Oxidative Stress Induces Parallel Autophagy and Mitochondria Dysfunction in Human Glioma U251 Cells

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Accumulation of reactive oxygen species (ROS) such as hydrogen peroxide (H2O2) is an oxidative stress response, which induced various defense mechanisms or programmed cell death (PCD). As one of the major types of PCD, autophagy has been observed in response to several anticancer drugs and demonstrated to be responsible for cell death. To date, however, the exact mechanism by which ROS regulates autophagy is still poorly understood. Thus, the purposes of this study were to elucidate how H2O2 exerts its cytotoxic effects on malignant glioma U251 cells and to uncover the molecular mechanism that might be involved. Here, we show that H2O2-induced autophagy and apoptosis in U251 cells are mediated through the Beclin 1 and Akt/mTOR pathways. Accumulation of ROS leads to changes in mitochondrial permeability with loss of mitochondrial membrane potential and disruption of mitochondrial dynamics at a transcriptional level of fission and fusion. Overexpression of cellular Bcl-2 partially inhibited autophagy through interference with the Beclin 1 and Akt/mTOR signaling pathways and is regulated by the anti-apoptotic gene Bcl-2 in glioma U251 cells.

Key Words: autophagy; mitochondria; Bcl-2; PI3K/Akt/mTOR; apoptosis.

Programmed cell death (PCD) is important in various developmental pathways. Apoptosis, the best known type, is now referred to as type I PCD; autophagy is characterized as type II PCD (Elliott and Reiners, 2008). Morphological manifestations are among the primary differences between types I and II PCD. Autophagy is characterized by the presence of abundant autophagic vacuoles that engulf bulk cytoplasm and cytosolic organelles such as mitochondria and endoplasmic reticulum, with subsequent degradation by cell’s own lysosomal system (Gozuacik and Kimchi, 2004). The relationship between autophagy and apoptosis is complex and varies between the cell types and the stress distinction. On occasion, autophagy and apoptosis occur instantaneously after stress; at other times, only autophagy or apoptosis is observed (Lockshin and Zakeri, 2004). The mechanisms responsible for autophagy are still not very clear. Human Beclin 1 that shares 24.4% amino acid identity with a yeast gene product, Apg6/Vps30p, was first identified in a yeast two-hybrid screen as a Bcl-2-interacting protein (Liang et al., 1998), i.e., frequently monoallelically deleted in sporadic breast, ovarian, and prostate tumors (Aita et al., 1999). As part of the class III phosphatidylinositol 3-kinase (PI3K) complex, Beclin 1 participates in autophagosome formation and is important in mediating the localization of other autophagic proteins to preautophagosomal membranes (Kihara et al., 2001). The autophagic function of Beclin 1 may play a role in the negative regulation of tumorigenesis, and some evidence suggests that functional inactivation of Beclin 1 may contribute to tumorigenesis (Liang et al., 1999; Yue et al., 2003). The anti-apoptotic gene Bcl-2 proteins bind to Beclin 1, and the Beclin 1-Bcl-2 complex functions as a brake on autophagy and autophagy-dependent cell death (Pattingre et al., 2005).

The signaling pathway composed of PI3K, protein kinase B (Akt), and mammalian target of rapamycin (mTOR), which plays a central role in the regulation of cell proliferation, differentiation, and survival (Oldham and Hafen, 2003; Vogt, 2001). The class I PI3K/Akt pathway, which is constitutively activated in glioma cells, is known to play an important role in cell survival (inhibition of apoptosis) and has been linked to various human cancers (Osaki et al., 2004; Wetzker and Rommel, 2004). Disruption of the PI3K/Akt pathway, culminating in inhibition of Akt, has been found to be associated with autophagy induced by a variety of antineoplastic agents in cancer cells (Takeuchi et al., 2005). Studies have indicated that Bcl-2 can be a strict mediator downstream of PI3K/Akt signaling and that Akt contributes to the positive regulation of mTOR signaling pathway, which can inhibit cell autophagic activity (Fresno Vara et al., 2004; Luo et al., 2003).

The purposes of this study were to determine whether hydrogen peroxide (H2O2) can induce PCD in human glioma U251 cells to examine the relationship between autophagy and apoptosis in this process and to approach the role of autophagy...
induced by oxidative stress in vitro. Our research shows that H$_2$O$_2$ indeed caused apoptosis, that autophagy occurs in U251 cells by means of class III PI3K/Bcl-2, and that the PI3K/Akt/mTOR signaling pathway decreases mitochondrial membrane potential and disrupts homeostasis of mitochondrial dynamics. Overexpressed Bcl-2 vector partially inhibited this autophagy; the small interfering RNA (siRNA) of Bcl-2 accelerated it. Inhibition of autophagy by the autophagic specific inhibitor 3-methyladenine (3-MA) enhanced the apoptosis induced by H$_2$O$_2$.

**MATERIALS AND METHODS**

**Cell culture and transfection.** Human glioma U251 cells were purchased from American Tissue Culture Collection (Rockville, MD). Cells were cultured at 37°C with 5% CO$_2$ in a humidified atmosphere in Dulbecco’s modified eagle media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

The Bcl-2 complementary DNA (cDNA) was amplified by PCR with appropriate oligonucleotide primers, sense primer: 5'-GAAGATCTAGGATGGCGCAGCTGG-3' and antisense primer: 5'-CGGATAATCTACTGTGGCCCGAATAGG-3', containing the restriction site for BglII and EcoRI during Bcl-2 amplification. The eGFP expression vector, pEGFP-C1 (Clontech, Mountain View, CA), was purchased from Clontech (Mountain View, CA), and the amplified cDNAs were cloned into the plasmid’s multicloning site. The plasmids were purified employing the HiSpeed Plasmid Maxi Kit (Qiagen Inc., Hilden, Germany). siRNA for human Bcl-2 (National Center for Biotechnology Information, accession numbers NM_000633) corresponded to the following sequence: 5'-ATTGTGATGAAGTACA-TCCA-3', pSilencer 3.1 H1vector, was purchased from Clontech (Mountain View, CA), and the amplified cDNAs were cloned into the plasmid’s multicloning site. The plasmids were transfected using Lipofectamine 2000 Plus reagent (Invitrogen) according to the manufacturer’s recommendations. Transfection efficiency was estimated by Western blot analysis. Cells were cultured for another 24 h after transfection before they were trypsinized and used for experiments.

**Cell viability assays.** The viability of the U251 cells was determined by MTT assays. Briefly, cells were plated for 24 h in a 96-well plate at a density of 1 $\times$ 10$^4$ cells/well in 200 μl of complete medium containing different concentrations of H$_2$O$_2$ (0.5, 1.0, 1.5 mM [30% stock solution; Calbiochem, San Diego, CA]) or 4% FBS (0, 6, 12, and 24 h) or treated with 1mM H$_2$O$_2$ and/or 10mM of the autophagy-specific inhibitor 3-MA (Sigma, St Louis, MO) or 25μM cell-permeable pan-caspase inhibitor ZVal-Ala-Asp-fluoromethylketone (Z-VAD-FMK, Sigma). Each treatment was repeated in three wells. The cells were incubated for 20 h at 37°C in a humidified chamber, and MTT reagent (10 μl, 5 mg/ml in PBS; Sigma) was added to each well and incubated for 4 h. The microtiter plate containing the cells was centrifuged at 300 $\times$ g for 5 min at 4°C. The MTT solution was removed from the wells by aspiration, and the formazan MTT assays. Briefly, cells were plated for 24 h in a 96-well plate at a density of 1 $\times$ 10$^4$ cells/well in 200 μl of complete medium containing different concentrations of H$_2$O$_2$ (0.5, 1.0, 1.5 mM [30% stock solution; Calbiochem, San Diego, CA]) or 4% FBS (0, 6, 12, and 24 h) or treated with 1mM H$_2$O$_2$ and/or 10mM of the autophagy-specific inhibitor 3-MA (Sigma, St Louis, MO) or 25μM cell-permeable pan-caspase inhibitor ZVal-Ala-Asp-fluoromethylketone (Z-VAD-FMK, Sigma). Each treatment was repeated in three wells. The cells were incubated for 20 h at 37°C in a humidified chamber, and MTT reagent (10 μl, 5 mg/ml in PBS; Sigma) was added to each well and incubated for 4 h. The microtiter plate containing the cells was centrifuged at 300 $\times$ g for 5 min at 4°C. The MTT solution was removed from the wells by aspiration, and the formazan crystals were dissolved in dimethylsulfoxide (150 μl). Absorbance was recorded at 570 nm wavelength.

**Lactate dehydrogenase activity-based cytotoxicity assays.** U251 cells (5 $\times$ 10$^4$ cells/well) were cultured in a 24-well plate overnight and then harvested with 1mM H$_2$O$_2$ and/or 10mM 3-MA or 25μM Z-VAD-FMK for 12 h. The lactate dehydrogenase (LDH) released in the culture medium (extracellular LDH or LDH) was used as an index of cell injury. The adherent and viable cells were lysed in 0.1% NP-40 for 15 min to release LDH (intracellular LDH or LDH). The percentage was calculated as follows:

\[ \text{LDH release rate} = \frac{\text{LDH}_{\text{extracellular}}}{\text{LDH}_{\text{total}} + \text{LDH}_{\text{extracellular}}} \times 100\% \]

**Electron microscopy.** Electron microscopy and morphometric analysis were performed as described previously (Han et al., 2007). Cells were fixed for 30 min with ice-cold 3% glutaraldehyde in 0.1M cacodylate buffer, embedded in Epon, and processed for transmission electron microscopy by standard procedures. Representative areas were chosen for ultra-thin sectioning and examined on transmission electron microscope at $\times 6000$ or $\times 12000$ magnification.

**Immunofluorescent confocal laser microscopy.** Cells cultured on coverslips were stained with nuclear stain Hoechst 33342 (2μM; Sigma) to reveal the cell chromatin condensation. Autophagy is characterized by development of autophagic vacuoles. Monodansylcadaverine (MDC) has been proposed as a tracer for autophagic vacuoles (Biederbick et al., 1995). U251 cells were cultured on coverslips overnight, then treated with different doses of stimuli for 12 h as described above, and rinsed with PBS. They were then stained with 50μM MDC at 37°C for 1 h. After incubation, the cells were fixed for 15 min with ice-cold 4% paraformaldehyde at 4°C, washed twice with PBS, and examined under Olympus FV1000 confocal laser microscopy.

The formation of acidic vesicular organelles (AVOs) is also related to cell autophagy (Komata et al., 2004). Acridine orange–stained cells show diffuse green fluorescence, whereas the acidic compartments, including AVOs, fluoresces bright red. Cells treated by different stimuli were incubated for 15 min with acridine orange (10μM in medium from a 10μM stock in water), washed with PBS, and then examined under Olympus FV1000 confocal laser microscopy.

**Flow cytometry analysis.** We used Rhodamine 123 (Rho123, 10μM; Sigma) to quantify the mitochondrial membrane potential ($\Delta$Ψ$_{m}$) as described previously (Ferlini and Scambia, 2007). 2,7-dichlorodihydro-fluorescein diacetate (DCFH-DA, 10μM; Sigma) staining was employed for reactive oxygen species (ROS) analysis as described previously (Huang et al., 2008). Propidium iodide (PI, 1 μg/ml) and Annexin V-FITC (1 μg/ml) were used for determination of cell death (Invitrogen). After exposure to the different experimental conditions, cells were trypanized and incubated with PI and Annexin V-FITC for 15 min at 37°C. The samples were then analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

**Coimmunoprecipitation analysis.** The Bcl-2 was immunoprecipitated with 5 μl of an anti-Bcl-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) from clear lysates of nontransfected U251 cells or from U251 cell lysates prepared 24 h after transfection with plasmids encoding Bcl-2 or Bcl-2 siRNA. Cells were lysed using 1 ml radioimmunoprecipitation (RIPA) buffer (50mM Tris-HCl [pH 6.8], 150mM NaCl, 0.1% SDS, 1mM EDTA, 0.1mM NaVO$_4$, 1mM sodium fluoride [NaF], 1% NP40, 1% Triton X-100, 1mM dithiobisreitol, and 1mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A), then scraped, and collected into an Eppendorf tube. After lysis for 40 min and centrifugation at 14,000 $\times$ g for 20 min, the supernatants were collected. A 25 μl aliquot of protein A-Sepharose CL-4B (GE Uppsala, Sweden) beads was added (50% in RIPA buffer) to immunoprecipitate the nonspecific antibody. Immune complexes were immobilized by adding 50 μl of protein A-Sepharose beads, washed three times, and 2$\times$ sample buffer was added. Western blot analysis for immunoprecipitated Bcl-2 and coimmunoprecipitated Beclin 1 was performed with an antibody raised against Beclin 1 (Santa Cruz Biotechnology).

**Reverse transcriptase PCR and Western blotting.** Total RNA was extracted from the cells using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. Three micrograms of total RNA was used for reverse transcription in a total volume of 20 μl with the SuperScript preamplification system (Promega, Madison, WI). PCR products were separated on a 1.5% agarose gel and viewed by ethidium bromide staining. These data were acquired with Tanon GIS gel imager system.

For protein analysis, the cells were harvested 12 h following different treatment as described above, washed with cold PBS, then incubated in ice-cold RIPA buffer. Cell lysates were sonicated for 30 s on ice and then lysed at 4°C for 60 min. The cell lysates were centrifuged for 30 min at 12,000 $\times$ g. Protein concentrations in the supernatants were determined by the BCA reagent (Pierce, IL). For Western blot analysis, lysate proteins (30-50 μg) were resolved over 8, 10, and 15% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose transfer membranes (Whatman, Maidstone, UK).
The membranes were blocked with 5% nonfat dry milk in buffer (10mM Tris-HCl [pH 7.6], 100mM NaCl, and 0.1% Tween 20) for 1 h at room temperature and then incubated with the desired primary antibody (all from Santa Cruz Biotechnology) overnight at 4°C, followed by incubation with horseradish peroxidase–conjugated secondary antibody (Thermo, Waltham, MA) at 1:2000 dilution for 1 h at room temperature. The immunoreactive bands were visualized by the diaminobenzidine (Sigma) coloration method. The representative bands of light chain 3 (LC3) were measured with a Tanon GIS gel imager system and analyzed as described previously (Klionsky et al., 2008; Koike et al., 2005). The levels of LC3-II and LC3-I were normalized to those of actin, and the ratios of normalized LC3-II to LC3-I were presented as a mean ± SD from three independent experiments.

**Statistical analysis.** Data are representative of at least three independent experiments each in triplicate determination. Statistical analysis of the data was performed using one-way ANOVA. The Tukey post hoc test was used to determine the significance for all pairwise comparisons of interest. p Values of less than 0.05 were considered to represent a statistically significant difference.

**RESULTS**

**H$_2$O$_2$ Induces PCD Including Apoptosis and Autophagy in U251 Glioma Cells**

As shown in Figure 1A, U251 cells treated with different concentrations of H$_2$O$_2$ for 6, 12, and 24 h exhibited dose-dependent cell death. Treatment with 1mM H$_2$O$_2$ for 12 h inhibited cell viability by 71.65 ± 5.45% (p < 0.05). H$_2$O$_2$ downregulated anti-apoptotic gene Bcl-2 expression and enhanced expression of pro-apoptotic gene Bax, cleaved caspase-3, and increased cytoplasm cytochrome c (Fig. 1B). These results indicated that the classic Bcl-2/Bax, caspase-3, and mitochondrial apoptosis pathways were involved in H$_2$O$_2$-induced U251 cell apoptosis.

Next, we raised the question whether H$_2$O$_2$ induced both autophagy and apoptosis at the same time. Cell autophagy was induced in nutrient-free medium—i.e., in the absence of serum and amino acids. Transmission electron microscopy revealed formation of autophagic vacuoles in both 12-h nutrient-free and 1mM H$_2$O$_2$-treated groups. As shown in Figure 2A, panel a (arrows), control cells exhibited normal nuclei with uniform and finely dispersed chromatin, surrounded by cytoplasm with normal appearing mitochondria. In contrast, culture for 12 h in nutrient-free medium or with 1mM H$_2$O$_2$ resulted in the appearance of abundant autophagic vacuoles (Fig. 2A, panels b and c, arrows). Then, MDC and Hoechst 33342 staining were used to detect the autophagic vacuoles and cell apoptotic chromatin condensation. Because MDC accumulates in mature autophagic vacuoles, such as autophagosomes but not in the early endosomal compartment, MDC staining can be used to detect autophagic vacuoles. In MDC staining, the bright green dots, which indicate autophagosomes, were increased in the cytoplasm. In this study, at 12 h, compared with the control, autophagosomes were increased and bigger in U251 cells after exposed to nutrient-free medium or H$_2$O$_2$ treatment (Fig. 2B). At 12 h compared with control group, 1mM H$_2$O$_2$-induced apoptotic chromatin condensation was assessed by Hoechst 33342 staining (Fig. 2B). We also examined the effect of H$_2$O$_2$ treatment on formation of AVOs in U251 cells by fluorescence microscopy. After staining with acridine orange, the protonated form of acridine orange accumulates in acidic compartments and forms aggregates, which are characterized by red fluorescence. As can be seen in Figure 2B, control U251 cells displayed primarily green fluorescence with minimal red fluorescence, indicating the absence of AVOs. Exposure to nutrient-free medium for 12 h produced an increase in red fluorescence. Treatment of U251 cells with 1mM H$_2$O$_2$ resulted in formation of red fluorescent AVOs that were relatively more pronounced at 12 h. These results provided further evidence that H$_2$O$_2$ treatment...
FIG. 2. H$_2$O$_2$ induced parallel autophagy and apoptosis at the same time in U251 cells. (A) H$_2$O$_2$ induces formation of autphagic vacuoles in U251 cells. Representative transmission electron microscopy photomicrographs of U251 cells in the nutrient-free condition or treated with 1mM H$_2$O$_2$ for 12 h. Nuclear and cell profile morphologies are normal in control cells (panel a, ×6000). Exposure to nutrient-free medium for 12 h resulted in the development of autophagic vacuoles (panel b, ×6000, arrows, autophagic vacuoles). The number of autophagic vacuoles was also markedly increased 12 h following H$_2$O$_2$ treatment (c, ×12,000, arrows, close-up of autophagic vacuoles). (B) MDC, Hoechst 33342, and acridine orange staining were used to detect the autophagic vacuoles, cell apoptotic chromatin condensation, and formation of autophagic vacuoles in U251 cells treated in the nutrient-free condition or treated with 1mM H$_2$O$_2$ for 12 h (confocal microscopy at ×800 magnification for MDC staining, ×600 magnification for Hoechst 33342 staining, and ×400 magnification for acridine orange staining). Representative results of three independent experiments. (C) The accumulation of LC3 in the nutrient-free condition or treated with 1mM H$_2$O$_2$ for 12 h. (D) The ratio of normalized LC3-II to LC3-I. The levels of LC3-II and LC3-I were measured as described above, normalized to those of actin, and the data were presented as a mean ± SD from three independent experiments. *p < 0.01 versus control group.
caused autophagy in U251 cells. Autophagy microtubule-associated protein LC3 is the mammalian equivalent of yeast Atg8; when autophagy is activated, LC3-I (18 kDa) is cleaved to proteolytic derived LC3-II (16 kDa). There are localized in the cytosol (LC3-I) or in autophagosomal membranes (LC3-II). Thus, detection of LC3-II can be used to estimate the abundance of autophagosomes (Gonzalez-Polo et al., 2007). As shown in Figures 2C and 2D, culture in nutrient-free medium or H2O2 treatment resulted in accumulation of LC3-II, and the ratios of the amounts of LC3-II to LC3-I were significantly higher than control group. All the above indicated that H2O2 induced simultaneous autophagy and apoptosis in U251 cells.

H2O2 Induces Autophagy through Class III PI3K/Beclin 1 and the PI3K/Akt/mTOR Pathway

Our next goal was to determine whether or not the autophagy induced by H2O2 was dependent on both the class III PI3K/Beclin 1 and the PI3K/Akt/mTOR pathways. After 0.5, 1, or 1.5 mM H2O2 treatment for 12 h, the U251 glioma cell protein was collected for Western blot analysis. The expression of autophagy-associated Beclin 1 protein was upregulated by treatment with 1 mM H2O2 (Figs. 3A and 3B). mTOR, which negatively mediates autophagy, is important in autophagy processing, and Akt contributes to the positive regulation of mTOR (Cao et al., 2006; Takeuchi et al., 2005). In order to determine whether H2O2-mediated induction of autophagy involved the PI3K/Akt/mTOR signaling pathway, we measured the phosphorylation of Akt at Ser-473, one of the downstream targets of Akt, and phosphorylation of FKHR at Ser-256 (Kuo et al., 2006). As shown in Figure 3C, treatment of U251 cells with 1 mM H2O2 resulted in a significant reduction of Akt, FKHR, mTOR, and p70S6K phosphorylation. Taken together, these results demonstrate that H2O2 induced autophagy in U251 cells by activation of the class III PI3K/Beclin 1 signaling pathway and by inhibition of Akt and mTOR activity through the PI3K/Akt/mTOR pathway.

Bcl-2 Participated in the Regulation of Autophagy Induced by H2O2

To evaluate the function of the anti-apoptotic gene Bcl-2 in autophagy induced by H2O2, U251 cells were transfected with pEGFP-C1-Bcl-2 or pSilencer 3.1-Bcl-2 plasmids. Transfection efficiency was estimated by Western blot analysis. As shown in Figure 4A, Bcl-2 protein expression significantly increased in U251 Bcl-2 cells (Fig. 4A). In siRNA-transfected Bcl-2 cells, endogenous level of Bcl-2 protein levels decreased by 73% (Fig. 4B).

To further address the function of Bcl-2 in the regulation of autophagy, we compared the levels of autophagy after H2O2 treatment in untransfected cells and cells transfected with Bcl-2 or Bcl-2 siRNA. As shown in Figure 4C, transfection of cells with Bcl-2 significantly inhibited the post-H2O2 function of autophagic vacuoles detected by MDC staining. In contrast, transfection with siRNA led to obviously increased MDC accumulation compared with untransfected U251 cells. We next assessed the effect of Bcl-2 transfection on cell acidification. As shown in Figure 4C, 1 mM H2O2 resulted in the appearance of autophagic vacuoles when cells were stained with acridine orange. Compared with U251 cells, overexpression of Bcl-2 inhibited the acidification induced by H2O2. In contrast, Bcl-2 siRNA increased the level of red fluorescence, which indicated cell acidification and formation of autophagic vacuoles.
Overexpression of Bcl-2 also reduced in LC3-II accumulation, in contrast, Bcl-2 silencing enhanced LC3 modification and increased the amount of LC3-I that were converted into LC3-II, a marker of autophagy (Figs. 4D and 4E). All these results corroborate the observation that Bcl-2 affected H2O2-induced autophagy, which was partially inhibited by overexpression of Bcl-2 and intensified by Bcl-2 siRNA.

**Bcl-2-Mediated Autophagy through Both Beclin 1 and Akt/mTOR Signaling**

To look for the possible pathways that might be involved in Bcl-2-regulated autophagy, we performed coimmunoprecipitation analysis to evaluate Beclin 1-Bcl-2 interactions. U251 cells transfected with Bcl-2 or Bcl-2 siRNA expressed similar levels of Beclin 1 compared with U251 control cells. Nevertheless, in
Bcl-2-transfected cells, Bcl-2 coimmunoprecipitated with more endogenous Beclin 1 than in control U251 cells (Fig. 5A). In contrast, little Beclin 1 was coimmunoprecipitated with Bcl-2 in cells transfected with Bcl-2 siRNA (Fig. 5A). To further confirm whether Bcl-2-mediated autophagy through a Beclin 1-dependent pathway in U251 cells, we measured Beclin 1 expression in U251 cells after H2O2 treatment. As shown in Figures 5B and 5C, the increase of Beclin 1 expression induced by H2O2 was inhibited by overexpression of Bcl-2 and reducing of Bcl-2 resulted in increased Beclin 1 activation. These results suggested that Bcl-2 has a direct interaction with endogenous Beclin 1. In Bcl-2-transfected cells, more Beclin 1 combined with Bcl-2, thereby inhibiting autophagy through the class III PI3K/Beclin 1 pathway, in contrast, Bcl-2 siRNA promoted autophagy, which related to Beclin 1 activation.

We also investigated whether PI3K/AKT/mTOR, which is important in regulating cell proliferation and autophagy, was involved in H2O2-induced Bcl-2-regulated autophagy. As previously shown (Fig. 3B), 1mM H2O2 treatment had been found to cause significant reduction in expression of the phosphorylation of Akt, FKHR, mTOR, and p70S6K. Thus, we speculated that Bcl-2-mediated autophagy might act through the PI3K/AKT/mTOR signaling pathway. To test our hypothesis, we measured the phosphorylation of Akt, FKHR, mTOR, and p70S6K after 12-h treatment of cells with 1mM H2O2. As shown in Figure 5D, compared with the untransfected cells, H2O2-induced downregulation of phosphorylation of Akt at Ser-473, FKHR at Ser-256, mTOR at Ser-2481, and p70S6K at Thr 389 was remarkably inhibited by overexpression of Bcl-2. In contrast, Bcl-2 siRNA decreased phosphorylation of Akt, FKHR, mTOR, and p70S6K. Collectively, these results indicated that autophagy is induced by H2O2 in U251 glioma cells through both the Beclin 1 and the Akt/mTOR signaling pathways.

H2O2 Induces Autophagy with Loss of Mitochondrial Membrane Potential and Disruption of Mitochondrial Dynamics

Accumulation of ROS in mitochondria leads to mitochondrial permeability and results in a collapse of mitochondrial membrane potential (Scherz-Shouval and Elazar, 2007). To investigate the mechanism by which H2O2, specific ROS,
induced autophagy in U251 glioma cells, the extent of cellular oxidative stress was estimated by monitoring the generation of ROS using the fluorescent dye DCFH-DA. As shown in Figure 6A, compared with the control group, exposure to 1mM H2O2 caused an increase of 21.0% in intracellular ROS accumulation in cultured U251 cells (p < 0.01). Overexpression of Bcl-2 partially inhibited accumulation of ROS to 12.4% above control levels. Abundant ROS accumulation to 58.8% was detected in Bcl-2 siRNA–transfected cells after H2O2 exposure (p < 0.05). Next, we evaluated the effects of H2O2 on mitochondrial membrane potential (ΔΨm) and correlated the changes in mitochondrial permeability. Quantitation of ΔΨm was determined by Rhodamine 123, a cationic fluorescent dye, which localizes in the mitochondria of viable cells because of the relatively high negative electrical potential across the mitochondrial inner membrane (Johnson et al., 1980). In this study, H2O2 treatment induced a drop in ΔΨm that reached 67.4% of control levels (p < 0.01). The overexpression of Bcl-2 prevented the effect of H2O2 on the loss in ΔΨm. A significant decrease in ΔΨm that reached 42.9% of control levels was observed in Bcl-2 siRNA–transfected cells after H2O2 exposure (Fig. 6B).

Mitochondrial dynamics are dictated by the equilibrium between fusion and fission of mitochondria, two processes that occur in normal nonapoptotic conditions. On stimulation of cell death, the balance between fusion and fission is disrupted and the mitochondrial fragmentation increased (Suen et al., 2008). To address the question whether mitochondrial dynamics are affected by H2O2 treatment, we examined fusion and fission activities at the transcriptional level. RT-PCR was performed with primers for the fusion factors Mfn1, Mfn2, and Opal and for the fission factors Fis1, Drp1, and MTP18 and GAPDH for normalization (Jendrach et al., 2008). Twelve hours after treatment with 1mM H2O2, messenger RNA levels of the fusion factor Mfn1 and the fission factors Fis1 and Drp1 were significantly upregulated compared with the control cells.
(Fig. 6C). Overexpression of Bcl-2 was associated with small increase in the levels of the Mfn1 transcript, but the levels of other factors were similar to the control group. By contrast, after treatment with H$_2$O$_2$, expression of the fission factors Fis1 and Drp1 was obviously enhanced in Bcl-2 siRNA–transfected cells, compared with U251 cells (Fig. 6D). Mfn2, Opal1, and MTP18 had no significant changes in this model. Taken together, H$_2$O$_2$ treatment increased generation and accumulation of ROS and loss of mitochondrial membrane potential. At the same time, H$_2$O$_2$ impaired the homeostasis of mitochondrial fusion and fission. Overexpression of Bcl-2 partially stabilized the mitochondria and led to recovery of the mitochondrial dynamics at the transcriptional level.

**Inhibition of Autophagy by 3-MA Accelerates the Apoptosis Induced by H$_2$O$_2$**

Autophagy has been shown to prolong cell survival under pharmacological stress conditions (Cuervo, 2004; Kelekar, 2005). Consequently, we examined the relationship between autophagy and apoptosis in our model, having demonstrated that H$_2$O$_2$ induced both autophagy and apoptosis in U251 cells. To investigate the interaction of these two processes, 10µM of the autophagy-specific inhibitor 3-MA, which has no significant toxic effect in some certain cells (Chen et al., 2008; Herman-Antosiewicz et al., 2006; Katayama et al., 2007), or 25µM cell-permeable pan-caspase inhibitor Z-VAD-FMK (Kunchithapautham and Rohrer, 2007) were used. MTT assay indicated that 3-MA or Z-VAD-FMK alone had no effect on cell viability. 3-MA treatment enhanced the cytotoxic effect of H$_2$O$_2$ ($p < 0.01$). Compared with 3-MA, the addition of Z-VAD-FMK increased cell viability of H$_2$O$_2$-treated cell but viability (Fig. 7A) and was still decreased compared with the control group ($p < 0.05$). LDH release assay also indicated that Z-VAD-FMK addition did not inhibited cell injury completely (Fig. 7B). As shown in Figure 7C, compared with H$_2$O$_2$ alone, 3-MA in combination with H$_2$O$_2$ inhibited H$_2$O$_2$-induced autophagy and Z-VAD-FMK had no significant effect.

Then, we analyzed caspase-3 activity and the release of cytochrome c from mitochondria to cytosol in U251 cells treated by H$_2$O$_2$ in combination with 3-MA or Z-VAD-FMK. These experiments showed that caspase-3 activation and cytochrome c release induced by H$_2$O$_2$ alone were at least two times higher than those in cells cotreated with H$_2$O$_2$-treated group by 3-MA. These events were largely inhibited in the presence of Z-VAD-FMK (Fig. 7D). To further confirm that the apoptosis was affected by inhibition of autophagy, cells were subjected to PI and Annexin V-FITC staining, followed by flow cytometry analysis in order to quantify the apoptotic cell populations in these cell samples. The results showed that H$_2$O$_2$ induced cell apoptosis to 9.9% ($p < 0.01$). The treatment with H$_2$O$_2$ combined with 3-MA resulted in an increase in the apoptotic cell population to 16.8% ($p < 0.01$). In contrast, Z-VAD-FMK blocked H$_2$O$_2$-induced classic apoptosis in U251 cells; apoptotic cells accounted for 3.4% of the total population (Figs. 7E and 7F). These results suggested that the autophagy inhibitor 3-MA aggravated the apoptosis induced by H$_2$O$_2$. The pan-caspase inhibitor Z-VAD-FMK protected the cells from classic apoptosis. Nevertheless, H$_2$O$_2$ induced cell death in a caspase-independent pathways, and cell vitality decreased significantly compared with the control group ($p < 0.05$) (Fig. 7A), correlates with LDH release increased (Fig. 7B), suggesting involvement of autophagy.

**DISCUSSION**

Gliomas are the most common primary brain tumors in adults (Weller, 1986). Malignant gliomas are resistant to various pro-apoptotic therapies, such as radiotherapy and conventional chemotheraphy (Stewart, 2002); glioma cells are also resistant to the conventional pro-apoptotic cancer therapeutics (Lefranc et al., 2005). Therefore, effective treatment of malignant gliomas may rely on the development of novel strategies for inducing nonapoptotic cell death, such as autophagic cell death or cell death through mitotic catastrophe, which has been recently described as alternative death pathways (Okada and Mak, 2004). Oxidative stress plays an important role in various pathological conditions such as neurodegenerative diseases and several cardiac diseases, which has been demonstrated to be related with autophagy. In Parkinson’s disease (PD), decreased dopamine level due to the degenerative changes in the substantial nigra neurons is one of the major culprit to mediate the enhanced oxidative stress in the pathogenesis of PD, and oxidation of dopamine induces activation of autophagy signaling (Gomez-Santos et al., 2003; Gurusamy and Das, 2009). Several pilot studies revealed that in the early stage of Alzheimer’s disease, oxidative injury to neurons and activation of autophagy have been identified; specifically, it has been noted to induce intralysosomal amyloid β-protein accumulation through activation of autophagy, those in vivo findings further indicating that these early neuronal changes might be interrelated (Nixon et al., 2005; Pratico, 2002; Zhu et al., 2004). In the other hand, autophagy signaling is also related with several cardiac diseases such as ischemic cardiomyopathy, dilated cardiomyopathy, cardiac hypertrophy, and reperfusion injury through the oxidative stress (Gurusamy and Das, 2009; Lefer and Granger, 2000; Nakai et al., 2007). However, the role of autophagy under those conditions is still controversial. Under oxidative stress, ROS including free radicals such as superoxide (O$_2^-$), hydroxyl radical (HO-), and nonradicals capable of generating free radicals (i.e., H$_2$O$_2$) are generated at the levels of high enough to induce oxidation and damage to DNA, lipids, proteins, and other macromolecules (Matsui et al., 2007; Pelicano et al., 2004; Scherz-Shouval et al., 2007). H$_2$O$_2$ administration in vitro imitates the cell injury in vivo due to increased ROS, and it has been reported that 1mM H$_2$O$_2$ triggered autophagy or apoptosis in U87 cells, HeLa cells, and M14 cells (Benassi et al., 2006; Chen et al.,
Various defense mechanisms can protect cells against oxidative stress, including the degradation and recycling of damaged cell proteins and organelles by autophagy (Kiffin et al., 2006). Atg4, an essential protease in the autophagic pathway, has been identified as a direct target for oxidation by H$_2$O$_2$ (Kirisako et al., 2000). However, these pathways remain to be elucidated. In this study, we have demonstrated that H$_2$O$_2$ induces apoptosis in U251 glioma cells, through classic Bax/Bcl-2, caspase-3, and mitochondrial apoptotic pathways. We have examined the process of autophagy in response to H$_2$O$_2$ and the underlying molecular mechanisms. Our results indicate that autophagy occurred with an increase in the protein level of Beclin 1, LC3-II, accumulation of autophagic vacuoles, and AVOs in the cytoplasm. We have further investigated the signaling pathways that are involved in the autophagy induced by H$_2$O$_2$ and have shown a role for class III PI3K/Beclin 1,
which is involved in the initial step of autophagosome formation, and the PI3K/Akt/mTOR pathway. These play important roles in upstream of autophagy induced by growth factors, nutrients, and in response to stress situations, including hypoxia and oxidative stress (Pattinigre et al., 2008). Recent studies have indicated that activation of Beclin 1 and inhibition of the Akt/mTOR pathway have consistently been associated with induction of autophagy in cancer cells (Paglin et al., 2005; Takeuchi et al., 2005). Our results show that H2O2 treatment increases the expression of Beclin 1 protein and decreases activation of Akt. The inhibitory effects were correlated with the loss of phosphorylation of the Akt downstream target FKHR. In addition, exposure to H2O2 also inactivated mTOR and reduced phosphorylation of its downstream target p70S6K. Together, these findings indicate that H2O2 induces autophagy through PI3K/Beclin 1 activation and PI3K/Akt/mTOR inhibition in human glioma U251 cells.

As a well-known anti-apoptotic gene, Bcl-2 interacts with Beclin 1 and downregulates Beclin 1-dependent autophagy (Klionsky, 2005). Recent studies have indicated that cellular and viral Bcl-2 inhibit Beclin 1-dependent autophagy by starvation in human MCF7 breast carcinoma cells by inhibiting the formation of the Beclin 1/hVps34 PI3K complex and Beclin 1-associated class III PI3K activity (Pattinigre et al., 2005). Other studies have suggested that Bcl-2 is also a regulator of PI3K/Akt signaling (Aziz et al., 2006). Takeuchi et al. (2005) have reported that the sensitivity of malignant glioma cells to rapamycin is closely tied to the extent of autophagy. Expression of dominant-negative Akt increased the incidence of autophagy; in contrast, expression of active Akt decreased it (Takeuchi et al., 2005). siRNA-mediated downregulation of Bcl-2 in MCF-7 cells dramatically enhanced the induction of autophagy by nutrient deprivation (Pattinigre et al., 2005). Our data show that the interaction between Bcl-2 and Beclin 1 was enhanced in cells overexpressing Bcl-2 cells where more Beclin 1 communoprecipitated with Bcl-2 and Beclin 1-dependent autophagy, induced by H2O2, was inhibited. Akt/mTOR signaling activity also decreased along with the phosphorylation of Akt, FKHR, mTOR, and p70S6K. Cell autophagy activity was also inhibited by transfection of Bcl-2, accumulation of MDC and LC3-II, and suppression of AVOs development. In contrast, transfection with Bcl-2 siRNA increased the autophagic event with opposite effects on all these observation.

As the main target of oxidative stress, mitochondrial function may represent the juncture of autophagy induced by H2O2; mitochondrial dysfunction and collapse of mitochondrial dynamics by accumulation of intracellular ROS have been associated with autophagy (Itoh et al., 2008; Twig et al., 2008b). For example, overexpression of the fusion factor Opal decreases mitochondrial autophagy (Twig et al., 2008a). As anti-apoptotic gene, Bcl-2 is effective in suppressing mitochondrial fragmentation and/or interacting with mitochondrial fission–fusion proteins (Brooks et al., 2007; Fuenzalida et al., 2007). In our study, after treatment with H2O2, generation and accumulation of ROS with loss of mitochondrial membrane potential were detected in U251 cells; fusion factors Drp1 and Fis1 and fusion factor Mfn1 increased. Overexpression of Bel-2 stabilized mitochondria and inhibited the upregulation of fusion factors, indicating that autophagy induced by H2O2 may be mediated by Bcl-2 by protecting mitochondria from oxidative stress and equilibrating mitochondrial dynamics.

The relationship between autophagy and apoptosis is still debated since there is overlap between autophagic and apoptotic cell death (Canu et al., 2005). In general, stress-inducing stimuli that promote apoptotic cell death also activate autophagy, and several studies revealed that autophagy as a protective response may prolong cell survival (Lum et al., 2005; Periyasamy-Thanavan et al., 2008). Autophagy may activate apoptosis, and conversely, genetic or chemical inhibition of autophagy can activate apoptotic cell death in nutrient-free mammalian cells (Baehrecke, 2003). Ceramide, which has been considered by many researchers to participate in the activation of apoptosis, is effective in establishing macroautophagy (Scarlett et al., 2004). Inhibition of autophagy by chemical or physiological methods triggers apoptosis depending on mitochondrial outer membrane permeabilization and subsequent caspase activation in HeLa cells (Boya et al., 2005). In our study, inhibition of the early stages of autophagy by the specific inhibitor 3-MA resulted in a decrease of cell autophagy, but exacerbated cytosolic release of cytochrome c and cleaved caspase-3 as well as apoptotic cell death, as revealed by PI/Annexin V-FITC staining. Inhibition of autophagy exacerbates apoptotic cell death, suggesting that autophagy may be a protective response that prolongs cell survival here. Previous studies have shown that sulforaphane induced the release of cytochrome c as well as apoptosis in PC-3 cells; these events were significantly exacerbated in the presence of the autophagy inhibitor 3-MA (Herman-Antosiewicz et al., 2006). It is reasonable to postulate that sulforaphane-induced autophagy sequesters mitochondria in autophagosomes resulting in delayed release of cytochrome c and activation of intrinsic caspase cascade. On the other hand, we speculate that inhibition of autophagy by 3-MA leads to abundant ROS accumulation in the cytoplasm, cell overacidification, and lipid peroxidation and accelerates apoptosis. As a well-known anti-apoptotic gene, overexpression of Bel-2 stabilized mitochondria, blocked the classic apoptotic pathways, effect on Beclin 1 and the Akt/mTOR signaling pathway, and inhibited apoptosis and autophagy. Z-VAD-FMK blocked classic apoptosis by inhibiting caspase, but still cell vitality decreased, suggesting involvement of autophagy.

In conclusion, our study reveals that H2O2 induces apoptosis and autophagy in U251 cells. Both the class III PI3K/Beclin 1 and the PI3K/Akt/mTOR signaling pathways are involved, resulting in change of cell membrane permeability and breakdown of the homeostasis of mitochondria fusion and fission. Overexpression of Bcl-2 partially inhibits this autophagy; siRNA of Bcl-2 exacerbates it. Inhibiting autophagy by the autophagic specific inhibitor 3-MA accelerates apoptosis induced by H2O2 in U251 glioma cells. Since mitochondria is
among the most important targets of ROS, both apoptosis and autophagy can be seen as a result of mitochondrial dysfunction, and it may be fruitful to further investigate the function of the membrane channels, mitochondrial proteins, or genes targeted to mitochondria, in order to further elucidate the mechanisms of autophagy.

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**REFERENCES**


Lefranc, F., Brotchi, J., and Kiss, R. (2005). Possible future issues in the treatment...