Enhancement of lysosomal proton permeability induced by photooxidation of membrane thiol groups

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Abstract

Effects of photooxidation of membrane thiol groups on lysosomal proton permeability were studied by measuring intralysosomal pH with fluorescein isothiocyanate–dextran and monitoring proton leakage with p-nitrophenol. Methylene blue-mediated photooxidation of lysosomes decreased their membrane thiol groups and produced cross-linking of the membrane proteins, which was established by the measurement of residual membrane thiol groups with 5,5'-dithio-bis(2-nitrobenzoic acid) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis, respectively. The cross-linking of proteins could be abolished by subsequent treatment of the photodamaged lysosomes with dithiothreitol, indicating that the proteins were linked via disulfide bonds. In addition, the photodamage of lysosomes raised the intralysosomal pH and caused leakage of the lysosomal protons, which could also be reversed by subsequent dithiothreitol treatment. This indicates that lysosomal proton permeability can be increased by photooxidation of the membrane thiol groups and recovered to the normal level by reduction of the groups. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Lysosome; Membrane thiol groups; Photooxidation; Proton permeability

Lysosomes are one of the main photodamage sites in living cells [1–4]. Some photosensitizers such as methylene blue (MB)\(^1\), acridine orange, nile blue, mono-L-aspartyl chlorin e6, and tetrarsulfonated tetraphenylporphine localize preferentially in the lysosomes [1–3]. In addition, hematoporphyrin can induce photodamage to lysosomes [5]. In recent years, interest in the mechanism of lysosomal photodestruction has heightened with the realization that lysosomotropic photosensitization plays an important role in killing tumor cells. The hydrolases leaked out of the photodestructed lysosomes can cause tumor cell death [6,7]. Additionally, the photoinduced lysis of lysosomes plays a role in either delivering some drugs or reducing cytoplasmic pH, which may augment the effects of some cancer treatments such as chemotherapy, hyperthermia, and PDT [8]. Recently studies suggested that the endonucleases [9] and cathepsins [10,11] released from oxidative stress-damaged lysosomes might induce apoptosis of living cells. In addition, acidification of the cytosol may be important for caspase-3 activation and therefore promotes apoptosis [12]. This evidence leads to active studies concerning how the lysosomes are photodestructed. Some investigators proposed that membrane lipid peroxidation is the immediate cause for the active oxygen-induced lysosomal destabilization [13,14]. Our recent studies established that membrane lipid peroxidation does not definitely destabilize lysosomes and that the direct cause for the lysosomal destabilization is the photoinduced osmotic imbalance across their membranes via an increased \(H^+/K^+\) exchange [15,16]. Lysosomal permeability to \(H^+\) can be increased by photodamage to their membranes. What alterations in the photodamaged membranes cause such an increase is still unclear. Elucidation of this issue may contribute to a better understanding of photoinduced lysosomal lysis.

\(^1\)Abbreviations used: CCCP, carbonyl cyanide m-chlorophenylhydrazone; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol; MB, methylene blue; FITC-dextran, fluorescein isothiocyanate–conjugated dextran; PDT, photodynamic therapy; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
As demonstrated previously, the thiol groups of membranes can affect their water permeability [17] and the membrane proton permeability correlates with the water permeability [18]. The lysosomal membranes are enriched in thiol groups [19]. It is likely that membrane thiol groups may influence the lysosomal proton permeability. Oxidation to protein thiol groups is normally associated with the impairment of various cell functions [20]. Since membrane thiol groups are prone to photooxidation [21,22], it may be a cause for the photoinduced increase in the lysosomal permeability to protons. The results of this study confirmed this hypothesis.

**Materials and methods**

**Chemicals.** FITC-dextran (average molecular mass, 71,200 Da), DTNB, CCCP, DTT, sodium dodecyl sulfate, acrylamide, and N,N'-methylenebisacrylamide were from Sigma (St. Louis, MO). The other chemicals used were of analytical grade from local sources. All aqueous solutions were prepared with deionized, glass-distilled water.

**Preparation of lysosomes.** Rat liver lysosomes were prepared by the method of Ohkuma et al. [23]. All procedures were carried out at 0–4°C. The intralysosomal pH measured with FITC-dextran is about 5.0. To prevent the leakage of lysosomal protons after preparation, the lysosomes were resuspended in 0.25 M sucrose medium (pH 5.0, adjusted with citric acid) at a final protein concentration of 21.3 mg/ml. Protein was determined according to Lowry et al. [24]. Measurements of the intralysosomal pH and proton leakage were performed within 3–4 h after the lysosomal preparation. During this period, the intralysosomal pH was maintained at 5.0.

**Light exposure procedure.** All lysosomal samples (19.2 mg protein/ml) prepared for photosensitization contained 0.1 mM MB and were exposed to light on ice bath for the indicated times. Control samples contained MB but were not exposed to light. Incident light was from a tungsten halogen lamp (24 V, 250 W, quartz envelope) with 660-nm filter (bandwidth 10 nm). The light intensity at the sample position was 10 mW/cm² measured using a BTY-8204 Solar Irradiation Meter made by the Institute of Solar Energy of Beijing.

**Photomodification of thiol groups.** Lysosomal membrane thiol groups were photooxidized by MB-mediated photosensitization. Since oxidized membrane thiol groups can be reduced by treatment with DTT [25], the photooxidized lysosomes were subsequently treated with 25 mM DTT at 37°C for 15 min to restore their membrane thiol groups.

**Determination of membrane thiol groups.** DTNB, which is used as Ellman’s reagent to determine thiol groups in proteins, forms its reaction product, 2-nitro-5-thiobenzoic acid (NTB), when the disulfide bond between the two halves of DTNB is replaced by another disulfide bond formed from the sulfhydryl of the protein. Thus, measurement of the absorbance of NTB at 412 nm provides a positive indication that reaction has taken place with the sulfhydryl group of the measured proteins [26]. A stock solution of 1 mM DTNB in 100 mM PBS (pH 7.4) was prepared on the day of the measurement. Lysosomal sample (480 μL, 9.6 mg protein/ml) was first ruptured in water and washed for three times at 4°C. The pellet of lysosomal membranes was resuspended in 80 μL 5% SDS for 5 min to disperse the membrane proteins, then 80 μL 0.25 M sucrose was added. For labeling, the suspension was mixed with 1.44 ml DTNB stock solution and incubated at 37°C for 10 min. The absorbance change at 412 nm was registered at 25°C on a Hitachi U-3200 spectrophotometer. For the calculation of SH content/mg protein, glutathione was used as a reference substance.

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis.** Lysosomal membranes were prepared as described under Determination of membrane thiol groups. Membrane proteins were thoroughly dispersed in 5% SDS solution and used for the electrophoresis. The SDS-PAGE of membrane proteins was carried out using a 3.9% acrylamide stacking gel and a 5% acrylamide separating gel. Protein was loaded at 60 μg/lane. Staining was accomplished with Coomassie blue R-250.

**Measurement of lysosomal pH.** According to the method of Ohkuma et al. [23], rats were injected intraperitoneally with FITC-dextran (20 mg of FITC-dextran/150 g of body weight), starved for 12 h, and then decapitated. The lysosomes loaded with FITC-dextran were prepared as described above. Fluorescence was measured at 25°C with excitation and emission wavelengths of 495 and 550 nm, respectively, on a Hitachi 850 fluorescence spectrophotometer. Intralysosomal pH was calculated from the fluorescence intensity of the lysosomal sample relative to that after addition of Triton X-100 to 0.02%, using a standard curve generated as described by Ohkuma et al. [23].

**Measurement of lysosomal proton leakage.** Lysosomal proton leakage can acidify their suspending medium. The acidification of assay medium by the proton leakage was measured as previously described [15]. Briefly, lysosomal sample was added to 2 ml assay medium (containing 0.25 M sucrose and 0.1 mM p-nitrophenol (Na salt), pH 7.0) at 0.479 mg protein/ml. The absorbance (400 nm) of the pH-sensitive dye p-nitrophenol was measured immediately at 25°C on a Hitachi U-3200 spectrophotometer. A decrease in the dye absorbance indicates an acidification of the assay medium, i.e., an increase in the lysosomal proton leakage. Changes in the assay medium pH were determined using a calculation curve generated from the absorbance of the above assay medium at 400 nm at various pHs.
Effects of photooxidation on the lysosomal membrane thiol groups

Effects of MB-mediated photooxidation on the thiol groups of lysosomal membrane proteins were observed by measuring the residual thiol content with DTNB. As shown in Fig. 1, the thiol groups of lysosomal membranes reduced with prolonged light exposure time. Irradiating the lysosomes in the absence of MB for 30 min or mixing the lysosomes with the same amount of MB but unirradiated did not change their membrane thiol content. To examine whether it is caused by a photooxidative action, the photodamaged lysosomes were treated with thiol reducing reagent DTT. The results show that treatment of the photooxidized lysosomes with DTT restored the decreased thiol groups. It thus confirms the effect of photooxidation on the membrane thiol groups.

The above conclusion was reexamined by SDS-PAGE of lysosomal membrane proteins. As shown in Fig. 2, several bands of low-molecular-mass polypeptide (range from 75,000 to 125,000 Da) appeared in the lanes of control lysosomes (lane a, lysosomes were irradiated in the absence of MB; lane b, lysosomes were mixed with MB but not irradiated). The lane of a control sample (not irradiated and not incubated with MB) did not differ from lanes a and b (data not shown). MB-mediated photooxidation to lysosomal membranes (lane d) abolished most of those polypeptide bands and produced large aggregates near the origin of the lane. It suggests that the photooxidation caused a cross-linkage of lysosomal membrane proteins. To examine whether the aggregated proteins in lane d were linked via disulfide bonds, a duplicate of the photodamaged lysosomal sample was treated with DTT prior to the electrophoresis. As shown in lane c, treatment of the photodamaged lysosomes with DTT abolished the large aggregates in lane d and restored the original gel patterns of control samples (lanes a and b). It confirms that the linkage of the proteins was caused by the photooxidation of membrane thiol groups, i.e., via the formation of disulfide bonds.

Effects of photooxidation of membrane thiol groups on the lysosomal pH

Influence of photooxidation of membrane thiol groups on the lysosomal proton permeability was studied by measuring the lysosomal pH with FITC-dextran. As shown in Table 1, control lysosomes maintained their internal pH at about 5.02 during a 2-min incubation in sucrose medium. This is similar to the value provided by Ohkuma et al. [23]. The photodamaged lysosomes exhibited a similar pH at the start of the incubation and a higher pH after the incubation. The increases of the lysosomal pH (maximal increase is about 0.2 pH units) during the incubation correlate with the times of light exposure. The elevation of lysosomal pH by photooxidation indicates the occurrence of proton leakage. Treating the photooxidized lysosomes with DTT aboli-
Effects of photodamage of lysosomes on the intralysosomal pH

Table 1

<table>
<thead>
<tr>
<th>Treatment of lysosomes</th>
<th>Intralysosomal pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>2 min</td>
</tr>
<tr>
<td>Light exposure for 0 min, then treated with DTT for 0 min (control)</td>
<td>5.02 ± 0.01</td>
</tr>
<tr>
<td>Light exposure for 15 min, then treated with DTT for 0 min</td>
<td>5.03 ± 0.01</td>
</tr>
<tr>
<td>Light exposure for 30 min, then treated with DTT for 0 min</td>
<td>5.03 ± 0.01</td>
</tr>
<tr>
<td>Light exposure for 30 min, then treated with DTT for 15 min</td>
<td>5.03 ± 0.00</td>
</tr>
</tbody>
</table>

Note. FITC-dextran-loaded lysosomal samples mixed with 0.1 mM MB (19.17 mg protein/ml) were exposed to light for the indicated times. The photodamaged lysosomes were subsequently treated with 25 mM DTT at 37°C for the indicated times. Then, the lysosomal samples (47.9 µg protein/ml) were incubated in 2 ml 0.25 M sucrose medium buffered at pH 7.0 with 20 mM Hepes/Tris. Intralysosomal pH was measured at indicated time of the incubation. Values are means ± SD, n = 4.

Fig. 3. Effect of photodestruction and reversing effect of DTT treatment on lysosomal proton leakage. Lysosomal sample was added to 2 ml assay medium (containing 0.25 M sucrose and 0.1 mM p-nitrophenol (Na salt)) at 0.479 mg protein/ml. Absorbance (400 nm) of p-nitrophenol was measured immediately. (a) Lysosomal samples containing 0.1 mM MB were exposed to light for (line 1) 30 min, assay medium pH was maintained at 7.0 with 0.2 M phosphate buffer, (2) 0 min (control), (3) 15 min, (4) 30 min, and (5) 0 min (control), assay medium containing 1 µM CCCP. (b) Lysosomes containing 0.1 mM MB were (lines 1) exposed to light for 0 min (control), (2) exposed to light for 30 min, and then treated with 25 mM DTT for 15 min, and (3) exposed to light for 30 min. A typical result from three experiments is shown.

Effects of photodestruction on the lysosomal proton leakage

Since ion flux through a membrane is proportional to the ion permeability [27], the lysosomal proton permeability can be observed by measuring their proton efflux-induced acidification of the suspending medium with the pH-sensitive dye p-nitrophenol as an indicator [15]. The measurements were carried out by monitoring the decrease in p-nitrophenol absorbance at 400 nm, which was based on the property of the dye that the unprotonated p-nitrophenol molecules have a sufficiently larger extinction coefficient at 400 nm than that of protonated molecules [28]. The conclusion obtained from Table 1 was reexamined by measuring the lysosomal proton leakage using p-nitrophenol. As shown in Fig. 3a, the dye absorbance of the photodamaged lysosomal samples decreased with prolonged exposure times (lines 2, 3, and 4). Addition of CCCP to the measuring medium caused a significant reduction in the absorbance (line 5). In addition, decrease in the absorbance of the photodamaged sample could be abolished by buffering the measuring medium (line 1). The results indicate that the decrease in the dye absorbance was due to a proton leakage-induced acidification of the lysosomal suspension and suggest that the proton permeability of lysosomes increased by the photodestruction of their membrane thiol groups.

The above conclusion was examined by the treatment of lysosomes with DTT. As shown in Fig. 3b, exposing the lysosomes to light for 30 min decreased the dye absorbance (compare line 3 with line 1). The magnitude of the absorbance decrease of the photodamaged lysosomal sample reduced markedly after the lysosomes were treated subsequently with DTT (line 2). The reversion of the photodestruction-induced proton leakage by the DTT treatment confirmed the effect of photodestruction of the membrane thiol groups on the lysosomal proton permeability.

Discussion

In the basic studies of the PDT of cancers, elucidating the mechanisms for the photodamage of subcellular targets continues to be an area of active investigation. In recent years, our studies focused on the mechanism of the photodamage of lysosomes. The results demonstrated that the photodamaged lysosomes could be destabilized by a loss in the Mg−ATP-dependent lysosomal proton translocation [29] and by an increase in their
Table 2
Changes in the assay medium pH

<table>
<thead>
<tr>
<th>Treatment of lysosomes</th>
<th>Assay medium pH</th>
<th>ΔpH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light exposure for 30 min, the medium buffered at pH 7.0</td>
<td>7.0</td>
<td>0</td>
</tr>
<tr>
<td>Light exposure for 0 min, then treated by DTT for 0 min</td>
<td>6.987 ± 0.001</td>
<td>21.0</td>
</tr>
<tr>
<td>Light exposure for 15 min, then treated by DTT for 0 min</td>
<td>6.976 ± 0.001</td>
<td>38.7</td>
</tr>
<tr>
<td>Light exposure for 30 min, then treated by DTT for 0 min</td>
<td>6.958 ± 0.001</td>
<td>67.7</td>
</tr>
<tr>
<td>Light exposure for 30 min, then treated by DTT for 15 min</td>
<td>6.984 ± 0.001</td>
<td>25.8</td>
</tr>
<tr>
<td>Control + CCCP</td>
<td>6.938 ± 0.001</td>
<td>100</td>
</tr>
</tbody>
</table>

Note. All experimental details were as for Fig. 3. The assay medium pH was determined using a calculation curve generated from the absorbance of the assay medium at 400 nm at various pHs. Changes in the assay medium pH (ΔpH) are expressed as a percentage of the change induced by CCCP. Values are means ± SD, n = 3.

membrane permeability to potassium ions [15]. The increased K⁺ uptake produces an osmotic imbalance across the lysosomal membranes, causing the photodamaged lysosomes of disintegrate. As demonstrated previously [15,30], an evident sign of photodamage to lysosomes is the leakage of their protons, which promotes the influx of K⁺ via an electroneutral K⁺/H⁺ exchange. Recently, we established that the photoinduced membrane rigidification increases the lysosomal permeability to potassium ions [31]. However, it is still unclear how the photodamage enhances the lysosomal proton permeability. The results of this study verify that the photooxidation of membrane thiol groups can increase the lysosomal proton permeability. It may contribute to a better understanding of the lysosomotropic photosensitization.

Lysosomes are acidic vacuoles containing a variety of acidic hydrolases which participate in the physiological turnover of cellular macromolecules such as nucleic acids, protein, lipids, and carbohydrates. Whether the lysosomal acidic pH can be maintained greatly affects the activities of their enzymes. In normal cases, the lysosomal membranes are relatively impermeable to protons [32]. An increase in lysosomal permeability to protons can elevate the intralysosomal pH via a proton leakage, which may cause cell death by a loss of the degradative capacity of lysosomes [33]. In addition to photodamage, attention has been also given to other factors affecting the lysosomal proton permeability such as the antineoplastic drugs lonidamine [34] and polyanion [35].

As demonstrated previously [19], lysosomal membranes are enriched in thiols, which play an important role in lysosomal activities such as sulfate transport [36] and ATPase-mediated proton translocation [29]. The thiol groups of membranes are susceptible to photooxidation [21] and crucially involved in the maintenance of their barrier functions such as membrane permeability to ions [17]. Oxidation of membrane thiol groups can cause a loss of the barrier function. This raises the possibility that photooxidation of lysosomal membrane thiol groups may affect their permeability to protons. The results of this study confirm this hypothesis and suggest that the membrane thiol groups are necessary to maintain the relative impermeability of lysosomes to protons. Since the thiol groups are ubiquitously present in the membranes, it is difficult to identify the proteins for which the thiol groups are responsible for the regulation of lysosomal proton permeability. As shown by the SDS–PAGE of lysosomal membrane proteins (Fig. 2), most of the low-molecular-mass protein bands disappeared after the lysosomes were photodamaged and some high-molecular-mass bands formed, which could be reversed by the treatment with DTT. The results suggest that these low-molecular-mass proteins contain thiol groups. It is consistent with the above evidence that lysosomal membrane proteins have abundant thiol groups. Since MB-mediated photosensitization occurs outside of the lysosomes [31], it is likely that the low-molecular-mass proteins taking part in the photoinduced cross-linkage are exposed to the cytoplasm. This is in line with the evidence provided previously [19] that most of the membrane proteins of lysosomes are exposed to the cytoplasm.

The thiol-reducing reagent DTT has been widely used to study the effect of protein thiol oxidation on various biological properties such as membrane permeability to K⁺ [25]. Since the photoinduced increase in the lysosomal permeability to protons could be restored by the treatment with DTT (Fig. 3, Tables 1 and 2), it can be concluded that the increase in the lysosomal permeability to protons was induced by the photooxidation of membrane thiol groups.

The photooxidation of lysosomal membrane thiol groups elevated the intralysosomal pH, indicating that the lysosomal proton permeability increased. As pointed out by Ohkuma [37], the pH within isolated lysosomes gradually increased (0.1–0.2 unit/4 min) in salt medium containing 100 mM KCl by a K⁺/H⁺ exchange. In sucrose medium, the lysosomal pH raises much more slowly. As shown in Table 1, photooxidizing the lysosomes with MB elevated their internal pH by 0.18 unit in sucrose medium (in 2 min), which is an evident increase in the pH compared with the pH change in salt medium.
The lysosomal membrane potential, which is generated by the internal protons, is negative inside [32]. The efflux of lysosomal protons must be electroneutrally accompanied by charge-compensating movements of other ions (either influx of external cations or efflux of internal anions). As reported previously [32], the lysosomes may contain chloride and phosphate. The anions may enter the lysosomes electroneutrally with the transportation of protons by $\text{H}^+\text{-ATPase}$. Since there are no cations in the sucrose medium used in this study, the efflux of lysosomal protons might be accompanied by the efflux of intralysosomal anions. This is supported by the previous observation [32] that leakage of lysosomal protons could be accompanied by the efflux of permeable anions such as chloride in sucrose medium.

It is generally believed that membrane permeability to water has a high correspondence with its proton permeability. There are at least four pathways or mechanisms for the water flux across membranes, including aqueous pores [38], solubility–diffusion mechanisms [38], and channel [39] and transient [27] defects in the membrane arising from thermal fluctuation. The permeation of protons across membranes is suggested as being along the hydrogen bonds of water crossing the membranes through aqueous pores [18] and transient defects [27]. The membrane thiol groups may be an important determinant of water permeability, which is supported by various lines of evidence that the water permeability of some membranes can be increased and decreased by modulating their membrane thiol groups [40,41]. The results of our recent study [42] suggest that photooxidation of the lysosomal membrane thiol groups may increase their water permeability. Another study [43] showed that oxidizing the thiol groups of erythrocyte membranes can increase the size of the aqueous pores of the membranes. This evidence suggests that changes in the redox states of membrane thiol groups may alter the water permeability and therefore affect the proton permeability. It appears that the thiol groups of some membrane proteins may control ground water permeability and maintain the membrane barrier function [44,45]. It was hypothesized that oxidation of membrane thiol groups may produce aqueous pores and water channels by aggregating membrane proteins [46]. Further studies are needed to confirm this hypothesis. As demonstrated previously, the oxidation of membrane thiol groups can produce disulfide bonds between the related proteins. The cross-linkage of proteins may change protein conformation and distribute phospholipid and proteins. The perturbation of the membrane structure may play a crucial role in the alteration of membrane permeability [17,44,47,48]. It may somewhat explain why oxidation of the membrane thiol groups can increase lysosomal permeability to protons.

Acknowledgments

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References