Life Sciences 229 (2019) 277–287

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Sparc, an EPS-induced gene, modulates the extracellular matrix and mitochondrial function via ILK/AMPK pathways in C2C12 cells

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ARTICLE INFO

Keywords:
SPARC
Extracellular matrix
Mitochondria
Electrical pulse stimulation
Muscle

ABSTRACT

Aims: Secreted protein acidic and rich in cysteine, (SPARC), is a matricellular protein implicated in the modulation of the extracellular matrix (ECM) and mitochondrial proteins expression.
Main methods: To study the mechanism through which SPARC is involved in the possible link between ECM and mitochondria, C2C12 myoblasts were cultured with/without the exogenous addition/inhibition of SPARC as well as activation/inhibition of adenosine monophosphate-activated protein kinase (AMPK). Electrical pulse stimulation (EPS), was applied for 2 days in myotubes.
Key findings: The expressions of ECM-related (integrin-linked kinase (ILK), glycogen synthase kinase-3 beta (GSK-3ß), phosphorylated-GSK-3ß (p-GSK-3ß) and collagen 1a1), mitochondrial-related (AMPK, phosphorylated-AMPK (p-AMPK), succinate dehydrogenase (SDHB) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Pgc1α)) and SPARC proteins and/or genes were measured after modulation of SPARC and/or AMPK as well as with or without EPS. The addition of SPARC in C2C12 myoblast increased the expression of ILK, p-GSK-3ß and p-AMPK whereas anti-SPARC antibody decreased them at different incubation times (0, 10, and 30 min, and 6 h). The AMPK activation increased SPARC, collagen 1a1, p-AMPK and SDHB proteins level, however, AMPK inhibition blunted the effects. EPS induced Sparc and Pgc1a genes expression.
Significance: Sparc, an EPS-induced gene, may be involved in the link between ECM remodeling and mitochondrial function in muscle via its interaction with ILK/AMPK.

1. Introduction

The process of human aging begins from the fourth decade (at age 30) and many age-related changes have been postponed [1]. One of the most striking effects of aging is, therefore, the involuntary loss of muscle mass, strength and function, “sarcopenia” [2]. The literature has indicated that exercise (resistance and aerobic) is an essential strategy for the prevention of sarcopenia [3]. To elucidate the molecular mechanisms underlying exercise-induced adaptations, some in vitro approaches have been developed such as electrical pulse stimulation (EPS) [4]. Indeed, several studies have used EPS of muscle cells as an in vitro exercise model [5] to induce hypertrophy of human muscle cells [6] as well as exercise-related responses including improved insulin sensitivity and increased oxidative capacity [7]. Furthermore, it is well known that mitochondria play crucial roles in redox homeostasis, energy production and regulation of catabolic pathways. Thus, the decline in mitochondrial function may be involved in the pathogenesis of sarcopenia [8]. It has been reported that sarcopenia is associated with

Abbreviations: 36B4, ribosomal protein gene; AICAR, 5-aminoimidazole-4-carboxamide-1-bêta-4-ribofuranoside;; AMPK, adenosine monophosphate-activated protein kinase; anti-SPARC, monoclonal antibody anti-osteonectin; CS, calf serum; DM, differentiation medium; DLW, density of each lane on the membrane; DMEM, Dulbecco's modified Eagle's medium; DLF, density of each lane on the film; ECM, extracellular matrix; EPS, electrical pulse stimulation; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GM, growth medium; GSK-3ß, glycogen synthase kinase-3 beta; ILK, integrin-linked kinase; MEM, minimum essential media; OXPHOS, oxidative phosphorylation; PC, positive control; p-AMPK, phosphorylated-AMPK; Pgc1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PBS, phosphate buffered saline; p-GSK-3ß, phosphorylated-GSK-3ß; PVDF, polyvinylidene fluoride; Q-RT-PCR, quantitative real-time PCR; RT, room temperature; rSPARC, recombinant mouse SPARC protein; SDHB, succinate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPARC, secreted protein acidic and rich in cysteine; TBS, protein-free T20

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https://doi.org/10.1016/j.lfs.2019.05.070
Received 10 March 2019; Received in revised form 24 May 2019; Accepted 27 May 2019
Available online 28 May 2019
0024-3205/

accumulations of mitochondrial dysfunction whose mechanisms are not yet fully understood [9]. For instance, a 40 to 50% decrease in oxidative phosphorylation (OXPHOS), a decrease in mitochondrial biogenesis represented by alterations in “mitophagy” and a reduction in the activities of the enzymatic complexes of the mitochondrial respiratory chain were detected in the aged muscle [10,11]. Indeed, the close relationship between aging, mitochondrial function and metabolic disorders has received a lot of attention and two key proteins appeared: adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) [12]. It has been demonstrated that PGC-1α, a master regulator of mitochondrial biogenesis, plays an important role in the regulation of mitochondrial proteostasis by promoting the expression of new mitochondrial proteins and keeping intact proteins in response to stress such as aging or exercise [13]. Furthermore, AMPK has been described as a metabolic sensor and shown to directly affect PGC-1α activity through phosphorylation and deacetylation [14].

The extracellular matrix (ECM) is a structured and complex network of secreted macromolecules and proteolytic enzymes. This scaffolding structure surrounding cells provides a structural and protective framework that makes the organization of muscles possible [15]. Matricellular proteins are non-architectural components of the ECM that play critical roles during muscle development and fiber repair [16]. Secreted protein acidic and rich in cysteine (SPARC, also known as osteonectin or BM-40), an extracellular matrix-associated glycoprotein, mediates interactions between cells and their ECM by its binding to structural matrix proteins[17]. Exercise training[18], heat shock [19], other forms of stress [20] as well as high-fat refeeding [21] and transforming growth factor beta 1 (TGF-β1)[22] are known to induce SPARC expression SPARC has also been shown to bind to collagen and serve as an intracellular collagen chaperone [17]. In addition, we have demonstrated that SPARC plays a crucial role in the differentiation of C2C12 muscle cells and could be involved in the possible link between ECM remodeling and mitochondrial function [23]. Although some mechanisms have been suggested, it is not known how SPARC can modulate ECM remodeling and mitochondria in muscle Integrin-linked kinase (ILK) has been identified as a potential SPARC binding partner [24], and it can participate in the mechanism by which SPARC regulates ECM organization [24]. Moreover,SPARC can interact with AMPK and may be involved in regulating glucose metabolism via AMPK activation [25]. On the other hand, skeletal muscle has been identified as an endocrine organ that synthesizes and secretes myokines [26], and it has been shown that the influence of myokines on whole body metabolism is potentially significant [27]. Indeed, Aoi et al. have identified SPARC as a novel myokine which inhibits the initiation of colon tumorigenesis via physical exercise [28]. In their study, SPARC was secreted into the extracellular medium after 60 min cyclic stretching of C2C12 myotubes [28], and SPARC secretion was also found to elevate in muscle following strength-training and after a single bout of exercise [28,29]. Furthermore, we have previously identified SPARC as one of the mild-exercise training specifically induced genes in elderly men [18,30]. Our transcriptomic analysis has revealed the modulation of 12 transcripts, some of which were related to ECM and OXPHOS [18]. Importantly, Hjorth et al. have demonstrated that SPARC had the highest expression level of all the upregulated genes after an acute and long-term physical activity in human [31].

Taken all together, we hypothesize that an exercise-induced gene, Sparc , modulates ECM remodeling and mitochondrial function via ILK and AMPK/PGC-1α pathways, respectively, and may play a key role in the link between the two compartments. Thus, we investigated if EPS can induce Sparc and if SPARC can modulate ILK and AMPK/PGC-1α pathways in C2C12 muscle cells. Furthermore, we studied the relationship between SPARC and AMPK.

2. Materials and methods

2.1. Materials

12-well plates (Falcon 35–3043) were from Corning (Corning et al, U.SA). Mouse skeletal muscle cell lines, C2C12 myoblasts were from American Type Culture Collection (ATCC® CRL1772™, ATCC, Manassas, USA). Dulbecco's modified Eagle's medium (DMEM), protein-free T20 (TBS) blocking buffer and phosphate buffered saline (PBS) were from Invitrogen (Carlsbad, USA). Fetal bovine serum (FBS) and calf serum (CS) were obtained from Hyclone (Utah, USA). Antibiotics were from Sigma-Aldrich (Oakville, Canada). Glutamax, Minimum Essential Media (MEM) essential amino acid, MEM non-essential amino acid and sodium pyruvate were from Gibco (Gaithersburg, USA). HEPES powder was obtained from Dojindo Molecular Technology (Rockville, USA). All antibodies for western blot and compound C were purchased from Santa Cruz Biotechnology (Texas, USA). TGX Stain-Free FastCast acrylamide kits, washing buffer (Tris buffer saline), western blot chemiluminescent solution (Clarity western ECL substrate solution) and film (sc-201697) were from Bio-Rad Laboratories (Mississauga, Canada). Monoclonal antibody anti-osteonectin (anti-SPARC) and recombinant mouse SPARC protein (rSPARC) were obtained R&D Systems (Ottawa, Canada). 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR, 99% free base) was from LC Laboratories (Woburn, USA).

2.2. Myoblasts culture

The C2C12 mouse adherent myoblasts were grown in growth medium (GM): DMEM supplemented with 10% FBS and 1% antibiotics. They were maintained in a 5% CO_2 atmosphere at 37 °C. GM was replaced with fresh 10% FBS-DMEM supplemented with different experimental conditions and cells were kept in GM until 80–90% of confluency.

2.3. Exogenous SPARC induction/inhibition

The rSPARC (8 μg/ml) and anti-SPARC (40 μg/ml) were directly added into GM. In the fourth experimental condition, anti-SPARC was first added into GM, and then rSPARC was appended 1 h later.

2.4. Identification of the molecular mechanism underlying the effect of SPARC on ECM and mitochondrial proteins expression

To identify the molecular mechanisms involved in the modulation of ECM and mitochondrial proteins expression, two independent experiments with three different passages were performed in 12-well plates. For the first experiment, cultured myoblasts were deprived of serum for 16 h, then incubated in fresh serum-free medium, followed by treatment with or without rSPARC and/or anti-SPARC. Proteins extractions were performed at 0 min, 10 min, 30 min and 6 h and cell extracts were used for western blotting. For the second independent experiment, C2C12 myoblasts were kept in serum-free medium for 16 h. AICAR (1 mM), compound C (20 μM) and/or rSPARC (8 μg/ml) were added (see S1 s) and cells were incubated with these different experimental conditions for 48 h. Proteins extraction followed by proteins quantification and western blot analyses were performed.

2.5. Electrical pulse stimulation

Myoblasts were seeded at 5×10^4 in eight-well plates without any coat, grown in 20% FBS-DMEM and maintained in a 5% CO_2 atmosphere at 37 °C [32]. When cells were at 80–90% confluent, they were transferred into differentiation medium (DM), 2% CS-DMEM, and DM was changed daily. Eight days later, C2C12 cells were fully differentiated and DM was replaced by EPS medium. EPS was applied for two days using C-Pace EP system from the IonOptix (Westwood, USA) as

previously described [32]. The frequency and voltage were changed each of day (1 Hz, 20 V for 8 h; then 50 Hz, 14 V for 16 h). The contractions of the myotubes were verified by examination under a microscope. RNA extraction and quantitative real-time PCR (Q-RT-PCR) were carried out.

2.6. RNA extraction

Total RNA was extracted using Trizol reagent according to manufacturer's instructions (Invitrogen) and quantified on a NanoDrop 2000 (Thermo Fisher Scientific). Reverse transcription was performed with 0.5 μg of RNA using Transcriptor First Standard cDNA synthesis kit (Roche Diagnostics Corporation, Indianapolis, USA).

2.7. Q_RT-PCR

Q_RT-PCR analysis was performed using a Light Cycler instrument and SYBR Green detection kit according to the manufacturer's protocol (Roche Diagnostics Corporation, Indianapolis, USA). All mRNA expression data were normalized to that of the ribosomal protein gene (36B4), a suitable reliable and consistent internal loading control for Q-RT-PCR [33]. See S2 Table for the primer sequences.

2.8. Immunoblot analysis

After the proliferation of C2C12 myoblasts, the resulting cultures were washed twice with PBS and scraped on ice by using cell lifter. Then, the PBS containing cells were centrifuged at 3000 rpm for 4 min at 4 °C. The pellets were resuspended in radio-immunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors cocktail and incubated on ice for 15 min. Lysates were sonicated 3× for 1 s, centrifuged at 14,000 rpm for 5 min at 4 °C. The whole cell extracts were stored at −80 °C until use. Pooled samples were used to determine the quantity of loading proteins (0–40 μg) and dilution of primary antibody (1/200–1/1600). The same pooled sample was loaded in each gel and used as a positive control (PC) to normalize the differences between each membrane. This will assure that the densitometric data for each target protein will be within the linear dynamic (quantitative) range to give accurate and reproducible results reflecting the true biology between samples in the study set [34]. The most popular loading controls include housekeeping such as beta-actin, tubulin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), however, these proteins are generally highly expressed in samples and are overloaded in the gel lane with the target protein such that they would not serve to normalize the loading [34]. Thus, we loaded the same pooled samples in each gel to be used as a control to normalize the difference between each membrane. Fifteen to 30 μg of proteins from each cell lysate including pooled samples were separated by electrophoresis through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the TGX Stain-Free FastCast acrylamide solutions, and trihalo compound in the gels was activated under UV as we mentioned before [35]. The total proteins were transferred onto polyvinylidene fluoride (PVDF) membrane by using Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories) with Mixed MW condition and visualized under UV using the AlphaImager™ 1220 (Alpha Innotech Co., San Leandro, USA). Then, membranes were blocked using suitable blocking buffer for 1 h at room temperature (RT) and incubated with primary antibodies for overnight at 4 °C (see S3 Table for western blot conditions). After washed (3×) with appropriates washing buffers, membranes were incubated with species-appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at RT, washed (3×) again and finally the visualization of the immune complexes was carried out with the Clarity™ Western ECL Blotting Substrate on a film. The visualized total proteins on the membranes (loading control) and target proteins on the films were quantified using the ImageJ software. The density of each lane on the membrane (DLM) and on the film (DLF) was expressed as a ratio to each PC on the same membrane/film. Then, the quantity of protein loaded was normalized by dividing DLF by DLM, as previously described [35,36].

2.9. Statistical analysis

All the data presented are repeated measures from three independent experiments (three different passages of C2C12 cells to measure the effect of SPARC on C2C12 phenotype. Statistical analyses were performed using two-way ANOVA with Fisher's protected LSD post-hoc test except that t-test was used for 0 min of time course experiment. P value was set at < 0.05. All results are reported as means ± SEM.

3. Results

3.1. Effects of SPARC on the modulation of ECM proteins expression through ILK/GSK-3ß pathway in C2C12 myoblasts

SPARC plays a critical role in the modulation of ECM remodeling [17]. Indeed, we have demonstrated that the addition of SPARC induces collagen 1a1 expression in proliferating, differentiating and differentiated C2C12 cells, whereas the addition of anti-SPARC has an opposite effect [23]. It is well known that ILK is a central regulator of ECM deposition [37] and is required in skeletal muscles for strengthening the integrin-ECM adhesion complex [38]. Moreover, a possible interaction between SPARC and ILK (SPARC regulates ECM assembly via modulation of ILK activity) has been suggested [24]. Furthermore, ILK inhibits one of ILK downstream targets, glycogen synthase kinase-3 beta (GSK-3ß), through its phosphorylation (p-GSK-3ß) conducting to the enhancement, stabilization and accumulation of ß-catenin which induces the activation of activator protein transcription factor [39]. SPARC is also known to inhibit adipogenesis through ILK/GSK-3ß pathway [40]. Thus, we studied the effect of exogenous rSPARC and anti-SPARC on ILK, GSK-3ß and p-GSK-3ß proteins expression in proliferating myoblasts at different incubation times (0 min, 10 min, 30 min and 6 h). ILK, GSK-3ß and p-GSK-3ß proteins expressions were analyzed by western blot. The obtained results showed that exogenous rSPARC increased the expression level of ILK and p-GSK-3ß proteins and decreased GSK-3ß protein expression, while exogenous anti-SPARC decreased ILK and p-GSK-3ß proteins level and increased GSK-3ß protein expression (Fig. 1). Thus, our previous study [23] and these data provide the evidence that SPARC can modulate ECM proteins expression via ILK/p-GSK-3ß pathway in skeletal muscle cells.

3.2. Involvement of SPARC in the modulation of mitochondrial proteins expression via its interaction with AMPK in muscle cells

We have previously reported that SPARC regulates mitochondrial proteins expression [23], whereas Song et al. have reported that SPARC is an AMPK-interacting protein [25]. For the first step to demonstrate if SPARC modulates mitochondrial proteins expression via AMPK in proliferating C2C12 myoblasts, we analyzed the effect of exogenous rSPARC and anti-SPARC on AMPK and p-AMPK proteins expression in murine myoblasts at several incubation times. For this experiment, we applied the same cell culture conditions as above, and the expression of AMPK and p-AMPK after the rSPARC and/or anti-SPARC additions were measured by western blot. In addition, the ratio of p-AMPK to total AMPK was evaluated. Our results demonstrated that rSPARC activated AMPK and anti-SPARC inhibited the effect (Fig. 2). Thus, SPARC may regulate mitochondrial proteins expression through the activation of AMPK.

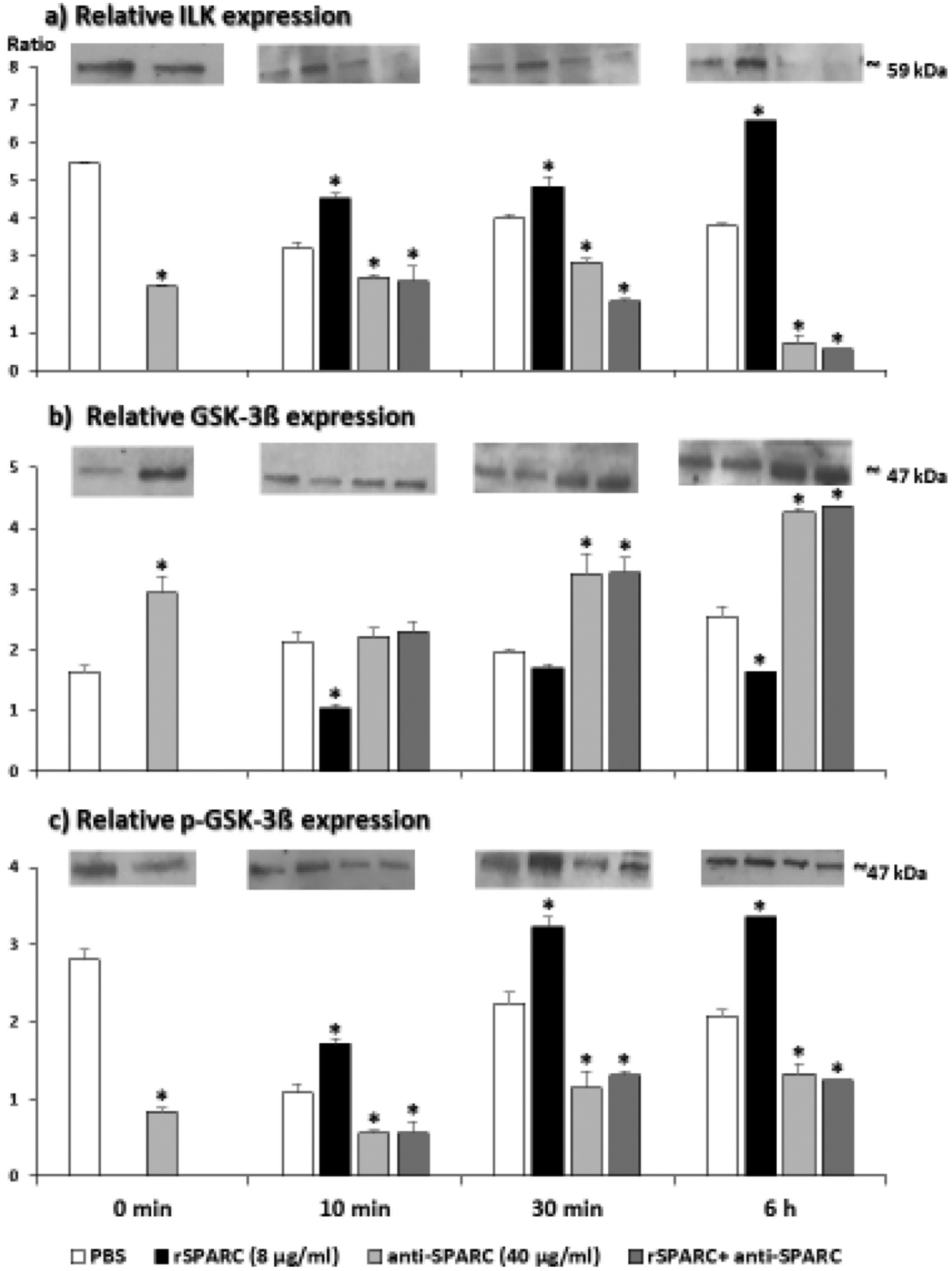

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Fig. 1. Exogenous rSPARC induced ILK expression and GSK-3ß phosphorylation in C2C12 myoblasts, whereas anti-SPARC inhibited it.
Cultured myoblasts were deprived of serum for 16 h, then incubated in fresh serum-free medium, followed by treatment with or without rSPARC (8 μg/ml) and/or anti-SPARC (40 μg/ml). Proteins extractions were performed at 0 min, 10 min, 30 min and 6 h, and cell extracts were used for proteins quantification. 15–30 μg of proteins were loaded for western blotting. Data were expressed as a ratio to a positive control (mean ± SEM, n = 3). The t-test (for each 0 min conditions) and 2-way ANOVA revealed significant effects of SPARC, time and interaction ($p < 0.05$). *Significant effects compared to PBS.
Abbreviations: anti-SPARC, anti-SPARC antibody; ILK, interleukin-linked kinase; GSK-3ß, glycogen synthase kinase; p-GSK-3ß, phosphorylated GSK-3ß; PBS, phosphate buffered saline; rSPARC, recombinant SPARC protein.

3.3. Implication of SPARC in the possible link between ECM and mitochondria

It is well known that ECM plays key roles in muscle function and development [15]. Importantly, skeletal muscle mitochondria are clearly implicated in muscle function because they are the main producers of free radicals and cellular energy [41] and involved in the modulation of cellular responses to different stimuli [42]. In general, mitochondria dysfunction is associated with aging [43], metabolic syndrome [44] as well as other diseases [45], whereas exercise is well known to activate mitochondrial biogenesis and function, which are impaired with aging [46]. We have already reported that mild-exercise training induced SPARC as well as ECM- and OXPHOS-related genes expression in elderly muscle [18], and suggested that SPARC may be involved in the possible link between ECM and mitochondria [23]. In order to illustrate the interaction between SPARC and AMPK as well as their effects on ECM and mitochondrial proteins expression, we treated C2C12 myoblasts with rSPARC, AICAR which activates AMPK [47] and compound C (AMPK inhibitor) [48]. Cells were incubated with these different experimental conditions for 48 h (see S1 Table). Our results showed that rSPARC induced collagen 1a1, p-AMPK and SDHB proteins levels (Fig. 3). Also, the addition of AICAR increased SPARC, p-AMPK and SDHB proteins expression. However, compound C decreased all these proteins levels (Fig. 3). These data suggest the involvement of SPARC in the possible link between ECM and mitochondria in muscle cells.

3.4. Sparc as an EPS-induced gene

Several studies have reported that EPS is an in vitro exercise model [6,32]. To study the effect of EPS on Sparc and Pgc1α genes expression in C2C12 cells, we applied EPS for 48 h with or without adding rSPARC and/or anti-SPARC. The frequency and voltage were changed each of day (1 Hz, 20 V for 8 h; then 50 Hz, 14 V for 16 h). Our results demonstrated that EPS increased both Sparc (by 10-fold) and Pgc1α genes expression (Fig. 4). Thus, Sparc, an EPS-induced gene, may play a critical role in muscle integrity via the modulation of mitochondrial genes expression.

4. Discussion

In this report, we studied the molecular mechanism whereby SPARC is involved in the modulation of ECM remodeling and mitochondrial proteins expression in muscle cells. Also, we investigated the effect of 48-h EPS on Sparc and Pgc1α genes expression. We provided evidence that Sparc, an EPS-induced gene, is implicated in the modulation of ECM remodeling and mitochondrial proteins expression in mouse myoblasts through ILK and AMPK pathways, respectively.

4.1. SPARC modulates ECM remodeling in muscle cells via ILK/GSK-3ß pathway

It is well recognized that several biological processes are regulated by ECM in muscle tissue [49]. In many cellular events, cell-ECM and cell-cell interactions play crucial roles [50]. Furthermore, SPARC, a non-collagenous multifunctional calcium-binding ECM glycoprotein, is known to modulate cell-ECM interactions [51] as well as ECM proteins expression [23]. We and others have already demonstrated that SPARC is involved in the myogenic process and it is an important participant in muscle ECM remodeling [23,52].

On the other hand, it has been reported that ECM can affect cell signaling via transmembrane receptors [53], integrins, which play key roles in ECM interactions [54]. Previous study has demonstrated that the cytoplasmic domains of integrins β1/β3 interact with ILK, a multifunctional pseudokinase, and modulate its activity, leading to the regulation of some signaling pathways [55]. Further, ILK is involved in the regulation of β-catenin accumulation and activation via the phosphorylation and thereby the inactivation of GSK-3β [39]. It is known that the phosphorylation of GSK-3ß is suppressed when ILK is downregulated [56]. In fact, the inhibition of GSK-3β leads to the stabilization of β-catenin [57] and also GSK-3ß has been shown to modulate the mediator effect of ILK [58]. An interesting report has described that ILK/GSK-3ß pathway is implicated in the regulation of dendrite initiation and growth [59].

Importantly, Barker et al. have reported that SPARC can regulate ILK activity in lung fibroblasts [24] and a mediated effect of ILK activity on SPARC has been demonstrated in glioma cells [60]. Also, it has been shown that SPARC is involved in the inhibition of adipogenesis via the induction of the nuclear transduction of ß-catenin in preadipocytes [40]. The same study has demonstrated that SPARC is implicated in the regulation of the production of ECM proteins and integrins through an ILK-dependent manner [40]. Furthermore, it has been suggested that SPARC has an ILK-dependent, anti-apoptotic function in cultured lens epithelial cells [61].

Although, to our knowledge, no report has shown the molecular mechanism via which SPARC is implicated in the modulation of ECM remodeling in muscle cells. Thus, we demonstrated here that the exogenous inhibition of SPARC decreased ILK and p-GSK-3ß proteins level and increased GSK-3ß protein expression. Indeed, the addition of rSPARC increased ILK and p-GSK-3ß proteins expression and decreased GSK-3ß. One possibility to explain the exogenous effect of SPARC on ILK protein expression in muscle cells is the identification of ILK as a potential SPARC-binding partner [24]. In fibroblasts, SPARC and ILK appear coincident and the validation of the interaction between these two proteins in a physiological context was shown by the colocalization and co-immunoprecipitation of a SPARC-ILK complex [24]. In a similar manner, our results support the data presented in glioma cells when the downregulation of SPARC with interfering RNA (siRNA) correlated with a reduction in ILK expression and activity, and when targeting ILK expression with siRNA reduced SPARC ability to stimulate invasion [60]. The same study has described that exogenous SPARC and SPARC overexpression increased the kinase activity associated with ILK which confirms our data [60]. In addition, ILK is known to directly phosphorylate and inactivate GSK-3ß [39]. In this context, Dorota et al. have described that blocking ILK signaling pathway activates GSK-3ß and concluded that GSK-3ß is a crucial downstream element of the ILK signaling [62]. Here, we analyzed the exogenous effect of SPARC on GSK-3ß and p-GSK-3ß proteins expression in C2C12 cells. The increased of p-GSK-3ß and the decreased GSK-3ß proteins level after exogenous SPARC induction as well as the decreased of p-GSK-3ß and the increased GSK-3ß proteins expression after the addition of anti-SPARC antibody are in agreement with the previous studies [61,63].

Taken all together, we have shown here that SPARC activates ILK that phosphorylates and inactivates GSK-3ß, leading to the modulation

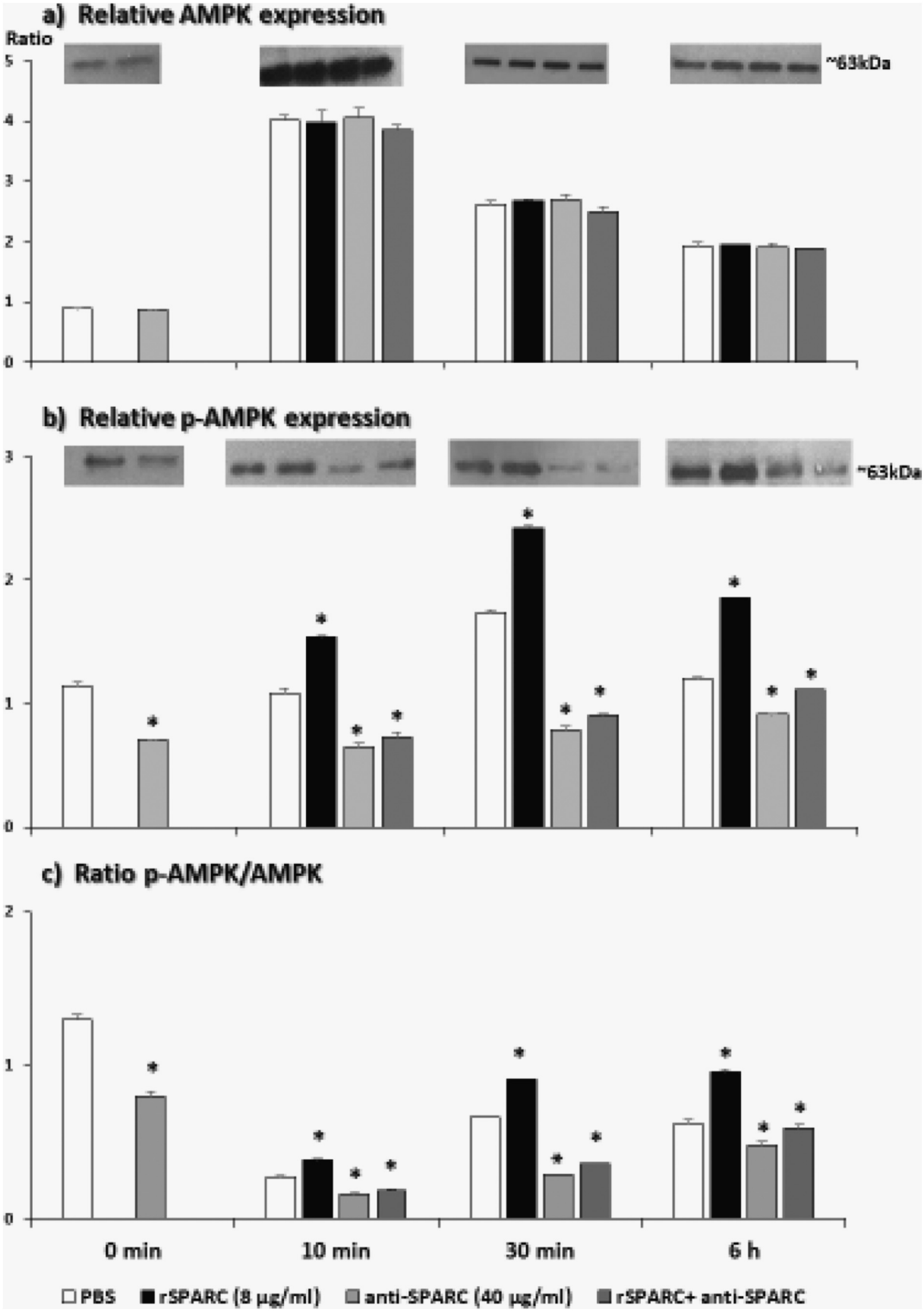

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Fig. 2. Addition of rSPARC induced AMPK phosphorylation in C2C12 myoblasts, however, anti-SPARC inhibited it.
|Confluent myoblasts were deprived of serum for 16 h, then incubated in fresh serum-free medium, followed by treatment with or without rSPARC (8 μg/ml) and/or anti-SPARC (40 μg/ml). Proteins extractions were performed at 0 min, 10 min, 30 min and 6 h. Expression of each protein was quantified by western blot and normalized to the total protein in its respective lane. The ratio of p-AMPK to total AMPK was evaluated. Data were expressed as a ratio to a positive control (mean ± SEM, n = 3). The t-test (for each 0 min conditions) and 2-way ANOVA revealed significant effects of SPARC, time and interaction ($p < 0.05$). *Significant effects compared to PBS.
Abbreviations: AMPK, 5′ adenosine monophosphate-activated protein kinase; anti-SPARC, anti SPARC antibody; p-AMPK, phosphorylated AMPK; PBS, phosphate buffered saline; rSPARC, recombinant SPARC protein.

of muscle ECM remodeling.

4.2. Implication of SPARC in the modulation of mitochondrial proteins expression through AMPK

In skeletal muscle, mitochondria are the main controllers of cellular metabolism and other physiological processes [64]. These fascinating organelles are the major source of reactive oxygen species (ROS) and are themselves the first target of these species [65]. Based on the key role of mitochondria in skeletal muscle physiology, many authors have mentioned the involvement of mitochondrial dysfunction in skeletal muscle disorders [66,67]. Moreover, it has been reported that AMPK is the master regulator of mitochondrial biogenesis via direct phosphorylation of PGC-1α [68]. In addition, previous literature has highlighted that SPARC knockdown mediates apoptotic cell death via the mitochondrial intrinsic pathway in human melanoma cells [69]. Indeed, a recent study has demonstrated a SPARC-mediated effect in the regulation of the mitochondrial apoptotic pathway through an interaction between SPARC and pro-caspase 8 [70]. Importantly, we have already shown an exogenous effect of SPARC on mitochondrial proteins expression in muscle cells [23]. All these data confirm the involvement of SPARC in the modulation of mitochondrial function.

However, we asked ourselves the question of how SPARC can regulate mitochondrial proteins level? In this context, an interesting study has demonstrated a relationship between SPARC and AMPK [25]. This study has reported that AMPK activation increases SPARC expression and knockdown of AMPK reduces SPARC protein levels, leading to conclude that SPARC is an AMPK-interacting protein, and possible implication of SPARC in glucose metabolism has been suggested [25]. Here, we measured AMPK and p-AMPK proteins expression in C2C12 myoblasts after exogenous addition and/or inhibition of SPARC at different incubation times (0, 10, 30 min and 6 h). Our results showed that the exogenous addition of SPARC increased p-AMPK protein level and anti-SPARC addition decreased it. Hence, our data support the findings of Song et al. in L6 myocytes where SPARC siRNA reduces AICAR-stimulated AMPK phosphorylation [25].

Thus, SPARC modulates mitochondrial proteins expression in C2C12 cells through direct interaction with AMPK.

4.3. Involvement of SPARC in the possible link between ECM and mitochondria in muscle

Increasing evidence suggests that changes in ECM composition may play an important role in the regulation of mitochondrial function. For instance, it has been reported that fibronectin, a key structural protein in the ECM, is essential for protecting fibroblasts from undergoing apoptosis through a caspase cascade [71]. In the same context, Wu et al. have observed that the loss of fibronectin induced mesangial cell apoptosis in a mitochondria-dependent manner [72]. Matrix metalloproteinases, modulators of ECM turnover, have been described to function as negative regulators for mitochondrial function in cardiac-specific matrix metalloproteinases transgenic mice under oxidative stress conditions [73]. In addition, a latent mitochondrial dysfunction and a spontaneous apoptosis in myofibers of collagen VI-deficient mice have been observed [74]. Recent studies have also mentioned that ECM mechanics and elasticity as well as tissue architecture impact mitochondrial function [75,76]. In the opposite direction, changes in the mitochondrial function may also affect ECM composition. For example, Waveren et al. described that specific OXPHOS is implicated in the modulation of ECM remodeling processes [77].

Consequently, a relationship between the ECM and mitochondria may exist as we suggested in our previous study where we proposed that SPARC may be involved in the possible link between ECM remodeling and mitochondrial function in muscle cells [23]. In the support of our hypothesis, a recent publication has reported that GSK-3β inhibition links mitochondrial dysfunction and ECM remodeling in chondrocytes [78].

Accordingly, to confirm the involvement of SPARC in the possible link between ECM remodeling and mitochondria function in muscle cells, we measured SPARC, collagen 1a1, AMPK, p-AMPK and SDHB proteins levels after the addition of rSPARC, AICAR and/or compound C (see S1table) to analyze the effect of rSPARC addition and/or the AMPK activation/inhibition on ECM and mitochondrial proteins expression as well as the SPARC-AMPK inter regulation. Our results revealed an increase of collagen 1a1, p-AMPK and SDHB proteins expression after rSPARC addition, however, compound C blunted this effect. Moreover, the activation of AMPK increased SPARC, SDHB and collagen 1a1 (a trend) proteins level. In fact, we showed here that: 1) AICAR induced AMPK phosphorylation [47], 2) compound C inhibited AMPK phosphorylation [79], 3) the addition of rSPARC increased collagen 1a1 and SDHB proteins level [23], and 4) the effect of rSPARC was blocked by compound C. Additionally, we investigated the effect of rSPARC addition and AMPK activation/inhibition on AMPK phosphorylation, SPARC, collagen 1a1 and SDHB proteins expression. Altogether, our results confirm the implication of SPARC in the possible link between ECM remodeling and mitochondrial function in muscle cells whereby its interaction with ILK and AMPK, respectively.

4.4. Sparc and Pgc1α as EPS-induced genes

Growing evidence has reported that the skeletal muscle is a secretory organ that releases bioactive proteins, myokines, in response to exercise and muscle contraction [80]. These myokines may exert either autocrine, paracrine, or endocrine functions [80]. Additionally, our team has characterized the skeletal muscle transcriptome after endurance exercise training in the elderly and identified new candidate genes that are mainly related to the extracellular matrix remodeling and mitochondrial function [18]. In our report, we have highlighted the importance of the mitochondrial OXPHOS and the ECM remodeling in the skeletal muscle adaptation [18]. We found that SPARC, an exercise-induced gene, was the main candidate gene [18]. On the other hand, it has been shown that EPS is an in vitro exercise model for human and murine muscle cells [6,32]. Ankie et al. have reported a direct effect of EPS on AMPK phosphorylation and myokine secretion [81]. The authors have also demonstrated that these modifications were mainly caused by EPS-induced contraction and not due to EPS-induced changes in cell culture media [81]. Thus, EPS can affect C2C12 myotube function and thereby alter gene expression in cells [81]. Accordingly, in vivo and in vitro reports are presently identified new myokines. For instance, Boström et al. have identified irisin as a new myokine using transgenic mice expressing PGC1-α which is already known as an exercise-induced myokine [82]. In addition, exercise has been also shown

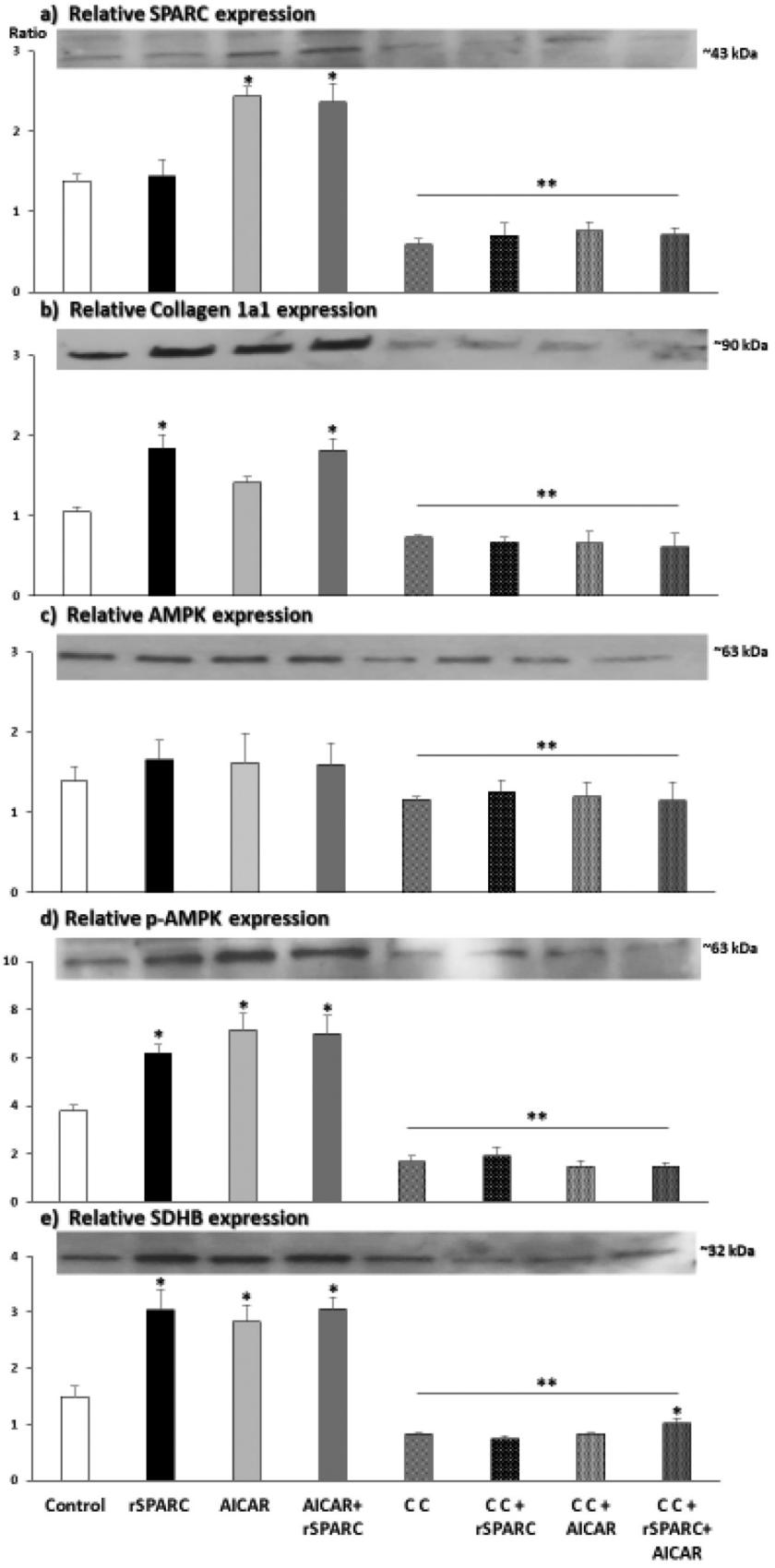

Fig. 3. Addition of rSPARC induced p-AMPK/SDHB as well as collagen 1a1 proteins expression, while AMPK activation increased SDHB and SPARC proteins level in C2C12 myoblasts. In addition, compound C blunted all these effects.

C2C12 myoblasts were deprived of serum for 16 h, then incubated in fresh serum-free medium, followed by treatment with or without rSPARC, AICAR and/or compound C. Proteins extractions were performed 48 h later and cell extracts were used for proteins quantification. 15 to 30 μg of proteins were loaded for western blotting. Data were expressed as a ratio to the control (mean ± SEM, n = 3). 2-way ANOVA represented significant effects of rSPARC/AICAR, compound C and interaction ($p < 0.05$) except for AMPK which had only a compound C effect. *Significant effects compared to control. **Compound C effect.

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside; AMPK, 5′ adenosine monophosphate-activated protein kinase; CC, compound C; p-AMPK, phosphorylated AMPK; PBS, phosphate buffered saline; rSPARC, recombinant SPARC protein; SPARC, secreted protein acidic and rich in cysteine; SDHB, succinate dehydrogenase.

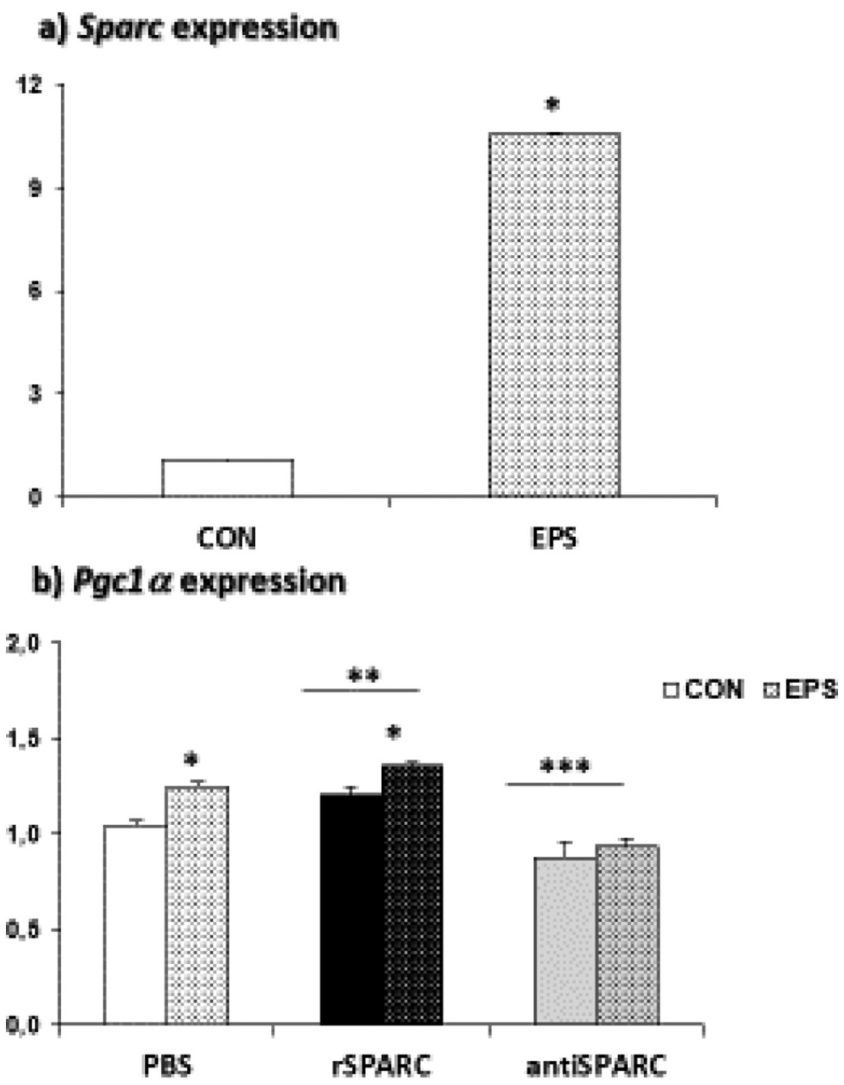

Fig. 4. EPS induced Sparc and Pgc1α genes expression. rSPARC induced Pgc1α expression and anti-SPARC inhibited both effects of EPS and rSPARC on Pgc1α expression.
C2C12 myoblasts were differentiated for 5 days. On day 6, rSPARC (4 μg/ml) and anti-SPARC (40 μg/ml) were added and myotubes were kept for 48 h (CON) or subjected to EPS (1 Hz for 8 h and 50 Hz for 16 h, then repeated another 24 h). Sparc (a) and Pgc1α (b) genes expression was measured by quantitative RT-PCR. Data were expressed as a ratio to the 36B4 gene (mean ± SEM, n = 3). The t-test (a) and 2-way ANOVA with Fisher's protected LSD post-hoc test (b) were applied ($p < 0.05$). *EPS effects. **rSPARC effect. ***anti-SPARC effect.
Abbreviations: anti-SPARC, anti-SPARC antibody; CON, control; EPS, electric pulse stimulation; PBS, phosphate buffered saline; Pgc1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; rSPARC, recombinant SPARC protein; SPARC, secreted protein acidic and rich in cysteine.

to stimulate SPARC secretion from muscle tissues [28].

Therefore, we analyzed the effect of 48-h EPS on Sparc and Pgc1α genes expression in C2C12 myotubes. We observed a greater increase in Sparc and Pgc1α genes expression after 48-h EPS (Fig. 4). In the support of our results, 60 min cyclic stretching of C2C12 myotubes was shown to stimulate SPARC secretion into the extracellular medium [28]. Moreover, Burch et al. have mentioned that EPS of C2C12 mouse muscle cells induces Pgc1α gene expression [83]. The increase in both Sparc and Pgc1α genes expression after 48 h in C2C12 myotubes confirmed our hypothesis regarding the involvement of SPARC in the modulation of muscle mitochondrial function and affirmed the possible link between ECM and mitochondria in muscle cells. Indeed, Sparc and Pgc1α, EPS-induced genes, may be potential targets of gene therapy in the identification of mechanisms by which exercise-induced metabolic changes in the muscle.

5. Conclusion

In conclusion, this report is the first to demonstrate how Sparc, an EPS-induced gene, may be implicated in the possible link between ECM remodeling and mitochondrial function in muscle cells. Our results demonstrated that SPARC activates ILK, which phosphorylates and inactivates GSK-3ß, leading to the stabilization of ß-catenin and the modulation of muscle ECM remodeling. Thus, SPARC/ILK/GSK-3ß pathway may be an important target in studying the involvement of ECM in muscle integrity. On the other hand, we showed that SPARC is involved in the modulation of mitochondrial proteins expression through the activation of AMPK. We also reported a possible link between ECM remodeling and mitochondrial function in muscle tissue and we believed that the new pathway SPARC-ILK/SPARC-AMPK may serve as a therapeutic target for treating diseases related to ECM/mitochondrial dysfunction such as sarcopenia (Fig. 5). To our knowledge, this pathway has not been explored in an animal model of age-related sarcopenia, however, our unpublished data of *Sparc* KO mice with decreased levels of ECM and mitochondrial proteins as well as lower skeletal muscle weight and glucose intolerance support well our in vitro results.

The induction of Sparc expression by EPS as well as by exercise training designates *Sparc* as one of the best candidate genes to develop “an exercise-pill” for sarcopenic elderly who are unable to make exercise. Finally, future studies are necessary to investigate the precise role of SPARC in the link between ECM and mitochondria and to explore different pathways involved in this link. We also anticipated that this therapeutic pathway will open the door to new directions in aging-related diseases therapy research.

Funding

This study was supported by the Canadian Institutes of Health Research (CIHR).

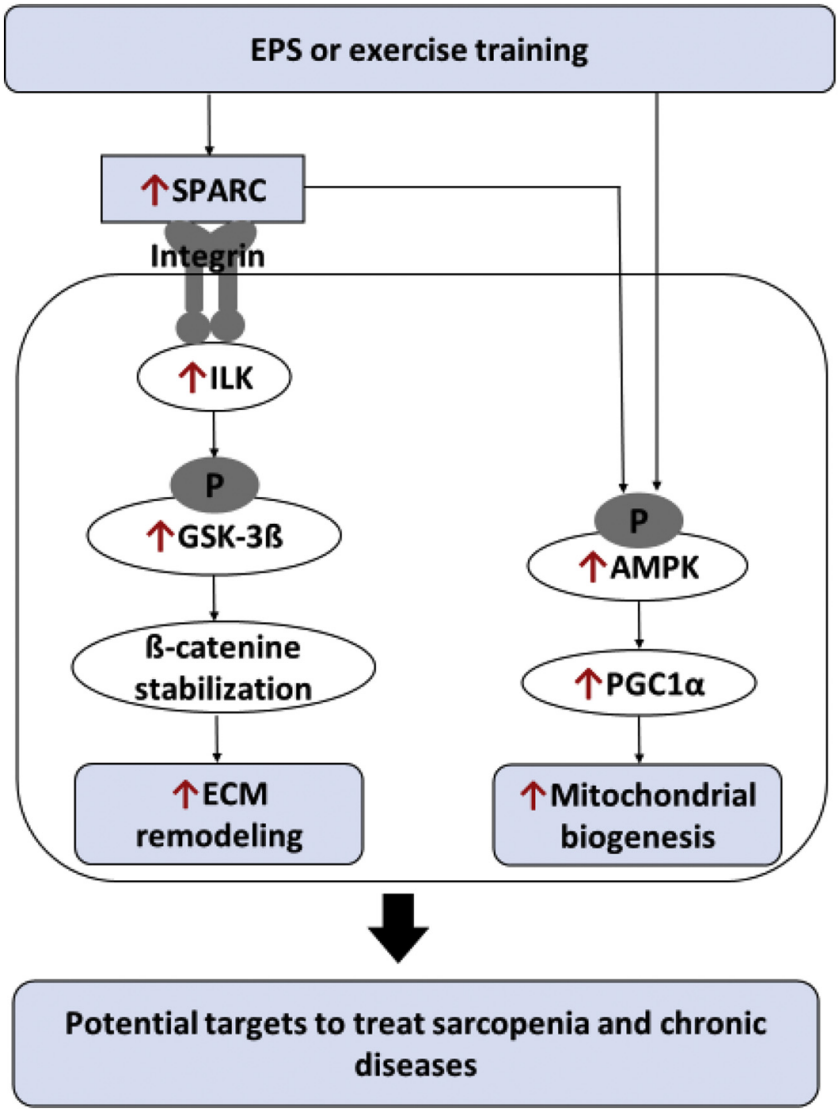

Fig. 5. The involvement of SPARC in the possible link between muscle ECM and mitochondria
Abbreviations: AMPK, adenosine monophosphate kinase; ECM, extracellular matrix; EPS, electrical pulse stimulation; ECM, extracellular matrix; ILK, integrin-linked kinase; GSK-3β, glycogen synthase kinase 3 beta; P, phosphate; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

Declaration of Competing Interest

A. Melouane, M. Yoshioka, M. Kanzaki and J. St-Amand have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.05.070.

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