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Increased In Vitro Lysosomal Function in Oxidative Stress-Induced Cell Lines

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Abstract Exposure of mammalian cells to oxidative stress alters lysosomal enzymes. Through cytochemical analysis of lysosomes with LysoTracker, we demonstrated that the number and fluorescent intensity of lysosome-like organelles in HeLa cells increased with exposure to hydrogen peroxide (H_2O_2), 6-hydroxydopamine (6-OHDA), and UVB irradiation. The lysosomes isolated from HeLa cells exposed to three oxidative stressors showed the enhanced antimicrobial activity against *Escherichia coli*. Further, when lysosomes that were isolated from HeLa cells exposed by oxidative stress were treated to normal HeLa cells, the viability of the HeLa cells was drastically reduced, suggesting increased in vitro lysosomal function (i.e., antimicrobial activity, apoptotic cell death). In addition, we also found that cathepsin B and D were implicated in increased in vitro lysosomal function when isolated from HeLa cells exposed by oxidative stress. Decrease in cathepsin B activity and increase in cathepsin D activity were observed in

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lysosomes isolated from HeLa cells after treatment with H_2O_2, 6-ODHA, or UVB, but cathepsin B and D were not the sole factors to induce cell death by in vitro lysosomal function. Therefore, these studies suggest a new approach to use lysosomes as antimicrobial agents and as new materials for treating cancer cell lines.

Keywords Oxidative stress · Lysosomes · In vitro function · Antimicrobial activity · Apoptotic cell death

Introduction

Lysosomes are heterogeneous cell organelles consisting of vacuoles that vary in size, form, and density [1]. Lysosomes are considered the stomach of the cell, and their importance in heterophagy and autophagy is recognized [1–3]. To treat waste in cells, lysosomes receive newly produced lysosomal enzymes, which are produced in the reticular network [2, 4]. In addition, lysosomes release lysosomal enzymes to digest intracellular and extracellular waste material [5]. Lysosomal enzymes degrade bacteria cell walls, demonstrating the antimicrobial activity of lysosomes [6, 7]. If lysosomes contact bacteria with a cell wall undergoing *N*-acetylation, the bacteria might be hydrolyzed as a result of the antimicrobial activity of the lysosomal enzyme released from the lysosomes [8, 9]. In contrast, lysosomes cannot degrade bacteria cell walls if the functional group of the cell wall is converted from *N*-acetylation to *O*-acylation [8]. All lysosomal functions previously identified occurred inside the cells (i.e., in vivo function). However, we found that lysosomes—acting as whole cell organelles, not lysosomal enzymes—isolated from hen's egg white and *Saccharomyces cerevisiae* showed antimicrobial activity in response to various kinds of microorganisms [6, 7], suggesting possible in vitro lysosomal applications.

Lysosomes are not silent organelles but dynamic organelles in which lysosomal enzymes are easily integrated or released when exposed to stressful conditions, especially oxidative stress [2, 10, 11]. Oxidative stress results from increased levels of reactive oxygen species (ROS) through oxidative metabolism or decreased clearance of ROS due to anti-oxidant deficiency, and apoptotic cell death can be induced under severe oxidative stress conditions [12]. Alteration of both lysosomal cysteine and aspartic proteases has been observed following oxidative stress-induced apoptosis [12], suggesting that lysosomal enzyme mediation is a key factor in inducing apoptotic cell death. A complete breakdown of the lysosomes with the release of high concentrations of lysosomal enzymes into the cytosol results in unregulated cell death when exposed to severe oxidative stresses [13]. If lysosome disruption was caused by oxidative stress, it would be impossible to evaluate in vitro lysosomal function as a cell organelle. Therefore, we used sublethal doses of oxidative stress, creating conditions for evaluating in vitro lysosomal function.

Understanding in vitro lysosomal function in cells following hydrogen peroxide (H_2O_2), 6-hydroxydopamine (6-OHDA), or UVB irradiation may elucidate lysosomal mechanisms associated with oxidative stress and lysosomal activation. We exposed HeLa cell lines to three oxidative-causing agents and isolated lysosomes from HeLa cells exposed to these stressors. Studies support the idea that cathepsin B and D activity was altered in lysosomes that were isolated from HeLa cells exposed to different oxidative stresses. Our data suggest that H_2O_2-, 6-OHDA-, and UVB-activated cells regulate lysosomes—at least in part—via cathepsin B and D. These studies provide

insight for the application of in vitro lysosomes as antimicrobial agents and as apoptosis-inducing materials.

Materials and Methods

Materials

Dulbecco's Modified Eagle Medium High Glucose (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, L-glutamine, and trypsin–EDTA solution for cell culture were obtained from Gibco-BRL (Grand Island, NY, USA). LysoTracker Green DND-26 was purchased from Molecular Probes Inc (Leiden, The Netherlands). H_2O_2 and 6-OHDA were supplied by Sigma-Aldrich Inc. Z-Arg-Arg-MCA was purchased from Peptides International Inc. (Louisville, KY, USA).

Cell Culture and Lysosome Isolation

HeLa cells were maintained in DMEM supplemented with 5% FBS at 37°C under a humidified atmosphere of 5% CO_2− 20% O_2 air. After the HeLa cells were grown to 60% confluence, the cells were treated with H_2O_2 or 6-OHDA (both at 0, 50, 100, 200, and 400 μM) for 24 h or were exposed to UVB irradiation by six 8-W UVB lamps (Vilber Lourmat Inc.). HeLa cells were treated with the UVB dose at 120 mJ/cm^2. The medium was removed before UVB treatment; control cells were maintained in the same conditions without UVB irradiation during UVB exposure. HeLa cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), treated with a lysis buffer (400 mM Na-phosphate, 75 mM NaCl, 4 mM EDTA, 0.25% Triton-X 100, pH 6.0) for 1 h on ice, ultrasonicated at 40 W for 1 min (1.0 s on/2.0 s off pulsed), and centrifuged at 3,000 rpm (10 min, 4 °C) to produce cell debris [7]. The total supernatant was centrifuged at 20,000 × *g* (30 min, 4 °C) to separate the lysosomes from the supernatant [7].

Antimicrobial Test and Apoptotic Cell Death

An antimicrobial test was performed to confirm the antimicrobial activity of the lysosomes against *Escherichia coli*. After the cell culture at OD_{600} reached 0.6–0.7, the cultures were diluted to 10^{-6} cells/ml in sterile water. One hundred microliters of the cell culture containing lysosomes in a 100-mM sodium phosphate buffer (900 μl) was spread over a LB plate medium. The antimicrobial activity of the lysosomes and their derivatives was measured using the colony count [6, 7]. Viability of HeLa cells treated by lysosomes or of controls was determined by trypan blue stain exclusion. Cells were harvested from dishes and transferred to centrifuge tubes. The cells were washed with PBS and resuspended in 1 ml of PBS. An equal amount of cell suspension was added to 2 × trypan blue stain, and the cells were counted in a hemocytometer. Grids were counted to quantitate the white cells [14].

Cytological Staining with LysoTracker

HeLa cells were grown in a cell culture dish, rinsed with 1×PBS and stained with 100 nM LysoTracker Green DND-26 in a medium without serum for 30 min at room temperature. The cells were washed with PBS. Sections were observed under confocal

laser scanning microscope (LSM 510 META); for our measurements, the 488-nm excitation line was used and images were generated with Zeiss LSM image Browser (Munich, German).

Cathepsin B and D Activity Assays

HeLa cells were grown in plates to 60–70% confluence and were treated with H_2O_2 or 6-OHDA (50–400 μM) for 24 h or were exposed to UVB for 1–50 min. After treatment, the cells were rinsed twice with ice-cold PBS, treated with lysis buffer (400 mM Na-phosphate, 75 mM NaCl, 4 mM EDTA, 0.25% Triton-X 100, pH 6.0) for 1 h on ice, ultrasonicated at 40 W for 1 min (1.0 s on/1.0 s off pulsed), and centrifuged at 3,000 rpm (10 min, 4 °C) to remove cell debris. Total protein was determined using the Bradford protein assay kit. Cathepsin B activity was determined fluorimetrically using the methyl-coumarylamide substrate z-Arg-Arg-MCA at pH 6.0, as previously described [15]. Fluorescence was measured with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Preparation and treatment were as previously described for cathepsin B activity. The cells were lysed in ice-cold assay buffer (100 mM Na acetate and 4 mM EDTA, 0.2% Triton X-100, pH 3.5) and incubated for 30 min on ice [6]. Cathepsin D was confirmed using acid-denatured hemoglobin (16.6 g/l) as a substrate in ammonium acetate buffer, pH 3.5. The reaction was stopped with TCA, and the absorbance at 750 nm was determined after the addition of Folin–Ciocalteau reagent [15].

Data Analysis

All data for error analysis were obtained from three independent samples carried out simultaneously, and the results are shown with the standard deviation and correlation between the cell mortality and experimental conditions. The data were analyzed using Sigma Plot (SPS, Chicago, IL, USA). A p value <0.05 was considered significant.

Results and Discussion

Involvement of Lysosomal Activation in HeLa Cells Following Exposure to H_2O_2, 6-OHDA, or UVB

Exposure of cells to oxidative stresses is the primary method for activating lysosomes [6]. H_2O_2, 6-OHDA, and UVB irradiation, effective agents for causing oxidative stress, were used to induce oxidative stress in HeLa cells for 24 h. LysoTracker green fluorescent dye was used to identify lysosomes inside the cytosol of HeLa. Treatment of HeLa cells with H_2O_2 increased the number of conspicuous, green lysosome-like organelles surrounding HeLa nuclei after 24 h of exposure to 50 μM H_2O_2 (Fig. 1a; 400 μM H_2O_2 showed relatively toxicity in HeLa cells as shown by the lower fluorescent intensity of lysosome-like organelles (Fig. 1a–d)). Exposure of HeLa cells to 50, 100, 200, and 400 μM 6-OHDA also increased the fluorescent intensities of lysosome-like organelles (Fig. 1f–j). In addition, lysosomes were examined after UVB exposure. Fluorescent staining of lysosomes showed marked increases after 10, 20, and 40 min of exposure to UVB irradiation (Fig. 2). However, lysosomes were not activated in response to UVB irradiation for 5 min, and lysosomal intensity decreased after 60 min of exposure to UVB because of the cytotoxicity of UVB in HeLa cells. These results indicate that, in HeLa cells, mild oxidative stress is related to the activation of the lysosomes; however, this activation is not observed under cytotoxic conditions.

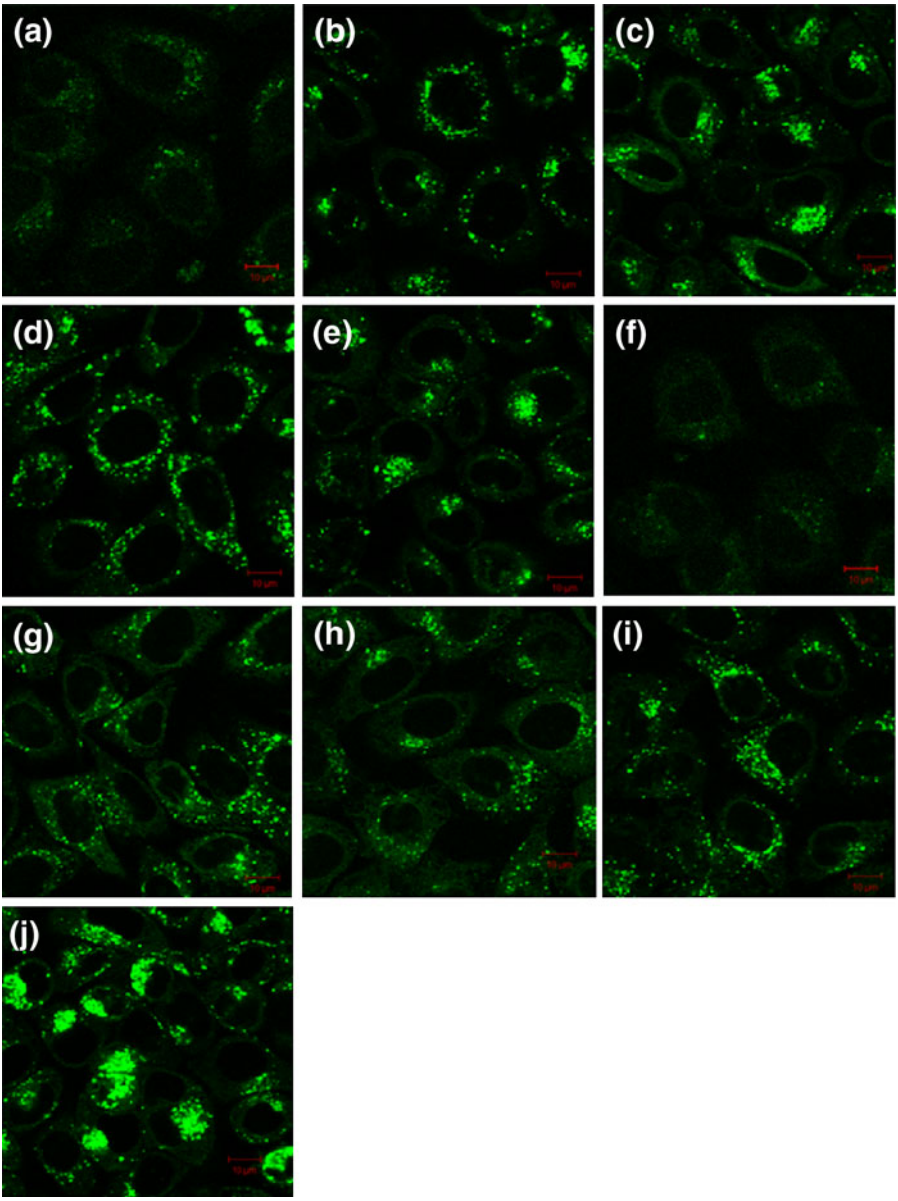

Fig. 1 Effect of hydrogen peroxide (H_2O_2) and 6-hydroxydopamine (6-OHDA) on the number of and green fluorescent intensity of lysosomes surrounding HeLa nuclei 24 h after exposure. HeLa cells were stained with LysoTracker Green DND-26; **a** control cells, **b** H_2O_2 50 μM, **c** H_2O_2 100 μM, **d** H_2O_2 200 μM, **e** H_2O_2 400 μM, **f** control cells, **g** 6-OHDA 50 μM, **h** 6-OHDA 100 μM, **i** 6-OHDA 200 μM, and **j** 6-OHDA 400 μM

Oxidative Stress Increased In Vitro Lysosomal Function in HeLa Cells

Lysosomes are active antimicrobial agents in vitro [6, 7]; therefore, we measured the lysosomal function via cell mortality of *E. coli* after treatment with lysosomes isolated from HeLa that were cultured under three different oxidative stress conditions. The antimicrobial

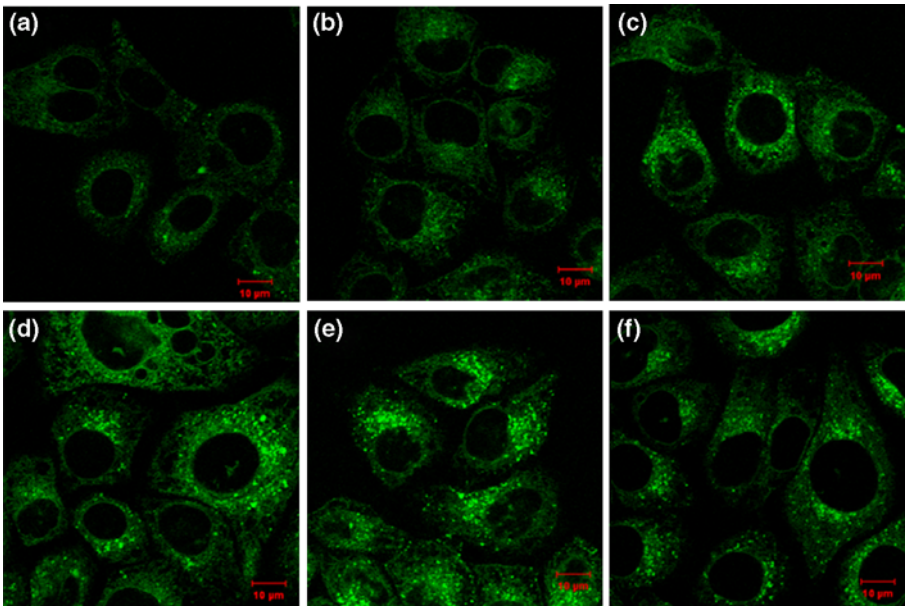

Fig. 2 Effect of UVB irradiation on the number of and green fluorescent intensity of lysosomes surrounding HeLa nuclei 24 h after exposure. HeLa cells were stained with LysoTracker Green DND-26; **a** control cells, **b** UVB exposure for 5 min, **c** 10 min, **d** 20 min, **e** 40 min, and **f** 60 min

efficiency of lysosomes isolated from normal HeLa resulted in 80% mortality of *E. coli* (L1 and L10 in Fig. 3). A 10% lysosome concentration was used (i.e., the ratio of lysosome solution isolated from HeLa cells to phosphate buffer with pH 6.0 was 1:9 (*w*/*w*)). Lysosomes isolated from HeLa cells exposed to UVB for 40 min slightly increased the cell mortality of *E. coli* by 1.2-fold ($p<0.01$). However, there were no significant differences in the other cases.

Lysosomes contain a number of proteases and acid hydrolases that kill bacteria cells, including the aspartic protease cathepsin D and the cystein protease cathepsin B, which are the most abundant and the most often reported apoptosis-inducers [12]. Cathepsins in the lysosomes were activated and then translocated to the cytosol during apoptosis induced by oxidative stress [12, 13]. Therefore, our study also aimed to evaluate apoptotic cell death caused by lysosomes isolated from HeLa cells subjected to oxidative stress. To evaluate apoptotic cell death caused by lysosomes, we used HeLa as the target mammalian cell line. We found that the in vitro function of lysosomes isolated from normal HeLa strongly affected the mortality of *E. coli* and HeLa cells.

As shown in L1 and L10 of Fig. 4, lysosomes isolated from normal HeLa cells did not affect the apoptotic death of HeLa cells (i.e., any proteases causing apoptotic cell death were not induced under normal conditions). However, in vitro lysosomal function was increased significantly in lysosomes isolated from HeLa cells exposed to oxidative stress (i.e., H_2O_2, 6-ODHA, or UVB irradiation). Lysosomes isolated from HeLa exposed to various concentrations of H_2O_2 or 6-ODHA were used to determine if cell death occurred via lysosomes from oxidative stress-induced HeLa cells. All concentrations used in this study were sublethal for HeLa. During exposure to H_2O_2, 6-ODHA, or UVB irradiation, selective disruption of lysosomes in HeLa cells was not observed (Figs. 1 and 2), suggesting that protease-like lysosomal enzymes are maintained in lysosomes.

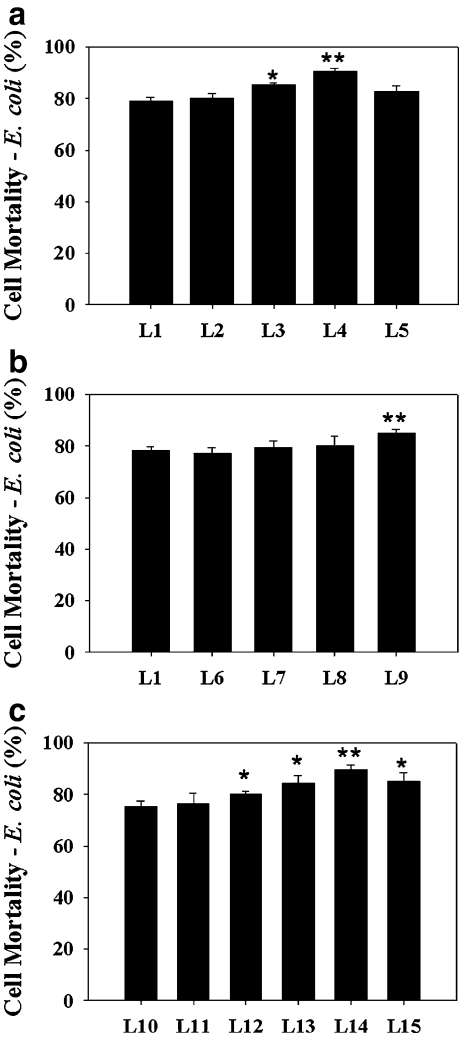

Fig. 3 Effect of oxidative stress on the mortality of *E. coli* treated with lysosomes that were isolated from HeLa cells exposed to hydrogen peroxide (H_2O_2) (**a**), 6-hydroxydopamine (6-OHDA) (**b**), or UVB irradiation (**c**). *L1*—lysosomes isolated from normal HeLa cells, *L2*—lysosomes isolated from HeLa cells exposed to H_2O_2 50 μM, *L3*—lysosomes isolated from HeLa cells exposed to H_2O_2 100 μM, *L4*—lysosomes isolated from HeLa cells exposed to H_2O_2 200 μM, *L5*—lysosomes isolated from HeLa cells exposed to H_2O_2 400 μM, *L6*—lysosomes isolated from HeLa cells exposed to 6-OHDA 50 μM, *L7*—lysosomes isolated from HeLa cells exposed to 6-OHDA 100 μM, *L8*—lysosomes isolated from HeLa cells exposed to 6-OHDA 200 μM, *L9*—lysosomes isolated from HeLa cells exposed to 6-OHDA 400 μM, *L10*—lysosomes isolated from normal HeLa cells without UVB irradiation, *L11*—lysosomes isolated from HeLa cells exposed to UVB for 5 min, *L12*—lysosomes isolated from HeLa cells exposed to UVB for 10 min, *L13*—lysosomes isolated from HeLa cells exposed to UVB for 20 min, *L14*—lysosomes isolated from HeLa cells exposed to UVB for 40 min, *L15*—lysosomes isolated from HeLa cells exposed to UVB for 60 min. Each *bar* represents the mean cell mortality ± S.D. and is statistically significant at $*P<0.05$ and $**P<0.01$ ($n=3$)

Therefore, lysosomal activity for apoptotic cell death was enhanced significantly by exposure to H_2O_2, 6-ODHA, or UVB irradiation in HeLa cells. Following exposure to 400 μM of H_2O_2, directly exposed HeLa lived, but mortality was 91% for HeLa cells treated with lysosomes isolated from HeLa exposed to 400 μM of H_2O_2. These data suggest that lysosomal proteases were effectively activated inside HeLa under oxidative stress conditions.

Cathepsin B and D Activity in Lysosomes Following Exposure to Oxidative Stress

Several reports observed that cathepsin D was activated and released from lysosomes after brief exposure to oxidative stress [11, 16]; however, cathepsin B, a cysteinyl proteinase of lysosomal origin, appears to be regulated in a manner quite unlike that of cathepsin D [17, 18]. We measured the enzymatic activity of cathepsin B and cathepsin D in lysosomes isolated from HeLa cells following exposure to H_2O_2, 6-ODHA, or UVB

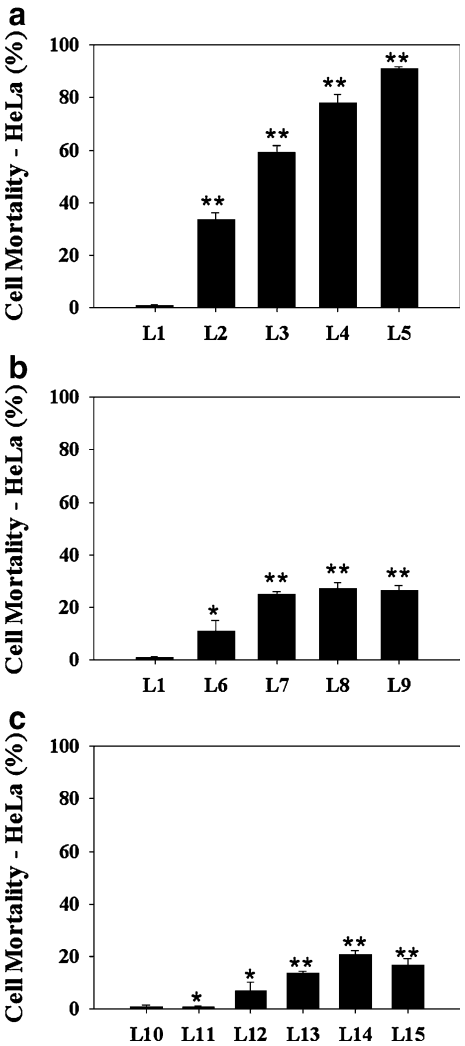

Fig. 4 Effect of oxidative stress on the mortality of HeLa cells treated with lysosomes that were isolated from HeLa cells exposed to hydrogen peroxide (H_2O_2) (**a**), 6-hydroxydopamine (6-OHDA) (**b**), or UVB irradiation (**c**). *L1*—lysosomes isolated from normal HeLa cells, *L2*—lysosomes isolated from HeLa cells exposed to H_2O_2 50 μM, *L3*—lysosomes isolated from HeLa cells exposed to H_2O_2 100 μM, *L4*—lysosomes isolated from HeLa cells exposed to H_2O_2 200 μM, *L5*—lysosomes isolated from HeLa cells exposed to H_2O_2 400 μM, *L6*—lysosomes isolated from HeLa cells exposed to 6-OHDA 50 μM, *L7*—lysosomes isolated from HeLa cells exposed to 6-OHDA 100 μM, *L8*—lysosomes isolated from HeLa cells exposed to 6-OHDA 200 μM, *L9*—lysosomes isolated from HeLa cells exposed to 6-OHDA 400 μM, *L10*—lysosomes isolated from normal HeLa cells without UVB irradiation, *L11*—lysosomes isolated from HeLa cells exposed to UVB for 5 min, *L12*—lysosomes isolated from HeLa cells exposed to UVB for 10 min, *L13*—lysosomes isolated from HeLa cells exposed to UVB for 20 min, *L14*—lysosomes isolated from HeLa cells exposed to UVB for 40 min, *L15*—lysosomes isolated from HeLa cells exposed to UVB for 60 min. Each *bar* represents the mean cell mortality ± S.D. and is statistically significant at $*P<0.05$ and $**P<0.01$ ($n=3$)

irradiation (Table 1). Following treatment with 5, 100, 200, and 400 μM of H_2O_2, cathepsin B activity was 3.83±0.24, 2.52±0.31, 0.63±0.19, and 0.43±0.23, respectively; the activity of normal lysosomes was 5.57±0.16. In HeLa cells, cathepsin B activity drastically decreased with increasing H_2O_2 concentration. Cathepsin B activity was also evaluated following exposure to 6-OHDA. Following treatment with 5, 100, 200, and 400 μM of 6-ODHA, cathepsin B activity was 2.86±0.23, 2.86±0.15, 0.98±0.13, and 0.08±0.26, respectively; the activity of normal lysosomes was 5.57±0.16. The level of cathepsin B activity decreased to a greater extent than that of cathepsin B when exposed to H_2O_2. As shown in Fig. 1, the lysosomal intensities of HeLa cells exposed to 6-ODHA were brighter than those of HeLa cells exposed to H_2O_2 at identical concentrations. These data showed consistent patterns with the decreasing level of cathepsin B. In vitro lysosomal function as related to apoptotic cell death contrasted with results from the above two cases. Previous reports demonstrated that, under oxidative stress conditions, large amounts of H_2O_2 diffused easily into lysosomes, where an increased production of ·OH leads to the oxidation of lysosomal contents [2, 19, 20]. Therefore, our results suggest that the residual H_2O_2 or ·OH in lysosomes synergistically affected the apoptotic cell death of HeLa via the in vitro activity of lysosomes isolated from HeLa exposed to H_2O_2. In addition, cathepsin D activity significantly increased as a function of H_2O_2 and 6-ODHA concentration and UVB exposure time. Therefore, these data suggest that cell death via lysosomes isolated from HeLa exposed to oxidative stress is partly cathepsin B and D activity dependent.

Table 1 Cathepsin B- and D-specific activity of lysosomes after exposure to hydrogen peroxide (H_2O_2), 6-hydroxydopamine (6-OHDA), or UVB in HeLa cells. Values are expressed as mean ± standard deviation

Specific activity unit	Chemicals									Irradiation					
	H_2O_2 (μM)					6-OHDA (μM)				UVB (Exposure time, min)					
	0	50	100	200	400	50	100	200	400	0	5	10	20	40	60
	L1[a]	L2	L3	L4	L5	L6	L7	L8	L9	L10[b]	L11	L12	L13	L14	L15
Cathepsin B (U/μg)	5.57 ± 0.16	3.83 ± 0.24	2.52 ± 0.31	0.63 ± 0.19	0.43 ± 0.23	2.86 ± 0.23	2.38 ± 0.15	0.98 ± 0.13	0.08 ± 0.26	5.64 ± 0.01	5.1 ± 0.09	3.70 ± 0.03	2.00 ± 0.10	1.28 ± 0.18	1.88 ± 0.05
Cathepsin D (μmol/mg)	10.1 ± 0.32	11.4 ± 0.45	12.6 ± 0.32	12.0 ± 0.26	13.1 ± 0.31	12.4 ± 0.61	13.1 ± 0.75	12.7 ± 0.36	9.86 ± 0.21	10.6 ± 0.57	12.6 ± 0.41	12.9 ± 0.40	13.4 ± 0.42	14.2 ± 0.94	13.3 ± 0.63

[a] L1 represents the lysosomes isolated from normal HeLa cells; L2—lysosomes isolated from HeLa cells exposed to H_2O_2 50 μM; L3—lysosomes isolated from HeLa cells exposed to H_2O_2 100 μM; L4—lysosomes isolated from HeLa cells exposed to H_2O_2 200 μM; L5—lysosomes isolated from HeLa cells exposed to H_2O_2 400 μM; L6—lysosomes isolated from HeLa cells exposed to 6-OHDA 50 μM; L7—lysosomes isolated from HeLa cells exposed to 6-OHDA 100 μM; L8—lysosomes isolated from HeLa cells exposed to 6-OHDA 200 μM; L9—lysosomes isolated from HeLa cells exposed to 6-OHDA 400 μM

[b] L10 represents the lysosomes isolated from normal HeLa cells, but this control was placed on the table, not inside the incubator during irradiation; L11—lysosomes isolated from HeLa cells exposed to UVB for 5 min; L12—lysosomes isolated from HeLa cells exposed to UVB for 10 min; L13—lysosomes isolated from HeLa cells exposed to UVB for 20 min; L14—lysosomes isolated from HeLa cells exposed to UVB for 40 min; L16—lysosomes isolated from HeLa cells exposed to UVB for 60 min

Conclusion

In conclusion, we found that lysosomes isolated from HeLa exposed to H_2O_2, 6-ODHA, and UVB irradiation showed increased in vitro activity as antimicrobial agents and that apoptotic cell death likely results from altered cathepsin B and D activity. To our knowledge, this is the first study of the relationship between in vitro lysosomal function and oxidative stress conditions. Increase in endogenous levels of lysosomes and their activity following oxidative stress exposure may have various applications (i.e., as antimicrobial agents, as apoptosis-inducing materials for cancer cells), and consequently it may be possible to use the organelles for improving in vitro functions.

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