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ERO1α KNOCKDOWN ATTENUATES PALMITIC ACID-MEDIATED INHIBITION OF TESTOSTERONE SECRETION BY INHIBITING ENDOPLASMIC RETICULUM STRESS IN TESTICULAR LEYDIG CELLS

Qi-Zhuang Lv[1,2], Ting Qin[1], Xin-Yun Qin[1], Xiao-Mei Liang[1], Ke-Yi Nong[1], Zi-Feng Gong[1], Li-Ying Liang[1], Lei Yang[3,*], Yu-lin Zhu[1,*]

[1]College of Biology & Pharmacy, Yulin Normal University, Yulin, PR China

[2]Guangxi Key Laboratory of Agricultural Resources Chemistry and Biotechnology, Yulin, PR China

[3]Key Laboratory of System Bio-medicine of Jiangxi Province, Jiujiang University, Jiujiang, Jiangxi, China

Corresponding authors: Lei Yang: yangleigeili@163.com; Yu-linZhu: gxzyl@163.com

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ABSTRACT

Background and objective

Palmitic acid (PA), the most common saturated free fatty acid (FFA) in food, is related to obesity-related male infertility. The possible mechanism is PA-mediated inhibition of testosterone secretion. Endoplasmic reticulum (ER) oxidoreductin-1alpha (ERO1α), an oxidase that is localized in the ER, plays an essential role in maintaining ER homeostasis and is related to hormone secretion. However, the role and underlying mechanisms of ERO1α in PA-mediated inhibition of testosterone secretion have not been reported.

Material and methods

Murine Leydig tumor cell line 1 (MLTC-1) cells were treated with different doses of PA. Cell viability, testosterone secretion, and ERO1α expression were measured by the Cell Counting Kit 8 (CCK-8) assay, enzyme-linked immunesorbent assay (ELISA), and Western blotting, respectively. Moreover, the expression of ER stress marker proteins (glucose-regulated protein 78 [GRP78] and CCAAT/enhancer-binding protein homologous protein [CHOP]) was also measured after treatment. Subsequently, the expression ofERO1α was knocked down, and cell viability, testosterone secretion, and ER stress were measured after treatment with the PA or the ER stress agonist thapsigargin (TG, an ER stress inducer). Also, testosterone secretion was measured by ELISA when ER stress was inhibited by 4-phenylbutyric acid (4-PBA, an ER stress inhibitor).

Results
PA treatment reduced cell viability, induced ERO1α expression, and enhanced the expression of the ER stress marker GRP78 and CHOP, while ERO1α knockdown inhibits ER stress marker expression, promotes testosterone secretion, and enhances cell viability in PA-treated MLTC-1 cells. In addition, ERO1α knockdown rescued the TG-induced the decrease in testosterone secretion and cell viability.

Conclusions
These findings suggest that PA inhibits testosterone secretion via ER stress and that ERO1α knockdown ameliorates PA-induced decreases in testosterone via ER stress in testicular Leydig cells. Our results indicate the necessity of exploring the potential applications of ERO1α as a target gene for restoring fertility in obese men.

Keywords: *endoplasmic reticulum oxidoreduclin-1 alpha; endoplasmic reticulum stress; palmitic acid; testicular Leydig cells; testosterone*

INTRODUCTION

Obesity is a serious social problem because overweight is often accompanied by the occurrence of various diseases, including infertility, especially in males.[1] The possible reason for obesity-induced male infertility is that saturated free fatty acids (FFAs) inhibit testosterone secretion.[2] Testosterone, which is mainly synthesized and secreted by Leydig cells and is generally induced in response to exogenous human chorionic gonadotropin (hCG), is recognized as an important indicator of male fertility, as testosterone plays essential roles in spermatogenesis, male sexual differentiation, and male secondary sex characteristics.[3–5] Palmitic acid (PA), the most common type of FFA, has been shown to induce cell apoptosis and decrease testosterone levels in Leydig cells via endoplasmic reticulum (ER) stress.[2,6]

The ER is the major component of the endomembrane system of eukaryotic cells and has different functions that influence the biogenesis, folding and trafficking of secretory and membrane proteins. ER stress or impaired ER homeostasis usually triggers the unfolded protein response, apoptosis, autophagy, and even cell death.[7–9] In recent years, there has been growing evidence suggesting that ER stress is closely associated with obesity and therefore with obesity-induced male infertility.[10,11]

Endoplasmic reticulum oxidoreductin-1 alpha (ERO1α), an oxidase that is localized in the ER, plays an important role in maintaining redox homeostasis in the ER and forming disulfide bonds in secreted and surface proteins.[12] Previous studies have shown that ERO1α is related to hormone secretion and ER stress. However, the role of ERO1α in PA-mediated inhibition of testosterone secretion has not been elucidated. In this study, we studied the role and mechanisms of ERO1α in PA-mediated inhibition of testosterone secretion in testicular Leydig cells.

METHODS

Cells, vectors, and reagents

Murine Leydig tumor cell line 1 cells were provided by Zhi Chen, PhD, Qiannan Normal University for Nationalities, PR China. HEK293T cells were a gift from professor Yan-ming Zhang, Northwest A&F University, PR China. The over-expression lentivector pCDH-CMV-MCS-EF1-GreenPuro (CD513B-1) (SBI), the knockdown lentivector pCDH-U6-MCS-EF1-GreenPuro (SBI), and packaging plasmids (pGag/Pol, pRev, and pVSV-G) were obtained from En-qi Du, PhD, Northwest A&F University, PR China. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), advanced DMEM and RPMI 1640 medium were

purchased from Gibco (Carlsbad, USA). Trizol, PrimeScript RT reagent Kit with gDNA Eraser and SYBR *Premix Ex Taq* II (Tli RNaseH Plus) were purchased from TaKaRa (Dalian, China). Dimethyl sulfoxide (DMSO), PA, hCG, bovine serum albumin (BSA), TG, 4-PBA, Polybrene, cholesterol, and yolk lecithin were procured from Sigma-Aldrich (St Louis, USA). TurboFect Transfection Reagent, enhanced chemiluminescence (ECL) reagent, and PageRuler Prestained Protein Ladder were procured from Thermo Scientific (Waltham, USA). Phenylmethylsulfonyl fluoride (PMSF), Total Protein Extraction Kit, and bicinchoninic acid (BCA) Protein Assay Kit and Phosphate Buffer solution (PBS) were purchased from Beyotime Biotechnology Co., Ltd. (Beijing, China). The testosterone ELISA Kit was purchased from Ji Yin Mei (Wuhan, China). Cell Counting Kit 8 (CCK-8) was purchased from Beijing Zoman Biotechnology Co., Ltd. (Beijing, China). Mouse anti-GAPDH, anti-CHOP, anti-GRP78 monoclonal antibody, and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody were purchased from Abcam (Cambridge, UK). Mouse anti-ERO1α monoclonal antibody was purchased from Abnova Biotechnology Co., Ltd. (Taiwan, China). Chemically Defined Lipid Concentrate was purchased from Invitrogen (Carlsbad, USA).

PA preparation

First, 20 mM PA in 0.01 M NaOH was incubated at 70°C for 30 min. Then, the fatty acid soaps were complexed with 5% fatty acid-free BSA in PBS in a 1:3 volume ratio to produce a 5 mM PA stock solution, which was then stored at −20°C. Before use, the stock solution was further diluted in a culture medium.

Cell culture and treatment

Murine Leydig tumor cell line 1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS at 37°C in a 5% CO_2 incubator. DMSO was used to prepare TG, which was diluted to a final concentration of 500 nM for use in the subsequent experiment. MLTC-1 cells (up to 70–80% confluence) were divided into seven groups, of which six groups were treated with 100–400 μM PA, 400 μM PA + shNC (MOI=10) + 6 μg/mL polybrene, 400 μM PA + shERO1α (MOI=10) + 6 μg/mL polybrene, 400 μM PA + 500 nM 4-PBA, 500 nM TG + shNC (MOI=10) + 6 μg/mL polybrene, and 500 nM TG+ shERO1α (MOI=10) + 6 μg/mL polybrene. After incubation for 24 h, 3 IU/mL hCG was added to each group, followed by incubation for another 12 h. Finally, the cells and supernatants were collected and used to measure cell viability, testosterone levels, and the protein expression levels of the ER stress markers GRP78 and CHOP.

Lentivector construction and lentivirus production

Recombinant lentiviruses expressing short hairpin RNAs (shRNAs) targeting murine ERO1α were constructed according to the procedures described in our previous study.[13] Briefly, the shRNA targeting murine ERO1α (Accession No.NM_015774.3)and a negative control shRNA were cloned into the knockdown lentivector pCDH-U6-MCS-EF1-GreenPuro to generate the shERO1α and shNC plasmids, respectively, using the primer pairs listed in Table 1. When the HEK293T cells reached 70–80% confluence, they were cotransfected with the recombinant plasmids and packaging plasmids (pGag/Pol, pRev, and pVSV-G) using TurboFect transfection reagent. After transfection for 16 h,the supernatants were removed and replaced with fresh advanced DMEM supplemented with 2% FBS and an appropriate concentration of cholesterol, yolk lecithin, and chemically defined lipid concentrate as previously described. After incubation for another 48 h, the supernatants containing lentiviruses were harvested for subsequent experiments. To confirm the validity of the constructed lentivectors, polymerase chain reaction (PCR) was performed by using the primer pair U6-F:5′-TTCTTGGGTAGTTTGCAGTT-3′/U6-R:5′-CGGAGCCAGTACACGACA-3′ as described in our recent study.[14]

TABLE 1 Short Hairpin RNA (shRNA) Inserts.

shRNA	Sequence (Loop in bold letters and Interference sequence in underscore characters) (5′–3′)
shERO1α-1F	GATCCGGACGACTGTACCTGTGATGT**CAAGAG**ACATCACAGGTACAGTCGTCCTTTTTG
shERO1α-1R	AATTCAAAAAGGACGACTGTACCTGTGATGT**CTCTTG**ACATCACAGGTACAGTCGTCCG
shERO1α-2F	GATCCGGAGGATATGGAGTGTCATCT**CAAGAG**AGATGACACTCCATATCCTCCTTTTTG
shERO1α-2R	AATTCAAAAAGGAGGATATGGAGTGTCATCT**CTCTTG**AGATGACACTCCATATCCTCCG
shERO1α-3F	GATCCGCAGCGCTTTGATGGGATTCT**CAAGAG**AGAATCCCATCAAAGCGCTGCTTTTTG
shERO1α-3R	AATTCAAAAAGCAGCGCTTTGATGGGATTCT**CTCTTG**AGAATCCCATCAAAGCGCTGCG
shNC-F	GATCCGCTTAAACGCATAGTAGGACT**CAAGAG**AGTCCTACTATGCGTTTAAGCTTTTTG
shNC-R	AATTCAAAAAGCTTAAACGCATAGTAGGACT**CTCTTG**AGTCCTACTATGCGTTTAAGCG

Measurement of cell viability

To determine the cytotoxicity induced by the various treatments, CCK-8 assays were used to measure the cell viability in the experimental groups. Briefly, MLTC-1 cells were cultured in 96-well culture dishes at adensity of 3×10^4 cells/well. After treatment, the cells were incubated for 24 h, 10 μL of CCK-8 solution was added, and the cells were incubated for another 2 h. Finally, the absorbance of each group was measured by using a microplate reader (Varioskan™ LUX, Thermo Scientific, USA) at 405 nm, and all experiments were performed three times independently.

Estimation of testosteronelevels

To explore the effects ofPA, TG, 4-PBA, and ERO1α on testosterone secretion, ELISA was utilized to determine the expression level of testosterone. Briefly, MLTC-1 cells were exposed to different treatments and incubated for 24 h, followed by coincubation with 3 IU/mL hCG for 12 h. Finally, the culture supernatants were harvested and testosterone concentrations were measured with the testosterone ELISA kit according to the manufacturer's instructions. The absorbanceat 450 nm of each group was measured by using a microplate reader, and all experiments were performed in triplicate.

Real-time quantitative RT-PCR

To measure the transcription levels of the ERO1α gene in different shRNA-treated MLTC-1 cells, real-time quantitative RT-PCR was performed as described in our previous study.[15] Briefly, total cell RNA was isolated using TRIzol. After reverse transcription with a PrimeScript RT reagent kit, the generated cDNA was used as a template for quantitative real-time PCR using SYBR *Premix Ex Taq* II (Tli RNaseH Plus) on a QuantStudio™ 6 and 7 Flex Real-Time PCR system (Thermo Scientific, USA) according to the manufacturer's recommendations. Finally, the relative expression level of the ERO1α gene was analyzed by using the 2-delta-delta Ct formula, with murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, as described in a previous study.[16] The primer pairs used for real-time quantitative RT-PCR are listed in Table 2.

Western blot analysis

To evaluate the protein expression levels of ERO1α, GAPDH, and the ER stress markers

TABLE 2 Sequences of Primer Pairs Used for Real-Time Quantitative RT-PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product(bp)	Accession No.
ERO1α	GACTGTGCCGTCAAACCCTG	CGCTCAGCTTGCTCACATTC	113	NM_015774.3
GAPDH	GGAGTCTACTGGTGTCTTC	GCTGACAATCTTGAGTGAGTT	157	NM_008084.3

GRP78 and CHOP, an immunoblotting analysis was performed as previously described with some modifications.[17] Briefly, cells were harvested, and total proteins were extracted using RIPA lysis buffer. The protein concentration was determined with a BCA protein assay kit, and equal amounts of protein (30 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto 0.45-μm polyvinylidene fluoride (PVDF) membranes. After being blocked with 5% skimmed milk for 2 h at room temperature, the membranes were then incubated with the relevant primary antibodies overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies for 2 h at room temperature. Finally, the immunoreactive bands were visualized with ECL reagents by using a gel imaging system (Bio-Rad, USA). The cellular protein GAPDH served as an internal control, and the amount of target protein was calculated by densitometry using ImageJ software (National Institutes of Health, USA) according to the manufacturer's instructions.

Statistical analysis

All data are presented as mean ± the standard deviations (SD) of three independent experiments. Statistical comparisons were analyzed using a Student's *t*-test, and $P < 0.05$ was considered statistically significant with "*", $P < 0.01$ was regarded as significant with "**", and $P < 0.001$ was regarded as highly significant with "***".

RESULTS

Expression of ERO1α is induced by PA

After different treatments with increasing concentrations of PA (100–400 μM), which were based on a previous report,[2] the expression levels of ERO1α and GAPDH in MLTC-1 cells

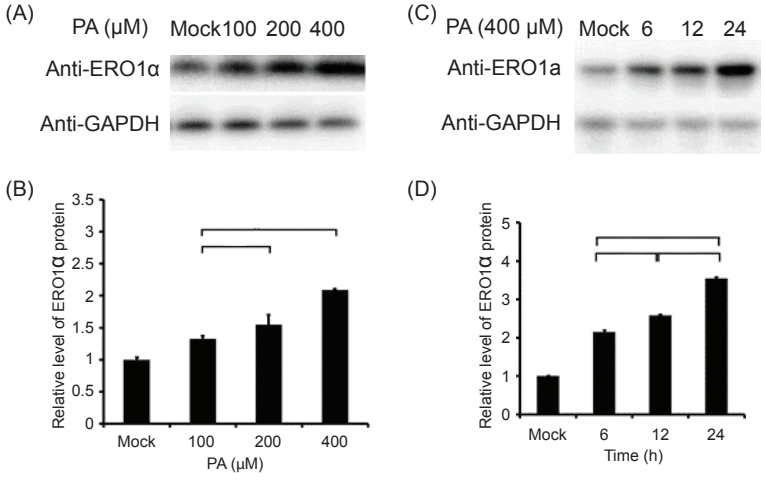

FIGURE 1 Palmitic acid induces the expression of ERO1α in MLTC-1 cells. (A, B) Protein levels of ERO1α in MLTC-1 cells that were subjected to different concentrations of PA (100–400 μM). (C, D) Protein levels of ERO1α in MLTC-1 cells treated with PA (400 μM) at 0 h, 6 h, 12 h, and 24 h. The expression levels of ERO1α in different groups are presented as the relative ratio of ERO1α to GAPDH and are shown in the bar graphs. $P < 0.05$ was considered statistically significant and is indicated by "*", $P < 0.01$ was regarded as significant and is indicated by "**", and $P < 0.001$ was regarded as highly significant and is indicated by "***".

were measured by Western blotting. As shown in Figure 1A, PA markedly induced ERO1α expression in a dose-dependent manner. To further explore the effects of PA on ERO1α expression, the expression levels of ERO1α and GAPDHin PA-treated MLTC-1 cells were measured again at different time points. As indicated in Figure 1B, PA (400 μM) also significantly enhanced ERO1α expression in a time-dependent manner.

PA reduces cell viability, inhibits testosterone secretion, and induces ER stress in MLTC-1 cells

As indicated in Figure 2A–E, PA (100–400 μM) significantly decreased cell viability and markedly promoted the protein expression of GRP78 and CHOP, which was similar to the findings of a previous study.[2] The results suggested that PA-induced inhibition of testosterone secretion was likely associated with ER stress in MLTC-1 cells.

Lentivirus-mediated ERO1α knockdown in MLTC-1 cells is effective

After the construction of the lentivectors, PCR was conducted to amplify the inserted shRNAs. As shown in Figure 3A, the DNA bands from each recombinant lentivector were all slightly (approximately 60 base pairs) higher than those of the mock lentivector, indicating that the lentivectors expressing shRNAs targeting ERO1α were successfully constructed. After infection with the different lentiviruses, the mRNA and protein expression levels of ERO1α were measured by using real-time quantitative RT-PCR and Western blotting, respectively. As indicated in Figure 3B, the infection efficiency was greater than 70%, which was sufficient for the present experiment. The RT-PCR and Western blot results revealed that knockdown of ERO1α in the shERO1α-2 group significantly decreased the expression of ERO1α

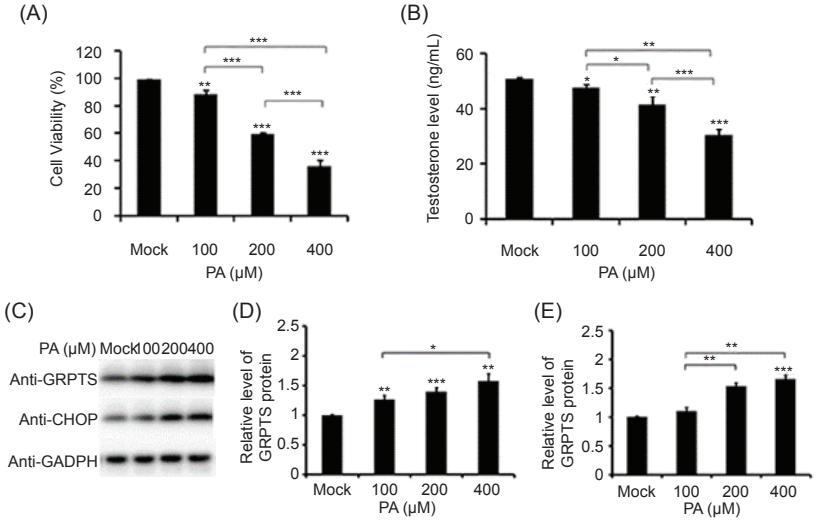

FIGURE 2 Palmitic acid reduces cell viability, inhibits testosterone secretion, and induces ER stress in MLTC-1 cells. (A) Cell viability was measured by CCK-8 assays. (B) Testosterone levels were determined via ELISA. (C, D) Protein levels of GRP78 in MLTC-1 cells treated with PA (100–400 μM) for 24 h. (C, E) Protein levels of CHOP in MLTC-1 cells treated with PA (100–400 μM) for24 h. The data are presented as the mean ± SD.P < 0.05 was considered statistically significant and is indicated by "*", P < 0.01 was regarded as significant and is indicated by "**", and P < 0.001 was regarded as highly significant and is indicated by "***".

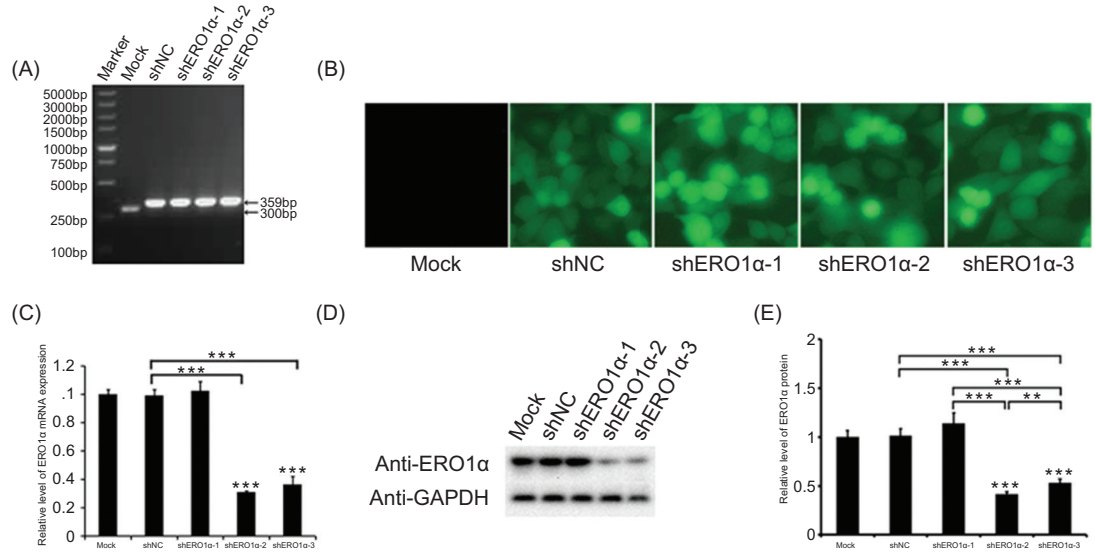

FIGURE 3 Effects of lentivirus-mediated ERO1α knockdown in MLTC-1 cells. (A) PCR analysis of recombinant lentiviral plasmids that interfere with ERO1α. (B) Fluorescence detection of MLTC-1 cells infected with each recombinant lentivirus construct. (C) ERO1α mRNA levels were measured viaRT-PCR. (D, E) Protein levels of ERO1α in MLTC-1 cells were measured by Western blotting. The data are presented as the mean ± SD. $P < 0.01$ was regarded as significant and is indicated by "**", and $P < 0.001$ was regarded as highly significant and is indicated by "***".

mRNA (Figure 3C) and protein (Figure 3D, E) compared with that of the shNC, shERO1α-2, and shERO1α-3 groups.

ERO1α knockdown ameliorates PA-induced cytotoxicity, ER stress, and inhibition of testosterone secretion in MLTC-1 cells

Murine Leydig tumor cell line 1 cells were treated with PA (400 μM) and infected with lentiviruses expressing shERO1α-2, and the cell viability, testosterone level, and expression of ER stress marker proteins (GRP78 and CHOP) were evaluated by CCK-8, ELISA, and Western blot analyses, respectively. As shown in Figure 4A–B, the PA-induced decreases in cell viability and testosterone secretion were partly restored in response to knockdown of ERO1α in MLTC-1 cells. A similar recovery was also observed in GRP78 and CHOP protein expression (Figure 4C–E).

ERO1α knockdown ameliorates TG-induced cytotoxicity, ER stress, and inhibition of testosterone secretion in MLTC-1 cells

TG, an ER stress inducer, is usually utilized to activate ER stress during various experiments. Here, we usedTG as a substitute for PA to replicate the cell microenvironment in which PA is added. As shown in Figure 5A–E, knockdown of ERO1α antagonized the TG-induced decreases in cell viability and testosterone secretion and the upregulation of GRP78 and CHOP protein expression.

4-PBA attenuates PA-induced inhibition of testosterone secretion in MLTC-1 cells

4-Phenylbutyric acid acts as an ER stress inhibitor by aiding in protein folding at the molecular level and preventing misfolded protein aggregation. A previous study showed that 4-PBA could attenuate PA-induced cytotoxicity and ER

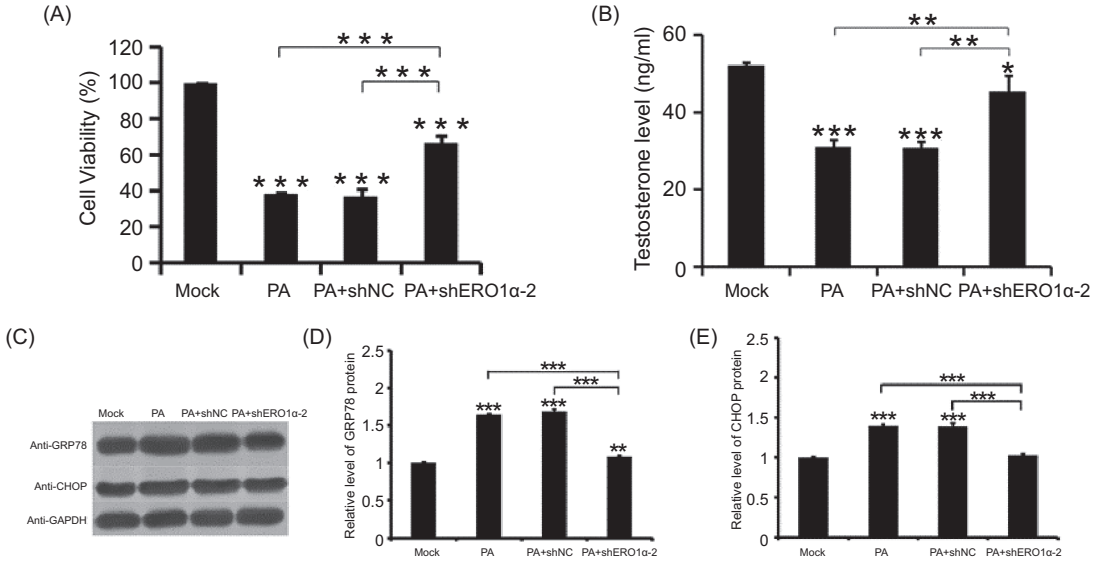

FIGURE 4 ERO1α knock down attenuates PA-induced ER stress and the inhibition of cell viability and testosterone secretion in MLTC-1 cells. (A) Cell viability was determined by CCK-8 assays. (B) Testosterone levels were detected by ELISA. (C, D) Western blot showing GRP78 expression and its relative expression level normalized to GAPDH are depicted. (C, E) Western blot showing CHOP expression and its relative expression level normalized to GAPDH are presented. The data are presented as the mean ± SD of three independent experiments. $P < 0.05$ was considered statistically significant and is indicated by "*", $P < 0.01$ was regarded as significant and is indicated by "**", and $P < 0.001$ was regarded as highly significant and is indicated by "***".

stress in MLTC-1 cells,[2] but the effect of 4-PBA on PA-induced inhibition of testosterone secretion in MLTC-1 cells is still unclear. Here, we demonstrated that 4-PBA could significantly attenuate PA-induced inhibition of testosterone secretion in MLTC-1 cells (Figure 6).

DISCUSSION

Since the occurrence of reduced testosterone levels, increased E_2 levels and reduced fertility associated with increased weight in men has been recognized in recent years,[18–20] the underlying mechanisms of obesity-induced male infertility have drawn public attention,[21] and many studies focusing on finding promising therapeutic agents to treat obesity-related male infertility have been reported.[2,22–24] A previous study reported that PA-induced cell apoptosis via the activation of ER stress in MLTC-1 cells and inhibiting ER stress protected against PA-induced Leydig cell injury and testosterone decrease, suggesting that PA is one of the causes of obesity-related male infertility.[2]

Endoplasmic reticulum oxidoreductin-1 alpha plays a key role in maintaining redox homeostasis in the ER and is related to hormone secretion and ER stress.[25–27] In this study, we explored the role of ERO1α in PA-mediated inhibition of testosterone secretion. The results showed that PA induced the expression of ERO1α in a dose- and time-dependent manner, enhanced the protein expression of ER stressmarkers, and reduced cell viability and testosterone production, suggesting that PA-induced inhibition of testosterone secretion may be associated with ER stress. To further explore the relationship between ER stress

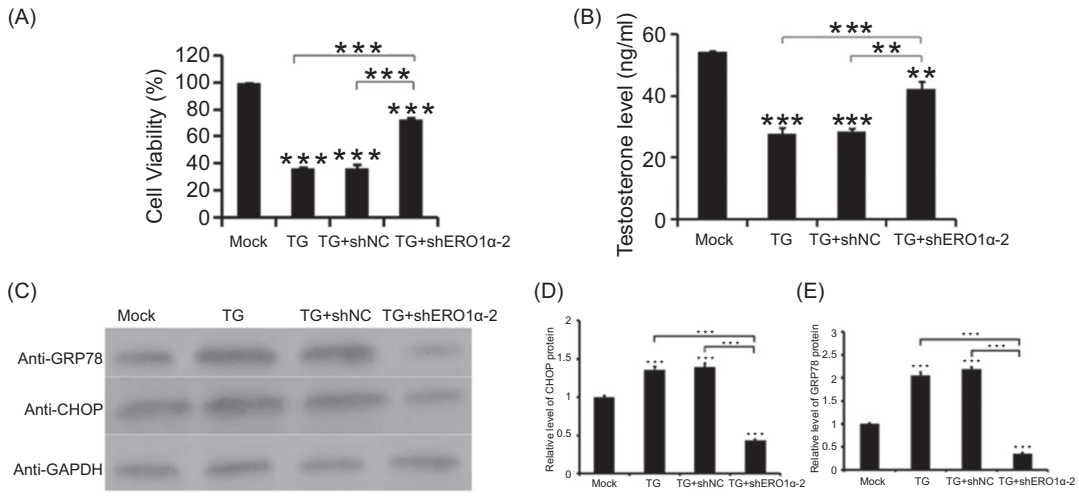

FIGURE 5 ERO1α knockdown attenuates TG-induced ER stress and the inhibition of cell viability and testosterone secretion in MLTC-1 cells. (A) Cell viability was analyzed using the CCK-8 assay. (B) Testosterone levels were measured by ELISA. (C, E) The relative expression level of GRP78 to GAPDH is presented. (C, D) The relative expression level of CHOP to GAPDH is shown. The data are presented as the mean ± SD of three independent experiments. $P < 0.01$ was regarded as significant and is indicated by "**", and $P < 0.001$ was regarded as highly significant and is indicated by "***".

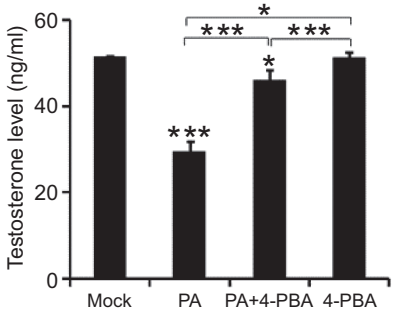

FIGURE 6 4-Phenylbutyric acid attenuates the PA-induced inhibition of testosterone secretion in MLTC-1 cells. The data are presented as the mean ± SD. $P < 0.05$ was considered statistically significant and is indicated by "*", and $P < 0.001$ was regarded as as highly significant and is indicated by "***".

and testosterone secretion, the ER stress inducer TG and inhibitor 4-PBA were used as positive and negative controls, respectively, and the results showed that testosterone secretion in the TG-treated group was significantly reduced compared with that in the mock group, while the PA-induced decrease in testosterone secretion was markedly attenuated in the presence of 4-PBA. These results suggested that PA-induced inhibition of testosterone secretion occurred through ER stress in MLTC-1 cells.

Considering the important role of ERO1α in the formation of disulfide bonds within secreted proteins at the post translational level, the results in Figure 1 show that PA can induce the expression of ERO1α in MLTC-1 cells; therefore, we hypothesize that ERO1α may have some impact on testosterone secretion. To confirm this hypothesis, we constructed recombinant lentiviruses to interfere with the ERO1α protein expression and infected MLTC-1 cells that had been treated with PA; the results indicated that cell viability and testosterone secretionin PA-treated MLTC-1 cells were significantly improved, while the protein expression levels of GRP78 and CHOP were markedly decreased, suggesting that knockdown of ERO1α antagonized PA-induced inhibition of cell survival and testosterone

secretion and activation of ER stress. To further corroborate these results, TG was used to replace PA as the inducer of ER stress, which is a typical manifestation of PA-induced cytotoxicity. Consistently, the results demonstrated that the TG-induced decreases in cell viability and testosterone secretion were significantly abrogated, while the TG-induced upregulation of GRP78 and CHOP protein expression in MLTC-1 cellswas significantly down regulated, suggesting that knockdown of ERO1α could significantly attenuate TG-induced inhibition of cell viability and testosterone secretion and activation of ER stress.

These data suggest that ERO1α protects against PA-induced inhibition of testosterone secretion by inhibiting ER stress. Hence, ERO1α may be a potential target gene to optimize and rogenic hormonal pathways, which is the goal of medical management for the treatment of obesity-associated male infertility.

CONCLUSIONS

In conclusion, ERO1α knockdown attenuates PA-induced inhibition of testosterone secretion by inhibiting ER stress in MLTC-1 cells. However, the details of the underlying mechanism by which ERO1α affects the secretion of testosterone still need further investigation.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This research has been submitted to and approved by the review board of the College of Biology & Pharmacy, Yulin Normal University.

CONFLICT OF INTEREST

The authors declare no competing interests.

FUNDING

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REFERENCES

1. Bieniek JM, Kashanian JA, Deibert CM, Grober ED, Lo KC, Brannigan RE, et al. Influence of increasing body mass index on semen and reproductive hormonal parameters in a multi-institutional cohort of subfertile men. Fertil Steril. 2016; 106:1070–5. https://doi.org/10.1016/j.fertnstert.2016.06.041
2. Chen Z, Wen D,Wang F, Wang CB, Yang L. Curcumin protects against palmitic acid-induced apoptosis via the inhibition of endoplasmic reticulum stress in testicular Leydig cells. Reprod Biol Endocrin. 2019; 17:71–80. https://doi.org/10.1186/s12958-019-0517-4
3. Haider SG. Cell biology of Leydig cells in the testis. Int Rev Cytol. 2014;233:181–241. https://doi.org/10.1016/S0074-7696(04)33005-6
4. Park SJ, Kim TS, Park CK, Lee SH, Lee DS. hCG-induced endoplasmic reticulum stress triggers apoptosis and reduces steroidogenic enzyme expression through activating transcription factor 6 in Leydig cells of the testis. J Mol Endocrinol. 2013;50(2):151–66. https://doi.org/10.1530/JME-12-0195
5. Yang L, Lei L, Zhao Q, Gong Y, Guan GP, Huang SX. C-type natriuretic peptide/natriuretic peptide receptor 2 is involved in cell proliferation and testosterone production in mouse Leydig cells. World J Mens Health. 2019;37(2): 186–98. https://doi.org/10.5534/wjmh.180041
6. Lu ZH, Mu YM, Wang BA, Li XL, Lu JM, Li JY, et al. Saturated free fatty acids, palmitic acid and stearic acid, induce apoptosis by stimulation of ceramide generation in rat testicular Leydig cell. Biochem Biophys Res Co. 2003;303(4):1002–7. https://doi.org/10.1016/s0006-291x(03)00449-2
7. Kim JH, Park SJ, Kim TS, Park HJ, Park J, Kim BY, et al. Testicular hyperthermia induces unfolded

protein response signaling activation in spermatocyte.Biochem Bioph Res Co. 2013;434(4):861–6. https://doi.org/10.1016/j.bbrc.2013.04.032
8. Rashid HO, Yadav RK, Kim HR, Chae HJ. ER stress: Autophagy induction, inhibition and selection. Autophagy. 2015;11(11):1956–77. https://doi.org/10.1080/15548627.2015.1091141
9. Sovolyova N, Healy S, Samali A, Logue SE. Stressed to death – Mechanisms of ER stress-induced cell death. Biol Chem. 2014;395(1):1–13. https://doi.org/10.1515/hsz-2013-0174
10. Cnop M, Foufelle F, Velloso LA. Endoplasmic reticulum stress, obesity and diabetes. Trends Mol Med. 2012;18(1):59–68. https://doi.org/10.1016/j.molmed.2011.07.010
11. Guzel E, Arlier S, Guzeloglu-Kayisli O, Tabak M, Ekiz T, Semerci N, et al. Endoplasmic reticulum stress and homeostasis in reproductive physiology and pathology. Int J Mol Sci. 2017;18(4):792. https://doi.org/10.3390/ijms18040792
12. Okumura M, Kadokura H, Hashimoto S, Yutani K, Kanemura S, Hikima T, et al. Inhibition of the functional interplay between endoplasmic reticulum (ER) oxidoreductin-1alpha (Ero1alpha) and protein-disulfide isomerase (PDI) by the endocrine disruptor bisphenol A. J Biol Chem. 2014;289(39):27004–18. https://doi.org/10.1074/jbc.m114.564104
13. Lv QZ, Guo KK, Zhang GF, Zhang YM. The ORF4 protein of porcine circovirus type 2 (PCV2) antagonizes apoptosis by stabilizing the concentration of ferritin heavy chain (FHC) through physical interaction. J Gen Virol. 2016;97(7):1636–46. https://doi.org/10.1099/jgv.0.000472
14. Lv QZ, Zhuo YL, Zhang YW, Deng JH, Tan XM, Lin Q. Construction of recombinant lentivirus vectors with porcine Hsp40 gene for over expression and interference. Chin Vet Sci. 2019;49(1):76–84. https://doi.10.16656/j.issn.1673-4696.2019.0220
15. Lv QZ, Guo KK, Xu H, Wang T, Zhang YM. Identification of putative ORF5 protein of porcine circovirus type 2 and functional analysis of GFP-fused ORF5 protein. PLoS One.2015;10(6):e0127859. https://doi.org/10.1371/journal.pone.0127859
16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta C(T)) method. Methods. 2001;25(4):402–8. http://dx.doi.org/10.1006/meth.2001.1262
17. Zhang CC, Kang KK, Ning PB, Peng YX, Lin Z, Cui HJ, et al. Heat shock protein 70 is associated with CSFV NS5A protein and enhances viral RNA replication. Virology. 2015;482:9–18. http://dx.doi.org/10.1016/j.virol.2015.02.014
18. Aggerholm AS, Thulstrup AM, Toft G, Ramlau-Hansen CH, Bonde JP . Is overweight a risk factor for reduced semen quality and altered serum sex hormone profile? Fertil Steril. 2008;90(3):619–26. https://doi.org/10.1016/j.fertnstert.2007.07.1292
19. Ramlau-Hansen CH, Hansen M, Jensen CR, Olsen J, Bonde JP, Thulstrup AM. Semen quality and reproductive hormones according to birthweight and body mass index in childhood and adult life: Two decades of follow-up. Fertil Steril. 2010;94(2):610–18. https://doi.org/10.1016/j.fertnstert.2009.01.142
20. Paasch U, Grunewald S, Kratzsch J, Glander HJ. Obesity and age affect male fertility potential. Fertil Steril. 2010;94(7):2898–901. https://doi.org/10.1016/j.fertnstert.2010.06.047
21. Katib A. Mechanisms linking obesity to male infertility. Cent European J Urol. 2015;68(1):79–85. https://doi.org/10.5173/ceju.2015.01.435
22. Kim ED, McCullough A, Kaminetsky J. Oral enclomiphene citrate raises testosterone and preserves sperm counts in obese hypogonadal men, unlike topical testosterone: Restoration instead of replacement. BJU Int. 2016;117(4):677–85. https://doi.org/10.1111/bju.13337
23. Roth MY, Amory JK, Page ST. Treatment of male infertility secondary to morbid obesity. Nat Clin Pract Endoc. 2008;4(7):415–19. https://doi.org/10.1038/ncpendmet0844
24. Raman JD, Schlegel PN. Aromatase inhibitors for male infertility. J Urol. 2002;167(2):624–9. https://doi.org/10.1016/s0022-5347(01)69099-2
25. Kajiwara T, Tanaka T, Kukita K, Kutomi G, Tamura Y. Hypoxia augments MHC class I antigen presentation via facilitation of ERO1-alpha-mediated oxidative folding in murine tumor cells. Eur J Immunol. 2016;46(12):2842–51. https://doi.org/10.1002/eji.201646525

26. Lee S, Lee E, Ko E, Ham M, Lee HM, Kim ES, et al. Tumor-associated macrophages secrete CCL2 and induce the invasive phenotype of human breast epithelial cells through upregulation of ERO1-alpha and MMP-9. Cancer Lett. 2018;437:25–34. https://doi.org/10.1016/j.canlet.2018.08.025

27. Lai L, Liu Y, Liu YY, Zhang N, Wu D. Role of endoplasmic reticulum oxidase 1α in H9C2 cardiomyocytes following hypoxia/reoxygenation injury. Mol Med Rep. 2020;22:1420–8. https://doi.org/10.3892/mmr.2020.11217