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Nerve growth factor stimulation of ERK1/2 phosphorylation requires both p75^{NTR} and α9β1 integrin and confers myoprotection towards ischemia in C2C12 skeletal muscle cell model

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ABSTRACT

The functions of nerve growth factor (NGF) in skeletal muscles physiology and pathology are not clear and call for an updated investigation. To achieve this goal we sought to investigate NGF-induced ERK1/2 phosphorylation and its role in the C2C12 skeletal muscle myoblasts and myotubes. RT-PCR and western blotting experiments demonstrated expression of p75^{NTR}, α9β1 integrin, and its regulator ADAM12, but not trkA in the cells, as also found in gastrocnemius and quadriceps mice muscles. Both proNGF and βNGF induced ERK1/2 phosphorylation, a process blocked by (a) the specific MEK inhibitor, PD98059; (b) VLO5, a MLD-disintegrin with relative selectivity towards α9β1 integrin; and (c) p75^{NTR} antagonists Thx-B and LM-24, but not the inactive control molecule backbone Thx. Upon treatment for 4 days with either anti-NGF antibody or VLO5 or Thx-B, the proliferation of myoblasts was decreased by 60–70%, 85–90% and 60–80% respectively, indicative of trophic effect of NGF which was autocrinically released by the cells. Exposure of myotubes to ischemic insult in the presence of βNGF, added either 1 h before oxygen-glucose-deprivation or concomitant with reoxygenation insults, resulted with about 20% and 33% myoprotection, an effect antagonized by VLO5 and Thx-B, further supporting the trophic role of NGF in C2C12 cells. Cumulatively, the present findings propose that proNGF and βNGF-induced ERK1/2 phosphorylation in C2C12 cells by functional cooperation between p75^{NTR} and α9β1 integrin, which are involved in myoprotective effects of autocrine released NGF. Furthermore, the present study establishes an important trophic role of α9β1 in NGF-induced signaling in skeletal muscle model, resembling the role of trkA in neurons. Future molecular characterization of the interactions between NGF receptors in the skeletal muscle will contribute to the understanding of NGF mechanism of action and may provide novel therapeutic targets.

1. Introduction

The physiology of skeletal muscle is controlled by a variety of growth factors such as insulin-like growth factor. These growth factors are responsible during development for myoblasts differentiation toward myotubes and upon maturation they regulate skeletal muscle contractility [1]. Increasing evidence suggests that the family of neurotrophic factors not only modulates survival and function of innervating motor neurons and proprioceptive neurons but also development and differentiation of myoblasts and muscle fibers. Neurotrophins and their receptors play a role in the coordination of muscle innervation and functional differentiation of neuromuscular junctions. However, neurotrophin receptors are also expressed in differentiating muscle cells, in particular at early developmental stages in myoblasts before they fuse. In adults with pathological conditions such as human degenerative and inflammatory muscle disorders, variations of neurotrophin expression are found, but the role of neurotrophins under such conditions is still not clear [2].

Abbreviations: ADAM12, disintegrin and metalloproteinase domain-containing protein 12; α9β1, integrin receptor composed of α9 and β1 sub-units; C2C12, a clonal cell derived from mouse skeletal muscle; EC_{50}, effective concentration 50%; ELISA, enzyme-linked immunosorbent assay; ERK1/2, mitogen-activated protein kinase type 1 or 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; K252a, $C_{27}H_{21}N_3O_5$, a TrkA inhibitor; LDH, lactate dehydrogenase; LM-24, 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1*H*-purin-7(6*H*)-yl)-*N*-(3-(dimethylamino)propyl)acetamide; myoD, myogenic regulatory factor type D; NGF, nerve growth factor; OGD, oxygen-glucose deprivation; p75^{NTR}, nerve growth factor receptor of 75 kDa; PC12, a pheochromocytoma tumor cell clone; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one, inhibitor of MEK; Reoxy, reoxygenation; Thx, 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1*H*-purin-7(6*H*)-yl) acetic acid; Thx-B, 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1*H*-purin-7(6*H*)-yl)-*N*-isopropyl-*N*-(isopropylcarbomyl) acetamide; trkA, a nerve growth factor tropomyosin-receptor-kinase; VLO5, *Vipera lebetina obtusa* disintegrin 5.

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Nerve growth factor (NGF), the prototype of the neurotrophins family of growth factors, is a conserved polypeptide neurotrophin, which plays a crucial role in the sympathetic and sensory nervous systems [3]. The biological functions of NGF are mediated through two classes of cell surface receptors: the p75 neurotrophin receptor ($p75^{NTR}$), common to all members of the neurotrophin family, and the tropomyosine kinase related receptor (trkA), belonging to the tyrosine kinase-neurotrophin receptor family [4]. The canonical MAPK signaling pathway induced by NGF [5] is involved in the majority of NGF's "neuronal effects" such as survival, proliferation, differentiation, growth, and maturation [6,7]. Recent studies described function and signaling of NGF in non-neuronal tissues such as smooth muscles, immune, endothelial cells and cardiomyocytes [8–11]. However, NGF receptors signaling and their role in skeletal muscle remain obscure. Emerging novel studies propose that the integrin α9 represents an additional receptor component for NGF in non-neuronal cells [12–15] proposing an interaction with the promiscuous neurotrophin $p75^{NTR}$ receptor [16].

The skeletal muscle tissue synthesizes and secretes NGF [17,18] and the expression of NGF and its $p75^{NTR}$ receptor in myoblasts is developmentally regulated during myogenesis [19]. Also, $p75^{NTR}$ and NGF binding sites have been observed in the developing muscle of chick and human embryos [20,21]. However, the expression of NGF, trkA, and $p75^{NTR}$ has been observed at relatively low levels, in adult rodent and human skeletal muscle [22–24] and their characteristics in different skeletal muscle fibers is unknown. Interestingly, an increased amount of NGF was measured in genetically dystrophic mice [25] and $p75^{NTR}$ was shown to be expressed in regenerating muscle fibers from patients affected by Duchene muscular dystrophy [22], proposing a role for NGF and its receptors in dystrophic muscle regeneration. Rat pups that were injected with anti-NGF as fetuses showed a reduction in the leg muscles size [26], resembling the anti-NGF transgenic mice [27]. These studies call for a re-evaluation of NGF receptor identity and signaling in skeletal muscles as a basic corner-stone before progressing with experimentation on skeletal muscle dystrophies and regeneration processes.

C2C12 mouse skeletal muscle myoblast cellular model expresses $p75^{NTR}$ and its expression is associated with signals promoting the myogenic program [28], survival and muscle strength [29]. trkA receptor was not found in C2C12 [28] thus providing a model system suitable for deciphering $p75^{NTR}$-related effects. Since the molecular mechanism of NGF action in skeletal muscle is unknown, the utilization of C2C12 model provides a suitable, non-genetically manipulated, pharmacological model to investigate *in vitro* how $p75^{NTR}$ induces signal transduction in the absence of trkA, and how it contributes to cell survival.

Integrin α9β1 was originally discovered in skeletal muscle and its presence was identified in other tissues as well [30]. Multiple ligands have been identified for this integrin, including the extracellular matrix proteins, such as tenascin C [31,32], osteopontin [33], thrombospondin-1 [34] and some members of ADAMs family (a disintegrin and metalloprotease) such as ADAM12 [35]. Both α9 integrin and its regulator ADAM12 are constitutively expressed during human myogenic cell differentiation and mediate a cell–cell interaction selectively involved in fusion of mononucleated myoblasts to myotubes [35]. ADAM12 expression parallels fusion and myogenin phenotype of C2C12 myogenic cells [36]. Therefore, we hypothesized that cells lacking trkA receptors, such as C2C12, but express $p75^{NTR}$, α9 and ADAM12, may respond to NGF by functional cooperation of $p75^{NTR}$ and α9 resulting with activation of MAPK phosphorylation pathway. To confirm this hypothesis we took advantage of two different classes of pharmacological tools: i) VLO5, a selective disintegrin antagonist of α9β1 integrin [37] and ii) Thx-B derivatives, small non-peptidic antagonists of $p75^{NTR}$ [38,39]. In the present study we provide evidences for the functional interaction between α9β1 integrin and $p75^{NTR}$ in stimulation of ERK1/2 phosphorylation, demonstrate autocrine release of NGF from the cells and characterize the involvement of NGF receptors in cell proliferation and myoprotection towards ischemia. We propose a functional model of NGF signaling in C2C12 skeletal muscle cells, in comparison to the neuron.

2. Materials and methods

2.1. Materials

Mouse β-NGF (βNGF) purified from murine submaxillary glands according to the procedure described earlier [40] and wild type human recombinant proNGF expressed in *Escherichia coli* and anti-NGF rabbit antibody were kindly provided by Alomone Labs, Jerusalem, Israel. The following polyclonal and monoclonal antibodies were used: anti-myogenin, anti-myoD, Anti-GAPDH, anti-β1, anti-ADAM12 and anti-trkA (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA), anti-fibronectin (Sigma-Aldrich, St. Louis, MO, USA), anti-$p75^{NTR}$ (Covance, La Jolla, CA, USA), anti-α9 (Chemicon, Temecula, CA, USA), phospho-specfic and pan-anti ERK1/2 (Cell Signaling Technology, Inc. Danvers, MA). Horseradish-peroxidase conjugated goat anti-rabbit or anti-mouse secondary antibodies were obtained from Jackson ImmunoResearch, West Grove, PA, USA. K252a was kindly provided by Fermentek, Jerusalem, Israel. PD98059 was purchased from Biomol Research Laboratories, Inc. Plymouth Meeting, PA, USA. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), glutamine, antibiotics: penicillin and streptomycin were purchased from Beit Haemek, Afula, Israel.

2.2. Pharmacological tools

VLO5 heterodimeric disintegrin was purified by two HPLC steps of chromatography from the *Vipera lebetina obtusa* venom, purchased from Latoxan Co. (Valence, France). VLO5 was found homogenous by SDS–PAGE electrophoresis and selective for its anti-α9β1 integrin activity as tested in α9K562 cell system, which overexpresses α9β1 integrin [37].

LM-24, a derivative of caffeine was synthesized and characterized according to Massa et al., 2006. Thx-B, (1,3-diisopropyl-1-[2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-purin-7-yl)-acetyl]-urea) and its derivatives of theophylline were synthesized by coupling theophylline-7-acetic acid to *N,N′*-diisopropyl carbodiimide in dimethyl formamide for 4 h at 60 °C. After cooling the solvent was evaporated in vacuum. The residue was dissolved in chloroform and purified by chromatography on Silica Gel 60A with chloroform:methanol (98/2), yielding 44% of the compound as a white powder. The structure was confirmed by LC/MS and NMR [38,39]. The compounds were dissolved in DMSO to generate stock solution of 4 mM.

2.3. Dissection of skeletal muscles from mice

Wild type mice (C57BL/6J) were maintained under standard conditions, 23 ± 1 °C, 12 h light cycle (7 a.m.–7 p.m.) with ad libitum access to food and drink at the Hebrew University germ free (SPF) animal housing facility. At the age of 10 weeks they were sacrificed after anesthesia with ketamine–xylazine. Tibialis anterior, gastrocnemius and quadriceps muscles were dissected. Muscle samples were frozen in liquid nitrogen and stored at −80 °C till use. The experiments were approved by the Hebrew University Animal Care and Use Committee.

2.4. Cell culture

C2C12 mouse myoblasts cell line was originally developed as a cell line which rapidly differentiates under serum starvation, forming contractile myotubes and producing characteristic muscle proteins [41]. C2C12 cells were grown in growth medium containing DMEM, supplemented with 20% FBS, 2 mM L-glutamine 100 μg/ml streptomycin, and 10,000 U/ml penicillin (Beit Haemek, Afula, Israel). The cultures were maintained in an incubator at 37 °C in a humidified atmosphere of