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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 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

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, fast skeletal muscle myosin heavy chain

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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 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, pH 7.5, 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 10^4 ×*g*, 15 min 4 °C and 15 μg of protein from total cell