

## RESEARCH ARTICLE

# Autophagic Degradation of Caspase-8 Protects U87MG Cells Against H<sub>2</sub>O<sub>2</sub>-induced Oxidative Stress

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### Abstract

Oxidative stress induces apoptosis in many cellular systems including glioblastoma cells, with caspase-8 activation was regarded as a major contribution to H<sub>2</sub>O<sub>2</sub>-induced cell death. This study focused on the role of the autophagic protein p62 in H<sub>2</sub>O<sub>2</sub>-induced apoptosis in U87MG cells. Oxidative stress was applied with H<sub>2</sub>O<sub>2</sub>, and cell apoptosis and viability were measured with use of caspase inhibitors or autophagic mediators or siRNA p62, GFP-p62 and GFP-p62-UBA (del) transfection. We found that H<sub>2</sub>O<sub>2</sub>-induced U87MG cell death was correlated with caspase-8. To understand the role of p62 in MG132-induced cell death, the levels of p62/SQSTM1 or autophagy in U87MG cells were modulated with biochemical or genetic methods. The results showed that the over-expression of wild type p62/SQSTM1 significantly reduced H<sub>2</sub>O<sub>2</sub> induced cell death, but knockdown of p62 aggravated the process. In addition, inhibition of autophagy promoted p62 and active caspase-8 increasing H<sub>2</sub>O<sub>2</sub>-induced apoptosis while induction of autophagy manifested the opposite effect. We further demonstrated that the function of p62/SQSTM1 required its C-terminus UBA domain to attenuate H<sub>2</sub>O<sub>2</sub> cytotoxicity by inhibition of caspase-8 activity. Our results indicated that p62/SQSTM1 was a potential contributor to mediate caspase-8 activation by autophagy in oxidative stress process.

**Keywords:** p62 - caspase-8 - U87MG - hydrogen peroxide - autophagy

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### Introduction

Reactive oxygen species (ROS), which leads to oxidative stress (OS) at excess amount, positively contributes to carcinogenesis and to malignant progression of tumor cells (Gerald et al., 2004). In contrast to normal cells, cancer cells, which exhibit an accelerated metabolism, demand higher ROS concentrations to maintain their high proliferation rate (Sosa et al., 2013). Importantly, several studies showed that OS production promotes cell apoptosis in cancer treatment with various radio (Mitsuishi et al., 2012) and chemotherapeutic drugs (Saha, 2012). However, the mechanism of apoptosis induced by OS is not clear.

H<sub>2</sub>O<sub>2</sub>, which mimics OS by producing excess ROS in cells, is applied for exploring the OS-induced cell death. Numerous studies have provided evidence that unwanted protein aggregates, such as  $\beta$ -amyloid, ubiquitinated proteins, which are involved in ROS generation process, bring out apoptosis ultimately (Pan et al., 2013; Vernon and Tang, 2013). Moreover, H<sub>2</sub>O<sub>2</sub>-induced apoptosis is directly through caspase-8 and is not through the mitochondria-dependent caspase-9 activation (Wu et al., 2011). In addition, it also induced autophagy in H<sub>2</sub>O<sub>2</sub>-treated cells, accompanied with a decreased level of p62 and the formation of autophagic vacuoles (Seo et al., 2011).

p62 is a central regulator of tumorigenesis due to its abilities to modulate autophagy, to control the levels of ROS and misfolded proteins, and to ensure a timely transit of cell through mitosis; all crucial factors in cancer (Moscat and Diaz-Meco, 2012). Previous studies have shown that p62 binds ubiquitin at its C-terminus, suggesting that it may serve as a storage compartment for ubiquitinated proteins (Yip et al., 2006). In addition, p62 binds specifically to ubiquitinated proteins, acting as a putative ubiquitin chain-targeting factor that shuttles these substrates for proteasome and autophagy degradation (Korolchuk et al., 2009). In particular, the UBA domain played a potential role in the p62 function as an adaptor or cargo receptor for degradation of ubiquitinated proteins (Long et al., 2010). In this study, we attempted to explore the role of caspase-8 in H<sub>2</sub>O<sub>2</sub>-induced associated apoptosis in U87MG cells, to evaluate the function of p62 in caspase-8 autophagic degradation response to oxidative insult by using the UBA domain deletion and knockdown of p62.

### Materials and Methods

#### *Plasmids, chemical reagents and antibodies*

The GFP-p62 plasmid was kindly provided by Dr. Terje Johansen (University of Tromsø, Tromsø, Norway).

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The GFP-LC3 plasmid was provided by professor Ying-Yu Chen (Peking University, Health Science Center, Beijing, China). A GFP-p62-UBA(del) plasmid was constructed by using CCGctcgag ATGGCGTCCG CTCACCGTGAAGGCCCT and CCGgaattc TCATGGCGG GAGATGTGG GTACAAG as PCR primers, and XhoI and EcoRI the restriction sites. All plasmids used in this study were confirmed by DNA sequencing.

3-methyladenine (3MA), rapamycin (RAPA) were purchased from Sigma-Aldrich (St Louis, MO, USA) was purchased from HyClone. AC-IETD-CHO, z-VAD-FMK, FAM-LETD-FMK, and FAM-LEHD-FMK was purchased from Biomol (Biomol. Hamburg, Germany). Antibodies of rabbit anti-LC3 or p62 (Sigma), mouse anti-actin (ZhongShan GoldenBridge Biotechnology, Beijing, China), mouse anti-Ub and anti-caspase-8 (p18) (P4D1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were applied following the vendors recommendation.

#### Cell culture and transfection

U87MG cells were purchased from the cell bank at Peking Union Medical University. U87MG cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (HyClone, Beijing, China) supplemented with 10% fetal bovine serum (FBS, Sijiqing, Zhejiang, China). Fugene HD reagent (Roche, Penzberg, Germany) was used for cell transfection following the manufacturer's standard protocols. The cells were observed and photographed under a DMIRB inverted fluorescent microscope (Leica, Solms, Germany).

#### Cell viability

The cell viability after H<sub>2</sub>O<sub>2</sub> treatment was determined using the CellTiter-96® Aqueous - One Solution Cell Proliferation (MTS) Assay kit (Promega, Madison, WI, USA). The cells were seeded in 96-well plates at 0.5~1x10<sup>4</sup> cells per well for 24 h and then treated with H<sub>2</sub>O<sub>2</sub> of necessary conditions according to the experimental design. The MTS reagents were applied for 1 h at 37 °C, and the plates were subjected to measures at 490 nm with a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT USA).

The cell apoptosis index was measured using Calcein-AM/PI method. The cells after H<sub>2</sub>O<sub>2</sub> treatments were incubated with 1 µg/ml Calcein-AM (Invitrogen, Eugene, OR, USA) and 10 µg/ml propidium iodide (PI, Invitrogen) for 30 min at 37°C. A double-blinded cell counting was performed for live (green) and dead cells under fluorescent microscope. At least three non-overlapped fields were acquired from each well under different treatment conditions, the number of stained cells was counted using ImageJ software and the percentage of PI-positive cells/total (both Calcein and PI positive cells) was calculated.

#### Caspase activity assay

Cells treated with H<sub>2</sub>O<sub>2</sub> for 3, 6, 9, 12 h were harvested and incubated in culture media with 10 µmol/L of FAM-LETD-FMK (caspase-8 fluorescent substrate) or FAM-LEHD-FMK (caspase-9 fluorescent substrate) for 1 h at 37°C. After washed 3 times with Apoptosis Wash Buffer, the cells were suspended in 300 µL buffers and analyzed

with a fluorescence microscope in three independent experiments.

#### RNA interference

The targeted fragment of siRNAs against p62 was 5'-GCATTGAAGTTGATATCGAT-3 as previously published (Pankiv et al., 2010). Cells were grown in 6-well plates and transfected using Fugene HD reagents with siRNA or the scrambled control (Augct, Beijing, China) at a final concentration 20 nmol/L. After 72 h, the p62 protein levels were examined by western blot and cells in parallel conditions were used for H<sub>2</sub>O<sub>2</sub> experiments.

#### Western blots

Cells in 6-well plates were lysed in 80-100 µL modified RIPA buffer (Thermo, Rockford, IL, USA) containing the full cocktail of protease inhibitors (Thermo). The protein concentrations were determined with the BCA protein assay kit (Novagen, San Diego CA, USA). Then proteins were separated by 10 or 15% SDS-PAGE and transferred to nitrocellulose filters, the blotted with accorded antibodies, secondary antibodies pre-labeled with IRDye800CW or IRDye700CW were used for the scanning by the Odyssey (LI-COR Biosciences, Lincoln, NE, USA).

#### Statistical analysis

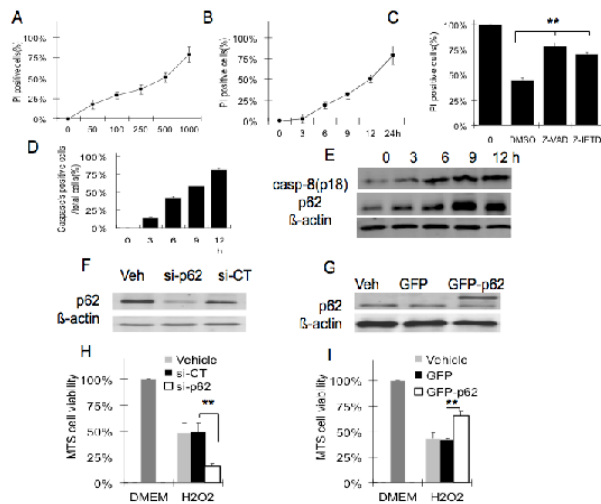
The experimental data presented in this study were from at least 3 independent experiments. The data values in cell viability are presented as means with SEM. Following one-way ANOVA and student T tests, the *p* value less than 0.05 was considered as statistical significance.

## Results

### *p62 attenuated H<sub>2</sub>O<sub>2</sub>-induced caspase-8 dependent apoptosis in U87MG cells*

In H<sub>2</sub>O<sub>2</sub>-treated U87MG cells, we observed that the amount of apoptotic cells were time and dose dependent on H<sub>2</sub>O<sub>2</sub> (Figure 1A and 1B). To explore the mechanism of apoptosis, we employed caspase-8 inhibitor AC-LETD-CHO or pan-caspase inhibitor z-VAD-FMK, and found that the caspase-8 inhibitor effectively attenuated H<sub>2</sub>O<sub>2</sub> induced cell death to the similar level as that of the pan caspase inhibitor (Figure 1C). Subsequently, as expected, caspase-8 activity and caspase-8 active component (p18) were correlative with H<sub>2</sub>O<sub>2</sub> treatment time course (Figure 1D). This suggested that caspase-8 appeared to be the predominant type of caspase in mediating H<sub>2</sub>O<sub>2</sub>-induced apoptosis. Interestingly, p62, which is autophagic relative protein, significantly increased with H<sub>2</sub>O<sub>2</sub> treatment (Figure 1E). Since p62 was thought to recruit poly-ubiquitinated caspase-8 and subsequently allow its full activation in TRAIL-treated cells (Jin et al., 2009), it indicated that p62 was a crucial molecule in H<sub>2</sub>O<sub>2</sub>-induced apoptosis.

To assess the function of p62, we modulated the p62 protein level in U87MG cell with the transfection of siRNA p62 and GFP-p62 (Figure 1F and 1G). As shown in Figure 1H, p62 deficiency promoted the significantly decrease of H<sub>2</sub>O<sub>2</sub>-treated cell viability from 49% reduced

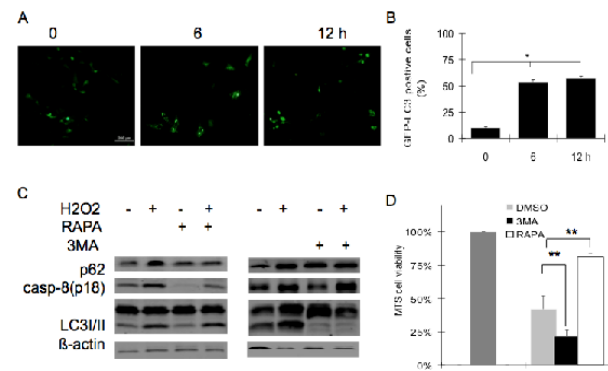


**Figure 1. Oxidative Stress Induced Caspase-8 Dependent Cell Death Modulated by p62 Protein Level in U87MG Cells.** (A) and (B) The dose and time course of H<sub>2</sub>O<sub>2</sub>-induced cell death in U87MG cells were characterized by calcein-AM/PI staining. Data was shown as the averaged percent of PI positive cells  $\pm$ SEM from three independent experiments by counting a total of 200 cells. (C) The cell death rate of U87MG cells treated with 500  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 12 h with pretreatments of pan-caspase (z-VAD-FMK) or caspase-8 (AC-IETD-CHO) inhibitors for 30 min. (D) The activities of caspase-8 were evaluated following H<sub>2</sub>O<sub>2</sub> treatment for 0, 3, 6, 9, 12 h. The cells were preloaded with FAM-LETD-FMK (caspase-8 fluorescent substrate) at 37 °C for 60 min. The numbers of positively stained cells were counted and shown as the averaged percentage  $\pm$ SEM from three independent experiments. (E) The western blot of caspase-8 activation in U87MG cells treated with 500  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 0, 3, 6, 9, 12 h. The protein levels of p62 and caspase-8 p18 were detected. (F) and (G) Transfection of siRNA targeted to p62/SQSTM1 for 72 h increase the cell death in U87MG cells after 500  $\mu$ M H<sub>2</sub>O<sub>2</sub> treatment for 12 h. (H) and (I) H<sub>2</sub>O<sub>2</sub>-induced cell death in U87MG cell with overexpression of p62/SQSTM1 for 24 h. The cell viability was assayed by MTS at 12 h. The results were normalized and presented as mean  $\pm$ SEM from five independent experiments. \**P* < 0.05, \*\**P* < 0.01

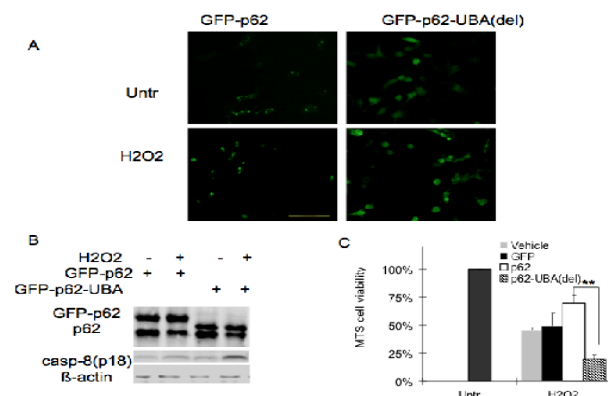
to 16%. Meanwhile, overexpression of p62 significantly increased the survival rate of U87MG from 42% to 65% (Figure 1I). The results suggested p62 inhibited the cytotoxicity of H<sub>2</sub>O<sub>2</sub> in U87MG cells.

#### Modulation of autophagy in U87MG cells altered H<sub>2</sub>O<sub>2</sub>-induced cell death with changes of p62/SQSTM1 and caspase-8 at protein levels

The caspase-8 and p62 protein degradation were mediated by autophagy. To explore whether autophagy occurred in H<sub>2</sub>O<sub>2</sub>-induced cell death, we tested autophagy using the GFP-LC3 conversion as the marker of the autophagosomes in U87MG cells. The cells with more than 3 fluorescent puncta were counted as positive for autophagy activation (Young et al., 2009). We found that approximately 53% and 57% of GFP-LC3 transfected U87MG cells were observed with autophagy activation after treated with 500  $\mu$ M/ml H<sub>2</sub>O<sub>2</sub> for 6 h and 12 h (Figure 2A and 2B). To investigate whether autophagy is functionally involved in H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity, we used the reagents to either activate or inhibit the autophagy process in U87MG cells. The results showed that the



**Figure 2. Activation or Inhibition of Autophagy Influenced H<sub>2</sub>O<sub>2</sub>-induced Cell Death and Associated with Changes of p62 Protein Levels and Caspases-8 Activity.** (A) H<sub>2</sub>O<sub>2</sub> treatments (500  $\mu$ M/ml) for 0, 6, 12 h induced GFP-LC3 aggregation in transfected U87MG cells. (B) From the confocal images in each sample, a random 50 GFP positive cells were analyzed for the percentage with at least three aggregated GFP-LC3 spectacles as positive for autophagy. U87MG cells were treated with autophagy inhibitor of 3MA at 5 mmol/L or autophagy activator rapamycin at 2.5  $\mu$ M/L for 3 h. (C) The protein levels of LC3 II, p62 and caspase-8 were determined by western blot at 12 h. (D) The cell viability was assayed by MTS at 12 h. The results were normalized and presented as mean  $\pm$ SEM from five independent experiments. \**P* < 0.05, \*\**P* < 0.01



**Figure 3. H<sub>2</sub>O<sub>2</sub> Induced Cell Death was Modulated by p62 Aggregation and Dependent on the UBA Domain.** (A) The p62/SQSTM1 UBA-deletion mutant was unable to form aggregated fluorescent speckles in U87MG cells. Cells were transfected for 24 h, and then exposed to H<sub>2</sub>O<sub>2</sub> for 12 h before photographed. (Bar=100  $\mu$ m) (B) Western blot for the comparison of caspase-8 activation following GFP-p62 and GFP-p62-UBA(del) transfection. After transfected for 24 h, the protein levels of caspase-8 p18 and p62 from 12 h H<sub>2</sub>O<sub>2</sub>-treated U87MG cells were detected by western blot. (C) Transfection of p62/SQSTM1 mutant attenuated the Mg132-induced cytotoxicity. U87MG cells were transfected with GFP-p62 or GFP-p62-UBA(del) for 24 h and assayed by MTS after 12 h exposure in 0.5  $\mu$ M H<sub>2</sub>O<sub>2</sub>. The results were presented as mean  $\pm$ SEM from three independent experiments. \*\**P* < 0.01.

inhibition of autophagy accelerated the H<sub>2</sub>O<sub>2</sub>-induced cell death, whereas the activation of autophagy with rapamycin exposure attenuated the cytotoxicity (Figure 2C and 2D).

#### UBA domain was key structure of p62/SQSTM1 which attenuated H<sub>2</sub>O<sub>2</sub>-induced cell death

UBA domain at the C-terminus of p62/SQSTM1

was suggested to be a key domain to bind and recruit ubiquitinated proteins, including polyubiquitinated caspase-8 (Laussmann et al., 2011). We used an overexpression plasmid of p62 mutant with the deletion of its UBA domain and tagged with GFP (Zhang et al., 2013). When transfected into the U87MG cells and compared with the wild type p62, we found that the UBA-deleted p62 largely abolished the ability to form intracellular aggregates (Figure 3A). The H<sub>2</sub>O<sub>2</sub>-induced cell death could be significantly aggravated by transfection of GFP-p62-UBA (del) (Figure 3C), as well as the caspase-8 cleavage and p18 production (Figure 3B).

## Discussion

Oxidative stress, which is a state with excess ROS induced by H<sub>2</sub>O<sub>2</sub>, lead to the production of peroxides and free radicals that damage mitochondria (Sun et al., 2012; Wu et al., 2012). However, numerous studies showed that H<sub>2</sub>O<sub>2</sub>-induced apoptosis was dependent on caspase-8 activation (Tartier et al., 2000; Yamakawa et al., 2000; Wu et al., 2011), not but on caspase-9 activation which is induced by mitochondria dysfunction. In this study, it also showed that caspase-8 activation was major to contribute to H<sub>2</sub>O<sub>2</sub> induced cell death in U87MG cells.

Recent studies suggested that caspase-8 activation was associated with p62/SQSTM1, an autophagic protein (Jin et al., 2009; Young et al., 2012; Zhang et al., 2013). The ubiquitin-binding protein p62 is required to complete the final activation by promoting aggregation of polyubiquitinated caspase-8. Meanwhile, p62 was an essential molecule to mediate the clearance of polyubiquitinated proteins in some diseases, such as Morbus Huntington (Kaniuk et al., 2007; Tung et al., 2010; Myeku and Figueiredo-Pereira, 2011). The ubiquitinated unwanted protein, caspase-8, which produced in oxidative stress process, was cleared by p62 adoption to autophagy (Bjorkoy et al., 2005; Pan et al., 2011). Moreover, p62-mediated autophagic degradation of active caspase-8 was a novel mechanism of cancer cell restraint to apoptosis (Hou et al., 2010). It prompted that p62 aggravation in cell plasma binding with active caspase-8 or other ubiquitinated proteins to neutralize their cytotoxicity and to degrade them by autophagy pathway. Therefore, it is controversial or complex to explain the role of p62 in caspase-8 activation. In H<sub>2</sub>O<sub>2</sub>-treated U87MG cells, we found that active caspase-8 level and/or cell viability was relative to the p62 protein level. Simultaneously, autophagy occurrence was together with p62 decreasing. In addition, UBA domain deletion leads to that p62 has no the ability to covalently bind ubiquitinated proteins (Ciani et al., 2003), which inhibited autophagic degradation of caspase-8 due to the loss of binding function. It suggested that harmful active caspase-8 was mediated by autophagy for the survival of cancer cells.

Autophagy and apoptosis are same important for regulating cell fate in response to cytotoxic stress (Levine and Kroemer, 2008). Recent reports indicated that autophagy could be a adaptive cytoprotection response before cell death (Hung et al., 2009; Ishida and Nagata, 2009; Han et al., 2012). The ability of autophagy even determined the

fate of different cell under same stress (Liang et al., 2013). The association between caspase-8 and p62 (Korolchuk et al., 2009; Ullman et al., 2011; Puissant et al., 2012) provide more evidences for the link of autophagy and apoptosis, which help us to better understand the complicated cell death processes. Therefore, H<sub>2</sub>O<sub>2</sub> mediated apoptosis could be a suitable cell model to explore for the role of autophagy in cell death process. The detailed molecular mechanism on how caspase 8 activation modulated by p62 needs further investigation.

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