Original Article

Lycium barbarum polysaccharide inhibits palmitic acid-induced BMSC apoptosis

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Abstract

Palmitic acid (PA) is the most common saturated fatty acid in plants and animals. Lycium barbarum polysaccharide (LBP) is a kind of famous traditional Chinese herbal medicine with potential cytoprotective effects. In this study, we aimed to explore the protective effects of LBP on PA-induced bone marrow mesenchymal stem cell (BMSC) apoptosis and to find the molecular mechanisms involved in this process. The results demonstrated that PA administration reduced cell viability, induce cell apoptosis, activated endoplasmic reticulum (ER) stress and inhibited PI3K/AKT signaling pathway. Moreover, LBP administration suppressed the decrease of PA-induced cell viability, cell apoptosis, ER stress-induced activation and PI3K/AKT inhibition. In addition, we found that LBP exerts its cytoprotective effects by alleviating ER stress-induced apoptosis via the activation of PI3K/AKT signaling pathway. In conclusion, we systematically explored the LBP's protection mechanisms against PA-induced BMSC apoptosis, which may provide a theoretical basis for the clinical application of LBP.

Keywords: apoptosis, endoplasmic reticulum, lycium barbarum, palmitic acid

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Introduction

Palmitic acid (PA, C16:0) as the most common saturated fatty acid in plants and animals can decrease insulin signaling, leading to insulin resistance and Type-2 diabetes (Reynoso *et al*., 2003; Benoit *et al*., 2009). Diabetes might be closely associated with the occurrence and development of osteoporosis. Bone marrow mesenchymal stem cells (BMSCs) are multipotent stem cells in the bone marrow, with strong potential for self-renewal and multi-lineage differentiation (Wang *et al*., 2018). As one of the main reasons causing diabetic osteoporosis due to the lipotoxicity of saturated fatty acids, PA may induce bone mesenchymal stem cell (MSC) apoptosis, inhibit the proliferation and differentiation of MSCs and trigger osteoblast apoptosis (Fillmore *et al*., 2015; Liu *et al*., 2018; Shan *et al*., 2010; Yang *et al*., 2018a).

Endoplasmic reticulum (ER) is an indispensable and elaborate eukaryotic organelle. ER homeostasis can be disrupted by many perturbations which will trigger a protective mechanism known as unfolded protein response (UPR). The primary objective of UPR is to re-establish homeostasis and alleviate ER stress through increasing protein folding capacity and decreasing unfolded protein load. However, excessive or prolonged activation of UPR may induce ER stress and lead to cell apoptosis.

Lycium barbarum is a kind of famous traditional Chinese herbal medicine with a history of thousands of years. Lycium barbarum has multiple biological activities, such as anti-oxidative activity and cytoprotection (Yang *et al*., 2018b). Lycium barbarum polysaccharide (LBP) in Lycium barbarum is the main component for these biological activities. The PI3K/AKT pathway can be mediated by the inhibition of ER stress (Hyoda *et al*., 2006). Moreover, LBP has a protective effect against cellular and/or tissue injuries via activating PI3K/AKT signaling or suppressing ER stress (Yang *et al*., 2018b).

However, it remains unknown whether LBP has cytoprotective activities against PA-induced BMSC apoptosis or whether the PI3K/AKT pathway and ER stress are involved in this process. This study aims to identify the effects and molecular mechanisms of LBP on PA-induced BMSC apoptosis, which may provide a theoretical basis for the clinical application of LBP.

Materials and Methods

***LBP preparation*:** Fresh Lyceum barbarum was dried at 60°C and ground into a fine powder which was further double refluxed to remove lipids with chloroform. Oligosaccharides were removed at 80°C with methanol solvent (2:1), followed by 80% ethanol solvent. After filtering, the residues were extracted and concentrated by a rotary evaporator at 60°C and then precipitated with 95% ethanol, 100% ethanol and 100% acetone, respectively. After filtering and centrifuging, the precipitate was collected and vacuum-dried.

***Materials*:** PA, LY294002 (PI3K/AKT inhibitor), thapsigargin (TG, ER stress agonist), Insulin-like growth factor 1 (IGF1, PI3K/AKT agonist) and 4-PBA (ER stress inhibitor) were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Both α-MEM medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). BMSCs of mouse were purchased from Cyagen Biosciences Co., Ltd. (Guangzhou, Guangdong, China). The Protein Extraction Kit and BCA Protein Assay Kit were purchased from Nanjing Keygen Biotech Co., Ltd. (Nanjing, Jiangsu, China). The Caspase-3 Activity Colorimetric Assay Kit was purchased from Nanjing JianCheng Bioengineering Institute (Nanjing, Jiangsu, China). The Cell Counting Kit-8 (CCK-8) was purchased from Beijing Zoman Biotechnology Co., Ltd. (Beijing, China). The Apoptosis Analysis Kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China).

***Cell culture and processing*:** The cells were cultured in the α-MEM medium supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin solution in a 37°C humidified incubator with 5% CO_2. When the cells reached 70-80% confluence, they were processed with different concentrations (100-800 μM) of PA or LBP (200-1600 mg/L) for 24 h. In other experiments, the cells were exposed to 400 μM PA in the presence or absence of LBP (200-1600 mg/L), 500 nM 4-PBA, 5 mM LY294002 or 500 nM TG for 24 h. The cells were then collected for analysis of their cell viability, flow cytometry and Western blot.

***Cell viability analysis*:** The cell viability was performed, according to the previously described protocol (Yang *et al*., 2018a).

***Western blot analysis*:** After the above-mentioned treatment, the cells were collected and Western blot was performed, according to the previously described protocol (Yang *et al*., 2018a). The primary antibodies used are listed in the Table 1.

Table 1 Antibody used in this study.

Target (diluted)	Catalogue number	Company
Akt (1:1000)	ab8805	Abcam
p-Akt (1:1000)	ab81283	Abcam
PI3K (1:1500)	ab154598	Abcam
p-PI3K (1:2000)	ab182651	Abcam
β-actin (1:2000)	sc-47724	Santa Cruz
BAX (1:500)	sc-4239	Santa Cruz
CHOP (1:1000)	ab10444	Abcam
GRP78 (1:1000)	ab32618	Abcam

***Apoptosis analysis*:** Upon completion of the above two steps, the apoptotic cells were quantified with an Annexin V-PE and PI apoptosis detection kit, according to the previously described protocol (Yang *et al*., 2018a).

***Statistical analysis*:** The experimental results were presented as the mean ± SEM of triplicate experiments. The data was analysed with one-way ANOVA, followed by Fisher's least significant different test (Fisher LSD) and Independent-Samples T test with the SPSS software. The differences were statistically significant when $P<0.05$. All the independent experiments were performed in triplicate and repeated three times, respectively.

Results

***LBP inhibited the decrease of cell viability and PI3K/AKT signaling caused by PA in BMSCs*:** As shown in Fig. 1A, the viability of BMSCs significantly decreased in a dose-dependent manner at 100-800 μM PA concentration for 24 h and decreased to 50% at 400 μM of PA concentration. By contrast, administration with 200-800 mg/L LBP for 24 h had no obvious cytotoxicity to BMSCs. The BMSC viability, however, was significantly decreased when the LBP concentration was up to 1600 mg/L (Fig. 1B). LBP (200-1600 mg/L) administration inhibited 400 μM PA-induced cell viability decrease in a dose-dependent manner and the optimum protective concentration was 800 mg/L (Fig. 1C). In addition, our result showed that the protein expression of pAKT (phospho S473) was significantly decreased in a dose-dependent manner when being administrated with 100-800 μM PA for 24 h (Fig. 1D). Unsurprisingly, the protein expression of pAKT (phospho S473) was inhibited by different concentrations of LBP administration from 200 to 1600 mg/L and up to the maximum at the concentration of 800 mg/L (Fig. 1E).

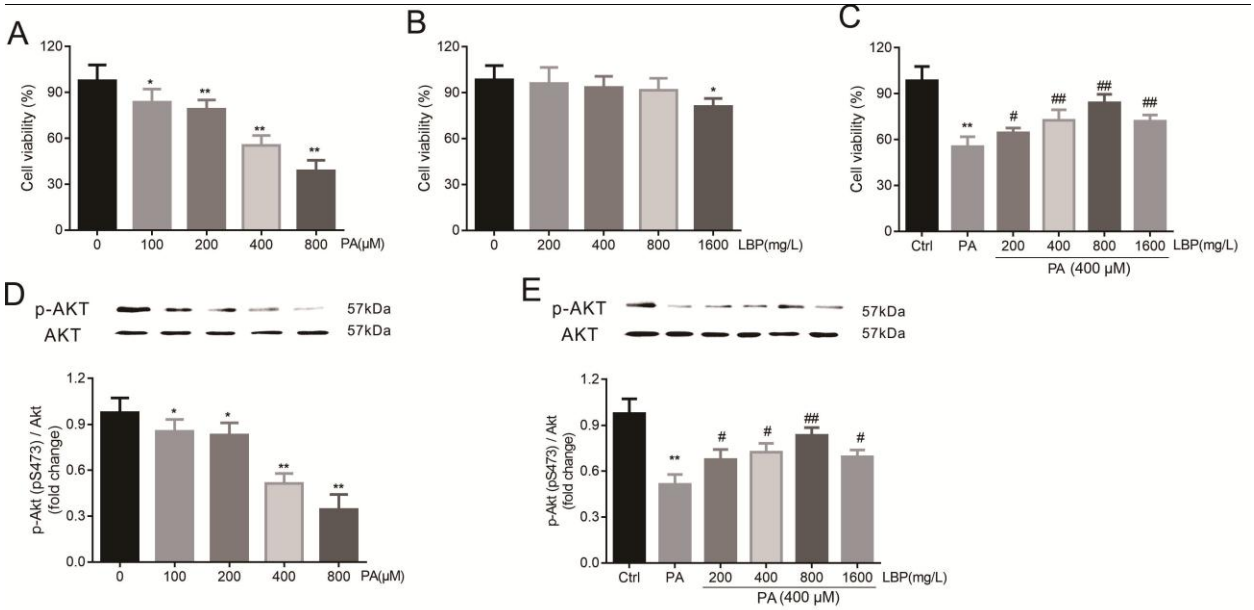

Figure 1 Cell proliferation and AKT signaling pathway after PA and LBP administration in BMSCs. (A) BMSCs were treated with different concentrations of PA (0, 100, 200, 400 and 800 μM) for 24 h and then evaluated with the CCK-8 method. (B) BMSCs were treated with different concentrations of LBP (200, 400, 800 and 1600 mg/L) for 24 h and then evaluated with the CCK-8 method. (C) BMSCs were exposed to 400 μM PA in the presence or absence of LBP (200-1600 mg/L) for 24 h and then evaluated with the CCK-8 method. (D) BMSCs were treated with different concentrations of PA (100, 200, 400 and 800 μM) for 24 h and then pAKT (phospho S473)/AKT protein expression was evaluated with the Western blot method. (E) BMSCs were exposed to 400 μM PA in the presence or absence of LBP (200-1600 mg/L) for 24 h and then pAKT (phospho S473)/AKT protein expression was evaluated with the Western blot method. All data was represented as the means ± SEM from three independent experiments. $^{*}P<0.05$ vs. Control group; $^{**}P<0.01$ vs. Control group; $^{\#}P<0.05$ vs. PA group; $^{\#\#}P<0.01$ vs. PA group.

***LBP attenuated PA-induced apoptosis in BMSCs*:** As shown in Fig. 2A, Caspase-3 activity was markedly increased in a dose-dependent manner in the process of administration with different concentrations of PA (100-800 μM) for 24 h. Moreover, LBP (200-800 mg/L) administration reduced the 400 μM PA-induced Caspase-3 activity in a dose-dependent manner and the optimum protective concentration was 800 mg/L. When LBP concentration was up to 1600 mg/L, Caspase-3 activity was significantly increased again (Fig. 2B). At the same time, the protein expression of BAX was significantly increased in a dose-dependent manner by the administration of PA at different concentrations (Fig. 2C). LBP (200-1600 mg/L) administration weakened the protein expression of BAX induced by 400 μM PA administration and the optimum protective dose was 800 mg/L. When LBP concentration was up to 1600 mg/L, the protein expression of BAX significantly rose again (Fig. 2D).

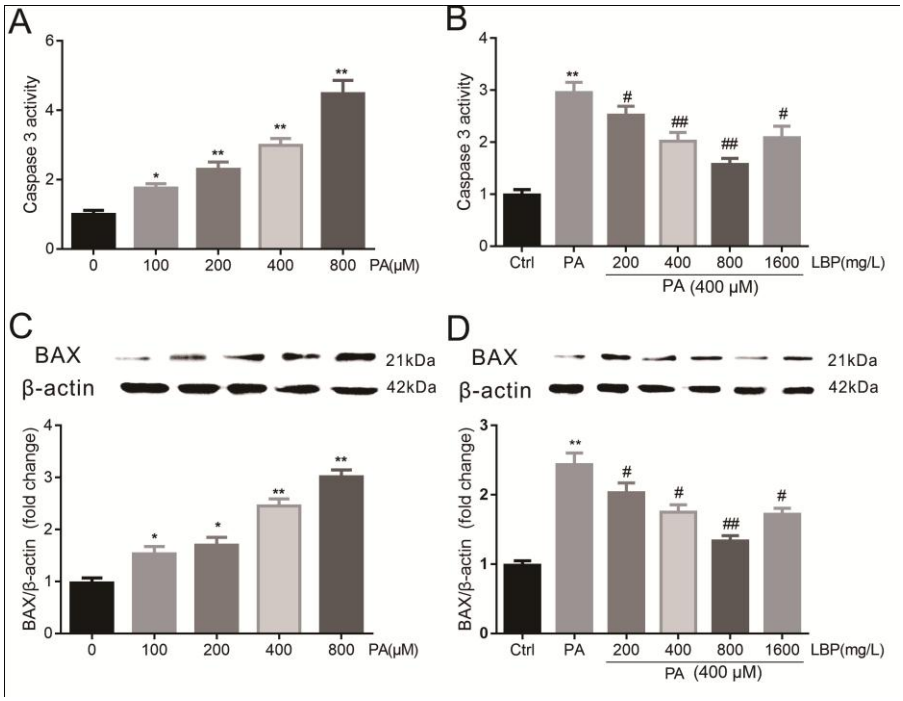

Figure 2 LBP reduced PA-induced BMSC apoptosis. (A) BMSCs were treated with different concentrations of PA (100, 200, 400 and 800 μM) for 24 h and then Caspase-3 activity was evaluated. (B) BMSCs were exposed to 400 μM PA in the presence or absence of LBP (200-1600 mg/L) for 24 h and then Caspase-3 activity was evaluated. (C) BMSCs were treated with different concentrations of PA (100, 200, 400 and 800 μM) for 24h and then BAX protein expression was evaluated with the Western blot method. (D) BMSCs were exposed to 400μM PA in the presence or absence of LBP (200-1600 mg/L) for 24 h and then BAX protein expression was evaluated with the Western blot method. All data was represented as the means ± SEM from three independent experiments. $^{*}P<0.05$ vs. Control group; $^{**}P<0.01$ vs. Control group; $^{\#}P<0.05$ vs. PA group; $^{\#\#}P<0.01$ vs. PA group.

***LBP attenuated PA-induced ER stress in BMSCs*:** ER stress marker proteins (GRP78 and CHOP) were up-regulated in a dose-dependent manner till peaking at 400 μM and then reduced (Fig. 3A) by 100-800 μM PA administration. LBP (200-1600 mg/L) administration weakened the protein expression of GRP78 and CHOP induced by 400 μM PA administration and the optimum protective dose was 800 mg/L. When LBP concentration reached 1600 mg/L, the GRP78 and CHOP expressions significantly rose again (Fig. 3B).

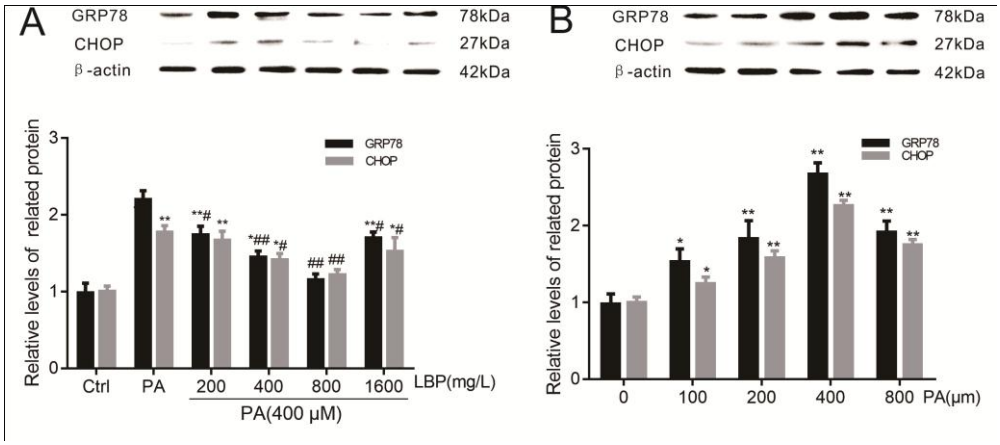

Figure 3 GRP78 and CHOP induced by LBP prevented the harmful effect of PA on BMSCs. (A) BMSCs were treated with different concentrations of PA (100-800 μM) for 24 h and then GRP78 and CHOP protein expressions were evaluated with the Western blot method. (B) BMSCs were exposed to 400 μM PA in the presence or absence of LBP (200-1600 mg/L) for 24 h and then GRP78 and CHOP protein expressions were evaluated with the Western blot method. All data was represented as the means ± SEM from three independent experiments. $^{*}P<0.05$ vs. Control group; $^{**}P<0.01$ vs. Control group; $^{\#}P<0.05$ vs. PA group; $^{\#\#}P<0.01$ vs. PA group.

***ER stress was involved in PA-induced BMSC apoptosis and PI3K/AKT inactivation*:** The results showed that 4-PBA (ER stress inhibitor) significantly reduced PA-induced BMSC apoptosis (Fig. 4A). At the same time, the cell viability declined and PA-induced Caspase-3 activity of BMSCs was also inhibited (Fig. 4B and C). Moreover, 4-PBA administration significantly reduced the expression of ER stress-related protein GRP78 and CHOP and apoptosis-related protein BAX. In addition, 4-PBA administration partly inhibited the decreased expression of PA-induced pAKT (phospho S473) in BMSCs (Fig. 4D and E).

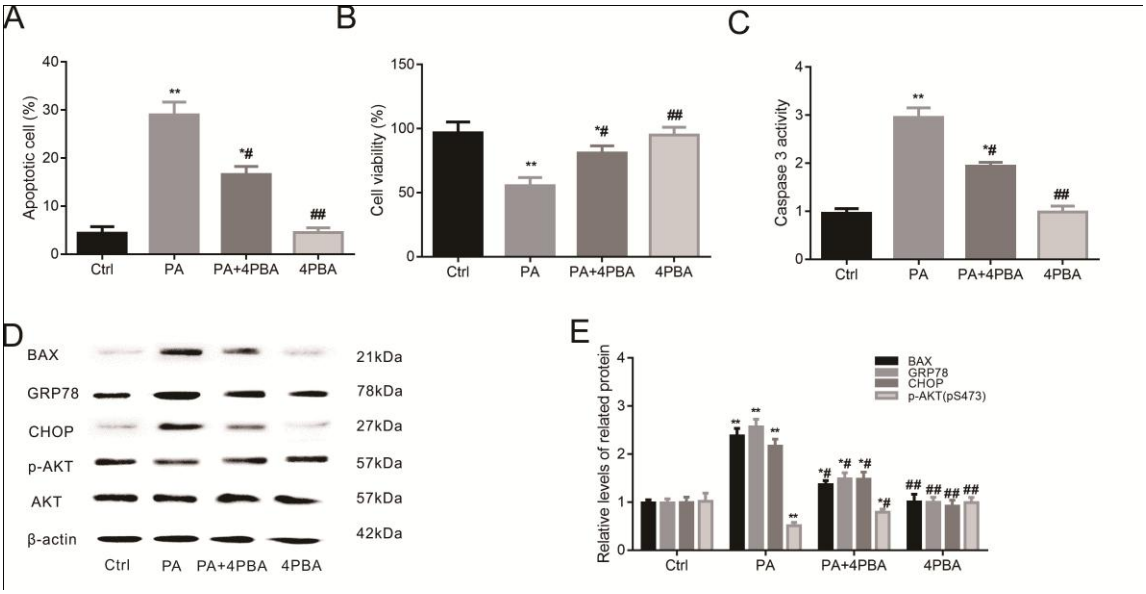

Figure 4 ER stress was involved in PA-induced BMSC apoptosis and PI3K/AKT inactivation. BMSCs were exposed to 400 μM PA in the presence or absence of ER stress inhibitor 4-PBA (500 nM) for 24 h. (A) The cell apoptosis rate was evaluated with flow cytometry. (B) Cell viability was evaluated with the CCK-8 method. (C) Caspase-3 activity was evaluated. (D) (E) BAX, GRP78, CHOP, pAKT (phospho S473), and AKT protein expressions were evaluated with the Western blot method. All data was represented as the means ± SEM from three independent experiments. $^{*}P<0.05$ vs. Control group; $^{**}P<0.01$ vs. Control group; $^{\#}P<0.05$ vs. PA group; $^{\#\#}P<0.01$ vs. PA group.

***LBP inhibited PA-induced cell apoptosis and ER stress via PI3K/AKT signaling pathways in BMSCs*:** LBP (200-800 mg/L) administration reduced PA-induced Caspase-3 activity which rose again by LY294002 (PI3K/AKT inhibitor) administration (Fig. 5B). At the same time, LBP attenuated PA-induced apoptosis of BMSCs, which were also abolished by LY294002 administration (Fig. 5A). Moreover, PA-induced Caspase-3 activity of BMSCs inhibited by LBP was reversed by LY294002 administration (Fig. 5C). Unsurprisingly, the expression of PA-induced ER stress-related protein GRP78 and CHOP and that of BAX protein significantly decreased by LBP were also reversed by LY294002 administration in BMSCs. In addition, LY294002 administration partly decreased the expression of PA-induced pAKT (phospho S473) in BMSCs (Fig. 5D and E).

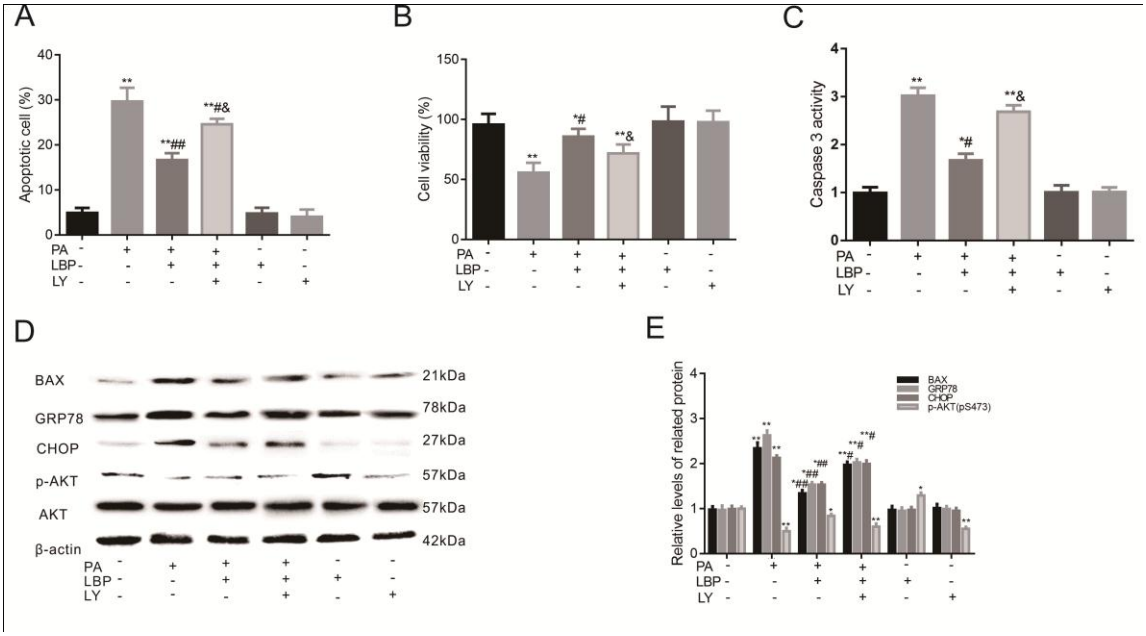

Figure 5 PI3K/AKT signaling pathway involved in LBP reduced PA-induced BMSC apoptosis. BMSCs were exposed to 400 μM PA in the presence or absence of PI3K/AKT signaling pathway inhibitor LY294002 (5 mM) and LBP (800 mg/L) for 24 h. (A) Cell apoptosis rate was evaluated with flow cytometry. (B) Cell viability was evaluated with the CCK-8 method. (C) Caspase-3 activity was evaluated. (D) (E) BAX, GRP78, CHOP, pAKT (phospho S473) and AKT protein expressions were evaluated with the Western blot method. All data was represented as the means ± SEM from three independent experiments. $^{*}P<0.05$ vs. Control group; $^{**}P<0.01$ vs. Control group; $^{\#}P<0.05$ vs. PA group; $^{\#\#}P<0.01$ vs. PA group; $^{\&}P<0.05$ vs. PA+LBP group.

***LBP inhibited ER stress-mediated cell apoptosis and PI3K/AKT inactivation in BMSCs*:** To further investigate the role of ER stress in LBP protection against PA injury in BMSCs, the ER stress activator TG was added to the medium along with or without LBP culture for 24 h. The results showed that LBP administration partly inhibited cell activity, apoptosis rate and Caspase-3 activity of BMSCs induced by TG (Fig. 6A-C). In addition, LBP administration inhibited the expression of proteins such as BAX, GRP78 and CHOP. Unsurprisingly, LBP administration prevented the inhibition of TG-induced pAKT (phospho S473) (Fig. 6D and E).

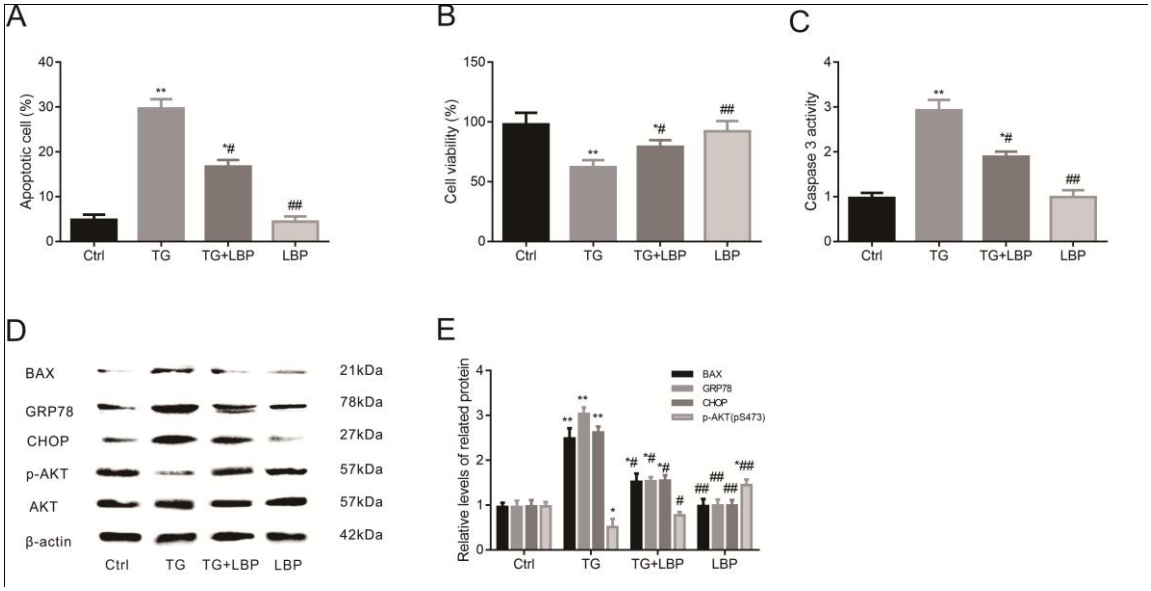

Figure 6 ER stress involved in LBP reduced PA-induced BMSC apoptosis. BMSCs were exposed to 400 μM PA in the presence or absence of ER stress activator TG (500 nM) and LBP (800 mg/L) for 24 h. (A) Cell apoptosis rate was evaluated with flow cytometry. (B) Cell viability was evaluated with the CCK-8 method. (C) Caspase-3 activity was evaluated. (D) (E) BAX, GRP78, CHOP, pAKT (phospho S473) and AKT protein expressions were evaluated with the Western blot method. All data was represented as the means ± SEM from three independent experiments. $^{*}P<0.05$ vs. Control group; $^{**}P<0.01$ vs. Control group; $^{\#}P<0.05$ vs. TG group; $^{\#\#}P<0.01$ vs. TG group.

***Activating PI3K/AKT pathway protected against TG- or PA-induced ER stress and apoptosis in BMSCs*:** To further investigate the relations of PA-induced PI3K/AKT and/or ER stress in BMSCs, the BMSCs were cultured with the PI3K/AKT activator IGF1 (50 ng) with or without TG and PA for 24 h. The results showed that IGF1 administration significantly inhibited the decrease of PA- and TG-induced cell viability (Fig. 7B), and the cell apoptosis rate and Caspase-3 activity increase (Fig. 7A and C). At the same time, IGF1 administration inhibited the expression of proteins such as BAX, GRP78 and CHOP induced by PA and TG administration in BMSCs. Consistently, IGF1 administration prevented the inhibition of pAKT (phospho S473) induced by TG and/or PA administration (Fig. 7D and E).

Discussion

It is well-known that diabetes leads to a decrease in the quantity of MSCs, which further causes the accumulation of osteoporosis (Hamann *et al*., 2012). Previous studies have reported that serum free fatty acids, especially PA, are one of the main reasons causing MSC apoptosis (Fillmore *et al*., 2015; Liu *et al*., 2018). Moreover, some studies have shown that LBP has a protective effect against cellular and/or tissue injuries (Yang *et al*., 2018b; Quan *et al*., 2011; Jing and Jia, 2018). This paper aims to explore the effects of LBP on PA-induced BMSC apoptosis and the underlying molecular mechanisms in the process. The data showed that the exposure of BMSCs to PA caused cell apoptosis, activated ER stress and inhibited PI3K/AKT, and LBP administration could reverse PA-mediated apoptosis, ER stress and the inhibition of PI3K/AKT signaling pathway.

Previous studies have reported that PA have induced apoptosis in different cell types, including liver cancer HepG2 cells, pancreatic islet B cells and H9C2 cardiomyocytes (Alnahdi *et al*., 2019; Huang *et al*., 2021; Yang *et al*., 2019). Our present study showed that PA administration markedly inhibited cell viability in a dose-dependent manner in BMSCs. Some previous studies indicated that LBP showed a good antioxidant effect in resisting neuronal and myocardial apoptosis (Zhao *et al*., 2016; Yu *et al*., 2019). This is consistent with our study that LBP protected BMSCs avoiding apoptosis. Our study also showed that 100-800 mg/L LBP increased cell viability, but 1600 mg/L LBP had the cell cytotoxicity. Therefore, LBP can inhibit the reduction in PA-induced cell viability in a dose-dependent manner. However, excessive amounts of LBP show a toxic effect.

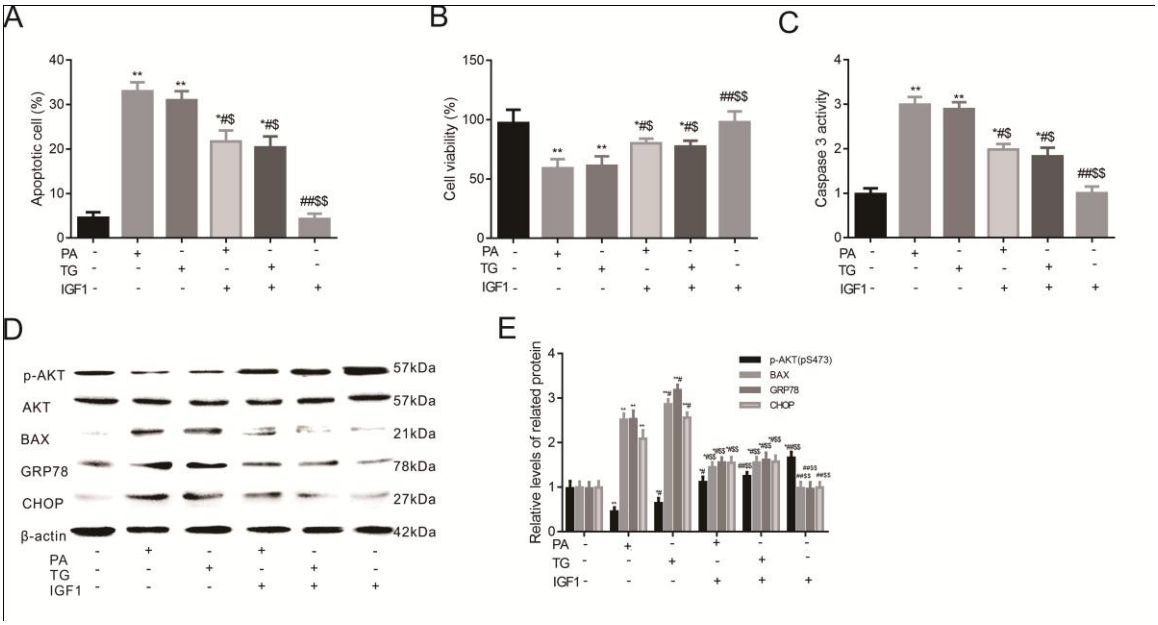

Figure 7 PI3K/AKT pathway was involved in TG or PA-induced ER stress and cell apoptosis. BMSCs were exposed to 400 μM PA or 500 nM TG in the presence or absence of PI3K/AKT signaling pathway activator IGF1 (50 ng) for 24 h. (A) Cell apoptosis rate was evaluated with flow cytometry. (B) Cell viability was evaluated with the CCK-8 method. (C) Caspase-3 activity was evaluated. (D) (E) BAX, GRP78, CHOP, pAKT (phospho S473) and AKT protein expressions were evaluated with the Western blot method. All data was represented as the means ± SEM from three independent experiments. $^{*}P<0.05$ vs. Control group; $^{**}P<0.01$ vs. Control group; $^{\#}P<0.05$ vs. PA group; $^{\#\#}P<0.01$ vs. PA group; $^{\$}P<0.05$ vs. TG group; $^{\$\$}P<0.01$ vs. TG group.

Previous studies have indicated that PA-induced cell apoptosis is realized through the activation of ER stress pathway or the inhibition of PI3K/AKT signaling pathway (Jing and Jia, 2016; Pardo *et al*., 2015; Beeharry *et al*., 2004; Liu *et al*., 2015; Morishita *et al*., 2015). It is now recognized that the activation of PI3K and the downstream activation of AKT play a key role in maintaining cell survival by blocking the apoptotic pathway. Apoptosis refers to a series of complex events initiated within a cell, which ultimately lead to cell destruction initiated by proteolytic enzymes called caspases. The PI3K/AKT pathway can block many pro-apoptotic proteins or have a positive effect on a variety of survival components (Duronio, 2008). According to the research, LBP can inhibit ER stress and active the PI3K/AKT signaling pathway to protect cells and tissues and thus avoid injury from outside (Jing and Jia, 2018; Yu *et al*., 2019; Zhao *et al*., 2016; Yang *et al*., 2014). So, we tested the involvement of ER stress and PI3K/AKT signaling pathway in PA-induced cell apoptosis. The results showed that PA stimulated the expressions of CHOP and GRP78 protein and inhibited the expression of pAKT protein (phospho S473) in BMSCs, which is consistent with the previous reports showing PA may stimulate ER stress and inhibit PI3K/AKT pathway (Jing and Jia, 2018; Alnahdi *et al*., 2019; Wente *et al*., 2006; Calvo-Ochoa *et al*., 2017). We also found that PA-mediated ER stress and the activation of PI3K/AKT could be reversed by LBP administration, which is in accordance with the results of a previous study that LBP inhibited ER stress in MC3T3-E1 cells and induced the activation of PI3K/AKT pathway (Jing and Jia, 2018; Zhao *et al*., 2016; Yang *et al*., 2014).

The importance of the PI3K/AKT pathway in mediating ER stress-induced cell apoptosis has been proved in previous studies (Hu *et al*., 2004; Wang *et al*., 2012). It has been confirmed in multiple systems and various diseases that the PI3K/AKT signaling pathway inhibits cell apoptosis by regulating the level of oxidative stress. For example, in patients with acute lung injury, Alzheimer's patients and cerebral ischemia patients during reperfusion, activation of PI3K/AKT signaling pathway significantly reduces the ER stress level, and ultimately controls apoptosis and reduces injury (Luo *et al*., 2019; Cui *et al*., 2017; Yuan *et al*., 2011). However, the interference between PI3K/AKT and ER stress signaling pathways in LBP protective effects has never been reported. In this study, the ER stress inhibitor PA or activator TG was added to the culture medium. It indicated that the ER stress inhibitor 4-PBA and LBP might prevent cell apoptosis, the activation of ER stress or the inhibition of PA- and/or TG-induced AKT. In addition, the processing of BMSCs with the PI3K inhibitor LY294002 may suppress the activation of AKT, result in higher levels of ER stress response protein CHOP and GRP78, and partially decrease the protective effects of LBP or IGF1. This data suggests that LBP administration increases cellular activity via the inhibition of ER stress and the cells with higher cellular activity allow for the higher activation of AKT in PA-induced cells. On the other hand, the PI3K/AKT pathway is necessary for mediating cellar survival under a variety of circumstances (Brunet *et al*., 1999; Brunet *et al*., 2001; Arslan *et al*., 2013). The pAKT (phospho S473) with a decreased level of ER stress as the activator of AKT inhibits apoptosis by phosphorylating the pro-apoptotic protein BAD, which further suppresses the intrinsic apoptosis pathways, including ER stress signaling pathway (Zhang *et al*., 2013; Li *et al*., 2017; Wolf *et al*., 2001; Zhang *et al*., 2015).

In conclusion, our study affirmed the protective effect of LBP in PA-induced BMSC apoptosis.

According to the study, LBP reduced PA-induced apoptosis in BMSCs via the inhibition of ER stress and the activation of the PI3K/AKT pathway. It was also concluded that LBP may attenuate ER stress-induced apoptosis by activating PI3K/AKT signaling pathway in PA-exposed BMSCs. These results suggest a potential therapeutic application in which LBP may be useful for attenuating ER stress, activing the PI3K/AKT signaling pathway and protecting against diabetic osteoporosis.

Acknowledgements

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