Article

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The Impact of Dibutyl Phthalate on Insulin Signaling in Human Skeletal Muscle Cells

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Background: In China, the environmental concern of Dibutyl Phthalate (DBP) exposure significantly endangers human health by inducing insulin resistance (IR). Skeletal muscle tissue plays a critical role in this process. However, the precise molecular mechanisms through which DBP interferes with the insulin signaling pathway remain to be fully elucidated. This study aims to explore the molecular mechanisms by which DBP induces IR in skeletal muscle, focusing on the phosphatidylinositol 3-kinase (PI3K)-serine/threonine kinase (AKT)-glucose transporter 4 (GLUT4) signaling pathway.

Methods: To investigate the molecular mechanisms underlying DBP-induced IR, an experimental study was established on a human skeletal muscle cell line (HSkMC). Expression levels of mRNA and proteins associated with key signaling genes within the insulin receptor (INSR)-insulin receptor substrate (IRS)-PI3K-AKT-GLUT4 pathway were assessed using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot techniques. Additionally, this study explored the effects of DBP alone and in combination with a PI3K inhibitor (BKM120) or phosphatase and tensin homolog (PTEN) overexpression lentivirus on these signaling components.

Results: Results from this study demonstrated that DBP exposure significantly decreased mRNA levels of *INSR*, *IRS1*, *PI3K*, *AKT2*, and *GLUT4* in HSkMC cells compared to untreated control cells. This reduction was exacerbated when DBP was combined with BKM120 or PTEN overexpression lentivirus, suggesting a synergistic effect. Furthermore, DBP treatment reduced the expression and phosphorylation of AKT2, indicating a disruption in the insulin signaling pathway.

Conclusions: This study elucidates a molecular mechanism by which DBP induces IR in skeletal muscle cells, primarily through the deregulation of the PI3K-dependent insulin signaling pathway. These insights enhance comprehension of the pathophysiological changes associated with IR caused by environmental pollutants like DBP, potentially guiding future strategies for prevention and intervention.

Keywords: Dibutyl Phthalate; skeletal muscle; insulin resistance; glucose transporters; insulin receptor substrate

Background

Phthalates (PAEs) are common environmental endocrine disruptors and are linked to a spectrum of health concerns including reproductive, neurologic, immunological disorders, and metabolic ailments [1]. Among these, Dibutyl Phthalate (DBP) is one of several compounds that raise concerns due to its potential toxicity, attributed to its small molecular weight and ubiquitous distribution in the environment. A survey conducted in China found that human exposure to DBP through dietary routes, indoor air, and dust is much higher than exposure to other types of PAEs [2]. In addition, exposure to DBP is an environmental problem that poses health threats, as evidenced by studies indicating its disruptive impact on male gonadal differentiation in frogs [3]. Therefore, it is necessary to conduct comprehensive research on the hazards of DBP to human health, alongside revealing the related molecular mechanisms.

Numerous clinical studies have shown that exposure to PAEs is closely associated with the development of insulin resistance (IR) [4,5], several metabolic syndromes, such as obesity, type II diabetes, polycystic ovary syndrome, several malignancies, and neurodegenerative diseases, share a common pathophysiological basis [6]. One meta-analysis identified a positive association between DBP exposure and IR [5]. Moreover, prospective cohort studies have demonstrated that childhood DBP exposure increases the likelihood of IR in adulthood [7]. Most studies have focused on the link between DBP exposure and IR. However, few studies have explored the molecular biological mechanisms involved in IR caused by DBP exposure. Consequently, further experiments are needed to explore the direct impact of DBP on IR and to uncover the related molecular mechanisms.

IR refers to reduced insulin sensitivity, resulting in resistance to insulin action [8]. IR can be categorized based

on its target organs, such as the liver, skeletal muscle, and adipose tissue [9]. Abnormalities at the molecular level cause IR, affecting insulin, insulin receptors, post-receptor signal transduction, and gene expression regulation [9,10].

Insulin activates two primary intracellular signaling pathways, namely the mitogen-activated protein kinases (MAPKs) pathway and the phosphatidylinositol 3-kinase (PI3K)/-serine/threonine kinase (AKT) signaling pathway. Of these, the PI3K/AKT signaling pathway plays an important role in the development of type 2 diabetes (T2DM). Insulin binds to its receptors to activate PI3K, which results in AKT phosphorylation. Subsequently, glycogen synthase kinase (GSK3β) is phosphorylated, inhibiting glycogenolysis and consequently lowering blood glucose levels. Furthermore, activated AKT promotes the transfer of glucose transporter 2 (GLUT2) and glucose transporter 4 (GLUT4) from the cytoplasm to the cell membrane, facilitating glucose uptake and thus lowering blood glucose. Adipocytes and muscle cells mainly take up glucose through GLUT4. Disruption of the PI3K/AKT pathway inhibits insulin-stimulated GLUT4 translocation and glucose transport, promoting IR [11]. The insulin receptor substrate (IRS) is a crucial molecule for insulin signaling. When its tyrosine residues are phosphorylated, they can form PI3K conjugation sites, which in turn bind to and activate PI3K. The IRS family comprises four members, with IRS-1 and IRS-2 being pivotal in insulin signaling [12]. Abnormal function or expression of signaling molecules can cause IR [11,13].

Therefore, to investigate whether DBP causes IR in skeletal muscle tissue by affecting the expression of the aforementioned signaling molecules, this study examined the mRNA and protein expression of the above molecules in the human skeletal muscle cell line (HSkMC) treated with DBP. In addition, to investigate whether PI3K/AKT activation directly mediates the effect of DBP on skeletal muscle cell IR, stable overexpression of phosphatase and tensin homolog (PTEN) was achieved in HSkMC cells via lentivirus, and the PI3K inhibitor BKMI20 was used for related experiments. This study aimed to reveal the molecular mechanisms of IR caused by DBP exposure and to provide an appropriate reference for its management.

Materials and Methods

Culture of Human Skeletal Muscle Cell Line HSkMC Cells

The human skeletal muscle cell line (HSkMC) (150-05A, Sigma–Aldrich, St Louis, MO, USA) underwent mycoplasma testing and was identified by Short Tandem Repeat (STR) analysis. HSkMC cells were thawed and centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and the cells were resuspended in high-glucose DMEM medium (C3132-0500, VivaCell, Shanghai, China) (containing 10% fetal bovine serum, 100,000 U/L penicillin, 100 mg/L streptomycin). After, cells were transferred to 6 cm cell culture dishes and cultured at 37 °C with 5% CO_2 and saturated humidity. The cells were passaged once every 2–3 days, approximately 2 or 3 times weekly, based on cell growth. Subsequent experiments were conducted when the cells reached the logarithmic phase, with observation facilitated using an ordinary inverted microscope (IX73, Olympus, Tokyo, Japan).

Construction of PTEN-Overexpressed Lentivirus

The forward and reverse primers for amplifying human PTEN (NM_000314, Gikech Gene Technology Co., Ltd., Beijing, China) coding sequence (CDS) were designed based on the National Center for Biotechnology Information (NCBI) database, and the AgeI cleavage site was added at the 5′ end of the primers. The CDS region of PTEN was amplified via polymerase chain reaction (PCR). Following PCR, agarose gel electrophoresis was employed to analyze the reaction results, with gels exhibiting bands of similar size to the target gene being excised and recovered for further processing. Concurrently, AgeI enzyme digestion was performed on the overexpressed vector GV358 (GCNL0149692, Shanghai GeneChem Co., Ltd., Shanghai, China), while the PCR product recovered from gel extraction. After digestion, the enzyme-digested PCR product was mixed with the GV358 linear vector and incubated at 37 °C for 30 min under the influence of recombinase ExnaseTM II (11791020, Invitrogen™, Carlsbad, CA, USA). Upon recombination of the PCR product with GV358, a circular recombinant plasmid was formed. The resultant recombinant plasmid was then transformed into competent cells, and monoclonal colonies were selected after 12–16 hours of incubation in culture plates supplemented with ampicillin for amplification.

The recombinant vector underwent sequencing, and monoclonal cultures with accurate sequencing were expanded for plasmid preparation. The GV358 vector plasmid containing PTEN-overexpressed sequence and two other virus-packing helper plasmids, pHelper1.0 and pHelper2.0 plasmid, were co-transfected into mycoplasma-negative HT293 cell lines (BIOCCBC2591, Zrbiorise, Shanghai, China), confirmed via Short Tandem Repeat (STR) analysis. The virus was harvested for 48–72 hours, tested for virus quality and titer, and stored in a refrigerator at –80 °C.

Drug Treatment of HSkMC Cells

HSkMC cells transfected with or without PTEN overexpression lentivirus were treated with 100 μM DBP (GBW(E)100481, Huinuo, Shanghai, China) with or without 1 μM concentration of PI3K inhibitor BKM120 (944396-07-0, ACMEC Biochemical Co., Ltd., Shanghai, China), while the control group was treated with an equal volume of dimethyl sulfoxide (DMSO) (S33102, Invitro-

Table 1. Primer sequences used for qRT-PCR.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
INSR	AAAACGAGGCCCGAAGATTTC	GAGCCCATAGACCCGGAAG
PTEN	TTTGAAGACCATAACCCACCAC	ATTACACCAGTTCGTCCCTTTC
IRS-1	ACAAACGCTTCTTCGTACTGC	AGTCAGCCCGCTTGTTGATG
AKT2	GGTGCAGAGATTGTCTCGGC	GCCCGGCCATAGTCATTGTC
GLUT4	GACCAGCACTCCCAAGGTTAC	CTGGCCCGGAAGACATCTG
PI3K	CTGCCTGCGACAGATGAGTG	TCCGATTACCAAGTGCTCTTTC
GAPDH	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG

qRT-PCR, quantitative real-time polymerase chain reaction; *IRS*, insulin receptor substrate; *PI3K*, phosphatidylinositol 3-kinase; *GLUT4*, glucose transporter 4; *INSR*, insulin receptor; AKT, serine/threonine kinase; *PTEN*, phosphatase and tensin homolog; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

gen™, Carlsbad, CA, USA). After 24 hours, cells were collected for subsequent quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analyses.

Construction of HSkMC Cells with Stable Overexpression of PTEN

HSkMC cells were inoculated in a 6-well plate at a density of 1×10^5/well. Following 24 hours of incubation, during which cells reached the logarithmic growth phase with a density of approximately 2×10^5 cells per well, the original medium was substituted with 2 mL of fresh medium supplemented with 6 µg/mL polybrene. Subsequently, an appropriate volume of viral suspension (MOI = 10) was added. After incubating at 37 °C for 12 h, the medium was replaced with the virus-free medium. The transfection efficiency was observed by the green fluorescence of the GFP antibody (YB48671, SAB Signalway Antibody) under an inverted fluorescence microscope (IX51, Olympus, Tokyo, Japan) after continued culture for 36 h. When the cell growth reached 80%, 1 µg/mL puromycin (A1113803, Solarbio, Beijing, China) was added to identify stably transfected cells.

qRT-PCR Experiment

Cells were lysed with TRIzol reagent (10296028, Invitrogen™, Carlsbad, CA, USA), and total RNA was extracted and reverse-transcribed into cDNA. Subsequently, qRT-PCR was performed using the SYBR Fluorescent Dye Kit (JBS-PCR-378, Enzo Life Sciences, Beijing, China) on an ABIQ6FLEX fluorescence quantitative PCR instrument (Applied Biosystems Pro, Thermo Fisher Scientific, Waltham, MA, USA). The qRT-PCR amplification conditions were as follows: 95 °C 30 s pre-denaturation 95 °C 15 s, 60 °C 30 s, a total of 40 cycles of PCR reaction. The primer sequences used in this study are shown in Table 1. The $\Delta\Delta$Ct value method was used to quantify the results using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. Each experiment was independently repeated, with each sample assessed in triplicate.

Western Blot

After various treatments, HSKMC cells were harvested, and cell lysates containing phenylmethylsulfonyl fluoride (PMSF) (RIPA:PMSF = 100:1) (ST507-10, Beyotime, Shanghai, China) were added to extract the total protein. The total protein concentration was quantified using the BCA method. After, SDS polyacrylamide gel electrophoresis was performed, and the proteins were transferred onto a PVDF membrane (88585, Thermo Fisher Scientific, Waltham, MA, USA). The membrane was then blocked with 5% BSA at room temperature for one hour. After being washed, the bands were incubated with primary antibodies, including PTEN rabbit monoclonal antibody (9559, Cell Signaling Technology, Danvers, MA, USA, 1:1000 dilution), β-actin mouse monoclonal antibody (A5441, Sigma-Aldrich, Bavaria, German, 1:5000 dilution), INSR rabbit polyclonal antibody (ab134115, Abcam, Eugene, OR, USA, 1:1000 dilution), IRS-1 rabbit polyclonal antibody (sc-559, Santa Cruz Biotechnology, Dallas, TX, USA, 1:1000 dilution), PI3K rabbit monoclonal antibody (4257, Cell Signaling Technology, Danvers, MA, USA, 1:1000 dilution), AKT2 rabbit monoclonal antibody (3063, Cell Signaling Technology, Danvers, MA, USA, 1:1000 dilution), p-AKT2 rabbit monoclonal antibody (4060, Cell Signaling Technology, Danvers, MA, USA, 1:1000 dilution), and GLUT4 rabbit polyclonal antibody (ab654, Abcam, Eugene, OR, USA, 1:1000 dilution). Following the primary antibody incubation, the membrane underwent three washes with TBST (5 min/time), followed by incubation with an HRP-labelled goat anti-rabbit IgG secondary antibody (catalog #ab205718, diluted 1:2000, Abcam, Eugene, OR, USA) at room temperature for one hour. After being washed three times with TBST (5 min/time), an ECL kit (PE0010, Solarbio, Beijing, China) was used for color development and exposure. The optical density of the target protein was corrected using β-actin and analyzed with Image J software (version 1.54h, National Institutes of Health, Bethesda, MD, USA).

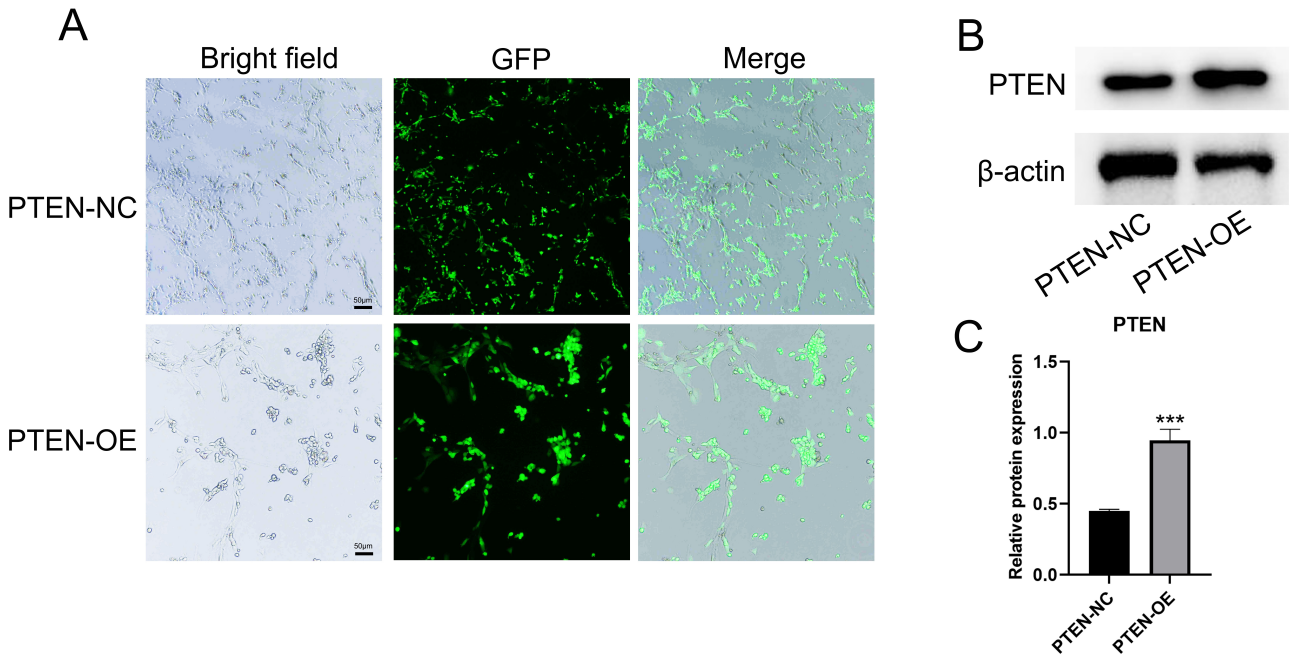

Fig. 1. Validation of the lentiviral vector-mediated PTEN-overexpressing human skeletal muscle cell line (HSkMC) cells. (A) The lentiviral transfection rate was monitored by GFP fluorescence under an inverted fluorescence microscope (all figures use the same scale bar, scale bar = 50 μm). (B) Detection of PTEN expression in lentivirus-transfected HSkMC cells. (C) Semiquantification of relative protein expression of PTEN in lentivirus-transfected HSkMC cells (***$p < 0.001$, n = 3).

Statistical Analysis

Statistical analysis was performed using SPSS 22.0 software (IBM Corp., Chicago, IL, USA). For datasets conforming to a normal distribution, the data were expressed as means ± standard deviation (SD). Student's *t*-test was utilized for two-group comparisons, while one-way Analysis of Variance (ANOVA) was applied for multiple-group comparisons. The Least Significant Difference (LSD) test was administered as a post hoc method for conducting multiple comparisons within the framework of one-way ANOVA. Conversely, for datasets deviating from a normal distribution, the rank sum test was employed for analysis. Statistical significance was defined as $p < 0.05$.

Results

In this study, HSkMC cells were engineered to stably overexpress PTEN. After screening, cells were photographed and imaged under an inverted fluorescence microscope, as shown in Fig. 1. The positive rate of cells with GFP exceeded 90% (Fig. 1A). Additionally, Western blot analysis revealed a significantly elevated relative PTEN expression in the overexpression group (PTEN-OE) compared to the control group (PTEN-NC), consisting of cells transfected with empty vectors (Fig. 1B,C).

Effect of DBP Treatment on mRNA Expression of Insulin-Related Signaling Molecules in HSkMC Cells

Using qRT-PCR, the effects of DBP treatment on the expression of *INSR*, *IRS-1*, *PI3K*, *AKT2*, *GLUT4*, and *PTEN* mRNAs in HSkMC cells were investigated. The results indicated that the mRNA levels of *INSR*, *IRS-1*, *PI3K*, *AKT2*, and *GLUT4* in HSkMC cells were all downregulated to different degrees after 15 minutes of 100 μM DBP treatment (DBP group) compared with the control group, and the differences were statistically significant ($p < 0.01$). However, the mRNA expression levels of *PI3K*, *AKT2*, and *GLUT4* were further reduced when 100 μM DBP was concurrently treated with 1 μM PI3K inhibitor BKMI20 (DBP + BKMI20 group) or transfected with *PTEN* overexpressing lentivirus (PTEN-OE + DBP group) ($p < 0.001$). Neither DBP treatment nor DBP + BKMI20 co-treatment affected the *PTEN* mRNA expression levels (Fig. 2A–F). However, *PTEN* mRNA expression was significantly increased in the cells transfected with lentivirus overexpression of *PTEN*, suggesting the effectiveness of transfection ($p < 0.0001$).

Effect of DBP Treatment on the Expression of Insulin-Related Signaling Proteins in HSkMC Cells

To demonstrate the regulatory effect of DBP on the expression of the aforementioned molecules in HSkMC cells, protein levels were also examined via Western blot analysis. The results showed that 100 μM DBP treatment significantly reduced the protein expression of PI3K,

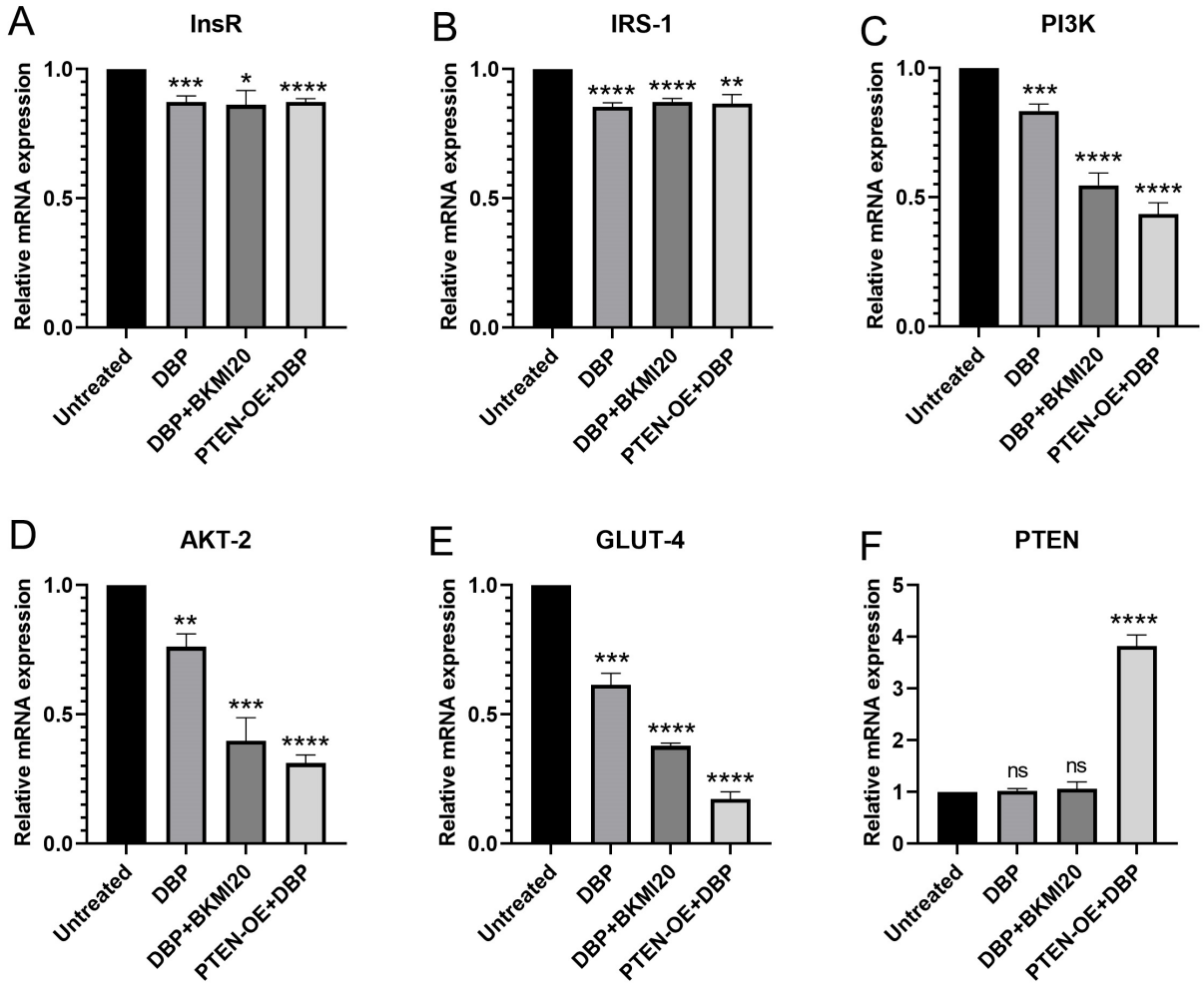

Fig. 2. qRT-PCR Analysis Reveals mRNA Expression Changes in HSkMC Cells Following Various Treatments. (A–F) qRT-PCR analysis for *INSR*, *IRS-1*, *PI3K*, *AKT2*, *GLUT4*, and *PTEN* genes. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns represents non-significance compared with untreated group (n = 3).

AKT2, phosphorylated AKT2, and GLUT4, with statistically significant differences observed ($p < 0.05$). Furthermore, concurrent treatment of DBP with BKMI20 or transfection with PTEN-overexpressing lentivirus led to a further reduction in the expression of these proteins, with statistically significant differences noted ($p < 0.05$). Although there was no statistically significant difference, the results also demonstrated that DBP treatment reduced the expression ratio of phosphorylated AKT2 to AKT2 (p-AKT2/AKT2), and DBP co-treatment with BKMI20 further reduced the ratio ($p > 0.05$). When DBP treatment was combined with PTEN overexpressed lentivirus vector, the p-AKT2/AKT2 ratio was further reduced ($p < 0.05$). Additionally, these treatments slightly reduced the protein expression of cellular INSR and IRS-1, although these differences were not statistically significant. Notably, these treatments had minimal impact on PTEN protein levels in cells, with significant upregulation of PTEN protein observed only in cells transfected with PTEN overexpressing lentivirus ($p < 0.001$). These results suggest that DBP treatment presents an overall inhibitory effect on INSR-IRS-1-PI3K-AKT-GLUT4 signaling in human skeletal muscle cell lines (Fig. 3).

Discussion

Skeletal muscle, the body's largest tissue, metabolizes glucose and fatty acids for energy. Moreover, skeletal muscle plays a crucial role in IR, characterized by diminished glucose uptake and utilization following meals. IR in skeletal muscle is linked to complex cellular and molecular mechanisms, including lipid mediator accumulation, mitochondrial dysfunction, and the activation of stress-responsive c-Jun-N-terminal kinase (c-JNK) and inflammation pathways [14]. DEHP and MEHP, two prevalent plasticizers in consumer products, have been found to cause impaired glucose tolerance and IR in skeletal muscle [15–17]. Additionally, DBP, a toxic component found in

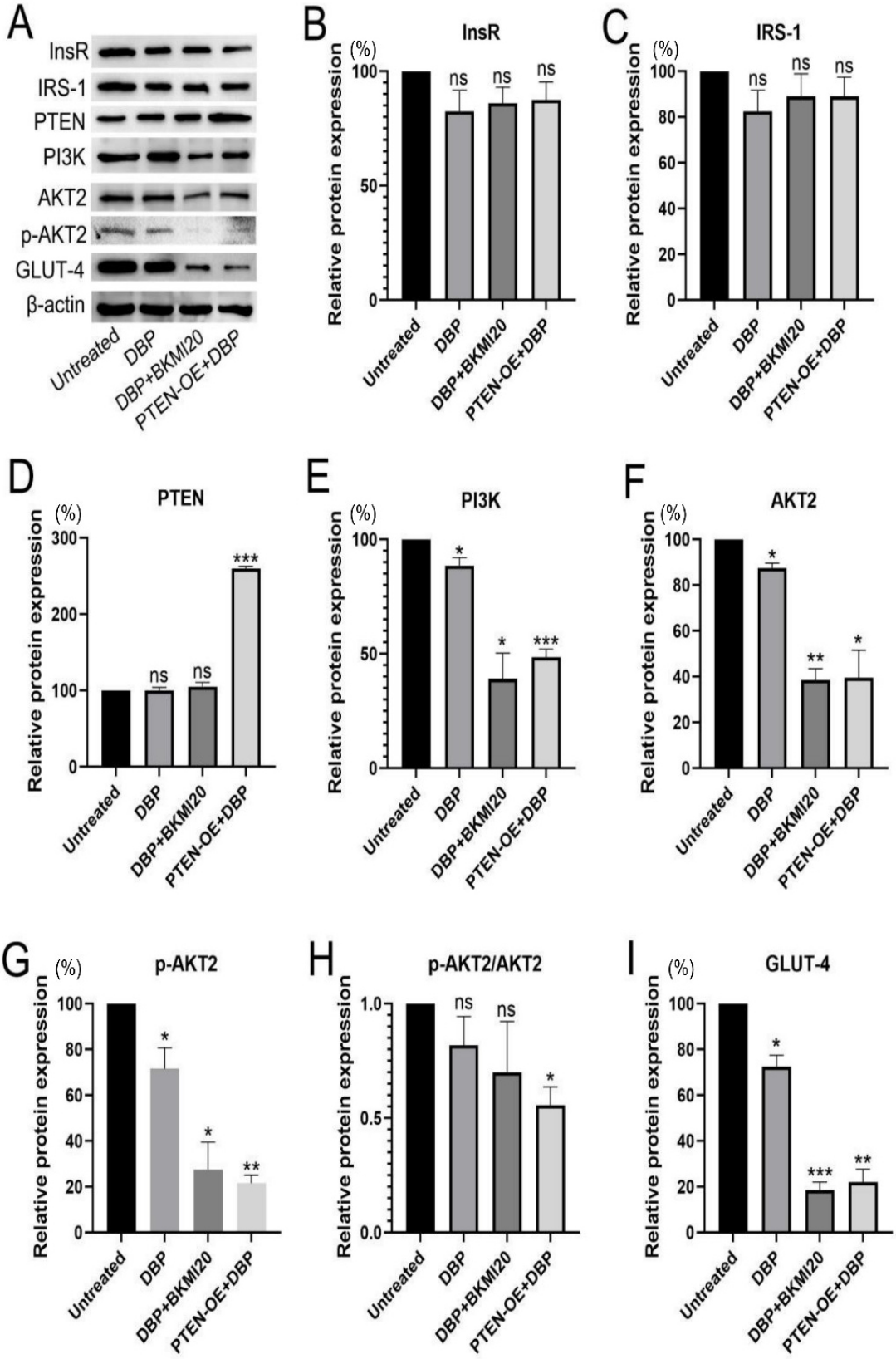

Fig. 3. Western blot detects the expression changes of each molecular protein level after treating HSkMC cells in different ways. (A) Protein expression of the insulin signaling pathway detected by Western blot. (B–I) Comparison of the relative expression levels of each molecule; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns represents non-significance, n = 3.

small molecular weight, can cause muscle IR by inhibiting the expression of INSR, IRS-1, PI3K, AKT, and GLUT4 in human skeletal muscle cell lines. A study by Merz *et al*. [18] demonstrated that abnormalities in the classical insulin signaling pathway, particularly via INSR-IRS1-PI3K-AKT-GLUT4 signaling, play an important role in skeletal muscle IR. DBP has been found to impair insulin signal transmission in skeletal muscle cells, causing IR. This was confirmed in a study that demonstrated DBP's effect on the INSR-IRS1-PI3K-AKT-GLUT4 signaling pathway in a human skeletal muscle cell line. Existing research suggests that DBP has an inhibitory effect on the PI3K-AKT signaling pathway, which is also observed in other cells. Li *et al*. [19] discovered that DBP can induce apoptosis in pancreatic β-cells by inhibiting PI3K/AKT/BCL2 expression. A study by Deng *et al*. [20] showed that DBP could inhibit the phosphorylation of PI3K and the expression of AKT and GLUT2 in pancreatic β-cells in a T2DM model in mice, induced by high-fat diet combined with low-dose streptozotocin (STZ). This disrupted the insulin signaling pathway and impaired insulin secretion in mice, exacerbating diabetes in mice. Additionally, Wang *et al*. [21] observed a significant increase in PTEN protein expression in Sertoli cells of the testis following 24-hour treatment with various concentrations of DBP compared to the control group. In contrast, the expression of p-PI3K, p-AKT, p70S6K, and 4E-BP1 proteins in the PI3K/AKT/mTOR signaling pathway was significantly decreased. Thus, it is hypothesized that the PTEN/PI3K/AKT/mTOR signaling pathway plays an important role in DBP-induced apoptosis in rat Sertoli cells of testis [21]. Moreover, treating HSkMC cells with 100 µm of DBP also reduced AKT phosphorylation levels. However, our study primarily focused on the impact of DBP on the IR of HSkMC cells. In future studies, we plan to examine the effect of DBP on apoptosis in HSkMC cells.

PTEN is a phosphatase that inhibits PI3K signaling by dephosphorylating PIP3, indirectly reducing insulin sensitivity [22]. Notably, experiments involving the deletion of the PTEN gene in skeletal muscle cells of mice revealed a counteraction against IR and diabetes induced by a high-fat diet [23]. In the present study, we observed the first instance of an inhibitory effect of DBP on PI3K/AKT/GLUT4 signaling in skeletal muscle cells. However, we did not find that the expression of PTEN in these cells was affected by DBP, suggesting that the inhibitory effect of DBP on PI3K-dependent pathways in skeletal muscle cells may not be mediated through PTEN.

The regulatory effect of DBP on PI3K/AKT signaling seems to be different from most other PAE substances, as substances like DINP, MEHP, and DEHP all exhibit a facilitative effect on PI3K/AKT signaling. Given the existence of multiple receptors for PAEs in cells (e.g., nuclear steroid hormone receptors, peroxisome proliferator-activated receptors, nuclear aerobiotic receptors) [24], we speculated that the diverse regulatory effects of different PAEs on the same signaling pathway may stem from interactions with different receptors. Signaling pathways undergo regulation through altered expression and function of molecules within the cascade. Modulations occur at all stages, with phosphorylation being the most important post-translational modification.

Conclusions

In this study, we found that DBP modulates the PI3K/AKT pathway in skeletal muscle cells, evident both transcriptionally and through phosphorylation, although the latter did not reach statistical significance. These findings suggest DBP's regulatory role in the pathway, mainly transcriptionally, shedding light on its contribution to IR mechanisms. However, insulin resistance's complexity, involving oxidative stress, inflammation, and epigenetics, remains only partially addressed by DBP's impact, highlighting the need for further research.

Availability of Data and Materials

The authors confirm that the data supporting the findings of this study are available within the article.

Author Contributions

KZ and DS contributed to the study concept and design. KZ, DS, and YC contributed to the acquisition of data. KZ and DS performed the statistical analysis. YC was involved in the interpretation of the data. All authors were involved in the drafting and critical revision of the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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