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Biphasic regulation of lysosomal exocytosis by oxidative stress

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Abstract

Oxidative stress drives cell death in a number of diseases including ischemic stroke and neurodegenerative diseases. A better understanding of how cells recover from oxidative stress is likely to lead to better treatments for stroke and other diseases. The recent evidence obtained in several models ties the process of lysosomal exocytosis to the clearance of protein aggregates and toxic metals. The mechanisms that regulate lysosomal exocytosis, under normal or pathological conditions, are only beginning to emerge. Here we provide evidence for the biphasic effect of oxidative stress on lysosomal exocytosis. Lysosomal exocytosis was measured using the extracellular levels of the lysosomal enzyme beta-hexosaminidase (β -hex). Low levels of oxidative stress stimulated lysosomal exocytosis, but inhibited it at high levels. Deletion of the lysosomal ion channel TRPML1 eliminated the stimulatory effect of low levels of oxidative stress. The inhibitory effects of oxidative stress appear to target the component of lysosomal exocytosis that is driven by extracellular Ca^{2+} . We propose that while moderate oxidative stress promotes cellular repair by stimulating lysosomal exocytosis, at high levels oxidative stress has a dual pathological effect: it directly causes cell damage and impairs damage repair by inhibiting lysosomal exocytosis. Harnessing these adaptive mechanisms may point to pharmacological interventions for diseases involving oxidative proteotoxicity or metal toxicity.

Graphical Abstract

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Co-author Approval Statement

- Publication of this manuscript has been approved by all authors.

Conflict of Interest Statement

The authors declare no conflicting interests.

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Keywords

Lysosomes; exocytosis; calcium; TRPML1; oxidative stress

1. Introduction

Production of reactive oxygen species (ROS) normally accompanies mitochondrial and cytoplasmic enzymatic activities [1, 2]. It can be accelerated by mitochondrial damage and by toxic compounds that promote ROS production [2]. The cellular defense system comprising catalases and superoxide dismutases effectively fights ROS at near-physiological conditions [3–8]. However, a significant imbalance towards the ROS accumulation leads to oxidative stress and to organellar and cytoplasmic damage.

Oxidative stress causes lipid peroxidation [9–11], and the ensuing loss of membrane integrity damages membrane organelles. The injured mitochondria and lysosomes accelerate the damage by stimulating ROS production and by leaking the digestive enzymes and pro-apoptotic factors [12–18]. The repair of oxidative damage necessitates a rapid elimination of the damaged organelles by autophagy, which depends upon proper lysosomal function [19–24]. Oxidative stress damages the cytoplasmic proteins as well, leading to protein aggregation. Elimination of such aggregates depends on autophagy, which, in turn, depends on lysosomes as well.

The endocytic/autophagic/lysosomal roles in cell health are not limited to degradation, but also involve the capture and rapid evacuation of organelles and cytoplasmic compartments. Indeed, lysosomal and autophagic exocytosis has recently emerged as an important cytoprotective mechanism [25–35]. Lysosomal exocytosis was observed more than 50 years ago as a mechanism to limit focal cellular injuries by sequestration followed by digestion or extrusion [36]. Although early studies focused upon its role in a plasma-membrane repair [37], exocytosis has recently re-emerged as an important component of the cellular clearance pathway. Upregulation of lysosomal exocytosis is proposed to underlie the therapeutic effect of TFEB overexpression in several disease models [25, 31, 33].

The lysosomal divalent cation channel TRPML1 was proposed to drive lysosomal exocytosis because of suppression of lysosomal exocytosis observed in mucopolipidosis type IV models [32, 38]. The proposed underlying mechanisms involves releasing the calcium ions stored in lysosomes [32], which actuates the conformational change in SNARE that drive the fusion of the lysosomes with the plasma membrane. A role of TRPML1 in lysosomal traffic and positioning [39] is likely to contribute to the exocytosis process as well. Finally, TRPML1 has been proposed to facilitate the expulsion of the copper-filled lysosomes [28], a process that is crucial for limiting oxidative stress [26, 40]. The recent evidence of TRPML1 activation by oxidative stress [41] raises the possibility that lysosomal, exocytosis responds to oxidative stress as well. Such a response would modulate the cellular clearance pathways in a manner integrating signals from oxidative stress and cellular energy sensing pathway due to the regulation of TRPML1 expression via mTORC1 and TFEB/TFE3.

As the details of the lysosomal exocytosis mechanisms are emerging, the complexity of the machinery that drives it is becoming apparent. This provides opportunities for pharmacological interventions into many diseases, as many additional targets of pathologies can now be recognized. We have recently shown that lysosomal exocytosis limits the oxidative damage caused by transition metal exposure [29, 40]. A surprising finding that emerged in the course of our previous studies is that while short-term exposure to transition metals stimulated lysosomal exocytosis, long-term exposure had an inhibitory effect [26, 40]. Since the long-term metal exposure is associated with oxidative stress [42, 43], here we investigate the role of oxidative stress in modulating lysosomal exocytosis and provide novel insights into its mechanisms.

2. Methods

Cell Culture and treatments

HeLa cells were maintained in DMEM (Dulbecco's modified Eagle's medium; Lonza) supplemented with 10% FBS (Atlanta Biologicals) at 37°C in the presence of 5% CO₂. tBHP was from Invitrogen (Carlsbad, CA) and ML-SA1 was from Sigma-Aldrich (St. Louis, MO). Primary cortical neuron cultures were prepared as previously described [44], from E15–16 C57BL/6 (Charles River) mouse embryos. The neurons were plated on poly-L-lysine-coated four-well chambered slides (Nunc Laboratory-Tek; Fisher Scientific, Agawam, MA, USA) at 3105 cells/cm² in serum-free Neurobasal media (Gibco, Thermo Fisher) and supplemented with 0.75mM L-glutamine (BioWhittaker, Walkersville, MD, USA) and B27 supplement (Gibco).

siRNA transfection

ON-TARGET plus siRNA probes were custom synthesized by Dharmacon (Lafayette, CO). The first siRNA probe was custom designed to target nucleotides 959–977 with the sequence 5'-CCCACATCCAGGAGTGTA-3' in human MCOLN1, as previously described (TRPML1 959 siRNA) [45, 46]. Non-targeting control siRNA purchased from Sigma was used as a negative control. For knockdown of endogenous TRPML1, cells were plated in 12 well plates and grown to ~70% confluency. As described by manufacturer's protocol, cells were transfected with either control or TRPML1-specific siRNA using RNAiMAX

(Invitrogen). To confirm TRPML1 knockdown, SYBR-green based qPCR was performed as described before [29, 40, 47].

β-Hex activity assay

The assay was performed as before [26, 29, 40]. In brief, HeLa cells on 12-well plates were washed once with regular buffer and 250 µl of buffer was added to each well. The buffer contained, in mM: 140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, pH adjusted to 7.4. The buffer was collected at the indicated times and incubated with 300 µl of 3 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide (N9376, Sigma-Aldrich) for 30 minutes at 37°C in 0.1 M citrate buffer (0.1 M sodium citrate, 0.1 M citric acid, pH 4.5). Reactions were stopped by adding 650 µl borate buffer (100 mM boric acid, 75 mM NaCl, 25 mM sodium borate, pH 9.8) and the absorbance was measured in a spectrophotometer at 405 nm. To determine total cellular β-hex content, cells were lysed with 250 µl of 1% Triton X-100 in PBS and after a 10,000 x g spin for 5 minutes at 4°C, 25 µl of the cell extracts were used for the enzyme activity reaction. Enzyme activity was determined as the amount of 4-nitrophenol produced. Absorbance was calibrated with different amounts of 4-nitrophenol (N7660, Sigma-Aldrich, St Louis, MO) in 0.1 M citrate buffer.

Statistical significance was calculated using a one-tailed, unpaired t-test with $p < 0.05$ considered significant. Data are presented as mean $\pm$ S.E.M.

3. Results

We have previously used β-hex assays in HeLa cells to study the role of lysosomal exocytosis on transition metal clearance and the effects of transition metals on lysosomal exocytosis [26, 29, 40]. β-hex is a lysosomal enzyme, the delivery of which to the extracellular medium is commonly used as a measure of lysosomal exocytosis. In the previous studies we performed a detailed analysis and verification of this process using LAMP1 exposure and siRNA for the components of the protein complex responsible for the lysosomal fusion with the plasma membrane [26].

Tert-Butyl hydroperoxide (tBHP), a stable form of hydrogen peroxide is commonly used to induce oxidative stress [48–50]. In our hands, tBHP changed the delivery of β-hex activity to the extracellular medium bathing HeLa cells in a concentration-dependent manner. Fig 1A shows that cells treated with 10 to 100 µM tBHP for 1 hr increased the rate of the lysosomal exocytosis. In this set of experiments, untreated cells released $2.81 \pm 0.32\%$ of their total β-hex content (measured by lysing cells with CHAPS) into the medium during the 1 hr exposure. Cells treated with 50 µM tBHP released $3.87 \pm 0.13\%$ of total enzyme, while cells treated with 100 µM tBHP released $4.20 \pm 0.09\%$ of the total β-hex (3 individual samples per condition per experiment, 6 experiments, $p < 0.05$ for 10, 50 and 100 µM tBHP). An independent series of experiments Fig 1B shows that the tBHP-induced increase was eliminated by siRNA-mediated knockdown of VAMP7, which was implicated in the lysosomal exocytosis [51, 52] (3 measurements per condition per experiment, 6 experiments, $p < 0.05$ for 10, 50 and 100 µM tBHP, $p < 0.05$ for all VAMP7-siRNA points). TRPML1 has been implicated in lysosomal exocytosis [32, 38]. Accordingly, TRPML1 activator ML-SA1 [32] stimulated lysosomal exocytosis (Fig 1C, 3 measurements per condition per

experiment, 3 experiments, $p < 0.05$ for 10, 50 and 100 μM ML-SA1). siRNA-mediated knockdown of TRPML1 eliminated the tBHP-dependent increase in the lysosomal exocytosis rate as well (Fig 1D, 3 measurements per condition per experiment, 6 experiments, $p < 0.05$ for 10, 50 and 100 μM tBHP). TRPML1 mRNA levels following the knockdown are shown in Supplementary Fig S1. These data suggest that a) TRPML1 activation by oxidative stress contributes to the increase in β -hex exocytosis in response to oxidative stress, and b) such an increase occurs according to the common paradigm of lysosomal exocytosis: Ca^{2+} release through TRPML1 actuating or facilitating VAMP7-dependent fusion events.

Interestingly, the trend of lysosomal exocytosis activation by oxidative stress reversed when tBHP concentration was raised over 200 μM . Fig 2A shows that while in cells treated with 100 μM tBHP, β -hex exocytosis averaged 180% of its levels in untreated cells ($n=9$, $p < 0.05$), increasing tBHP concentration to 300 μM dropped the exocytosis to about 100% of control cells. At 400 μM tBHP β -hex exocytosis averaged 60% of its levels in control cells (Fig 2A, $n=9$, $p < 0.05$). The total β -hex cellular activity measured after the lysing of the cells using Triton X-100 did not show the same degree of suppression (Supplementary Fig S2).

The lysosomal exocytosis decline in response to oxidative stress was detected in the mouse primary cortical neuron cultures as well. In this set of experiments, β -hex exocytosis rate in cells treated with 400 μM tBHP averaged 67% of its levels in untreated cells ($n=6$, $p < 0.05$) (Fig 2B). We conclude that at high levels oxidative stress suppresses lysosomal exocytosis in both transformed and primary cells. As an additional treatment we used the mitochondrial uncoupler rotenone [53, 54], known to increase the mitochondrial electron-leak causing oxidative stress. Fig 2B shows that rotenone suppressed levels of lysosomal exocytosis as well (72% of control levels, $n=9$, $p < 0.05$). These tBHP and rotenone concentrations are routinely used to study the effects of oxidative stress in neuronal cultures *in vivo* and *in vitro* [19, 49, 50, 55].

In the next set of experiments, we sought to determine the source of lysosomal exocytosis inhibition by oxidative stress. Lysosomal exocytosis probably involves multiple steps including vesicle positioning, delivery, docking and fusion. The previous evidence and data in Fig 1C,D show that TRPML1 is critical for this process. However, whether it controls all faucets of lysosomal exocytosis is unclear. Indeed, we have recently shown that Ca^{2+} influx from the extracellular medium has an important role in lysosomal exocytosis [26]. Accordingly, the removal of Ca^{2+} from the extracellular buffer inhibited the basal rate of lysosomal exocytosis (Fig 3A). When measured in Ca^{2+} -free buffer, basal lysosomal exocytosis averaged 34% or its levels in regular buffer containing 1 mM Ca^{2+} (3 experiments, 3 individual samples in each experiment, $p < 0.06$). In these experiments, Ca^{2+} was removed at the onset of the experiment. In our hands, ML-SA1 did not induce β -hex exocytosis in Ca^{2+} -free buffer (Fig 3B). The removal of extracellular Ca^{2+} suppressed the lysosomal exocytosis activation by tBHP as well (Fig 3Ci). Under these conditions, ionomycin induced some β -hex exocytosis, although its magnitudes were significantly suppressed compared with the ionomycin effect in normal Ca^{2+} buffer (Fig 3Cii) The nonspecific plasma-membrane Ca^{2+} channel blocker La^{3+} (100 μM) also suppressed the β -hex exocytosis activation by tBHP (Fig 3D).

Increasing the extracellular Ca^{2+} levels to 2 mM raised the basal β -hex exocytosis rates by about 40% (Fig 4A; 3 experiments, 3 individual samples in each experiment, $p < 0.06$). The magnitude of β -hex signal under the high- Ca^{2+} conditions was comparable with that induced by ionomycin or tBHP under normal conditions. Under the high- Ca^{2+} conditions, the tBHP concentrations that activated β -hex exocytosis in normal Ca^{2+} produced little or no increase in β -hex exocytosis (Fig 4B; 3 experiments, 3 individual samples in each experiment, $p < 0.06$). However, the tBHP concentrations that inhibited β -hex exocytosis in normal Ca^{2+} , produced marked decrease in β -hex exocytosis. Based on this, we propose that extracellular Ca^{2+} influx is important for the lysosomal exocytosis. While the exocytosis activation by low levels of ROS depends on TRPML1, lysosomal exocytosis inhibition by oxidative stress involves inhibiting of plasma membrane Ca^{2+} influx.

4. Discussion

Oxidative stress drives pathological processes in many diseases including ischemic stroke, reperfusion injury, heart failure, Alzheimer's disease, ALS, genetic and toxic forms of Parkinson's disease, autism and Asperger's syndrome [20, 53, 56–65]. A better understanding of the mechanisms of oxidative damage and the recovery from oxidative stress is crucial for better treatments for these diseases. An important component of the cellular defense against oxidative stress is the recovery from oxidative damage. The removal of damaged organelles from the cytoplasm is a function of the autophagic/lysosomal pathway [19, 24]. Beyond the degradation of damaged organelles, the expulsion of damaged cellular components via exocytosis has recently emerged as an important component of the cellular clearance mechanism [21, 31, 66–71]. Therefore, the lysosomal exocytosis pathway is a potentially important pharmacological target for modulating the effects of oxidative stress.

The recent evidence of TRPML1 activation by ROS [41] raised the possibility that TRPML1-dependent processes are regulated by ROS as well. The initial evidence for the role of TRPML1 in lysosomal exocytosis comes from resting unstimulated human skin fibroblasts. TRPML-deficient fibroblasts from mucopolipidosis type IV patients were found to have lower exocytosis rates than age-matched heterozygous controls [38]. TRPML1 has been implicated in the exocytosis of Cu-filled lysosomes [28]. Finally, inhibition of lysosomal exocytosis and the resulting membrane deficits were used to explain phagocytosis suppression in TRPML1-deficient cells [32]. Our data on the stimulation of the lysosomal exocytosis by low and moderate levels of the pro-oxidant tBHP in a manner requiring TRPML1 agrees with this evidence.

Our data show that oxidative stress (high levels of ROS) suppresses the process of lysosomal exocytosis, which would be predicted to impair the expulsion of toxic compounds from cells. Therefore, we propose that ROS have a biphasic effect on the lysosomal clearance: consistent with their role as a second messenger moderate levels of ROS stimulate the lysosomal exocytosis, but pathological levels of ROS inhibit the lysosomal exocytosis. Therefore, the impact of oxidative stress pathology on cells is two-pronged: beyond direct damage, it impairs the ability of the cell to limit and repair damage by extrusion of damaged materials. Indeed, an accumulation of damaged mitochondria and protein aggregates, which

in turn can further amplify oxidative stress [24], is observed in multiple human diseases. An impact of oxidative stress on the cellular clearance pathway has been studied in many models; its specific effects are being disputed. An inhibitory effect of oxidative stress on the cellular clearance is also supported by data emerging from studies of ALS [72] and Alzheimer's disease [66, 73].

Several candidate mechanisms for the observed effects can be discussed. Ca^{2+} release through TRPML1 has been implicated in lysosomal exocytosis [32]. Lysosomal exocytosis probably involves the stages of vesicle delivery to the plasma membrane and their fusion. TRPML1 has been implicated in vesicle positioning and motility [39] and membrane fusion [32]. Impairments in either of these processes would impact lysosomal exocytosis. Both processes appear to require Ca^{2+} . Our previously published data [26] and the results presented here show that extracellular Ca^{2+} is necessary for the lysosomal exocytosis as well. These data suggest a contribution of the extracellular Ca^{2+} influx into cellular clearance and repair processes driven by lysosomal exocytosis. Oxidative stress was shown to inhibit the store-operated Ca^{2+} entry component Orai [74–76], and thus the inhibition of the store-operated Ca^{2+} entry by oxidative stress may be responsible for some of the loss of lysosomal exocytosis under oxidative stress. Since cell signaling involving G protein-coupled receptors drives the store-operated Ca^{2+} entry, this may serve as evidence connecting G protein-coupled receptor signaling and the cellular clearance via lysosomal exocytosis.

We show that increasing extracellular Ca^{2+} stimulates lysosomal exocytosis to the extent that renders oxidative stress ineffective (Fig 4). We think that under these conditions, the increased Ca^{2+} influx triggers the processes normally actuated by TRPML1-dependent Ca^{2+} release, such as lysosomal positioning or fusion. Since the loss of lysosomal exocytosis was proposed to contribute to mucopolipidosis type IV pathology [32, 38], it would be interesting to answer whether increased Ca^{2+} influx reverses some aspects of this pathology.

5. Conclusions

Oxidative stress drives pathological processes in many diseases including ischemic stroke, heart attack and neurodegenerative diseases. Lysosomal exocytosis has emerged as an important cellular clearance pathway. We show that oxidative stress suppresses lysosomal exocytosis. Therefore, beyond direct damage, oxidative stress suppresses cellular repair by inhibiting the expulsion of toxic compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Oxidative stress regulated lysosomal exocytosis in a biphasic manner.
- Stimulation of lysosomal exocytosis by low levels of oxidative stress requires TRPML1.
- Inhibition of lysosome exocytosis by high levels of oxidative stress involves calcium influx.

Fig 1. Moderate ROS levels activate lysosomal exocytosis

A. β -hex exocytosis in cells treated with low and moderate tBHP concentrations. β -hex activity in the extracellular medium expressed as the percentage of total cellular β -hex content at different tBHP concentrations. **B.** β -hex exocytosis normalized to its levels in untreated cells, which were taken as 100%. VAMP7 knockdown using siRNA suppresses the basal and the tBHP-stimulated β -hex exocytosis. **C.** β -hex exocytosis expressed as in panel B as a function of ML-SA1 concentration. **D.** β -hex exocytosis analyzed as in panel B in mock- and TRPML1 siRNA-transfected cells. β -hex assays in HeLa cells performed as in our previous publications [26, 29, 40]. β -hex activity in the culture medium was analyzed one hour after the fresh medium was introduced. The activity was normalized to the total β -hex content, which was analyzed by lysing cells; total β -hex content in cell lysates of untreated cells was taken for 100% (shown in Supplementary Fig S2). tBHP and ML-SA1 were added immediately after the recording had begun. siRNA transfections were performed 24 hours before the experiment. Data represent 3 experiments, 3 independent biological replicates each; * denotes $p < 0.05$ relative to untreated control.

Fig 2. Inhibition of lysosomal exocytosis by high levels of oxidative stress
 β -hex assays performed in HeLa cells (**A**) and mouse primary cortical neuron cultures (**B**). All treatments are as in Fig 1. As in our recent publication [26], 100 μM Cu stimulated lysosomal exocytosis. Rotenone (Rot, 20 μM) was applied 12 hours before the experiment. Data represent 3 experiments, 2 independent biological replicates each; * denotes $p < 0.05$ relative to untreated control.

Fig 3. Involvement of extracellular Ca^{2+} in lysosomal exocytosis

β -hex assays performed in HeLa cells as in Fig 1 and 2. **A.** Ca^{2+} removal from the extracellular medium suppresses lysosomal exocytosis. Data are expressed as percentage of lysosomal exocytosis in cells exposed to normal 1 mM Ca^{2+} . **B.** ML-SA1 (100 μ M) added immediately after beginning of the experiment as in Fig 1C is not effective in stimulating β -hex exocytosis in the absence of extracellular Ca^{2+} . The data are expressed as percentage of β -hex exocytosis relative to untreated cells exposed to Ca^{2+} -free solution. **C.** (i) Cells exposed to Ca^{2+} -free medium immediately after the beginning of experiment and treated as in Fig 1C and 2A do not show an increase in β -hex exocytosis in response to tBHP. (ii) Comparison of the ionomycin effect on lysosomal exocytosis in Ca^{2+} -containing and Ca^{2+} -free buffers. Exocytosis is expressed as in panel B. **D.** tBHP is ineffective in inducing lysosomal exocytosis in the presence of 100 μ M $LaCl_3$. The experiments were performed in a normal Ca^{2+} buffer to which La^{3+} was added, and the data are expressed as percentage of β -hex exocytosis relative to untreated cells in La^{3+} buffer. Data represent 3 experiments, 3 independent biological replicates each; * denotes $p < 0.05$ relative to untreated control.

Fig 4. Extracellular Ca²⁺ influx and lysosomal exocytosis

β-hex assays performed in HeLa cells that were bathed in 2 x Ca²⁺ media (2 mM Ca²⁺) starting immediately after beginning of the experiment. **A.** Raising extracellular Ca²⁺ increases lysosomal exocytosis. Data are expressed as percentage of lysosomal exocytosis in cells exposed to normal 1 mM Ca²⁺. **B.** High levels of tBHP cause significant suppression of lysosomal exocytosis in 2x Ca²⁺ buffer. Cells were treated and the data are expressed essentially the same as in Fig 3C. Data represent 3 experiments, 3 independent biological replicates each; * denotes p<0.05 relative to untreated control.