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Vacuolar H$^+$-ATPase Inhibitor Induces Apoptosis *via* Lysosomal Dysfunction in the Human Gastric Cancer Cell Line MKN-1

Shigehito Nakashima[1], Yusuke Hiraku[2], Saeko Tada-Oikawa[2], Terutoshi Hishita[4], Esteban C. Gabazza[1], Shigenori Tamaki[1], Ichiro Imoto[3], Yukihiko Adachi[1] and Shosuke Kawanishi[*,2]

[1]Third Department of Internal Medicine, [2]Department of Environmental and Molecular Medicine and [3]Department of Endoscopic Medicine, Mie University School of Medicine, Tsu, Mie, 514-8507; and [4]Department of Hematology / Oncology, Graduate School of Medicine, Kyoto University, Kyoto, 606-8397

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We investigated the mechanism of apoptosis induced by bafilomycin A_1, an inhibitor of vacuolar H$^+$-ATPase. Bafilomycin A_1 significantly inhibited the growth of MKN-1 human gastric cancer cells. Bafilomycin A_1 induced apoptosis as demonstrated by DNA ladder formation and the TUNEL method. We designed a flow cytometric assay to detect the alteration in lysosomal pH using a fluorescent probe, fluorescein isothiocyanate-conjugated dextran. This assay revealed that bafilomycin A_1 dramatically increased lysosomal pH. However, bafilomycin A_1 induced neither significant decrease in mitochondrial transmembrane potential nor the release of mitochondrial cytochrome *c* into the cytoplasm. Western blotting showed that cathepsin D, but not cathepsin L, was released into the cytoplasm. The activity of caspase-3 was significantly increased by bafilomycin A_1. However, cathepsin D did not directly cleave procaspase-3. These findings suggest that bafilomycin A_1-induced apoptosis in MKN-1 cells is mediated by other proteases released after lysosomal dysfunction followed by activation of caspase-3 in a cytochrome *c*-independent manner. The present study showed that flow cytometric analysis of lysosomal pH can be useful to evaluate lysosomal protease-mediated apoptosis.

Key words: apoptosis, bafilomycin A_1, cathepsin D, flow cytometry, lysosome.

Abbreviations: $\Delta\Psi m$, mitochondrial transmembrane potential; V-ATPase, vacuolar H$^+$-ATPase; FITC-dex, fluorescein isothiocyanate–conjugated dextran; Ac-DEVD-AFC, Ac-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin; $DiOC_6(3)$, 3,3′-dehexyloxacarbocyanine iodide; PBS, phosphate-buffered saline; HRP, horseradish peroxidase.

Mitochondria are known to play an important role in apoptosis (*1*, *2*). The loss of mitochondrial transmembrane potential ($\Delta\Psi m$) mediates the release of cytochrome *c* into the cytosol, followed by activation of caspase-3 (*3–6*). On the other hand, the role of cytochrome *c*-independent mechanisms in apoptosis has been the focus of recent studies. Several studies have shown that lysosomal proteases mediate apoptosis in certain cell lines. Cathepsin D, a lysosomal aspartic protease, mediates apoptosis induced by cytokines (*7*, *8*), oxidative stress (*9*) and serum deprivation (*10*). Cathepsin L, a lysosomal cysteine protease, has also been reported to mediate apoptosis (*11*, *12*). These cathepsins are released from lysosomes into the cytosol, where they exert their catalytic activity. Recently, a novel lysosomal protease, lysoapoptase, has been reported to increase caspase-3 generation by cleaving procaspase-3, resulting in apoptosis (*13*, *14*).

In this study, we focused on the role of lysosomal dysfunction caused by disruption of vacuolar H$^+$-ATPase (V-ATPase) in apoptosis using bafilomycin A_1, which is a potent inhibitor of V-ATPase (*15*). We designed a method for flow cytometric analysis of lysosomal pH in bafilomycin A_1-treated MKN-1 human gastric cancer cell line using a pH-dependent fluorescent probe. We have previously reported that lysosomal pH increases during early apoptosis in P39 cells treated with etoposide (*16*). Apoptosis induction was detected by DNA ladder formation and the TUNEL method. Western blotting was performed to detect cathepsins released into the cytoplasm. To clarify whether cytochrome *c*-dependent mechanism is involved in apoptosis, we examined the loss of $\Delta\Psi m$ by flow cytometry and the release of cytochrome *c* into the cytosol by Western blotting.

MATERIALS AND METHODS

Materials—Bafilomycin A_1 was purchased from Wako Life Science (Osaka). E-64d and pepstatin A were obtained from Peptide Institute (Osaka). Fluorescein isothiocyanate-conjugated dextran (FITC-dex) and cathepsin D were from Sigma Chemical (St. Louis, MO, USA). 3,3′-Dihexyloxacarbocyanine iodide [$DiOC_6(3)$] was from Molecular Probes (Eugene, OR, USA). Ac-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) was obtained from Biomol (Plymouth Meeting, PA, USA). Procaspase-3 was purchased from Calbiochem (La Jolla, CA, USA)

Growth Inhibition of MKN-1 Cells by Bafilomycin A_1—MKN-1 cells (1.5×10^4 cells/ml, the Institute of Physical and Chemical Research, Tsukuba) were incubated with

*To whom correspondence should be addressed. Tel: +81-59-231-5011, Fax: +81-59-231-5011, E-mail: kawanisi@doc.medic.mie-u.ac.jp

bafilomycin A_1 in Dulbecco's modified essential medium (Gibco/BRL, New York, NY, USA) supplemented with 10% fetal bovine serum (Gibco/BRL). Relative number of viable cells was measured using a CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI, USA). We measured the absorbance of a formazan product, formed by bioreduction of a tetrazolium compound, at 490 nm, which was used as the relative cell number. Statistical analysis was carried out using Student's *t*-test, and $p < 0.05$ was considered as statistically significant.

Detection of Apoptotic Cells by the TUNEL Method—After treatment of MKN-1 cells (1×10^5 cells) with 100 nM bafilomycin A_1 for 24 h, the cells were fixed in 4% formaldehyde, washed with phosphate-buffered saline (PBS), then incubated with fluorescein-12-dUTP and terminal deoxynucleotidyl transferase. The cells were observed using a fluorescent microscope (*17*).

Detection of DNA Ladder Induced by Bafilomycin A_1—MKN-1 cells (1×10^6 cells) were treated with bafilomycin A_1 for 72 h, then washed twice with PBS. The cells were lysed and treated with RNase and proteinase K as described previously (*18*). DNA was extracted with phenol-chloroform and water-saturated ether, and then precipitated with ethanol. The DNA was then electrophoresed on a 1.4% agarose gel containing ethidium bromide.

Flow Cytometric Measurement of Lysosomal pH and $\Delta\Psi m$—MKN-1 cells (1×10^6 cells) were incubated with bafilomycin A_1 at 37°C. To measure lysosomal pH, 0.65 mg/ml FITC-dex, a pH-dependent fluorescent probe, was added to the media, and the cells were washed once with PBS and analyzed using a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA). FITC-dex is taken up by fluid phase endocytosis and accumulates in lysosomes (*19*, *20*). To detect changes in $\Delta\Psi m$, the cells were treated with 40 nM $DiOC_6(3)$ for 15 min at 37°C. The cells were then washed and analyzed as described above.

Detection of Cathepsin and Cytochrome c by Western Blotting—Bafilomycin A_1–treated MKN-1 cells (1×10^7 cells) were washed twice with PBS and suspended in 100 μl of Tris-HCl (pH 7.4) containing 250 mM sucrose. The cells were chilled on ice for 15 min, then frozen in liquid nitrogen, thawed and disrupted by douncing 8 times using a microhomogenizer with a Teflon-coated pestle. The cell lysate was centrifuged at 50,000 ×*g* for 30 min at 4°C to precipitate lysosomes. SDS-PAGE and Western blotting were performed as described previously (*21*). To detect cathepsin D, the polyvinylidene difluoride membrane was treated with goat anti-human cathepsin D antibody (1:500, Santa Cruz, Biotechnology, CA, USA) and then with horseradish peroxidase (HRP)–conjugated anti-goat IgG antibody (1:2,000, Santa Cruz Biotechnology). Quantitative analysis of cathepsin D was carried out using a laser densitometer (LKB 2222 UltroScan XL). To detect cathepsin L, rabbit anti-human cathepsin L antiserum (1:500, RD Laboratorien, Mühlstraße, Germany) and HRP-conjugated anti-rabbit IgG antibody (1:2,000, New England Biolabs, Beverly, MA, USA) were used. To examine the release of mitochondrial cytochrome *c* into the cytoplasm, cytosolic extracts were prepared from MKN-1 cells treated with 100 nM bafilomycin A_1 by precipitating mitochondria. Western blotting was performed as reported previously (*22*).

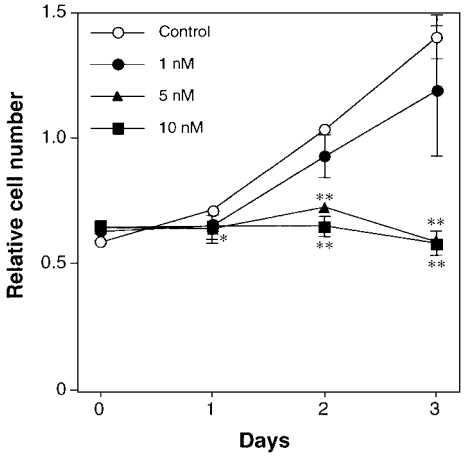

Fig. 1. **Effect of bafilomycin A_1 on the growth of MKN-1 cells.** MKN-1 cells were treated with the indicated concentrations of bafilomycin A_1 for 72 h. The relative cell number (absorbance at 490 nm) was measured as described in "MATERIALS AND METHODS." Values represent means ± SD of three independent experiments. $^*p < 0.01$ and $^{**}p < 0.001$, compared to untreated cells.

Measurement of Caspase-3 Activity—MKN-1 cells (1×10^6 cells) were incubated with bafilomycin A_1 for 48 h at 37°C, then washed twice with PBS and resuspended in 100 μl of assay buffer [0.1 M HEPES (pH 7.4), 2 mM DTT, 0.1% CHAPS and 1% sucrose]. The cell suspensions were frozen in liquid nitrogen and thawed at 37°C. This freeze-thaw procedure was repeated three times. The cell lysates were centrifuged, then the reaction with Ac-DEVD-AFC, a fluorescent substrate of caspase-3, was performed as described previously (*5*, *6*). The amount of AFC released was measured using a Shimadzu FC 5300 spectrofluorometer with excitation at 400 nm and emission at 505 nm. To detect caspase-3, Western blotting was also performed as reported previously (*22*).

Cleavage of Procaspase-3 by Cathepsin D—Cleavage of procaspase-3 by cathepsin D was evaluated in a reaction mixture containing 5 μM human procaspase-3 and 1 μM cathepsin D. After incubation for 4 h at 37°C, the mixture was electrophoresed on a 7.5% polyacrylamide gel containing 0.1% SDS.

RESULTS

Growth Inhibition of MKN-1 Cells Induced by Bafilomycin A_1—Figure 1 shows the effects of bafilomycin A_1 on growth of MKN-1 cells. The relative number of viable cells after treatment with 5 and 10 nM bafilomycin A_1 was significantly smaller than that of untreated control cells at 48 h (day 2) and 24 h (day 1), respectively. The cells showed no growth at these concentrations of bafilomycin A_1. Cell growth was not significantly inhibited at the concentration of 1 nM. E-64d, a cathepsin L inhibitor, and pepstatin A, a cathepsin D inhibitor, did not block the inhibitory effect of bafilomycin A_1 on cell growth (data not shown).

Detection of Apoptotic Cells by the TUNEL Method—We examined bafilomycin A_1-induced apoptosis by the

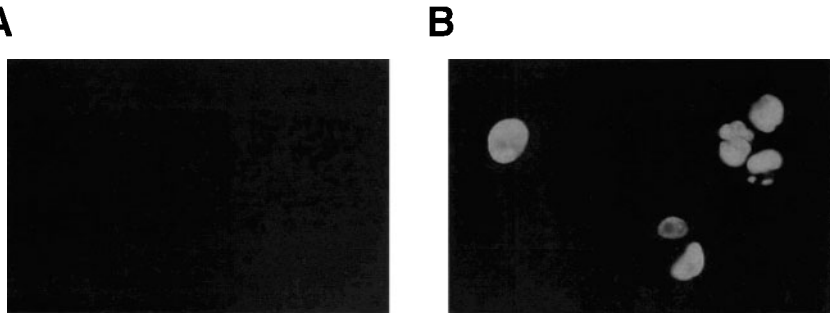

Fig. 2. **TUNEL method in MKN-1 cells treated with bafilomycin A_1.** MKN-1 cells were treated with no (A) or 100 nM bafilomycin A_1 (B) for 24 h. TUNEL-positive nuclei were observed in bafilomycin A_1-treated cells (B), whereas no fluorescence was observed in untreated cells (A).

TUNEL method. TUNEL-positive nuclei were observed at 10 nM bafilomycin A_1, and apparent fluorescence of the nuclei was observed at 100 nM (Fig. 2B). No fluorescence was observed in untreated control cells (Fig. 2A).

Bafilomycin A_1-Induced DNA Ladder Formation in MKN-1 Cells—Figure 3 shows DNA ladder formation in MKN-1 cells treated with bafilomycin A_1. Bafilomycin A_1 induced DNA ladder formation in a dose-dependent manner; faint ladders were observed at concentrations of 10 and 20 nM, and distinct ladders were observed at concentrations of 50 and 100 nM.

Alteration in Lysosomal pH and $\Delta\psi$m in Bafilomycin A_1-Treated MKN-1 Cells—Figure 4 shows the effect of bafilomycin A_1 on lysosomal pH and $\Delta\psi$m in MKN-1 cells as analyzed by flow cytometry. Lysosomal pH was dramatically increased by bafilomycin A_1 at 12 h (1.6-fold, geometric mean) and 48 h (4.4-fold) (Fig. 4A). However, bafilomycin A_1 did not induce a significant change in $\Delta\psi$m at 12 h (1.1-fold) and 24 h (0.81-fold) (Fig. 4B).

Detection of Cathepsins and Cytochrome c—Figure 5 shows the release of cathepsin D into the cytosol in MKN-1 cells treated with bafilomycin A_1. Lysosomal cathepsin D was released into the cytosol, and its release increased in a time-dependent manner (Fig. 5B). However, the release of cathepsin L into the cytosol was not observed at 24 h (data not shown). In addition, cytochrome *c* was not released from mitochondria into the cytosol at 48 h (data not shown).

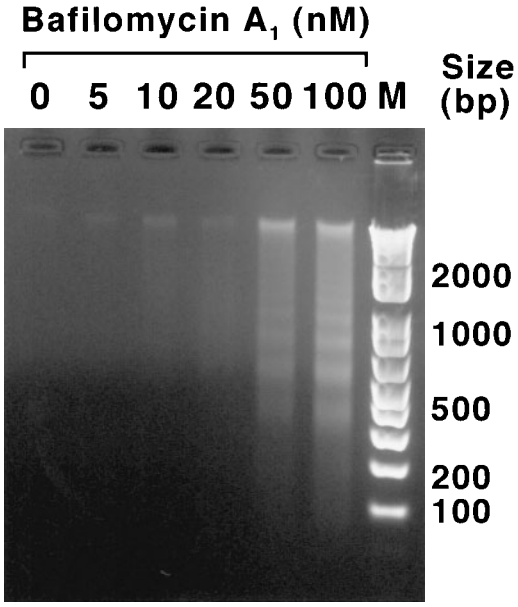

Fig. 3. **DNA ladder formation in MKN-1 cells treated with bafilomycin A_1.** MKN-1 cells were treated with the indicated concentrations of bafilomycin A_1 for 72 h. DNA was extracted and electrophoresis was carried out as described in "MATERIALS AND METHODS." M, 1Kb Plus DNA Ladder (Gibco/BRL)

Caspase-3 Activity in Bafilomycin A_1-Treated Cells—Caspase-3 activation was assessed using its substrate, DEVD-AFC. The activity of caspase-3 was significantly increased after 48-h treatment with 10 and 50 nM bafilomycin A_1 compared to untreated cells ($p < 0.001$, Fig. 6). Activated caspase-3 was detected in MKN-1 cells treated with bafilomycin A_1 by Western blotting (data not shown). However, cathepsin D did not cleave procaspase-3 into caspase-3 in a cell-free system (data not shown).

DISCUSSION

In the present study, we investigated the mechanism of apoptosis induced by bafilomycin A_1, a V-ATPase inhibitor. Bafilomycin A_1 significantly suppressed the cell growth of MKN-1 cells at low concentrations, and also induced apoptosis as demonstrated by DNA ladder formation and TUNEL-positive cells. Flow cytometry revealed that bafilomycin A_1 dramatically increased lysosomal pH without inducing a significant decrease in $\Delta\Psi$m. Release of mitochondrial cytochrome *c* into the cytosol was not observed in MKN-1 cells, suggesting that bafilomycin A_1-induced apoptosis is cytochrome *c*–independent. These findings suggest that bafilomycin A_1 induces apoptosis in MKN-1 cells via lysosomal dysfunction leading to activation of caspase-3. A recent study showing that leakage of lysosomal cysteine proteases into the cytosol leads to activation of caspase-3-like protease supports these findings (*23*, *24*). On the other hand, we found that cytochrome *c* is released into the cytosol of HL-60 cells treated with bafilomycin A_1 (unpublished observation). A recent study has shown that concanamycin A, a V-ATPase inhibitor, also induces the release of cytochrome *c* into the cytosol of human submandibular gland ductal cells (*25*). Therefore, the involvement of cytochrome *c* in apoptosis induced by V-ATPase inhibitors may depend on the cell type.

The mechanism of bafilomycin A_1–induced apoptosis cannot be explained simply by the increase in lysosomal pH, because a recent study has demonstrated that ammonium chloride increases lysosomal pH and inhibits cell growth without inducing apoptosis (*26*). In addition to the increase in lysosomal pH, lysosomal enzymes (*e.g.*, lysoapoptase) and cathepsins, which are released into the

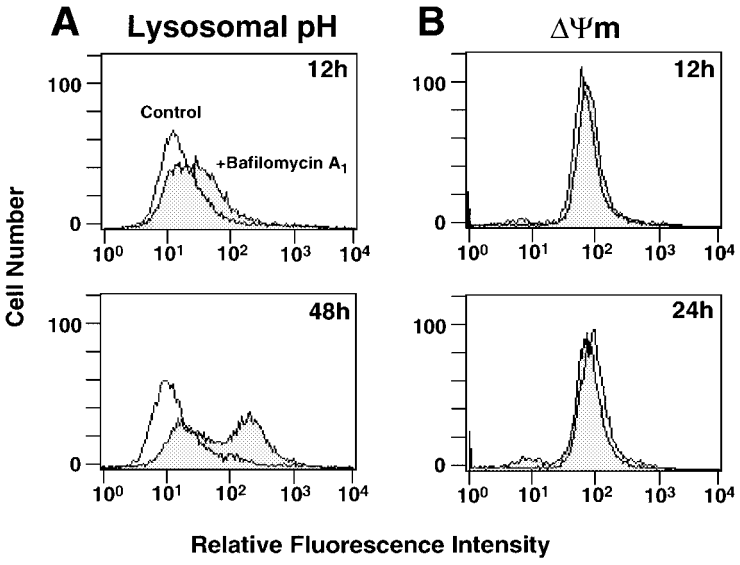

Fig. 4. **Flow cytometric fluorescence distributions of MKN-1 cells treated with bafilomycin A_1.** MKN-1 cells were treated with 100 nM bafilomycin A_1 for the indicated durations. The cells were then treated with FITC-dex (A) or $DiOC_6(3)$ (B) and analyzed with a FACScan flow cytometer as described in "MATERIALS AND METHODS." Lysosomal pH was dramatically increased by bafilomycin A_1 (A). However, bafilomycin A_1 did not induce a significant change in $\Delta\psi m$ (B). Open peaks, control cells; shaded peaks, bafilomycin A_1–treated cells.

cytosol through an unknown mechanism that is associated with bafilomycin A_1–induced inhibition of V-ATP, play important roles in apoptosis. Cathepsin D mediates apoptosis induced by a variety of factors, such as cytokines (*7*, *8*), oxidative stress (*9*) and serum deprivation (*10*). Ceramide directly binds to and activates the endosomal cathepsin D (*27*). Cathepsin D protein expression is induced by adriamycin, etoposide and γ-radiation (*28*). In this study, bafilomycin A_1 induced the release of cathepsin D into the cytosol, and significantly increased caspase-3 activity. However, pepstatin A did not block the inhibitory effect of bafilomycin A_1 on cell growth. In addition, cathepsin D did not directly cleave procaspase-3. Cathepsin L has also been reported to induce apoptosis (*11*, *12*). However, in this study, bafilomycin A_1 did not induce the release of cathepsin L into the cytosol. Therefore, proteases other than cathepsins may be involved in bafilomycin A_1-induced apoptosis. A recent study has demonstrated that a novel lysosomal enzyme, lysoapoptase, mediates apoptosis through caspase-3 activation (*13*, *14*). The present study demonstrated that bafilomycin A_1 induces apoptosis in MKN-1 cells by increasing lysosomal pH without affecting mitochondria. Lysoapoptase is probably involved in bafilomycin A_1-induced apoptosis in MKN-1 cells. We have recently reported that etoposide and bafilomycin A_1, which specifically target lysosomes, induce apoptosis in myelodysplastic syndrome-derived P39 cells (*16*). Overall, these observations suggest that the function of H^+-ATPase and/or lysosomal membrane are affected in certain cell types by lysosome-targeting agents including bafilomycin A_1.

There is no flow cytometric method capable of assessing the impairment of lysosomal function and the role of

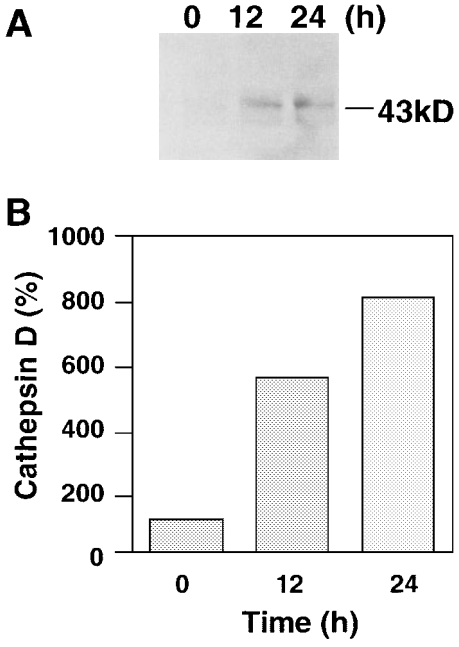

Fig. 5. **Release of cathepsin D into the cytosol induced by bafilomycin A_1.** (A) Western blotting analysis of cathepsin D in the cytosol of MKN-1 cells. MKN-1 cells were treated with 100 nM bafilomycin A_1 for the indicated durations. The cytosolic extract was then prepared by centrifugation to precipitate lysosomes. SDS-PAGE and Western blotting were performed as described in "MATERIALS AND METHODS." (B) Relative activity of cathepsin D in MKN-1 cells. The activity of cathepsin D in untreated cells is 100%.

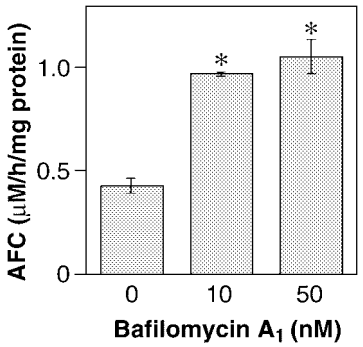

Fig. 6. **Caspase-3 activity in MKN-1 cells treated with bafilomycin A_1.** Caspase-3 activation was assessed using the substrate of caspase-3, DEVD-AFC. The activity of caspase-3 was significantly increased after the 48-h treatment with bafilomycin A_1 compared to untreated cells ($*p < 0.001$).

lysosomal enzymes in apoptosis. Low internal pH is maintained in lysosomes *via* the membrane-bound H^+-ATPase for optimal activity of lysosomal enzymes. Bafilomycin A_1 and concanamycin A_1 are potent selective inhibitors of V-ATPase (*15*). In the present study, we have designed a flow cytometric assay to measure lysosomal pH using a sensitive indicator, FITC-dex, which is internalized into lysosomes by endocytosis. Since a low pH is an optimal condition for endocytosis (*29*, *30*), FITC-dex uptake may be smaller in bafilomycin A_1-treated cells than in control cells. Nevertheless, the fluorescence intensity of FITC-dex was increased in the treated cells. This enhanced fluorescence intensity seems to reflect a significant increase in lysosomal pH. Our results demonstrated that the use of FITC-dex is a valid technique for measuring dynamic changes in lysosomal pH during lysosome-dependent apoptosis. This method may be useful to evaluate apoptosis occurring *via* lysosome-dependent mechanisms.

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