIDA, to J. S.).

1 Bok D. and Young R. W. (1979) In: The Retinal Pigment Epithelium. Marmor M. F. and Zinn K. M. (eds), pp. 148–174, Harvard University Press, Cambridge, Mass.
2 Besharse J. C. and Defoe D. M. (1998) In: The Retinal Pigment Epithelium: Function and Disease. Marmor M. F. and Wolfensberger T. J. (eds), pp. 152–172, Oxford University Press, New York
3 LaVail M. M., Sidman R. L. and Gerhardt C. O. (1975) Congenic strains of RCS rats with inherited retinal dystrophy. J. Here. **66:** 242–244
4 Duncan J. L., LaVail M. M., Yasumura D., Matthes M. T., Yang, H. et al. (2003) An RCS-like retinal dystrophy phenotype in mer knockout mice. Invest. Ophthalmol. Vis. Sci. **44:** 826–838
5 Rakoczy P. E., Zhang D., Robertson, T., Barnett, N. L., Papadimitriou, J., Constable, I. J. et al. (2002) Progressive age-related changes similar to age-related macular degeneration in a transgenic mouse model. Am J Pathol **161:**1515–1524
6 Hasson T. (1999) Molecular motors: sensing a function for myosin-VIIa. Curr Biol **9:** R838–R841
7 Edwards R. B. and Flaherty P. M. (1986) Increased phagocytosis of outer segments in the presence of serum by cultured normal, but not dystrophic, rat retinal pigment epithelium. J. Cell. Physiol. **127:** 293–296
8 Heth C. A. and Marescalchi P. A. (1994) Inositol triphosphate generation in cultured rat retinal pigment epithelium. Invest. Ophthalmol. Vis. Sci. **35:** 409–416
9 Heth C. A. and Schmidt S. Y. (1991) Phagocytic challenge induces changes in phosphorylation of retinal pigment epithelium proteins. Curr. Eye. Res. **10:** 1049–1057
10 Ershov A. V., Lukiw W. J. and Bazan N. G. (1996) Selective transcription factor induction in retinal pigment epithelial cells during photoreceptor phagocytosis. J. Biol. Chem. **271:** 28458–28462
11 Feng W., Yasumura D., Matthes M. T., LaVail M. M. and Vollrath D. (2002) Mertk triggers uptake of photoreceptor outer segments during phagocytosis by cultured retinal pigment epithelial cells. J. Biol. Chem. **277:** 17016–17022
12 Scott R. S., McMahon E. J., Pop S. M., Reap E. A., Caricchio R., Cohen P. L. et al. (2001) Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature **411:** 207–211
13 Finnemann S. C. and Rodriguez-Boulan E. (1999) Macrophage and retinal pigment epithelium phagocytosis: apoptotic cells and photoreceptors compete for alphavbeta3 and alphavbeta5 integrins, and protein kinase C regulates alphavbeta5 binding and cytoskeletal linkage. J. Exp. Med. **190:** 861–874
14 D'Cruz P. M., Yasumura D., Weir J., Matthes M. T., Abderrahim H., LaVail M. M. et al. (2000) Mutation of the receptor tyrosine kinase gene Mertk in the retinal dystrophic RCS rat. Hum. Mol. Genet **9:** 645–651
15 Gal A., Li Y., Thompson D. A., Weir J., Orth U., Jacobson S. G., Apfelstedt-Sylla E. et al. (2000) Mutations in MERTK, the human orthologue of the RCS rat retinal dystrophy gene, cause retinitis pigmentosa. Nat. Genet. **26:** 270–271
16 Boyle D., Tien L. F., Cooper N. G., Shepherd V. and McLaughlin B. J. (1991) A mannose receptor is involved in retinal phagocytosis. Invest. Ophthalmol. Vis. Sci. **32:** 1464–1470
17 Finnemann S. C., Bonilha V. L., Marmorstein A. D. and Rodriguez-Boulan E. (1997) Phagocytosis of rod outer segments by retinal pigment epithelial cells requires alpha(v)beta5 integrin for binding but not for internalization. Proc. Natl. Acad. Sci. USA **94:** 12932–12937
18 Lin H. and Clegg D. O. (1998) Integrin alphavbeta5 participates in the binding of photoreceptor rod outer segments during phagocytosis by cultured human retinal pigment epithelium. Invest. Ophthalmol. Vis. Sci. **39:** 1703–1712
19 Ryeom S. W., Sparrow J. R. and Silverstein R. L. (1996) CD36 participates in the phagocytosis of rod outer segments by retinal pigment epithelium. J. Cell Sci. **109:** 387–395
20 Hall M. O., Burgess B. L., Abrams T. A., Ershov A. V. and Gregory C. Y. (1996) Further studies on the identification of the phagocytosis receptor of rat retinal pigment epithelial cells. Exp. Eye. Res. **63:** 255–264
21 Allikmets R., Singh N., Sun H., Shroyer N. F., Hutchinson A., Chidambaram A. et al. (1997) A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Stargardt macular dystrophy. Nat. Genet **15:** 236–246
22 Mata N. L., Tzekov R. T., Liu X., Weng J., Birch D. G. and Travis G. H. (2001) Delayed dark-adaptation and lipofuscin accumulation in abcr+/– mice: implications for involvement of ABCR in age-related macular degeneration. Invest. Ophthalmol. Vis. Sci. **42:** 1685–1690
23 Young R. W. (1987) Pathophysiology of age-related macular degeneration. Surv. Ophthalmol. **31:** 291–306
24 Beatty S., Koh H., Phil M., Henson D. and Boulton M. (2000) The role of oxidative stress in the pathogenesis of age-related macular degeneration. Surv. Ophthalmol. **45:** 115–134
25 Hjelmeland L. M., Cristofolo V. J., Funk W., Rakoczy E. and Katz M. L. (1999) Senescence of the retinal pigment epithelium. Mol. Vis. **5:** 33
26 Zimmerman W. F., Godchaux W. 3rd and Belkin M. (1983) The relative proportions of lysosomal enzyme activities in bovine retinal pigment epithelium. Exp. Eye. Res. **36:** 151–158
27 Hoppe G., Marmorstein A. D., Pennock E. A. and Hoff H. F. (2001) Oxidized low density lipoprotein-induced inhibition of processing of photoreceptor outer segments by RPE. Invest. Ophthalmol. Vis. Sci. **42:** 2714–2720
28 Ball R. Y., Bindman J. P., Carpenter K. L. and Mitchinson M. J. (1986) Oxidized low density lipoprotein induces ceroid accumulation by murine peritoneal macrophages in vitro. Atherosclerosis **60:** 173–181
29 Coffey M. D., Cole R. A., Colles S. M. and Chisolm G. M. (1995) In vitro cell injury by oxidized low density lipoprotein involves lipid hydroperoxide-induced formation of alkoxyl, lipid, and peroxyl radicals. J. Clin. Invest. **96:** 1866–1873

30 Hoff H. F., Zyromski N., Armstrong D. and O'Neil J. (1993) Aggregation as well as chemical modification of LDL during oxidation is responsible for poor processing in macrophages. J Lipid Res **34:** 1919–1929

31 Sparrow J. R., Krane S., Jang Y. P., Zhou J., Cai B., Vollmer H. R. et al. (2003) In: Annual ARVO Meeting, pp. 3144, Fort Lauderdale, Fla.

32 Li W., Yuan X. M. and Brunk U. T. (1998) OxLDL-induced macrophage cytotoxicity is mediated by lysosomal rupture and modified by intralysosomal redox-active iron. Free Radic. Res. **29:** 389–398

33 Burke J. M., Skumatz C. M., Irving P. E. and McKay B. S. (1996) Phenotypic heterogeneity of retinal pigment epithelial cells in vitro and in situ. Exp. Eye Res. **62:** 63–73

34 Hatch F. T. and Lees R. S. (1968) Practical method for plasma lipoprotein analysis. Adv. Lipid Res. **6:** 2–63

35 Hoppe G., O'Neil J. and Hoff H. F. (1994) Inactivation of lysosomal proteases by oxidized low density lipoprotein is partially responsible for its poor degradation by mouse peritoneal macrophages. J. Clin. Invest. **94:** 1506–1512

36 Brown M. S., Basu S. K., Falck J. R., Ho Y. K. and Goldstein J. L. (1980) The scavenger cell pathway for lipoprotein degradation: specificity of the binding site that mediates the uptake of negatively-charged LDL by macrophages. J. Supramol. Struct. **13:** 67–81

37 Hall M. O. (1978) Phagocytosis of light- and dark-adapted rod outer segments by cultured pigment epithelium. Science **202:** 526–528

38 Boulton M., McKechnie N. M., Breda J., Bayly M. and Marshall J. (1989) The formation of autofluorescent granules in cultured human RPE. Invest. Ophthalmol. Vis. Sci. **30:** 82–89

39 Kamido H., Kuksis A., Marai L. and Myher J. J. (1993) Identification of core aldehydes among in vitro peroxidation products of cholesteryl esters. Lipids **28:** 331–336

40 Bilheimer D. W., Eisenberg S. and Levy R. I. (1972) The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. Biochim. Biophys. Acta **260:** 212–221

41 Hoff H. F., O'Neil J., Wu Z., Hoppe G. and Salomon R. L. (2003) Phospholipid hydroxyalkenals: biological and chemical properties of specific oxidized lipids present in atherosclerotic lesions. Arterioscler. Thromb. Vasc. Biol. **23:** 275–282

42 Krook A., Whitehead J. P., Dobson S. P., Griffiths M. R., Ouwens M., Baker C. et al. (1997) Two naturally occurring insulin receptor tyrosine kinase domain mutants provide evidence that phosphoinositide 3-kinase activation alone is not sufficient for the mediation of insulin's metabolic and mitogenic effects. J. Biol. Chem. **272:** 30208–30214

43 Finnemann S. C. and Silverstein R. L. (2001) Differential roles of CD36 and alphavbeta5 integrin in photoreceptor phagocytosis by the retinal pigment epithelium. J. Exp. Med. **194:** 1289–1298

44 Endemann G., Stanton L., Madden K., Bryant C., White R. and Protter A. (1993) CD36 is a receptor for oxidized low density lipoprotein. J. Biol. Chem. **268:** 11811–11816

45 Rakoczy P. E., Lai M. C., Baines M. G., Spilsbury K. and Constable I. J. (1998) Expression of cathepsin S antisense transcripts by adenovirus in retinal pigment epithelial cells. Invest. Ophthalmol. Vis. Sci. **39:** 2095–2104

46 Bajkowski A. S. and Frankfater A. (1975) Specific spectrophotometric assays for cathepsin B1. Anal Biochem **68:** 119–127

47 Vieira O. V., Botelho R. J. and Grinstein S. (2002) Phagosome maturation: aging gracefully. Biochem J **366:** 689–704

48 Hoppe G., O'Neil J., Hoff H. F. and Sears J. (2004) Products of lipid peroxidation induce missorting of the principal lysosomal protease in retinal pigment epithelium. Biochim. Biophys. Acta. 1689 (in press)

49 Miller Y. I., Worrall D. S., Funk C. D., Feramisco J. R. and Witztum J. L. (2003) Actin polymerization in macrophages in response to oxidized LDL and apoptotic cells: role of 12/15-lipoxygenase and phosphoinositide 3-kinase. Mol. Biol. Cell **14:** 4196–4206

50 Vieira O. V., Botelho R. J., Rameh L., Brachmann S. M., Matsuo T., Davidson H. W et al. (2001) Distinct roles of class I and class III phosphatidylinositol 3-kinases in phagosome formation and maturation. J. Cell Biol. **155:** 19–25

51 Greenberg S. (1999) Modular components of phagocytosis. J. Leukoc. Biol. 66, 712–717

52 Winkler B. S., Boulton M. E., Gottsch J. D. and Sternberg P. (1999) Oxidative damage and age-related macular degeneration. Mol. Vis. **5:** 32

53 Esterbauer H. (1993) Cytotoxicity and genotoxicity of lipid-oxidation products. Am. J. Clin. Nutr. **57:** 779S–785S; discussion 785S–786S

54 Sundelin S., Wihlmark U., Nilsson S. E. and Brunk U. T. (1998) Lipofuscin accumulation in cultured retinal pigment epithelial cells reduces their phagocytic capacity. Curr. Eye. Res. **17:** 851–857

55 Kornfeld S. and Mellman I. (1989) The biogenesis of lysosomes. Annu. Rev. Cell Biol. **5:** 483–525

56 Mukherjee S., Soe T. T. and Maxfield F. R. (1999) Endocytic sorting of lipid analogues differing solely in the chemistry of their hydrophobic tails. J. Cell Biol. **144:** 1271–1284

57 O'Neil J., Hoppe G. and Hoff H. F. (2003) Phospholipids in oxidized low density lipoproteins perturb the ability of macrophages to degrade internalized macromolecules and reduce intracellular cathepsin B activity. Atherosclerosis **169:** 215–224

58 Finnemann S. C., Leung L. W. and Rodriguez-Boulan E. (2002) The lipofuscin component A2E selectively inhibits phagolysosomal degradation of photoreceptor phospholipid by the retinal pigment epithelium. Proc. Natl. Acad. Sci.. USA **99:** 3842–3847

59 Holz F. G., Schutt F., Kopitz J., Eldred G. E., Kruse F. E., Volcker H. E. et al. (1999) Inhibition of lysosomal degradative functions in RPE cells by a retinoid component of lipofuscin. Invest. Ophthalmol. Vis. Sci. **40:** 737–743

60 Bermann M., Schutt F., Holz F. G. and Kopitz J. (2001) Does A2E, a retinoid component of lipofuscin and inhibitor of lysosomal degradative functions, directly affect the activity of lysosomal hydrolases? Exp. Eye Res. **72:** 191–195

61 Fratti R. A., Backer J. M., Gruenberg J., Corvera S. and Deretic V. (2001) Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. J. Cell Biol. **154:** 631–644

62 Blommaart E. F., Krause U., Schellens J. P., Vreeling-Sindelarova H. and Meijer A. J. (1997) The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. Eur. J. Biochem. **243:** 240–246

63 Cox D. and Greenberg S. (2001) Phagocytic signaling strategies: Fc(gamma)receptor-mediated phagocytosis as a model system. Semin. Immunol. **13:** 339–345

64 Venkateswarlu K. and Cullen P. J. (2000) Signalling via ADP-ribosylation factor 6 lies downstream of phosphatidylinositide 3-kinase. Biochem. J. **345:** 719–724

65 Christoforidis S., Miaczynska M., Ashman K., Wilm M., Zhao L., Yip S. C. et al. (1999) Phosphatidylinositol–3-OH kinases are Rab5 effectors. Nat Cell Biol **1:** 249–252

66 Siddhanta U., McIlroy J., Shah A., Zhang Y. and Backer J. M. (1998) Distinct roles for the p110alpha and hVPS34 phosphatidylinositol 3'-kinases in vesicular trafficking, regulation of the Actin cytoskeleton, and mitogenesis. J. Cell Biol. **143:** 1647–1659

67 Simonsen A., Lippe R., Christoforidis S., Gaullier J. M., Brech A., Callaghan J. et al. (1998) EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature **394:** 494–498

68 Patki V., Virbasius J., Lane W. S., Toh B. H., Shpetner H. S. and Corvera S. (1997) Identification of an early endosomal protein regulated by phosphatidylinositol 3-kinase. Proc. Natl. Acad. Sci. USA **94:** 7326–7330
69 Merithew E., Stone C., Eathiraj S. and Lambright D. G. (2003) Determinants of Rab5 interaction with the N terminus of early endosome antigen 1. J. Biol. Chem. **278:** 8494–8500
70 Corvera S. and Czech M. P. (1998) Direct targets of phosphoinositide 3-kinase products in membrane traffic and signal transduction. Trends Cell Biol. **8:** 442–446
71 Mills I. G., Jones A. T. and Clague M. J. (1998) Involvement of the endosomal autoantigen EEA1 in homotypic fusion of early endosomes. Curr. Biol. **8:** 881–884
72 Vieira O. V., Bucci C., Harrison R. E., Trimble W. S., Lanzetti L., Gruenberg J. et al. (2003) Modulation of Rab5 and Rab7 recruitment to phagosomes by phosphatidylinositol 3-kinase. Mol. Cell Biol. **23:** 2501–2514
73 Eldred G. E. (1998) In: The Retinal Pigment Epithelium. Function and Disease. (Marmor M. F. and Wolfensberger T. J. (eds), pp. 651–668, Oxford University Press, New York