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The functional role of Bax/Bak in palmitate-induced lipoapoptosis

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ABSTRACT

Induction of programmed cell death, mainly apoptosis (lipoapoptosis) is a major cellular consequence of the lipotoxicity, a harmful effect resulting from the overload of lipids. Both Endoplasmic reticulum (ER) stress and autophagy have been suggested to play important role in the regulation of lipoapoptosis. However, the exact mechanisms underlying lipoapoptosis remain unclear. In the present study, we aimed to investigate the functional role of Bax/Bak in lipoapoptosis using mouse embryonic fibroblasts (MEFs) cell culture model. Results showed that palmitate induced caspase-dependent apoptosis in wild-type Bax/Bak MEF cells, whereas a caspase-independent cell death was induced by palmitate in Bax/Bak knockout MEF cells, suggesting requirement of Bax/Bak in palmitate-induced caspase activation. More importantly, we found that the status of Bax/Bak is a determinant that governs the decision between the pro-survival or pro-death function of autophagy in response to palmitate exposure, and Bax/Bak is required for palmitate-induced activation of endoplasmic reticulum (ER) stress and subsequently ER stress-mediated apoptosis. The findings of the present study provided novel insights into understanding the mechanisms involved in the regulation of palmitate-induced lipoapoptosis.

1. Introduction

Lipid accumulation in non-adipose tissues such as liver, heart, kidney, muscle, and pancreatic-islets causes a harmful effect on these organs or systems, named lipotoxicity (Schaffer, 2016). Induction of programmed cell death, mainly apoptosis (lipoapoptosis) is a major cellular consequence of the lipotoxicity. Proposed mechanisms involved in the lipoapoptosis include the induction of ER stress and subsequently activation of the CCAAT/enhancer-binding protein-homologous protein (CHOP) and JNK-mediated apoptotic signaling pathways (Cazanave and Gores, 2010).

Activation of the mitochondrial pathway plays a central role in apoptosis process. The disruption of mitochondrial membrane potential (MMP) is a hallmark and a key step for activating mitochondrial pathway (Akazawa et al., 2010). MMP is strictly controlled by the Bcl-2 family proteins including the anti-apoptotic members of this family Bcl-2, Bcl-xl, Mcl-1, and pro-apoptotic members of this family Bax, Bak, Bim, Puma, Bid and Bad etc. Among them, Bax and Bak are the two key pro-apoptotic proteins with multiple BH domains and can be activated by the BH3-only proteins and inhibited by the anti-apoptotic proteins. Once activated, they undergo conformation changes leading to oligomerization, which is required for the permeabilization of the mitochondrial outer membrane (Jiang et al., 2014). In addition, Bax and Bak have been found to play role in the regulation of autophagy and ER stress (Lindqvist et al., 2014; Mukhopadhyay et al., 2014).

Palmitate, a commonly used fatty acid for lipotoxicity induction, is able to induce apoptosis in multiple types of cells including hepatocytes, myoblasts and pancreatic β cells (Leamy et al., 2014; Borradaile et al., 2004; Jung et al., 2015). In the meanwhile, autophagy is either activated or inhibited depending on the duration of exposure to palmitate (González-Rodríguez et al., 2014). Inhibition of autophagy results in an enhanced apoptosis in a series of studies, suggesting autophagy functioning as suppressor of palmitate-induced apoptosis (Cai et al., 2014). In the present study, we aimed to investigate the functional role of Bax/Bak in palmitate-induced caspase activation, autophagy and ER stress. Results showed that palmitate induced caspase-dependent apoptosis in mouse embryonic fibroblasts (MEFs) cells, but caspase-independent cell death in Bax/Bak knockout MEF cells, suggesting requirement of Bax/Bak in palmitate-induced activation of caspase. Furthermore, we identified that the status of Bax/Bak is a

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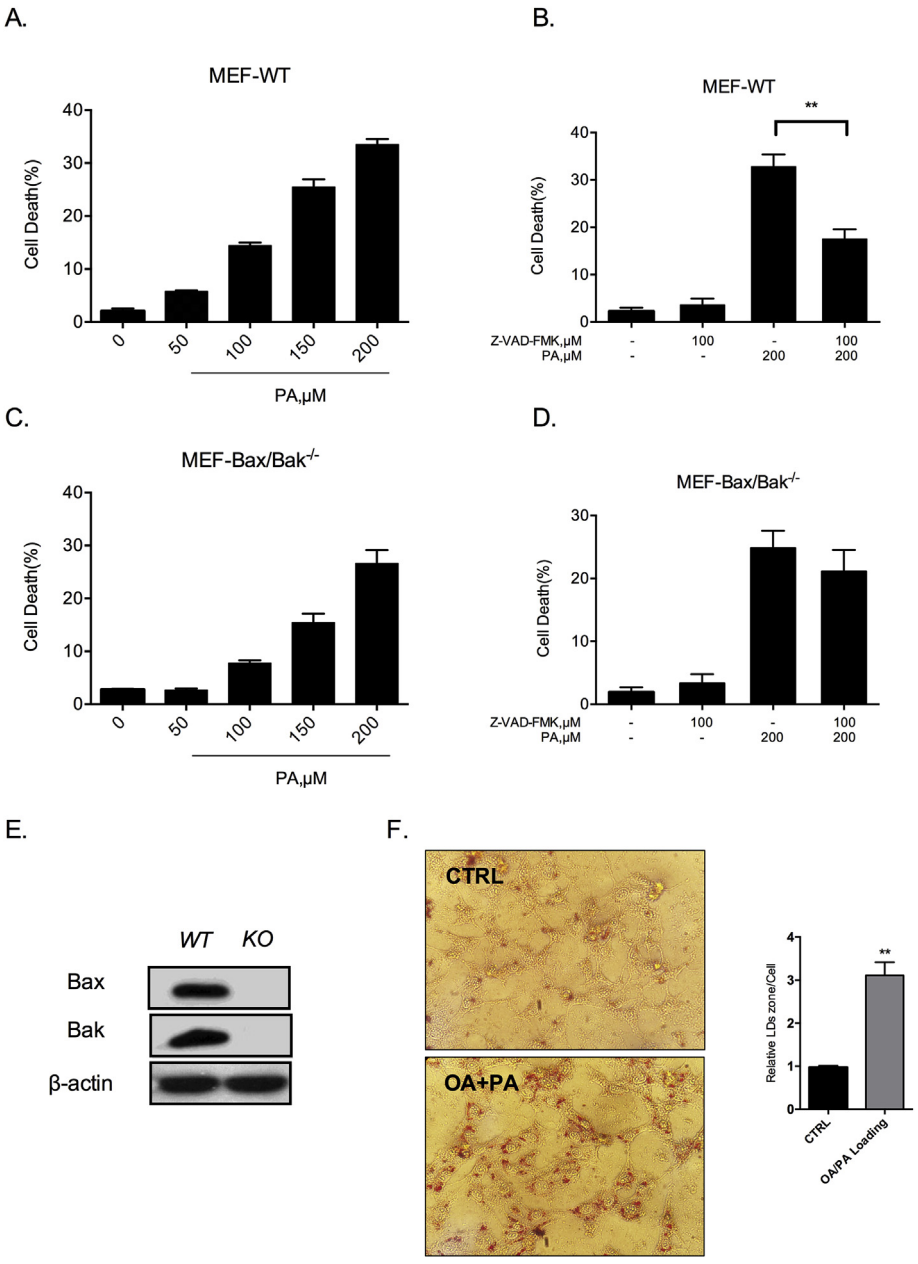

Fig. 1. Bax/Bak is required for palmitate-induced caspase-dependent apoptosis in MEF cells. A. Palmitate induces dose-dependent cell death in wild-type MEF cells. B. Effects of pan-caspase inhibitor on palmitate-induced cell death in wild-type MEF cells. C. palmitate induces dose-dependent cell death in Bax/Bak knockout cells. D. Effects of pan-caspase inhibitor on palmitate-induced cell death in Bax/Bak knockout cells. E. Western blotting analysis of Bax/Bak in the cell lines used. F. palmitate/oleic acid induces lipid accumulation in MEF cells. $p < 0.01$ (**), ($n = 3$).

determinant for autophagy exerting the pro-survival or pro-death function in response to palmitate exposure, and Bax/Bak is required for palmitate-induced ER stress activation. Our findings provided novel insights into understanding the mechanisms involved in the regulation of pamitate-induced lipoapoptosis.

2. Materials and methods

2.1. Chemicals and reagents

Sodium palmitate, 3-methyladenine (3-MA) and bafilomycin A1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary antibodies of phospho-EIF2α and phospho-PERK were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibody Phospho-IRE1α was purchased from Abcam (Cambridge, UK). The primary antibody LC3, pan-caspase inhibitor z-VAD-fmk and horseradish peroxidase-conjugated secondary antibodies were purchased from MBL International Corporation (Woburn, MA, USA). Primary antibody specific for β-actin was purchased from Action Biotech. Tauroursodeoxycholic acid (TUDCA) was purchased from Cayman Chemical (Ann Arbor, MI, USA).

2.2. Cell and cell culture

The MEF cells (kindly provided by Professor Feng Zhu, Department of Biochemistry and Molecular Biology, Tongji Medical College, Huazhong University of Science and Technology) were cultured in high glucose DMEM (Thermo, SH30022.01B) supplemented with 10% fetal bovine serum without antibiotics. When the cells reached about 50% confluence, the medium was changed before the starting treatment with

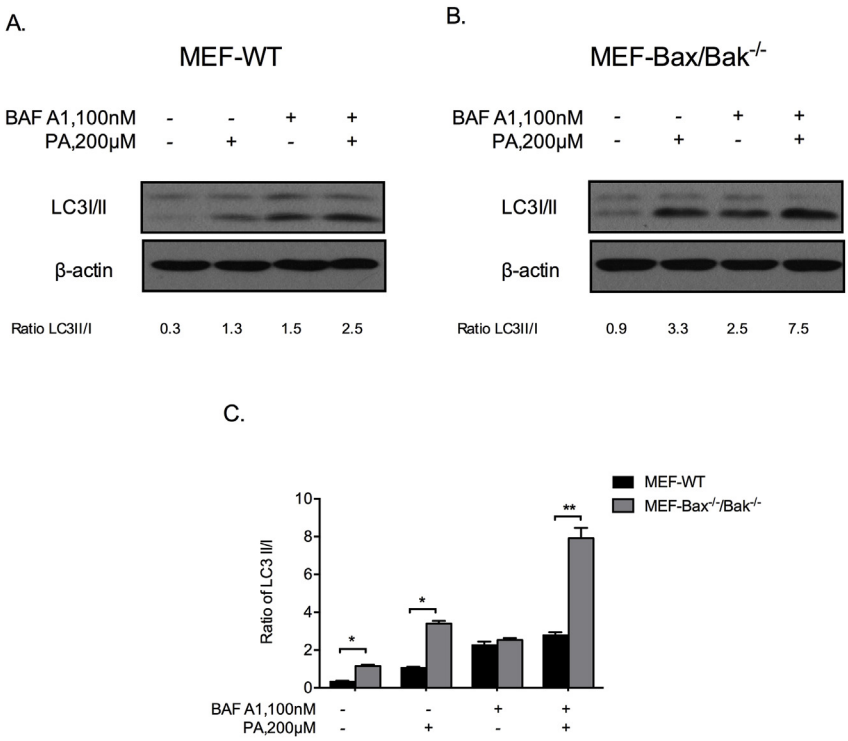

Fig. 2. Palmitate induces a stronger activation of autophagy in Bax/Bak knockout MEF cells than that in Bax/Bak wild-type MEF cells. A-C. The conversion of the microtubule-associated protein 1 light chain 3 (LC3) -I (18kD) to LC3-II (16kD) induced by palmitate in the presence or absence of bafilomycin A1 (BAF), an inhibitor of autophagosome degradation, was measured by western blotting in wild-type MEF (A) and Bax/Bak knockout cells (B), and its quantitative analysis (C). $p < 0.05$ (*), $p < 0.01$ (**), ($n = 3$).

palmitate and/or the other agents.

2.3. Cell death evaluation

Cell death was determined by flow cytometry following Annexin V/PI double staining of externalized phosphatidyl-serine (PS) and DNA fragmentation in the dead cells using Annexin V/PI staining kit from MBL International Corporation (Hu et al., 2009).

2.4. Autophagy detection

Autophagy was analyzed by western blotting analysis of conversion of the microtubule-associated protein 1 light chain 3 (LC3) -I (18kD) to LC3-II (16kD) (Herman-Antosiewicz et al., 2006).

2.5. Transfection

siRNAs targeting ATG5 and the non-targeting siRNA were purchased from Life Technologies. The MEF cells were transfected with 10 nM of ATG5-siRNAs or the negative control siRNAs using INTERFERin siRNA transfection reagent according to the manufacturer's instructions (Polyplus-Transfection). 24 h post-transfection, the cells were used in subsequent experiments.

2.6. Western blotting

The cells were lysed with ice-cold radioimmunoprecipitation assay buffer. The cell lysates were resolved by SDS-PAGE and electro-blotted onto PVDF membranes. The membranes were blocked with 5% defatted milk then incubated with selective primary antibodies followed by incubation HRP-conjugated secondary antibodies. The blots were visualized by enhanced chemiluminescence (Fisher/Pierce, Rockford, IL, USA).

2.7. Statistical analysis

Data were presented as mean ± SD. These data were analyzed with the two way ANOVA with appropriate post-hoc comparison among means with Graph Pad Prism 6.0. $p < 0.05$ (*) was considered statistically significant.

3. Results

3.1. Bax/Bak is required for palmitate-induced caspase-dependent apoptosis in MEF cells

MEF cells were exposed to palmitate at concentrations ranging from 50 to 200 μM for 24 h and the cell death induction was measured by Annexin v/PI straining. As shown in Fig. 1A, palmitate induced a concentration-dependent cell death in MEF cells, which is consistent with that found in other cell lines tested previously (Zhang et al., 2016). To determine the role of caspase activation in cell death induction by palmitate, we assessed effect of caspase inactivation by a pan-caspase inhibitor zVAD-fmk on palmitate-induced cytotoxicity of MEF cells. As shown in Fig. 1B, a significant decreased palmitate-induced cytotoxicity was observed in the presence of the pan-caspase inhibitor, suggesting the caspase activation plays an important role in palmitate-induced cell death in MEF cells. To investigate the functional role of Bax/Bak in palmitate-induced cell death, we measured the cell death induction in Bax/Bak knockout MEF cells and results are shown in Fig. 1C. Under the condition of Bax/Bak deficiency, palmitate was still able to induce cell death in MEF cells, but the magnitude of cell death induction was less than that found in Bax/Bak wild-type MEF cells. To examine whether caspase activation is also involved in palmitate-induced cell death in Bax/Bak wild-type MEF cells, we then assessed the influence of the pan-caspase inhibitor on cell death induction of Bax/Bak knockout MEF cells. As shown In Fig. 1D, the pan-caspase inhibitor failed to offer significant protection against palmitate-induced cell death in the absence of Bax/Bak, indicating that Bax and Bak are necessary for palmitate-induced caspase-dependent cell death in MEF cells, and both caspase-dependent and independent cell death were involved in palmitate-induced cytotoxicity in MEF cells. To test the knockout efficiency of cell line used in the study, western blotting was employed to measure the expression of Bax and Bak, As shown in Fig. 1E, neither

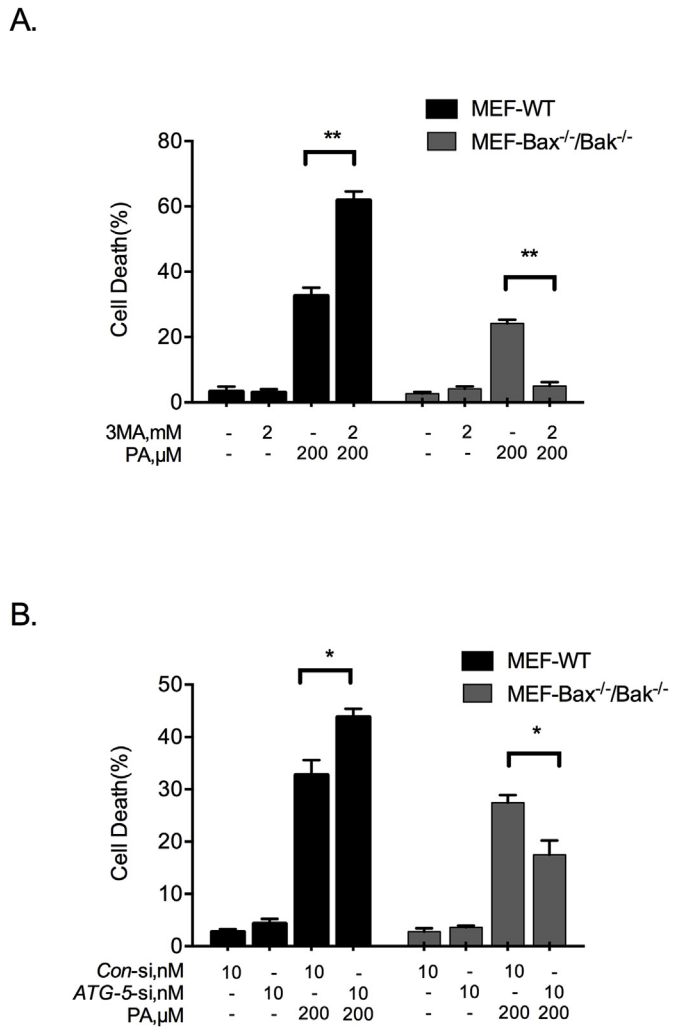

Fig. 3. Bax/Bak is required for autophagy exerting the protective effect on palmitate-induced apoptosis. A. Effects of autophagy inhibitor 3-MA on palmitate-induced cell death in wild-type and Bax/Bak knockout MEF cells. B. Effects of ATG5 knockdown on palmitate-induced cell death in wild-type and Bax/Bak knockout MEF cells. $p < 0.05$ (*), $p < 0.01$ (**), ($n = 3$).

Bax nor Bak was dateable in Bax/Bak knockout MEF cells. To establish the relevance of MEF cell line as a model for investigating lipotoxicity, the levels of lipid accumulation in MEF cells were measured by Oil-O-Red staining in response to palmitate/oleic acid exposure. As shown in Fig. 1F, exposure of MEF cells to palmitate/oleic acid resulted in a significant lipid accumulation, which is similar to that found in the physiologically relevant liver cells (Zhang et al., 2017).

3.2. Palmitate induces a stronger activation of autophagy in Bax/Bak knockout MEF cells than that in Bax/Bak wild-type MEF cells

It has been shown that Bax and Bak play a role in the regulation of autophagy in certain conditions (Lindqvist et al., 2014; Mukhopadhyay et al., 2014; Buytaert et al., 2006). We asked whether the status of Bax/Bak influenced autophagy induction in response to palmitate exposure. Western blotting was employed to analyze the lipidated form of microtubule-associated protein 1 light chain 3 (LC3), LC3-II, a well-known autophagic marker. As shown in Fig. 2A–C, a relatively higher basal LC-3II/I ratio has been found in Bax/Bak knockout MEF cells than that in Bax/Bak wild-type MEF cells, suggesting that Bax/Bak knockout MEF cells have higher basal levels of autophagy. Palmitate exposure caused an increased LC3-II in both Bax/Bak wild-type and knockout MEF cells. To determine the increased LC3-II was due to the induction of autophagosome formation or the inhibition of autophagosome degradation, the autophagic flux was measured. Treatment with bafilomycin A1 (BAF), an inhibitor of autophagosome degradation, led to elevated accumulation of LC3-II because of the degradation inhibition. In the presence of BAF, palmitate exposure caused a further increased LC3-II level in these two types of MEF cells with a higher level in Bax/Bak knockout MEF cells. The results suggest that autophagic flux was augmented, and autophagy was activated in response to palmitate exposure in these two types of MEF cells. Moreover, a stronger autophagy was induced in Bax/Bak knockout MEF cells than that found in Bax/Bak wild-type MEF cells.

3.3. Bax/Bak is required for autophagy exerting the protective effect on palmitate-induced apoptosis

Having found the activation of autophagy by palmitate in MEF cells, we then examined the functional role of autophagy activation in palmitate-induced cell death. We first measured effect of autophagy inactivation by its inhibitor 3-methyladenine (3-MA) on palmitate-induced cell death. As shown in Fig. 3A, Inhibition of autophagy by its inhibitor resulted in increased cell death induction by palmitate exposure in Bax/Bak wild-type MEF cells, suggesting that autophagy exerted a pro-survival function to suppress palmitate-induced cell death in MEF cells with the wild-type Bax/Bak. In contrast, inhibition of autophagy by the inhibitor led to significantly decreased cell death induction by palmitate in Bax/Bak knockout MEF cells, indicating the involvement of autophagy-mediated cell death in palmitate-induced cytotoxicity under Bax/Bak defective condition. To further validate this controversial role of autophagy in MEF cells with or without Bax/Bak, the RNAi approach was employed to inactivate autophagy by knocking down autophagy-related 5 (ATG5), an essential gene for autophagy induction. Under such condition, the cell death induction of these two types of MEF cells was measured. As shown in Fig. 3B, autophagy inhibition by silencing ATG5, promoted palmitate-induced cell death in Bax/Bak wild-type MEF cells, but reduced palmitate-induced cell death in Bax/Bak knockout MEF cells. Together, these data clearly suggest that the decision between the pro-survival and pro-death activity of autophagy is depending on the availability of Bax/Bak in palmitate-induced cell death in MEF cells.

3.4. Increased cell death induction by autophagy inhibition is attributed to the enhancement of ER stress in Bax/Bak wild-type MEF cells

It has been shown that ER stress was activated by palmitate, and the activation of ER stress plays a critical role in palmiate-induced apoptosis (Pardo et al., 2015). We first confirmed the ER stress activation by palmitate in Bax/Bak wild-type MEF cells (Fig. 4A). We next questioned whether the protective effect of autophagy on palmitate-induced cell death was associated with its inhibitory effect on the ER stress activation. Autophagy was inhibited by knocking down ATG5 (Fig. 4B), and the changes of key ER stress markers were analyzed by western blotting. As shown in Fig. 4C, under the condition of autophagy inhibition, the phosphorylation of IRE1, PERK and EIF2α was further increased by palmitate exposure compared with these found in con-si/palmitate-treated cells. These results indicate that the protective effect of autophagy on palmitate-induced cell death is due to its ability to suppress ER stress activation in MEF cells.

3.5. Bax/Bak is required for palmitate-induced ER stress in MEF cells

As shown in Fig. 4A, ER stress was activated by palmitate in MEF cells. We next asked whether Bax/Bak was involved in palmitate-induced ER stress. Bax/Bak knockout MEF cells were exposed to palmitate for 24 h, and the ER stress markers were analyzed by western blotting as shown in Fig. 5A, the changes of key ER stress markers by

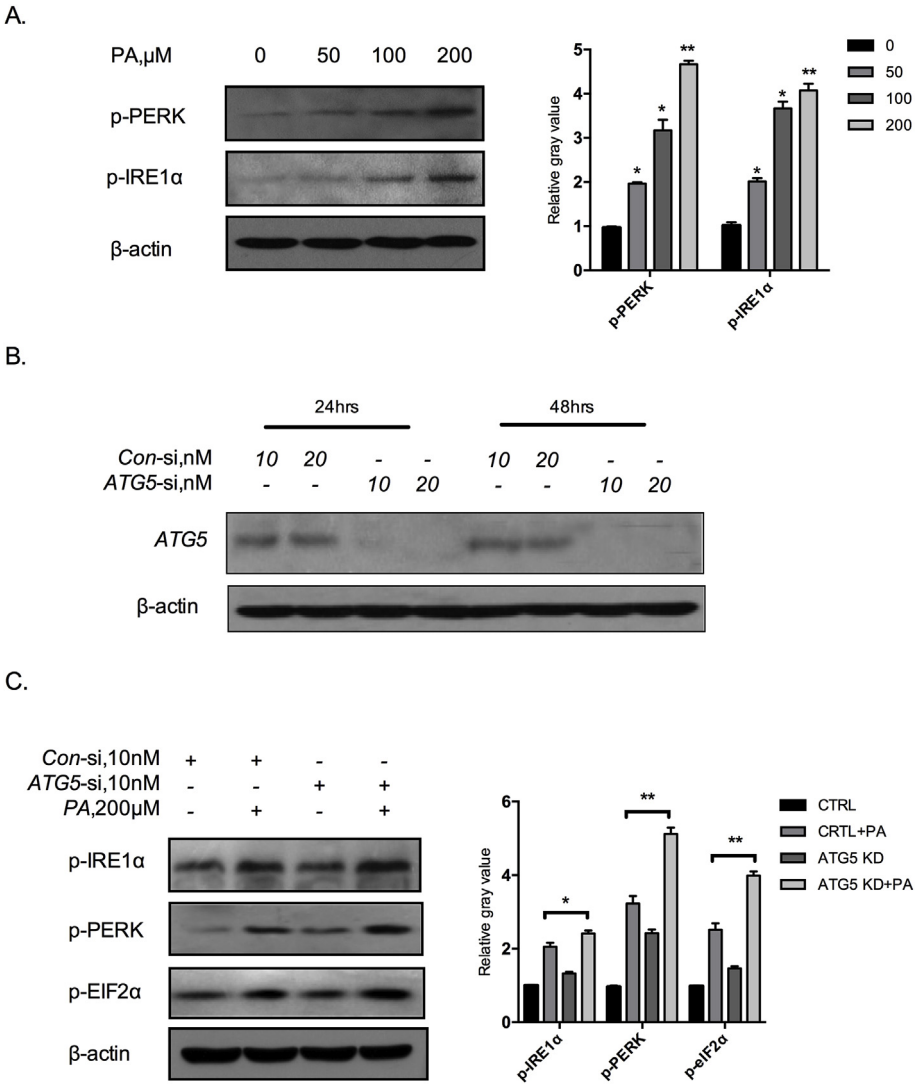

Fig. 4. Increased cell death induction by autophagy inhibition is attributed to the enhancement of ER stress in Bax/Bak wild-type MEF cells. A. Palmitate induces ER stress in wild-type MEF cells. B. The knockdown efficiency of ATG5 siRNA measured by western blotting. C. Effects of autophagy inhibition on palmitate-induced ER stress in MEF cells. $p < 0.05$ (*), $p < 0.01$ (**), (n = 3).

palmitate exposure found in Bax/Bak wild-type MEF cells were not observed in Bax/Bak knockout MEF cells. The results suggest that Bax/Bak is required for palmitate-induced ER stress activation in MEF cells. Accordingly, inhibition of ER stress activation by its inhibitor tauroursodeoxycholic acid (TUDCA) rescued significantly cell death induction by palmitate in Bax/Bak wild-type MEF cells, whereas, no significantly protective effect of the ER stress inhibitor on palmitate-induced cell death in Bax/Bak knockout MEF cells (Fig. 5B). These data further support the requirement of Bax/Bak in palmitate-induced ER stress activation in MEF cells.

4. Discussion

Lipoapoptosis is a key event of lipotoxicity. ER and Oxidative stress-mediated mitochondrial signaling pathway is suggested to play the critical role in lipoapoptosis induction (Cazanave and Gores, 2010; Akazawa et al., 2010; Win et al., 2015). In the present study, we have addressed the role of Bax/Bak in lipoapoptosis using Bax/Bak wild-type/knockout MEF cell system and demonstrated for the first time that Bax/Bak played an important role in regulating the types of cell death induction in response to palmitate exposure.

Autophagy is an intracellular material degradation process. Numerous studies have shown that autophagy plays an important role in the regulation of cell death induction (Booth et al., 2014). Autophagy induction may serve either pro-survival or pro-death functions depending on the context. In most cases, the activation of autophagy promotes cell survival via suppression of apoptosis induction; however, a growing body of evidence has indicated that the autophagy activation may function as a pro-death signal to promote apoptosis or to trigger an alternative non-apoptotic form of programmed cell death, termed autophagic cell death (Kroemer and Levine, 2008). The determinants that govern the decision between pro-survival or pro-death autophagy remain unclear. It is generally believed that excessive or prolonged autophagy may promote cell death induction (Fulda and Kögel, 2015). Previous studies have demonstrated that inhibition of autophagy led to increased apoptosis induction by palmitate in number types of cells (Park et al., 2015). Our present data confirmed these results in MEF cells, suggesting that autophagy serves pro-survival function to suppress palmitate-induced apoptotic signaling. Regarding the mechanisms of apoptosis inhibition by autophagy, our data showed that autophagy inhibition by knocking down ATG5 resulted in an enhanced ER stress activation, which has been shown to play a pivotal role in lipoapoptosis (Cazanave and Gores, 2010; Akazawa et al., 2010). Our results suggest that autophagy protects MEF cells from palmitate-induced apoptosis

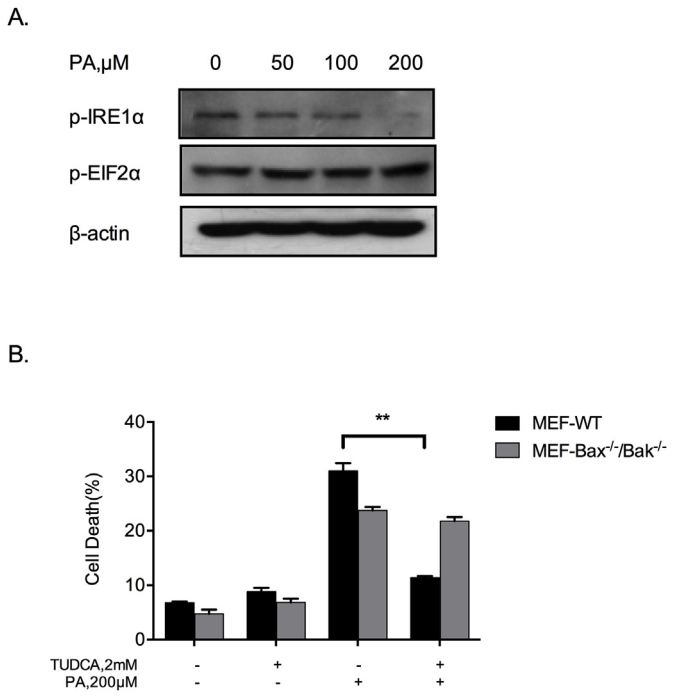

Fig. 5. Bax/Bak is required for palmitate-induced ER stress in MEF cells. A. Palmitate failed to induce ER stress in Bax/Bak knockout MEF cells. B. Effects of ER stress inhibitor on palmitate-induced cell death in wild-type and Bax/Bak knockout MEF cells. $p < 0.01$ (**), (n = 3).

possibly through compromising the ER stress activation. Interestingly, our study further demonstrated that autophagy inhibition by either chemical inhibitor or genetic approach resulted in a significant decreased cell death induced by palmitate in Bax/Bak knockout MEF cells, clearly indicating autophagy functioned as pro-death signal to trigger autophagy-mediated cell death in the absence of Bax/Bak in response to palmitate exposure. It was worth to point out that palmitate induced a higher level of autophagy in Bax/Bak knockout MEF cells than that in Bax/Bak wild-type cells, supporting that Bax/Bak was involved in the regulating autophagy induction in response to palmitate exposure. These results implicate that the level of autophagy as a possible factor contributed to the opposite action of autophagy on palmitate-induced cell death. The detailed mechanisms of the status of Bax/Bak affecting function of pro-survival and pro-death of autophagy need to be further investigated in the future study.

The activation of ER stress is suggested to be a key event in palmitate-induced apoptosis (Cazanave and Gores, 2010; Akazawa et al., 2010). In the present study, we demonstrated that the phosphorylation of IRE1, PERK and EIF2α, the key markers of ER stress, was induced by palmitate, indicating ER stress was activated by palmitate in MEF cells. The activation of ER stress is supposed to trigger JNK activation and CHOP up-regulation, which in turn led to mitochondrial-dependent apoptosis through regulation of Bcl-2 family proteins. It has been suggested that induction of ER stress is associated with impaired autophagy in lipotoxicity (Munch et al., 2014). However, in the present study, we found that autophagy was activated instead of inhibition by palmitate in the current experimental condition, suggesting that autophagy inhibition is unlikely the reason for ER stress activation in palmitate-induced apoptosis in the present study. It has been shown that Bax/Bak can translocate to the ER in addition to the mitochondria (Scorrano et al., 2003; Zong et al., 2003; Wang et al., 2011). Our data showed that palmitate induced the activation of ER stress in Bax/Bak wild type MEF cells, but failed to trigger this event in Bax/Bak knockout MEF cells, indicating the involvement of Bax/Bak in palmitate-induced ER stress. We speculated that the ER translocation of Bax/Bak might take place in response to palmitate exposure, which in turn resulted in the activation of ER stress. We will test this hypothesis in the future study.

In summary, Bax/Bak is required for palmitate-induced activation of ER stress and caspases in MEF cells. The status of Bax/Bak is a factor that determines the function of pro-survival and pro-death of autophagy in response to palmitate exposure in MEF cells. Autophagy protects against palmitate-induced apoptosis through suppression of ER stress. The findings of the present study extend our understanding of the mechanisms underlying palmitate-induced lipoapoptosis.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Transparency document

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