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Approaches for detecting lysosomal alkalinization and impaired degradation in fresh and cultured RPE cells: evidence for a role in retinal degenerations

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Abstract

Lysosomes contribute to a multitude of cellular processes, and the pH of the lysosomal lumen plays a central mechanistic role in many of these functions. In addition to controlling the rate of enzymatic degradation for material delivered through autophagic or phagocytotic pathways, lysosomal pH regulates events such as lysosomal fusion with autophagosomes and the release of lysosomal calcium into the cytoplasm. Disruption of either the steady state lysosomal pH or of the regulated manipulations to lysosomal pH may be pathological. For example, chloroquine elevates the lysosomal pH of retinal pigmented epithelial (RPE) cells and triggers a retinopathy characterized by the accumulation of lipofuscin-like material in both humans and animals. Compensatory responses to restore lysosomal pH are observed; new data illustrate that chronic chloroquine treatment increases mRNA expression of the lysosomal/autophagy master transcription factor TFEB and of the vesicular proton pump vHATPase in the RPE/choroid of mice. An elevated lysosomal pH with upregulation of TFEB and vHATPase resembles the pathology in fibroblasts of patients with mutant presenilin 1 (PS1), suggesting a common link between age-related macular degeneration (AMD) and Alzheimer's disease. While the absolute rise in pH is often small, elevations of only a few tenths of a pH unit can have a major impact on both lysosomal function and the accumulation of waste over decades. Accurate measurement of lysosomal pH can be complex, and imprecise measurements have clouded the field. Protocols to optimize pH measurement from fresh and cultured cells are discussed, and indirect measurements

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to confirm changes in lysosomal pH and degradative capacity are addressed. The ability of reacidifying treatments to restore degradative function confirms the central role of lysosomal pH in these functions and identifies potential approaches to treat diseases of accumulation like AMD and Alzheimer's disease. In summary, various approaches to determine lysosomal pH in fresh and cultured cells, as well as the potential to restore pH levels to an optimal range, can help identify and repair pathologies associated with lysosomal defects in RPE cells and perhaps also suggest new approaches to treat lysosomal storage diseases throughout the body.

Keywords

Retinal pigmented epithelium; lysosome; autophagy; Alzheimer's disease; lipofuscin; cathepsin D; aging

1. Chronic retinal degenerations, ion transport, autophagy and lysosomal pH

Lysosomes are best known for their acidity and their ability to degrade material. While this places them at a critical point in processing endogenous material delivered via the autophagic system in all cells, the prodigious phagocytosis of photoreceptor outer segment tips by RPE cells places an increased load on lysosomes of the RPE. In recent years it has become clear that lysosomal alkalinization may contribute to the pathologies in a number of chronic diseases and that reacidification of the lysosome is an important target for intervention (Appelqvist et al., 2013; Guha et al., 2014; Wolfe et al., 2013) (Fig. 1A). This review will present evidence for a pathological role for the chronic lysosomal alkalinization in RPE cells and will focus on different approaches to assess lysosomal pH in fresh and cultured cells.

1.1 Chronic lysosomal disruption and chloroquine retinopathy

Healthy lysosomes are acidic; the enzymes responsible for both degrading waste material and for storing signaling molecules function optimally at a pH of 4-5-5.0 (Barrett, 1977). The regulation of lysosomal pH is complex and dynamic, with the vHATPase proton pump, anion channels and transporters, cation channels, and membrane voltage all influencing the accumulation of protons in the lysosomal lumen (Mindell, 2012). These transport mechanisms are regulated by a network of factors including cellular energetics and trafficking rates (Cang et al., 2013; Lee et al., 2010). Within RPE cells, steady state activity of the vHATPase pump is regulated by a β-crystallin anchor and by mTOR (Valapala et al., 2014), while transient fluctuations in pH levels are regulated by a variety of plasma membrane receptors and second messengers (Guha et al., 2013; Guha et al., 2012; Guha et al., 2014).

The pH of RPE cell lysosomes can be pathologically elevated by several factors. The best known of these alkalinizing agents is chloroquine. Chloroquine has been used for over 50 years to treat malaria and autoimmune disorders like rheumatoid arthritis and lupus (Goldman et al., 1953; Rinehart et al., 1957). Reports of chloroquine retinopathy have been around for nearly as long as chloroquine itself (Ben-Zvi et al., 2012; Hobbs et al., 1959;

Lloyd and Hiltz, 1965; Shinjo et al., 2007; Walter, 1961). Chloroquine diffuses into acidic vesicles, becomes protonated, and gets trapped, thereby raising the pH (Homewood et al., 1972). The lysosomes of RPE cells are particularly susceptible to chloroquine because chloroquine has an affinity for pigmented cells and is retained in RPE lysosomes long after drug treatment has stopped (Bernstein et al., 1963). This affinity, combined with the high degradative load of RPE cells, leads to considerable damage to RPE cells and, secondarily, to the photoreceptors.

Chloroquine retinopathy shares parallels with other retinal degenerations. Treatment of patients with chloroquine led to central visual loss and macular cone dysfunction, pigment changes and Bull's eye maculopathy, in which RPE cells are lost in an expanding circle of hyperfluorescence (Kellner et al., 2006; Michaelides et al., 2011; Shinjo et al., 2007). Bull's eye maculopathy has also been reported in patients with mutations in the retinoid flipase ABCA4, a mutation associated with the early onset retinal degeneration in Stargardt's disease (Michaelides et al., 2007), and has some similarities with geographic atrophy, in that RPE cells are lost in an expanding ring. Interestingly, only ~7% of patients receiving chloroquine treatment display retinopathy (Scherbel et al., 1965), suggesting an additional factor, perhaps genetic makeup, predisposes some patients to an exacerbated loss of vision in response to lysosomal alkalinization.

Animal models of chloroquine retinopathy also show RPE damage and have proven useful in understanding the morphological changes induced by alkalinization of the RPE lysosomes. Chronic treatment of primates with chloroquine led to lipid accumulations in the RPE, a thickened basement membrane with collagen fibrils, and increased choroidal macrophages (Rosenthal et al., 1978). In cats, extended chloroquine treatment led to RPE hypertrophy followed by loss of photoreceptors (Meier-Ruge, 1965). In rats, chloroquine led to an accumulation of lysosomal-associated organelles in RPE cells and to lipid deposits throughout Bruch's membrane (Ivanina et al., 1983; Peters et al., 2006). The more pronounced pathologies seen with chloroquine, as compared to analogue hydroxychloroquine, were attributed to the greater effect of chloroquine on lysosomal alkalinization (Mahon et al., 2004; Sundelin and Terman, 2002). This provides additional support for the role of lysosomal alkalinization in chloroquine retinopathy. A chronic elevation of lysosomal pH may induce both detrimental and protective changes, and compensatory changes in gene expression may occur. We report here that the RPE/choroid of mice treated with chloroquine for 3 weeks had an increased expression of mRNA for the vesicular proton pump vHATPase, and the master lysosomal/autophagy transcription factor TFEB (Fig. 1B). These rises in gene expression parallel those detected in fibroblasts from patients with a mutation in presenilin 1 (PS1) associated with elevated lysosomal pH (Coffey et al., 2014; Lee et al., 2010), and suggest that cellular attempts to compensate for chronic lysosomal alkalinization are a general phenomenon. We hypothesize that the cellular damage is more pronounced when these endogenous compensations to reacidify lysosomes are attenuated by age or secondary mutations.

1.2 Acute lysosomal disruption

Acute disruption of lysosomes can lead to more cataclysmic responses. At high concentrations, chloroquine can perturb fusion of autophagic vesicles, increase levels of LC3-II and p62, and even lead to death of ARPE-19 cells (Chen et al., 2011; Yoon et al., 2010). Direct disruption of the RPE lysosome leads to activation of the inflammasome (Tseng et al., 2013), and has been implicated in inflammasome activation by retinoid byproduct N-retinylidene-N-retinylethanolamine (A2-E) (Anderson et al., 2013). These acute responses clearly have a role to play in sudden death of RPE cells at the hands of experimentalists *in vitro*, and may even contribute to the end-stage death seen in geographic atrophy.

In contrast to these acute responses, most aging diseases represent moderate changes in cell physiology that build up over many years, eventually contributing to a cascade of pathological events. We believe that moderate elevations of lysosomal pH represent a common step in many disorders of aging, including age-related macular degeneration and Alzheimer's disease. While elevation of lysosomal pH may be a primary defect in some cases, this alkalinization may also be a side effect of waste accumulation in other lysosomal storage diseases. As RPE cells filled with lipofuscin represent a type of lysosomal storage disease, any alkalinizing effects of lipofuscin on lysosomal pH would exacerbate accumulation, leading to a "snowball" effect (Fig. 1C). As such, repairing lysosomal pH is predicted to reduce the storage disorder regardless of the initial cause.

1.3 Pharmacological alkalinization of RPE lysosomes

Chloroquine is a reliable method for inducing chronic lysosomal alkalinization in RPE cells; the decades of documented chloroquine retinopathy in patients, combined with the propensity of chloroquine to accumulate in pigmented cells, makes it an ideal drug to examine the consequences of lysosomal alkalinization of RPE cells (Bernstein et al., 1963; Hobbs and Calnan, 1958). Lysosomal alkalinizing agents other than chloroquine lack the attraction to pigment and as such are less specific for RPE cells, with profoundly detrimental effects observed when given systemically. For example, systemic addition of NH_4^+ induces coma or metabolic acidosis (Nowik et al., 2010). Intravitreal injection may provide a compromise, however; the injection of vHATPase-inhibitor bafilomycin into the rat vitreous led to the accumulation of opsin-loaded phagolysosomes (Deguchi et al., 1994). The similar pathologies observed after chloroquine treatment and intravitreal bafilomycin injections imply that lysosomal alkalinization is itself sufficient to induce an accumulation of lipofuscin-like material.

Given the advantages of using chloroquine to alkalinize RPE lysosomes in animal models, the drug is an obvious choice for in vitro experiments. Low concentrations can stably alkalinize lysosomes for 10 days without inducing cell death (Baltazar et al., 2012). However, the central role of lysosomal alkalinization is best determined when the effects of multiple agents are compared. For example, the ability of both the vHATPase inhibitor bafilomycin and the retinoid A2-E to inhibit intra-lysosomal accumulation of methylamine was attributed to lysosomal alkalinization by A2-E following vHATPase inhibition (Bergmann et al., 2004). Likewise, the ability of both NH_4^+ and A2-E to reduce

photoreceptor degradation in vitro was also attributed to lysosomal alkalinization (Bergmann et al., 2004). Both NH_4^+ and bafilomycin were used to alkalinize RPE lysosomes in vitro to examine interactions with the proteasome (Ryhanen et al., 2009). While chloroquine, NH_4^+, bafilomycin and tamoxifen were all capable of acute lysosomal alkalinization in our hands, tamoxifen was the most reliable and rapid, and showed the best dose-response curve (Liu et al., 2008), although tamoxifen had slightly different effects on lysosomal enzyme activity than chloroquine in pig RPE cells (Toimela et al., 1998). Of note, the alkalinizing actions of tamoxifen are unrelated to the estrogen receptor but attributed to actions specifically on the lysosomal membrane (Altan et al., 1999; Chen et al., 1999).

1.4 Genetic links between lysosomal pH and macular degeneration

It is likely that most common forms of AMD in elderly patients result from the interaction between environmental insults and genetic predisposition; age-dependent changes in the inflammatory response and chemical modulations from time-dependent accumulations are unlikely to be fully recapitulated in patients with mutations that directly elevate lysosomal pH as systemic issues would predominate. While mutations in transporters controlling lysosomal pH are not among the recognized genetic risk factors for AMD, this probably has more to do with the central role such transporters have in cellular function throughout the body than the lack of a role for lysosomal pH in the disease. The overall absence of diseases caused by mutations in transporters controlling lysosomal pH suggests that such mutations may be embryonic lethal.

The loss of certain genes associated with lysosomal ion transport is linked to retinal degenerations in mice, however. For example, the ClC-7 gene codes for a lysosomal Cl^-/proton exchanger that is expressed in the RPE; mice missing this gene display retinal and neural degeneration that resembles neuronal ceroid lipidosis (Kasper et al., 2005; Kornak et al., 2001). Recent work stresses the central role of the transporter in regulating lysosomal pH (Ishida et al., 2013), although older work could not detect a pH change (Kornak et al., 2001).

Lysosomal pH may be elevated secondarily to an imbalance of lysosomal lipids. In Gaucher's disease, the accumulation of cholesterol was associated with a secondary elevation in lysosomal pH (Sillence, 2013). Interestingly, reducing glucosylceramide synthase either chemically (Sillence, 2013) or molecularly (van der Poel et al., 2011) also alkalinized lysosomes, with the latter study suggesting glucosylceramide stimulates the vHATPase pump. This suggests that optimal levels of the protol pump require a balanced intermediate level of glucosylceramide.

The theory that activity of lysosomal transporters is modulated by levels of lysosomal lipids is supported by patch clamp studies showing sphingomyelin dramatically inhibits current through the lysosomal cation channel TRPML1 (Shen et al., 2012). In RPE cells with reduced TRPML1 levels, Fe^{2+} exposure leads to an increase in reactive oxygen species and to mitochondrial damage (Coblentz et al., 2014); given the accumulation of lipid-rich lipofuscin in aging RPE cells (Sparrow and Boulton, 2005) and the likelihood of Fe^{3+} imbalance in macular degeneration (Song and Dunaief, 2013), it is possible that lipid-mediated modulation of lysosomal transporters may alter lysosomal pH in disease.

1.5. Autophagy, proteasomes and other lysosomal functions

Lysosomes were traditionally thought of as just the “garbage disposals” of the cell, but research over the past few years has identified lysosomal involvement in a large number of cell functions, with lysosomal pH a key component to these functions (Settembre et al., 2013). In some cell types, lysosomes act as storage sites for signaling molecules like ATP and cytokines, and can influence lipid oxidation (Dou et al., 2012; Pivtoraiko et al., 2009; Stanley and Lacy, 2010; Zhang et al., 2007). Investigations to determine whether lysosomes serve similar functions in RPE cells are underway.

The actions of lysosomes and proteasomes can be interrelated in RPE cells. For example, inhibition of the proteasome by MG-132 increases the number of LAMP2-stained, and enhances autophagic clearance of protein (Ryhanen et al., 2009; Viiri et al., 2013). While proteasomes may help clear excess protein upon lysosomal alkalinization, the lack of a similar backup mechanism to clear lipids may explain the predominantly lipid composition of the waste lipofuscin retained in defunct lysosomes.

Degradation of material by lysosomal enzymes can be thought of as an end-stage step in the autophagic process. Autophagy plays a central role in the health of ocular cells and defective autophagy has been recently implicated in age-related macular degeneration, photoreceptor degradation and the visual cycle (Frost et al., 2014; Kaarniranta et al., 2013; Kim et al., 2013; Mitter et al., 2012). The elevation of lysosomal pH interferes with upstream steps in autophagy and impairs the fusion of autophagosomes with lysosomes (Kawai et al., 2007). As such, lysosomal pH is a key determinant in autophagic clearance and we propose that treatment to reacidify lysosomes may enhance autophagy in compromised cells, regardless of the cause of the initial defect.

2. Measurement of lysosomal pH

2.1 Complexities in the measurement of lysosomal pH

While it is clear that elevating the lysosomal pH of RPE cells can lead to an AMD-like damage, the accurate measurement of lysosomal pH is complex. The accumulation of H^+ inside lysosomes at high concentrations requires the active pumping of an ATPase enzyme, so gradients are rapidly dissipated in dying cells, making accurate pH measurement a challenge as free protons can be hard to maintain in fixed sections. This favors the assessment of lysosomal pH in living cells, but even in live cells, the lack of an ideal reporter probe complicates measurements. For example, the LysoTracker dye readily diffuses into acidic organelles and is useful to show colocalization with compounds in lysosomes, such as the demonstration that the lipofuscin component A2-E is localized to the lysosomes of RPE cells (Sparrow et al., 1999). While the lack of LysoTracker staining can be used to demonstrate relatively large elevations in pH (Avrahami et al., 2013), LysoTracker is a single wavelength dye and therefore changes in the amount of emitted light can arise either from an elevation in pH or from a change in the number and/or area of the lysosomes. As lysosomes can change size in response to numerous signals (Bakker et al., 1997), the LysoTracker output can be misleading. We have recently found the LysoTracker signal indistinguishable in control fibroblasts and those with the PS1 mutation linked to

familial Alzheimer's disease even though lysosomes in the mutant fibroblasts were significantly alkalinized by 0.25 units (Coffey et al., 2014). Overall we feel LysoTracker is not a particularly sensitive probe for determining small changes in lysosomal pH, although it remains an excellent tool for localizing components to acidic vesicles and for detecting larger shifts in pH.

Dextran-linked rhodamine sensors are another tool used to assess pH, but come with their own set of complications. As the large dexran groups encourage endocytosis, dextran-rhodamine probes are ideal for measuring phagocytosis, but the pK_a of rhodamine means that the signal is largely saturated at pH 5.0–5.5, making the probes of limited use in the accurate measurement of lysosomal pH near 4.5; small pH elevations from this baseline can consequently go undetected. The pH_{Rodo} probe is one example of such a tool that provides excellent reporting of pH in endosomes but loses sensitivity once the pH falls below 5.0 (Life Technologies; Molecular Probes handbook Figure 20.3.3); the dextran-rhodamine probes are far from ideal when it comes to detecting small differences in lysosomal pH levels.

Fluorescein isothiocyanate (FITC)-based sensors suffer from a different problem, as the dye has a pK_a of 6.3–6.7. While the FITC dye has been used for pH measurements in lysosomes, a shift in pH from 5.0 to 4.5 reduced fluorescence output by only ~2%, from 5% to 3% of maximum levels (Geisow, 1984), making this dye less than ideal for the pH range found in healthy lysosomes. This is of particular concern given the number of experiments in which photoreceptor outer segments loaded with FITC were visualized as they progressed through the phagocytotic and endocytotic pathways of RPE cells. Disappearance of the FITC signal in acidic environments is a physical characteristic of the dye, and should not be confused with photoreceptor degradation. We have found that the pH-insensitive dye calcein provides a reasonable substitute (Liu et al., 2008).

One alternative to directly labeling the material to be phagocytosed is to tag pH-sensitive fluorescent reporters to dextran spheres that are themselves labeled with a fluorophore of a second color. These dual-tagged probes offer an ideal way to study the phagocytotic and endocytotic pathways. However, dextran-conjugated probes can only report from organelles actively involved in the endocytosis process. Compromised lysosomes in RPE cells with elevated pH are less likely to participate in fusion with freshly endocytosed dyes than healthy lysosomes (Chen et al., 2011). The pH readout from the dextran-conjugated reporters will thus be shifted to more acidic levels and the probe may miss the very lysosomes that are most problematic. This of course is fine for protocols in which the pH is manipulated exogenously, but in measurements to determine lysosomal pH from diseased or older cells with heterogeneous populations of lysosomes, the use of a dextran-conjugated probe can underestimate the degree of lysosomal alkalinization.

2.2. Optimization of protocols for the use of LysoSensor Yellow/Blue DND 160

The LysoSensor Yellow/Blue DND 160 (subsequently referred to as LysoSensor Yellow/Blue) offers some advantages for pH measurement, but like the other probes, it is far from ideal. In our hands it only provides a reliable output when a complex series of controls are employed. LysoSensor Yellow/Blue is ratiometric, so the ratio of light excited at 340 nm to

380 nm and emitted at >527 nm is proportional to lysosomal pH. Like the ratiometric calcium reporter Fura-2, the ratiometric aspect of LysoSensor Yellow/Blue means that the signal is independent of dye concentration, and thus output should not greatly affected by changes in lysosomal size. Importantly, the dye is particularly sensitive over acidic pH values, making it a preferable alternative to rhodamine or FITC probes. However, LysoSensor Yellow/Blue is not particularly stable and can itself modulate pH if retained for an extended period of time. To control for this, we have performed a series of measurements to optimize parameters like loading time, concentration and post-loading interval (Fig. 2A). We have found that in cultured ARPE-19 cells, optimal results are obtained with 2–5 μM dye loaded for 3 min with measurements made 15–19 min after dye removal (Guha et al., 2012; Liu et al., 2012). For reference, best results were obtained from cultured human fibroblasts when they were incubated with 2 μM LysoSensor Yellow/Blue for 3 min and measurements made 12–14 min later (Coffey et al., 2014). Investigators are encouraged to determine optimum conditions for their own cell types.

Confidence in results from the LysoSensor Yellow/Blue probe is greatest for comparisons between simultaneous measurements; this allows any time- or dye-dependent shifts to be controlled for. While output from a microscope-based platform gives reasonable qualitative output and provides satisfying images, we have found it difficult to accurately compare the absolute responses between different preparations using a microscope. As such, we perform all quantitative measurements on a plate reader using 96- or 384-well plates. The dye is loaded and removed from all cells almost simultaneously, and the distribution of different cells and/or drug conditions is randomized across the plate to further minimize bias. Repetitive sweeps of the plate and alternating excitation at 340nm and 380nm provide measurements.

While the changes in fluorescent ratio provided by this protocol can be reliably compared to others within the plate, baseline levels for each plate can differ. To compensate for this difference, ratios across plates can be normalized to a control value in each plate, or can be calibrated. This calibration is somewhat analogous to that used for the calcium reporter Fura-2, for which the cell is bathed in solutions of various calcium concentrations and a selectively permeant ionophore enables clamping of the intracellular calcium concentration to the extracellular solution. However, calibrating the ion concentration of intracellular vesicles is topographically more complex as the concentration must be matched across two sets of membranes (Fig. 2B). To calibrate lysosomal pH, wells are perfused with a series of solutions composed of 20 mM 2-(N-morpholino)ethane sulfonic acid (MES), 110 mM KCl, and 20 mM NaCl and adjusted to pH 4.0 to 6.0 with HCl/NaOH; the intravesicular pH is then clamped using the H^+/Na^+ ionophore/transporter monensin and the H^+/K^+ ionophore/transporter nigericin, as reported (Lin et al., 2003). Calibration measurements are made simultaneously with the pH measurements in adjacent wells, ensuring that any time or dye-dependent effect is controlled for. While the relationship between the ratio of light emitted at 340/380 nm and pH is usually linear over the range examined in RPE cells (Liu et al., 2008), the calibration is subject to numerous issues involved in delivering the ionophore/transporters to both cytoplasmic and vesicular membranes and is not always reliable. In addition, the need for designated wells can be limiting when cell number is low, such as

with freshly isolated RPE cells. As such, quantification is often performed on ratios with calibration performed only on an example (Coffey et al., 2014).

2.3. Modifications for lysosomal pH measurements from freshly isolated RPE cells and primary cultures

While indirect measures of pH are possible in fixed cells or *in vivo*, we have found it difficult to accurately measure lysosomal pH levels in RPE cells *in vivo*. As the most accurate comparisons of lysosomal pH are performed simultaneously on a plate reader, cultured ARPE-19 cells have been used for the majority of the experiments as they can be produced in sufficient number for screening. However, results are always confirmed in primary RPE cultures or freshly isolated cells. This is particularly true for experiments in which the status of RPE lysosomes in their endogenous state is being investigated, such as in the presence of the ABCA4$^{-/-}$ mutation and aging. In such cases, *it is critical that lysosomal pH be measured only from freshly isolated RPE cells*; cells that have divided, even once, should not be used.

When freshly isolated RPE cells are used, several adjustments to the protocol are necessary. The best results with mouse RPE cells were obtained when cells were incubated in 2–5μM LysoSensor Yellow/Blue for 5 min. Because of the reduced number of cells, and therefore reduced overall signal, available from a pair of mouse eyes, cells are distributed into the smaller volume wells of 384-well plates and drugs added ex vivo. The use of plates made of plastic with low excitation at 340 nm, such as the UV Star from Greiner Bio-One, improves the signal/noise. It should be noted that while the RPE cells from ABCA4$^{-/-}$ mice are themselves autofluorescent, the signal from the LysoSensor Yellow/Blue dye was ~100 fold greater (Liu et al., 2008). This dye-to background signal should be checked for each preparation, however.

The measurement of lysosomal pH from RPE cells of the ABCA4$^{-/-}$ mouse highlights several key issues in the measurement of lysosomal pH from fresh cells. In this model, the primary defect is found in the photoreceptors, and for the consequences of the mutation to impact the RPE cells, the two cell types must be adjacent (Weng et al., 1999). The delivery of excess A2-PE from photoreceptors to RPE cells is required for the production of A2-E by RPE cells and, presumably, the subsequent damage to the lysosomes. As such, even a single cell division by primary RPE cells in culture will result in the production of new lysosomes, untainted by the A2-E as they have been removed from their association with the mutant photoreceptors. In these new lysosomes from daughter cells, the altered phenotype is no longer present, or at best the magnitude of the defect is diluted out. In experiments comparing lysosomal pH in RPE cells in ABCA4$^{-/-}$ versus control mice (Liu et al., 2008), measurements are always performed from freshly isolated RPE cells. Likewise, in trials demonstrating that activators of the lysosomal chloride channel CFTR (Liu et al., 2012) or agonists for the D5 dopamine receptor (Guha et al., 2012) were capable of restoring lysosomal pH, measurements were always performed on RPE cells that had been removed from the eye of ABCA4$^{-/-}$ mice less than 3 hrs previously. Preliminary findings suggesting an age-dependent elevation in lysosomal pH were likewise performed on freshly isolated RPE cells that had not yet divided (Mitchell and Laties, 2012).

With regards to the age of the cultures, it may be relevant that lysosomal pH measurement of control and mutant PS1 fibroblasts was most consistent on cells grown for at least 6 days on their substrate (Coffey et al., 2014). While a detailed analysis of culture duration and response has yet to be performed for RPE cells, the “snowballing” hypothesis illustrated in Figure 1C implies that some time is needed to accumulate waste material before the lysosomal pH is altered. This requirement for some delay is also consistent with the findings that 24hr exposure of RPE cells to the retinoid A2-E had no immediate effect on lysosomal pH (Lakkaraju et al., 2007; Liu et al., 2008) but that three weeks of exposure to A2-E did elevate the lysosomal pH (Holz et al., 1999; Liu et al., 2008). The alkalinizing effect of a three-week nextended exposure to A2-E has recently been confirmed (Poliakov et al., 2014). These observations are also consistent with the age-dependent lysosomal alkalinization found in ABCA4$^{-/-}$ mice (Liu et al., 2008). The mechanisms by which A2-E associated accumulations lead to lysosomal alkalinization are currently being investigated.

3. Indirect approaches to support the detection of lysosomal alkalinization

3.1. Assessment of cathepsin D activity

Given that the direct measurement of lysosomal pH is complex and is best performed in live cells, additional support for pH disruption is both necessary and informative. While degradation, detailed in the section below, is perhaps the most relevant assay, additional protocols can provide support for the detection of defective lysosomes and some can be translated to the *in vivo* condition more readily than direct measurement of lysosomal pH.

The assays used most effectively in our laboratory involve the lysosomal protease cathepsin D. The maturation of cathepsin D is pH-sensitive, as catalytic enzymes require an acidic milieu for effective cleavage of pro forms into active forms (Richo and Conner, 1994). Western blotting has confirmed that the ratio of mature to pro-cathepsin isoforms to immature pro forms is greater in cells with an acidic lysosome than in those in which the lysosomal pH is chronically alkalinized (Coffey et al., 2014). As this approach uses standard immunoblots, it has the advantage that it can be performed from preserved tissue and does not require live cells.

The BODIPY FL-pepstatin A assay provides a similar output from live cells. Not only is the production of mature cathepsin D dependent upon an acidic lumen, but the protease activity is also optimal at an acidic pH, with degradative activity decreasing by 80% when the pH rises from 4.5 to 5.3 (Barrett, 1977). Access to the binding site can be measured with fluorescent BODIPY FL-pepstatin A; the fluorescent signal is greatly increased when pH falls to 4.5 (Chen et al., 2000). In ARPE-19 cells, the fluorescent signal of BODIPY FL-pepstatin A is greater under control conditions than in cells treated with chloroquine to raise lysosomal pH (Baltazar et al., 2012). Likewise, stimulation of the $P2X_7$ receptor increased lysosomal pH, and reduced the BODIPY FL-pepstatin A signal (Guha et al., 2013). Again, human cells with mutant PS1 show decreased BODIPY FL-pepstatin A staining compared to control, consistent with their elevated lysosomal pH (Coffey et al., 2014)., It should be kept in mind that under chronically pH elevation, a loss of Bodipy pepstatin A fluorescence can result from either a decrease in the amount of mature cathepsin D or a decrease in the pH-dependent access to the binding site; both factors will sum.

Standard biochemical measures of lysosomal enzyme activity should be approached with caution, as most of these kits and assays measure enzyme activity in a pre-made solution of fixed pH. This will prevent the detection of any change in enzyme activity caused solely by a shift in lysosomal pH. This may explain why addition of A2-E had no direct effect on the activity of lysosomal enzymes when tested in lysed suspensions (Bermann et al., 2001); indirect effects on enzyme activity arising from its ability to raise lysosomal pH would be missed by this approach. Of course, for enzymes like cathepsin D where acidity is needed for enzyme maturation in addition to direct activity, such measurements may detect evidence for chronic alkalinization. A fluorometric assay was recently used to demonstrate a decline in cathepsin D activity in mice missing the *Cryba1* gene, a defect that led to lysosomal alkalinization (Valapala et al., 2014); presumably levels of active enzyme were reduced by the chronic rise in lysosomal pH.

3.2. Lysosomal alkalinization and degradation

Lysosomal alkalinization can damage RPE cells by impairing degradation; the lysosomal enzymes responsible for degrading ingested outer segments and autophagic material are impaired by alkalinization. As mentioned, cathepsin D activity decreases dramatically with a modest rise in lysosomal pH(Barrett, 1977). Because cathepsin D is the enzyme primarily responsible for the breakdown of opsin (Feeney-Burns et al., 1987), a moderate lysosomal alkalinization can slow outer segment clearance. RPE cells also have a large load of photoreceptor-derived lipids to degrade, and the activity of lysosomal lipases needed to handle this lipid burden is also very pH-dependent, with a 75% drop in the ability to degrade triglycerides observed when pH goes from 4.8 to 5.4 (Ameis et al., 1994).

Graphic evidence that lysosomal alkalinization interferes with outer segment clearance is shown by electron microscopy showing the accumulation of photoreceptor outer segment debris in RPE cells of rats treated with chloroquine (Peters et al., 2006). However, *in vitro* assays can be more quantitative while also isolating the effect of lysosomal pH changes more directly. Monitoring the disappearance of fluorescently labeled outer segments has been performed for many years. However, the pH dependence of FITC makes the classic techniques of labeling outer segments with FITC problematic, as discussed above. Labeling outer segments with the pH-insensitive dye calcein avoids this issue; elevating lysosomal pH led to an increased retention of calcein fluorescence, while treatment to restore acidity reduced the calcein fluorescence (Liu et al., 2012; Liu et al., 2008).

The lipofuscin-like autofluorescence of incorrectly processed photoreceptor outer segments also provide a reasonable measure by which clearance can be quantified. Detection is improved by using a flow cytometer and repeated feeding of cultured cells for at least a week with isolated outer segments. This outer segment-derived autofluorescence co-localizes with lysosomes, consistent with accumulation of partially digested remains in lysosome-like organelles (Guha et al., 2012). The autofluorescence of cultured RPE cells showed a small increase after treatment with chloroquine alone, suggesting that lysosomal alkalinization increased an autofluorescent signal arising from the autophagic degradation of endogenous RPE material (Baltazar et al., 2012; Guha et al., 2013). The addition of outer segments and chloroquine together to cultured RPE cells increased autofluorescence

substantially. Critically, this autofluorescence decreases when cells are treated with agents to reacidify lysosomal pH, such as D5 dopamine receptor agonists or acid nanoparticles (Baltazar et al., 2012; Guha et al., 2012). This implies that the impaired degradation was due to a rise in lysosomal pH. A similar reduction in autofluorescence was observed when lysosomal pH was reacidified with the $P2X_7$ receptor antagonist BBG (Guha et al., 2013). Together these experiments demonstrate that increased clearance of lipofuscin-like autofluorescence is a general response to lysosomal acidification and that drugs to reacidify lysosomes have promise as treatment for diseases of accumulation like AMD.

While levels of an added fluorescent label like calcein to outer segments, or detection of autofluorescence levels provides an index of turnover, it is important to confirm that the accumulated material being measured is of outer segment origin. In this regard, measuring the protein opsin with immunoblots provides a specific assay of outer segment degradation, as the protein is derived only from added outer segments. Treatment of ARPE-19 cells with outer segments over 7 days dramatically increased the 40 kDa band of opsin; the ability of acid nanoparticles to reduce this opsin band by over 90% provided a clear support for the theory that lowering lysosomal pH improves the degradation of photoreceptor outer segments by RPE cells and reduces lipofuscin (Baltazar et al., 2012).

4. Summary

In summary, increasing evidence implicates lysosomal alkalinization in diseases of accumulation such as AMD and Alzheimer's disease. For example, raising lysosomal pH increases markers associated with retinal degeneration, stimuli associated with RPE damage elevate lysosomal pH, and lysosomal alkalinization alone leads to a macular degeneration. Perhaps most importantly, reacidifying lysosomes with drugs or nanoparticles can reverse signs of retinal degeneration. The complexities in accurately measuring lysosomal pH and degradation may explain some of the controversy over the relative contribution of defective lysosomal pH to AMD and Alzheimer's disease. These complexities also stress the need for numerous complementary approaches when evaluating the presence of a perturbed lysosomal pH or of potential treatments. The preference for live cells when performing accurate measurements highlights a complementary role for both cultured and freshly isolated cells. Analysis of enzymes like cathepsin D can provide an indirect support that can be performed on protein samples from fixed tissue. The ability of chloroquine to induce maculopathy provides *in vivo* support for a role for lysosomal alkalinization, while analysis of mRNA from mice treated with chloroquine assigns modern markers to this classic model. The analysis of lysosomal degradation from RPE cells is facilitated by their uptake of exogenous photoreceptor outer segments; the effect of lysosomal pH on the processing and turnover of photoreceptor outer segments can be monitored by using a pH-insensitive label such as calcein-AM, by quantifying the autofluorescence present after prolonged exposure to outer segments, or by probing immunoblots for a specific marker like opsin. The convergence of all these approaches from cultured, fresh and *in situ* material provides compelling evidence that chronic alkalinization of the lysosomal lumen is a major issue for RPE cells. Given the emerging role for lysosomes in the storage and release of cellular signals, additional pathways impacted by lysosomal alkalinization are sure to emerge. Our

ability to restore an acidic lysosomal pH with a number of treatments may help repair many of these functions.

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Highlights

- Evidence for contribution of RPE lysosomal alkalinization in retinal degenerations
- Elevated expression of TcFEB and vHATPase in RPE cells in vivo after chronic lysosomal alkalinization with chloroquine
- Advantages and optimization of different techniques for measuring lysosomal pH
- Detection of secondary markers of lysosomal alkalinization in fresh and fixed tissue
- Linkage between lysosomal alkalinization, impaired degradation and lipofuscin-like autofluorescence

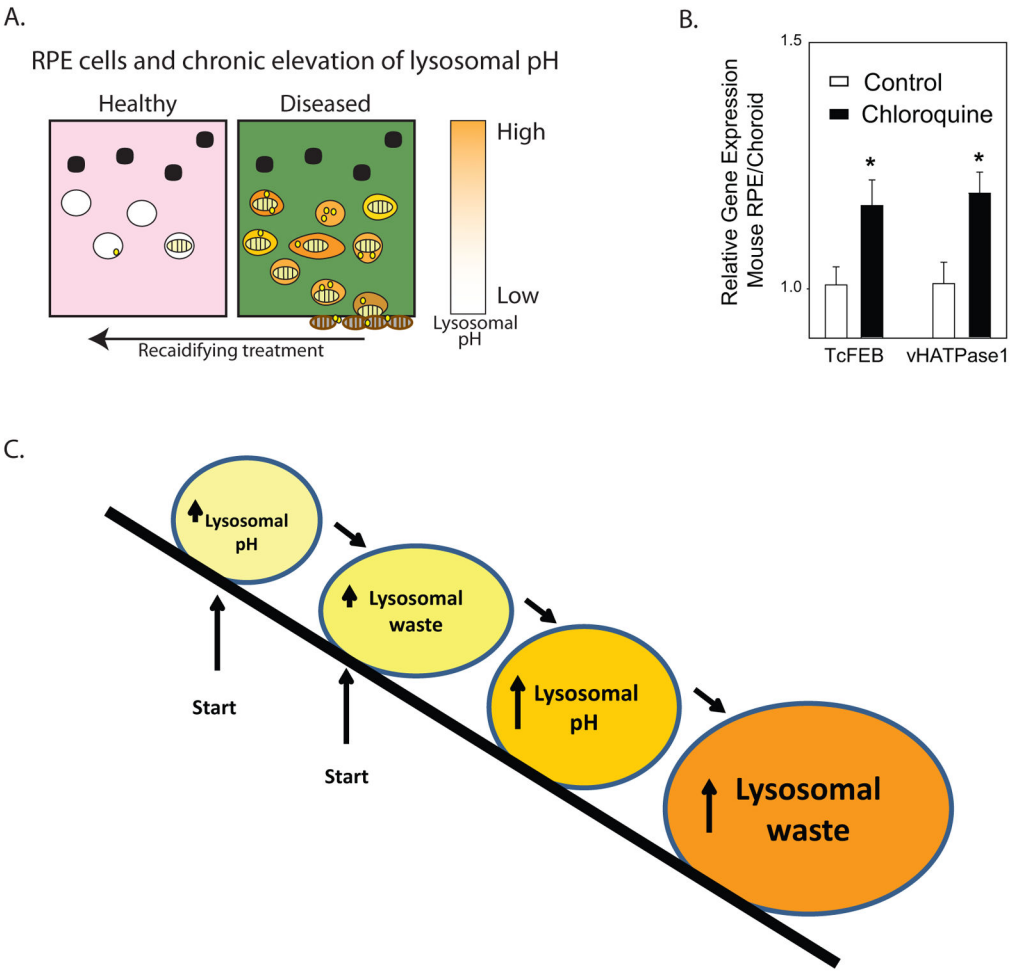

Figure 1. Lysosomal pH and accumulation of outer segment material

A. Model of predicted effects of chronic lysosomal alkalinization on RPE cell function. In healthy RPE cells, the acid pH of the lysosomes enables efficient degradation of phagocytosed photoreceptor outer segments (striped ovals) in addition to autophagic material (yellow spheres). An elevation of lysosomal pH in diseased RPE cells impedes the enzymatic clearance of both outer segment material and autophagosomes, leading to cellular accumulation and extrusion of improperly degraded material. Treatment to reacidify lysosomes can improve clearance of outer segment material. B. Mice injected I.P. with 50 mg/kg chloroquine 3x per week for 4–6 weeks showed a compensatory increase in mRNA for the lysosomal/autohpagy transcription factor TcFEB and the lysosomal proton pump vHATPase (lysosomal V1 subunit B1, gene Atp6v1b1), as determined using the qPCR reaction. n=11–12, * $p<0.05$, all animals treated according to approved protocols. C. The “Snowballing” model of lysosomal alkalinization, in which an elevation in lysosomal pH leads to the accumulation of oxidized lipids which in turn leads to an elevation on lysosomal pH etc. The initial event can be either lysosomal acidification or lysosomal storage.

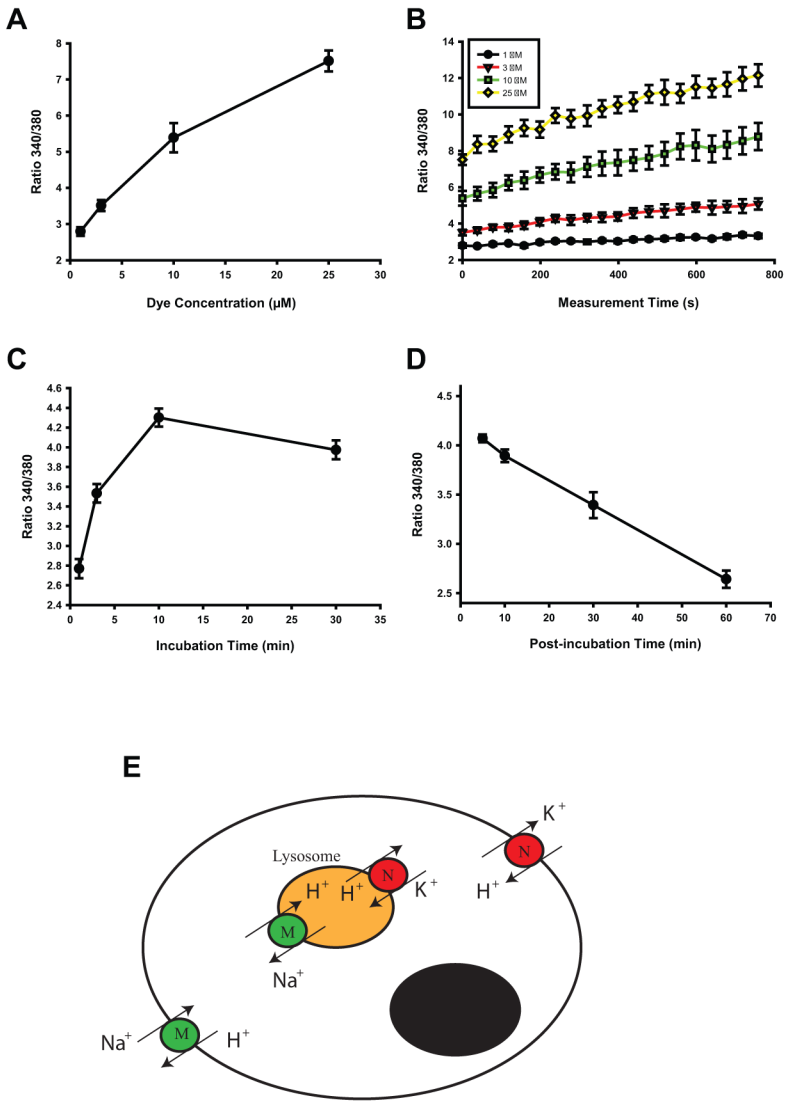

Figure 2.

Technical challenges in optimizing the measurement of lysosomal pH.

A–D. Optimization of the parameters for measuring lysosomal pH in ARPE-19 cells with LysoSensor Yellow/Blue showing the effect of dye concentration on (A) the strength of the signal emitted at >500 nM, defined as the ratio of light excited at 340 and 380 nm, (B) the rate of change in the signal; the effect of incubation time on the signal size and (D) the decay of the signal (C) the raand emitted (A) E. Model of calibration using dual ionophores/ transporters monensin (M) and nigericin (N) illustrating the complexities of calibrating across two sets of membranes.