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Rescue of compromised lysosomes enhances degradation of photoreceptor outer segments and reduce lipofuscin-like autofluorescence

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Abstract

Healthful cell maintenance requires the efficient degradative processing and removal of waste material. Retinal pigmented epithelial (RPE) cells have the onerous task of degrading both internal cellular debris generated through autophagy as well as phagocytosed photoreceptor outer segments. We propose that the inadequate processing material with the resulting accumulation of cellular waste contributes to the downstream pathologies characterized as age-related macular degeneration (AMD). The lysosomal enzymes responsible for clearance function optimally over a narrow range of acidic pH values; elevation of lysosomal pH by compounds like chloroquine or A2E can impair degradative enzyme activity and lead to a lipofuscin-like autofluorescence. Restoring acidity to the lysosomes of RPE cells can enhance activity of multiple degradative enzymes and is therefore a logical target in early AMD. We have identified several approaches to reacidify lysosomes of compromised RPE cells; stimulation of beta-adrenergic, A_{2A} adenosine and D5 dopamine receptors each lowers lysosomal pH and improves degradation of photoreceptor outer segments. Activation of the CFTR chloride channel also reacidifies lysosomes and increases degradation. These approaches also restore the lysosomal pH of RPE cells from aged ABCA4^{-/-} mice with chronically high levels of A2E, suggesting that functional signaling pathways to reacidify lysosomes are retained in aged cells like those in patients with AMD. Acidic nanoparticles transported to RPE lysosomes also lower pH and improve degradation of outer segments. In summary, the ability of diverse approaches to lower lysosomal pH and enhance outer segment degradation support the proposal that lysosomal acidification can prevent the accumulation of lipofuscin-like material in RPE cells.

Keywords

Retinal pigment epithelium; age-related macular degeneration; lipofuscin; autophagy; lysosomal pH; Stargardt's disease

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14.1 AMD, lysosomes and pH

The cardinal features of age-related macular degeneration (AMD) pathogenesis have been defined. A series of observations have clarified a step-wise progression consequent to inadequate internal maintenance of the RPE cell [1]. Failure to properly degrade and/or recycle protein and lipid derived from the daily ingestion of phagosomes, as well as internal cell membranes by specific organelles within the RPE cell, leads to the slow accumulation of lipoprotein aggregates [2]. Such aggregates are in part retained and in part exocytosed basolaterally. In time those that are retained impede essential cell function. Perhaps more important, the exocytosed material forms deposits in the subretinal space and infiltrates into Bruch's membrane. Characterized by pathologists as basal laminar deposits, basal linear deposits and drusen, these aggregates engender a series of local reactions that include incitement of innate immunity [3]. By their volume the subretinal deposits lengthen the distance between the choroidal circulation and the inner segments of photoreceptors. Further, lipoprotein infiltration of Bruch's membrane leads to a series of pathologies that include breakdown of the elastic lamina, swelling and loss of permeability. Since the RPE, as one of its essential functions, aids in the maintenance of retinal transparency by constantly acting as an outward water pump - a necessary prerequisite for transparency, the impedance to outward water movement is responsible for the frequently observed pigment epithelial detachment noted in subjects with AMD.

While the phenotypes associated with AMD are likely due to numerous overlapping pathologies, we propose that the inadequate breakdown of material by RPE lysosomes provides a common locus that may explain many of the subsequent effects. The sharp pH sensitivity of lysosomal enzymes, combined with evidence that this pH is elevated by compounds associated with AMD-like retinopathies, make lysosomal pH an ideal target in addressing AMD at its early stages (Figure 1).

14.2 Pathological elevation of lysosomal pH in RPE cells

The lysosomal pH of RPE cells can be elevated by numerous insults. For example, the antimalaria drug chloroquine has long been known to alkalinize lysosomes [4]. This tertiary amine accumulates in acidic organelles, freely diffusing across the membrane and then becoming trapped upon protonation. Tamoxifen also elevates lysosomal pH in RPE cells independently of its estrogenic actions; while a similar trapping mechanism is suspected a detergent-like effect may also contribute to the alkalinization [5, 6].

Of particular interest to RPE cells is the observation that lysosomal pH may also be increased by chronic exposure to N-retinylidene-N-retinylethanolamide (A2E), a by product of the visual cycle. A2E accumulates with age in most individuals, but rises to higher levels at an earlier age when the ABCA4 transporter is defective as in recessive Stargardt's disease [7]. A2E rapidly localizes to the lysosomes of RPE cells [8]. Although short term exposure to high levels can impair cholesterol and phospholipid metabolism, it does not change lysosomal pH [9, 10]. However, chronic exposure to A2E does raise lysosomal pH; several weeks of A2E reduces the ATPase activity of isolated lysosomes, slows phagocytosis and degradation of photoreceptor outer segments and reduces autophagy [11, 12]. Further

evidence for an alkalinizing effect of A2E comes from the RPE cells of ABCA4^{-/-} mice, whose lysosomes have a substantially higher pH as compared to that of age matched controls [6]. Whether the delayed alkalinization by A2E is caused by its detergent-like effects in the membrane, by changes to cholesterol metabolism or changes in expression of lysosomal genes remains unclear, but as quaternary amines like A2E do not display the rapid protonation in acidic environments seen by tertiary amines like chloroquine, the lack of rapid alkalinization is expected.

14.3 Consequences of lysosomal alkalinization on degradation

Lysosomal enzymes function optimally over a narrow range of acidic pH values and the predominant lysosomal enzymes of the RPE reflect this tight pH dependence. For instance, activity of the major RPE enzyme lysosomal acid lipase decreases by 60% when the pH is raised from 4.5 to 5.2, while activity of major protease cathepsin D falls by 80% when the pH rises from 4.5 to 5.0 [13, 14]. This sharp pH dependence of enzyme activity implies that alkalinizing lysosomes of RPE cells will lower the activity of multiple enzymes and interfere with the degradation of internalized outer segments. Autofluorescence associated with photoreceptor outer segments was increased in RPE cells exposed to chloroquine [15]. Lysosomal alkalinization decreased staining of Bodipy-pyostatin-A, suggesting the lysosomal enzyme cathepsin D was less effective in RPE cells with perturbed lysosomes [15, 16]. The clearance of outer segments is also decreased when the lysosomal pH is elevated with tamoxifen [17]. Thus experimental lysosomal alkalinization leads to decreased activity of degradative enzymes and accumulation of partially degraded photoreceptor outer segment debris.

Lysosomal alkalinization by chloroquine also disrupts outer segment degradation by RPE cells *in vivo*. Chronic treatment of rats with chloroquine leads to the accumulation of lysosomal-associated organelles and multilamellar bodies within the RPE cells [18, 19]. Of particular relevance is the accumulation of partially degraded material of photoreceptor origin in and around Bruch's membrane. Together, these *in vivo* and *in vitro* studies imply that lysosomal alkalinization is itself sufficient to impair outer segment degradation by RPE cells and promote deposition of exocytosed debris onto Bruch's membrane.

14.4 Restoration of an acidic lysosomal pH to compromised RPE cells

We have proposed that the restoration of an acidic lysosomal pH to compromised RPE cells simultaneously increases activity of numerous degradative enzymes. Our laboratory employed a screening approach to identify drugs capable of reacidifying lysosomes in damaged RPE cells. Epinephrine, norepinephrine and beta adrenergic agonist isoproterenol reacidified lysosomes; while the alpha adrenergic receptor agonist phenylephrine had no effect, and beta receptor antagonist timolol blocked the reacidification induced by norepinephrine [6]. The non-specific adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA) and the A_{2A} adenosine receptor agonist CGS21680 reacidified lysosomes, while A₁ adenosine receptor agonists were not effective [6]. Dopamine agonists A68930, A77636 and SKF81297 all reacidified lysosomes in compromised RPE cells; siRNA identified the D5 dopamine receptor as mediating the

response [16]. SKF81297 was particularly effective and produced a sustained reacidification of at least 12 days.

Beta adrenergic receptors, A_{2A} adenosine receptors and D5 dopamine receptors are all linked to the G_s protein. As activation of G_s leads to elevation of cytoplasmic cAMP, the second messenger was implicated in the reacidification. This was confirmed when the direct elevation of cytoplasmic cAMP with membrane permeant cpt-cAMP reacidified lysosomes in treated cells [6]. Protein kinase A was also identified as involved in the pathway leading to lysosomal reacidification.

Lysosomal reacidification is partially dependent upon enhanced Cl⁻ influx into the lysosomes [17]. The entry of anions into the lysosomal lumen can minimize the change in electrical potential that accompanies an accumulation of protons, allowing a higher concentration of protons, and thus a lower pH, to be established. Specific activators of the Cl⁻ channel CFTR restored lysosomal pH. The effect of CFTR-associated drugs was larger in cells with alkalinized lysosomes, consistent with the pH dependence of channel selectivity. This suggests that the transport mechanisms underlying this receptor-mediated reacidification may be distinct from those that set the baseline pH levels of the lysosome. Of importance, this also implies that activation of the cAMP-dependent pathway may target damaged lysosomes with some degree of selectivity, with little effect on the healthy organelle.

It is important to stress that this approach was effective on RPE cells from older ABCA4^{-/-} mice. Direct activation of cAMP substantially reacidified lysosomes of RPE cells from 6 month old ABCA4^{-/-} mice [6], while dopamine D5 receptor agonists A68930, A77636 and SKF81297 lowered lysosomal pH in RPE cells from 11–12 month old ABCA4^{-/-} mice [16]. The A_{2A} agonist CGS21680 restored lysosomal acidity in cells treated with A2E for 4 weeks [6]. Together these findings imply that the cAMP pathway and receptors can function in damaged cells from older animals. This is critical, as the majority of patients with AMD are likely to have lysosomes that have been perturbed for many years; any putative treatment must be effective on cells that have been perturbed for an extended period.

In addition to the pharmacological approaches above, acidic nanoparticles have also been used to reacidify lysosomes from compromised RPE cells [15]. Poly DL-lactide spheres were rapidly taken up into the RPE cells and colocalized with lysosomes within 60 minutes. These acidic nanospheres reduced the pH in cells exposed to chloroquine when measured after 1 hr. Remarkably, lysosomal pH remained significantly acidified 12 days after one application of nanoparticles.

14.5 Functional effects of lysosomal reacidification

The lysosomal reacidification induced by the above treatments induces functional improvements in RPE cells. For example, exposure of RPE cells to photoreceptor outer segments for a week increased the lipofuscin-like autofluorescence, but treatment with the D5 dopamine receptor agonist SKF81297 completely prevented this. SKF 81297 also restored access to the cathepsin D binding sites, consistent with the improved degradation [16]. Stimulation of A_{2A} adenosine and beta adrenergic receptors, and activation of CFTR,

also enhanced the degradation of photoreceptor outer segments [6, 17]. Poly-DL lactide nanospheres restored access to the cathepsin D binding site and reduced the lipofuscin-like autofluorescence from outer segments. The nanospheres also reduced opsin levels by over 90%, confirming their role in degradation of outer segments. The ability of acidic nanoparticles to induce analogous enhancement in outer segment degradation clearly demonstrates that functional effects are downstream of lysosomal acidification and not due to non-specific actions of cAMP.

14.6 Summary

In conclusion, these studies have identified several different approaches to reacidify compromised lysosomes in RPE. Reacidification enhances the degradation of spent photoreceptor outer segments and lessens lipofuscin-like autofluorescence. This strategy potentially may have general benefit in prevention treatment of AMD at an early stage.

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Figure 1.