Article

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Notopterol Inhibits TNF-α-Induced C2C12 Myoblast Apoptosis by Stimulating the PI3K/AKT Pathway

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Background: Qianghuo is the root and rhizome of the umbelliferae plant *Notopterygium incisum* Ting ex H.T. Chang (NI) or *N. franchetii* H. de Boiss. Notopterol (linear furocoumarin) is the most effective component of *Notopterygium incisum*. Notopterol exhibits antipyretic, analgesic, anti-inflammatory, anti-oxidation, and anti-apoptosis effects. However, the effects of notopterol on skeletal muscle cells are unclear. This study aims to evaluate the influence of notopterol on the activity of myoblast as well as its inhibiting effect on apoptosis induced by tumor necrosis factor-alpha (TNF-α).

Method: We assessed the effect of notopterol on TNF-α-induced C2C12 apoptosis and its underlying mechanisms. Cell viability and apoptosis were evaluated using the CCK-8 and Annexin V-FITC/PI, respectively. The expressions of apoptosis-related proteins and pathways were assessed utilizing western blot (WB) analysis. Additionally, the role of notopterol in the phosphatidylinositol 3-kinases/AKT (PI3K/AKT) pathway was explored using specific inhibitors of PI3K.

Results: We observed that notopterol increased the activity of TNF-α-treated myoblasts ($p < 0.05$). Furthermore, flow cytometry, Hoechst-33258 staining, and WB analysis revealed that notopterol reduced TNF-α-induced apoptosis of C2C12 myoblasts by increasing B Cell Lymphoma 2 (BCL-2) levels and decreasing BCL-2-associated X protein (BAX), caspase-3, and caspase-9 levels ($p < 0.05$). Additionally, notopterol can activate the PI3K/AKT pathway in the first place, then can increase AKT phosphorylation and BCL-2 expression and decrease BAX and caspase-3 expression sequentially. These effects were reversed with the introduction of LY294002, which is a specific inhibitor of PI3K ($p < 0.05$).

Conclusions: Notopterol can affect C2C12 through PI3K/AKT, thus protecting against TNF-α-induced C2C12 myoblast apoptosis. Therefore, notopterol can be used to treat amyotrophy, providing insights into Sarcopenia prevention and treatment.

Keywords: notopterol; myoblast; apoptosis; PI3K/AKT; TNF-α

Introduction

Sarcopenia is characterized by a decrease in muscle mass and function, increasing the risk of falls, disability, and even death over a patient's lifetime [1–3]. Moreover, sarcopenia is associated with heart diseases [4], respiratory diseases [5], cognitive disorders [6], and movement disorders [7–9]. Therefore, recognizing its effect on the quality of life, this disease was incorporated into the International Classification of Diseases ICD-10 disease code (M62.85) in 2016 [10]. Due to the huge cost associated with its pathogenesis and treatment, which is borne by patients and their families, even the society at large, urgent improvement is required.

Sarcopenia is primarily caused by chronic low-grade inflammation, which may occur in patients with various chronic diseases, including cancer, chronic obstructive pulmonary disease, obesity, Acquired Immune Deficiency Syndrome (AIDS), and type 2 diabetes [11–15]. Among them, tumor necrosis factor-alpha (TNF-α) is a major pro-inflammatory factor. Long-term chronic secretion of high concentration of TNF-α *in vivo* is closely linked to skeletal muscle depletion [16]. At the pathological level, TNF-α inhibits protein synthesis in skeletal muscle cells, adversely impacting muscle integrity and function, thereby leading to sarcopenia [17]. TNF-α can inhibit the repair and regeneration of skeletal muscle by inhibiting myoblast differentiation and inducing apoptosis [18]. Moreover, it can also inhibit muscle differentiation by downregulating Myogenic differentiation (MyoD) through the activation of the nuclear factor kappa B (NF-κB) pathway [19]. Additionally, upon binding to the TNF receptor1 (TNFR1) receptor on the cell membrane, it induces the recruitment of Fas-related death domain protein (FADD) and caspase-8 to form death-inducing complex II. This activation affects caspase-3, triggering the cascade reaction of cysteine aspartic proteases (caspase) [20]. Meanwhile, the pro-apoptotic protein BCL-2-associated X protein (BAX) is upregulated, changing the proportion of B Cell Lymphoma 2 (BCL-2) family members. In contrast, the anti-apoptotic protein BCL-

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2 is downregulated, thereby promoting the permeability of the mitochondrial membrane. This process leads to the release of cytochrome c (Cyt c) and activation of caspase-9, ultimately affecting caspase. Finally, the executive protein caspase-3 transmits the apoptotic signal, leading to cell death upon occurrence of cell-specific nuclear events [21].

Muscle stem cells (MuSCs), also called Satellite cells (SCS), undergo differentiation and proliferation to form new muscle fibers under the stimulation of external physiology or pathology. Undifferentiated C2C12 cells (myoblasts) are recognized muscle satellite cells *in vitro* [22–24], actively participating in muscle growth and repair processes. When muscle atrophy occurs due to injuries (inflammation), the Myoblast changes from a static to an active state, leading to the initiation of myoblast differentiation to form new muscle tubes through proliferation and expression of various myoblast factors [25,26]. They fuse to form new muscle clusters.

Apoptosis is a programmed cell death that can induce satellite cell death. Apoptosis-induced satellite cell death depletes the pool of satellite cells [27,28]. As a result, damaged skeletal muscle fails to obtain sufficient reserves for regeneration and repair, leading to sarcopenia. Therefore, sarcopenia can be prevented by inhibiting myoblast apoptosis [29]. The phosphatidylinositol 3-kinases/AKT (PI3K/AKT) pathway plays an essential role in the apoptosis in mouse skeletal muscle cells [30–32]. PI3K, the upstream regulatory protein of AKT, can activate and phosphorylate the AKT molecule on the cell membrane. Phosphorylated AKT can further regulate apoptotic signaling by targeting the expression of downstream apoptosis-related proteins, especially the BCL-2 family members [33–35].

Notopterygium incisum, a traditional Chinese herbal medicine, is predominantly used to treat rheumatoid arthritis, headaches, and colds due to its anti-rheumatic and analgesic effects. For example, Jiuwei Qiang Live Decoction, a classic Chinese prescription where *Notopterygium incisum* serves as the main ingredient, has been used to treat limb soreness induced by wind cold. Notopterol is the most effective bioactive ingredient in *Notopterygium incisum*, exhibiting anti-inflammatory, antioxidant, anticancer, and anti-apoptotic effects [36–38]. Notopterol can downregulate caspase-3 and BAX proteins while increasing AKT phosphorylation and the expression of BCL-2 protein in cardiomyocytes in a model of cardiomyocyte injury [39–41]. Therefore, notopterol can inhibit cardiomyocyte apoptosis through AKT phosphorylation. However, the effect of Notopterol on skeletal muscle remains unknown. This study aimed to assess the anti-apoptosis effect of Notopterol in skeletal muscle and elucidate the associated mechanisms using an apoptosis model of TNF-α-stimulated C2C12 myoblasts *in vitro*.

Materials and Methods

Chemicals and Reagents

C2C12 cells (Cat#CL-0044), DMEM (Cat#PM150210), 10% FBS (Cat#164210-50), and 1% P/S (Cat#PB180120) were sourced from Procell (Wuhan, China). Enhanced Cell Counting Kit 8 (WST-8/CCK-8, Cat#E-CK-A362) and Annexin V-FITC/PI (Cat#E-CK-A211) were obtained from Elabscience (Wuhan, China). Hoechst-33258 (Cat#C0003) and LY294002 (Cat#S1737, PI3K inhibitor) were obtained from Biotechnology (Shanghai, China). TNF-α (Cat#50349-MNAE) was sourced from Sino Biological (Beijing, China). Notopterol (Cat#88206-46-6) was obtained from Aladdin Biochemical Technology Company (Shanghai, China). RIPA (Cat#P0013B) lysis buffer and BCA (Cat#P0010S) protein detection kit was sourced from Biosharp Biotechnology (Hefei, China). A resistant diluent (Cat#MI00609B) and rapid sealing liquid (Cat#MI00610B), ECL luminescent liquid enhanced version (Cat#MI00607B) were obtained from Mishushengwu Biology (Xi'an, China). BCL-2 (Cat#YT0470), BAX (Cat#YT0459), PI3 Kinase (Cat#YM3503), and phosphorylated AKT (Cat#YP0006) antibodies were sourced from Immunoway Biotechnology (Plano, TX, USA). Rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cat#60004-1-Ig) was obtained from Proteintech (Wuhan, China). Caspase-3 (Cat#AF0081) and Phospho-PI3K *p*85α (Cat#AF5905), total AKT (Cat#AA326), were sourced from Beyotime Biotechnology (Shanghai, China).

Cell Culture

The C2C12 cell line was validated through short tandem repeat analysis and underwent mycoplasma testing. The cells were incubated in a DMEM medium containing 10% FBS and 1% P/S (penicillin plus streptomycin) at 37 °C and 5% CO_2.

CCK-8 Assay

The C2C12 cells were incubated with different concentrations of TNF-α (0, 5, 10, 20, 40, and 80 ng/mL) and notopterol (0, 5, 10, 20, and 40 μM) at 37 °C for 24 hours. C2C12 cells were seeded in 96-well plates (1×10^4/well). After incubation, treated cells were exposed to CCK-8 solution. Cell viability was observed by measuring the OD values at 450 nm using BioTek Synergy HTX (BioTek Instruments, Inc., Winooski, VT, USA). Each experiment was replicated three times.

Grouping and Processing

The TNF-α stock solution was diluted to a concentration of 100 ng/mL using sterile DMEM. Similarly, notopterol was dissolved to a concentration of 50 nM using DMSO and subsequently stored at –20 °C. The cells were divided into five groups: the blank control group and four treatment groups, including 40 μM notopterol, 20 ng/mL

TNF-α, and 20 ng/mL TNF-α+40 μM notopterol treatments, as well as 20 ng/mL TNF-α+40 μM notopterol+5 μM LY294002 inhibitor treatments. The cells underwent treatment for 24 hours.

Flow Cytometry

Flow cytometry was used to assess the apoptosis rate via an annexin V-FITC/PI apoptosis detection kit. For this purpose, C2C12 myoblasts were inoculated in a 6-well plate and subsequently treated as described in section *Grouping and Processing*. The cells in each group were centrifuged at 300 ×g for 5 minutes, and resuspended with phosphate belanced solution (PBS). The cell suspension was centrifuged at 300 ×g for 5 minutes and the supernatant was discarded. After washing with PBS, 100 μL of diluted 1× Annexin V Binding Buffer was added. After this, 2.5 μL of Annexin V-FITC reagent and 2.5 μL of PI Reagent (50 μg/mL) were added into the cell suspension, followed by incubation at room temperature for 15 minutes in the dark. The sample was placed on ice and immediately analyzed using Flow cytometer (Cyto FLEX, BD Biosciences, San Jose, CA, USA). Annexin V-FITC-monopositive cells were identified as early apoptotic cells, whereas Annexin V-FITC and PI double-positive cells were categorized as necrotic and late apoptotic cells, respectively. The cells in quadrant 3 were used as controls, while those in quadrants 1 and 4 were apoptotic cells. The percentage of apoptotic cells was calculated.

Hoechst 33258 Staining

The cells were inoculated into 12-well plates and treated as described in section *Grouping and Processing*. Following treatment, the cells were fixed with 4% paraformaldehyde for 15 minutes and subsequently washed twice with PBS. After this, 0.25 mL of Hoechst 33258 staining solution was added to each well and incubated at room temperature for 5 minutes, followed by two PBS washes. The cells were immediately observed using a fluorescence microscope (Leica DM IL, Leica Microsystems, Wetzlar, Germany) at an excitation wavelength of 350 nm. Six microscope fields were randomly selected for analysis within each treatment group. The apoptosis index (AI) was assessed by calculating the percentage of apoptotic nuclei among the total number of apoptotic nuclei using ImageJ software (5.2.1 127847, NIH, Bethesda, MD, USA).

Western Blot Analysis

The cells were washed twice with ice-cold PBS and lysed on ice using RIPA (1:100) buffer containing protease inhibitor for 45 minutes. Subsequently, the cells were thoroughly scraped and collected in the EP tube. Following centrifugation at 13,400 ×g for 20 minutes, the resulting supernatant was retained for further analysis. After this, the proteins were quantified utilizing a BCA kit. Denatured proteins (40 μg) were resolved using SDS-PAGE with 10% polyacrylamide gel and then transferred onto the PVDF membrane. The membrane was blocked by a rapid sealing solution at room temperature for 30 minutes. GAPDH protein was used as the internal reference. The membrane was incubated overnight with primary antibodies against BCL-2 (1:1000), BAX (1:1000), caspase-3 (1:1000), caspase-9 (1:1000 dilution), PI3K (1:1000), AKT (1:1000), phospho-AKT (1:1000), GAPDH (1:2000). The next day, the membrane was washed three times with TBST (15 minutes for each wash), followed by incubation with secondary antibody HRP (1:500) at room temperature for 2 hours. Finally, protein bands were analyzed using a Bio-rad camera and imaging system (Image Station 2000 MM, Kodak, Rochester, NY, USA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9.4.1 software (GraphPad Software Inc., San Diego, CA, USA). The multiple-group comparisons were assessed using a one-way ANOVA according to the homogeneity of variance, and Tukey's test was applied for pairwise comparisons. The statistical comparison between the two groups was performed using the student's *t*-test. Data were expressed as mean ± standard deviation (n ≥3). A p-value < 0.05 was considered statistically significant.

Results

TNF-α Inhibits C2C12 Activity and Induces Apoptosis, and Notopterol Increases Cell Viability of TNF-α-Stimulated C2C12 Myoblasts

To investigate the influence of TNF-α on the activity of myoblast, the cells were treated with different concentrations of TNF-α for 24 hours and 48 hours. In both incubation periods, the outcomes showed that myoblast and TNF-α exhibited time and concentration-dependent effects. Based on the time-concentration curve and statistical analysis, the activity of cells did not show a significant difference when the concentration of TNF-α was below 20 ng/mL in 24 hours. However, the activity of cells reduced when the conditions were set beyond this range ($p < 0.05$, Fig. 1A). Furthermore, significant apoptosis was observed when the concentration of TNF-α reached 20 ng/mL in 24 hours ($p < 0.01$, Fig. 1B); hence, this condition was maintained in the following experiments ($p < 0.05$, Fig. 1D). With increasing notopterol concentration, positive changes in cell activity were found at 24 hours. However, at 48 hours, cell activity increased at the concentrations below 5 μM but decreased at higher concentrations ($p < 0.05$, Fig. 1C). To maintain the optimal cell activity, notopterol was applied in 24 hours at 40 μM concentration in the following experiment. This experimental condition was validated by the literature (Fig. 2A).

We speculated that the decrease in cell activity caused by high concentration of notopterol for 48 hours may be due to the accumulation of drug toxicity.

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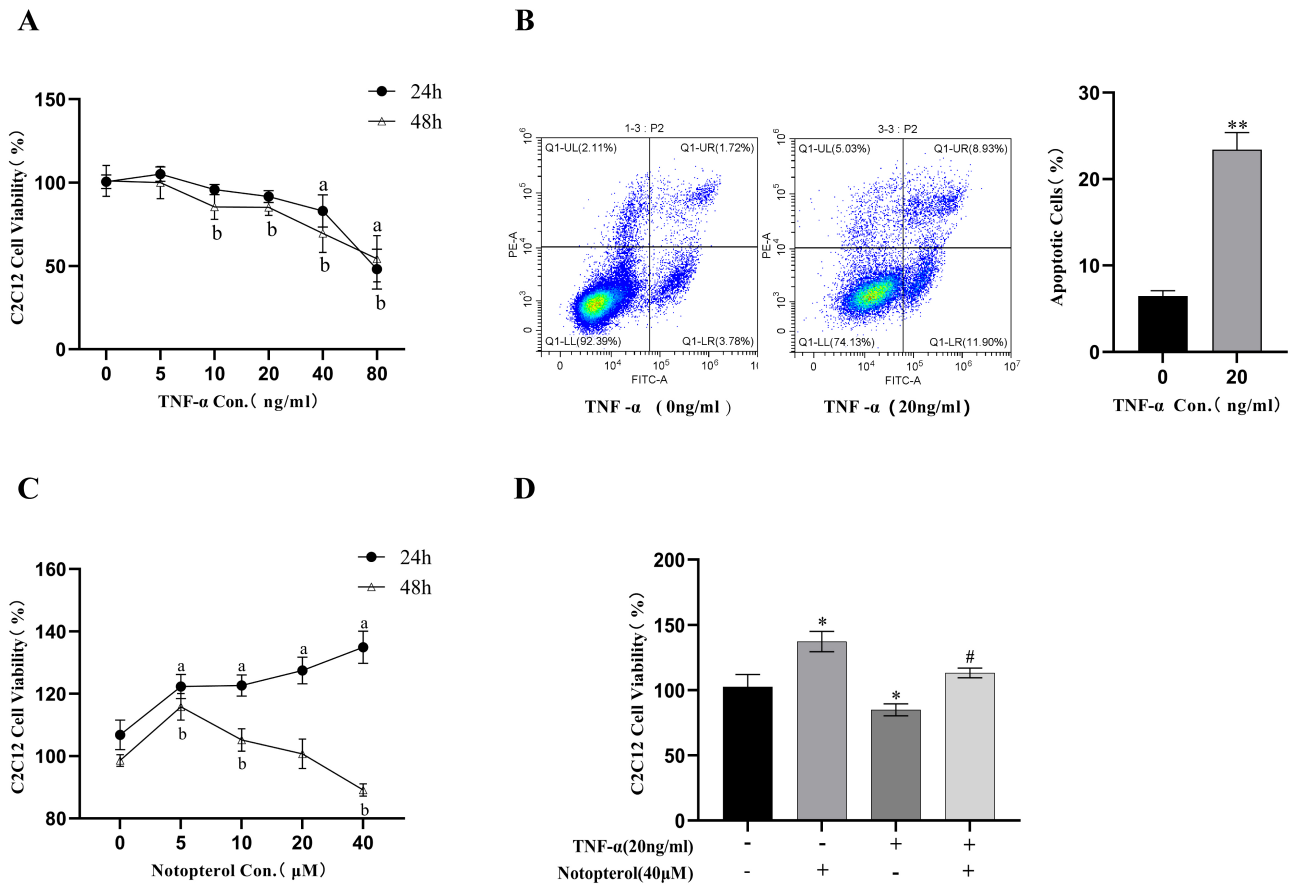

Fig. 1. Effects of tumor necrosis factor-alpha (TNF-α) and notopterol on cell viability. (A) The activity of C2C12 myoblasts after treatment with different concentrations of TNF-α. (B) TNF-α (20 ng/mL) can induce apoptosis in C2C12 myoblasts. Analysis of apoptosis ratio using flow cytometry. (C) Effect of nototerol on myoblast activity following 24 hours and 48 hours of exposure. (D) The cell viability after exposure of TNF-α-stimulated C2C12 myoblasts to 40 μM notopterol for 24 hours. Data were expressed as mean ± standard deviation (SD), n = 6, $^{a}p < 0.05$, different concentrations of TNF or notopterol following 24 hours vs. Control. $^{b}p < 0.05$, different concentrations of TNF or notopterol at 48 hours vs. Control. $^{**}p < 0.01$, TNF-α group vs. Control. $^{*}p < 0.05$, notopterol or TNF-α group vs. Control. $^{\#}p < 0.05$, TNF-α+notopterol vs. TNF-α group.

Notopterol Inhibits TNF-α-Induced Apoptosis in C2C12 Myoblasts

We investigated whether notopterol could protect cells from TNF-α-induced apoptosis. The cells were divided into four groups: the control group, the 40 μM notopterol treatment group, the 20 ng/mL TNF-α treatment group, and 20 ng/mL TNF-α combined with the 40 μM notopterol treatment group. Cells within these four groups were stained using Hoechst-33258 and demonstrated that neither the blank control group nor the notopterol group showed significant apoptosis. However, the cells treated with TNF-α showed apoptotic features such as nuclear shrinkage and DNA fragmentation. Additionally, the combination of TNF-α and notopterol effectively inhibited apoptosis ($p < 0.05$, Fig. 2A,B).

To verify our results, we evaluated apoptosis rates utilizing flow cytometry with Annexin V/PI staining ($p < 0.05$, Fig. 2C,D). We observed an elevation in apoptosis rate in the group of cells treated with TNF-α, whereas a significant reduction was found in the fourth group, indicating a decrease in TNF-α-induced apoptosis in C2C12 myoblasts. This result was consistent with Hoechst-33258 staining outcomes. Western blot analysis revealed that compared to the blank control group, the ratio of BCL-2/BAX decreased, while the expression levels of caspase-3 and caspase-9 increased in the experimental group. In contrast, notopterol treatment resulted in an increase in the BCL-2/BAX ratio and a decrease in caspase-3 and caspase-9 levels ($p < 0.05$, Fig. 2E,F). These findings suggest that notopterol can effectively inhibit TNF-α-induced apoptosis in C2C12 myoblasts.

Notopterol Inhibits TNF-α-Induced Myoblast Apoptosis by Activating the PI3K/AKT Pathway and Upregulating Phosphorylated AKT

The PI3K/AKT pathway plays a crucial role in apoptosis [30]. Moreover, AKT is crucial for cell survival, differentiation, and energy metabolism, indicating that AKT

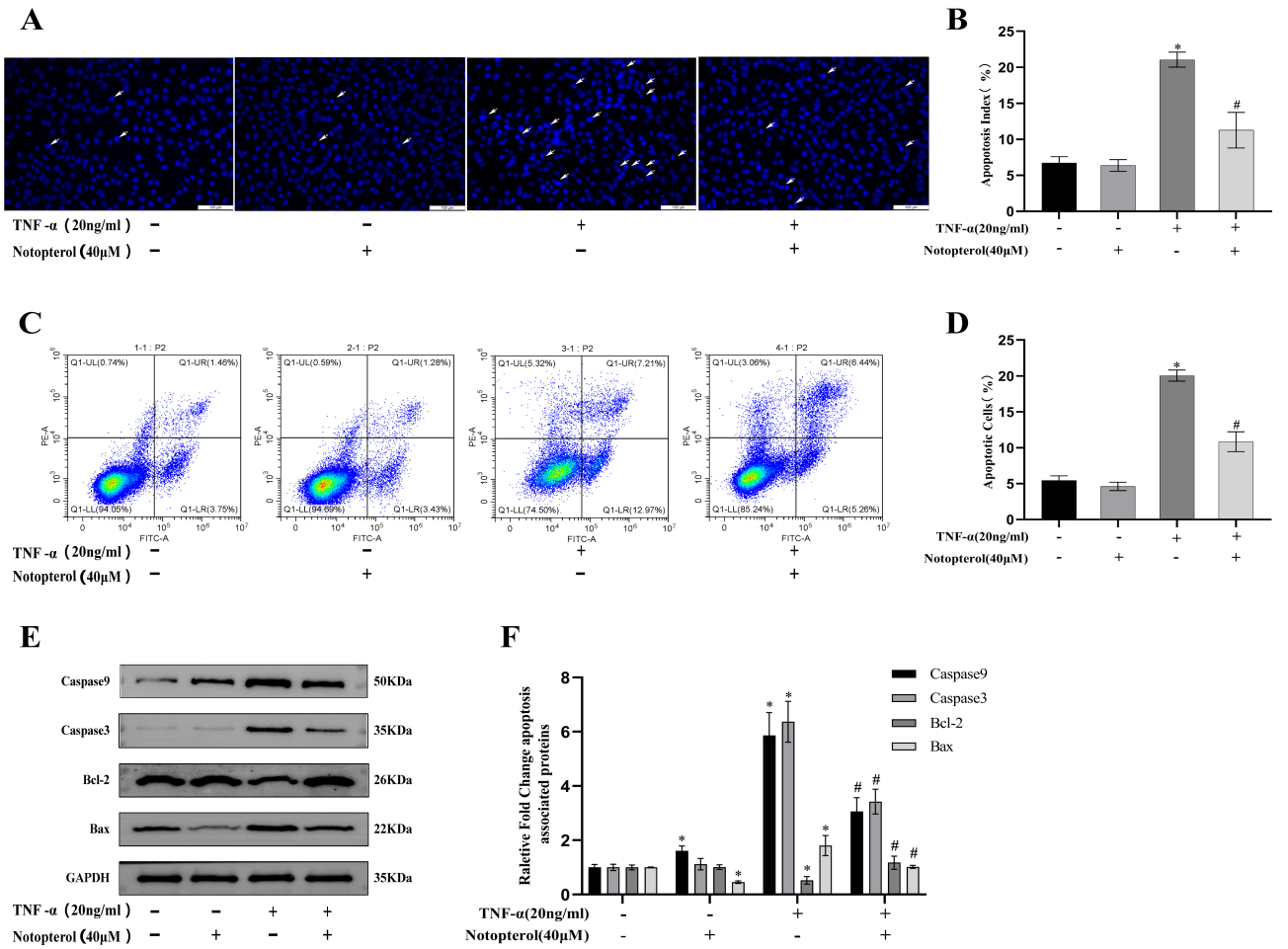

Fig. 2. Notopterol inhibits TNF-α-induced apoptosis in C2C12 myoblasts. (A) C2C12 myoblasts in the control group, 40 μM notopterol group, 20 ng/mL TNF-α group, and 20 ng/mL TNF-α combined with 40 μM notopterol under a 100-fold fluorescence microscope after Hoechst-33258 staining (scale bar = 100 μm). The cells indicated by the arrow are apoptotic cells. (B) Apoptosis index of C2C12 myoblasts in four groups. (C) Flow cytometry results of cell apoptosis. (D) Analysis of apoptosis ratio using flow cytometry. (E) Notopterol regulates the expression level of apoptosis-related proteins. The expressions of B Cell Lymphoma 2 (BCL-2), BCL-2-associated X protein (BAX), caspase-3, and caspase-9 in four groups were assessed utilizing western blot analysis. (F) Expression levels of apoptosis-related proteins. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference, and the data were expressed as mean ± standard deviation. n = 3, *$p < 0.05$, TNF-α group vs. Control. $^{\#}p < 0.05$, TNF-α+notopterol vs. TNF-α group.

phosphorylation can regulate cell apoptosis [34]. In this study, TNF-α downregulated PI3K ($p85\alpha$) and p-AKT, while increasing the phosphorylation of PI3K ($p85\alpha$) and AKT after treatment with notopterol ($p < 0.05$, Fig. 3A–C). Furthermore, LY294002 downregulated PI3K ($p85\alpha$) and p-AKT, while it did not significantly affect the expression levels of total AKT ($p < 0.05$, Fig. 3D–F). Nonetheless, LY294002 upregulated BAX and caspase-3 and downregulated BCL-2 ($p < 0.05$, Fig. 3G–J). These results suggest that notopterol can inhibit TNF-α-induced apoptosis in C2C12 cells by activating the PI3K/AKT pathway.

Discussion

Sarcopenia is characterized by adverse changes in muscle tissue that can occur over the course of life, increasing the risk of fall, disability, hospitalization, death, and other adverse events. Currently, the primary treatment for this disorder in the world includes exercise and nutritional support, as no effective and safe diagnosis or drug has been approved for this disease. Therefore, it is eager to uncover viable drugs capable of potentially preventing or delaying sarcopenia [42,43].

TNF-α can induce apoptosis in various cell types. In this study, the concentration range of TNF-α was established based on previous studies to verify the concentration and duration required for TNF-α to induce apoptosis in

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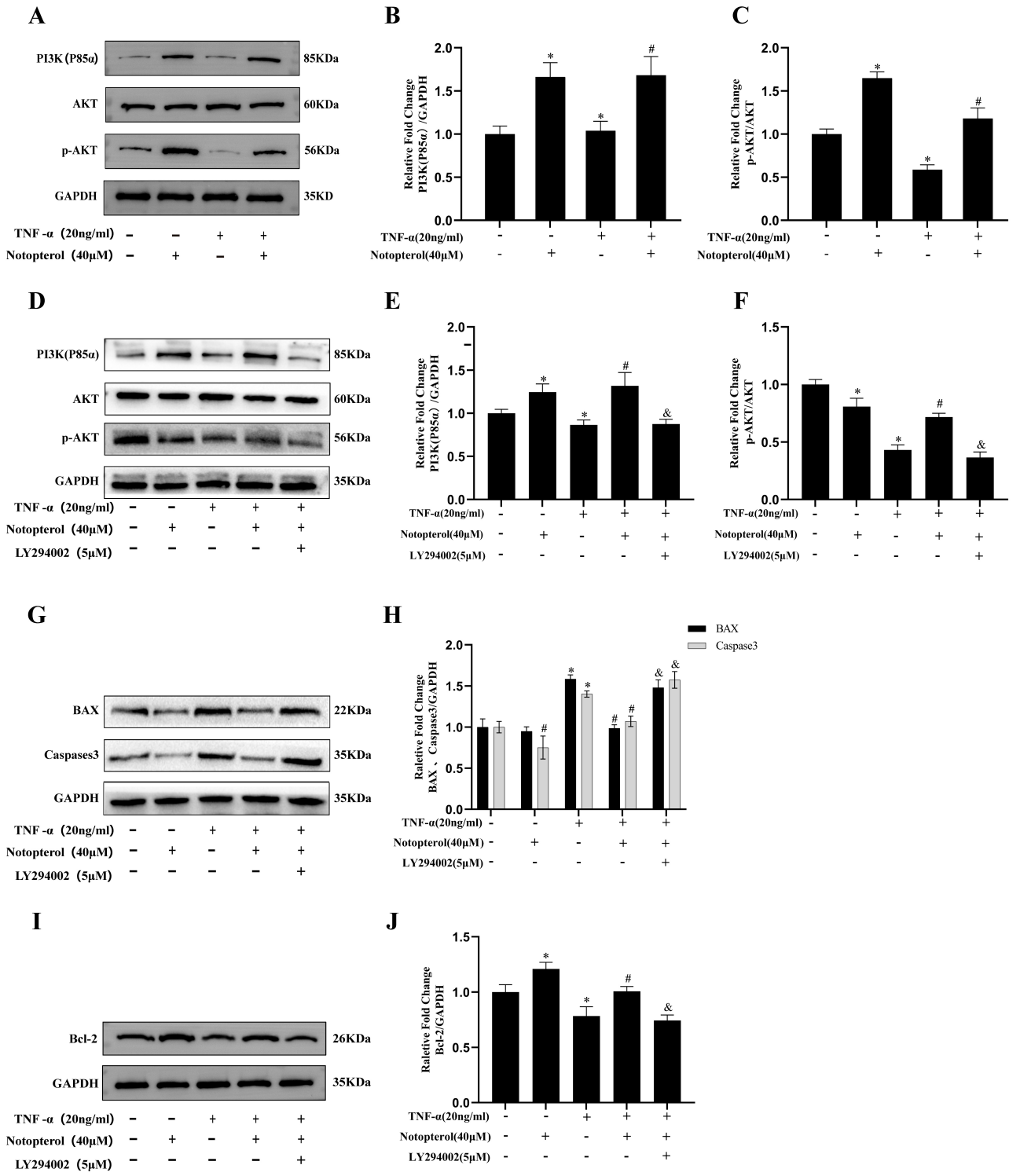

Fig. 3. Notopterol activates the phosphatidylinositol 3-kinases/AKT (PI3K/AKT) pathway and increases PI3K (*p*85α) and phosphorylated AKT levels. (A) Western blot analysis of protein expression of PI3K (*p*85α), total AKT, and *p*-AKT without PI3K inhibitor. (B) Relative Fold Chang of PI3K (*p*85α)/GAPDH in (A). (C) Relative Fold Chang of *p*-AKT/AKT in (A). (D) Western blot analysis of the expressions of PI3K (*p*85α), total AKT, and *p*-AKT after LY294002 treatment. (E) Relative Fold Chang of PI3K (*p*85α)/GAPDH in (D). (F) Relative Fold Chang of *p*-AKT/AKT in (D). (G) Western blot analysis of protein expression levels of BAX and caspase-3 after LY294002 treatment. (H) Relative Fold Chang of BAX and caspase-3. (I) Western blot analysis of protein expression levels of BCL-2 after LY294002 treatment. (J) Relative Fold Chang of BCL-2. GAPDH was used as an internal reference. The data were expressed as mean ± standard deviation of three independent experiments, n = 3, $^{*}p < 0.05$, notopterol or TNF-α group vs. Control. $^{\#}p < 0.05$, TNF-α+notopterol vs. TNF-α group. $^{\&}p < 0.05$, TNF-α+notopterol+LY29400Y vs. TNF-α+notopterol.

myoblasts. The time-concentration curve showed a gradual decrease in cell activity with increasing TNF-α concentration. Furthermore, cell activity significantly decreased at 24 hours when TNF-α concentration exceeded 20 ng/mL. Therefore, treatment with 20 ng/mL TNF-α for 24 hours was selected to ensure optimal cell activity. Flow cytometry was used to analyze cell apoptosis, and the effect of 20 ng/mL TNF-α treatment for 24 hours on apoptosis was confirmed through a literature review [44].

This study aimed to evaluate the inhibitory effect of Notopterol on TNF-α-mediated apoptosis of myoblasts. Previous studies have indicated the effect of 40 μM Notopterol on cardiomyocytes and human acute myeloid leukemia HL-60 cells [37,39], but its impact on C2C12 myoblasts remained uninvestigated. In our study, cell activity was assessed using the CCK-8 assay, elucidating the relationship between cell viability and notopterol concentration. We found a positive association between those two variables in the initial 24 hours; conversely, a negative relationship was observed from 24 to 48 hours. Specifically, at 48 hours, cell viability elevated with notopterol concentration ranging from 0 to 5 μM, followed by a persistent decrease with increasing Notopterol concentration from 5 to 40 μM. This phenomenon indicates potential drug toxicity. When a high concentration of notopterol was used to interfere with C2C12 cells, the enhancing effect of a high concentration of notopterol on cells was decreased after incubation for 48 hours, possibly because of limited cellular metabolism within the culture environment. As the drug reached a specific concentration, its toxicity exceeded the cellular capacity to withstand it, thus reducing the cell activity. Unlike animals or human bodies with metabolic organs like the liver or kidney, cells have a “detoxification” effect. Similar phenomena have been observed in many Traditional Chinese medicine monomers. This finding is instructively significant for clinical application in the future, indicating careful consideration of toxicity and concentration when applying drugs clinically.

Notopterol intervention increased cell activity within the TNF-α-induced myoblast apoptosis model. Furthermore, both flow cytometry analysis and Hoechst-33258 staining showed that notopterol could reduce the number and proportion of myoblast apoptosis. TNF-α induces apoptosis through exogenous and endogenous pathways. The transduction of these two apoptotic signals occurs in the cell interlacing and overlapping. Members of the BCL-2 family play a crucial role in the mitochondrial apoptosis pathway (endogenous pathway). BCL-2 and BAX exist as a dimer, and the ratio of BCL-2/BAX changes during the activation of the caspase signal. A high ratio of BCL-2/BAX inhibits apoptosis, whereas a low ratio promotes apoptosis. BCL-2 maintains the integrity of mitochondria. When BCL-2 is downregulated, it destroys the mitochondrial membrane, leading to the release of cytochrome C from the mitochondrial mesomembrane into the cytoplasm. Furthermore, upregulation of BAX accelerates cytochrome C release, consequently activating caspase-3, the executor of apoptosis [18–20]. In this study, Notopterol alone increased cell activity and the ratio of BCL-2/BAX, while the expression levels of caspase-3 and caspase-9 were not changed. Furthermore, when combined with TNF-α, notolithol was found to decrease the ratio of BCL-2/BAX and upregulate mitochondrial apoptotic protein caspase-9 and apoptotic executive protein caspase-3, suggesting that Notopterol can inhibit apoptosis through the mitochondrial pathway.

The PI3K/AKT pathway becomes activated in mouse skeletal muscle, thereby inhibiting the expression of apoptosis-related proteins [31,32]. In this study, Notopterol increased the expression levels of PI3K and *p*-AKT in myoblasts, indicating its capacity to activate PI3K and increase AKT phosphorylation. However, TNF-α treatment downregulated PI3K and *p*-AKT. Notably, Notopterol upregulated PI3K and *p*-AKT in TNF-α-induced apoptotic cells but did not significantly affect total AKT levels. These results suggest that the anti-apoptotic effect of Notopterol is correlated with the PI3K/AKT pathway. Further findings showed that Notopterol failed to activate PI3K after treatment with a PI3K inhibitor (LY294002). Moreover, Notopterol downregulated PI3K and *p*-AKT after LY294002 treatment while increasing the expressions of pro-apoptotic proteins BAX and caspase-3. Notably, LY294002 decreased the expression levels of anti-apoptotic proteins BCL-2 after LY294002 treatment. These findings suggest that Notopterol can induce an anti-apoptotic effect in TNF-α-induced myoblasts by activating the PI3K/AKT pathway, consequently promoting apoptosis.

In summary, this study aimed to examine the anti-apoptotic effect and underlying mechanism of notopterol by establishing an *in vitro* apoptosis model. These outcomes can be summarized as follows. Firstly, notopterol can positively increase the activity of myoblasts. Secondly, notopterol can potentially inhibit TNF-α-induced apoptosis of myoblast. Thirdly, notopterol can activate the PI3K/AKT pathway and positively modulate the expression of *p*-AKT, thereby inhibiting the apoptosis of myoblasts to certain extents.

The experiment mentioned in this study confirmed that notopterol has the positive impact on myoblasts and potential valuation in the treatment of sarcopenia in the first time. However, this study was limited to cellular level due to the lack of resources to simulate the animals or humans in real-life conditions. Thus, huge differences exist between *in vitro* tissue and *in vivo* cells. To fully understand the function and mechanism of notopterol in sarcopenia, it is necessary to perform animal trials.

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Conclusions

Our results suggest that notopterol exhibits an inhibitory effect on TNF-α-mediated myoblast apoptosis. In the appropriate concentration and duration, notopterol demonstrates an anti-apoptotic role to adjust the expression of apoptosis-related proteins through the PI3K/AKT pathway. These findings provide potential research directions and ideas for preventing and treating sarcopenia. However, further investigations into the mechanism of notopterol need animal trials.

Availability of Data and Materials

All the data had been collected in this document, and the original data, could be accessed from the corresponding author upon reasonable requirement.

Author Contributions

Substantial contribution on design and concept: YYLZ; data gathering, analysis: XQZ, QX; graphing: YYLZ, YXW, YW, YS. Writing, reviewing, drafting and editing: all authors. All authors gave final approval of the version to be published. 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

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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