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Developmental endothelial locus-1 attenuates palmitate-induced apoptosis in tenocytes through the AMPK/autophagy-mediated suppression of inflammation and endoplasmic reticulum stress

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Aims

Myokine developmental endothelial locus-1 (DEL-1) has been documented to alleviate inflammation and endoplasmic reticulum (ER) stress in various cell types. However, the effects of DEL-1 on inflammation, ER stress, and apoptosis in tenocytes remain unclear.

Methods

Human primary tenocytes were cultured in palmitate (400 μM) and palmitate plus DEL-1 (0 to 2 μg/ml) conditions for 24 hours. The expression levels of ER stress markers and cleaved caspase 3, as well as phosphorylated 5' adenosine monophosphate-activated protein kinase (AMPK) and autophagy markers, were assessed by Western blotting. Autophagosome formation was measured by staining with monodansylcadaverine, and apoptosis was determined by cell viability assay and caspase 3 activity assay.

Results

We found that treatment with DEL-1 suppressed palmitate-induced inflammation, ER stress, and apoptosis in human primary tenocytes. DEL-1 treatment augmented LC3 conversion and p62 degradation as well as AMPK phosphorylation. Moreover, small interfering RNA for AMPK or 3-methyladenine (3-MA), an autophagy inhibitor, abolished the suppressive effects of DEL-1 on inflammation, ER stress, and apoptosis in tenocytes. Similar to DEL-1, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), an activator of AMPK, also attenuated palmitate-induced inflammation, ER stress, and apoptosis in tenocytes, which 3-MA reversed.

Conclusion

These results revealed that DEL-1 suppresses inflammation and ER stress, thereby attenuating tenocyte apoptosis through AMPK/autophagy-mediated signalling. Thus, regular exercise or administration of DEL-1 may directly contribute to improving tendinitis exacerbated by obesity and insulin resistance.

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Keywords: DEL-1, ER stress, Apoptosis, AMPK, Autophagy, Tenocytes

Article focus

- How does developmental endothelial locus-1 (DEL-1) affect apoptosis in palmitate-treated tenocytes?
- How does DEL-1 affect inflammation and endoplasmic reticulum (ER) stress in palmitate-treated tenocytes?
- How does DEL-1 affect 5' adenosine monophosphate-activated protein kinase

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(AMPK) phosphorylation and autophagy in palmitate-treated tenocytes, and do they participate in the effects of DEL-1?

Key messages

- Developmental endothelial locus-1 (DEL-1) suppresses inflammation and ER stress in palmitate-treated human tenocytes.
- DEL-1 prevents palmitate-induced tenocyte apoptosis.
- The AMPK/autophagy axis contributes to the suppressive effects of DEL-1 on inflammation, ER stress, and apoptosis in tenocytes.

Strengths and limitations

- For the first time, the current study investigates the effects of DEL-1 on inflammation, ER stress, and apoptosis in human tenocytes under hyperlipidemic conditions.
- This study explored molecular pathways regarding the protective effects of DEL-1 on inflammation, ER stress, and apoptosis in palmitate-treated tenocytes.
- Further animal studies should be conducted to investigate the effects of DEL-1 on tendinitis in obese and insulin resistance models.

Introduction

Obesity has been documented as a risk factor for deteriorated recovery ability in patients with tendinopathy due to metabolic stresses, such as chronic inflammation and oxidative stress.[1,2] In the same context, obesity and insulin resistance positively correlate with the development of tendinopathy and aggravated tendon healing.[3,4] Adipose tissue suffering from chronic low-grade inflammation (due to obesity) releases various proinflammatory cytokines leading to apoptosis in various tissues,[5] thereby developing chronic disorders, such as insulin resistance, type 2 diabetes, cardiovascular diseases (CVDs), and autoimmune diseases, which are suggested as risk factors for musculoskeletal diseases.[6-8] In the same context, more apoptotic tendon cells were observed in tendinopathy.[9]

The endoplasmic reticulum (ER), which is evolutionarily conserved in eukaryotic cells, is a cellular protein-folding site responsible for synthesizing, folding, and assembling numerous proteins.[10] However, due to stimuli within and around the cell, excessive protein synthesis may be required or incorrect folding of assembled proteins, resulting in accumulation in the ER. This state is known as ER stress.[11] Prolonged ER stress causes apoptosis.[12] Obesity causes ER stress and inflammation, leading to the development of various chronic diseases.[13,14] Notably, ER stress and apoptosis have been documented to play a causative role in the pathogenesis of tendinopathy.[15,16] Furthermore, Zhang and Kaufman[17] found that ER stress enhances inflammatory responses through several pathways. Therefore, prevention of apoptosis by simultaneously regulating ER stress and inflammation could be a therapeutic strategy for tendinopathy treatment.

Developmental endothelial locus-1 (DEL-1) is identified as a secretory glycoprotein from endothelial cells and a ligand for integrin.[18] Several studies have identified the anti-inflammatory properties of DEL-1 in several cell types. For example, Eskan et al[19] demonstrated that DEL-1 ameliorates inflammation-mediated bone loss. In addition, DEL-1 attenuates inflammatory pulmonary fibrosis.[20] Furthermore, Kwon et al[21] reported that DEL-1 improves insulin resistance in adipocytes through the 5' adenosine monophosphate-activated protein kinas (AMPK)-mediated suppression of inflammation. In contrast, proinflammatory cytokines suppress DEL-1 expression, causing endothelial cell inflammation.[19] Kwon et al[21] recently identified DEL-1 as a myokine through microarray and RNAseq using human skeletal muscle tissue. Moreover, DEL-1 suppresses ER stress in skeletal muscle under hyperlipidemic conditions.[22] Hence, the present study investigated the effects of DEL-1 on inflammation, ER stress, and apoptosis in palmitate-treated tenocytes and explored the underlying molecular mechanisms.

Methods

Tenocyte culture and treatments. Human tenocytes (ZenBio, USA) were cultured with tenocyte culture medium (ZenBio) supplemented with 10% fetal bovine serum (FBS) (HyClone, USA) and 1% antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin) (HyClone) at 37°C in a humidified atmosphere containing 5% CO_2. The cells were cultured on collagen type I-coated (Sigma-Aldrich, USA) culture plates and rinsed with phosphate-buffered saline (PBS). Tenocytes were subcultured when they reached approximately 90 to 95% confluence. Tenocytes at passages 4 or 5 were used and confirmed to be free of contamination with mycoplasma. Sodium palmitate (Sigma-Aldrich) was conjugated with 5% bovine serum albumin (fatty acid-free grade; Sigma-Aldrich). Human recombinant DEL-1 (Abcam, USA) was reconstituted in PBS. Tenocytes were treated with bovine serum albumin (BSA)-conjugated palmitate (400 μM), DEL-1 (0 to 2 μg/ml), 3-methyladenine (3-MA) (0.5 mM) (Sigma-Aldrich), and/or 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (0.1 mM) (Sigma-Aldrich) at the same time for 24 hours. BSA was used as a vehicle control.

Total protein extraction and Western blotting analysis. Cells were washed twice with PBS and then scraped with cell scrapers for harvesting. Harvested cells were suspended in PRO-PREP (iNtRON Biotechnology, South Korea) and incubated at 4°C for one hour. The cell suspension was centrifuged at 13,000 rpm for 30 minutes at 4°C to collect supernatants as total proteins. Total protein extracts (30 μg/well) containing sodium dodecyl-sulphate (SDS) loading buffer (Biosesang, South Korea) were separated by performing 7 or 12% SDS–polyacrylamide gel electrophoresis (PAGE). Total

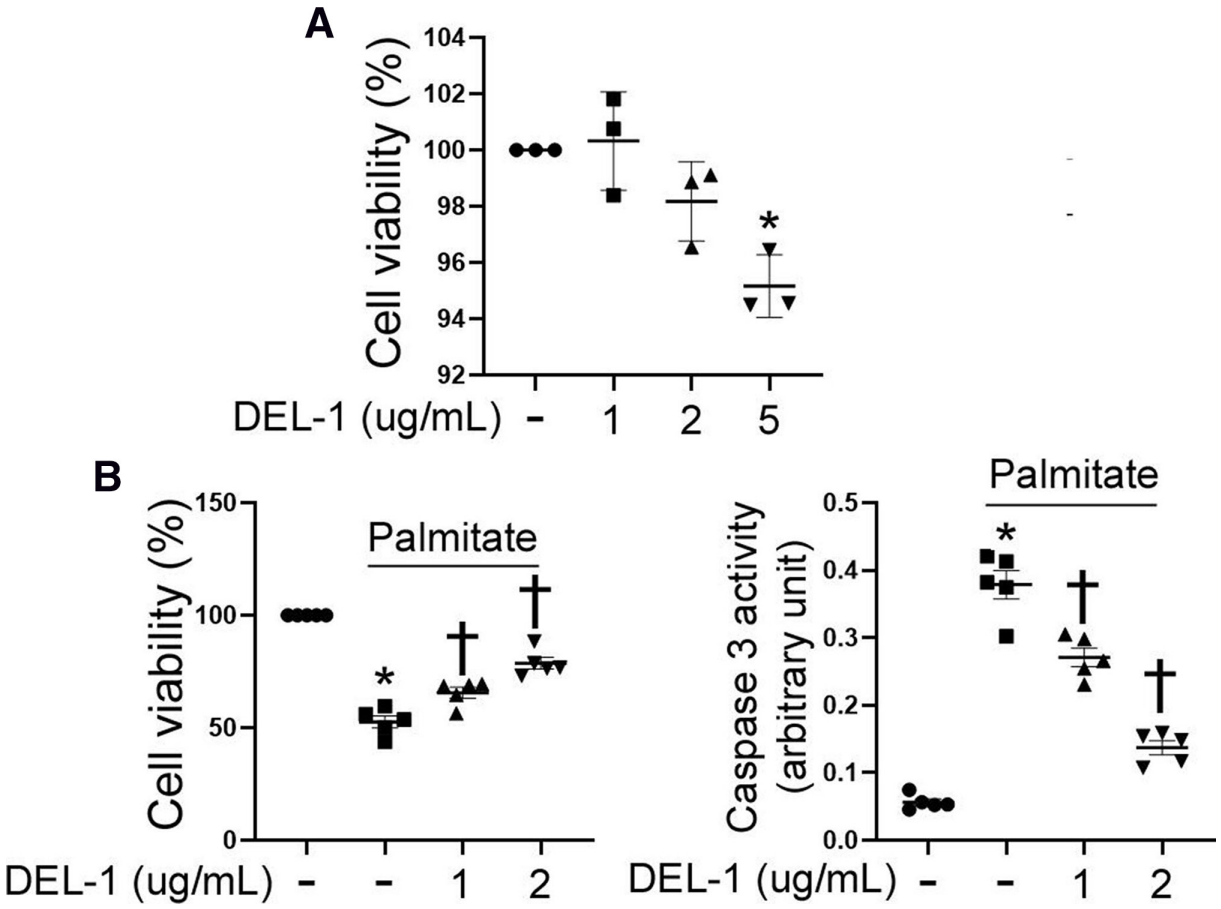

Fig. 1

Developmental endothelial locus-1 (DEL-1) prevents apoptosis in palmitate-treated tenocytes. a) Cell viability assay in tenocytes treated with DEL-1 (0 to 2 μg/ml) for 24 hours. b) Cell viability assay and caspase 3 activity assay in tenocytes treated with palmitate (400 μM) and DEL-1 (0 to 2 μg/ml) for 24 hours. Means and standard deviations were calculated from three or five independent experiments. Significance ($p < 0.05$). *: vs control.†: vs palmitate.

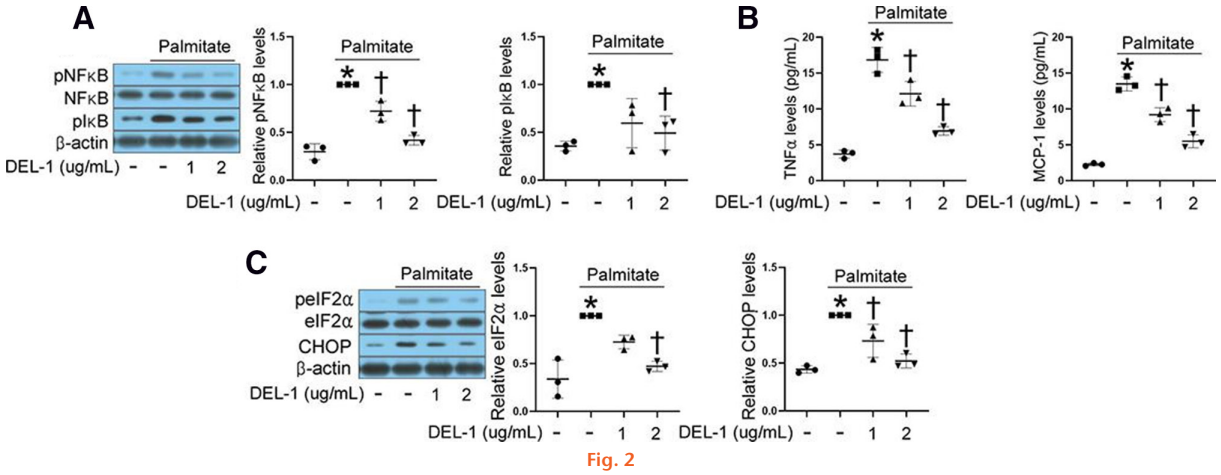

Fig. 2

Developmental endothelial locus-1 (DEL-1) suppresses inflammation and endoplasmic reticulum (ER) stress in tenocytes treated with palmitate. a) Western blotting of NFκB and IκB phosphorylation in tenocytes treated with palmitate (400 μM) and DEL-1 (0 to 2 μg/ml) for 24 hours. b) Enzyme-linked immunosorbent assay for determining tumour necrosis factor alpha (TNFα) and monocyte chemoattractant protein-1 (MCP-1) concentrations in the culture medium of tenocytes treated with palmitate (400 μM) and/or DEL-1 (0 to 2 μg/ml) for 24 hours. c) Western blotting of eIF2α phosphorylation and CHOP expression in tenocytes treated with palmitate (400 μM) and/or DEL-1 (0 to 2 μg/ml) for 24 hours. Means and standard deviations were calculated from three independent experiments. Significance ($p < 0.05$). *: vs control. †: vs palmitate.

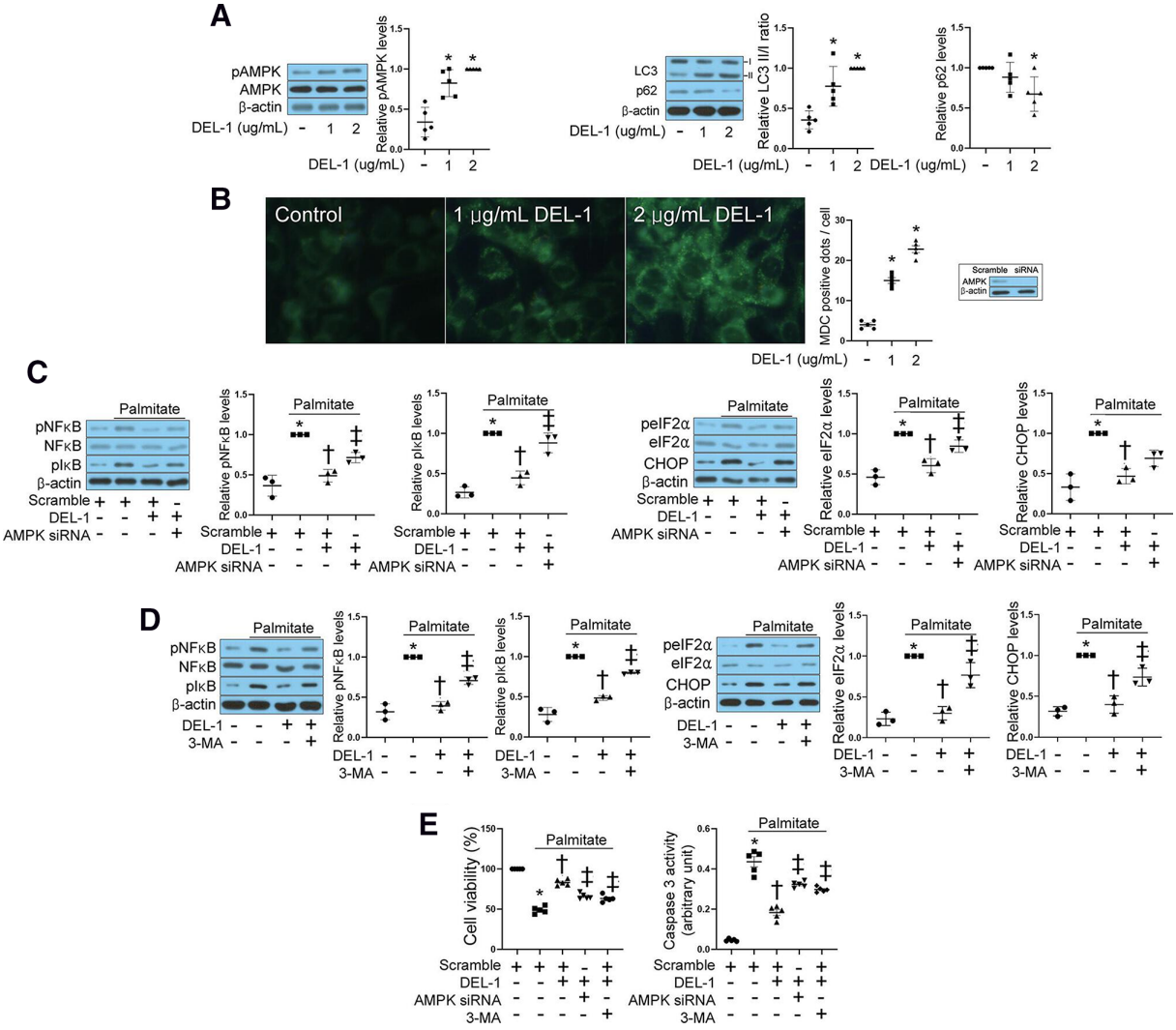

Fig. 3

The phosphorylated 5' adenosine monophosphate-activated protein kinase (AMPK)/autophagy axis participates in the effects of developmental endothelial locus-1 (DEL-1) on inflammation, endoplasmic reticulum (ER) stress, and apoptosis in palmitate-treated tenocytes. a) Western blotting of AMPK phosphorylation as well as LC3 and p62 expression and b) monodansylcadaverine (MDC) staining in tenocytes treated with DEL-1 (0 to 2 μg/ml) for 24 hours. c) Western blotting of phospho-NFκB, IκB, eIF2α, and CHOP expression in AMPK small interfering RNA (siRNA)-transfected tenocytes treated with palmitate (400 μM) and/or DEL-1 (2 μg/ml) for 24 hours. d) Western blotting of phospho-NFκB, IκB, eIF2α, and CHOP expression in tenocytes treated with palmitate (400 μM), 3-MA (0.5 mM), and/or DEL-1 (2 μg/ml) for 24 hours. e) Cell viability assay and caspase 3 activity assay in AMPK siRNA-transfected tenocytes treated with palmitate (400 μM), 3-MA (0.5 mM), and/or DEL-1 (2 μg/ml) for 24 hours. Means and standard deviations were calculated from three independent experiments. Significance ($p < 0.05$). *: vs control. †: vs palmitate. ‡: vs palmitate plus DEL-1.

protein-transferred nitrocellulose membranes were blocked with 5% skim milk solution, reacted with primary antibodies, and then bound with a matched secondary antibody. A detectable signal was generated following the binding of an antibody specific to the protein of interest. Information on the antibodies used is provided as follows: anti-phospho NFκB (1:1,000), anti-phospho IκB (1:2,000), anti-phospho eIF2α (1:1,000), anti-phospho AMPK (1:1,000), anti-AMPK (1:2,000), and anti-p62 (1:2,000) antibodies were procured from Cell Signalling Technology (Billerica, USA). Anti-NFκB (1:2,000), anti-eIF2α (1:2,000), and anti-β-actin (1:5,000) antibodies were purchased from Santa Cruz Biotechnology (USA). Anti-LC3 (1:2,000) antibody was acquired from Novus Biologicals (Littleton, USA).

Measurement of proinflammatory cytokine concentrations. The supernatants of tenocyte cultures were collected after experimental treatments. The samples were stored at -80°C for the subsequent experiments. The concentrations of tumour necrosis factor alpha (TNFα) and monocyte chemoattractant protein-1 (MCP-1) in culture supernatants were quantitated using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA) according to their protocols.

Autophagosome staining and counting. Autophagosome formation was determined by monodansylcadaverine

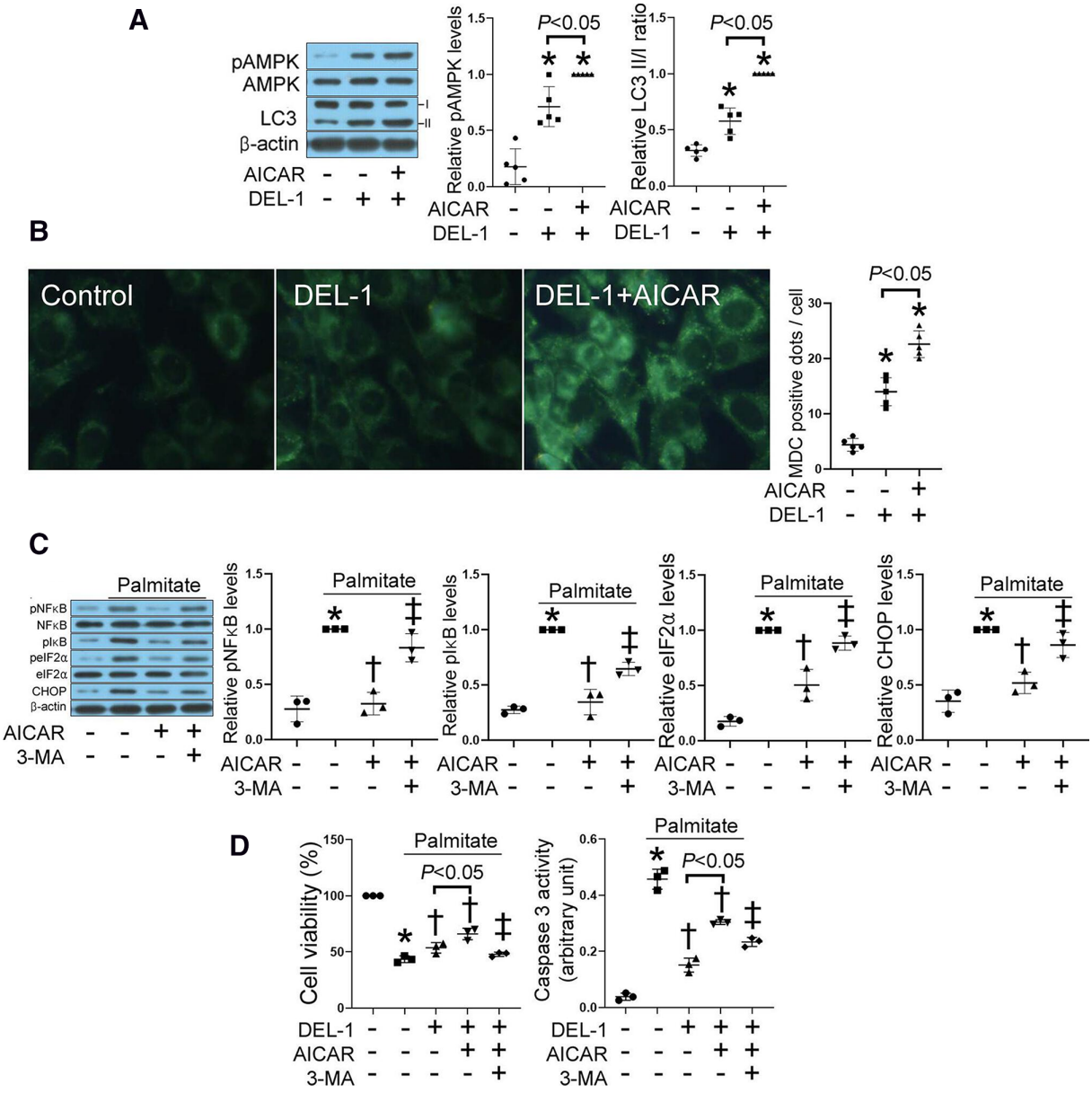

Fig. 4

Stimulation of phosphorylated 5' adenosine monophosphate-activated protein kinase (AMPK)/autophagy signalling plays a crucial role in the effects of developmental endothelial locus-1 (DEL-1) on inflammation and endoplasmic reticulum (ER) stress, as well as apoptosis in palmitate-treated tenocytes. a) Western blotting of phospho-AMPK and LC3 expression and b) MDC staining in tenocytes treated with DEL-1 (1 µg/ml) and/or AICAR (0.1 mM) for 24 hours. c) Western blotting of phospho-NFκB, IκB, eIF2α, and CHOP expression in tenocytes treated with palmitate (400 µM), 3-MA (0.5 mM), and/or AICAR (0.1 mM) for 24 hours. d) Cell viability assay and caspase 3 activity assay in tenocytes treated with palmitate (400 µM), 3-MA (0.5 mM), DEL-1 (1 µg/ml), and AICAR (0.1 mM) for 24 hours. Means and standard deviations were calculated from three independent experiments. Significance ($p < 0.05$). *: vs control. †: vs palmitate. ‡: vs palmitate plus 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) or palmitate, DEL-1, plus AICAR.

(MDC) staining. Tenocytes were incubated with dansylcadaverine (0.2 mM) (Sigma-Aldrich) for ten minutes at 37°C and observed through green fluorescence microscopy. Autophagosomes (green dots) were counted in a randomly selected cell.

Suppression of gene expression. Following the manufacturer's directions, tenocytes were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, USA) to suppress target gene expression. Small interfering (si) RNA oligonucleotides (20 nmol/l) specific for AMPK (Santa Cruz Biotechnology) were applied. Scrambled siRNA (Santa Cruz Biotechnology) was used as a control.

Determination of apoptosis. Cell viability was determined by performing an MTT assay. Experimental tenocyte cultures in 96-well plates were incubated with MTT solution (Sigma-Aldrich) for two hours at 37°C. The red formazan inside the cells was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) for ten minutes at room temperature with gentle agitation. The optical density

was measured using a microplate reader (Bio-Rad, USA) at 550 nm. A caspase 3 activity assay was conducted using a colorimetric caspase 3 activity assay kit (Abcam).

Statistical analysis. All statistical analyses were performed using GraphPad Prism version 6 software (USA). The results are presented as the relative ratio compared to the highest values (mean and standard deviation (SD)). The quantifiable results of caspase 3 activity, cytokine levels, and autophagosome staining are presented as the absolute values (mean and SDs). All experiments were independently performed three or five times. Statistical significance was assessed by one-way repeated analysis of variance (ANOVA) and Tukey post hoc tests. Protein expression levels were quantitated using ImageJ software (National Institutes of Health, USA). A p-value less than 0.05 indicates statistical significance.

Results

DEL-1 alleviates apoptosis in tenocytes treated with palmitate. Sun et al[22] first determined the DEL-1 treatment concentration by performing a cell viability assay. DEL-1 at a concentration rate of 0 to 2 μg/ml did not show cell toxicity in tenocytes (Figure 1a). We next examined the effect of DEL-1 on apoptosis in palmitate-treated tenocytes. A 400 μM palmitate significantly ($p < 0.001$, one-way ANOVA and Tukey post hoc test) reduced cell viability and increased caspase 3 activity. However, treatment with DEL-1 dose-dependently reversed these changes in tenocytes (Figure 1b).

DEL-1 ameliorates inflammation and ER stress in palmitate-treated tenocytes. Treatment with DEL-1 attenuated palmitate-induced expression of inflammatory markers, such as phospho-NFκB and IκB (Figure 2a). The release of proinflammatory cytokines, such as TNFα and MCP-1, from palmitate-treated tenocytes was also suppressed by DEL-1 treatment (Figure 2b). Furthermore, treatment of tenocytes with DEL-1 dose-dependently ameliorated ER stress markers, such as phospho-eIF2α and C/EBP homologous protein (CHOP), in the presence of palmitate (Figure 2c).

DEL-1 suppresses inflammation and ER stress via AMPK/autophagy signalling, resulting in apoptosis in tenocytes. AMPK[23] and autophagy[24] have been reported to suppress inflammation and ER stress in various cell types. Moreover, AMPK directly increases autophagy.[25] DEL-1 augmented AMPK phosphorylation and autophagy markers, such as LC3 conversion and p62 degradation, as well as autophagosome formation in a dose-dependent fashion (Figures 3a and 3b). Small interfering RNA (siRNA)-mediated inhibition of AMPK or 3-methyladenine (3-MA) abrogated the effects of DEL-1 on inflammation and ER stress as well as apoptosis in palmitate-treated tenocytes (Figures 3c to 3e).

The AMPK/autophagy pathway mainly participates in the effects of DEL-1 on apoptosis in tenocytes. Treatment with AICAR, an AMPK activator, enhanced the effects of DEL-1 on LC3 conversion and p62 degradation, as well as the formation of autophagosomes (Figures 4a and 4b). Similar to the effects of DEL-1, AICAR treatment ameliorated palmitate-induced markers of inflammation and ER stress as well as apoptosis in tenocytes. However, 3-MA mitigated the effects of AICAR in tenocytes (Figure 4c). AICAR treatment amplified the effects of DEL-1 on apoptosis. However, 3-MA partially reversed these changes (Figure 4d).

Discussion

Herein, we confirmed the following novel results: 1) DEL-1 ameliorates apoptosis in palmitate-treated tenocytes; 2) DEL-1 suppresses palmitate-treated inflammation and ER stress in tenocytes; 3) DEL-1 increases AMPK phosphorylation and autophagy markers; and 4) AMPK siRNA or 3-MA abolishes the effects of DEL-1 on inflammation and ER stress as well as apoptosis in tenocytes. These results show the potential of DEL-1 for treating obesity or insulin resistance associated with tendinopathy.

Apoptosis is a form of 'programmed' cell death that contributes to cellular homeostasis. However, excessive apoptosis in a tendon causes chronic diseases, including tendinopathy. For instance, in these two clinical studies, Lian et al[26] showed that increased apoptotic cell death is a hallmark of patellar tendinosis. Furthermore, it was suggested that the regulation of apoptosis might have an influential role in treating tendinopathy.[27] Moreover, Lundgreen et al[28] showed the general relevance of the rotator cuff in the presence of supraspinatus rupture and the potential role of both p53-dependent and p53-independent apoptosis. Therefore, these studies reveal that apoptosis in tenocytes exacerbates the severity of tendinopathy. This study found that treatment with DEL-1 recovered cell viability and caspase 3 activity in palmitate-treated tenocytes. These results may introduce DEL-1 as a potential therapeutic agent for tendinopathy in an obese state.

AMPK is a phylogenetically conserved serine/threonine kinase responsible for maintaining cellular energy homeostasis by sensingadenosine monophosphate (AMP) and the adenosine diphosphate (ADP)/adenosine triphosphate (ATP) ratio.[29] Several studies have suggested AMPK as a therapeutic target for various metabolic diseases.[30,31] AMPK has been documented to have anti-inflammatory effects. In the same context, many studies have demonstrated that AMPK activation ameliorates metabolic disorders by suppressing inflammation and ER stress. Kim et al[32] reported that valdecoxib ameliorates skeletal muscle insulin resistance through AMPK-mediated suppression of inflammation and ER stress. Additionally, ginsenoside Rb2 activates AMPK-mediated suppression of inflammation and ER stress, thereby attenuating atherosclerotic responses.[33] Although there is controversy about the effect of AMPK on apoptosis, several studies have supported the idea that AMPK might have a protective effect against apoptosis under inflammatory or ER

stress conditions. In this way, AMPK activation by AICAR ameliorates apoptosis in human bronchial cells treated with cigarette smoke extracts by relieving ER stress.[34] The current study demonstrated that treatment with DEL-1 augmented AMPK phosphorylation in tenocytes. Furthermore, AMPK siRNA abolished the effects of DEL-1 on inflammation, ER stress, and apoptosis in tenocytes in the presence of palmitate. These results reveal that DEL-1 attenuates inflammation and ER stress through an AMPK-dependent pathway, thereby ameliorating apoptosis in palmitate-treated tenocytes.

Autophagy plays a central role in lysosomal catabolic signalling by degrading cellular proteins and small broken organelles,[35] and serves as a defense mechanism against inflammation and ER stress.[36] Furthermore, autophagy is positively regulated by AMPK through the phosphorylation of autophagy-related proteins.[25] In this context, Ansari et al[37] suggested that Butein stimulates autophagy via AMPK-mediated signalling to suppress chondrocyte inflammation. Pyun et al[38] reported that kynurenic acid alleviates nonalcoholic fatty liver disease via AMPK/autophagy-associated suppression of ER stress. In addition, aucubin prevents apoptosis in osteoblasts via the AMPK-regulated autophagy pathway.[39] Therefore, we selected autophagy as a downstream signalling pathway of AMPK to resolve palmitate-induced apoptosis in tenocytes. DEL-1 treatment upregulates autophagy markers and autophagosome biogenesis. 3-MA reversed DEL-1-mediated suppression of inflammation, ER stress, and apoptosis in palmitate-treated tenocytes. Therefore, these results suggest that the AMPK/autophagy axis participates in the effects of DEL-1 on inflammation, ER stress, and apoptosis in tenocytes under hyperlipidemic conditions.

To reconfirm the central role of AMPK/autophagy signalling in DEL-1-related effects in tenocytes, AICAR was added in the presence of DEL-1. AICAR treatment amplified the effect of DEL-1 on AMPK phosphorylation and autophagy markers. AICAR treatment ameliorated palmitate-induced inflammation and ER stress, whereas 3-MA reversed these changes. AICAR treatment enhanced the effects of DEL-1 on cell viability and caspase 3 activity in palmitate-treated tenocytes. Moreover, the amplified effects of DEL-1 by AICAR were partially mitigated by 3-MA. These results indicate that the AMPK/autophagy axis mainly contributes to the effects of DEL-1 in palmitate-treated tenocytes.

This study demonstrates that DEL-1 improves inflammation and ER stress, thereby attenuating tenocyte apoptosis under hyperlipidemic conditions through AMPK-regulated autophagy-mediated signalling (Supplementary Figure a). Of course, to reinforce this study's conclusions, it is necessary to reproduce the in vitro results through animal experiments. Finally, the present study provides a basis for developing therapeutic agents for tendinopathy in patients with obesity or insulin resistance by stimulating muscle DEL-1 secretion through regular exercise or by administration of DEL-1. Moreover, t will be more effective if the use of DEL-1 is additionally considered in the previously reported treatment methods[40–43] for musculoskeletal disorders.

Supplementary material

A schematic diagram showing how developmental endothelial locus-1 affects apoptosis in tenocytes under hyperlipidemic conditions.

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