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Effects of phospholipase A_2 on the lysosomal ion permeability and osmotic sensitivity

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Abstract

In this study, we investigated the mechanism of PLA_2-induced lysosomal destabilization. Through the measurements of lysosomal β-hexosaminidase free activity, their membrane potential, the intra-lysosomal pH and the lysosomal latency loss in hypotonic sucrose medium, we established that PLA_2 could increase the lysosomal membrane permeability to both potassium ions and protons. The enzyme could also enhance the organelle osmotic sensitivity. The increases in the lysosomal ion permeability promoted influx of potassium ions into the lysosomes via K^+/H^+ exchange. The resulted osmotic imbalance across the lysosomal membranes osmotically destabilized the lysosomes. In addition, the enhancement of the lysosomal osmotic sensitivity caused the lysosomes to become more liable to destabilization in the osmotic stress. The results explain how PLA_2 destabilized the lysosomes.

Keywords: Lysosome; Phospholipase A_2; Permeability; Potassium ion; Proton; Osmotic sensitivity

1. Introduction

Lysosomes play important roles in the physiological turnover of cellular macromolecules such as proteins, lipids, nucleic acids and carbohydrates. The maintenance of lysosomal integrity is of the utmost importance for the organelle to carry out its functions. The destabilization of lysosomes influences their normal activities, may even lead to cell death. Forty years ago, lysosomes were named 'suicide bag' of cells, since the leaked lysosomal hydrolases had been shown to be cytotoxic (de Duve and Wattiaux, 1966). In recent years, interest in the lysosomal destabilization has heightened with the knowledge that leakage of lysosomal enzymes, especially cathepsins, can cause apoptosis or necrosis (Brunk et al., 1997; Erdal et al., 2005; Cirman et al., 2004). The leaked lysosomal enzymes can also bring about harmful effects in the pathogenesis of many diseases such as prion encephalopathies (Laszlo et al., 1992), Alzheimer's disease (Nixon et al., 1992), myocardial ischemia (Decker et al., 1980), poliovirus infection (Guskey et al., 1970), complement activation-produced lung injury (Hatherill et al., 1989), and acute tissue injury (Fell and Dingle, 1963). Since lysosomal integrity is critical for living cells, a great number of studies investigated the factors that are detrimental to lysosomal integrity. To elucidate

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Oxonol VI, bis(3-propyl-5-oxoisoxazol-4-yl)pentamethine-oxonol; FITC-Dextran, fluorescein isothiocyanate-dextran; PLA_2, phospholipase A_2

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the lysosomal destabilizing mechanisms is important for understanding apoptosis, necrosis and lysosomal pathophysiology (Ferri and Kroemer, 2001; Kroemer and Jaattela, 2005).

Lysosomal membrane is a barrier responsible for its integrity. Damages to lysosomal membranes or changes in the membrane structure may cause the organelle destabilization. A line of evidence indicates that phospholipase A_2 may destabilize lysosomes in the presence of mercury and copper (Marchi et al., 2004). The enzyme activated by the elevated cytosolic calcium concentration can destabilize lysosomes in leukocyte and mussel blood cell (Marone et al., 1983; Burlando et al., 2002). Recent studies showed that PLA_2 destabilized lysosomes under some apoptotic stimuli, the resulting leakage of lysosomal enzymes can induce apoptosis (Zhao et al., 2001; Brunk et al., 1997). However, it is still unclear how PLA_2 destablizes lysosomes. In this study, we established that PLA_2 increased the lysosomal membrane permeability to K^+ and H^+, and enhanced their osmotic sensitivity for the first time. These changes in the lysosomal membrane properties may account for the destabilization of the organelle.

2. Experimental procedures

2.1. Materials

Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), fluorescein isothiocyanate-dextran (FITC-Dextran, $M_r = 70{,}000$), 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide, phospholipase A_2 (from honey bee venom) and Valinomycin were from Sigma (St. Louis, MO). Bis(3-propyl-5-oxoisoxazol-4-yl)pentamethine-oxonol) (oxonol VI) was from Molecular Probes. Percoll was purchased from Amersham (Uppsala, Sweden). Other chemicals used were of analytical grade from Beijing Chemical Factory.

2.2. Preparation of lysosomes

Male Wistar rats were starved for 24 h and killed by decapitation. Rat liver lysosomes were isolated by the Percoll gradient centrifugation methods of Jonas with a minor modification to increase lysosomal purity (Jonas et al., 1983). Briefly, rat liver was homogenized in 0.25 M sucrose and centrifuged at 3000 × *g* for 8 min. The supernatant was incubated at 37 °C for 5 min in the presence of 1 mM $CaCl_2$ to promote separation of lysosomes from mitochondria (Yamada et al., 1984). Then, the supernatant was centrifuged for 20 min at 20,000 × *g*. The pellet was resuspended in sucrose and mixed with Percoll (2:1, by vol.), and centrifuged at 40,000 × *g* for 90 min. The lower 1/4 volume of the gradient (lysosomal fraction) was pooled and mixed with 10 volumes of 0.25 M sucrose, and centrifuged at 10,000 × *g* for 13 min to remove Percoll. The purified lysosomes were resuspended in 0.25 M sucrose medium at 2.12 mg protein/ml for use. All performances were carried out at 0–4 °C. Protein was determined according to (Lowry et al., 1951).

2.3. Assay of lysosomal integrity

Lysosomal integrity was assessed by the measurement of lysosomal enzyme latency. The latency of a lysosomal enzyme refers to the percent of intact lysosomes as revealed by the inability of substrate to reach the lysosomal enzyme until the organelles are deliberately ruptured (Greene and Schneider, 1992). 4-Methylumbelliferyl *N*-acetyl-β-D-glucosaminide, the substrate of lysosomal β-hexosaminidase, was used at 1 mM to measure the enzyme activity (Bird et al., 1987). The liberated 4-methylumbelliferone was determined by measuring its fluorescence (excitation 365 nm, emission 444 nm) on a Hitachi F-4010 fluorescence spectrophotometer. Activities of the enzyme measured in the absence and presence of 0.36% Triton X-100 was designated the free activity and the total activity, respectively. 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 increased percentage free activity or loss of lysosomal enzyme latency.

2.4. Assay of lysosomal permeability to K^+

The lysosomal permeability to K^+ was assessed by the osmotic protection method. It is the most widely used method to determine whether a solute can enter the lysosomes (Lloyd and Forster, 1986; Forster and Lloyd, 1988; Reign and Tager, 1977). According to the principle of this method, an impermeable solute can provide perfect osmotic protection to the lysosomes suspended in the isotonic solution. However, the solute that penetrates into the lysosomes can break the initial osmotic balance across the lysosomal membranes. A progressive osmotic imbalance develops with increasing the solute concentration inside the lysosomes. As a result, the lysosomes swell and burst. Thus the rupture of the lysosomal membrane induced by swelling, and hence the permeability to a solute including ions, can be monitored by measuring changes in the latency of a lysosomal enzyme after incubating the lysosomes in a solution of that solute. This approach gives a semiquantitative measure of relative

rate of entry of permeant ions (Casey et al., 1978). In short, a 22.5-μl PLA_2-treated or control lysosomal sample was incubated in 180 μl incubation medium (0.125 M K_2SO_4, buffered at pH 7.0 with 20 mM Hepes/KOH) at 37 °C for the indicated time. Then, an 80-μl portion of this lysosomal suspension was used for the assay of lysosomal integrity. Enhancement of the lysosomal permeability to K^+ was determined as increased loss of lysosomal enzyme latency.

2.5. Measurement of lysosomal membrane potential

The lysosomal K^+ permeability can be assessed by the measurement of membrane potential using membrane potential-sensitive probe oxonol VI (Zhong et al., 2000). The assay medium contained 0.25 M sucrose (pH adjusted to 6.7 with imidazole), oxonol VI (2 μM) and CCCP (2 μM). A 20-μl PLA_2-treated or control lysosomal sample was added to 1.8 ml assay medium. The contents of blank cuvette were same as that of sample cuvette except addition of lysosomal sample. Two hundred microliters of 0.75 M K_2SO_4 was added to the sample and blank cuvettes at indicated time. Membrane potential was registered by the absorbance difference $\Delta A_{625-587}$ (Loh et al., 1984). All measurements were performed at 25 °C on a Hitachi 557 spectrophotometer.

2.6. Measurement of lysosomal proton leakage

Lysosomal proton leakage can acidify the suspending medium. Based on the property of *p*-nitrophenol that the unprotonated molecules of the dye have a sufficiently larger extinction coefficient at 400 nm over that of protonated molecules, acidification of the assay medium induced by lysosomal proton leakage can be measured by monitoring the decrease in *p*-nitrophenol absorbance at 400 nm (Yao and Zhang, 1997; Zhang et al., 2000). The assay medium contained 0.25 M sucrose and 0.04 mM *p*-nitrophenol (Na salt) (pH was adjusted to 7.0 with KOH). PLA_2 and CCCP were added to the medium as indicated. Lysosomal sample was added to 2 ml assay medium at 0.45 mg protein/ml. Absorbance of the pH sensitive dye *p*-nitrophenol was measured at 400 nm upon addition of 200 μl 0.75 M K_2SO_4 and 10 μl 1 mM valinomycin to the medium. All measurements were carried out at 25 °C on a UNIC-2100 spectrophotometer.

2.7. Measurement of intra-lysosomal pH

According to the method of Ohkuma, the intra-lysosomal pH was measured using pH sensitive probe FITC-Dextran (Ohkuma et al., 1982). The fluorescence excitation spectrum of FITC-Dextran varies with pH. It is widely used to measure cell pH and organelle pH. Lysosomal pH can be measured directly by allowing cells to take up the impermeant dye linked to high-molecular-weight dextran. The advantages of this measurement of intravesicular pH are that the technique is relatively rapid, not toxic, and subject to few artifacts (Van Adelsberg et al., 1989). Briefly, rat was injected intraperitoneally with FITC-Dextran (20 mg FITC-Dextran/150 g body weight) and starved for 16 h before decapitation. Lysosomes were prepared as described above. The measurement medium was composed of 0.25 M sucrose or 0.125 M K_2SO_4, buffered at pH 7.0 with 20 mM Hepes/Tris. Fluorescence was measured at 25 °C with excitation and emission wavelengths of 495 and 550 nm, respectively, on a Hitachi F-4010 fluorescence spectrophotometer. Intra-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 calibration curve generated as described by (Ohkuma et al., 1982). In short, a calibration curve is constructed by measuring the intensity of fluorescence emission of FITC-Dextran in phosphate-buffered saline at various pH values.

2.8. Assay of lysosomal osmotic sensitivity

Lysosomal osmotic sensitivity was assessed as described previously by examining their integrity after incubation in hypotonic sucrose medium (Wan et al., 2002). Briefly, lysosomal samples (0.45 mg protein/ml) were treated with 333 mU PLA_2 at 37 °C for 2 min. Then, the PLA_2-treated lysosomes or control lysosomes were incubated in sucrose medium at indicated concentration at 37 °C for the indicated time. After the incubation, an 80-μl portion of the lysosomal suspension was used for the assay of lysosomal integrity. Increases in free enzyme activity of the lysosomes indicate increases in the lysosomal osmotic sensitivity.

3. Results

3.1. Effects of PLA_2 on the lysosomal K^+ permeability

Normal lysosomes show only a limited permeability toward K^+, which is favorable for the maintenance of lysosomal osmotic stability (Harikumar and Reeves, 1983). The effect of PLA_2 on the lysosomal K^+ permeability was examined using the widely used osmotic protection assay (Casey et al., 1978; Ruth and Weglicki, 1982). In this assay, the lysosomes are suspended in an

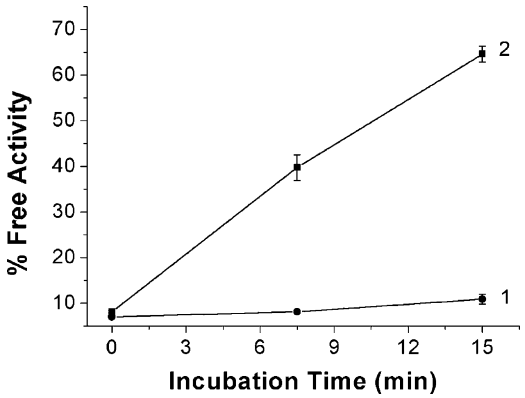

Fig. 1. PLA_2 treatment enhanced lysosomal permeability to potassium ions. A 7.5-μl lysosomal sample was treated with 333 mU PLA_2 at 37 °C for 2 min. Then, the sample was incubated in 180 μl 0.125 M K_2SO_4 (buffered at pH 7.0 with 20 mM Hepes/KOH) at 37 °C for the indicated time. Lysosomal free enzyme activity was measured immediately after the incubation. Line 1: control lysosomes; line 2: PLA_2-treated lysosomes. Values are means ± S.D. of three measurements.

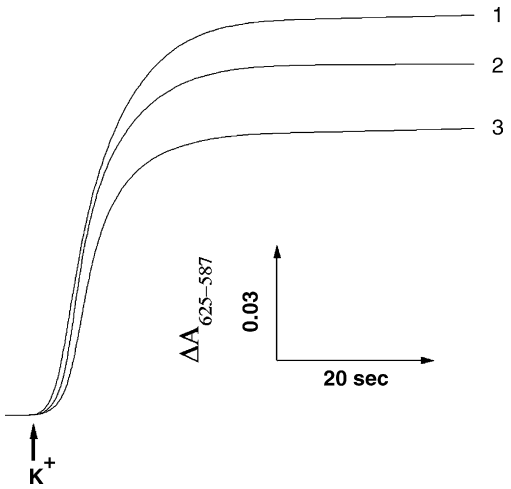

Fig. 2. Effects of PLA_2 treatment on the lysosomal membrane potential. Lysosomes were treated with 333 mU PLA_2 (at 0.45 mg protein/ml) at 37 °C for 2 min before the measurement. Assay medium contained 0.25 M sucrose, 2 μM oxonol VI and 2 μM CCCP, pH adjusted to 6.7 with imidazole. K_2SO_4 (200 μl, 0.75 M) was added at indicated time. Line 1: control lysosomes, assay medium contained 5 μM valinomycin; line 2: PLA_2 treated lysosomes; line 3: control lysosomes. Measurement of lysosomal membrane potential was as described in Section 2. A typical result out of three measurements is shown.

isotonic K^+-containing medium. The entry of K^+ into the lysosomes will cause an osmotic imbalance across the lysosomal membranes. The increases in the lysosomal K^+ permeability induce progressive accumulation of K^+ inside the lysosomes, which causes the lysosomes to swell and leads to the loss of lysosomal integrity. Therefore, the permeability to K^+ can be examined by measuring changes in the latency of a lysosomal enzyme (Lloyd and Forster, 1986; Forster and Lloyd, 1988; Reign and Tager, 1977). As shown in Fig. 1, free enzyme activity of PLA_2-treated lysosomes increased from 8.1% to 64.5% after 15 min incubation in the isotonic K^+-containing medium (line 2). In contrast, the control lysosomes maintained their enzyme latency after the incubation (line 1). The marked latency loss of the PLA_2-treated lysosomes suggests that the enzyme treatment increased the lysosomal K^+ permeability and induced lysosomal destabilization.

The effect of PLA_2 on the lysosomal permeability to K^+ was further assessed by the measurement of membrane potential using oxonol VI as a probe (Zhong et al., 2000; Van Walraven et al., 1985). This membrane potential-sensitive dye has been used to indicate the relative permeability of K^+ through its absorbance changes. The diffusion potential applied is the result of K^+ influx (Van Walraven et al., 1985). An increase in the differential absorbance ($\Delta A_{625-587}$) of oxonol VI will be observed when potassium ions are allowed to enter the lysosomes (Zhong et al., 2000). As shown in Fig. 2, the differential absorbance of PLA_2-treated lysosomes (line 2) increased more markedly than that of control lysosomes (line 3) upon addition of K^+ to the medium. It implicates that the PLA_2 treatment enhanced the lysosomal permeability to K^+. Compared to control lysosomal sample (line 3), the dye absorbance of PLA_2-treated lysosomes (line 2) is closer to the valinomycin-permeabilized lysosomes (line 1). It indicates that the PLA_2 treatment increased the lysosomal K^+ permeability to that of the valinomycin-permeablized lysosomes approximately. This result further established that PLA_2 increased the lysosomal K^+ permeability. The protonophore CCCP was used in the above measurements to permeablize all of the lysosomes to protons. We believe this treatment is important for the following reason: The pH gradient across lysosomal membranes produces an inside negative membrane potential. An increase in the lysosomal H^+ permeability causes the internal membrane potential to become more negative (Harikumar and Reeves, 1983). As shown in the following experiments, the PLA_2 treatment could increase the lysosomal H^+ permeability (cf. Fig. 3 and Table 1). To assess changes in the lysosomal K^+ permeability by the measurement of membrane potential, we must eliminate the difference between the proton permeability of the control and the PLA_2-treated lysosomes. In other words, their permeabilities to H^+ are maintained at the same level. Thus, changes in the membrane potential will depend solely on their own K^+ permeability.

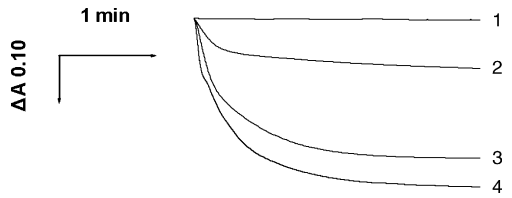

Fig. 3. PLA_2 treatment caused leakage of lysosomal protons. Lysosomes were added to 2 ml assay medium containing 0.25 M sucrose (adjusted to pH 7.0 with KOH) and 0.04 mM *p*-nitrophenol at 0.45 mg protein/ml. The assay medium of line 1 was buffered at pH 7.0 with 20 mM Hepes/KOH. 333 mU PLA_2 was added to the assay medium of lines 1 and 3, no PLA_2 was added to the assay medium of lines 2 and 4. The assay medium of line 4 contained 1 μM CCCP. Absorbance of *p*-nitrophenol was measured at 400 nm upon addition of 200 μl 0.75 M K_2SO_4 and 10 μl 1 mM valinomycin to the medium. A typical result out of three measurements is shown.

3.2. Effects of PLA_2 on the lysosomal H^+ permeability

Normally, lysosomes exhibit only a limited permeability toward H^+ (Harikumar and Reeves, 1983). The increase in the lysosomal H^+ permeability can cause leakage of their protons, which may acidify the suspending medium of the lysosomes. Using the pH-sensitive dye *p*-nitrophenol, we examined the acidification of the assay medium to assess the lysosomal H^+ permeability. As shown in Fig. 3, the absorbance of PLA_2-treated lysosomal sample decreased more markedly (line 3) than that of control lysosomes (line 2). It suggests that the PLA_2 treatment increased the lysosmal proton leakage. To validate the effect of leaked protons on the dye absorbance, we used CCCP and valinomycin to permeablize the lysosomes to H^+ and K^+, respectively. A maximal proton leakage could be produced through K^+/H^+ exchange. The results showed that the ionphores caused the largest decrease in the absorbance (line 4), while the absorbance decrease was abolished in buffered measuring medium (line 1). It confirmed the effect of leaked H^+ on the dye absorbance. These results indicated that the PLA_2 treatment increased the lysosomal proton leakage, i.e. the lysosomal H^+ permeability was increased. The efflux of lysosomal protons must be accompanied by the charge-compensating ions to maintain the electroneutrality of the ion movement. Previous study demonstrated that the lysosomal protons leaked out via K^+/H^+ exchange in the presence of external K^+ (Casey et al., 1978). In the above measurements, we suspended the lysosomes in the K^+-containing medium to fully exhibit the lysosomal proton leakage. Knowing that PLA_2 could increase the lysosomal K^+ permeability, we used valinomycin to permeablize the lysosomes to K^+ to keep their K^+ permeability at the same level. Thus, the extent of K^+/H^+ exchange and the amount of leaked protons of differently treated lysosomes (control lysosomes and PLA_2-treated lysosomes) will depend solely on their own H^+ permeability.

Table 1
Effects of PLA_2 treatment on the intra-lysosomal pH

Treatment of lysosomes	Assay medium	0 min pH	8 min pH
Control	Sucrose	5.53 ± 0.01	5.55 ± 0.01
PLA_2 treatment	Sucrose	5.53 ± 0.01	5.63 ± 0.01
Control	K_2SO_4	5.53 ± 0.01	5.64 ± 0.01
PLA_2 treatment	K_2SO_4	5.53 ± 0.01	6.27 ± 0.03

A 7.5 μl lysosomes were incubated in 1 ml 0.25 M sucrose medium or 0.125 M K_2SO_4 medium (both buffered at pH 7.0 with 20 mM Hepes/Tris) in the presence or absence of 333 mU PLA_2. Intra-lysosomal pH was measured at indicated time. All procedures were as described in Section 2. Values are means ± S.D. of three measurements.

Leakage of lysosomal protons can elevate the intra-lysosomal pH. To confirm the influence of PLA_2 on the lysosomal H^+ permeability, we measured the lysosomal pH. As shown in Table 1, the control lysosomes maintained their pH in sucrose medium. The lysosomal pH increased only by 0.11 unit in K_2SO_4 medium. In contrast, the internal pH of the PLA_2-treated lysosomes increased by 0.1 unit and 0.74 unit in the sucrose medium and K_2SO_4 medium, respectively. It suggested that the PLA_2 treatment caused a loss of lysosomal protons, especially in the K^+-containing medium. In sucrose medium, the lysosomal pH increased slightly, possibly due to an electroneutral co-efflux of lysosomal H^+ with the intra-lysosomal anions such as chloride and phosphate (Reeves, 1984). The greater elevation of lysosomal pH in the K_2SO_4 medium was caused by an exchange of lysosomal protons for the external K^+. In other words, potassium ions promoted the efflux of lysosomal H^+ through K^+/H^+ exchange. In contrast, the control lysosomes maintained their pH either in the sucrose medium or in the K_2SO_4 medium, presumably due to their limit permeability toward H^+ and K^+. The maintenance of lysosomal acidic pH in living cells (cytosol contains 140 mM K^+) is partly attributed to the limited K^+/H^+ exchange. These results further established that the PLA_2 treatment enhanced the lysosomal permeability to H^+ and K^+.

3.3. K^+/H^+ exchange of PLA_2-treated lysosomes

The passive diffusion of K^+ into lysosomes must be accompanied by the charge-compensating ions to maintain the electroneutrality of the ion movement. It has been proven that K^+/H^+ exchange is a major pathway for the entry of K^+ into lysosomes (Casey et al., 1978). To

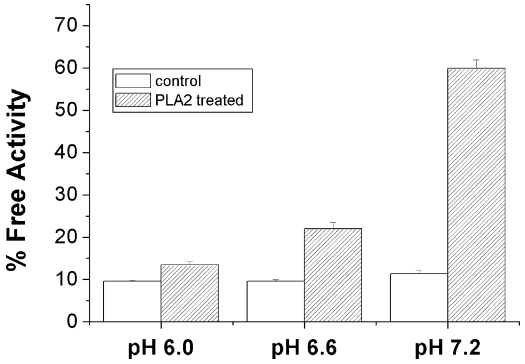

Fig. 4. Effects of medium pH on the integrity of PLA_2-treated lysosomes. A 7.5-μl lysosomal sample was treated with 333 mU PLA_2 at 37 °C for 2 min, then the lysosomes were incubated in 180 μl 0.125 M K_2SO_4 medium at 37 °C for 15 min. The medium was buffered at indicated pH with 20 mM citric acid/Hepes or KOH/Hepes. Lysosomal free enzyme activity was measured immediately after the incubation. Open bar: control lysosomes, not treated with PLA_2; hatched bar: lysosomes were treated with PLA_2. Values are means ± S.D. of three measurements.

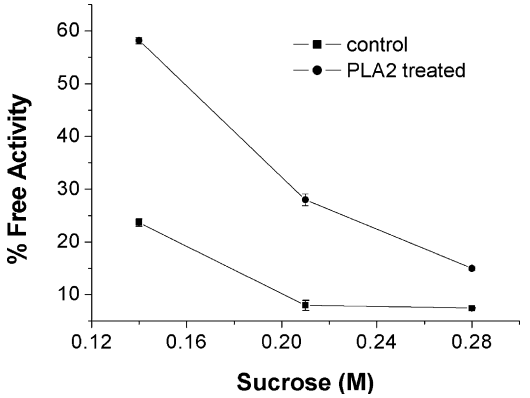

Fig. 5. Effects of sucrose concentration on the osmotic stability of PLA_2-treated lysosomes. Lysosomes were treated with 333 mU PLA_2 (at 0.45 mg protein/ml) at 37 °C for 2 min. Then, the lysosomal sample was incubated in the sucrose medium at indicated concentrations for 5 min. Lysosomal free enzyme activity was measured immediately after the incubation. Detailed procedures were as described in Section 2. Values are means ± S.D. of three measurements.

what extent K^+ enters lysosomes correlates to the lysosomal permeability toward both K^+ and H^+. As shown above, the lysosomal protons leaked out through K^+/H^+ exchange. To determine whether potassium ions entered the PLA_2-treated lysosomes via K^+/H^+ exchange, the lysosomes were suspended in the K^+-containing mediums that were buffered at different pH. The different pH gradient across the lysosomal membranes provided different driving force for the exchange of lysosomal protons with external K^+. Since the PLA_2 treatment increased the lysosomal permeability to H^+ and K^+, larger ΔpH should cause greater influx of K^+ into the lysosomes. As a result, the lysosomes should lose their enzyme latency more markedly. As show in Fig. 4, free enzyme activity of the PLA_2-treated lysosomes increased markedly with elevating the medium pH from 6.0 to 7.2, indicating that more K^+ entered the lysosomes at higher pH of the medium. In contrast, free enzyme activity of the control lysosomes increased slightly at higher pH of the medium. The results suggested that K^+ entered the PLA_2-treated lysosomes via a K^+/H^+ exchange pathway.

3.4. Effects of PLA_2 on the lysosomal osmotic sensitivity

The lysosomes are named intracellular "osmometer" due to their relatively higher sensitivity to osmotic stress (Lloyd and Forster, 1986). The osmotic sensitivity of lysosomes reflects their ability to resist hypotonic pressures. An increase in the lysosomal osmotic sensitivity can cause the organelle to become more susceptible to osmotic shock. It may induce an increased influx of water into the lysosomes in osmotic stresses and cause osmotic lysis of the organelle. To assess the effect of PLA_2 on the lysosomal osmotic sensitivity, we measured free activity of lysosomal marker enzyme β-hexosaminidase after incubating the lysosomes in hypotonic sucrose medium for a period of time. As shown in Fig. 5, the PLA_2-treated lysosomes exhibited a greater percentage of free enzyme activity than the control lysosomes at each hypotonic concentration of sucrose medium. The treated lysosomes lost their latency in low sucrose concentration more dramatically than the control ones. It indicated that the

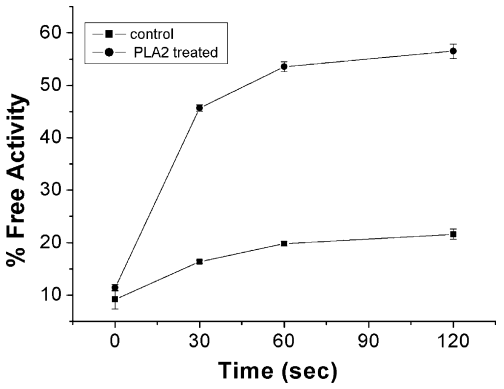

Fig. 6. Effects of incubation duration on the osmotic stability of PLA_2-treated lysosomes in hypotonic sucrose medium. Lysosomes were treated with PLA_2 as described in Fig. 1. Then, the lysosomes were incubated in 0.14 M sucrose medium at 37 °C for the indicated time. Lysosomal free enzyme activity was measured immediately after the incubation. Values are means ± S.D. of three measurements.

PLA_2-treated lysosomes were more susceptible to the increased osmotic imbalance across their membranes. The effect of PLA_2 on the lysosomal osmotic sensitivity was further established by the evidence that the PLA_2-treated lysosomes lost their enzyme latency more markedly and rapidly than the control ones in the hypotonic sucrose (Fig. 6). The results suggested that the PLA_2 treatment caused the lysosomes to lose their ability to resist hypotonic pressure and to become more sensitive to osmotic stresses.

4. Discussion

Lysosomal destabilization can cause leakage of their protons, loss of the membrane potential and increase in their membrane permeability. It has been established that the lysosomal membrane ΔpH and membrane potential play important roles in the across-membrane transportations of many molecules such as some amino acids and inorganic ions (Gahl, 1992; Vadgama and Jonas, 1992; Pisoni and Thoene, 1991). The loss of lysosomal membrane ΔpH and membrane potential affects the lysosomal transport, which may cause serious metabolism disorder. An elevation in the lysosomal pH may inactivate the acidic hydrolases therein, resulting in the loss of lysosomal functions (Futerman and van Meer, 2004). Lysosomes show a limited permeability to various ions and the molecules with low molecule weight. The increases in their membrane permeability promote the lysosomal uptake of external ions and molecules, causing osmotic stresses and destabilization of the lysosomes. The most serious change in the destabilized lysosomes is the loss of their enzyme latency. In recent years, a number of studies demonstrated that cathepsins, a family of lysosomal proteases, could modify Bid (a member of Bcl-2 family) and directly activate caspase-3 after leaking into the cytosol (Cirman et al., 2004). These events could induce apoptosis (Erdal et al., 2005). In addition, the leaked lysosomal hydrolases often caused necrosis if the enzyme leakage was extensive and serious (Brunk et al., 1997). The leaked lysosomal enzymes also brought about harmful effects in the pathogenesis of many diseases (Laszlo et al., 1992; Nixon et al., 1992; Decker et al., 1980). In the past years, these evidences have drawn attentions to the factors that destabilize lysosomes, but the mechanisms for the lysosomal destabilization are not well studied.

As demonstrated previously, phospholipase A_2 may destabilize lysosomes under various conditions (Marchi et al., 2004; Marone et al., 1983; Burlando et al., 2002). Recent study revealed that PLA_2 might destabilize lysosomes in apoptosis (Zhao et al., 2001). The leaked lysosomal enzymes could induce apoptosis (Brunk et al., 1997). The above studies emphasized the destructive effects of PLA_2 on the lysosomal integrity. How the enzyme destabilized lysosomes is unclear. Without knowing this mechanism, biologists cannot understand how PLA_2 induces apoptosis through a lysosome-mediated pathway. In this study, we first demonstrate that PLA_2 can increase the lysosomal permeability to K^+ and H^+, and enhance their osmotic sensitivity. These changes in the lysosomal membrane properties destabilize the organelle. The results provide new information concerning the mechanism for the PLA_2-induced lysosomal destabilization. It may provide valuable clues for the studies of apoptosis and necrosis.

It is well known that a variety of damages to membranes can perturb their phospholipid order and affect the membrane integrity. The lysosomal membranes contain abundant phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and sphingomyelin (Bode et al., 1976). The lipids play important roles in the maintenance of lysosomal integrity. As depicted above, PLA_2 could increase the lysosomal ion permeability and osmotic sensitivity, presumably due to the PLA_2-produced hydrolysis of lysosomal membrane lipids. It was established that PLA_2 could hydrolyze membrane phospholipids and loosen the membrane structure (Hyvonen et al., 2001). Atomic force microscope data showed that the enzyme produced numerous narrow channels in the membrane. The membrane also exhibited changes in orientation and branching of numerous channels, which might lead to fragmentation of the membrane (Grandbois et al., 1998). In addition, PLA_2 could produce larger holes in membranes (Jensen and Simonsen, 2005) and cause local spontaneous curvature on membranes (Grandbois et al., 1998). The accumulation of PLA_2-produced products at the hydrolyzed region perturbed lipid packing, leaving space for water molecules to penetrate deeper into the lipid bilayer (Staneva et al., 2004). A lines of evidence demonstrated that PLA_2 could increase membrane permeability to H^+, Ca^{2+} and water (Eriksson and Saris, 1989; Donlon et al., 1979; Utsumi et al., 1985). The enzyme could also affect membrane potential (Cecchini et al., 2005) and membrane fluidity (Kinjo et al., 1988). Treating liposomes with PLA_2 increased their osmotic sensitivity (Kinjo et al., 1988). The results of this study are consistent with previous findings. By now, how PLA_2 affects the membranes is not well elucidated. We also cannot explain how the enzyme increased the lysosomal permeability and osmotic sensitivity. Further studies are required to elucidate these issues. In living cells, many factors and events such as lipid per-

oxidation (McLean et al., 1993), complement (Panesar et al., 1997), increased cytosol calcium ions (Burlando et al., 2002) and caspases (Wising et al., 1997) can activate PLA_2. The activated PLA_2 may cause various pathological changes in the cells, including the lysosomal destabilization-induced pathogenesis. The results of this study will remind researchers to notice the integrity of lysosomes when PLA_2 is activated in a pathological event.

Generally, the lysosomes can be destabilized by two kinds of mechanisms. One type of the mechanism attributes the lysosomal destabilization to the alterations in their membranes. In this respect, the effects of membrane lipid peroxidation on the lysosomal stability have been extensively studied (Desai et al., 1964; Fong et al., 1973). Interestingly, several fat-soluble vitamins and some hormones can destabilize the lysosomes (de Duve et al., 1962). The hydrolysis of lysosomal membrane lipids by their internal phospholipases may destabilize isolated lysosomes under acidic conditions (Weglicki et al., 1974). As demonstrated recently, the activated phospholipase A_2 may destabilize lysosomes in living cells (Marchi et al., 2004; Zhao et al., 2001), but the mechanism for this event is unclear. Another mechanism for the lysosomal destabilization correlates to the occurrence of osmotic stresses. The lysosomes are named intracellular 'osmometer' owing to their susceptibility to osmotic stress (Lloyd and Forster, 1986). An osmotic imbalance across the lysosomal membranes can osmotically destabilize the organelle. Our previous studies demonstrated that the changes in the physical state of lysosomal membranes and the photooxidation of lysosomal membrane thiol groups could increase the lysosomal osmotic sensitivity (Wan et al., 2002; Yang et al., 2000). As shown above, PLA_2 increased the lysosomal osmotic sensitivity, causing the lysosomes to become more liable to disrupt in an osmotic shock. At present, the mechanism by which the lysosomal osmotic sensitivity increases is unclear. It may be caused by the increases in their permeability to water and the enhancement of the membrane osmotic fragility.

Lysosomes in vivo are surrounded by a high concentration of cytosolic K^+ (140 mM). In the past years, a number of studies paid attentions to the K^+-induced lysosomal destabilization. As reported by Ruth and Weglicki, an increase in the lysosomal K^+ permeability promoted the lysosomal uptake of K^+, which might produce an osmotic stress to the membranes and eventually disintegrate the lysosomes (Ruth and Weglicki, 1982). The maintenance of lysosomal integrity in the cytosol correlates to their limited permeability toward K^+ (Harikumar and Reeves, 1983). However, some factors or events such as low temperature (Reijngoud and Tager, 1977) and the oxidation to the lysosomal membranes (Zhang and Yao, 1997) can increase the lysosomal K^+ permeability. As shown in this study, the PLA_2 treatment increased the lysosomal K^+ permeability. As a result, potassium ions flowed into the lysosomes, causing progressive accumulation of K^+ inside the lysosomes. The resulted osmotic imbalance across the lysosomal membranes caused the lysosomes to become swell. According to the concept of the osmotic protection method (Lloyd and Forster, 1986; Forster and Lloyd, 1988; Reign and Tager, 1977), the loss of the lysosomal integrity induced by swelling, and hence the permeability to K^+, can be examined by measuring changes in the latency of a lysosomal enzyme. The increases in the lysosomal K^+ permeability could produce an osmotic shock to the lysosomes, while the enhancement of the lysosomal osmotic sensitivity induced by the PLA_2 treatment caused the lysosomes to become more sensitive to the osmotic shock. Previous study showed that the extent of lysosomal destabilization caused by the entry of K^+ correlated to the lysosomal permeability to both K^+ and H^+ (Casey et al., 1978). It was proved that K^+/H^+ exchange was a major pathway for the lysosomal uptake of K^+ (Casey et al., 1978; Henning, 1975). The lysosomal membranes normally exhibited a limited permeability to H^+ (Harikumar and Reeves, 1983). Since the PLA_2 treatment could increase the lysosomal H^+ permeability, it thus promoted the influx of K^+ into the lysosomes through K^+/H^+ exchange. In other words, the increase in the lysosomal H^+ permeability might contributes to the K^+-induced lysosomal destabilization.

By now, there is no evidence showing the existence of K^+ channels, K^+ carrier and K^+ pump on the lysosomal membranes. As demonstrated by the studies of lysosomal K^+ permeability, K^+ entered the lysosomes by a passive diffusion mechanism (Casey et al., 1978; Ruth and Weglicki, 1982). It was proposed that the transient defect on the membranes is a pathway for solute permeation (Trauble, 1971). Because PLA_2 can hydrolyze the membrane phospholipids, the resultant defects may act as a potential channel that carries K^+ across the lysosomal membranes. In this study, we have found that the membranes of the PLA_2-treated lysosomes are slightly permeable to sucrose compared to normal lysosomes. It is likely that the enzyme may also increase the lysosomal permeability to other molecules and ions. We cannot rule out the possibility that other cytosolic molecules enter simultaneously the lysosomes and then play a role in the lysosomal destabilization.

In summary, PLA_2 can increase the lysosomal membrane permeability to K^+ and H^+, and enhance their

osmotic sensitivity. These changes in the lysosomal membrane properties caused losses of the lysosomal integrity. It explains how PLA_2 destabilizes lysosomes.

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