

Role Of VEGFB In Electrical Pulse Stimulation Inhibits Apoptosis In C2C12 Myotubes

LanLan Guo

Beijing Normal University

YanJun Li

Beijing Normal University

Zheng Xing

Beijing Normal University

JingBo Zhang

Beijing Normal University

Jing Zhang (✉ zhangjing@bnu.edu.cn)

Beijing Normal University

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Abstract

Skeletal muscle is the major effector organ for exercise. It has been proposed that the expression of VEGFB in skeletal muscle is significantly related to apoptosis. However, whether the decrease in VEGFB in skeletal muscle participates in the occurrence of skeletal muscle apoptosis and whether exercise inhibits apoptosis by promoting the expression of VEGFB in skeletal muscle cells have not been reported thus far. Here, we developed in vitro experiments to mimic the effect of exercise through electrical pulse stimulation (EPS) to observe the effect of EPS on apoptosis and the change in VEGFB expression in differentiated myotubes. In addition, we employed RNA interference experiments to explore whether VEGFB is directly involved in the regulation of myotube apoptosis during EPS. Our results showed that exogenous VEGFB₁₆₇ significantly inhibited C2C12 myotube apoptosis induced by TNF- α treatment and that endogenous VEGFB in differentiated C2C12 myotubes was upregulated significantly by EPS. In addition, EPS decreased the expression of the apoptotic indicators Bax and Bcl-2 at the mRNA level and downregulated the protein expression of cleaved caspase-3. The antiapoptotic effect of EPS weakened substantially as VEGFB in C2C12 myotubes was inhibited. Taken together, exercise-like EPS inhibits apoptosis by promoting the expression of C2C12 myotube-derived VEGFB.

Introduction

Vascular endothelial growth factor B (VEGFB) is a new member of the vascular endothelial growth factor family discovered in 1996 and is mainly distributed and highly expressed in tissues and cells with a strong metabolic phenotype, such as cardiomyocytes, skeletal muscle, brown adipose tissue, and brain[1]. It has been reported that VEGFB can promote the transport of fatty acids by endothelial cells, which are involved in the ectopic deposition of fat in the myocardium and skeletal muscle[2, 3]. Moreover, VEGFB plays a critical role in inhibiting apoptosis and promoting survival. VEGFB can significantly promote survival and inhibit apoptosis in many types of cells, such as vascular endothelial cells, smooth muscle cells, cerebral cortical neurons and retinal neurons[4–6]. To date, the effect of VEGFB on cardiomyocyte apoptosis has been confirmed[7]. Exogenous addition of VEGFB can reduce myocardial injury by inhibiting cardiomyocyte apoptosis in hypoxia-, adriamycin- or isoproterenol-induced cardiomyocyte injury models in vitro or in myocardial ischemia reperfusion-, diabetic cardiomyopathy- and TAC-induced disease models in vivo[8–11].

Skeletal muscle atrophy was closely related to some pathological conditions. Our previous study showed that in rats with heart failure induced by abdominal aortic coarctation, skeletal muscle atrophy and apoptosis increased evidently, and the expression of VEGFB in skeletal muscle decreased significantly. We also confirmed that eight weeks of treadmill exercise apparently inhibited skeletal muscle apoptosis in heart failure rats, and the expression of VEGFB in skeletal muscle increased significantly. Our previous research results suggested that the expression of VEGFB was markedly correlated with apoptosis in skeletal muscle. However, reports on whether the decrease in VEGFB expression in skeletal muscle participates in the occurrence of skeletal muscle cell apoptosis and whether exercise inhibits apoptosis by promoting the expression of VEGFB are limited thus far. Therefore, we designed a series of in vitro

experiments in which TNF- α treatment was conducted to induce apoptosis in differentiated C2C12 myotube cells. We also used electrical pulse stimulation (EPS) to imitate the exercise effect of skeletal muscle contraction in vitro to observe the effect of exercise on apoptosis of C2C12 myotubes and the changes in VEGFB expression. Furthermore, we employed RNA interference to verify the effect of EPS on apoptosis of C2C12 myotubes when the expression level of VEGFB was knocked down and the role of VEGFB in exercise against skeletal muscle apoptosis.

Materials And Methods

Cell culture and groups

C2C12 myoblasts (Cell Center of the Basic Institute of the Chinese Academy of Medical Sciences, Beijing, China) were seeded into six-well plates at a density of 10^5 per well, with 2 mL growth medium comprised of Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and 4 nM L-glutamine (HyClone, Logan, USA), supplemented with 10% fetal bovine serum (Gene X, Australia) and 1% antibiotics containing 100 U/mL penicillin–streptomycin (Solarbio, Beijing, China). After the cells reached 70~80% confluence, differentiation was induced by switching from 10% FBS to 2% horse serum. The medium was changed every day until differentiated myotubes were visualized under a light microscope (4~5 days). Cells were placed in an incubator under the constant conditions of 37 °C and 5% carbon dioxide.

Groups: The differentiated C2C12 myotubes were divided up for respective treatments: control (CON); C2C12 myotubes treated with 20 ng/ml TNF- α from the fifth day of differentiation for 24 hr (TNF- α); C2C12 myotubes cotreated with 20 ng/ml TNF- α and 100 ng/ml VEGFB₁₆₇ from the fifth day of differentiation for 24 hr (TNF- α + VEGFB); C2C12 myotubes treated with 20 ng/ml TNF- α from the fifth day of differentiation for 24 hr followed by EPS for 12 hr (TNF- α + EPS); C2C12 myotubes transfected with the negative control, followed by addition of 20 ng/ml TNF- α on the fifth day of differentiation for 24 hr (TNF- α + NC); C2C12 myotubes transfected with the negative control or siRNA, followed by the addition of 20 ng/ml TNF- α incubated for 24 hr, followed by EPS for 12 hr (TNF- α + NC+EPS/TNF- α + siRNA+EPS).

TNF- α treatment

C2C12 myotubes were treated with TNF- α from the fifth day of differentiation for 24 hr at a concentration of 20 ng/ml to induced apoptosis. In detail, 10 μ g recombinant mouse TNF- α (R&D, Minneapolis, USA, CS1417071) was fully dissolved in 100 μ l sterile PBS containing 0.1% BSA. The solution was filtered with a 0.22 μ m filter. TNF- α (100 μ g/ml) solution was fully dissolved in sterile PBS and diluted 50 times to achieve a concentration of 2 μ g/ml, which was then added to 2 ml DMEM medium directly and incubated with the cells to induce apoptosis.

VEGFB treatment

C2C12 myotubes were treated with recombinant mouse VEGFB₁₆₇ from the fifth day of differentiation to observe the effect of VEGFB on myotube apoptosis. In detail, 25 μ g mouse VEGFB₁₆₇ (R&D, Minneapolis,

USA, HF00217011) was reconstituted at 100 µg/ml in sterile 4 mM HCl containing 0.1% BSA. The solution was filtered with a 0.22 µm filter. The sterile solution was added to 2 ml DMEM and incubated with the cells for 24 hr at a concentration of 100 ng/ml.

Electrical pulse stimulation (EPS)

EPS was employed to mimic the effect of exercise in vitro. Differentiated C2C12 myotubes were seeded into six-well plates with 2 ml differentiation medium per well, which were connected to a C-Dish electrode (IonOptix, Milton, MA, USA). Electrical currents were discharged from a carbon electrode immersed in the medium. Electrical pulse stimulation (20 V, 1 Hz, 2 ms) was applied to the cells using a C-Pace pulse generator (C-PACE 100, IonOptix) for 12 hours in an incubator at 37°C and 5% CO₂. The contraction can be observed under microscope. Myotubes were harvested immediately after EPS.

siRNA transfection

Myoblasts were seeded into 6-well plates at a density of 5×10^5 cells/well with growth medium as described above. Myoblasts were replaced with differentiation medium the following day when they reached 70-80% confluence. Differentiated C2C12 myotubes were transfected on Day 4 after being cultured in differentiation medium containing 2% horse serum. Serum-free and antibiotic-free medium was replaced 24 hours before transfection. siRNA against VEGFB or negative control siRNA was transfected using Lipofectamine® RNAiMAX Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. Stealth RNAi duplex(No.11645679) or Stealth RNAi Negative Control duplex (No.1984719) was added for 48 hours at a concentration of 50 nM for transfection. The siRNA sense sequences were as follows, and the anti-sense sequence is completely complementary to the sense sequence.

Si-1: 5'-GCGGAAUCUCAAUCUACAUAUUUG-3'

Si-2: 5'-GGAUAUGGCUCAGGGUCGAAGUUAA-3'

Si-3: 5'-GAGAGGCUUUUAUAAUAGCAAUGCA-3'

Hoechst staining

Hoechst staining was conducted immediately after the respective treatments to observe cell apoptosis. Myotubes were washed twice with precooled sterile PBS to remove debris. Then, 4% paraformaldehyde was added to the six-well plate for 15 min to fix the cells. After that, PBS was used to wash the cells 2-3 times. Hoechst 33342 staining solution (Solarbio, Beijing, China, C0030) was added to six-well plates at a concentration of 10 µg/ml. Staining at room temperature was carried out in a dark environment for 15 min. The maximum excitation wavelength of Hoechst 33342 is 461 nm, and the maximum emission wavelength is 460 nm. After staining, myotubes were washed with PBS 2-3 times for 3 minutes each time. The cells were observed and imaged under a fluorescence microscope.

Protein extraction and western blotting

Proteins were collected immediately after the respective treatments. RIPA lysis buffer (Applygen, Beijing, China) containing 1% PMSF was added at 5 times the cell volume. Lysis was carried out at a low temperature for 20 min. The collected mixture was centrifuged at 12000 rpm for 10 min. The total cellular protein was collected in the supernatant. A BCA Protein Assay kit (Thermo, USA) was used to establish a BSA standard curve according to the manufacturer's instructions. The absorbance was detected at 562 nm by an enzyme labeling instrument. The total protein concentration was calculated according to the standard curve. The sample volume was calculated according to the obtained protein concentration, and 20 µg protein was added to each well. The target proteins were separated via a 10% gel and a 5% concentrating gel followed by transfer to a PVDF membrane by electroblotting in transfer buffer containing 20% methanol under cold conditions. The membrane was sealed in a bag with 5% skimmed milk powder at room temperature for 1-2 h. The antibody was diluted with TBST according to the instructions. The PVDF membrane was incubated overnight at 4°C with 1:1000 VEGFB antibody (Abcam, UK, EPR455), 1:1000 cleaved caspase-3 antibody (Cell Signaling Technology, Boston, USA, CST9664) and 1:8000 GAPDH antibody (Cell Signaling Technology, Boston, USA, CST5174). The PVDF membrane was incubated with HRP goat anti-rabbit IgG as secondary antibody (EARTH, #E030120) for one hour at room temperature. Subsequently, the target proteins were visualized by chemiluminescence (WBKLS0100) using a Tanon5200 imager and ImageJ for gray analysis. The relative protein expression of VEGFB and cleaved caspase-3 was analyzed based on the gray value of GAPDH.

mRNA isolation and quantitative PCR analyses

Total RNA was isolated with an RNAPrep Pure Cell Kit (TianGen Biotech, Beijing, China). Reverse transcription RNA was conducted with the FastKing RT Enzyme Mix (TianGen Biotech, Beijing, China). The Real-time PCR total reaction volume was 20 µL, which included 15 µl Super Real Pre Mix Plus 30 (SYBR Green) system with 0.5 µl each of the 10 mmol/L upstream and downstream primers, plus 5 µl of the cDNA template. Real-time PCR was performed with a LightCycler 96 real-time fluorescence quantitative PCR instrument (Roche, USA), using GAPDH as the internal reference. The PCR procedure was predenaturation at 94 °C for 30 s, denaturation at 94 °C for 5 s, annealing at 60 °C for 30 s and extension at 72 °C for 45 s, with 40 thermal cycles in total. All of the primers were synthesized by Sangong Bioengineering (Shanghai, China). The primer sequences for real-time PCR are shown in Table 1.

Table 1
Primer Sequences for real-time PCR Gene Expression

Gene	Primer sequences	
VEGFB	Forward	5'-GGAGGTGGTGGTACCTCTGA-3'
	Reverse	5'-GCATTCACATTGGCTGTGTT-3'
Bcl-2	Forward	5'-TCTTTGAGTTCGGTGGGGTC-3'
	Reverse	5'-AGTTCCACAAAGGCATCCCAG-3'
Bax	Forward	5'-CTGGATCCAAGACCAGGGTG-3'
	Reverse	5'-CCTTTCCCCTTCCCCATTTC-3'
GAPDH	Forward	5'-ACAGCAACAGGGTGGTGGAC-3'
	Reverse	5'-TTTGAGGGTGCAGCGAACTT-3'

Statistical analysis

The experimental data are expressed as the mean \pm standard deviation ($\bar{x} \pm s$). One way ANOVA was conducted by Prism 6.0 software. The mean values of each group were compared with each other. A Newman Keuls test was used to assess the difference between each group. The test confidence interval was 0.05; that is, $P < 0.05$ indicates that the difference was statistically significant, $P < 0.01$ indicates that the difference was very significant, and $P < 0.001$ indicates that the difference was extremely significant.

Results

VEGFB inhibited apoptosis induced by TNF- α in C2C12 myotubes

TNF- α may induce apoptosis or atrophy in skeletal muscle cells. C2C12 myotubes on the fifth day of differentiation were treated with TNF- α or cocultured with VEGFB₁₆₇ and TNF- α for 24 hr. According to Hoechst staining results (Fig. 1A), we observed obvious apoptosis in the TNF- α group in the nucleus, which was manifested by nuclear fusion, morphological changes and increased brightness. Notably, exogenous administration of VEGFB₁₆₇ alleviated nuclear fusion. The mRNA results showed that TNF- α incubation upregulated the expression of the apoptotic gene Bax (Fig. 1B) and downregulated the expression of the antiapoptotic gene Bcl-2 by nearly 50% in C2C12 myotubes (Fig. 1C). The decrease in the Bcl-2/Bax ratio suggested that the antiapoptotic ability decreased, while the exogenous administration of VEGFB increased the antiapoptotic activity in C2C12 myotubes (Fig. 1D). These experimental results suggested that VEGFB may play a critical role in inhibiting apoptosis induced by TNF- α in C2C12 myotubes.

Electrical pulse stimulation on C2C12 myotube apoptosis and VEGFB expression

Electrical pulse stimulation (EPS) is used to induce muscle contraction in vitro to mimic the effect of exercise. We used TNF- α treatment on the fifth day of differentiation to construct an apoptotic cell model in C2C12 myotubes, followed by EPS to explore whether EPS can upregulate the VEGFB expression level and inhibit the apoptosis induced by TNF- α treatment. According to our experimental results, TNF- α treatment significantly increased the apoptosis of differentiated C2C12 myotubes (Fig. 2A). EPS attenuated myotube apoptosis, decreased the apoptotic gene Bax (Fig. 2B), and increased the antiapoptotic gene Bcl-2 more than three-fold (Fig. 2C). The Bcl-2 to Bax relative mRNA ratio was used to evaluate the apoptosis status of cells, which was significantly downregulated by TNF- α treatment but returned to the level of the CON group through 12 hr of EPS (Fig. 2D). In addition, EPS apparently decreased cleaved caspase-3 protein levels (Fig. 2E), which is an identified index of cell apoptosis. These results suggested that EPS can inhibit TNF- α -induced myotube apoptosis at the mRNA and protein levels. Our experimental results also revealed that EPS significantly upregulated the expression levels of VEGFB at the protein level (Fig. 2F) and the mRNA level (Fig. 2G) in the CON and TNF- α groups. Furthermore, whether the upregulation of VEGFB in myotubes induced by EPS is directly related to the decrease in myotube apoptosis remains unclear. We will verify this scientific question through subsequent experiments.

Role of VEGFB in electrical pulse stimulation against apoptosis of C2C12 myotubes

According to previous experiments, we speculate that the VEGFB produced by myotube contraction may be related to the inhibition of apoptosis. To verify the role of VEGFB in EPS resistance to cell apoptosis, we employed an RNA interference experiment to observe whether the effect of exercise in restraining apoptosis occurs when VEGFB is knocked down in myotubes. Our experimental results showed that the mRNA expression of VEGFB decreased by approximately 70% and that the protein expression level decreased by an average of 60% after transfection compared with the negative control group (Fig. 3, B to D). Hoechst staining indicated that nuclear staining was increased, as shown by the enhanced blue fluorescence in the TNF- α +NC treatment group (Fig. 3E), which suggested that apoptosis had increased in the myotubes. However, the nuclear staining of the negative control decreased after 12 hours of EPS, illustrating that EPS played a realistic role in the inhibition of apoptosis in the negative control myotubes. However, nuclear staining and aggregation increased significantly in the myotubes of the siRNA+EPS group compared with the negative control group. Molecular test results showed that when VEGFB was knocked down in myotubes, the apoptotic gene Bax increased 1.5-fold (Fig. 3G), and the Bax/Bcl-2 ratio, which is used to reflect the antiapoptotic function, decreased by 35% (Fig. 3I) compared with negative control group after 12 hours of EPS. Similarly, the protein expression level suggested that VEGFB was negatively correlated with cleaved caspase-3 (Fig. 3J-I). Taken together, these data imply that when the expression of VEGFB in myotubes was inhibited, apoptosis increased significantly, while the antiapoptotic effect of EPS decreased. Accordingly, our results confirmed that VEGFB authentically plays a protective role in EPS against C2C12 myotube apoptosis.

Discussion

VEGFB is widely expressed in tissues and cells with a strong metabolic phenotype, including cardiac and skeletal myocytes and endothelial cells[1]. It has two different subtypes: heparin-binding VEGFB₁₆₇ and diffusible VEGFB₁₈₆ isoforms. VEGFB₁₆₇ has a heparin-binding carboxy-terminus structure, allowing it to bind specifically to heparin-like sulfated protein polysaccharide on the surface of the cell membrane[12]. The biological functions of VEGFB include promoting angiogenesis[13], promoting fatty acid transport[14] and resisting apoptosis[4]. Notably, VEGFB plays an important role in inhibiting apoptosis and promoting survival in some pathophysiological conditions. For instance, Li et al. proposed that revascularization of the ischemic border zone was impaired in VEGFB^{-/-} mice in a model of acute myocardial infarction (MI), whereas VEGFB₁₆₇ therapy enhanced ischemic myocardial revascularization[15]. Additionally, the expression of cleaved caspase-3 in cardiomyocytes decreased significantly after treatment with adeno-associated virus serotype 9-VEGFB for 4 weeks in mice with heart failure induced by aortic coarctation[16]. VEGFB was proven to inhibit apoptosis in cardiomyocytes treated with hypoxic conditions or epirubicin or in the myocardial infarction area induced by left anterior descending coronary artery ligation[17]. Similarly, VEGFB treatment increased Bcl-2 expression while reducing Bax expression in a hypoxia-reoxygenation-induced H9c2 cardiomyocyte injury model in a dose-dependent manner, mechanistically VEGFB promoted cardiomyocyte survival and inhibited autophagy by activating the PI3K/Akt/Bcl-2/Beclin1 signaling pathway[18]. Lal et al. revealed that incubation with exogenous VEGFB (100ng/ml) for 1 h inhibited cardiomyocyte apoptosis by reducing the Bax-to-Bcl-2 gene expression ratio, which has been used as an index of cell apoptosis[19]. In addition, inhibition of VEGFB through siRNA abolished its protective effects in DOX-treated cardiomyocytes[20]. In this experiment, we treated differentiated C2C12 myotubes co-incubated with 20 ng/ml TNF- α and 100 ng/ml VEGFB for 24 hours. TNF- α is a critical inflammatory cytokine elevated in heart failure and one of the key cytokines inducing skeletal muscle atrophy. We observed that VEGFB prominently inhibited TNF- α -induced apoptosis in C2C12 myotubes by upregulating various survival genes and inhibiting apoptosis-related genes.

Skeletal muscle is a tissue with a high level of expression of VEGFB[21]. Exercise has been authenticated to stimulate the expression of VEGFB in skeletal muscle. Kivelä et al. reported the mRNA level of VEGFB in the lateralis muscle of an exercised leg in healthy men was upregulated at 48 h postexercise, which may be one of the early mechanisms involved in skeletal muscle remodeling after high mechanical loading[22]. Long-term endurance exercise was shown to upregulate VEGFB gene expression in male rats, which may result in disturbance to angiogenesis in muscles[23]. VEGFB mRNA expression increased in healthy skeletal muscle at 6 h postexercise[24]. Birot et al. reported that VEGFB mRNA levels increased by 90%, while the VEGFB protein content increased by 72% in rat muscle fibers at the end of an acute running session. In brief, previous studies have indicated that VEGFB is highly expressed in skeletal muscle and markedly regulated by exercise as well[25]. However, the specific role of VEGFB in skeletal muscle remains unclear. Therefore, our team has been conducting relevant experiments to understand the effect of VEGFB in skeletal muscle during exercise. Our previous studies found that aerobic exercise promotes VEGFR1, which is a specific receptor of VEGFB expression in obese mice induced by a high-fat diet. Additionally, chronic aerobic exercise can significantly increase the mRNA and protein expression of

VEGFB in the skeletal muscle of rats with heart failure. We also investigated whether EPS promoted the expression of VEGFR1 in C2C12 myotubes incubated with oleic acid and fatty acid. In this experiment, we revealed that the mRNA and protein expression levels of VEGFB in mature C2C12 myotubes increased prominently due to muscle contraction induced by EPS, suggesting that exercise can regulate the expression of VEGFB in muscle cells. Taken together, VEGFB is highly expressed in skeletal muscle, and exercise can induce the expression of VEGFB both in vivo and in vitro, which suggests that VEGFB is a novel myokine regulated by muscle contraction.

Exercise has a strong antiapoptotic effect. Caspase-3, one of the key apoptotic regulators, cleaves myofibrillar proteins, thereby providing substrates for the ubiquitin–proteasome system[26]. Moreover, proapoptotic molecules, such as B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), and antiapoptotic molecules (e.g., Bcl-2) are involved in skeletal muscle apoptosis[27]. Aerobic exercise has been confirmed to inhibit the expression of caspase-3 and Bax and increase the expression of Bcl-2 in TBI-induced apoptotic neuronal cells, indicating a strong effect in resisting apoptosis[28]. HIIT is an effective exercise strategy that has been demonstrated to downregulate the protein levels of caspase-3 and Bax and to upregulate the Bcl-2 and Bcl-2 to Bax ratios in gastrocnemius muscle of 4T1 breast cancer-bearing mice [29]. In the gastrocnemius muscle, 8 weeks of treadmill exercise increased the Bcl-2/Bax ratio but decreased the caspase-3 mRNA content to inhibit skeletal muscle apoptosis in aging rats[30]. Long-term aerobic exercise training also prevents aging-induced apoptosis in cardiac muscles by decreasing Bax and cleaved caspase-3 and increasing Bcl-2 protein levels while decreasing the Bax/Bcl-2 ratio[31]. Kwak et al. revealed that aging may target the Bcl-2 pathway of apoptosis in the heart; however, 12 weeks of exercise in aging reduced caspase levels and the Bax/Bcl-2 ratio by lowering Bax protein expression while increasing Bcl-2 levels compared with the age-matched sedentary group[32].

Electrical pulse stimulation (EPS), an in vitro model for skeletal muscle contraction, has been a major model used for the study of in vitro exercise to understand skeletal muscle and its role in the complex multidirectional crosstalk between different tissues and its implications for metabolic regulation[33]. Nedachi *et al.* reported that EPS for 24 hr at 1 Hz induced visible contraction of myotubes and exercise-like effects[34]. Horie *et al.* reported that EPS (40 V, 1 Hz, 2 ms) markedly decreased the ROS/RNS redox potential and cell viability and increased the expression of the apoptosis marker annexin V in C2C12 myotubes[35]. Zhao *et al.* stimulated C2C12 mouse skeletal muscle cells with EPS, and conditioned medium (CM) was collected after 12 h of EPS at 20 V, 1 Hz, and 2 ms. The phosphorylation of NF- κ B, which is a protein associated with cell inflammation and apoptosis that indicates apoptosis in endothelial cells, was significantly reduced by the CM-EPS[36]. In this experiment, EPS (20 V, 1 Hz, 2 ms) for 12 h significantly downregulated apoptosis indicator, including Bax and cleaved caspase-3, but promoted the antiapoptotic index Bcl-2, suggesting that EPS could repress apoptosis in skeletal muscle cells.

It has been confirmed that exercise stimulates skeletal muscle contraction to secrete a great variety of myokines, which may play important roles in the benefit of exercise[37]. VEGFB is a novel myokine regulated by muscle contraction. However, whether it is involved in the antiapoptotic effect of exercise is

not known. To understand the role of VEGFB in skeletal muscle apoptosis, we used RNAi to interfere with the expression of VEGFB in differentiated C2C12 myotubes. Both the mRNA and protein levels of VEGFB were decreased in myotubes by siRNA. Our results showed that the EPS-mediated prevention of apoptosis weakened when the expression of VEGFB in myotubes was knocked down, suggesting that VEGFB is involved in exercise resistance to skeletal muscle apoptosis.

Conclusion

Exercise like electrical pulse stimulation irritates VEGFB expression in C2C12 myotubes, which plays a positive role in the inhibition of skeletal muscle cell apoptosis induced by TNF- α treatment.

Abbreviations

ANOVA:	Analysis of variance
Bax:	Bcl-2-associated X protein
Bcl-2:	B-cell lymphoma 2
BSA:	Bovine serum albumin
CON:	Control
DMEM:	Dulbecco's modified Eagle's medium
EPS:	Electrical pulse stimulation
FBS:	Fetal bovine serum
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
NC:	Negative control
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PMSF:	Phenylmethylsulfonyl fluoride
PVDF:	Poly(vinylidene fluoride)
siRNA:	Short-interfering RNA
TNF- α :	Tumor Necrosis factor α
VEGFB:	Vascular endothelial growth factor B

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets in the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests. All data were generated in-house and that no paper mill was used.

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Authors' contributions

All authors participated in the design and interpretation of the studies. Specifically, J.Z designed of the study and approved the submitted version, LL.G performed the experiments, analyzed data, and wrote the manuscript. YJ.L performed the experiments and analyzed data. Z.X and JB.Z performed the experiments and consult relevant materials. All authors have read, edited, and approved the final manuscript.

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Figures

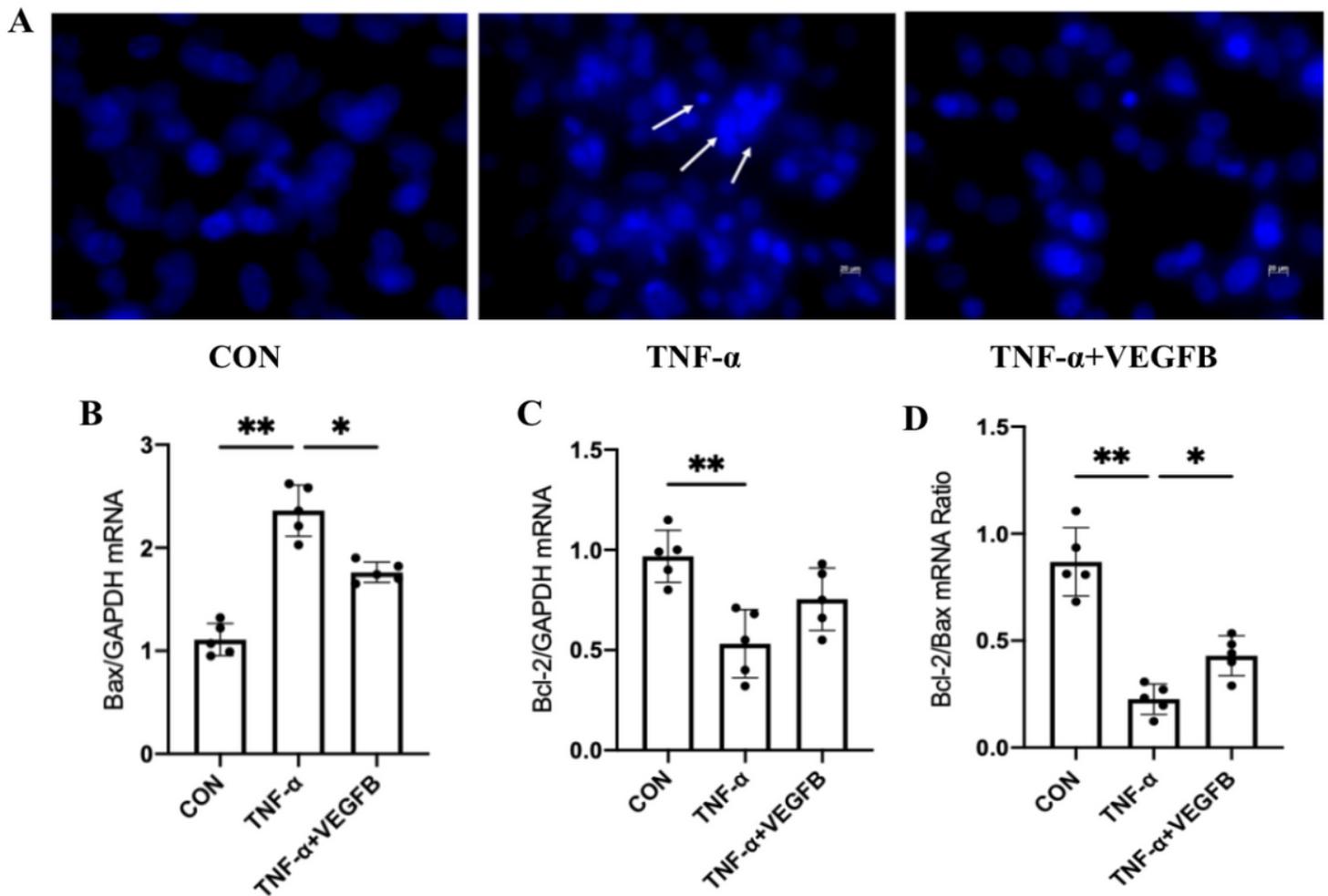


Figure 1

VEGFB inhibited C2C12 myotube apoptosis induced by TNF- α treatment

(A) Fluorescent images of Hoechst-stained differentiated C2C12 myotubes, including the CON, TNF- α treatment at 20 ng/ml for 24 hr and TNF- α coinubation with VEGFB₁₆₇ at 100 ng/ml for 24 hr groups (scale bar, 20 μ m).

(B-C) Total RNA was obtained from each group of differentiated C2C12 myotubes collected immediately after the respective treatments, and the relative mRNA expression levels of Bax and Bcl-2 were determined

by real-time PCR.

(D) Bcl-2 to-Bax mRNA relative ratio for evaluation of the apoptosis status of the cells.

Error bars show the mean \pm SD, n =5 per group. *P<0.05, **P<0.01, ***P<0.001, Student's test.

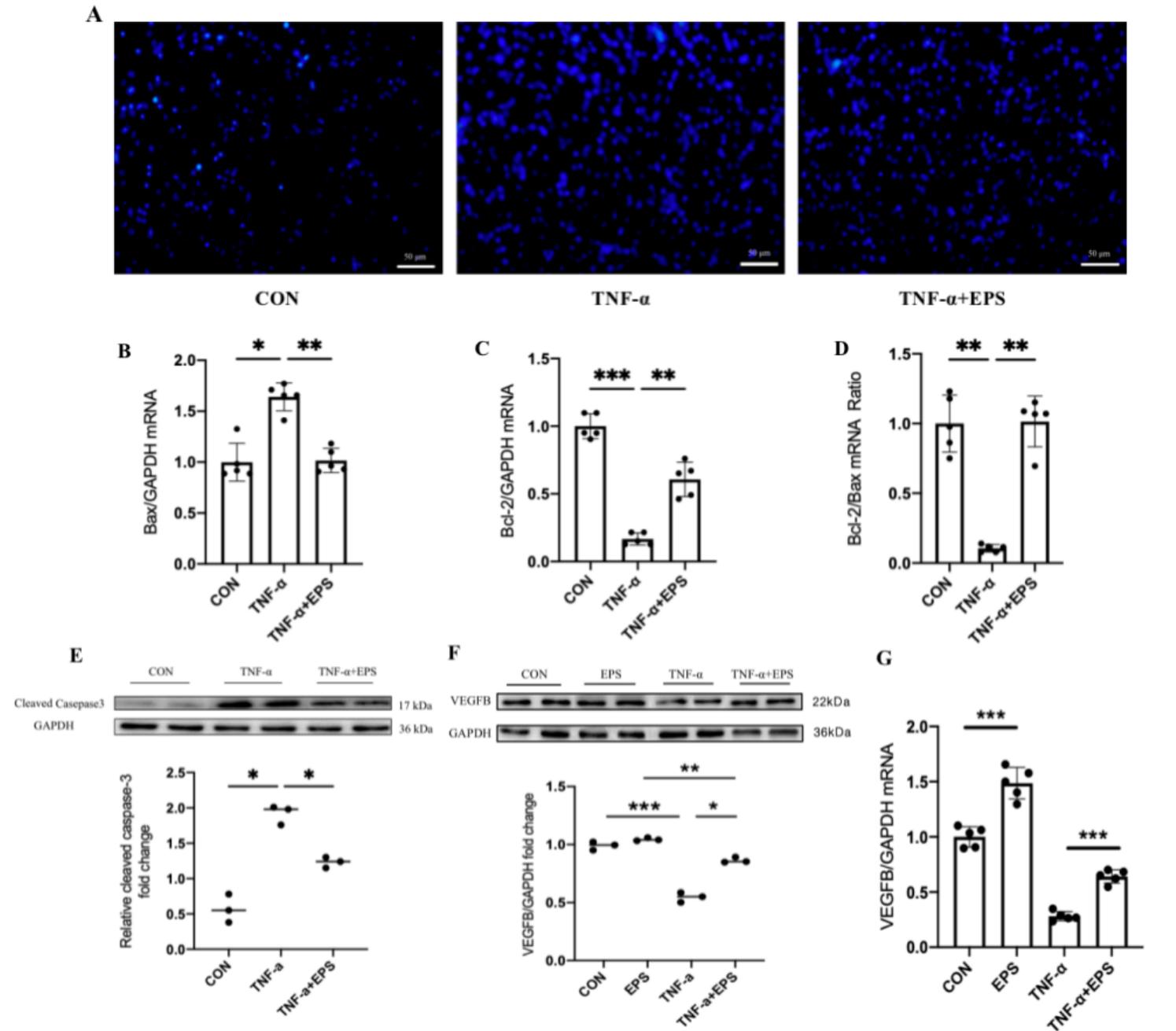


Figure 2

Effect of EPS on C2C12 myotube apoptosis and VEGFB expression

(A) Fluorescent images of Hoechst-stained differentiated C2C12 myotubes, including the CON, TNF- α treatment at 20 ng/ml for 24 hr and TNF- α treatment followed by EPS for 12 hr at 20 V, 1 Hz, 2 ms groups,

(scale bar, 50 μm).

(B-D) Total RNA was collected from each group of differentiated C2C12 myotubes immediately after treatment. The relative mRNA levels of Bax and Bcl-2, base on the value of GAPDH, were determined by real-time PCR analysis, and the Bcl-2-to-Bax mRNA relative ratio were used to evaluate the apoptosis status of cells.

(E) Cleaved caspase-3 and GAPDH were measured by Western blotting. The gray value of cleaved caspase-3 was analyzed based on the gray value of GAPDH. These blots are representative of 3 independent experiments, each containing 2 samples.

(F) The relative protein expression level of VEGFB in each group of differentiated C2C12 myotubes. The gray value was analyzed based on the protein expression of GAPDH. These blots are representative of 3 independent experiments, each containing 2 samples.

(G) The relative mRNA expression level of VEGFB in each group of differentiated C2C12 myotubes.

Error bars show the mean \pm SD, n=5 per group for the mRNA analysis, n=3 per group for the protein analysis. *P<0.05, **P<0.01, ***P<0.001, Student's test.

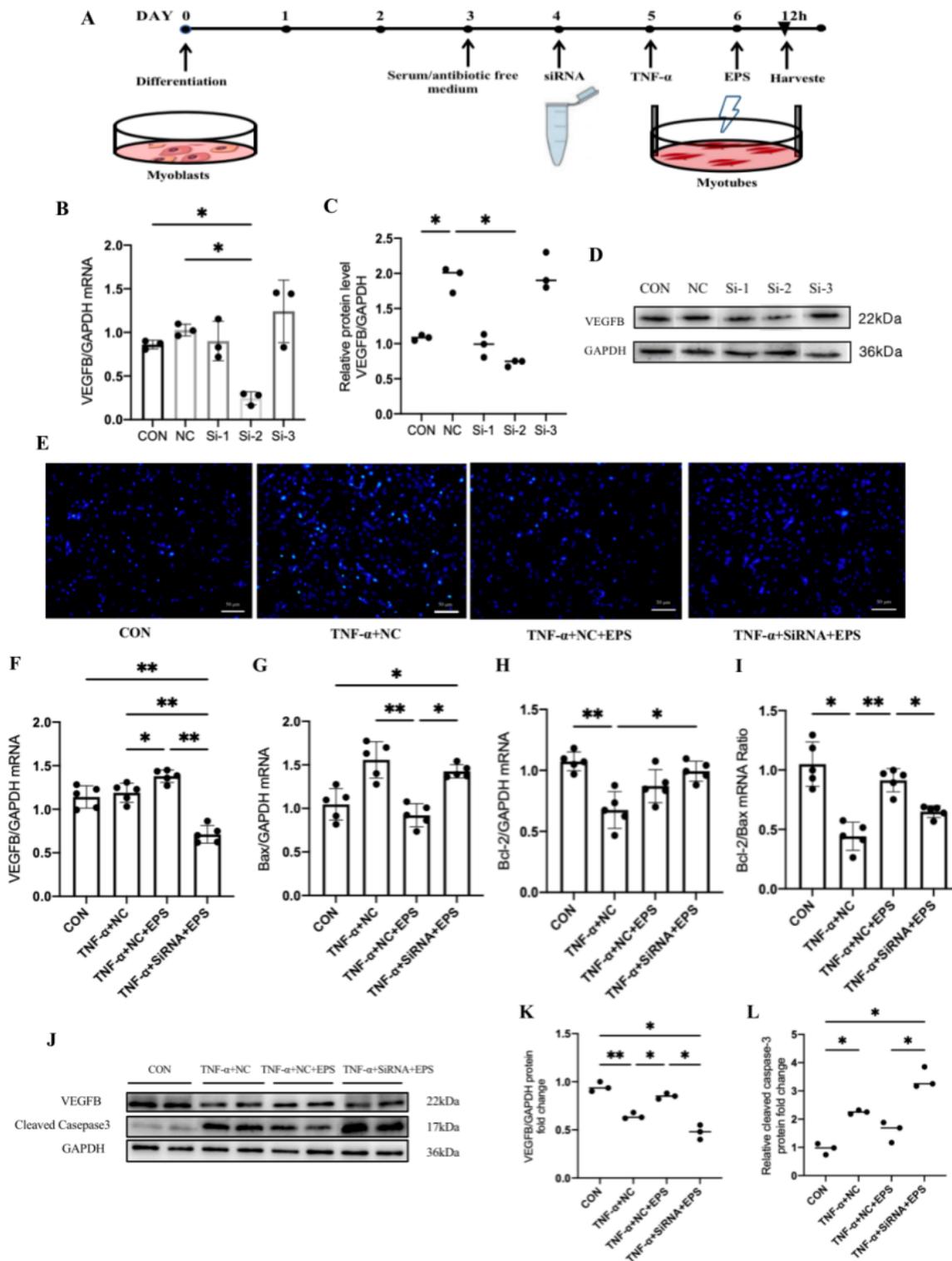


Figure 3

VEGFB in EPS protected against C2C12 myotube apoptosis induced by TNF-α treatment

(A) Experimental process of VEGFB RNA interference in the EPS-induced inhibition of apoptosis in differentiated C2C12 myotubes.

- (B) Cell interference efficiency verification of VEGFB mRNA relative expression in differentiated C2C12 myotubes. The cells were transfected with 50 nM Stealth RNAi duplex or Stealth RNAi Negative Control duplex for 48 hr.
- (C-D) Cell interference efficiency verification of VEGFB protein relative expression in differentiated C2C12 myotubes. The VEGFB protein content was analyzed based on the gray value versus GAPDH. These blots are representative of 3 independent experiments, each containing one sample.
- (E) Fluorescent images of Hoechst-stained differentiated C2C12 myotubes, including the CON, TNF- α +NC, TNF- α +NC+EPS, and TNF- α +siRNA+EPS groups (scale bar, 50 μ m).
- (F) The relative mRNA expression of VEGFB in each group of differentiated C2C12 myotubes.
- (G) The relative mRNA expression of Bax in each group of differentiated C2C12 myotubes.
- (H) The relative mRNA expression of Bcl-2 in each group of differentiated C2C12 myotubes.
- (I) Bcl-2/Bax mRNA relative ratio based on GAPDH to evaluate the apoptosis status of the cells.
- (J) VEGFB, cleaved caspase-3 and GAPDH were measured by western blotting. The gray value of the blots was analyzed based on the gray value of GAPDH. These blots are representative of 3 independent experiments, each containing 2 samples. (K-L) Relative gray value analysis of target proteins based on the gray value of GAPDH. Error bars show the mean \pm SD. *P<0.05, **P<0.01, ***P<0.001, Student's test.