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Biochimica et Biophysica Acta 1284 (1996) 35–40

BBA Biochimica et Biophysica Acta

Loss of lysosomal integrity caused by the decrease of proton translocation in Methylene blue-mediated photosensitization

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Received 11 March 1996; accepted 15 May 1996

Abstract

Loss of lysosomal integrity is a critical event for killing tumor cells in the photodynamic therapy of cancers. To elucidate the mechanism of photodamage induced lysosomal disintegration, we investigated the role of losing lysosomal proton translocation in latency loss of photosensitized lysosomes. Isolated rat liver lysosomes were light exposed in the presence of Methylene blue. Through monitoring lysosomal ΔpH with Acridine orange and measuring its membrane potential with 3,3′-dipropylthiadicarbocyanine iodide, loss of Mg-ATP dependent proton translocation and decrease in electrogenicity of the proton pump were observed after lysosomes were photosensitized. When normal lysosomes were incubated for 60 min in K^+ contained medium, percentage free activity of lysosomal enzyme β-galactosidase increased, i.e. lysosomal latency decreased. In the presence of Mg-ATP, the latency loss of incubated lysosomes reduced. Addition of *n*-ethylmaleimide, a potent inhibitor of lysosomal H^+-ATPase, abolished the effect of Mg-ATP on lysosomal latency. It suggests a role of proton translocation in protecting lysosomal integrity. Under the same conditions, Methylene blue photosensitized lysosomes increasingly lost latency of β-hexosaminidase and β-galactosidase with light exposure, presumably due to the photodamage induced loss of proton pumping. In contrast, the photosensitization did not decrease lysosomal latency in the absence of Mg-ATP, implying that lysosomal integrity might not be impaired via other photodamage effects under the conditions of this study. These results indicate that lysosomal integrity can be photodestructed via the loss of proton translocation.

Keywords: Lysosome; Latency loss; Photosensitization; H^+-ATPase; Potassium ion

1. Introduction

Lysosomes is one of the main cellular photodamage sites [1–3]. Since leakage of hydrolytic enzymes from photodestructed lysosomes may lead to cell death [4,5], lysosomotropic photosensitization has been emphasized in the basic studies of photodynamic therapy of cancers [6]. Although great efforts have been made to investigate photodamage induced lysosomal disruption, exact mechanism of the lysis has not been completely elucidated.

Cytoplasm contains abundant potassium ions. Constant entry and accumulation of K^+ in lysosomes decreases lysosomal osmotic stability [7–11]. It has been suggested that the electrogenic proton translocation by lysosomal H^+-ATPase may protect lysosomes against K^+ entry induced osmotic disruption [10–13]. Therefore, lysosomal proton pump plays important protective role. A number of enzymes are susceptible to photooxidation [14]. Lysosomal H^+-ATPase may also be prone to photodamage due to its thiol group, therefore losing its function. In this paper, we demonstrated that proton translocation by lysosomal H^+-ATPase and the electrogenicity of the pump are photodamaged in Methylene blue mediated photosensitization. The osmotic protection to lysosomes provided by proton pumping in K^+ contained medium is also lost. Thus, we propose a new mechanism of photodamage induced loss of lysosomal integrity.

2. Materials and methods

2.1. Chemicals

$DiSC_3(5)$ and $DiOC_6(3)$ were from Molecular Probes (Eugene, OR); UMBG, 4-methylumbelliferyl *N*-acetyl-β-

Abbreviations: UMBG, 4-methylumbelliferyl-β-D-galactoside; $DiSC_3(5)$, 3,3′-dipropylthiadicarbocyanine iodide; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; MB, Methylene blue; AO, Acridine orange; NEM, *n*-ethylmaleimide; TBARS, thiobarbituric acid reactive substance(s); LOOH(s), lipid hydroperoxide(s)

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PII S0005-2736(96)00105-8

D-glucosaminide, AO, ATP, Hepes and NEM were from Sigma. The other chemicals used were of analytical grade from local sources. All aqueous solutions were prepared with deionized, glass-distilled water.

2.2. Preparation of lysosomes

Male wistar rats starved for 24 h were killed by decapitation. Rat liver lysosomes were prepared by the method of Ohkuma et al. [15]. All procedures were carried out at 0–4°C. Lysosomes were resuspended in 0.25 M sucrose medium at a final protein concentration of 25 mg/ml. Protein was determined according to Lowry et al. [16].

2.3. Light exposure procedure

All photoreaction mixture consisted of MB (0.01 mM) and lysosomes (22.5 mg protein/ml) in 0.25 M sucrose. Incident light was from a REFLECTA slide reflector with 670 nm filter. The light intensity at the sample position was 5 mW/cm^2. Samples were light exposed on ice bath.

2.4. Incubation of lysosomes

The incubation medium contained 0.13 M sucrose, 0.05 M K_2SO_4, 1 mM $MgSO_4$ and was buffered at pH 7.0 with 0.02 M Hepes/KOH. A 30 μl photosensitized or control lysosomal sample was suspended in 1 ml incubation medium and incubated at 37°C for 60 min. ATP or NEM was added to incubation medium as designated in the figure legends. After incubation, a 50 μl portion of this lysosomal suspension was used for the assay of lysosomal integrity.

2.5. Assay of lysosomal integrity

Lysosomal integrity can be assessed by measuring lysosomal enzyme latency or its enzyme sedimentability [17–19]. In this study, we used the method of assessing lysosomal enzyme latency.

The activity of lysosomal enzymes was measured fluorometrically by the method of Bird et al. [20]. β-Galactosidase was assayed using UMBG as substrate at a final concentration of 0.5 mM in the assay medium [21]. β-Hexosaminidase was assayed using 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide as substrate at a final concentration of 2 mM in the assay medium [22]. The liberated 4-methylumbelliferone was determined by measuring the fluorescence (excitation: 365 nm, emission: 444 nm) on a Hitachi 850 fluorescence spectrophotometer.

The activities of the enzyme measured in the absence and presence of Triton X-100 are designated the free activity and the total activity respectively [20]. Percentage free activity was calculated as (free activity/total activity) × 100. Lysosomal enzyme latency can be defined as [1-(free activity/total activity)] × 100. Loss of lysosomal integrity was determined as loss of lysosomal enzyme latency or increased percentage free activity.

2.6. Measurement of proton pumping

The medium of measuring proton pumping contained 0.1 M KCl, 1 mM $MgCl_2$, buffered at pH7.0 with 0.02 M Hepes/Tris. Proton translocation by lysosomal H^+-ATPase was initiated by the addition of ATP. As previously described, the proton pumping was monitored by measuring differential absorbance ($\Delta A_{492-540\ nm}$) of AO at 25°C on a Hitachi 557 dual wavelength spectrophotometer [23]. Detailed procedures of the measurement are designated in the figure legends.

2.7. Measurement of lysosomal membrane potential

Carbocyanine dye $DiSC_3(5)$ was used to measure lysosomal membrane potential [12]. The assay medium contained 0.5 μM $DiSC_3(5)$, 0.25 M sucrose, buffered at pH 7.0 with 0.02 M Hepes/Tris. A 10 μl lysosomal sample was used for the assay. Fluorescence measurements were conducted at 25°C with excitation and emission wavelength of 622 and 670 nm, respectively.

3. Results

3.1. Photodamage to lysosomes decreases Mg-ATP dependent H^+ translocation

Lysosomal proton pumping was assessed by using AO to monitor the increase in lysosomal ΔpH upon addition of Mg-ATP. As shown in Fig. 1, after initiating proton translocation with ATP, lysosomes of three control samples exhibit largely increased ΔpH (curves 1–3), indicating a higher level of H^+ translocation. In contrast, the increase in ΔpH of photosensitized lysosomes declines with prolonging exposure time (curves 4 and 5). Treatment of lysosomes with NEM, a potent inhibitor of lysosomal H^+-ATPase, causes a more significant decline in the ΔpH increase (curve 6), indicating that proton pumping is strongly inhibited. To clarify if the photosensitization caused lysosomal H^+ leakage which may partly counteract H^+ pumping, therefore inducing the decline in ΔpH increase (curves 4 and 5), we used pH sensitive dye *p*-nitrophenol to examine H^+ release [24]. The unprotonated *p*-nitrophenol molecules have a sufficiently larger extinction coefficient at 400 nm over that of protonated molecules. A 50 μl lysosomal sample was added to 2 ml assay medium containing 0.25 M sucrose and 0.1 mM *p*-nitrophenol, pH 6.5. The decrease in the dye absorbance was monitored for 3 min upon addition of lysosomal samples to detect proton leakage in the assay samples. Uptake of AO depends on lysosomal ΔpH [25], quenching of the fluorescence of $DiSC_3(5)$ depends on the internal

potential of lysosomes [12]. A lower ΔpH and a more negative interior potential induced by increased H^+ release can be detected in comparison with normal lysosomes by measuring the decrease in $\Delta A_{492-540\ nm}$ of AO [26] and the quenching of the fluorescence of $DiSC_3(5)$ [12] upon addition of lysosomal samples. A decline in the $\Delta A_{492-540\ nm}$ decrease and an increase in the magnitude of the fluorescence quenching designate a fall in the ΔpH and a decrease in the interior potential, respectively. The medium for AO assay is the same as that of Fig. 1 and contained 2.5 μM AO. The medium for $DiSC_3(5)$ assay is the same as that of Fig. 2. There are no ATP addition in both assays. Similar results were observed in these assays for normal and photodamaged lysosomes, indicating that the photosensitization did not cause a detectable increase in lysosomal H^+ leakage.

3.2. Photodamage to lysosomes decreases electrogenicity of lysosomal H^+-ATPase

A number of investigations have demonstrated that lysosomal H^+-ATPase is electrogenic, which is designated by an elevated internal potential during H^+ pumping [12,26,27]. Lysosomal membrane potential can be measured by carbocyanine dye $DiSC_3(5)$. The negative potential inside lysosomes quenches fluorescence of the dye, while H^+ pumping induced elevation of internal potential

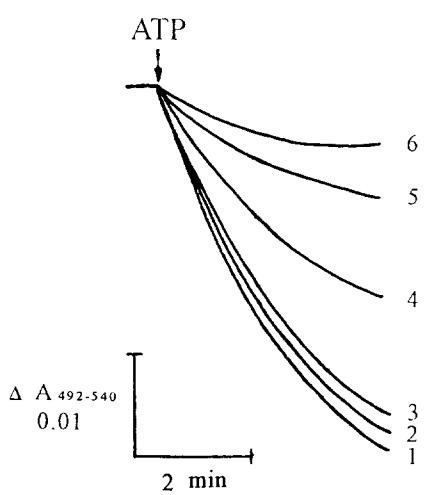

Fig. 1. Loss of Mg-ATP dependent H^+ translocation on photodamaged lysosomes. Medium contained 0.1 M KCl, 1 mM $MgCl_2$, buffered at pH 7.0 with 0.02 M Hepes/Tris. 50 μl lysosomes (1.125 mg protein) was incubated in 2 ml medium for 4 min, followed by addition of AO at a final concentration of 2.5 μM. Then ATP was added at indicated time with 1 mM concentration in the medium. H^+ translocation was monitored by measuring AO differential absorbance ($\Delta A_{492-540\ nm}$). Photosensitive conditions were described in Section 2. Lysosomes were differently prepared before adding to the medium: (1) normal, (2) treated by same amount of MB but no light exposure, (3) light exposed for 8 min in the absence of MB, (4) photosensitized for 4 min, (5) photosensitized for 8 min, (6) medium contained 0.1 mM NEM, lysosomes were not photosensitized. (1–3) are control samples. A typical result out of three experiments is shown.

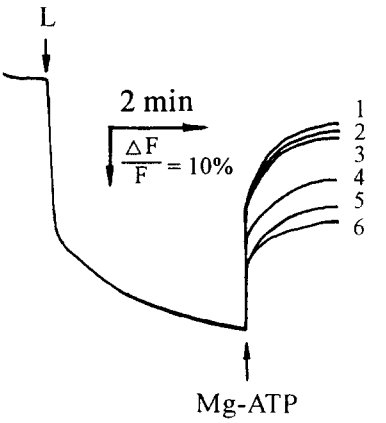

Fig. 2. Effect of photodamage on Mg-ATP depolarized lysosomal membrane potential. Medium contained 0.25 M sucrose and 0.5 μM $DiSC_3(5)$, buffered at pH 7.0 with 0.02 M Hepes/Tris. 10 μl lysosomes (0.225 mg protein) was added to 2 ml medium at indicated time. $MgSO_4$ and ATP were added at the arrow point to a final concentration of 0.5 mM. Fluorescence of $DiSC_3(5)$ was measured with excitation and emission wavelength of 622 and 670 nm, respectively. The fluorescence intensity is expressed as a percentage of its intensity just before addition of the lysosomes (ΔF/F). Preparation of lysosomal samples in (1–6) are the same as that in (1–6) of Fig. 1. A typical result out of three experiments is shown.

increases the fluorescence [12,27]. As shown in Fig. 2, Mg-ATP initiated H^+ pumping increases the fluorescence of $DiSC_3(5)$. Compared with the fluorescence of three control samples (curves 1–3), the magnitude of the fluorescence increase of photosensitized samples decreases in an exposure time dependent manner (curves 4 and 5). It indicates that the electrogenicity of lysosomal proton pump is photodamaged. Addition of NEM caused similar effect (curve 6), suggesting that the decline in electrogenicity of the pump is due to a decrease in H^+ translocation.

3.3. Operation of proton pump promotes lysosomal osmotic stability

It has been reported that lysosomes exhibit a low, but significant, permeability toward K^+ and are less stable in K^+ contained medium than in impermeable sucrose medium [8,12,28]. As shown in Table 1, lysosomes are incubated in the K^+ contained medium. A 60 min incubation causes percentage free activity of β-galactosidase to increase from 8% to 34%, showing an osmotic destabilization of lysosomes. Under the same incubation conditions, operating lysosomal proton pump by Mg-ATP significantly decreases the enzyme free activity to 16%, indicating a promotion of lysosomal osmotic stability. In the presence of 0.1 mM NEM, Mg-ATP loses its protective effect on lysosomes (32% free activity), suggesting the key role of proton pumping in the protection. As shown in the table, NEM alone does not increase the percentage free activity. Therefore, lysosomal integrity is not affected by the reagent under such conditions. In separate experiments,

Table 1
Promotion of lysosomal stability by Mg-ATP dependent H^+ translocation

Incubation time (min)	Addition in incubation medium		Percentage free activity (%)	P * (<)
	ATP	NEM		
0	–	–	8.1 ± 0.6	0.001
60	–	–	33.9 ± 2.6	0.001
60	+	–	15.7 ± 1.9	–
60	+	+	32.2 ± 2.7	0.001
60	–	+	33.4 ± 1.4	0.001

30 μl normal lysosomal sample (0.75 mg protein) was incubated at 37°C for the indicated time in 1 ml medium containing 0.13 M sucrose, 0.05 M K_2SO_4, 1 mM $MgSO_4$ and 0.02 M Hepes/KOH, pH 7.0. Aliquots (50 μl) of the suspension were removed for measurement of β-galactosidase activity as described in Section 2. For initiating H^+ translocation, 25 μl 0.2 M ATP was added to the incubation medium (5 mM final concentration) at 0 min and 30 min of the incubation period, respectively. NEM was added in incubation medium at 0.1 mM final concentration. Values are means ± S.D. of four measurements

* t-Tested against the effect of adding ATP alone.

we examined if 0.1 mM NEM inhibits β-galactosidase. 30 μl lysosomal sample treated by 0.2% Triton X-100 was incubated in the medium of Table 1 for 60 min in the presence of 0.1 mM NEM or not (no ATP addition). After incubation, activity of β-galactosidase was measured by the same method. The results show that 0.1 mM NEM does not inhibit the enzyme in 60 min incubation.

3.4. Photodamage to lysosomes decreases osmotic protection effect of H^+-ATPase

Differently photosensitized lysosomes were incubated in the K^+ contained medium for 60 min, then the activity of β-galactosidase and β-hexosaminidase was measured. As shown in Fig. 3, percentage free activity of two enzymes of unirradiated lysosomes increases by more than 20% in 60 min incubation (compared between left end point of curve 2 and point a), while addition of Mg-ATP to the incubation medium significantly decreases the percentage free activity (compared between left end point of curve 1 and curve 2). It is consistent with the results in Table 1, showing a protection of lysosomal integrity by proton pumping. When Mg-ATP is present in the incubation medium, percentage free activity of photosensitized lysosomes is higher than that of unirradiated lysosomes and increases with prolonging light exposure time (curve 1), showing a loss of protective effect of the pump. It is presumably due to the photodamage induced decrease in proton pumping. In contrast, when the proton pump is not operated by Mg-ATP, prolonging light exposure up to 8 min does not increase the percentage free activity of photosensitized lysosomes (curve 2). It suggests that other photodamage effects on lysosomes, if any, might not be strong enough for decreasing lysosomal latency. The results indicate that lysosomal Mg-ATP dependent proton translocation is sensitive to photodamage and loss of proton pumping impairs lysosomal integrity in K^+ contained medium.

4. Discussion

Cytoplasm contains abundant potassium ions. Although lysosomal membrane shows only a limited permeability toward K^+, constant entry and accumulation of K^+ in lysosomes may cause its osmotic disruption [7–11]. A

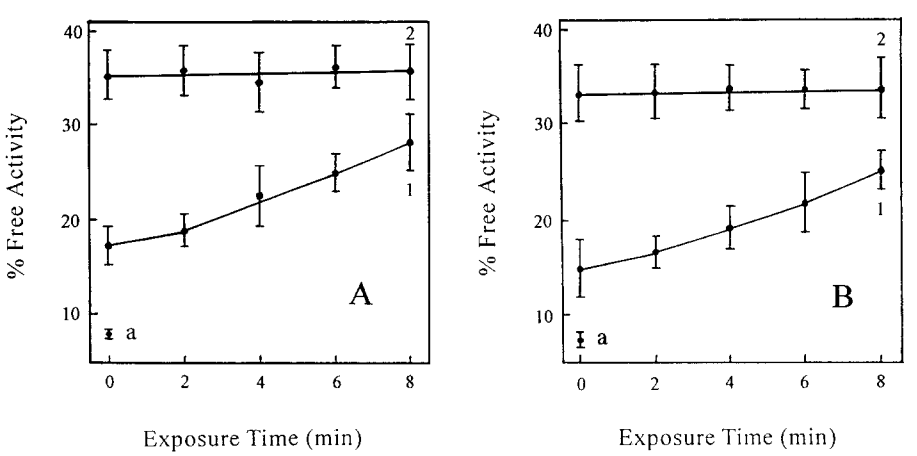

Fig. 3. Effect of photodamage on lysosomal stability in the presence and absence of Mg-ATP. 30 μl lysosomes (0.675 mg protein) photosensitized for indicated time were incubated at 37°C in 1 ml medium containing 0.13 M sucrose, 0.05 M K_2SO_4, 1 mM $MgSO_4$ and 0.02 M Hepes/KOH, pH 7.0. After 60 min incubation, 50 μl suspension was assayed for enzymes free and total activity by the method described in Section 2. (a) Control sample: unirradiated lysosomes with 0 min incubation. (1) 25 μl 0.2 M ATP was added to incubation medium (final concentration of 5 mM) at 0 min and 30 min of the incubation period, respectively. (2) no ATP addition. (A) Percentage free activity of β-galactosidase. (B) Percentage free activity of β-hexosaminidase. Values are means ± S.D. of four measurements. For unirradiated lysosomes (light exposure 0 min), Mg-ATP provided protection (decrease in percentage free activity) was significant at $P < 0.001$ for both (A) and (B). In the presence of Mg-ATP, loss of Mg-ATP provided protection (increase in percentage free activity) induced by 8 min light exposure was significant at $P < 0.001$ in (A) and $P < 0.01$ in (B).

number of investigators suggested that the electrogenicity of lysosomal proton pump may promote osmotic stability of lysosomes by lowering its K^+ content. Although direct evidence has never been obtained due to the experimental difficulties, two possible mechanisms were widely proposed [10–13]. First, depolarization of lysosomal membrane potential via proton pumping provides a means of excluding K^+ from lysosomal interior. Second, elevation of internal potential of lysosomes may electrically prevent K^+ entry. Therefore, lysosomes can be osmotically stabilized by maintaining a relatively lower internal K^+ concentration.

Thiol is susceptible to active oxygen. Enzyme with SH-group in its active center, such as glyceraldehyde-3-phosphate dehydrogenase, can be inactivated by photooxidation [14]. Since lysosomal H^+-ATPase is strongly inhibited by sulfhydryl reagent NEM due to its essential thiol group, the enzyme is presumably prone to photooxidation and loses its function. Furthermore, photooxidation may probably modify the microenvironment of lysosomal H^+-ATPase, therefore decreasing the enzyme activity.

As shown in this study, Mg-ATP dependent proton translocation is photodamageable (Fig. 1) and loss of proton pumping impairs lysosomal integrity (Fig. 3, curve 1). There seems no other photodamage effects which may decrease lysosomal latency (Fig. 3, curve 2). However, a number of investigations emphasized the role of membrane lipid peroxidation in lysosomal disruption [29–31], although the mechanism has not been elucidated. In addition, photodamage to membrane is generally linked to increased ion permeability [32,33]. To confirm the implication of Fig. 3 (curve 2), we examined whether these alterations were produced on lysosomes under the conditions of this study. Lysosomal lipid peroxidation products LOOHs and TBARS were measured by the iodometric method of Girotti et al. [34] and by the method of Ohkawa et al. [35], respectively. Lysosomal permeability to K^+ was assessed using $DiOC_6(3)$ to monitor membrane potential upon addition of K_2SO_4 [12,36,37] and assessed using osmotic protection method [28,38]. There are no detectable increased K^+ permeation and lipid peroxidation when lysosomes are photosensitized from 0 min to 8 min, while Mg-ATP dependent proton translocation decreases under the same conditions (Fig. 1). Since photodamage effects are generally dependent on the sensitizer dose, light dose, photosensitizing property of sensitizers, photosensitivity of biological targets and other conditions [1–3], it is likely that the photosensitive condition of this study (0.01 mM MB, light exposure 8 min, light intensity 5 mW/cm^2) is not strong enough for producing the alterations. When lysosomes were photosensitized under potentiated condition (0.1 mM MB, light exposure 30 min, light intensity 10 mW/cm^2), we detected significantly increased K^+ permeation, lipid peroxidation and lysosomal disintegration. Compared with lysosomal K^+ permeability and membrane lipid, Mg-ATP dependent proton translocation might be more sensitive to photodamage. Similarly, some membrane structures and functions of mitochondria also exhibit differential sensitivity to photodamage [39]. H^+-ATPase mediated proton transport is photodamaged more easily than some other targets on mitochondria. Photodestruction of the targets is also sensitizer dose and light dose dependent.

Finally, some sensitizers mediated photosensitization causes photodamage to mitochondria [39,40]. The decrease of ATP synthesis induces decline in cytoplasmic ATP level [41–43]. In such case, lysosomal proton pump is probably not in full operation due to insufficient ATP supply. It may also decrease the osmotic protection effect of the pump.

The major purpose of this study is to propose a new mechanism of photodamaged lysosomal disintegration. Biochemical analysis on photooxidation of lysosomal H^+-ATPase remains for further investigation.

Acknowledgements

We would like to thank professors Wang, Zhi-Xin and Li, Qi-Long for their many helpful suggestions.

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