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Bradykinin mediates myogenic differentiation in murine myoblasts through the involvement of SK1/Spns2/S1P$_2$ axis

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ABSTRACT

Skeletal muscle tissue retains a remarkable regenerative capacity due to the activation of resident stem cells that in pathological conditions or after tissue damage proliferate and commit themselves into myoblasts. These immature myogenic cells undergo differentiation to generate new myofibers or repair the injured ones, giving a strong contribution to muscle regeneration. Cytokines and growth factors, potently released after tissue injury by leukocytes and macrophages, are not only responsible of the induction of the initial inflammatory response, but can also affect skeletal muscle regeneration. Growth factors exploit sphingosine kinase (SK), the enzyme that catalyzes the production of sphingosine 1-phosphate (S1P), to exert their biological effects in skeletal muscle. In this paper we show for the first time that bradykinin (BK), the leading member of kinin/kallikrein system, is able to induce myogenic differentiation in C2C12 myoblasts. Moreover, evidence is provided that SK1, the specific S1P-transporter spinster homolog 2 (Spns2) and S1P$_2$ receptor are involved in the action exerted by BK, since pharmacological inhibition/antagonism or specific down-regulation significantly alter BK-induced myogenic differentiation. Moreover, the molecular mechanism initiated by BK involves a rapid translocation of SK1 to plasma membrane, analyzed by time-lapse immunofluorescence analysis. The present study highlights the role of SK1/Spns2/S1P receptor 2 signaling axis in BK-induced myogenic differentiation, thus confirming the crucial involvement of this pathway in skeletal muscle cell biology.

1. Introduction

Skeletal muscle retains a remarkable regenerative capacity that is responsible for tissue repair. Skeletal muscle regeneration is characterized by an early and transient phase of inflammation that occurs in injured tissue to ensure the removal of dead cells and debris [1–4]. In the damaged microenvironment resident quiescent stem cells, called satellite cells, become activated, proliferate and migrate towards the site of the lesion, where they differentiate and fuse with each other or with injured myofibers in order to repair the tissue [5]. Both resident and infiltrating cells release soluble factors that affect muscle regeneration, however, their exact effects on muscle recovery remain unknown. A deeper knowledge of the mechanisms underlying skeletal muscle regeneration becomes crucial to identify possible innovative interventions to efficaciously treat skeletal muscle diseases.

Cytokines and growth factors, produced and released after damage by the injured tissue or by infiltrating leukocytes and macrophages, are not only responsible for the generation/amplification of the initial inflammatory response, but also affect skeletal muscle regeneration [4,6–8]. A role for the nonapeptide bradykinin (BK), the leading member of kinin/kallikrein system, in skeletal muscle vasodilation is well established during contraction, when the peptide is actively released [9,10]. BK exerts its biological action after the ligation to two distinct GPCR, named bradykinin B1 receptor (B1R) and bradykinin B2 receptor (B2R) based on their distinct pharmacology [11]. B2R subtype was found to

Abbreviations: SK, Sphingosine kinase; S1P, sphingosine 1-phosphate; BK, bradykinin; Spns2, spinster homolog 2; S1P$_2$, S1P receptor 2; B1R, bradykinin B1 receptor; B2R, bradykinin B2 receptor; SPL, sphingosine 1-phosphate lyase; MyHC, skeletal fast myosin heavy chain.

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be constitutively expressed in myocytes, while B1R is barely detectable in normal healthy skeletal muscle, being induced after injury [9,12–15]. The molecular mechanism of BK action improve oxygen delivery [16]. In addition, in skeletal muscle BK is involved in nutrient disposal, since it has been shown that the peptide enhances insulin signal and potentiates insulin-stimulated glucose uptake through the B2R in cultured myoblasts [17]. Moreover, genetic polymorphism for B2R correlates with skeletal muscle performance, muscle strength or size [18]. Finally, BK is degraded by ACE and hence it is inversely linked with the angiotensin II system, which plays a major role in skeletal muscle wasting and sarcopenia [19].

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid generated by sphingomyelin catabolism, physiologically present in plasma and serum, capable of regulating multiple cellular processes, including proliferation and survival, cell motility and differentiation [20]. S1P is synthesized from sphingosine through the ATP-dependent phosphorylation catalyzed by sphingosine kinase 1 (SK1) or 2 (SK2), which are activated by various stimuli, and reversibly degraded by specific S1P phosphatases and non-specific lipid phosphate phosphatases or irreversibly cleaved by S1P lyase (SPL) [21]. The sphingolipid can exert its function as an intracellular messenger, even if it acts mainly as ligand of specific receptors ($S1P_{1-5}$) [22], after its release via specific and unspecific transporters, spinster homolog 2 (Spns2) and ATP-binding cassette (ABC) family members, respectively [23]. The signaling pathways downstream the different S1P receptors can influence many biological processes, such as angiogenesis, immune response, tumorigenesis and embryonic development [24]. Of note, the biological role exerted by S1P signaling axis in skeletal muscle has been described in literature. Indeed, the bioactive sphingolipid stimulates proliferation of satellite cells and cell motility [25], whereas it behaves as pro-myogenic [26] and anti-motogenic [27] cue in murine myoblasts. Experimental evidence has been provided that many growth factors affecting skeletal muscle regeneration in physiological or pathological conditions require the so-called inside-out S1P signaling [28]. For this reason, the regulation of S1P-synthesizing enzymes becomes crucial for the modulation of S1P levels. In this regard, PDGF and IGF1 exploit SK activation and S1P receptor engagement, to exert their biological actions towards a reduced proliferation/enhanced cell motility and differentiation, respectively [29,30]. A complex cross talk between TGFβ and S1P signaling axis has also been highlighted, that accounts for the detrimental pro-fibrotic and pro-apoptotic effects of the cytokine [31,32].

In this study we have identified BK as promyogenic cue in C2C12 myoblasts. BK-induced myoblast differentiation has been found to rely on SK1 translocation to plasma membrane. Moreover, the specific transporter Spns2 and $S1P_2$ engagement appear to be required for the biological response of BK. The present results contribute to the characterization of the physiological action of pro-inflammatory cue BK on skeletal muscle regeneration, confirming at the same time the crucial role of S1P inside-out signaling in skeletal muscle cell biology.

2. Materials and methods

2.1. Materials

Biochemicals, TRI Reagent® RNA Isolation Reagent, cell culture reagents, protease inhibitor cocktail, bovine serum albumin (BSA), monoclonal anti-fast skeletal muscle myosin heavy chain (MyHC, clone MY-32), bradykinin (BK), mouse skeletal muscle C2C12 cells, Dulbecco's modified Eagle's medium (DMEM), Dulbecco's modified phosphate-buffered saline (DPBS) and Fetal calf serum (FCS) were purchased from Sigma-Aldrich (St. Louis, MO). Pharmacological inhibitors of SKs (VPC96091 and VPC96047) were kindly provided by Prof. K. Lynch, University of Virginia, USA. pcDNA3 plasmid encoding B2R was kindly provided by Dr. Andree Blaukat (formerly University of Heidelberg, Germany). GFP-SK1-S225A encoding plasmid was kindly provided by Stuart Pitson University of South Australia. The specific $S1P_{1/3}$ antagonist, VPC23019, and the selective $S1P_2$ antagonist, JTE013, were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Short interfering RNA (siRNA) duplexes targeting specific gene of interest such as mouse SK1 (SASI_Mm01_00033983 and SASI_Mm01_00033984), mouse SK2 (SASI_Mm01_00050883 and SASI_Mm01_00050884), mouse $S1P_2$ (SASI_Mm01_00082880 and SASI_Mm01_00082881) mouse Spns2 (EMU148461) and scrambled (SCR)-siRNA (Mission Universal Negative control no. 1) were from Sigma-Proligo (The Woodlands, TX, USA). Lipofectamine RNAiMAX® Transfection Reagent and all reagents and probes used to perform real-time PCR were obtained from Life Technologies (Carlsbad, CA, USA). SK2 (N-terminal region) rabbit polyclonal and SK1 (central region) rabbit polyclonal antibodies were purchased from ECM Biosciences LLC (Versailles, KY USA). Secondary antibodies conjugated to horseradish peroxidase, monoclonal anti-β-actin and monoclonal anti-myogenin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-caveolin-3 antibody was from BD Biosciences Transduction Laboratories (Lexington, KY, USA). Enhanced chemiluminescence (ECL) reagents were obtained from GE Healthcare Europe GmbH (Milan, Italy). Fluorescein-conjugated anti-mouse secondary antibody and Vectashield® mounting medium were purchased from Vector Laboratories (Burlingame, CA, USA).

2.2. Cell culture

Murine C2C12 myoblasts were maintained in DMEM containing 10% FCS, 2mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin at 37 °C in 5% CO_2. For myogenic differentiation, cells were seeded in p35 and when 90% confluent, the proliferating medium was replaced with DMEM without serum supplemented with 1 mg/ml BSA and incubated in the presence or absence of 1 μM BK for 24 or 48 h. In some of the experiments, cells were pre-treated with 1 μM JTE013, 1 μM VPC23019, 5 μM VPC96047 or 5 μM VPC96091, 30 min before agonist stimulation.

2.3. Cell transfection

For siRNA transfection, C2C12 cells were transfected with Lipofectamine RNAi-MAX according to the manufacturer's instructions. Briefly, Lipofectamine RNAiMAX was incubated with siRNA in DMEM without serum and antibiotics at room temperature for 20 min, and afterwards the lipid/RNA complexes were added with gentle agitation to C2C12 cells to a final concentration of 50 nM in serum containing DMEM. After 24 h, cell medium was changed with serum-free DMEM containing 1 mg/ml BSA and then used for the experiments within 48 h from the beginning of the transfection. The specific gene knockdown was evaluated by real-time PCR.

For live cell imaging fluorescence experiments, C2C12 cells were transiently co-transfected with plasmids encoding for bradykinin B2 receptor (B2R) and GFP-SK1 or GFP-SK1-S225A using Lipofectamine® 2000 Reagent according to the manufacturer's instructions. Transfection with GFP-SK1 or GFP-SK1-S225A plasmids has been previously described [33]. Briefly, cells were seeded onto 8-well poly-L-lysin-coated glass coverslips, and then transfected with a total amount of 0,5 μg of plasmid DNA per well. After 24 h, transfection medium was replaced with proliferating medium for other 24 h. Cells were than starved overnight before experiments. Transfection efficiency for GFP-SK1 and GFP-SK1-S225A was analyzed by fluorescence microscopy of living cells, and cells were used for the experiments within 72 h from the beginning of the transfection.

2.4. Western blot analysis

C2C12 myoblasts after 24 and 48 h of BK treatment were collected and lysed 30 min at 4 °C in a buffer containing 50 mM Tris, pH7.5, 120 mM NaCl, 1 mM EDTA, 6 mM EGTA, 15 mM $Na_4P_2O_7$, 20 mM NaF, 1% Nonidet and protease inhibitor cocktail. Then lysates were centrifuged at 10ˆ4×*g*, 15 min 4 °C and 15 μg of protein from total cell lysates were used to perform a SDS-polyacrylamide gel electrophoresis and Western blot (WB) analysis [26] in order to evaluate the different expression of SK1, SK2, the myogenic differentiation markers, myogenin and caveolin-3. PDVF membranes were incubated overnight with the primary antibodies at 4 °C and then with specific secondary antibodies for 1 h at room temperature. Binding of the antibodies with the specific proteins has been detected by chemiluminescence.

2.5. Quantitative real-time RT-PCR

Total RNA from C2C12 myoblasts was extracted using a TRI Reagent® RNA Isolation Reagent. Then, 1 μg of RNA was reverse transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems). TaqMan gene expression assays were used to perform real-time PCR in order to quantify SK1, SK2, $S1P_2$ and Spns2 mRNA expression. Each measurement was carried out in triplicate, using the automated ABI Prism 7700 Sequence Detector System (Applied Biosystems) as described previously [31], by simultaneous amplification of the target sequence (SK1 Mm00448841_g1, SK2 Mm00445021_m1, $S1P_2$ Mm01177794_m1, Spns2 Mm01249328_m1; Applied Biosystems) together with the housekeeping gene 18S rRNA. The analysis of the results was performed by ABI Prism Sequence Detection System software, version 1.7 (Applied Biosystems). The 2ˆ(-ΔΔCt) method was applied as a comparative method of quantification [34], and data were normalized to ribosomal 18S RNA expression.

2.6. Immunostaining and fluorescence microscopy

C2C12 myoblasts were seeded on pre-coated (2% gelatin) microscope slides. After 24 h the proliferating medium was replaced with DMEM without FCS supplemented with 1 mg/ml BSA and cells were treated or not with 1 μM BK. In some of the experiments, cells were pre-treated with 5 μM VPC96047 or 5 μM VPC96091, 30 min before agonist stimulation. After 96 h, cells were fixed in 2% paraformaldehyde in PBS for 20 min and then permeabilized in 0.1% Triton X-100–PBS for 30 min. For blocking step cells were kept in agitation with 3% BSA in DPBS for 1 h. Then, C2C12 were incubated with anti-MyHC antibody for 2 h and fluorescein-conjugated anti mouse secondary antibody for 1 h. Finally, cells were incubated with propidium iodide solution (0.5 μM PI in 0.3 M NaCl, 0.03 M sodium citrate, pH7.0) for 15 min for nuclear staining. Images were taken using a Leica SP5 laser scanning confocal microscope with 63× objective.

2.7. Live cell imaging

C2C12 cells were seeded onto 8-well poly-L-lysin-coated glass coverslips and co-transfected as described above. During the live imaging acquisition, C2C12 cells were incubated in HBSS (containing 118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 15 mM HEPES, pH7.4) and treated with 1 μM BK. Excitation laser lines/emission filter were set up at 488/505 nm for GFP. Fluorescence images were obtained using a Zeiss LSM510 inverted confocal laser scanning microscope and a Plan-Apochromat 63×1.4 oil immersion objective (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

The translocation half-time was calculated by measuring the decrease of fluorescence intensity in the cytosolic region and fitting of an exponential function to this decay [33].

2.8. Determination of sphingolipid concentrations by high-performance liquid chromatography tandem mass spectrometry

Cell pellets were suspended with 100 μl extraction buffer (citric acid 30 mM, disodium hydrogen phosphate 40 mM) and 20 μl of the internal standard solution containing sphingosine-d7, sphinganine-d7 (30 ng/ml each), sphingosine-1-phosphate-d7 (40 ng/ml, all avanti polar lipids, Alabaster, USA). The mixture was extracted once with 500 μl methanol/chloroform/hydrochloric acid (15:83:2, v/v/v). The lower organic phase was evaporated at 45 °C under a gentle stream of nitrogen and reconstituted in 50 μl of methanol.

Analytes were separated using an Agilent 1260 series binary pump (Agilent technologies, Waldbronn, Germany) equipped with a Kinetex EVO C18 HPLC column (50 mm × 2.1 mm ID, 1.7 μm particle size, 100 Å pore size; Phenomenex, Aschaffenburg, Germany). The column temperature was 55 °C. The HPLC mobile phases consisted of water with 0.5% formic acid and (mobile phase A) and acetonitrile/isopropanol/acetone (50:30:20, v/v/v) with 1% formic acid (mobile phase B). For separation, a gradient program was used at a flow rate of 0.3 ml/min. The total running time was 7.5 min and the injection volume was 20 μl. After every sample, ethanol was injected for washing the column. The MS/MS analyses were performed using a triple quadrupole mass spectrometer 5500QTRAP (Sciex, Darmstadt, Germany) equipped with a Turbo V Ion Source operating in positive electrospray ionization mode. The MS parameters were set as follows: Ionspray voltage 4500 V, source temperature 500 °C, curtain gas 35 psi, collision gas 6 psi, nebulizer gas 50 psi and heating gas 70 psi. The analysis was done in Multiple Reaction Monitoring (MRM) mode.

Data Acquisition was done using Analyst Software V 1.6 and quantification was performed with MultiQuant Software V 3.0 (both Sciex, Darmstadt, Germany), employing the internal standard method (isotope dilution mass spectrometry). Variations in accuracy of the calibration standards were less than 15% over the whole range of calibration, except for the lower limit of quantification, where a variation in accuracy of 20% was accepted.

2.9. Glucose uptake assay

To determine glucose uptake, C2C12 cells were seeded in 6-well plates, serum-starved for 18 h and then stimulated with 1 and 10 μM BK and 10 nM insulin for 60 min. Briefly, following BK or insulin challenge, a buffered solution (140 mM NaCl, 20 mM Hepes, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$ and 5 mM KCl, pH7,4) containing 1 μCi of ^{3}H–glucose (1 mCi/0.1 mmol) was added to each well and glucose uptake was allowed at 37 °C for 15 min. Cells were then washed with cold PBS and lysed in 0.1 M NaOH. Incorporated radioactive glucose was quantified by liquid scintillation counting of aliquots of the lysates, and normalized on protein content.

2.10. Statistical analysis

ImageJ software was used to perform densitometric analysis of the Western Blot bands. The images obtained in live fluorescence were edited using the programs LSM Image Browser or ZEN (www.zeiss.com/micro). Graphical representations were obtained by GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Statistical analysis was performed using Student's *t*-test, one-way and two-way ANOVA analysis of variance followed by Bonferroni's *post-hoc* test. Asterisks show statistical significance as reported in figure legends.

3. Results

In order to study the possible action of BK on myogenic differentiation, a concentration-response effect of BK on myogenic marker expression was analyzed in confluent serum starved C2C12 myoblasts, after the addition of different concentrations of BK for 24 h. Total cell lysates were subjected to western blot analysis to measure the relative content of myogenic marker myogenin. As reported in Fig. 1A, 24 h BK treatment was capable of inducing myogenin expression in a dose-dependent manner, being efficacious both at 1 μM and 10 μM concentration. Taking into consideration that myogenin is an early transcriptional myogenic marker, we then employed confocal immunofluorescence analysis to quantify the expression of a later functional myogenic marker such as myosin heavy chain (MyHC). The confocal microscopy images illustrated in Fig. 1B clearly demonstrate that 1 μM BK potently enhanced the expression of MyHC after three day-incubation and induced a more mature phenotype in myotubes.

Moreover, with the specific aim to study glucose uptake by BK in C2C12 myoblasts, we performed ^{3}H-glucose incubation for 15 min after the stimulation with the peptide at 1 and 10 μM for 60 min. We established that glucose uptake was not significantly affected by BK challenge, whereas it was significantly increased after 10 nM insulin stimulation (Fig. 2), ruling out a role for BK in glucose disposal in C2C12 myoblasts.

Since SK1/S1P axis exerts a pro-myogenic action in murine myoblasts [35], the involvement of this signaling pathway in BK-mediated effect was studied. For this reason, a pharmacological approach was used to inhibit SK activity and analyze its involvement in BK-induced myogenic differentiation. The treatment of C2C12 myoblasts with specific SK1 inhibitor VPC96091 and pan-inhibitor VPC96047 for 18 h caused a dramatic decrease of S1P levels measured by high-performance liquid chromatography tandem mass spectrometry (Fig. 3A). The blockade of SK1 by VPC96091 significantly decreased the expression of myogenin and the late myogenic marker caveolin-3 induced by 1 μM BK for 24 and 48 h, respectively. On the other hand, the treatment with VPC96047 potently reduced myogenin expression, whereas it affected to a lesser extent caveolin-3 levels after BK challenge (Fig. 3B).

The involvement of SK in BK-mediated differentiation was further studied by measuring the expression of MyHC to confirm the results obtained by WB for myogenin and caveolin-3 expression. Immunofluorescence analysis performed in three-day serum-starved murine myoblasts treated with 1 μM BK after pre-treatment with VPC96091 (Fig. 4) showed that the expression of MyHC was significantly reduced by SK1 inhibition, thus demonstrating that the enzyme is involved in BK-induced myogenic differentiation. Pre-incubation with SK pan-inhibitor VPC96047 less strongly inhibited MyHC expression.

To further establish the role exerted by each SK isoform, RNA interference approach was used. Efficient and selective down-regulation of SK1 (Fig. 5A and B) in myoblasts transfected with specific murine SK1-siRNA, beside significantly reducing the basal level of myogenic marker expression, abrogated BK-mediated myogenin and caveolin-3 expression, as illustrated in Fig. 5C. Conversely, specific SK2 down-regulation (Fig. 5A and B) did not alter the effect brought about by BK (Fig. 5C).

Then to dissect the molecular mechanism by which SK1 is involved in BK action, we analyzed the effect of BK on SK1 intracellular localization. Taking into account that the biological actions of BK are transmitted by its B2 type receptor (B2R), being the B1R physiologically absent [14–17], C2C12 myoblasts were co-transfected with B2R and GFP-SK1 (Fig. 6A) encoding plasmids. By immunofluorescence microscopy analysis, we examined the effect of 1 μM BK treatment showing that the peptide was capable of inducing a rapid translocation of SK1 from cytosol to the plasma membrane. SK1 translocation to plasma membrane was measured by time-lapse laser-scanning confocal microscopy. The decrease of fluorescence in the cytosol after BK treatment was used to calculate the translocation half-time of the enzyme that ranged between 4 and 7 s (Fig. 6B), sustaining the view that the BK challenge provokes a very robust translocation of SK1.

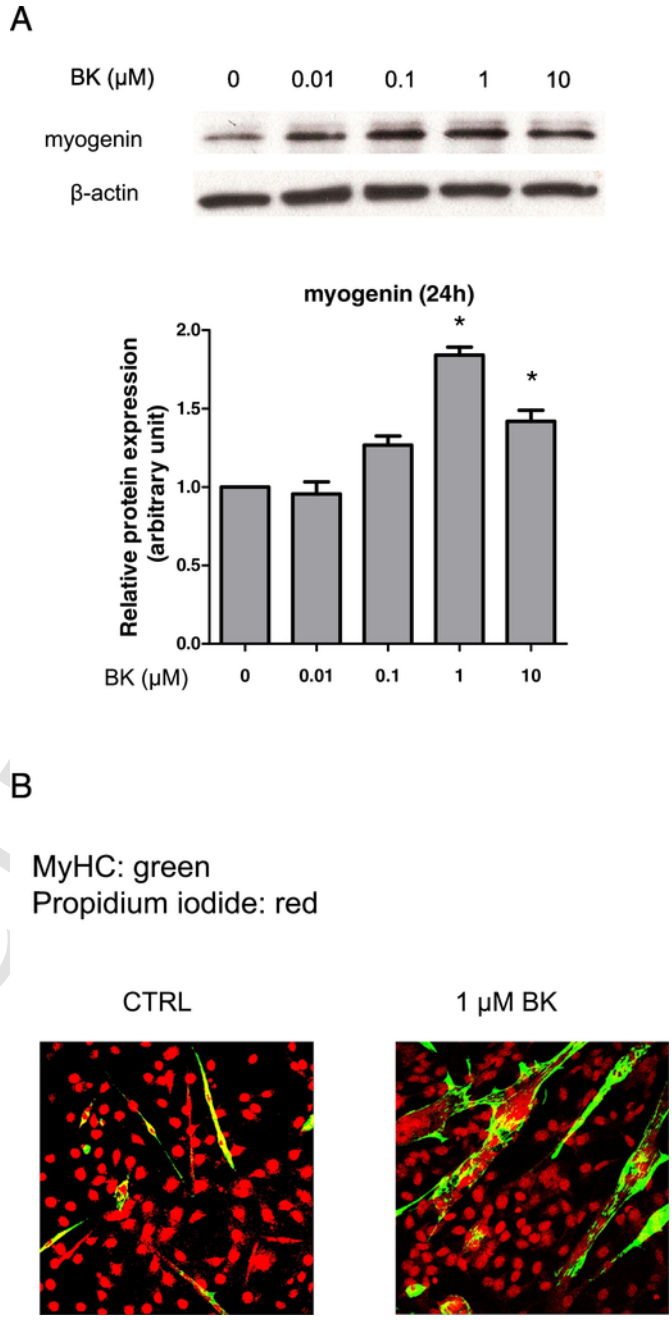

Fig. 1. Concentration-dependent effect of Bradykinin on myogenin expression in murine myoblasts.**A.** Total cell lysates (15 μg) of C2C12 cells stimulated with the indicated concentration of BK for 24 h, were subjected to western blotting to measure myogenin expression. Blot is representative of three experiments with similar results. The effect of 1 and 10 μM BK on myogenin expression, normalized to that of beta isoform of actin (β-actin), was statistically significant by one-way Anova, followed by Bonferroni's post-hoc test, $P < 0.05$. **B.** Confocal immunofluorescence analysis was performed in three-days serum-starved C2C12 cells in the presence or absence of 1 μM BK, using anti-fast skeletal muscle myosin heavy chain (MyHC) antibody, fluorescein-conjugated anti-mouse secondary antibody and propidium iodide staining. The immunofluorescence images here reported are representative of three independent experiment with each condition performed in duplicate with similar results.

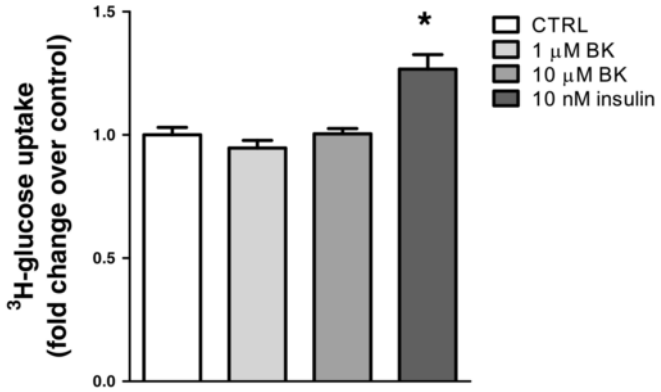

Fig. 2. Effect of Bradykinin on glucose uptake in murine myoblasts.C2C12 cells stimulated with the indicated concentration of BK or 10 nM insulin for 60 min were incubated with radiolabeled ^{3}H-glucose for 15 min to measure glucose uptake. Data are mean ± SEM obtained from three independent experiment each performed in triplicate, and reported as fold increase over control, set as 1. The effect of 10 nM insulin on glucose uptake was statistically significant compared to control untreated by one-way Anova followed by Bonferroni's post-hoc test, $*P < 0.05$.

Pitson and colleagues previously demonstrated that SK1 translocation from the cytosol to plasma membrane is induced by Ser225 phosphorylation [36]. To analyze the contribution of Ser225 phosphorylation to SK1 translocation, the above-described experiments were performed in C2C12 myoblasts co-transfected with plasmids encoding for B2R and for the phosphorylation-defective mutant of hSK1, GFP-SK1-S225A. Translocation half-time measured by fluorescence variation in the cytosol of GFP-SK1-S225A after BK treatment was unaltered compared to GFP-SK1, suggesting that Ser225 phosphorylation was not necessary to induce membrane translocation of the enzyme after BK challenge (Fig. 6A and B).

Myogenic differentiation brought about by exogenously added S1P in murine myoblasts has been reported to be accomplished through $S1P_2$ receptor engagement [26]. The possible involvement of S1P receptors in BK mediated action was analyzed by using specific antagonists for S1P1/3 VPC23019 or $S1P_2$ JTE013. The specific blockade of $S1P_2$ was capable of abrogating BK-induced caveolin-3 and myogenin expression, demonstrating that this receptor subtype is involved in the action mechanism of BK (Fig. 7). However $S1P_{1/3}$ blockade did not alter myogenin expression, it slightly enhanced caveolin-3 basal level even if in a not significant manner (Fig. 7).

The engagement of $S1P_2$ in BK-dependent differentiation was further analyzed by RNA interference approach. Specific $S1P_2$ siRNA was transfected in murine myoblasts to knockdown the expression of this receptor subtype. $S1P_2$ downregulation, assessed by Real-Time PCR in Fig. 8A, caused an impairment of BK-induced caveolin-3 and myogenin expression as depicted in Fig. 8B, confirming the results obtained with the pharmacological approach. Morphological analysis by optical microscopy was used to analyze the effect of BK on myogenic differentiation in $S1P_2$-siRNA transfected skeletal muscle cells after four days of serum deprivation. As shown in Fig. 8C, $S1P_2$ knocked-down murine myoblasts were unable to differentiate towards mature myotubes in response to 1 μM BK, thus claiming a crucial role for this specific receptor subtype in BK action.

The participation of both SK1 and $S1P_2$ in BK-induced myogenic effect could be explained via the "inside-out" mechanism of action, in which S1P produced within the cell can be exported via S1P transporters in order to act as ligand of S1P receptors. To address this issue, RNA interference was used to investigate the role of the specific transporter Spns2 in BK-dependent myogenic differentiation. The specific down-regulation of Spns2, checked by Real-Time PCR in siRNA transfected myoblasts (Fig. 9A), fully abrogated the augmented expression of myogenin and caveolin-3 induced by BK at 24 and 48 h, respectively (Fig. 9B). These latter results clearly demonstrate that BK stimulates myoblast differentiation towards myotubes through the involvement of the specific S1P transporter responsible for the export of the bioactive lipid that acts in autocrine or paracrine manner.

4. Discussion

Skeletal muscle is a stable tissue that retains a good regenerative capacity, which is responsible for tissue repair after injury. Skeletal muscle regeneration is characterized by an early phase of acute inflammation that accounts for the clearance of dead cells and cell debris [37–40]. Soluble factors produced and released by resident and infiltrating cells, constitute a microenvironment that affects later phases of skeletal muscle regeneration, when the tissue is repaired by resident stem cell activation, proliferation and differentiation [41]. However, in chronic human diseases such as dystrophies and other myopathies repeated cycles of degeneration of newly formed myofibers progressively exhaust the stem cell pool and, in association with chronic inflammation, cause persistent tissue damage and fibrosis [42].

BK is a vasoactive nonapeptide that is involved in the maintaining of circulation homeostasis, exerting its function mainly through its specific binding to G protein coupled receptors inducible B1R and constitutive B2R [43,44]. Within skeletal muscle, the peptide promotes an increase of muscle blood flow and glucose uptake [16,17]. A role for BK in tissue inflammation and vasodilatation, plasma extravasation has been extensively addressed in literature [45]. During skeletal muscle contraction, the rise of interstitial BK is relevant for the increase in blood flow and vasodilation, improving oxygen delivery that meets the elevated oxygen demand during exercise [10]. In inflammatory states, BK promotes both the migration of inflammatory cells from blood to tissues and the activation of various tissue components such as macrophages [46]. However, whether BK plays a role in skeletal muscle regeneration has not been yet investigated.

In this study, satellite cell-derived murine cell line, C2C12 myoblasts, has been employed, which is extensively used as a model to study skeletal muscle biology *in vitro*. These cells display a myogenic behavior, differentiating into syncytial cells, known as myotubes. Here we show for the first time that BK increases the myogenic differentiation of myoblasts that was analyzed by using a molecular and morphological approach. On this basis, we can speculate that during skeletal muscle regeneration BK, besides being released after a damage to affect macrophage and lymphocyte recruitment, can also promote skeletal muscle repair by inducing the differentiation of myogenic precursor cells. Intriguingly, a specific allelic polymorphism of B2R was associated with greater skeletal muscle metabolic efficiency in both athletes and non-athletes [47] and with muscle hypertrophy [18]. Notably, experiments aimed at measuring BK-induced glucose uptake showed that the peptide did not significantly affect this process in C2C12 myoblasts. Although this is not in agreement with previous results that identify a role for BK in GLUT4 translocation and glucose uptake in different cell models [48,49], it can be explained by the absence of GLUT4 in C2C12 cell line [50].

It is well established that several cytokines and growth factors modulate S1P signaling axis to exert their biological functions. In this regard, it has been previously demonstrated that in murine myoblasts the recognized pro-myogenic cue IGF1 acts via the involvement of SK/$S1P_2$ [29]. The molecular mechanism brought about by IGF1 implicates the rapid translocation of both SK1 and SK2 to plasma membrane. Conversely, in the present study, we show that BK action depends on the regulation of SK1, since when the enzyme was inhibited or specifically down-regulated by siRNA treatment, BK effect was potently reduced. Inhibition or downregulation of SK2 did not influence BK promyogenic effect.

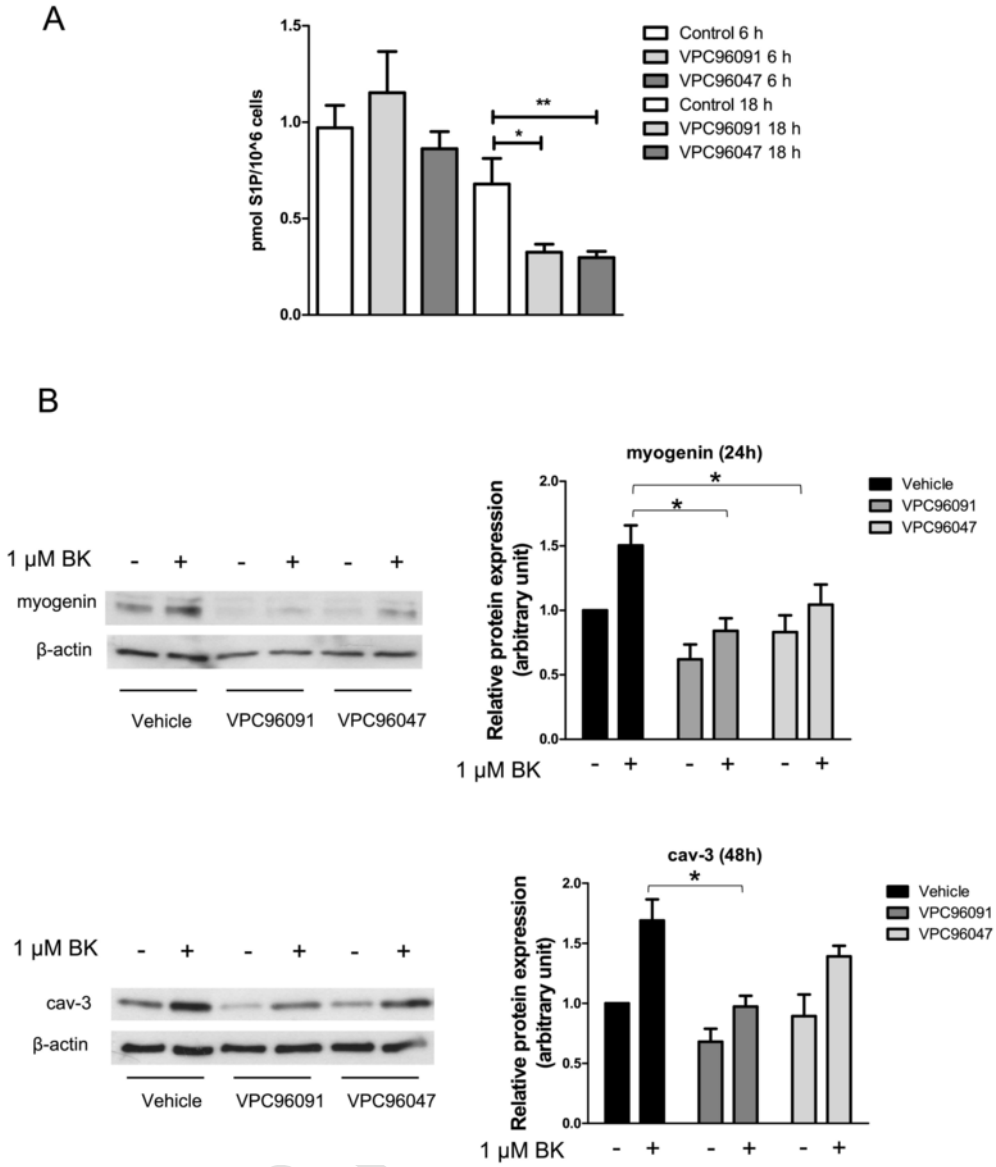

Fig. 3. Role of SK1 in Bradykinin-induced myogenic differentiation of C2C12 myoblasts (I)**A.** Serum-starved C2C12 cells were treated with SK1 specific inhibitor (VPC96091) or SK pan-inhibitor (VPC96047) for 6 and 18 h and cell lysates were analyzed for S1P content by high-performance liquid chromatography tandem mass spectrometry as described in Materials and Methods. Results are reported as mean ± SEM of pmol S1P/10^6 cells. The effect of VPC96091 and VPC96047 on S1P levels was statistically significant by one-way Anova, followed by Bonferroni's post-hoc test, $*P < 0.05$, $**P < 0.01$.**B.** C2C12 cells were stimulated with 1 μM BK for 24 and 48 h after the treatment with SK1 specific inhibitor (VPC96091) or SK pan-inhibitor (VPC96047) for 30 min, and cell lysates were subjected to western blotting to measure myogenic differentiation markers myogenin and caveolin-3 (cav-3), normalized to the expression of non-muscle beta isoform of actin (β-actin). Blot is representative of three experiments with similar results. The effect of VPC96091 and of VPC96047 on BK-induced myogenin expression was statistically significant by two-way Anova, followed by Bonferroni post-hoc test, $*P < 0.05$. The effect of VPC96091 on BK-induced caveolin-3 expression was statistically significant by two-way Anova, followed by Bonferroni post-hoc test, $*P < 0.05$.

It has been previously demonstrated that SK1 activation requires phosphorylation and translocation to the plasma membrane [36], where the substrate sphingosine is located. Results presented here, however, clearly show that phosphorylation is not necessary for B_2 receptor-induced membrane translocation of SK1 in C2C12 myoblasts, since the translocation half-time of the GFP-SK1-S225A mutant after BK challenge was similar to that of the wild-type enzyme. Phosphorylation-independent translocation of SK1 has been shown before in HEK-293 cells for the M_3 muscarinic receptor, which also is a G_q-coupled receptor [33]. Thus, it might be speculated that G_q-mediated activation of SK1 involves phosphorylation-independent signaling pathways, however, it cannot be excluded that phosphorylation of SK1, while dispensable for translocation, is still necessary for its activation.

During acute inflammation in response to injury or invading pathogens cytokines, BK and TNFα among others, are released to mobilize effector cells and molecules to the site of injury and to promote tissue healing. Low-dose TNFα is physiologically involved in the adaptation of healthy skeletal muscle to intensive exercise by promoting the differentiation of muscle progenitors [51,52]. The here reported biological action of BK on myoblast differentiation is similar to the effect of low-dose TNFα in the biology of murine myoblasts, where it has been previously demonstrated that the cytokine exerts a pro-differentiating effect through the involvement of SK1/$S1P_2$ axis [53].

The observation that BK-induced myogenic effect is dependent on $S1P_2$-engagement is in accordance with previous findings indicating that $S1P_2$ is responsible for transducing the pro-differentiating action of exogenous and endogenous S1P in these cells [27]. Noteworthy, we hy-

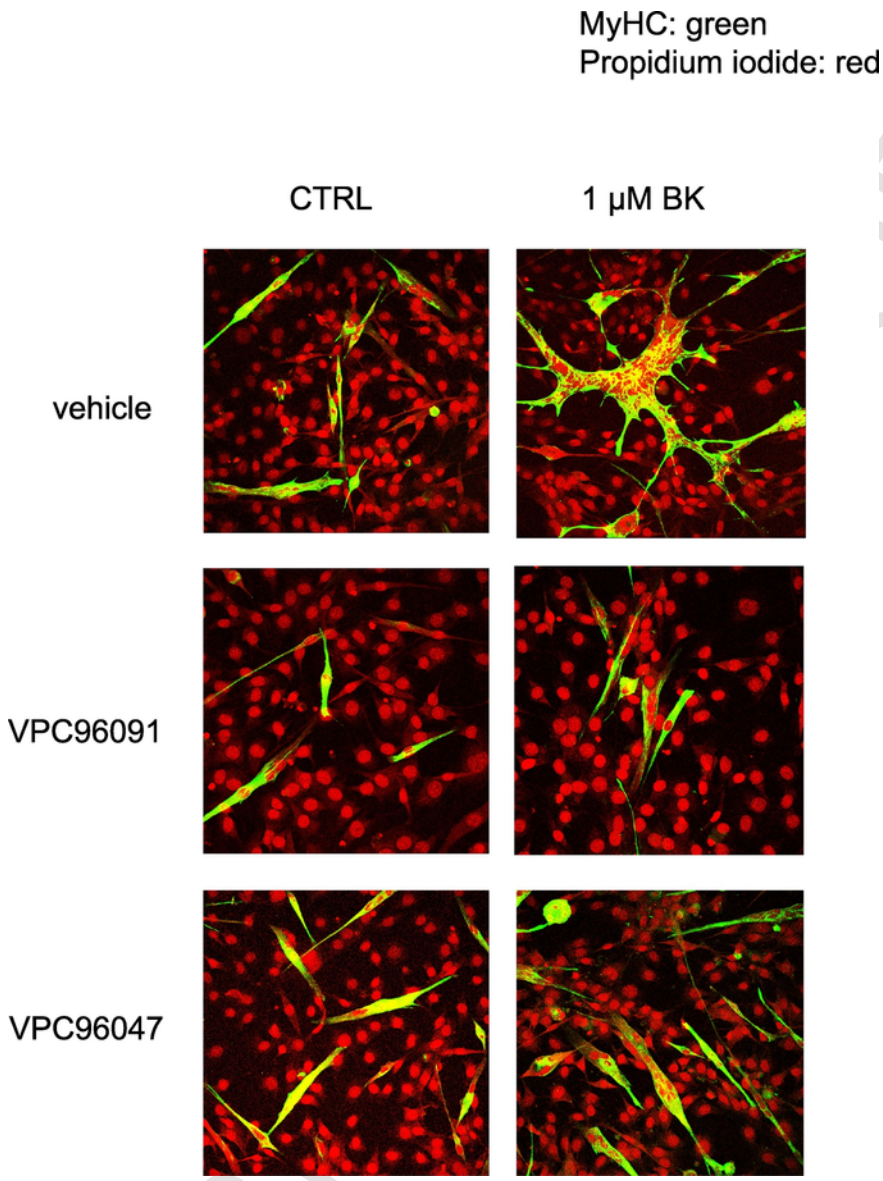

Fig. 4. Role of SK1 in Bradykinin-induced myogenic differentiation of C2C12 myoblasts (II)Immunofluorescence analysis of the myogenic marker myosin heavy chain (MyHC) was performed in three day-differentiated myoblasts in the presence or absence of 1 μM after the treatment with SK1 specific inhibitor VPC96091 and SK pan-inhibitor VPC96047, using a specific mouse anti-myosin heavy chain (MyHC) antibody, fluorescein-conjugated anti-mouse secondary antibody and propidium iodide staining (images are representative of three independent experiments).

pothesized that the transactivation of $S1P_2$ by BK occurs via the well-known S1P inside-out signaling, that implicates the involvement of an S1P transporter. Indeed, we found out that Spns2 is expressed in murine myoblasts in agreement with a previous publication [54] and that, the pro-differentiating effect of BK was completely abolished when Spns2 was specifically down-regulated, thus implicating that S1P export is necessary in BK-dependent action.

Notably, we observed that SK1 blockade or knock-down were capable of dramatically decreasing basal myogenic markers expression, in agreement with the critical role of SK1 in myogenic differentiation [35]. However, the same effects were not observed targeting Spns2 or S1P2, maybe due to the spatially and temporally restricted engagement of the transporter and the receptor by BK to transmit its specific pro-myogenic effect.

Intensive research aimed at addressing the molecular mechanisms of skeletal muscle regeneration will feasibly identify innovative targets to treat acute muscle injuries and chronic muscle diseases. These findings may have relevant implications for therapeutic purposes, since a treatment for such skeletal muscle diseases is not yet available.

5. Conclusion

The here reported results describe for the first time a pro-myogenic effect of BK in murine myoblasts. Moreover, BK has been here shown to be capable of inducing a rapid SK1 translocation to plasma membrane that is required for BK-induced differentiation of myoblasts.

The presented data support the hypothesis that a precise regulation of S1P inside-out signaling takes place in the pro-myogenic action of BK. In particular, this pathway involves Spns2 transporter and the $S1P_2$ transactivation that are both responsible for BK-induced myogenic differentiation.

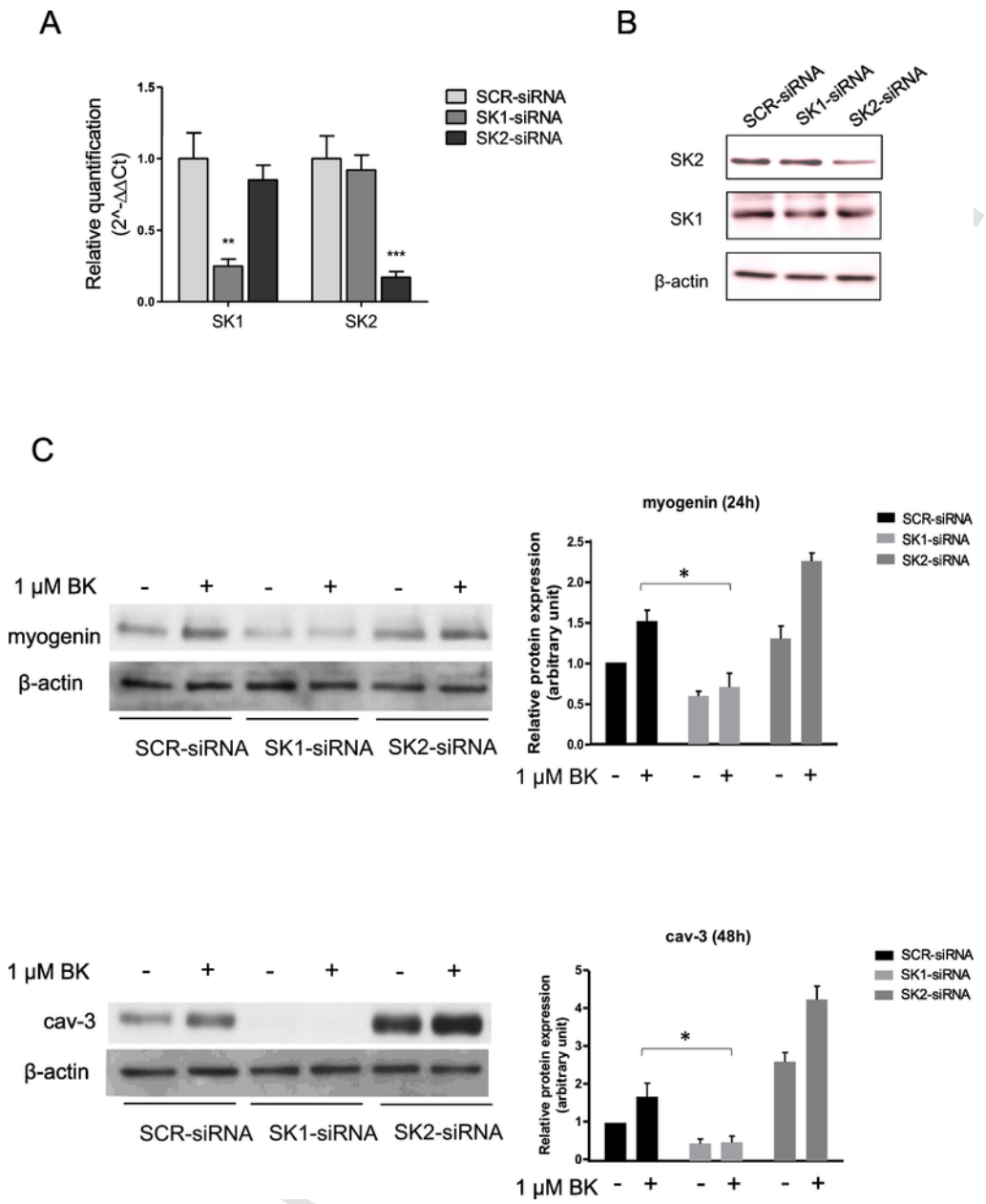

Fig. 5. Role of SK1 in Bradykinin-induced myogenic differentiation of C2C12 myoblasts (III)**A**. Real-time PCR analysis was performed to assess the down-regulation of SK1 and SK2 mRNA in SK1- or SK2-siRNA transfected myoblasts. Results are reported as mean ± SD of three independent experiments performed in triplicate, using the 2^(-ΔΔCt) method as described in Materials and Methods section. The effect of siRNA transfection on SK1 and SK2 down-regulation was statistically significant by student's *t*-test, $^{**}P < 0.01$. $^{***}P < 0.001$**B.** Cell lysates from SK1- or SK2-siRNA transfected C2C12 cells were subjected to western blotting to measure both SK1 and SK2. Results were checked for the expression of non-muscle beta isoform of actin (β-actin). Blot is representative of three experiments with similar results.**C.** Cell lysates from SK1- or SK2-siRNA transfected C2C12 cells, incubated with 1 μM BK for 24 and 48 h, were subjected to western blotting to measure myogenic differentiation markers myogenin and caveolin-3, respectively. Results were normalized to the expression of non-muscle beta isoform of actin (β-actin) and reported as mean ± SEM, fold change over control, set as 1. Blot is representative of three experiments with similar results. The effect of SK1 down-regulation on BK-induced myogenic differentiation was statistically significant by two-way Anova, followed by Bonferroni's post-hoc test, $^{*}P < 0.05$.

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Conflict of interest disclosure

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2018.02.001.

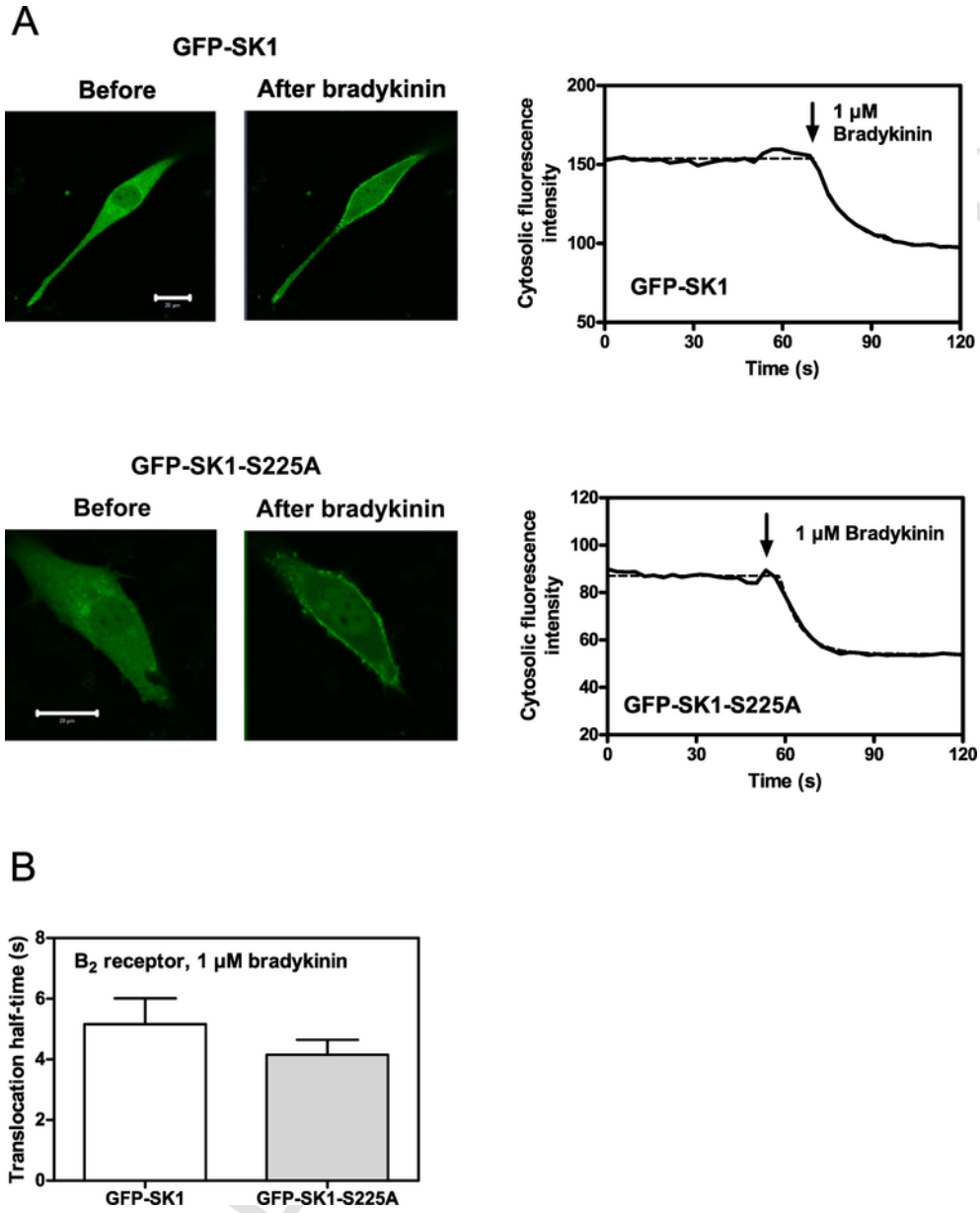

Fig. 6. Bradykinin-induced SK1 translocation in C2C12 myoblasts.The cells had been transfected with the bradykinin B2 receptor and either GFP-SK1 wild-type or GFP-SK1-S225A. A, Representative experiments showing transfected C2C12 myoblasts before and 1 min after addition of 1 μM bradykinin. The traces show the corresponding time courses of cytosolic fluorescence intensity (solid lines) and the fitted exponential curves (dashed lines). B, Translocation half- time of GFP-SK1 wild-type and GFP-SK1-S225A in C2C12 myoblasts stimulated with 1 μM bradykinin (means ± SEM, $n = 7$ cells each).

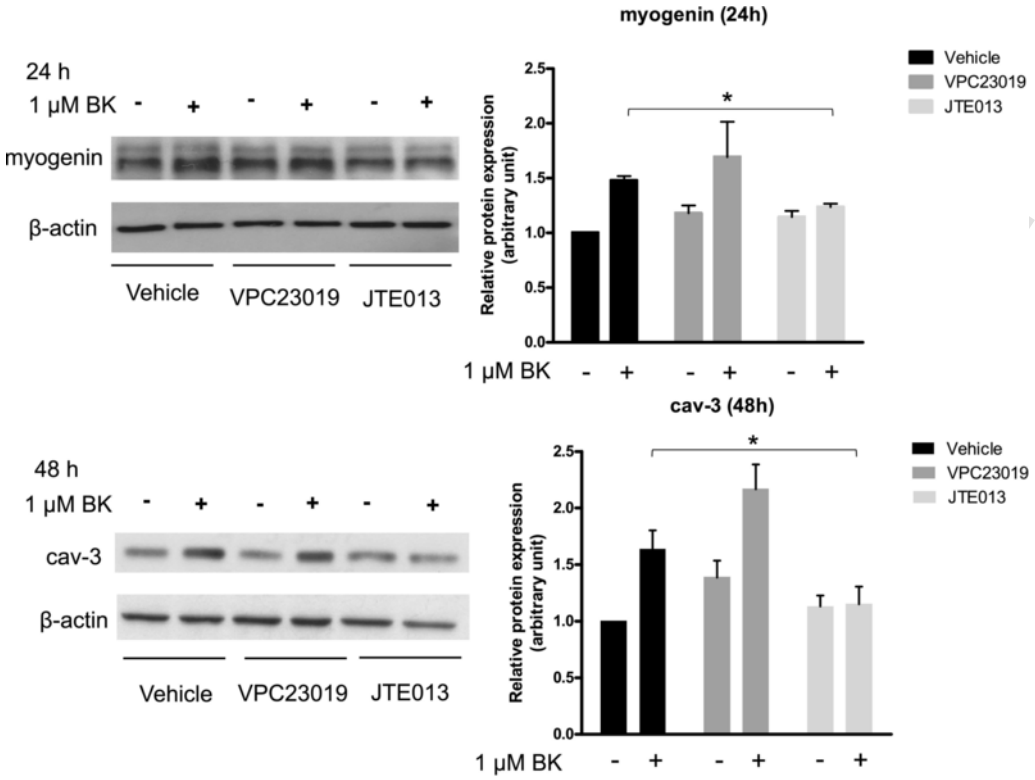

Fig. 7. $S1P_2$ is involved in Bradykinin-mediated myogenic differentiation of C2C12 myoblasts (I)C2C12 myoblasts were stimulated with 1 μM BK for 24 h after the treatment with S1PR specific antagonists (VPC23019 or JTE013) and cell lysates were subjected to western blotting to measure myogenic differentiation markers myogenin and caveolin-3, normalized to the expression of non-muscle β-actin. Blot is representative of three experiments with similar results. The effect of JTE013 on BK-induced myogenic differentiation was statistically significant by two-way Anova, followed by Bonferroni post-hoc test, $*P < 0.05$.

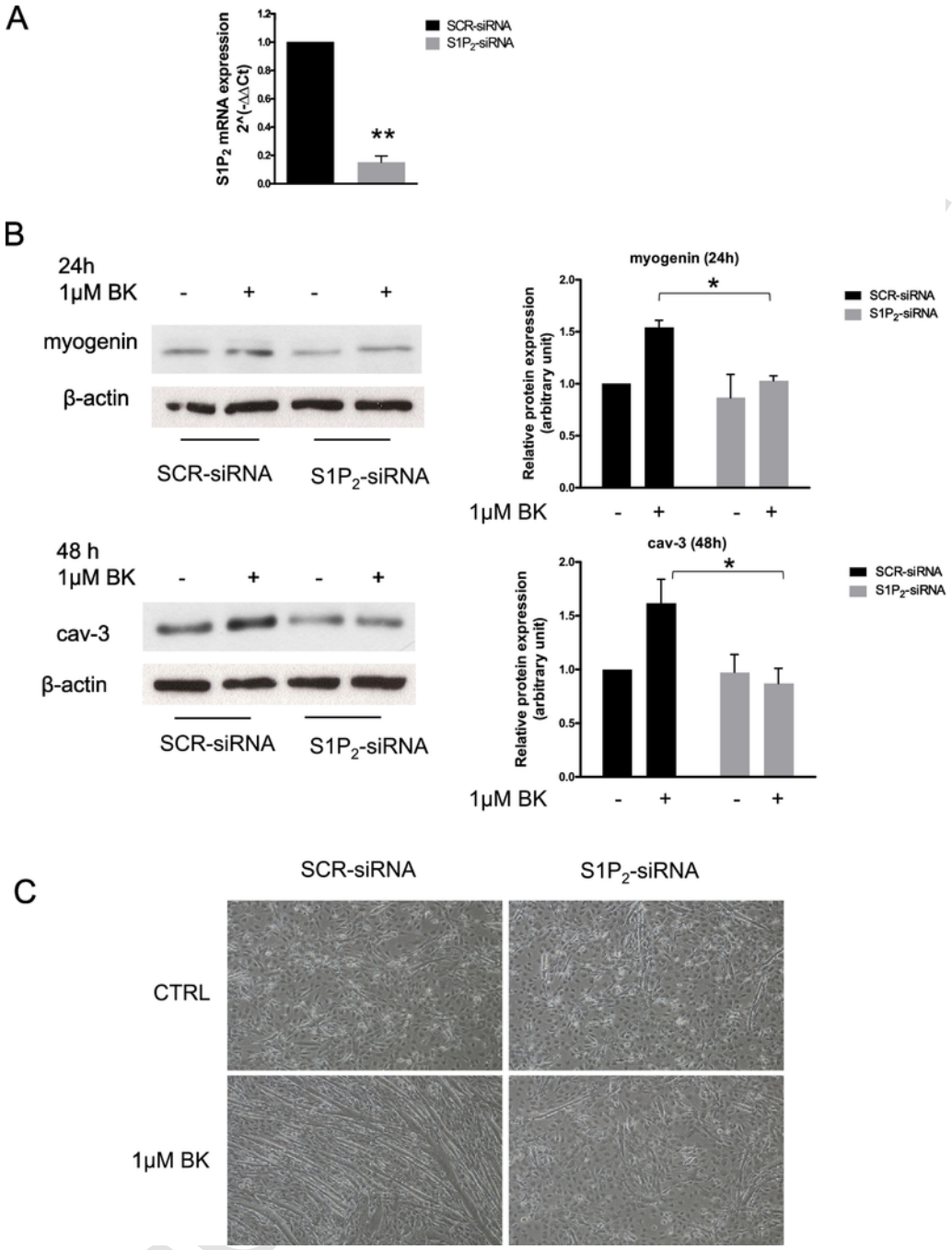

Fig. 8. $S1P_2$ is involved in Bradykinin-mediated myogenic differentiation of C2C12 myoblasts (II)**A**. Real-time PCR analysis was performed to assess the down-regulation of $S1P_2$ mRNA in $S1P_2$-siRNA transfected myoblasts. Results are reported as mean ± SD of three independent experiments performed in triplicate, using the 2^(-ΔΔCt) method as described in Materials and Methods section. The effect of siRNA transfection on $S1P_2$ down-regulation was statistically significant by student's *t*-test, $^{**}P < 0.01$.**B**. $S1P_2$-siRNA and SCR-siRNA transfected C2C12 myoblasts were treated with 1 μM BK for 24 h and 48 h and cell lysates were analyzed by western blotting to measure myogenic differentiation markers, myogenin and caveolin-3. Results were normalized to the expression of non-muscle β-actin. Blot is representative of three experiments with similar results.**C**. Optical microscopy images of myoblasts transfected with SCR-siRNA and $S1P_2$-siRNA incubated with 1 μM BK in DMEM containing 0.1% BSA for four days. The images were representative of three experiments with similar results.

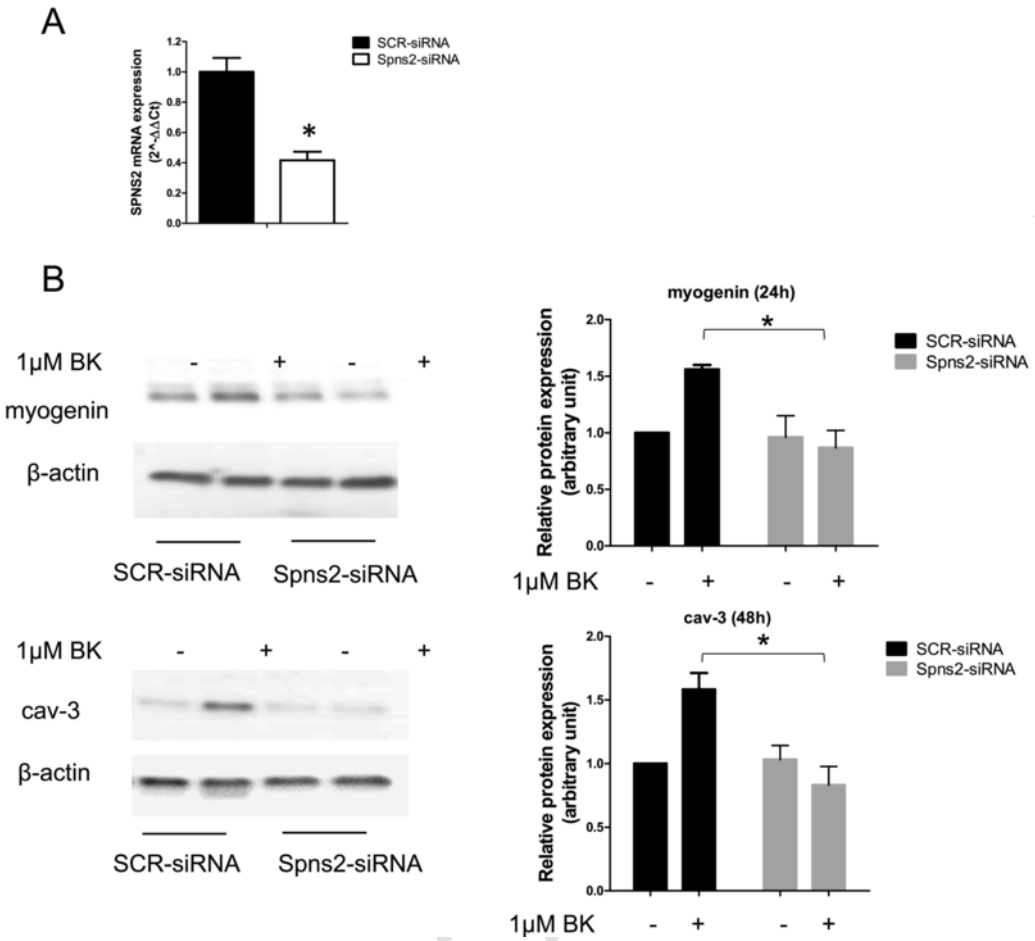

Fig. 9. Bradykinin-mediated myogenic differentiation is dependent on Spns2 expression**A**. Real time PCR analysis was performed to assess the down-regulation of Spns2 mRNA in SPNS2-siRNA transfected myoblasts. The effect of siRNA transfection on Spns2 down-regulation was statistically significant by student's *t*-test, $*P < 0.05$.**B**. Spns2-siRNA and SCR-siRNA transfected C2C12 myoblasts were treated with 1 μM BK for 24 h and 48 h and cell lysates were analyzed by western blotting to measure myogenic differentiation markers, myogenin and caveolin-3. Results were normalized to the expression of non-muscle β-actin, and are reported as mean ± SEM, fold change over control. Blot is representative of three experiments with similar results.

References

[1] B. Chazaud, C. Sonnet, P. Lafuste, G. Bassez, A.-C. Rimaniol, F. Poron, F.-J. Authier, P.A. Dreyfus, R.K. Gherardi, Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth, J. Cell Biol. 163 (2003) 1133–1143, https://doi.org/10.1083/jcb.200212046.

[2] L. Lescaudron, E. Peltékian, J. Fontaine-Pérus, D. Paulin, M. Zampieri, L. Garcia, E. Parrish, Blood borne macrophages are essential for the triggering of muscle regeneration following muscle transplant, Neuromuscul. Disord. 9 (1999) 72–80, https://doi.org/10.1016/S0960-8966(98)00111-4.

[3] T.A. Robertson, M.A.L. Maley, M.D. Grounds, J.M. Papadimitriou, The role of macrophages in skeletal muscle regeneration with particular reference to chemotaxis, Exp. Cell Res. 207 (1993) 321–331, https://doi.org/10.1006/excr.1993.1199.

[4] J.G. Tidball, Inflammatory processes in muscle injury and repair, Am. J. Physiol. Regul. Integr. Comp. Physiol. 288 (2005) R345–R353, https://doi.org/10.1152/ajpregu.00454.2004.

[5] H. Yin, F. Price, M.A. Rudnicki, Satellite cells and the muscle stem cell niche, Physiol. Rev. 93 (2013) 23–67, https://doi.org/10.1152/physrev.00043.2011.

[6] V. Prisk, J. Huard, Muscle injuries and repair: the role of prostaglandins and inflammation, Histol. Histopathol. 18 (2003) 1243–1256, https://doi.org/10.14670/HH-18.1243.

[7] U. Späte, P.C. Schulze, Proinflammatory cytokines and skeletal muscle, Curr. Opin. Clin. Nutr. Metab. Care 7 (2004) 265–269 http://journals.lww.com/co-clinicalnutrition/Abstract/2004/05000/Proinflammatory_cytokines_and_skeletal_muscle.5.aspx.

[8] G.J. Dietze, M. Wicklmayr, K. Rett, S. Jacob, E.J. Henriksen, Potential role of bradykinin in forearm muscle metabolism in humans, Diabetes 45 (1996) S110–S114 http://diabetes.diabetesjournals.org/content/45/Supplement_1/S110.short.

[9] H. Langberg, C. Bjørn, R. Boushel, Y. Hellsten, M. Kjaer, Exercise-induced increase in interstitial bradykinin and adenosine concentrations in skeletal muscle and peritendinous tissue in humans, J. Physiol. 542 (2002) 977–983, https://doi.org/10.1113/jphysiol.2002.018077/full.

[10] C. Blais, A. Adam, D. Massicotte, F. Péronnet, Increase in blood bradykinin concentration after eccentric weight-training exercise in men, J. Appl. Physiol. 87 (1999) 1197–1201 http://jap.physiology.org/content/87/3/1197, (accessed August 23, 2017).

[11] D. Regoli, J. Barabé, Pharmacology of bradykinin and related kinins, Pharmacol. Rev. 32 (1980) 1–46 http://pharmrev.aspetjournals.org/content/32/1/1, (accessed August 23, 2017).

[12] J.B. Calixto, D.A. Cabrini, J. Ferreira, M.M. Campos, Kinins in pain and inflammation., Pain 87 (n.d.) 1–5. https://insights.ovid.com/pubmed?pmid=10863040 (accessed August 23, 2017).

[13] C.D. Figueroa, G. Dietze, W. Müller-Esterl, Immunolocalization of bradykinin B2 receptors on skeletal muscle cells, Diabetes 45 (Suppl. 1) (1996) S24–28.

[14] F. Marceau, D.R. Bachvarov, Kinin receptors, Clin. Rev. Allergy Immunol. 16 (1998) 385–401, https://doi.org/10.1007/BF02737658.

[15] R. Couture, M. Harrisson, R.M. Vianna, F. Cloutier, Kinin receptors in pain and inflammation, Eur. J. Pharmacol. 429 (2001) 161–176, https://doi.org/10.1016/S0014-2999(01)01318-8.

[16] Y. Hellsten, M. Nyberg, L.G. Jensen, S.P. Mortensen, Vasodilator interactions in skeletal muscle blood flow regulation, J. Physiol. 590 (2012) 6297–6305, https://doi.org/10.1113/jphysiol.2012.240762/full.

[17] T. Miyata, T. Taguchi, M. Uehara, S. Isami, H. Kishikawa, K. Kaneko, E. Araki, M. Shichiri, Bradykinin potentiates insulin-stimulated glucose uptake and enhances insulin signal through the bradykinin B2 receptor in dog skeletal muscle and rat L6 myoblasts, Eur. J. Endocrinol. 138 (1998) 344–352 http://www.eje-online.org/content/138/3/344.short.

[18] J.Z.P. Gacesa, M. Momcilovic, I. Veselinovic, D.A. Brodie, N.G. Grujic, Bradykinin type 2 receptor-9/−9 genotype is associated with triceps brachii muscle hypertrophy following strength training in young healthy men, BMC Musculoskelet. Disord. 13 (2012) 217, https://doi.org/10.1186/1471-2474-13-217.

[19] L. Campins, M. Camps, A. Riera, E. Pleguezuelos, J.C. Yebenes, M. Serra-Prat, Oral drugs related with muscle wasting and sarcopenia. A review, Pharmacology 99 (2017) 1–8 http://www.karger.com/Article/PDF/448247.

[20] A.V. Thuy, C.-M. Reimann, N.Y. Hemdan, M.H. Gräler, Sphingosine 1-phosphate in blood: function, metabolism, and fate, Cell. Physiol. Biochem. 34 (2014) 158–171 http://www.karger.com/Article/PDF/362992.

[21] S. Spiegel, S. Milstien, Sphingosine-1-phosphate: an enigmatic signalling lipid, Nat. Rev. Mol. Cell Biol. 4 (2003) 397–407, https://doi.org/10.1038/nrm1103.
[22] H. Rosen, E.J. Goetzl, Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network, Nat. Rev. Immunol. 5 (2005) 560–570, https://doi.org/10.1038/nri1650.
[23] K. Takabe, S.W. Paugh, S. Milstien, S. Spiegel, "Inside-out" signaling of sphingosine-1-phosphate: therapeutic targets, Pharmacol. Rev. 60 (2008) 181–195 http://pharmrev.aspetjournals.org/content/60/2/181.short.
[24] M. Maceyka, K.B. Harikumar, S. Milstien, S. Spiegel, SPHINGOSINE-1-phosphate signaling and its role in disease, Trends Cell Biol. 22 (2012) 50–60, https://doi.org/10.1016/j.tcb.2011.09.003.
[25] S. Calise, S. Blescia, F. Cencetti, C. Bernacchioni, C. Donati, P. Bruni, Sphingosine 1-phosphate stimulates proliferation and migration of satellite cells: role of S1P receptors, Biochim. Biophys. Acta BBA - Mol. Cell Res. (2012) 439–450, 1823 https://doi.org/10.1016/j.bbamcr.2011.11.016.
[26] C. Donati, E. Meacci, F. Nuti, L. Becciolini, M. Farnararo, P. Bruni, Sphingosine 1-phosphate regulates myogenic differentiation: a major role for S1P2 receptor, FASEB J. 19 (2005) 449–451 http://www.fasebj.org/content/19/3/449.short.
[27] L. Becciolini, E. Meacci, C. Donati, F. Cencetti, E. Rapizzi, P. Bruni, Sphingosine 1-phosphate inhibits cell migration in C2C12 myoblasts, Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids. 1761 (2006) 43–51, https://doi.org/10.1016/j.bbalip.2006.01.006.
[28] C. Donati, F. Cencetti, P. Bruni, Sphingosine 1-phosphate axis: a new leader actor in skeletal muscle biology, Front. Physiol. 4 (2013) https://doi.org/10.3389/fphys.2013.00338.
[29] C. Bernacchioni, F. Cencetti, S. Blescia, C. Donati, P. Bruni, Sphingosine kinase/sphingosine 1-phosphate axis: a new player for insulin-like growth factor-1-induced myoblast differentiation, Skelet. Muscle 2 (2012) 15, https://doi.org/10.1186/2044-5040-2-15.
[30] P. Nincheri, C. Bernacchioni, F. Cencetti, C. Donati, P. Bruni, Sphingosine kinase-1/S1P1 signalling axis negatively regulates mitogenic response elicited by PDGF in mouse myoblasts, Cell. Signal. 22 (2010) 1688–1699, https://doi.org/10.1016/j.cellsig.2010.06.009.
[31] F. Cencetti, C. Bernacchioni, P. Nincheri, C. Donati, P. Bruni, Transforming growth factor-β1 induces transdifferentiation of myoblasts into myofibroblasts via up-regulation of sphingosine kinase-1/S1P3 axis, Mol. Biol. Cell 21 (2010) 1111–1124 http://www.molbiolcell.org/content/21/6/1111.short.
[32] F. Cencetti, C. Bernacchioni, F. Tonelli, E. Roberts, C. Donati, P. Bruni, TGFβ1 evokes myoblast apoptotic response via a novel signaling pathway involving S1P4 transactivation upstream of Rho-kinase-2 activation, FASEB J. 27 (2013) 4532–4546 http://www.fasebj.org/content/27/11/4532.short.
[33] M. ter Braak, K. Danneberg, K. Lichte, K. Liphardt, N.T. Ktistakis, S.M. Pitson, T. Hla, K.H. Jakobs, D. Meyer zu Heringdorf, Galpha(q)-mediated plasma membrane translocation of sphingosine kinase-1 and cross-activation of S1P receptors, Biochim. Biophys. Acta 1791 (2009) 357–370.
[34] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method, Methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.
[35] E. Meacci, F. Nuti, C. Donati, F. Cencetti, M. Farnararo, P. Bruni, Sphingosine kinase activity is required for myogenic differentiation of C2C12 myoblasts, J. Cell. Physiol. 214 (2008) 210–220, https://doi.org/10.1002/jcp.21187.
[36] S.M. Pitson, P.A.B. Moretti, J.R. Zebol, H.E. Lynn, P. Xia, M.A. Vadas, B.W. Wattenberg, Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation, EMBO J. 22 (2003) 5491–5500, https://doi.org/10.1093/emboj/cdg540.
[37] B. Chazaud, C. Sonnet, P. Lafuste, G. Bassez, A.-C. Rimaniol, F. Poron, F.-J. Authier, P.A. Dreyfus, R.K. Gherardi, Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth, J. Cell Biol. 163 (2003) 1133–1143 http://jcb.rupress.org/content/163/5/1133.abstract.
[38] L. Lescaudron, E. Peltékian, J. Fontaine-Pérus, D. Paulin, M. Zampieri, L. Garcia, E. Parrish, Blood borne macrophages are essential for the triggering of muscle regeneration following muscle transplant, Neuromuscul. Disord. 9 (1999) 72–80 http://www.sciencedirect.com/science/article/pii/S0960896698001114.
[39] T.A. Robertson, M.A.L. Maley, M.D. Grounds, J.M. Papadimitriou, The role of macrophages in skeletal muscle regeneration with particular reference to chemotaxis, Exp. Cell Res. 207 (1993) 321–331 http://www.sciencedirect.com/science/article/pii/S0014482783711997.
[40] J.G. Tidball, Inflammatory cell response to acute muscle injury, Med. Sci. Sports Exerc. 27 (1995) 1022–1032 http://europepmc.org/abstract/med/7564969.
[41] H. Yin, F. Price, M.A. Rudnicki, Satellite cells and the muscle stem cell niche, Physiol. Rev. 93 (2013) 23–67 http://physrev.physiology.org/content/93/1/23.short.
[42] C.J. Mann, E. Perdiguero, Y. Kharraz, S. Aguilar, P. Pessina, A.L. Serrano, P. Muñoz-Cánoves, Aberrant repair and fibrosis development in skeletal muscle, Skelet. Muscle 1 (2011) 21, https://doi.org/10.1186/2044-5040-1-21.
[43] C. Golias, A. Charalabopoulos, D. Stagikas, K. Charalabopoulos, A. Batistatou, The kinin system - bradykinin: biological effects and clinical implications. Multiple role of the kinin system - bradykinin, Hippokratia 11 (2007) 124–128 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2658795/.
[44] J. Howl, S.J. Payne, Bradykinin receptors as a therapeutic target, Expert Opin. Ther. Targets 7 (2003) 277–285, https://doi.org/10.1517/14728222.7.2.277.
[45] Contact system: a vascular biology modulator with anticoagulant, profibrinolytic, antiadhesive, and proinflammatory attributes | Blood J., (n.d.). http://www.bloodjournal.org/content/90/10/3819.long?sso-checked=true (accessed August 24, 2017).
[46] S. Böckmann, I. Paegelow, Kinins and kinin receptors: importance for the activation of leukocytes, J. Leukoc. Biol. 68 (2000) 587–592.
[47] A.G. Williams, S.S. Dhamrait, P.T.E. Wootton, S.H. Day, E. Hawe, J.R. Payne, S.G. Myerson, M. World, R. Budgett, S.E. Humphries, H.E. Montgomery, Bradykinin receptor gene variant and human physical performance, J. Appl. Physiol. 96 (2004) 938–942, https://doi.org/10.1152/japplphysiol.00865.2003.
[48] M. Wicklmayr, G. Dietze, H. Brunnbauer, K. Rett, H. Mehnert, Dose-dependent effect of bradykinin on muscular blood flow and glucose uptake in man, Hoppe-Seyler's Z. Für Physiol. Chem. 364 (2009) 831–834, https://doi.org/10.1515/bchm2.1983.364.2.831.
[49] T. Taguchi, H. Kishikawa, H. Motoshima, K. Sakai, T. Nishiyama, K. Yoshizato, A. Shirakami, T. Toyonaga, T. Shirontani, E. Araki, M. Shichiri, Involvement of bradykinin in acute exercise-induced increase of glucose uptake and GLUT-4 translocation in skeletal muscle: studies in normal and diabetic humans and rats, Metabolism 49 (2000) 920–930.
[50] D. Mangnall, C. Bruce, R.B. Fraser, Insulin-stimulated glucose uptake in C2C12 myoblasts, Biochem. Soc. Trans. 21 (1993), 438S–438S https://doi.org/10.1042/bst021438s.
[51] A.M.W. Petersen, B.K. Pedersen, The anti-inflammatory effect of exercise, J. Appl. Physiol. 98 (2005) 1154–1162, https://doi.org/10.1152/japplphysiol.00164.2004.
[52] Y.-P. Li, R.J. Schwartz, TNF-α regulates early differentiation of C2C12 myoblasts in an autocrine fashion, FASEB J. 15 (2001) 1413–1415, https://doi.org/10.1096/fj.00-0632fje.
[53] C. Donati, P. Nincheri, F. Cencetti, E. Rapizzi, M. Farnararo, P. Bruni, Tumor necrosis factor-α exerts pro-myogenic action in C2C12 myoblasts via sphingosine kinase/S1P2 signaling, FEBS Lett. 581 (2007) 4384–4388, https://doi.org/10.1016/j.febslet.2007.08.007.
[54] A.S. de la Garza-Rodea, D.M. Baldwin, B. Oskouian, R.F. Place, P. Bandhuvula, A. Kumar, J.D. Saba, Sphingosine phosphate lyase regulates myogenic differentiation via S1P receptor-mediated effects on myogenic microRNA expression, FASEB J. 28 (2014) 506–519, https://doi.org/10.1096/fj.13-233155.