The influence of oxidation of membrane thiol groups on lysosomal proton permeability

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The influence of oxidation of membrane thiol groups on lysosomal proton permeability was studied by measuring lysosomal pH with FITC-conjugated dextran, determining the membrane potential with 3,3′-dipropylthiadicarbocyanine iodide and monitoring their proton leakage with *p*-nitrophenol. Residual membrane thiol groups were measured with 5,5′-dithiobis-(2-nitrobenzoic acid). The lysosomal membrane thiol groups were modified by treatment with diamide and dithiothreitol. SDS/PAGE revealed aggregations of the membrane proteins induced by the treatment of lysosomes with diamide. The cross-linkage of proteins could be abolished by subsequent treatment with dithiothreitol, indicating that the proteins were linked via disulphide bonds. Treating the lysosomes with diamide decreased their membrane thiol groups and caused increases in lysosomal pH, membrane potential and proton leakage, which could be reversed by treatment of the lysosomes with dithiothreitol. This indicates that the lysosomal proton permeability can be increased by oxidation of the membrane thiol groups and restored to the normal level by reduction of the groups. Treatment of the lysosomes with *N*-ethylmaleimide reduced their membrane thiol groups but did not change the lysosomal pH or their degree of proton leakage. It suggests that protein aggregation may be an important mechanism for the increase in lysosomal proton permeability. The results raise the possibility that the proton permeability of lysosomes *in vivo* may be affected by the redox states of their membrane thiol groups.

Key words: lysosomal pH, membrane potential, proton leakage.

INTRODUCTION

The acidic interior of lysosomes is favourable for the activities of their various acidic hydrolases which participate in the physiological turnover of cellular macromolecules [1,2]. The pH gradient of lysosomes plays an important role in some carrier-mediated transport across their membranes [3]. So far it is widely accepted that the maintenance of intralysosomal pH *in vivo* may be due to the dynamic equilibrium between the influx of protons by H^+-ATPase, their efflux in exchange for the monovalent cations in the cytoplasm [1] and a Donnan equilibrium [4]. Since lysosomes *in vivo* are surrounded by a high concentration of cytoplasmic K^+ (140 mM) [5], the opposing transmembrane concentration gradients of H^+ and K^+ can drive an exchange of lysosomal H^+ for external K^+, resulting in an elevation of the intralysosomal pH. Moreover, the H^+/K^+ exchange may osmotically destabilize lysosomes by the entry of K^+ [2,6–8]. In addition to the effects on the acidification and osmotic stability of lysosomes, the outward diffusion of lysosomal protons, which must be accompanied by charge-compensating migrations of either cations or anions, may play a role in lysosomal ion homoeostasis. There are many lysosomal transport systems that have been shown to be sensitive to the transmembrane pH gradient, including cystine, cysteine, vitamin B_{12}, sulphate and calcium [9]. An increase in lysosomal proton permeability, which decreases the pH gradient by proton leakage, can affect these transport and other lysosomal activities. The above evidence indicates that the membrane permeability to protons is an important property of lysosomes. In normal cases, the lysosomal membrane is relatively impermeable to H^+ compared with its permeability to some monovalent cations such as Cs^+, K^+ and Na^+ [2,6], but exhibits an increased permeability towards H^+ after the lysosomes are photodamaged [7] or treated by the anti-neoplastic drug lonidamine [10]. Recently [11] we established that the physical state of membranes can influence lysosomal proton permeability, but what lysosomal membrane structures can regulate their proton permeability is unknown.

Thiol groups are ubiquitous in the membranes of living cells. These groups are of great importance for a variety of membrane functions, such as the transport by simple channels of water and urea, gated chloride-dependent K^+ channel flux and transport by a variety of mobile carriers for glucose, nucleosides, choline, phosphate, amino acids, fatty acids, anions and cations [12]. In addition, the redox states of the thiol groups of membranes are crucially involved in the maintenance of their barrier functions such as membrane permeability [13]. Oxidation of membrane thiol groups can cause a loss of the barrier function. In living cells, superoxide radical anions and hydrogen peroxide are physiologically produced as normal metabolites, which can oxidize lysosomal membrane components [14,15]. As demonstrated previously [16], lysosomal membranes are enriched in thiol groups. It is of interest to establish whether the redox states of membrane thiol groups of lysosomes affect their proton permeability. In the past, the thiol reagent diamide was used widely to study the effects of thiol groups on some membrane properties such as ion permeability [13]. Diamide is a highly specific thiol oxidant, and this precludes selective interactions with other membrane component [17]. In this study, we used diamide and dithiothreitol (DTT) to oxidize and reduce the lysosomal membrane thiol groups, respectively, and examined the resulting changes in lysosomal proton permeability. The

Abbreviations used: $DiSC_3(5)$, 3,3′-dipropylthiadicarbocyanine iodide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; NEM, *N*-ethylmaleimide; NTB, 2-nitro-5-thiobenzoic acid.

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results show that the proton permeability of lysosomes can be increased and decreased by respectively oxidizing and reducing their membrane thiol groups.

EXPERIMENTAL

Materials

3,3′-Dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] was purchased from Molecular Probes (Eugene, OR, U.S.A.); diamide, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), DTT, FITC-conjugated dextran (average molecular mass, 71200 Da), SDS, *N*-ethylmaleimide (NEM), acrylamide, *N*,*N*′-methylenebisacrylamide, 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide and Hepes were from Sigma (St. Louis, MO, U.S.A.). The other chemicals used were of analytical grade from local sources.

Preparation of lysosomes and modulation of lysosomal membrane thiol groups

Male Wistar rats were used. Normal liver lysosomes and lysosomes loaded with FITC-conjugated dextran were prepared by the method of Ohkuma et al. [18]. All procedures were carried out at 0–4 °C. Lysosomes were resuspended in 0.25 M sucrose medium at 21.3 mg of protein/ml. Protein was determined by the method of Lowry et al. [19].

Lysosomal membrane thiol groups were modified by the treatment of lysosomes with diamide and DTT. To oxidize the thiol groups, the lysosomes were incubated (10.65 mg of protein/ml) in the presence of diamide at the indicated concentration for the indicated period. Reduction of the oxidized thiol groups was accomplished by subsequent incubation in the presence of 25 mM DTT for 10 min. The incubations were carried out at 0 °C.

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 disulphide bond formed between the two halves of DTNB is replaced by another disulphide bond formed from the thiol of the protein. Thus measurement of the absorbance of NTB indicates whether a reaction has taken place with a thiol group [20]. A stock solution of 1 mM DTNB in 100 mM PBS (pH 7.4) was prepared on the day of the experiment. The lysosomal samples were ruptured and washed three times with 2 ml of cold water. To solubilize the membranes, the pellet was resuspended in 80 μl of 2% SDS for 2 min, and then 80 μl of 0.25 M sucrose was added. For labelling, the lysosomal suspension was mixed with 1.44 ml of 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 calculation of the thiol content/mg of protein, glutathione was used as a reference substance.

SDS/PAGE

Lysosomal membranes were prepared as described in the previous section. Membrane proteins were dispersed thoroughly in 5% SDS solution and used for electrophoresis. 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 70 μg/lane. Staining was accomplished with Coomassie Brilliant Blue R-250.

Measurement of lysosomal pH

According to the method of Ohkuma et al. [18], rats were injected intraperitoneally with FITC-conjugated dextran (20 mg of FITC-conjugated dextran/150 g of body weight), starved for 12 h and then decapitated. The lysosomes loaded with FITC were also prepared as described in [18]. Fluorescence was measured at 25 °C with excitation and emission wavelengths of 495 and 550 nm, respectively, on a Hitachi 850 fluorescence spectrophotometer. Lysosomal 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. [18].

Measurement of lysosomal membrane potential

The recording of membrane diffusion potential provides a means of probing the proton permeability of some membranes [21]. The proton permeability of lysosomes can be assessed by measuring their membrane potential using the carbocyanine dye $DiSC_3(5)$ as a probe [22,23]. The assay medium contained 0.25 M sucrose/0.5 μM $DiSC_3(5)$, buffered at pH 7.0 with 0.02 M Hepes/Tris. A 2.5–5 μl lysosomal sample was used for the assay. The fluorescence measurements were carried out at 25 °C with excitation and emission wavelengths of 622 and 670 nm, respectively, on a Hitachi 850 fluorescence spectrophotometer.

Measurement of lysosomal proton leakage

Lysosomal proton leakage can acidify the surrounding medium. The acidification of assay medium by proton leakage was measured as described previously [7]. Briefly, a lysosomal sample was added to 2 ml of assay medium (containing 0.25 M sucrose and 0.1 mM *p*-nitrophenol) at 0.479 mg of protein/ml. The absorbance (400 nm) of the pH-sensitive dye *p*-nitrophenol was measured immediately. All measurements were carried out at 25 °C on a Hitachi U-3200 spectrophotometer.

Assay of lysosomal latency

Lysosomal latency was assessed by measuring the activity of lysosomal β-hexosaminidase using 2 mM 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide as the substrate [8]. The liberated 4-methylumbelliferone was determined by measuring the fluorescence (excitation, 365 nm; emission, 444 nm) on a Hitachi 850 fluorescence spectrophotometer.

The diamide-treated lysosomes and control samples were incubated in either 0.25 M sucrose medium or 0.125 M K_2SO_4 medium (buffered at pH 7.0 with Hepes/KOH) at 37 °C for 30 min. After the incubation, a 50 μl portion of the lysosomal suspension (10.65 mg of protein/ml) was used for the assay of lysosomal latency. The activities of the enzyme measured in the absence and presence of Triton X-100 were designated the free activity and the total activity, respectively. The 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 latency was determined by increases in the percentage free activity.

RESULTS

Modulation of lysosomal membrane thiol groups

The effects of diamide-induced oxidation on the thiol groups of lysosomal membrane proteins were observed by measuring the residual thiol group content with DTNB. As shown in Table 1, lysosomal membrane thiol groups decreased with increasing

Table 1 Effects of diamide and DTT treatments on the membrane thiol groups of lysosomes

Lysosomes were treated with diamide as indicated. To reduce the oxidized membrane thiol groups, a subset of diamide-treated lysosomes was treated subsequently with 25 mM DTT for 10 min. All treatments were carried out at 0 °C. Membrane thiol groups were measured immediately after treatment. All procedures were as described in the Experimental section. Residual thiol groups were expressed as a percentage of the control value. Values are means ± S.D., $n = 3$ (except for *, where $n = 10$).

Treatment	Residual thiol groups (nmol of thiol/mg of protein)	Residual thiol groups (%)
Control	102.02 ± 1.88*	100
10 mM Diamide (5 min)	91.1 ± 4.28	89.3 ± 4.2
10 mM Diamide (10 min)	76.62 ± 3.06	75.1 ± 2.9
10 mM Diamide (20 min)	64.17 ± 3.06	62.9 ± 3.0
10 mM Diamide (20 min) then 25 mM DTT (10 min)	90.29 ± 1.84	88.5 ± 1.8
Control	102.02 ± 1.88*	100
5 mM Diamide (10 min)	89.17 ± 2.24	87.4 ± 2.2
10 mM Diamide (10 min)	76.62 ± 3.06	75.1 ± 2.9
15 mM Diamide (10 min)	68.05 ± 2.45	66.7 ± 2.4
15 mM Diamide (10 min) then 25 mM DTT (10 min)	95.59 ± 2.75	93.7 ± 2.7

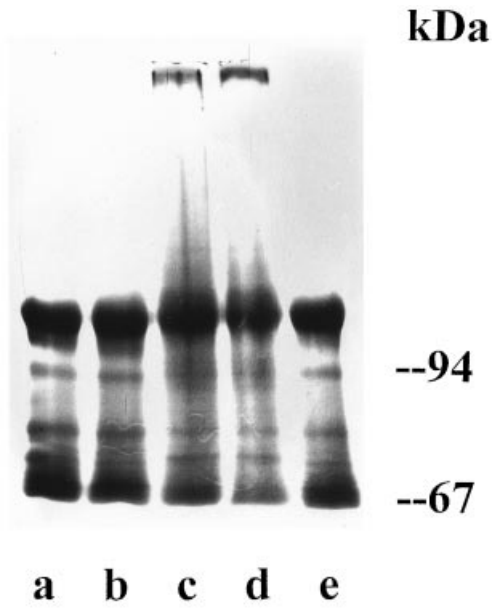

Figure 1 Diamide-induced cross-linkage of lysosomal membrane proteins

SDS/PAGE of lysosomal membrane proteins and other treatment conditions are described in the Experimental section. Lane a, control; lanes b–e, lysosomal samples treated with: (b) 15 mM diamide for 10 min and then 25 mM DTT for 10 min; (c) 15 mM diamide for 10 min; (d) 10 mM diamide for 20 min; (e) 10 mM diamide for 20 min and then 25 mM DTT for 10 min. Protein was loaded at 70 μg/lane. A typical result from three measurements is shown. The markers shown are albumin (67 kDa) and phosphorylase *b* (94 kDa).

duration and concentration of diamide treatment. To examine whether this effect was caused by oxidative action, the lysosomes treated with diamide were subsequently incubated with the thiol-reducing reagent DTT. The results show that treatment of the diamide-treated lysosomes with DTT restored their decreased thiol groups to a considerable extent. It thus confirms the oxidative effect of diamide on the membrane thiol groups.

Additional evidence in favour of this conclusion came from striking oxidation-induced changes in the SDS/PAGE pattern of lysosomal membrane proteins. As shown in Figure 1, several bands of low-molecular-mass polypeptide appeared in the lane containing the control sample (lane a). The treatment of lysosomes with diamide at either 15 mM for 10 min (Figure 1, lane c) or 10 mM for 20 min (Figure 1, lane d) abolished most of the bands and produced large aggregates near the origin of lanes. This suggested that diamide treatment caused the cross-linkage of lysosomal membrane proteins. To examine whether the aggregated proteins in Figure 1, lanes c and d, were linked via disulphide bonds, two duplicates of the diamide-oxidized lysosomal samples were treated with DTT prior to the electrophoresis. As shown in Figure 1, lanes b and e, treatment of diamide-treated lysosomes with DTT abolished the large aggregates in lanes c and d, and restored the original gel pattern of the control sample (lane a). This confirms that the linkage of the proteins in Figure 1, lanes c and d, was caused by oxidation of the lysosomal membrane thiol groups, i.e. via the formation of disulphide bonds.

Table 2 Measurement of lysosomal pH with FITC-conjugated dextran

Lysosomes suspended in 0.25 M sucrose medium (10.65 mg of protein/ml) were exposed to diamide and DTT as indicated. The treated and control lysosomal samples (21.3 μg of protein/ml) were then incubated in 2 ml of 0.25 M sucrose medium buffered at pH 7.0 with 20 mM Hepes/Tris. Lysosomal pH was measured at the indicated times. All procedures were as described in the Experimental section. Values are means ± S.D., $n = 3$.

	Lysosomal pH	
Treatment	0 min	2 min
Control	4.95 ± 0.01	4.98 ± 0.01
10 mM Diamide (5 min)	4.97 ± 0.01	5.07 ± 0.01
10 mM Diamide (10 min)	4.97 ± 0.01	5.16 ± 0.01
10 mM Diamide (20 min)	4.98 ± 0.01	5.22 ± 0.01
10 mM Diamide (20 min) then 25 mM DTT (10 min)	4.97 ± 0.01	5.00 ± 0.01
Control	4.95 ± 0.01	4.98 ± 0.01
5 mM Diamide (10 min)	4.97 ± 0.01	5.07 ± 0.01
10 mM Diamide (10 min)	4.97 ± 0.01	5.16 ± 0.01
15 mM Diamide (10 min)	4.97 ± 0.01	5.18 ± 0.01
15 mM Diamide (10 min) then 25 mM DTT (10 min)	4.97 ± 0.01	4.99 ± 0.01

Effects of the oxidation of membrane thiol groups on lysosomal pH

The influence of oxidation of membrane thiol groups on lysosomal proton permeability was studied by measuring the lysosomal pH with FITC. As shown in Table 2, control lysosomes maintained their internal pH at about 4.95 during a 2 min incubation in sucrose medium, which is similar to the value provided by Ohkuma et al. [18]. The diamide-treated lysosomes exhibited

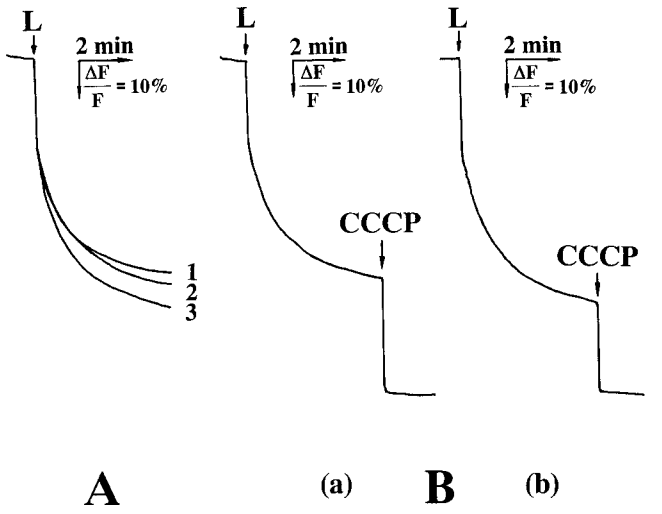

Figure 2 Effects of the duration of diamide treatment on lysosomal membrane potential

Procedures for the measurement of lysosomal membrane potential and treatment of lysosomes with diamide were as described in the Experimental section. The assay medium contained 0.25 M sucrose and 0.5 μM $DiSC_3(5)$, buffered at pH 7.0 with 0.02 M Hepes/Tris. The lysosomal sample (L) and CCCP (2 μM) were added to the medium as indicated. Fluorescence of $DiSC_3(5)$ was measured at 25 °C with excitation and emission wavelengths of 622 and 670 nm, respectively. The fluorescence intensity is expressed as a percentage of its intensity just before addition of the lysosomes ($\Delta F/F$). Lysosomes were treated with 10 mM diamide for the following times. (**A**) Curve 1, 0 min (control); curve 2, 5 min; curve 3, 20 min; (**B**) curve a, 0 min (control); curve b, 20 min. A typical result from three experiments is shown.

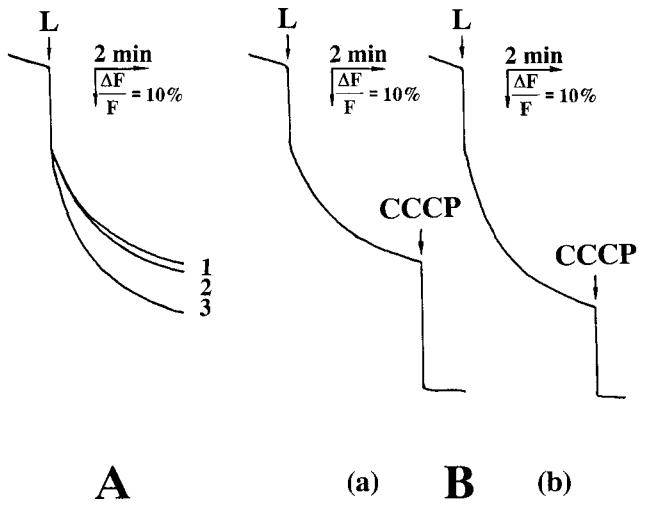

Figure 3 Effects of diamide concentration on lysosomal membrane potential

All experimental details were as for Figure 2. Lysosomes were treated with the following concentrations of diamide for 10 min. (**A**) Curve 1, 0 mM (control); curve 2, 5 mM; curve 3, 15 mM; (**B**) curve a, 0 mM (control); curve b, 15 mM. A typical result from three experiments is shown.

a similar pH to the controls at the start of the incubation and a higher pH after the incubation. The increases in the lysosomal pH (the maximal increase was about 0.2 pH units) during the incubation correlated with either the duration of diamide treatment or the diamide concentration, depending on which variable was changed. The elevation of lysosomal pH by diamide treatment indicates the occurrence of proton leakage. Treating the diamide-treated lysosomes with DTT abolished the pH increase that occurred during incubation, suggesting that the elevation of lysosomal pH, i.e. an increase in lysosomal proton permeability, was caused by oxidation of the membrane thiol groups.

Effects of oxidation of membrane thiol groups on lysosomal membrane potential

Lysosomal proton permeability can be assessed by measuring lysosomal membrane potential using the carbocyanine dye $DiSC_3(5)$ as a probe [22]. This positively charged dye accumulates within the lysosome, which is electrically negative inside with respect to the outside, leading to quenching of the dye's fluorescence. An increase in the fluorescence quenching of the dye, indicating that the lysosomal interior has become more negative, will be observed when the lysosomal proton permeability is increased. Effects of the modulation of membrane thiol groups on the proton permeability of lysosomes were assessed using this method. As shown in Figure 2(A), the decrease in dye fluorescence of lysosomal samples was postively correlated with the length of diamide treatment. Also, dye fluorescence quenching increased with increasing diamide concentration (Figure 3A). Based on the results in Table 1, this suggests that the proton permeability of the lysosomes increased with the oxidation of their membrane thiol groups.

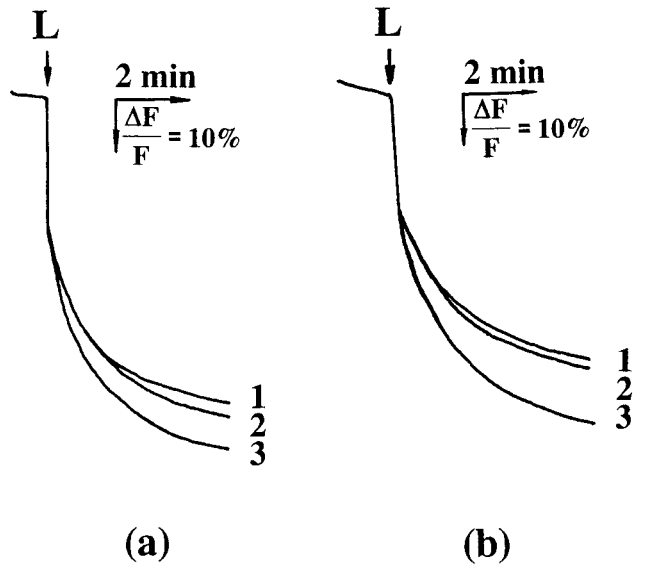

Figure 4 Effect of DTT treatment on the membrane potential of diamide-treated lysosomes

The assay medium contained 0.25 M sucrose and 0.5 μM $DiSC_3(5)$, buffered at pH 7.0 with 0.02 M Hepes/Tris. The lysosomal sample (L) was added to the medium as indicated. $DiSC_3(5)$ fluorescence was measured and expressed as described in Figure 2. Treatments of lysosomes were as follows: (**a**) curve 1, no treatment (control); curve 2, 10 mM diamide for 20 min, then 25 mM DTT for 10 min; curve 3, 10 mM diamide for 20 min; (**b**) curve 1, no treatment (control); curve 2, 15 mM diamide for 10 min, then 25 mM DTT for 10 min; curve 3, 15 mM diamide for 10 min. Procedures for the lysosome treatments were as described in the Experimental section. A typical result from three experiments is shown.

The effect of diamide treatment on the lysosomal proton permeability was further established by using protonophores in the fluorescence measurements. Since protonophores such as CCCP and carbonyl cyanide *p*-trifluoromethoxyphenylhydra-

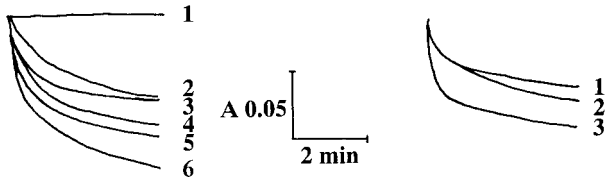

Figure 5 Effect of diamide treatment for different times and the reversing effect of DTT treatment on lysosomal proton leakage

The lysosomal sample was added to 2 ml of assay medium (containing 0.25 M sucrose and 0.1 mM *p*-nitrophenol) at 0.479 mg of protein/ml. Absorbance (400 nm) of *p*-nitrophenol was measured immediately. Left-hand panel, lysosomes were treated with 10 mM diamide for the following times. Curve 1, 20 min, assay medium pH was maintained at 6.0 with 0.1 M citrate buffer; curve 2, 0 min (control); curve 3, 5 min; curve 4, 10 min; curve 5, 20 min; curve 6, 0 min (control), assay medium contained 1 μM CCCP. Right-hand panel, treatments of lysosomes were as follows. Curve 1, no treatment (control); curve 2, 10 mM diamide for 20 min, then 25 mM DTT for 10 min; curve 3, 10 mM diamide for 20 min. Procedures were as described in the Experimental section. A typical result from three experiments is shown.

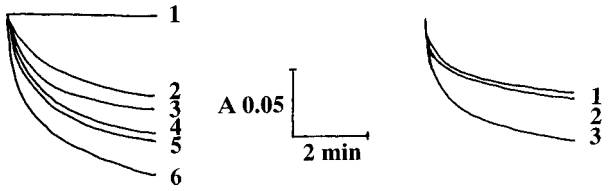

Figure 6 Effect of diamide concentration and the reversing effect of DTT treatment on lysosomal proton leakage

Assay details were as for Figure 5. Left-hand panel, lysosomes were treated with the following concentrations of diamide for 10 min. Curve 1, 15 mM, assay medium pH was maintained at 6.0 with 0.1 M citrate buffer; curve 2, 0 mM (control); curve 3, 5 mM; curve 4, 10 mM; curve 5, 15 mM; curve 6, 0 mM (control), assay medium contained 1 μM CCCP. Right-hand panels, lysosomes were treated as follows. Curve 1, 0 mM diamide for 10 min (control); curve 2, 15 mM diamide for 10 min, then 25 mM DTT for 10 min; curve 3, 15 mM diamide for 10 min. Procedures were as described in the Experimental section. A typical result from three experiments is shown.

zone (FCCP) can make the lysosomal membrane permeable to protons and produce an equilibrium membrane potential for the protons, an additional decrease in the fluorescence of $DiSC_3(5)$ can be produced by these agents during the measurement of lysosomal membrane potential. The total fluorescence quenching of the dye, i.e. the sum of the fluorescence quenching before and after addition of protonophores, indicates the proton permeability of the H^+-permeable membrane, or the equilibrium membrane potential for protons [22]. As shown in Figures 2(B) and 3(B), the fluorescence decrease in the diamide-treated lysosomal sample (curves b) was larger than that of the control sample (lines a) in the absence of CCCP. However, the magnitude of CCCP-induced additional fluorescence quenching of the former sample was smaller than that of the latter. The total magnitudes of fluorescence decrease were similar, although the extents of fluorescence quenching of these two samples were different before and after adding CCCP. The results indicate that diamide treatment did not alter the CCCP-induced lysosomal permeability to protons but increased the intrinsic proton permeability of the organelle and made it approach that induced by CCCP.

Based on the results shown in Table 1, the effect of the diamide treatment on lysosomal proton permeability was presumably due to oxidation of the membrane thiol groups. To examine whether this conclusion is correct, the diamide-oxidized lysosomes were treated subsequently with DTT. The magnitude of dye fluorescence quenching of the diamide-treated lysosomes at either 10 mM for 20 min (Figure 4a, curve 3) or 15 mM for 10 min (Figure 4b, curve 3) was decreased by DTT treatment (Figures 4a and 4b, curves 2). This indicates that the proton permeability of the diamide-treated lysosomes was decreased by DTT treatment. Thus the effect of oxidation of the membrane thiol groups on lysosomal proton permeability can be confirmed.

Effects of oxidation of lysosomal membrane thiol groups on proton leakage

Since ion flux through a membrane is proportional to the ion permeability [24], lysosomal proton permeability can be studied by measuring proton-efflux-induced acidification of the lysosomes suspension medium with the pH-sensitive dye *p*-nitrophenol as an indicator [7]. The measurements were carried out by monitoring the decrease in *p*-nitrophenol absorbance at 400 nm, based on the property of the dye that the unprotonated *p*-nitrophenol molecules have a sufficiently greater absorption coefficient at 400 nm than protonated molecules [25]. The conclusion obtained from the results shown in Table 2 and Figures 2–4 was re-examined by measuring the proton leakage of diamide-treated lysosomes using *p*-nitrophenol as an indicator. The dye absorbance of diamide-treated lysosomal samples decreased with treatment duration (Figure 5, left-hand panel, curves 3–5) and increasing diamide concentration (Figure 6, left-hand panel, curves 3–5). Addition of CCCP to the medium caused a significant reduction in absorbance (Figures 5 and 6, left-hand panels, curves 6). In addition, the decrease in absorbance of diamide-treated samples could be abolished by buffering the medium (Figures 5 and 6, left-hand panels, curves 1). The results indicate that the decrease in dye absorbance was due to a proton-leakage-induced acidification of the lysosomal suspension and suggest that the lysosomal proton permeability increased by oxidation of the membrane thiol groups.

The above conclusion was examined by treatment of lysosomes with DTT. Treating the lysosomes with 10 mM diamide for 20 min (Figure 5, right-hand panel) or 15 mM diamide for 10 min (Figure 6, right-hand panel) decreased the dye absorbance (Figures 5 and 6, right-hand panels, compare curve 3 with curve 1 in each Figure). The magnitude of the absorbance decrease of the diamide-treated lysosomal sample was reduced markedly when the lysosomes were treated subsequently with DTT (Figures 5 and 6, right-hand panels, curves 2). The reversal of the diamide-induced proton leakage by DTT treatment confirmed the effect of oxidation of membrane thiol groups on lysosomal proton permeability.

Effects of alkylation of membrane thiol groups on lysosomal proton permeability

To determine whether the diamide-induced increase in lysosomal proton permeability was due to either the loss of thiol groups or protein aggregations, we used NEM to alkylate the lysosomal membrane thiol groups and examined proton permeability. Treating lysosomes with 0.6 mM NEM for 30 min reduced the thiol groups by 30% (similar to the extent of thiol-group loss produced by diamide treatment, see Table 1), but the lysosomal pH and the level of lysosomal proton leakage did not change (results not shown). This suggests that protein aggregation may be an important mechanism for the increase in lysosomal proton permeability.

Effects of oxidation of lysosomal membrane thiol groups on lysosomal latency

Lysosomal enzyme latency refers to the percentage of intact lysosomes as revealed by the inability of a substrate to reach the enzymes until the organelles are deliberately ruptured [9]. The effects of oxidation of membrane thiol groups on the lysosomal latency were observed by measuring the latency of the lysosomal marker enzyme β-hexosaminidase. The lysosomes (10.65 mg of protein/ml) were treated with 10 mM diamide for 20 min. Then both the diamide-treated lysosomes and control samples were incubated in either 0.25 M sucrose medium or 0.125 M K_2SO_4 medium (buffered at pH 7.0 with Hepes/KOH) at 37 °C for 30 min. After the incubation, the lysosomal latency was examined. The diamide-treated and control lysosomes exhibited the same percentage of free enzyme activity (13 %) after incubation in sucrose medium. This indicates that lysosomal enzyme latency was not affected by diamide treatment if the lysosomes were suspended in isotonic sucrose medium. In contrast, percentage free enzyme activity of the diamide-treated lysosomes increased by 8 % above control samples after the incubation in K_2SO_4 medium. Thus the oxidation of membrane thiol groups slightly increased the loss of lysosomal latency in the K^+-containing medium. Taken together, the above results suggest that the proton permeability of lysosomes can be regulated by the redox states of their membrane thiol groups.

DISCUSSION

Thiol groups play an important role in a variety of activities of cell membranes. In the past few years, clarifying the influence of various factors on membrane thiol groups, the effects of thiol groups on membrane activities and the related mechanisms has continued to be an active area of investigation. In this respect, erythrocyte membranes have been studied extensively [13]. Membrane thiol groups are liable to be modified by oxidants or alkylating agents. Alkylation or oxidation of thiol groups of erythrocyte membranes can increase their permeability to ions such as K^+, Na^+ and Cl^-, indicating that membrane permeability to these ions is correlated with membrane thiol groups [13]. Until now, little information has been available concerning the influence of membrane thiol groups on proton permeability. For lysosomes, it is less known what factor and/or component of the membranes affect their proton permeability. The results of this study suggest that the redox states of membrane thiol groups may modulate lysosomal proton permeability. It provides some insights into which properties of the lysosomal membrane are correlated with lysosomal proton permeability.

Thiol groups are also of importance to the transport of protons via the lysosomal H^+-ATPase for the MgATP-driven proton pump, which was found to be a kind of thiol enzyme [18]. Lysosomal H^+-ATPase is sensitive to thiol reagents like NEM. The sensitivity of lysosomal H^+-ATPase to NEM is often used to help identify it and to discriminate it from others such as the ATPase of mitochondria [26]. A previous study of ours [23] showed that lysosomal H^+-ATPase was prone to lose its function by photo-oxidation due to its essential thiol groups. In addition, the photo-oxidation of lysosomal membranes may modify the microenvironment of the enzyme, therefore decreasing its activity [23]. At present, little information is available concerning the influence of oxidative stress *in vivo* on lysosomal H^+-ATPase. Whether the redox states of thiol groups of the enzyme can regulate its activity *in vivo* is still unclear. The elucidation of this issue is important for a better understanding of lysosomal acidification.

As described by Brunk and Terman [14], oxidative stress inevitably occurs in most tissues as a result of normal oxygen metabolism. The reactive oxygen species such as superoxide radical anions and hydrogen peroxide are produced mainly by mitochondria in living cells. Excess production of reactive oxygen is toxic, causing membrane damage and activating pathways of cell death. Lysosomes contain traces of heavy metals such as iron, which can catalyse oxidative reactions intralysosomally when cellular oxidative stress diffuses into lysosomes. Moreover, catalases and superoxide dismutases are possibly absent in lysosomes [27]. Thus the intracellular formation of significant quantities of reactive oxygen would result in oxidative damage to lysosomes [28,29]. This raises the possibility that lysosomal proton permeability may be affected under such conditions. As proposed previously, the enhancement of lysosomal proton permeability can elevate lysosomal pH by proton leakage, which may cause cell death by a decrease in lysosomal degradative capacity [14]. In addition, the leakage of lysosomal protons can destabilize the lysosomal osmotic balance by H^+/K^+ exchange. The lysis of lysosomes may cause cell apoptosis and necrosis by the leakage of hydrolases [14]. The results of this work confirm that oxidation of membrane thiol groups can increase lysosomal proton permeability, which may have some significance for studies of lysosomal pathophysiology.

The maintenance of lysosomal integrity is of the utmost importance for their function. It was of interest to examine whether oxidation of membrane thiol groups affected lysosomal enzyme latency. The results show that the lysosomal enzyme latency was not affected by diamide treatment if the lysosomes were suspended in isotonic sucrose medium. The latency loss of the diamide-treated lysosomes in the K_2SO_4 medium was caused presumably by an exchange of external K^+ for the intralysosomal H^+. The increase in lysosomal proton permeability by the oxidation of membrane thiol groups may promote such an exchange. It suggests that oxidizing membrane thiol groups might osmotically destabilize the lysosomes in cytoplasm, which contains abundant K^+.

In this work, we used diamide and DTT to oxidize and reduce lysosomal membrane thiol groups, respectively. Diamide, first used by Kosower et al. [30], is a highly specific thiol reagent and mild oxidant. It is well known that active oxygen can oxidize not only membrane thiol groups but also other membrane components such as membrane lipids. Since the products of membrane lipid peroxidation can increase proton permeability [31], it is difficult to determine whether oxidation of membrane thiol groups also affects proton permeability when active oxygen is used as an oxidant. For this reason, diamide is used widely to study membrane thiol groups instead of active oxygen. Thus the effect of membrane lipid peroxidation on proton permeability can be ruled out in this study.

The measurement of lysosomal pH reflects the fact that oxidation of lysosomal membrane thiol groups influences their permeability to protons. As pointed out by Ohkuma [1], the pH within isolated lysosomes gradually increases (0.1–0.2 units/4 min) in salt medium containing 100 mM KCl by K^+/H^+ exchange. In sucrose medium, the lysosomal pH rises much more slowly. As shown in Table 2, treating lysosomes with diamide elevated their internal pH by 0.24 units in 2 min in sucrose medium, which is a larger increase in pH compared with the changes seen in salt medium [1].

It is believed that the water permeability of a membrane has much correspondence with its proton permeability. The precise mechanism for such a correlation is still not clarified, but some plausible explanations have been provided. As proposed by Deamer and Nichols [32], proton permeation involves the

transient formation of hydrogen-bonded chains of water within the membrane. A small fraction of the water in the bilayer might be associated through hydrogen bonding, thereby providing a conductance pathway unique to protons. At least four pathways or mechanisms for the flux of water across membranes have been proposed, including aqueous pores [33], the solubility–diffusion mechanism [33], channels [34] and transient defects in the membrane arising from thermal fluctuation [24]. The permeation of protons across membranes is suggested to be along the hydrogen bonds of water crossing the membranes through aqueous pores [35] and transient defects [24]. Membrane thiol groups may be an important determinant of water permeability, a notion 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 [36,37]. We recently established that lysosomal osmotic sensitivity can be enhanced by photo-oxidation of their membrane thiol groups (F.-Y. Wan, L. Yang, Y.-G. Zhong, W. Zhu, Y.-N. Wang and G.-J. Zhang, unpublished work). Another study showed that oxidating erythrocyte membranes could increase the size of the aqueous pores [17]. This evidence suggest that changes in redox states of membrane thiol groups may alter their water permeability and therefore affect their proton permeability. Why redox states of membrane thiol groups can affect water permeability is still unclear. It appears that the thiol groups of some membrane proteins may control ground water permeability and maintain the membrane barrier function [38,39]. It has been hypothesized that oxidation of membrane thiol groups may produce aqueous pores and water channels by aggregating membrane protein [40]. Further studies are needed to confirm this hypothesis. As demonstrated previously, oxidation of membrane thiol groups can produce disulphide bonds between the related proteins. The cross-linkage of proteins may change protein conformation and the distribution of phospholipids and proteins. Perturbation of the membrane structure may play a crucial role in the alteration of membrane permeability [13,17,41,42]. It may partly explain why oxidation of the membrane thiol groups increases the lysosomal proton permeability.

As demonstrated previously [16], lysosomal membranes are enriched in thiol groups, which play an important role in the lysosomal activities such as sulphate transport [12] and ATPase-mediated proton translocation [23]. In this study, we have established that membrane thiol groups are also of importance to lysosomal proton permeability. As shown by SDS/PAGE of lysosomal membrane proteins (Figure 1), some bands of low-molecular-mass protein disappeared after the lysosomes were treated with diamide, and some high-molecular-mass bands were formed. The reversal of protein aggregation by DTT treatment suggests that these low-molecular-mass proteins contain thiol groups. It is likely that lysosomal proton permeability is affected by the redox states of membrane thiol groups located on different proteins. Finally, the above results and evidence suggest that lysosomal proton permeability *in vivo* might be affected by the intracellular redox state.

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