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A preliminary study on the role of Piezo1 channels in myokine release from cultured mouse myotubes

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ABSTRACT

It has long been known that regular physical exercise induces short and long term benefits reducing the risk of cardiovascular disease, diabetes, osteoporosis, cancer and improves sleep quality, cognitive level, mobility, autonomy in enderly. More recent is the evidence on the endocrine role of the contracting skeletal muscle. Exercise triggers the release of miokines, which act in autocrine, paracrine and endocrine ways controlling the activity of muscles but also of other tissues and organs such as adipose tissue, liver, pancreas, bones, and brain. The mechanism of release is still unclear.

Neuromuscular electrical stimulation reproduces the beneficial effects of physical activity producing physiological metabolic, cardiovascular, aerobic responses consistent with those induced by exercise. *In vitro*, Electrical Pulse Stimulations (EPS) of muscle cells elicit cell contraction and mimic miokine release in the external medium.

Here we show that, in cultured mouse myotubes, EPS induce contractile activity and the release of the myokine IL-6. Gadolinium highly reduces EPS-induced IL-6 release, suggesting the involvement of mechanical activated ion channels. The chemical activation of mechanosensitive Piezo1 channels with the specific agonist Yoda1 stimulates IL-6 release similarly to EPS, suggesting the involvement of Piezo1 channels in the control of the myokine release. The expression of Piezo1 protein in myotubes was confirmed by the Western blot analysis.

To the best of our knowledge, this is the first evidence of a Piezo1-mediated effect in myokine release and suggests a potential translational use of specific Piezo1 agonists for innovative therapeutic treatments reproducing/enhancing the benefits of exercise mediated by myokines.

1. Introduction

During the past decade numerous studies have described the endocrine role of skeletal muscle, able to produce and release several myokines during contraction including interleukins IL-6, IL-8, IL-15, Insulin-like growth factor, BDNF, myostatin, irisin and others, which bind to their receptors and activate specific signaling pathways [1]. Circulating myokine levels increase during physical exercise and decrease with physical inactivity. In particular, skeletal muscles are considered the dominant source of IL-6 production secreted into the bloodstream in response to muscle contraction [2] and, therefore, mostly responsible for the systemic increase of IL-6 induced by exercise [3,4]. IL-6 binds to the transmembrane receptor IL-6R or a soluble receptor sIL-6R. Signaling is mediated through the glycoprotein named gp130, activating intracellular signaling including cytosolic Ca^{2+} increase, production of reactive oxygen species and NF-kB pathway [5]. Higher levels of plasma IL-6 characterize intensive and long activity exercises with the use of larger muscle mass. IL-6, more known as inflammatory cytokine, is involved in a dual effect. A transient short-term action is related to its autocrine and paracrine activity required to modulate proliferation and differentiation of muscle cells [6–9]. A persistent pro-inflammatory activity is related to higher levels of the circulating miokine with a long-lasting effect associated to muscle damage

Abbreviations: EPS, Electrical pulse stimulation; IL-6, interleukin 6.

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[10,11]. Conventional and noisy Pulse Electro Stimulations (EPS) mimick the nerve activity in cultured skeletal muscle cells [12–14]. EPS elicit time-locked Ca^{2+} increases, cell contractions [13,15] and IL-6 release [16,17].

Sustaining muscle cell contractions, physical exercise as well as neuromuscular electrical stimulation are supposed to activate cation-permeable mechano-activated channels (MAs) at the skeletal muscle level eliciting Ca^{2+}-dependent signaling [18] but the mechanism promoting the miokine release during muscle contractions is still unknow. Piezo1 channels belong to the large family of the mechanically-activated (MA) channels [19]; they are activated either by mechanical and chemical stimuli [20]. The chemical activation is mediated by the specific exogenous agonist Yoda1, offering the advantage to induce the opening of Piezo1 channels in the absence of any mechanical stimulation [21].

This study aimed to investigate the possible contribution of MA channel activity in the IL-6 release in EPS stimulated skeletal muscle cells, with a particular attention on Piezo1 channels.

2. Materials and methods

2.1. Cell cultures

Primary myotube cultures were obtained starting from satellite cells derived from the hind-leg muscles of a 7-day-old male Balb/c mouse [22] killed by cervical dislocation as approved by Local Animal Care Committee and in agreement with the European legislation.

Myoblasts were seeded at 70.000 cells per dish onto matrigel-coated (0.5 mg/ml) coverslips and grown for 24 h in a Growth Medium (GM) consisting of HAM F-10 plus 20% foetal bovine serum (FBS), L-Glutamine (4 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml). To induce myoblast fusion into myotubes, GM was replaced with Differentiation Medium (DM) composed by DMEM high glucose enriched with Horse Serum (2%), L-Glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml). Dishes were maintained in an incubator at 37 °C in saturated humidity and in CO_2 (5%)-enriched air. The culture medium was replaced every 48 h.

2.2. EPS of cultured myotubes

EPS was carried out on day 7 differentiated myotubes plated on matrigen-coated 6-well plates. Before the EPS, in each well the medium was replaced with 1 ml Tyrode's Salt solution enriched with FBS (10%) and penicillin and streptomycin as above. During EPS, the contractile activity of myotubes was assessed by an inverted Axiovert microscope (Carl Zeiss, Germany). EPS was performed by field electrostimulation delivered by the Grass S88 stimulator (Grass Instruments, Quincy, MA) to the myotubes kept at 37 °C in saturated humidity and in CO_2 (5%)-enriched air. In each well, two handmade connection cards connected to a couple of parallel platinum-iridium electrodes (0.2 mm in diameter) were present. The couples of electrodes were placed 2 cm apart and positioned 1–2 mm over the cells. Biphasic single conventional 1 ms pulses, were delivered at the frequencies of 1, 5 and 10 Hz. The stimulus strength (strengths 6–7 V peak to peak) was set to approximately 10% above the mechanical threshold in order to induce visible myotube contractions, without causing cell damage.

2.3. Electrophysiological recordings

Changes in membrane potential elicited by EPS were recorded by single myotubes by patch-clamp technique under current clamp conditions with perforated patch clamp method. Mouse myotubes that occasionally exhibited spontaneous contractions, were discharged.

Changes in voltage were recorded by an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). Extracellular recording solution contained (in mM): 100 NaCl, 2.8 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, 10 glucose, pH 7.3. Pipette solution contained (in mM): 140 K-aspartate, 10 NaCl, 2 $MgCl_2$, 10 Hepes and 150 μg/ml amphotericin B, pH 7.3. Voltage recordings were sampled at 100 kHz and low-pass filtered at 1 Hz.

2.4. Cytotoxicity assay

During EPS, lactate dehydrogenase activity (LDH) was measured by spectrophotometric measurements of the supernatant and lysate using a LDH assay kit (In Vitro Toxicology Assay Kit, Lactic Dehydrogenase based, Sigma Aldrich) and following the manufacturer's instructions. In order to obtain the absorbance measure, the value of the background absorbance of the multi-well plates measured at 690 nm was subtracted from the absorbance measured at a wavelength of 490 nm. LDH levels (%) were calculated by dividing the amount of LDH detected in the supernatant by the total amount of LDH (detected in supernatant and lysate). Each experimental point was performed in triplicate.

2.5. IL-6 ELISA quantification

Immediately after the EPS, supernatants (1 ml) were collected from both the electrically-stimulated and control dishes, precooled at 4 °C and spun 5 min at 20,000 *g* to remove non adherent cells. Surnatants were stored at −80 °C. Cells were lysed with 400 μl lysis buffer/well (PBS + 1% TritonX-100) collected and stored at −80 °C.

IL-6 concentrations in supernatants were measured with commercially available Quantikine® ELISA kit (R&D Systems Minneapolis, MN, USA) in a Microplate reader (Synergy, BioTeck Winooski, VT).

Total proteins were extracted with lysis buffer and the protein concentration was quantified by Coomassie (Bradford) protein assay (Thermo Fisher Scientific) and used to normalize against the values obtained for cytokine release. Each experimental point was repeated at least in duplicate.

2.6. Western blotting

Myotubes were washed twice with warm PBS and incubated for 30 min at 4 °C in RIPA buffer (25 mM Tris-HCl pH = 7.6, 150 mM NaCl, 1% NP40, 0,5% Sodium Deoxycholate, 0,1% SDS, 1 mM EDTA) supplemented with 1 × EDTA-free protease inhibitor cocktail tablets (Sigma-Aldrich). After removing the insoluble material by centrifugation (13,000 *g* for 10 min at 4 °C), protein concentrations were determined by Bio-Rad DC Protein Assay. Thirty-microgram aliquots of proteins were separated by SDS-PAGE and subject to Western blotting. Proteins were transferred onto 0.22-μm nitrocellulose membranes (Amersham). Binding of nonspecific proteins to membranes was blocked by incubating the membranes in the blocking buffer consisting of 5% non-fat milk in TBS plus 0.1% Tween 20 (TBST) for 1 h at room temperature. Membranes were then incubated overnight at 4 C with rabbit polyclonal antibody against Piezo1 (NBP1-78446) diluited 1:1000 in blocking buffer. After three washes with TBST, membranes were incubated in horseradish peroxidase-conjugated secondary antibodies for 1 h. After three more washes with TBST, immunoreactive proteins on the membranes were detected by WesternBright ECL (Advasta, San Jose, CA).

2.7. Chemicals

L-Glutamine, penicillin and streptomycin were purchased from Euroclone (Milano, Italy); FBS from Gibco (Burlington, ON, Canada). Matrigel from Corning (Tewksbury, MA, USA). Unless otherwise stated, all the other chemicals were from Sigma-Aldrrich (St. Louis, MA, USA). Stock solutions of Yoda1 were reconstituted in DMSO and stored at −20 °C. According to the experimental protocol, when applied, cells were preincubated for 30 min with Yoda1 and the agonist was maintained in the bathing solution for all the duration of the experiment.

2.8. Statistical analysis

Data of *n* experiments are expressed as mean ± standard error (SEM). All statistical tests and curve fitting were performed using the software package GraphPad Prism (CA, USA) Version 8.00. Unpaired Student's *t*-test was used to compare data between two groups of normally distributed values. Mann-Whitney non parametric test was used to compare two groups of non-parametric values. The significance level was set at $P < 0.05$.

3. Results

3.1. EPS induce contractions

In a set of experiments, 7 day differentiated mice myotubes were stimulated for 4 h with regular bipolar 1 ms pulses at constant frequencies of 1, 5 and 10 Hz. At all frequencies, action potentials were observed, time-locked with EPS (Fig. 1A). As previously reported, each action potential was associated to a cell twitching [13]. Upon EPS delivery, LDH was measured in the external medium. LDH release did not differ significantly in media from stimulated compared with unstimulated myotubes (Fig. 1B). Accordingly, the observation of stimulated cells excluded a visible cell damage. The results indicated that none of the EPS protocols, used and delivered for 4 h, induced cytotoxicity.

3.2. EPS induce IL-6 production

The content of IL-6 release in the supernatants derived from EPS stimulated myotubes was measured as picrograms per total milligrams of proteins detected in the cell extract (pg IL-6/mg proteins) and normalized to that derived from the unstimulated cells.

A set of experiments was carried out to investigate on the time-dependency of the secretion. IL-6 content was assessed at 1 h and 4 h of regular 1 ms EPS delivered at 10 Hz. The entity of IL-6 released was unchanged after 1 h but it resulted significantly increased after 4 h EPS in respect to unstimulated cells (Fig. 2A).

No increase of IL-6 release was observed after 4 h at 1 Hz EPS (259.1 ± 30.03 pg/mg after 4h EPS vs 218.5 ± 25.96 pg/mg in controls); while EPS delivered at 5 Hz significantly increased the IL-6 production (216.1 ± 31.71 pg/mg vs 119.0 ± 26.19 pg/mg observed in controls) as well as at 10 Hz (315.3 ± 54.45 pg/mg protein vs 136.6 ± 18.52 pg/mg in controls, Fig. 2B).

3.3. The role of mechanosensitive Piezo1 channels in the release of IL-6

When myotubes underwent EPS at 10 Hz for 4 h in the presence of the MA channel blocker gadolinium (Gd^{3+}) [19], the release of the myokine severely decresed. In more detail, the pre-incubation (30 min) with Gd^{3+} 30 μM reduced by ~65% the EPS-induced release of IL-6 (Fig. 3A) suggesting the involvement of the MA channels in the control of the myokine secretion.

Interestingly, Piezo1 protein expression was detected in myotubes by WB analysis (Fig. 3 B). Moreover, in the absence of EPS stimulation, the pharmacological activation of Piezo1 channels by the specific agonist Yoda1 (for 4 h at 3 μM and 10 μM) increased the IL-6 release (Fig. 3C). The content of IL-6, detected at the two agonist concentrations, revealed that the chemical activation of Piezo1 mimics the effect of the EPS-induced contractile activity (see Fig. 2B).

4. Discussion

IL-6 is the cytokine with the highest plasma increase after acute physical exercise [23,24] and skeletal muscle is mostly responsible

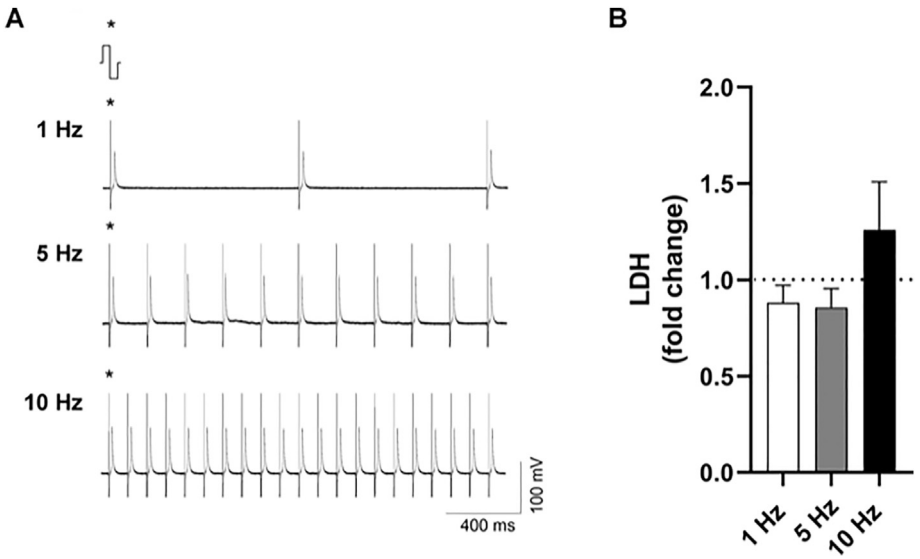

Fig. 1. EPS elicit action potentials. A. Representative traces of action potentials elicited in 7 day - differentiated myotubes by regular stimulations (1 ms biphasic pulses) at the frequences of 1, 5 and 10 Hz as indicated. Traces were sampled at 100 kHz and band-pass filtered at 1 Hz. B. LDH content in the external medium of myotubes after similar protocols of EPS ($n = 5$). LDH contents are expressed as fold changes to control values. No significant variation was observed ($P > 0.05$ vs. unstimulated).

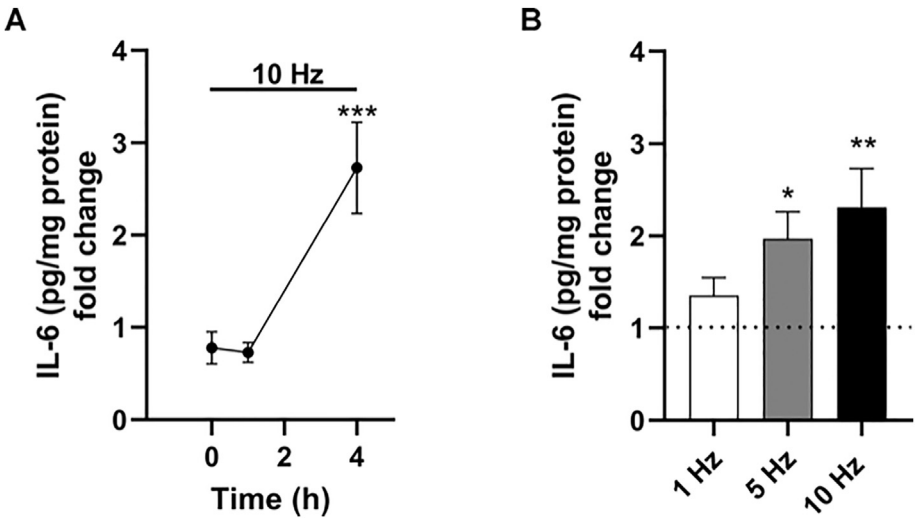

Fig. 2. EPS elicit IL-6 release. A. IL-6 release after 4 h EPS (1 ms biphasic pulses) at 1, 5 and 10 Hz expressed as fold increase to unstimulated conditions. EPS at 5 and 10 Hz significantly (*$P < 0.05$, **$P < 0.01$) increase the IL-6 secretion from 7 day - differentiated myotubes ($n = 5–10$). B. IL-6 secretion assessed at 1 and 4 h of EPS at 10 Hz expressed as fold increase in respect to unstimulated conditions, indicate a significant IL-6 secretion starting from 4 h of EPS.

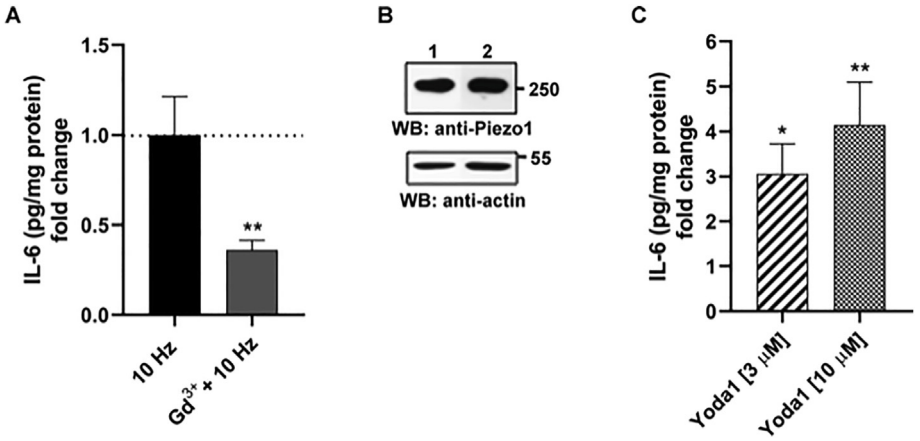

Fig. 3. Piezo1 channel expression and Yoda1-induced IL-6 release in myotubes. A. Gd3+ inhibitory effect on IL-6 release induced by 10 Hz EPS. Myotubes were stimulated with EPS at 10 Hz with and without 30 min preincubation with 30 μM Gd3+ (**$P < 0.01$). B. Western blot analysis revealed the Piezo1 expression in myotubes derived from two indipendent cultures (lane 1 and lane 2). Actin was used as loading control. C. Yoda1 at 3 μM and 10 μM increased the IL-6 production. Data are normalized to unstimulated conditions. (*$P < 0.05$, **$P < 0.01$).

for this phenomenon [3,4]. Indeed, the magnitude of the IL-6 increase is related to the individual muscle mass, duration and intensity of the exercise [25].

We previously detected in mouse myotubes the efficiency of electrostimulation protocols in promoting intracellular Ca^{2+} changes, time-locked with electric pulses and contractile activity [13]. This *in vitro* exercise model offers the advantage to explore the muscle release of myokines, without cell damage avoiding other possible myokine sources present in the intact muscles (e.g. neuronal, endocrine, immune system cells). Various studies reported electrostimulation-mediated increase in IL-6 expression and release in muscle cell lines such as C2C12 (8,26,27,28], primary human myotubes [29–31] and rodent myotubes [16,32,33]. Variability in the amount and time-course of IL-6 release has been related to the different experimental animal species, stimulation protocols, or protein detection.

The mechanisms regulating IL-6 production and secretion are not completely known. Intracellular IL-6-positive vesicles-like structures have been detected in muscle fibers at both the sarcolemma and inside the fibers in the T-tubules area, reduced in number after contraction [34]. Furthermore, exercise was recently proposed to activate lactate-dependent pH sensitive-proteases, which would alter the extracellular matrix, inducing membrane conformational changes, responsible for IL-6 release [35]. Independently from the mechanism of release, a beneficial role of IL-6 acting in autocrine/paracrine way on skeletal muscle regeneration was observed [5,8].

Regarding the mechanisms controlling IL-6 expression/release triggered by EPS, previous reports indicate that field electrostimulation of skeletal muscle cells promotes the release of extracellular ATP through pannexins [14–16] favouring IL-6 expression via IP_3-dependent intracellular Ca^{2+} signals [16]. IL-6 was suggested to be released and to autocrinally promote, though a positive feedback, IL-6 production acting on IL-6R and activation of the JAK2/STAT3 signaling [16].

During contraction, muscle cells undergo mechanical strain, causing the activation of specific signaling controlling regulation of gene expression, cell differentiation and hypertrophy [36,37]. The

contractile activity causes MA channels activation; being them Ca^{2+} permeable, they could potentially control a wide number of intracellular signaling pathways including the myokine production.

In this report, for the first time we show that MA channel activity sustains to a large extent (~65%) the IL-6 release. The lacking of specific MA channel blockers/antagonists, does not allow the identification of the specific MA channel subtypes involved, considering that many of them could be likely expressed in myotubes [38]. Recently, we identified a time-window in which the chemical activation of the mechanical sensitive Piezo1 activity promotes differentiation and fusion of the myogenic precursors [39]. Here we unveil the role of Piezo1 in controlling IL-6 release in skeletal muscle cells similarly to what reported in cardiac fibroblasts where Piezo1 activation by Yoda 1 was found to be coupled to IL-6 secretion via a p38 MAPK-dependent pathway [40]. However, patient immobilization was recently found to be associated to a downregulation of Piezo1, and an upregulation of atrophy-related genes including *Il6* via a signaling pathway mediated by the transcription factor Krüppel-like factor 15 (KLF15) [41]. The discrepancy between the reported results requires further investigation.

The comparison between the amount of IL-6 released by EPS and by the chemical activation of Piezo1 channel suggests that the chemical activation of Piezo1 channels by Yoda1 could be even more efficient than the EPS at 10 Hz in inducing the myokine release. Thus, according to that, specific pharmacological Piezo1 activation could represent a new powerfull tool to promote IL-6 release in physiological and pathological contests [42,43].

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Author contributions

MS, PL, and AB conceived and designed research; MS, GM and PZ, FT performed experiments; MS, GM analyzed data; MS, FT interpreted results of experiments; MS, GM and PZ prepared figures; MS and AB drafted manuscript; MS, PL and AB edited and revised manuscript; MS, GM, PZ, PL, FT and AB approved final version of manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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