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Sphingosine-1-phosphate pretreatment amends hypoxia-induced metabolic dysfunction and impairment of myogenic potential in differentiating C2C12 myoblasts by stimulating viability, calcium homeostasis and energy generation

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Abstract Sphingosine-1-phosphate (S1P) has a role in transpiration in patho-physiological signaling in skeletal muscles. The present study evaluated the pre-conditioning efficacy of S1P in facilitating differentiation of C2C12 myoblasts under a normoxic/hypoxic cell culture environment. Under normoxia, exogenous S1P significantly promoted C2C12 differentiation as evident from morphometric descriptors and differentiation markers of the mature myotubes, but it could facilitate only partial recovery from hypoxia-induced compromised differentiation. Pretreatment of S1P optimized the myokine secretion, intracellular calcium release and energy generation by boosting the aerobic/anaerobic metabolism and mitochondrial mass. In the hypoxia-exposed cells, there was derangement of the $S1PR_{1-3}$ expression patterns, while the same could be largely restored with S1P pretreatment. This is being proposed as a plausible underlying mechanism for the observed pro-myogenic efficacy of exogenous S1P preconditioning. The present findings are an invaluable addition to the existing knowledge on the pro-myogenic potential of S1P and may prove beneficial in the field of hypoxia-related myo-pathologies.

Keywords Normoxia · Hypoxia · S1P · Differentiation · Myogenesis

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Introduction

Oxygen (O_2) homeostasis being essential to survival, a suboptimal oxygen level elicits physiological disturbances and pathological responses [1, 2]. Skeletal muscle is a dynamic biological system whose oxygen demand varies according to its functional activity, and it responds to hypoxia by eliciting orchestrated metabolic remodeling to optimize oxygen utilization and energy production [3]. However, sustained exposure to severe hypoxic stress leads to mal-acclimatization-mediated patho-physiological responses [4]. Insufficient energy production and uncontrolled inflammation along with a disrupted redox balance are the underlying causes for decreased muscular activity even during sub-chronic hypoxia exposure. On the other hand, muscle tissues possess robust regenerative capabilities to repair tissue damage, a process that involves cooperative activity of immune cells, resident myogenic stem cells and fibroblasts. These myogenic stem cells are the satellite myoblasts that get activated, differentiate and fuse to generate multinucleated myofibers, which assist in the repair of damaged muscle fibers [5, 6]. However, as mentioned earlier, sustained hypoxic insult leads to a plethora of patho-physiological responses leading to loss of the satellite cell functions that hamper the muscle repair and

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regeneration process, leading to exponential muscle loss under chronic hypoxia [7, 8]. Specifically, myoblasts respond to hypoxia exposure by inhibiting terminal myogenic differentiation and myotube formation via downregulation of myogenic regulatory factors [9]. This hypoxia-induced muscle fatigability and hampered regenerative repair lead to loss of endurance and skeletal muscle strength. One of the best examples is of chronic hypoxia-induced reduction in fiber size and mass during high altitude exposure. However not only high altitude, but also several other patho-physiological conditions involving systemic or localized hypoxia, such as peripheral limb ischemia, are also known to disrupt tissue homeostasis and result in muscle tissue damage [10]. In view of the paucity of effective interventions to promote muscle repair and regeneration to recoup from hypoxia-induced muscle pathologies, there is an unmet need to investigate novel and effective pharmacological/biological interventions.

Research outcomes of the past two decades have established S1P signaling pathways as central to the molecular and cellular pathogenesis of several human diseases including atherosclerosis, inflammation, cancer and multiple sclerosis, etc. [11, 12]. This has placed S1P and related investigational drugs at the center stage of the recent drug discovery programs. S1P is a bioactive lipid mediator that acts intra and extracellularly via specific G protein-coupled receptors (GPCR)-S1P receptors (S1PR$_{1-5}$) and regulates fundamental processes such as cell proliferation, migration, cytoskeleton re-arrangement, angiogenesis, endothelial cell chemotaxis, immune cell trafficking and mitogenesis, etc. [13]. Both in vitro and in vivo studies by our group have also evidenced the preclinical efficacy of exogenous S1P in improving systemic and vital organ acclimatization to simulated environmental hypoxia as well as coping with the associated pathological disturbances such as redox imbalance, inflammation and energy deficits, etc. [14–16]. However, its effect on the restoration of skeletal muscle physiology under hypoxia remains to be elucidated. Here, we have investigated the efficacy of S1P pre-conditioning on myogenic differentiation, a key facet of skeletal muscle physiology, in C2C12 myoblast cells under normoxic and hypoxic environment. These cells offer an established model for studying the muscle differentiation and growth process in vitro, producing reliable data with an advantage of controlled and synchronized differentiation of myogenic stem cells [17].

The study findings highlight the merits of S1P preconditioning in facilitating myogenic differentiation in normoxia and its partial recovery in hypoxia, optimization of adaptive aerobic/anaerobic energy remodeling and calcium homeostasis while balancing the acute myokine responses following hypoxic challenge to C2C12 myoblasts. The dynamics of exogenous S1P-mediated GPCR's

expression patterns have been proposed as one of the plausible mechanisms underlying the observed benefits, although we do not rule out other receptor-independent effects of S1P. In view of the unifying observation of S1P pre-conditioning in the restitution of several aspects of hypoxia-induced loss of myogenic potential in C2C12 cells, this signaling lipid holds the potential to join the league of exploratory drug candidates worth studying for clinical applications in muscle pathologies.

Materials and methods

Materials

All chemicals and culture reagents (Dulbecco's modified Eagle's high-glucose medium—DMEM HG, fetal bovine serum—FBS) were purchased from SIGMA-Aldrich. Horse serum (HS) was purchased from Thermo Fisher Scientific Inc. Assay kits and ELISA kits were purchased from BD Biosciences, Randox Laboratories and Thermo Fisher Scientific Inc. Antibodies were purchased from Merck Millipore and Santa Cruz Biotechnology.

C2C12 myoblast culture and differentiation

Murine C2C12 myoblast cells (purchased from NCCS Pune, India) were grown in DMEM HG supplemented with 10% FBS, 150 mg/l ampicillin and 150 mg/l streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. For all the experimental groups, the same number of cells (0.2×10^5/cm^2) were seeded and induced to differentiate at 90–95% confluence in differentiation medium (DM)—DMEM HG with 2% HS.

Exogenous S1P pre-conditioning, hypoxia exposure and sample collection

S1P was diluted initially in 10 mM NaOH to prepare a stock solution of 1 mM. This stock solution was diluted using the vehicle solution, 0.1% BSA prepared in normal saline (pH 7.8), to be dispensed to the cultures at the final dose of 1 μM S1P. The C2C12 myoblast cells were treated with either 1 μM S1P or vehicle (VC) when the culture had attained 90–95% confluence following which the media were replaced with DM, as described earlier [18]. After 30 min of S1P or VC treatment, separate groups of cells were maintained under either hypoxia (0.5% O_2) or normoxia (21% O_2) until the fully differentiated stage (8th day). Samples were collected at undifferentiated 0 day (0D) (90–95% confluence stage prior to drug treatment), 2nd day (2D), 4th day (4D), 6th day (6D) and 8th day (8D) from the normoxia (N) and hypoxia (H) groups,

respectively. The groups were denoted as: 0D, 2NVC, 2NS1P, 2HVC, 2HS1P, 4NVC, 4NS1P, 4HVC, 4HS1P, 6NVC, 6NS1P, 6HVC, 6HS1P, 8NVC, 8NS1P, 8HVC and 8HS1P. Various parameters were assessed in each group as described in the following sections.

Cell lysate preparation

Following exposure, C2C12 cells were harvested on ice, thoroughly washed with cold PBS and lysed in NP-40 buffer (150 mM NaCl, 50 mM Tris and 1% NP-40, pH 7.4) fortified with protease inhibitor cocktail. Protein estimation was performed using Bradford's method [19]. This sample was used for Western blot and bio-energetic marker analysis.

Morphometric changes, assessment of viability and cell damage during C2C12 differentiation

Cell proliferation, myoblast fusion, myotube formation and viability were observed by adding 100 nM of calcein-AM fluorescence dye (SIGMA-Aldrich, 17783) to the culture media. Briefly, $0.2 \times 10^5/cm^2$ cells were seeded in each group and induced to differentiate, as described earlier, following which the cells were incubated with the fluorescence dye under respective culture conditions for 30 min, washed twice with incomplete DMEM and visualized using a fluorescence microscope (Olympus inverted fluorescence microscope, Olympus IX2-KSP) at $10\times$ magnification. Calcein-AM fluorescence signal intensity, an indicator of cell viability, was also measured using a multimode plate reader (FLUOstar Omega) and reported as average fluorescence signal intensity relative to 0D. Extracellular lactate dehydrogenase (LDH) activity, an indicator of cellular damage, was estimated in media supernatant according to a previously described method [20].

Myokines estimation

Specific myokines viz. IL-6, IFN-γ and TNF-α were quantified in the media supernatant collected at each of the mentioned time points using BD OptEIA^{TM} sandwich ELISA kits according to the manufacturer's prescribed protocol.

Intracellular calcium quantification

Intracellular calcium was estimated using the Fluo-4 NW calcium assay kit (Thermo Fisher Scientific Inc., F36206). The fluorescent signal was quantified using a multimode plate reader (FLUOstar Omega) at an excitation and emission wavelength of 490 and 520 nm, respectively.

Quantitative fluorescence data were reported as relative fluorescence units with respect to 0D.

Markers of cellular bioenergetics

Glucose and lactate were quantified by GLUC-PAP (Randox Laboratories, GL2614) and LAC (Randox Laboratories, LC2389) in cell lysate samples. LDH [20], hexokinase (HK) [21], citrate synthase (CS) [22] and creatine kinase (CK) (Randox, CK-NAC335) enzyme activities were measured in cell lysate using previously described methods.

Intracellular ATP quantification and mitochondrial imaging

Intracellular ATP content was measured in cell lysate using the ATP Estimation Kit (Thermo Fisher Scientific Inc., A22066). Mitochondrial imaging was performed as described earlier; a cell-permeable mitochondrial stain, MitoTracker® Red CMXRos (Thermo Fisher Scientific Inc., M7512), was added to the incomplete DMEM at a final concentration of 1 μM at each time point. The cells were incubated under respective culture conditions for 30 min, washed twice with incomplete DMEM and imaged by fluorescence microscopy at $10\times$ magnification. Mitochondrial mass (mitotracker intensity) was also quantified by a multimode plate reader (FLUOstar Omega), and the average signal intensity relative to 0D was reported [23].

Myotube differentiation marker: myosin heavy chain (MHC)

A 25-μg protein sample was mixed with $6\times$ Laemelli buffer (0.25 M Tris–HCl pH 6.8, 10% SDS, 0.5% bromophenol blue, 0.5 M di-thiothretiol and 50% glycerol) and boiled for 10 min. These samples were resolved on a 10% polyacrylamide gel following which the resolved proteins were blotted on a nitrocellulose membrane and blocked in 5% BSA solution for 2 h. Blocking solution and antibody dilutions were prepared in Tris-buffered saline with 0.1% Tween-20. Blot was incubated with anti-myosin heavy chain (MHC, Merck Millipore, MHC A4.1025) primary antibody dilution (1:1000) overnight at 4°C and then with HRP labeled-secondary antibody (1:40,000, Santa Cruz Biotechnology) for 2 h at room temperature. An enhanced chemiluminescence detection kit (SIGMA-Aldrich, CPS1300-1KT) was used to develop the blots and capture them on X-ray film (Fuji Films). β-Actin was used as the loading control (Santa Cruz Biotechnology, sc-47778) (1:1000). Densitometry was done using ImageJ software [24].

S1PR$_{1-5}$ expression in C2C12 cells: RNA isolation, cDNA synthesis and real-time PCR

Total RNA was isolated from differentiating C2C12 cells at each time point using the Total RNA Isolation kit (SIGMA-Aldrich, RTN70) and was reverse transcribed as per the manufacturer's protocol (Thermo Fisher Scientific Inc., 12574-035). cDNA of the respective sample was used to determine the expression of S1PR$_{1-5}$ mRNA with Power SYBR Green PCR Master Mix (Applied Biosystem, 4367659) using real-time PCR (Applied Biosystem, 4376598). The PCR conditions used were initial denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C denaturation for 60 s, 60 °C primer annealing for 60 s and extension at 72 °C for 60 s, and product formation was confirmed by melting curve analysis (55–94 °C). Relative mRNA expression of the respective receptor was calculated after normalization to an endogenous reference gene (β-actin). Results are reported as fold changes with respect to undifferentiated myoblasts (0D) at each time point according to the comparative CT method ($2^{-\Delta\Delta C_T}$ method) as previously described [25]. Details of the specific gene primers used are listed in Supplementary Data Table 1.

Morphometric analysis of mature myotubes

Morphometric analysis was done in calcein-AM-stained differentiated C2C12 myotubes at 8D in normoxia VC and S1P pretreated cells (8NVC vs. 8NS1P). To quantify the morphometric parameters of differentiated myotubes, three random microscopic fields from three independent cultures were analyzed. Myotubes more than 600 μm long were counted per field, and each of these myotubes was analyzed further [26]. Myotubular branchings (Y shaped structures) were counted manually, and the fiber diameter and length were measured by ImageJ software.

Statistical analysis

Data are reported as mean ± standard deviation (SD) from three independent experiments carried out in triplicate. Statistical analysis was performed using the one-way ANOVA/post hoc Bonferroni's analysis method. Image analysis and densitometry were performed using ImageJ software. Statistical significance was denoted by NVC vs. HVC ($\$p < 0.05$, $*p \leq 0.005$) and HVC vs. HS1P ($@p < 0.05$, $\#p \leq 0.005$) at respective time points. The statistical analysis for morphometric study was performed at 8D using unpaired Student's t test. Statistical significance was denoted by NVC vs. NS1P ($**p < 0.05$, $*p \leq 0.005$).
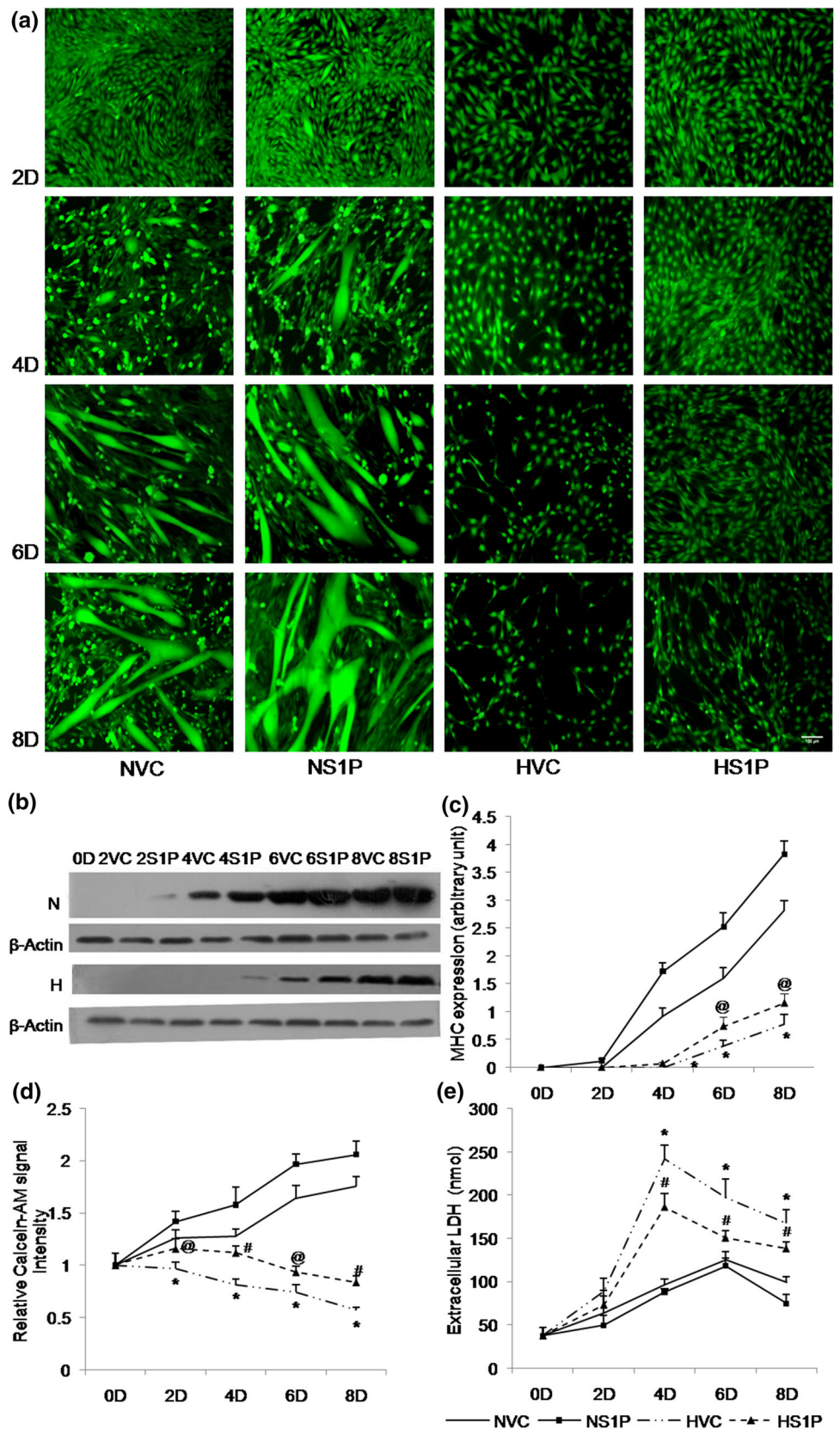
Results

Pre-conditioning with exogenous S1P in C2C12 myoblast cells partially remediates hypoxia-mediated impaired differentiation

The chronological changes in the morphology of C2C12 cells during differentiation were imaged using calcein-AM dye. In the NVC group, the flat star/fusiform-shaped undifferentiated myoblasts (0D) transformed into elongated fusing myoblast at 2D. These cells further differentiated into myocytes and nascent myotube like structures at 4D, where a large number of thin myotubes were interspersed scantily with thick myotubes. A robust myogenesis was observed by 6D with the emergence of an even larger number of thicker and longer syncytial myotubes that at 8D terminally differentiated into branched, mostly Y-shaped myotubes that even elongated in multi-directions in several microscopic fields (Fig. 1a). In the Western blot analysis, the expression of MHC protein could also be captured earliest at 4D (Fig. 1b) with the emergence of short length nascent myotubes (Fig. 1a).

The detrimental effect of exposure to hypoxia (0.5% O$_2$) on the differentiation of C2C12 was captured as the inability of these hypoxia-exposed C2C12 cells to fuse and form nascent myotube-like structures at 4D (Fig. 1a). Also, the prevailing hypoxic environment, up to 8D, led to severely compromised viability as evident from a steep fall in the calcein-AM signal intensity (Fig. 1d) and raised LDH activity in the culture medium (Fig. 1e). Toward the end stages of differentiation there was an appearance of scant, thin and short nascent myotubes interspersed with remnant myoblasts that had failed to differentiate. As evidenced by morphometric analysis, the compromised myogenesis in HVC cells co-existed with the earliest detection of MHC expression only at 6D (Fig. 1b, c).

The present study, for the first time, demonstrates the efficacy of S1P pre-conditioning to facilitate recovery from hypoxia-mediated injury and retarded differentiation in C2C12 cells. Interestingly, even under normoxic conditions, with S1P pre-conditioning, myogenic differentiation could be observed at 2D along with the appearance of MHC (Fig. 1b, c). The cells in this group appeared densely populated and relatively more elongated compared to respective vehicle control cells at 2D. Also, their fusion resulted in an early appearance of thicker, longer and more branched myotubes as observed during 4–8D. Certainly, this pro-myogenic efficacy of S1P was an advantage during the hypoxic challenge, wherein the MHC signal was detectable as early as 4D, although it was only accompanied by the emergence of thin nascent myotubes (with nearly 1.5-fold higher MHC expression by 8D (w.r.t. HVC

Fig. 1 Effect of S1P pre-conditioning on hypoxia-impaired differentiation: $0.2 \times 10^5/cm^2$ C2C12 cells were seeded in each group, maintained in DMEM-HG media and induced to differentiate at 90–95% confluence by switching to DM (DMEM HG with 2% HS). **a** S1P (1 μM) and VC pretreated differentiating C2C12 cells under normoxia (N) and 0.5% hypoxia (H) at various time points (2D, 4D, 6D and 8D) were stained with calcein-AM (100 nM) and imaged by an inverted fluorescence microscope at ×10 magnification. *Scale bar* 100 μm. The groups were: 2NVC, 2NS1P, 2HVC, 2HS1P, 4NVC, 4NS1P, 4HVC, 4HS1P, 6NVC, 6NS1P, 6HVC, 6HS1P, 8NVC, 8NS1P, 8HVC, 8HS1P. **b** MHC (molecular weight: 220 kDa) expression was determined in cell lysate by standard Western blotting protocol. **c** Densitometric analysis of MHC Western blot normalized against the loading control (β-actin). **d** Calcein-AM fluorescence signal intensity, an indicator of cell viability, was measured using a multimode plate reader (FLUOstar Omega), and average values relative to 0D were reported. **e** Cell damage marker, extracellular LDH activity, was estimated in cell culture supernatant by the previously described method. Data are reported as mean ± SD from three independent experiments carried out in triplicate. Statistical analysis was performed using the one-way ANOVA/post hoc Bonferroni's analysis method. Image analysis and densitometry was performed using ImageJ software. Statistical significance is denoted by NVC vs. HVC ($\$p < 0.05$, $*p \leq 0.005$) and HVC vs. HS1P ($@p < 0.05$, $\#p \leq 0.005$) at respective time points

cells, $p < 0.05$; Fig. 1a, c).The nascent myotubes in the HS1P group were evidently longer and displayed a higher formation propensity (Fig. 1a). Also, the hypoxia-mediated cell injury was curtailed in these cells as indicated by the higher calcein-AM signal ($p < 0.05$; Fig. 1d) and lower LDH activity ($p \leq 0.005$; Fig. 1e) in the media in S1P-pretreated cells.

Morphometric analysis of terminally differentiated myotubes

Morphometric analysis of end-stage (8D) VC- and S1P-treated myotubes, under normoxia, revealed that pre-conditioning with S1P facilitates the formation of long, wide and branched fibers. In hypoxic cultures (HVC and HS1P), mature myotubes, like in normoxic culture, were absent; only nascent myotubes were observed, which were comparatively shorter, thinner, scanty and indistinct. As shown in Fig. 1a, counting nascent myotubes was not feasible and incomprehensible in hypoxia.

In normoxic culture we found S1P mediated a two fold ($p < 0.05$) increment in the number of myotubes per field under normoxic conditions in comparison to the NVC group (Fig. 2a). Further, analysis of individual myotubes (>600 μm length) in each of the groups indicated a longer length, diameter and branching ($p < 0.05$) as an outcome of S1P pre-conditioning (Fig. 2b–d). Specifically, in the NS1P group nearly 14.8% of the counted myotubes superseded the NVC myotubes in all the above-mentioned morphometric indices, indicating a higher propensity of myoblasts to fuse and differentiate into myotubes. A scatter plot of morphological parameters makes it more evident that S1P pretreatment improved the quality of mature myotubes (Supplementary Data Figure 1). However, morphometric analysis could not be performed in hypoxic culture even though microscopically we observed that S1P could assist the formation of thin and short nascent myotubes under hypoxia by the 8th day, which were significantly scanty in the hypoxia control group (Fig. 1a).

S1P-mediated temporal secretion of myokines IL-6, IFN-γ and TNF-α assisted recovery of differentiation in hypoxic C2C12 cells

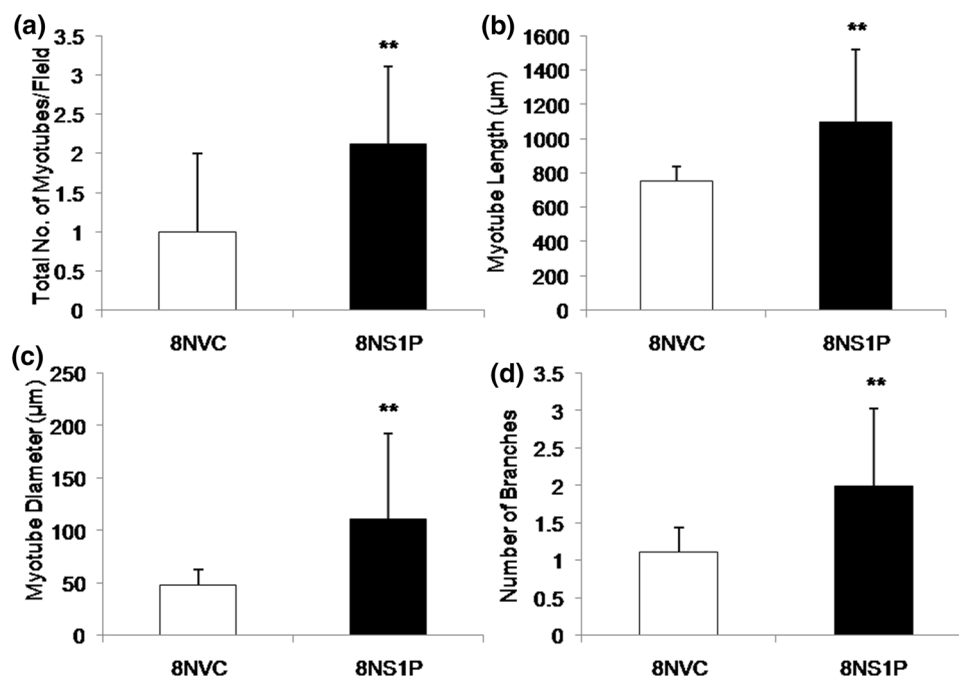Myokines IL-6, IFN-γ and TNF-α are the cytokines secreted by skeletal muscle cells that regulate the immune/

Fig. 2 Morphometric analysis of terminally differentiated myotubes: $0.2 \times 10^5/cm^2$ C2C12 myoblast cells were seeded and induced for myotube formation as described previously. Morphometric analysis was done in calcein-AM stain differentiated C2C12 myotubes (>600 μm length) at 8D in normoxia VC and S1P pretreated cells (8NVC vs. 8NS1P). **a** Number of myotubes/field was counted manually. **b** Myotube length was measured by using ImageJ software. **c** Myotube diameter was measured by using ImageJ software. **d** Number of branches (Y shaped structures) was counted manually. Data are reported as mean ± SD from three independent experiments carried out in triplicate. Statistical analysis was performed using unpaired Student's t test. Statistical significance is denoted by NVC vs. NS1P (**$p < 0.05$, *$p \leq 0.005$)

metabolic responses linked to muscle functioning. Of these, IL-6 and IFN-γ are major determinants of the proliferation, differentiation potential and metabolic homeostasis [27–29]. Time series evaluations of secreted myokines show that IL-6, IFN-γ and TNF-α are increased along with differentiation of C2C12 cells under normoxia (Fig. 3), whereas hypoxia exposure robustly increases the IL-6 secretion at 2D to nearly 1.4-fold (w.r.t. NVC, $p \leq 0.005$) followed by decreased secretion toward 8D (HVC) (Fig. 3a). Interestingly, in S1P-pretreated hypoxia-exposed cells (HS1P), two fold boosts in IL-6 secretion (w.r.t. 0D, $p < 0.05$ vs. HVC) were observed on 2D followed by relatively higher IL-6 secretion toward 8D (w.r.t. HVC, $p < 0.05$). IFN-γ secretion under hypoxic conditions is boosted until 2D in HVC and HS1P cultures, and thereafter the secretions are stabilized until 8D. The IFN-γ secretion in the HS1P group was higher than in HVC cultures at all time points ($p < 0.05$ vs. HVC). TNF-α secretion, indicative of a pro-apoptotic milieu, was also observed to be upregulated across at all time points in hypoxic conditions; however, S1P-treated cells secreted significantly lower levels of TNF-α ($p < 0.05$, HVC vs. HS1P). Moreover, we speculate a plausible reciprocal

regulation of TNF-α secretion by the S1P-mediated IFN-γ secretion (Fig. 3c) [30].

S1P pre-conditioning boosts intracellular calcium to facilitate recovery from hypoxia-mediated loss of myogenic differentiation in C2C12 cells

The $i[Ca^{2+}]$ in myogenic cells is a key regulator of cellular differentiation especially for molecular synthesis (energy metabolism) and cell behavior (alignment and fusion) [31]. In the present study, a robust and temporal increase in the $i[Ca^{2+}]$ accompanied the myogenic differentiation of C2C12 cells with a maximum 7.9-fold increase of the Fluo-4 signal in 8D NVC cells as compared to 0D (Fig. 4). In C2C12 cells pretreated with pro-myogenic S1P, fluorimetric analysis revealed significantly high $i[Ca^{2+}]$ levels at all time points compared to the NVC group (Fig. 4).

There was a notable fall in the differentiation potential of these cells along with a steep reduction in $i[Ca^{2+}]$ levels following hypoxia exposure ($p \leq 0.005$, NVC vs. HVC) with a mere 2.7-fold maximum boost in the calcium level at 8D HVC (w.r.t. 0D). However, S1P pretreatment, despite hypoxia exposure, could remarkably boost the $i[Ca^{2+}]$
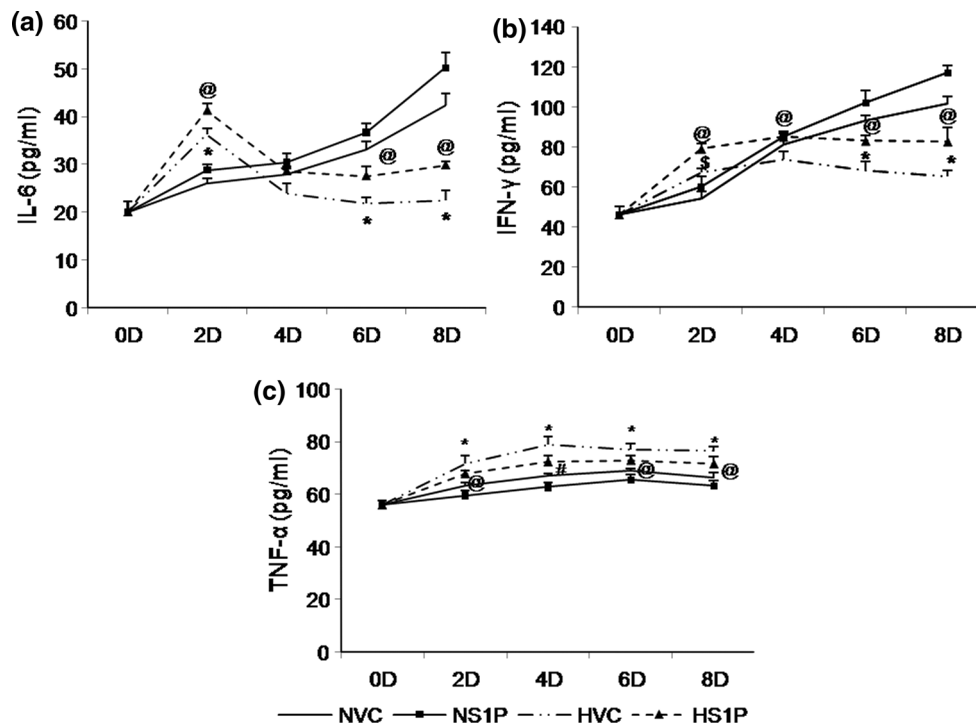

Fig. 3 S1P mediates temporal secretion of myokines in hypoxic differentiating C2C12 cells. Media supernatant from S1P (1 μM) and VC pretreated differentiating C2C12 cells under normoxia (N) and 0.5% hypoxia (H) at various time points was analyzed for the composition of secreted myokines—IL-6 (**a**), IFN-γ (**b**) and TNF-α (**c**); $0.2 \times 10^5/cm^2$ C2C12 cells were seeded and induced to differentiate as described. Following this the media supernatant was snap-frozen for myokine content analysis using a sandwich ELISA-based commercial kit. Data are reported as mean ± SD from three independent experiments carried out in triplicate. Statistical analysis was performed using the one-way ANOVA/post hoc Bonferroni's analysis method. Statistical significance is denoted by NVC vs. HVC ($\$p < 0.05$, *$p \leq 0.005$) and HVC vs. HS1P (@$p < 0.05$, #$p \leq 0.005$) at respective time points
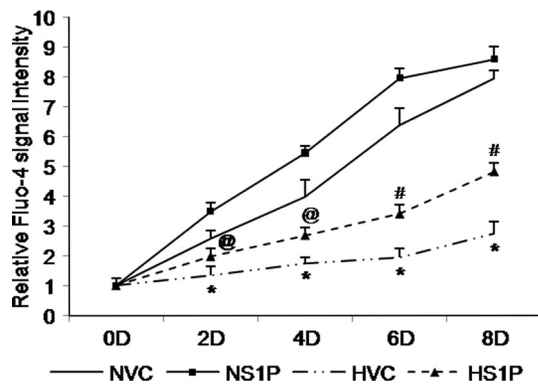
Fig. 4 S1P pre-conditioning boosts intracellular calcium in hypoxia-impaired C2C12 cell differentiation. S1P (1 μM) and VC pretreated differentiating C2C12 cells under normoxia (N) and 0.5% hypoxia (H) were stained with Fluo-4 dye at pre-defined study time points. Intracellular calcium was quantified by a fluorimeter. Data are reported as relative fluorescence signal intensity with respect to 0D, which is proportional to the calcium binding to Fluo-4. Data are reported as mean ± SD from three independent experiments carried out in triplicate. Statistical analysis was performed using the one-way ANOVA/post hoc Bonferroni's analysis method. Statistical significance is denoted by NVC vs. HVC ($\$p < 0.05$, $*p \leq 0.005$) and HVC vs. HS1P ($@p < 0.05$, $\#p \leq 0.005$) at respective time points

levels with a 4.8-fold maximal boost in 8D HS1P (w.r.t. 0D) ($p < 0.05$, HVC vs. HS1P; Fig. 4).

S1P pre-conditioning facilitates efficient substrate utilization, boosts both aerobic and anaerobic energy production, and increases mitochondrial mass to recover from the hypoxia-induced energy deficit

S1P pre-conditioning significantly remediated the ATP deficit imposed by hypoxic challenge in differentiating the C2C12 culture ($p \leq 0.005$, HVC vs. HS1P; Fig. 6c). This S1P-mediated recovery from energy deficiency was an outcome of several adaptive metabolic modifications—foremost being the increased intracellular glucose availability in HS1P cells in comparison to HVC cells ($p < 0.05$; Fig. 5a). Further, this increased intracellular glucose could be sourced to the upregulated anaerobic glycolysis (oxygen-independent energy-producing pathway) indicated by the significantly increased enzyme activities of LDH and HK ($p < 0.05$; Fig. 5c, d) to replenish the cellular ATP. An effect known as Pasteur's effect was evident here, which led to accumulation of higher intracellular lactate levels, especially in the sub-chronic duration of 4D, where besides being a metabolic end product, lactate could also play pro-myogenic signaling functions ($p < 0.05$; Fig. 5b) [32]. The apparent boost of Pasteur's effect following S1P pretreatment conferred an adaptive advantage to the cells under hypoxia.
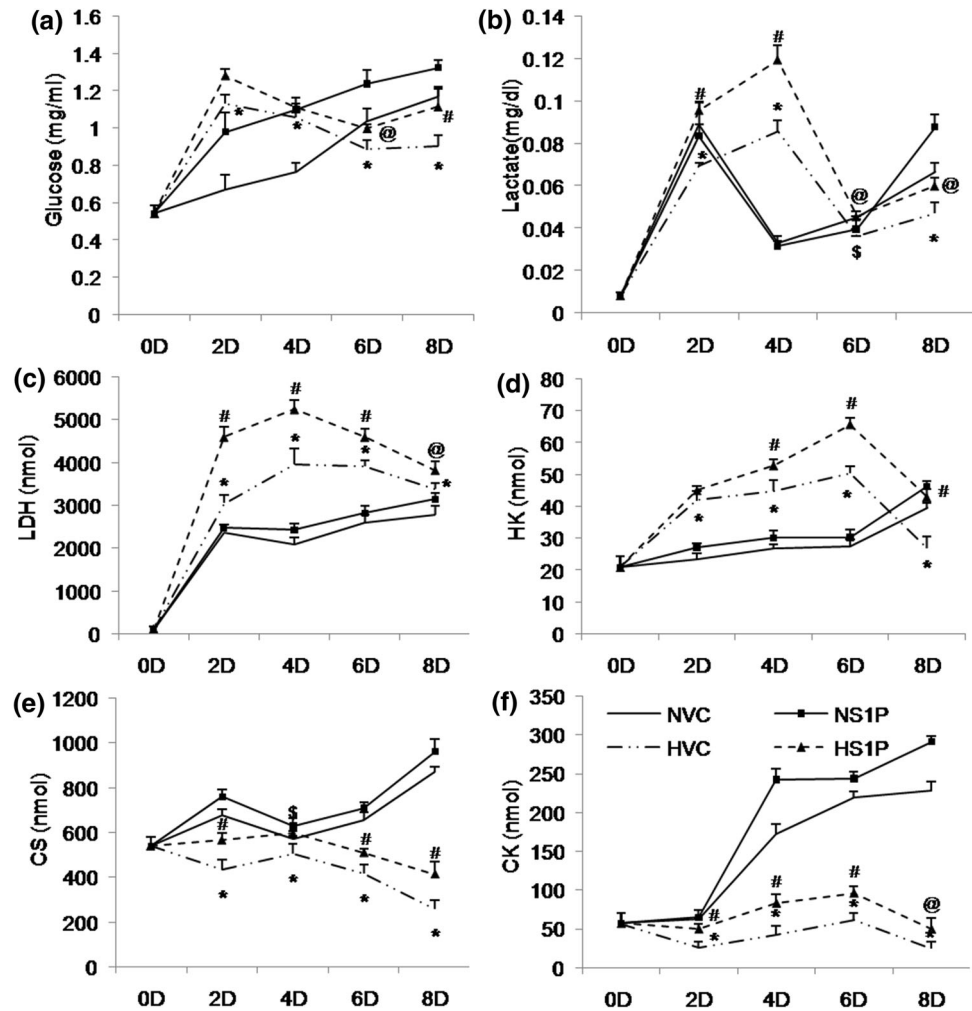
Along with an anaerobic boost, the S1P pre-conditioning positively influenced the aerobic energy generation machinery as well. Since a rise in mitochondrial mass is mandatory for fulfilling the ever-rising energy demands during myogenesis, the pro-myogenic response of S1P was accompanied with a significantly higher mitochondrial density compared with NVC (Fig. 6a, b). Hypoxia exposure drastically affected the myogenic potential of C2C12 cells and also the mitochondrial biogenesis, which resulted in a significant deficit in the mitochondrial mass of C2C12 cells. This is evident from a 20% deficit in the MitoTracker intensity of the HVC group in comparison with the NVC group at 2D reaching a maximum deficit of 70% in HVC at 8D ($p \leq 0.005$; Fig. 6b) with a compromised myogenic differentiation in the HVC group at all time points (Fig. 1a). The hypoxia-induced loss of mitochondrial mass could be restored to a significant extent, with S1P pre-conditioning of C2C12 cells prior to hypoxia exposure as evident from the relatively higher signal of MitoTracker dye after 2D in HS1P compared with HVC ($p < 0.05$; Fig. 6a, b). At the functional level, the S1P-mediated boost in the mitochondrial mass observed via the increased aerobic metabolism was also validated by the increased activities of the aerobic enzyme markers CS and CK (Fig. 5e, f). Also, the S1P-mediated significant increase in CK activity ($p < 0.05$) is a pro-myogenic indicator as the cellular CK content appears only after the myoblasts have been triggered to differentiate into myotubes.

The property of S1P pre-conditioning to boost mitochondrial mass is observed to be an adaptive advantage in the hypoxia-challenged C2C12 culture. The mitochondrial mass signal in the HS1P group is increased nearly 1.2–1.5 fold with respect to HVC from 2D to 8D and is coincident with pro-myogenic stimuli despite the ambient hypoxia.

Modulation of S1P receptor expression in C2C12 differentiating cells

The assessment of relative gene expression using real-time PCR revealed that hypoxia exposure temporally modulated the $S1PR_{1-4}$ receptor expression in a way to suggest the occurrence of cellular injury and loss of the myogenic potential of the differentiating C2C12 cells. Interestingly, S1P preconditioned C2C12 myocytes expressed a distinct $S1PR_{1-4}$ receptor expression profile, appearing to assist recovery from hypoxia-induced cellular injury and loss of myogenic potential (Fig. 7). Explicitly, the $S1PR_1$ was continuously upregulated until 8D in the normally differentiating NVC group, whereas, under hypoxia, $S1PR_1$ expression started to decline from 6D onwards ($p < 0.05$, NVC vs. HVC; Fig. 7a). With S1P pre-conditioning prior to hypoxia exposure (i.e., the HS1P group), though a similar depression of $S1PR_1$ expression was observed from 6D onwards, interestingly, its

Fig. 5 S1P pre-conditioning boosts energy metabolism in hypoxia-exposed differentiating C2C12 myoblast cells. C2C12 myoblast cells were seeded and induced to differentiate as described earlier. S1P (1 μM) and VC pretreated differentiating C2C12 cells under normoxia (N) and 0.5% hypoxia (H) were analyzed for various cellular bioenergetics markers at predefined time points in all study groups. Differentiating C2C12 cell lysate was analyzed for intracellular glucose (**a**), lactate (**b**), lactate dehydrogenase activity (LDH) (**c**), hexokinase activity (HK) (**d**), citrate synthase activity (CS) (**e**) and creatine kinase activity (CK) (**f**). Data are reported as mean ± SD from three independent experiments carried out in triplicate after normalization with cell lysate (mg/ml). Statistical analysis was performed using the one-way ANOVA/post hoc Bonferroni's analysis method. Statistical significance is denoted by NVC vs. HVC ($p < 0.05$, *$p \leq 0.005$) and HVC vs. HS1P (@$p < 0.05$, #$p \leq 0.005$) at respective time points

expression was maintained at a significantly higher level compared to the hypoxia control group at all the time points ($p \leq 0.005$; HVC vs. HS1P). In all the study groups, the S1PR$_2$ followed a similar trend of gene expression as S1PR$_1$ since its level showed a continuous upregulation in the normally differentiating NVC group, while with hypoxia exposure (both HVC and HS1P) its level started to fall after an initial boost until 4D. However, as with S1PR$_1$, S1P preconditioning assisted in the maintenance of a significantly higher level of S1PR$_2$ expression despite hypoxia exposure at all the study time points in comparison to the HVC group ($p \leq 0.005$, HVC vs. HS1P; Fig. 7b). The effect of S1P preconditioning on S1PR$_1$ and S1PR$_2$ expression per se could be vividly observed in the NS1P group where a robust increase in the expressions of both these receptors was documented at all the time points in comparison to the NVC as well as HVC groups ($p < 0.05$ NVC vs. NS1P, $p < 0.05$ HVC vs. HS1P).

In the NVC group, there was only a subtle upregulation in the expression of S1PR$_3$ and S1PR$_4$ on 4D, following which they were maintained at a comparable level until

8D; however, following hypoxia exposure in the HVC groups, they were continuously downregulated until the study end point ($p < 0.05$, NVC vs. HVC; Fig. 7c, d). As with S1PR$_{1,2}$, S1P preconditioning prior to hypoxia exposure (HS1P group) maintained a significantly higher level of S1PR$_{3,4}$ expression in comparison to the HVC group throughout the study ($p \leq 0.005$, HVC vs. HS1P), which was indeed due to the S1PR$_{1-4}$ expression boosting effect of S1P alone as observed in the NS1P group. The expression of S1PR$_5$ could not be detected in any of the experimental groups in the present study.

Discussion

Exposure to acute or chronic hypoxia damages skeletal muscle tissues to varying degrees by affecting the morphological and cellular homeostasis. In the present longitudinal study, we evaluated the in vitro preclinical efficacy of S1P in promoting myogenic differentiation of C2C12
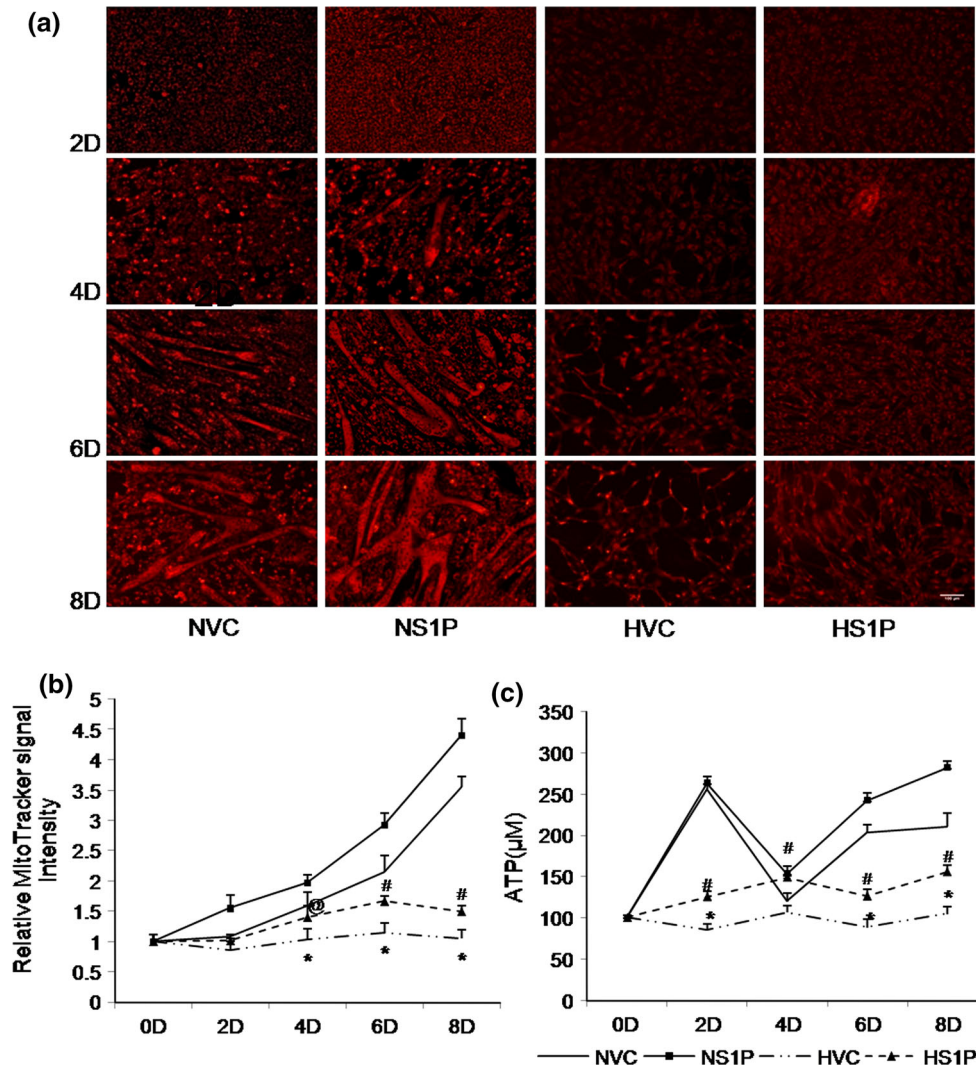
Fig. 6 S1P pre-conditioning boosts ATP production by enhancing mitochondrial density; $0.2 \times 10^5/cm^2$ C2C12 cells were seeded and maintained in DMEM-HG media and induced to differentiation at 90–95% confluence by shifting to DM (DMEM HG with 2% HS). **a** S1P (1 μM) and VC pretreated differentiating C2C12 cells under normoxia (N) and 0.5% hypoxia (H) at pre-defined study time points were stained by MitoTracker and imaged by an inverted fluorescence microscope at ×10 magnification. *Scale bar* 100 μm. **b** Mitochondrial mass (mitotracker intensity) was also quantified by a multimode plate reader (FLUOstar Omega), and average values relative to 0D were reported. **c** ATP was measured in differentiating C2C12 cell lysate using a commercial kit as per the manufacturer's protocol. Data are reported as mean ± SD from three independent experiments carried out in triplicate after normalization with cell lysate (mg/ml). Statistical analysis was performed using the one-way ANOVA/post hoc Bonferroni's analysis method. Statistical significance is denoted by NVC vs. HVC ($\$p < 0.05$, $*p \leq 0.005$) and HVC vs. HS1P ($@p < 0.05$, $\#p \leq 0.005$) at respective time points

myoblast cells under normoxia and hypoxia (0.5% O_2) over a period of 8 days [33]. This is the first study, to the best of our knowledge, in which the temporal myokine secretary responses and the bioenergetic changes congruent with C2C12 myoblasts differentiation have been elucidated. Under normoxic culture conditions, the S1P pre-conditioning significantly improved the terminal differentiation morphometric descriptors of the myotubes (especially the length and diameter) as evident from Figs. 1a and 2. A separate set of experiments was conducted under chronic hypoxia (up to 8 days), an extreme stress leading to critical

loss of the myogenic differentiation potential in the C2C12 cells. Here too exogenous S1P pre-conditioning was observed to promote the formation of a relatively larger number of nascent myotubes, although their morphometric comparison in HS1P vs. HVC groups was not possible because of either complete loss or extremely scanty nascent myotubules in the HVC group (Fig. 1a). The formation of mature myotubes is the end stage of differentiation, although there are several preceding stages and factors influencing the final outcome of differentiation. Here, in the present study, the benefit of S1P preconditioning has
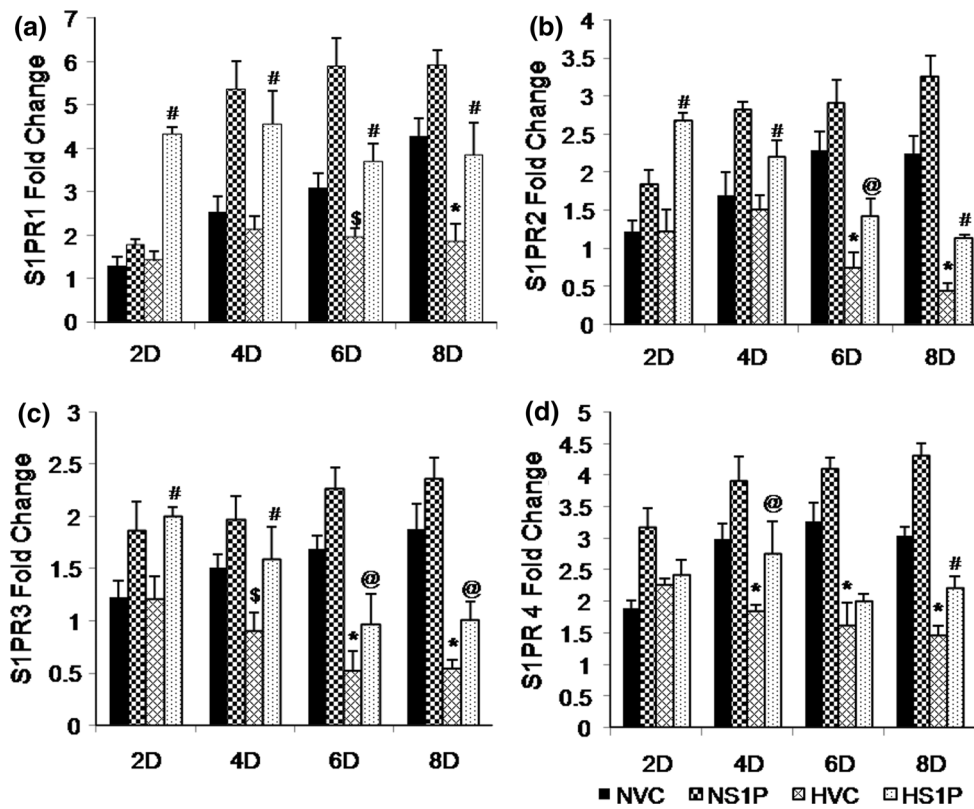
Fig. 7 Hypoxia modulates S1P receptor expression in C2C12 differentiating cells. S1P (1 μM) and VC pretreated differentiating C2C12 cells under normoxia (N) and 0.5% hypoxia (H) were analyzed for S1P receptor expression. Quantitative mRNA analysis was performed by using real-time PCR of the target genes $S1PR_1$ (**a**), $S1PR_2$ (**b**), $S1PR_3$ (**c**) and $S1PR_4$ (**d**). $S1PR_5$ expression was not detected. Relative mRNA expression of the respective receptor was calculated after normalization to an endogenous reference gene (β-actin). Results are reported as fold changes (mean ± SD) from three independent experiments carried out in triplicate with respect to undifferentiated myoblasts (0D) at each time point according to the comparative CT method ($2^{-\Delta\Delta C_T}$ method). Statistical analysis was performed using the one-way ANOVA/post hoc Bonferroni's analysis method. Statistical significance is denoted by NVC vs. HVC ($\$p < 0.05$, $*p \leq 0.005$) and HVC vs. HS1P ($@p < 0.05$, $\#p \leq 0.005$) at respective time points

been observed for several factors (viz., $i[Ca^{2+}]$, myokines, MHC expression, bioenergetics and mitochondrial density, etc.) that affect various stages of differentiation.

In the present study, we hypothesize that pre-conditioning with S1P to undifferentiated C2C12 myoblast cells confers an adaptive advantage in early differentiation and triggers the differentiation signaling cascade in normoxia/hypoxia culture. Myokine remodeling and calcium signaling are the early factors that determine the fate of differentiation by activating the residual undifferentiated myogenic satellite cells [34]. S1P enhances the IL-6 secretion and stabilizes the IFN-γ level, which prevents TNF-α-induced apoptosis and leads to activation of the undifferentiated myoblasts in early differentiation and maintains the metabolic homeostasis during metabolic demand alteration [28, 29], while reduced TNF-α and upregulated $S1PR_2$ expression have the anti-apoptotic and mitogenic ability that boosts the differentiation protein MHC [30, 35]. However, upregulated $S1PR_1$ mRNA expression differentially regulates the early C2C12 differentiation by enhancing the proliferation into reserve cells and commitment in myoblasts to differentiate [36]. In agreement with this, the present study indicated S1P pretreated C2C12 cells secreted lower levels of TNF-α, despite hypoxia exposure, and displayed a higher level of differentiation promoting $S1PR_{1-2}$ m-RNA along with MHC expression (Fig. 1b) [37]. Meanwhile, along with myokine secretion, a temporal increase in $i[Ca^{2+}]$ corroborated the facilitation of differentiation in normoxia, whereas following hypoxia exposure its decreased level co-existed with hampered differentiation [31, 34]. An important observation was that all the study time points in S1P pretreatment, in both the normoxia and hypoxia groups, could retain the $i[Ca^{2+}]$ levels above that of HVC cells and also co-existed with improved C2C12 differentiation (Figs. 1a, 2, 4) [31, 38, 39]. Remarkably, downregulation of $S1PR_2$ and $S1PR_3$ receptor expression could abolish the calcium mobilization during differentiation in hypoxia.

The S1P-mediated boost in the expression of $S1PR_2$ and $S1PR_3$, especially under hypoxia, is being proposed to be a potential underlying factor behind the improved $i[Ca^{2+}]$ levels in these cells (Figs. 2, 7). It has been previously reported that S1P elicits the mobilization of calcium via. $S1PR_2$ and $S1PR_3$ mediated opening of calcium channels to reorient the localized cytoskeleton architecture at sites of intercellular adherens junctions between myoblasts and also to activate cell contraction [40, 41].

Being an energy-intensive biological process, the fate and rate of differentiation are strictly regulated by the metabolic substrate and oxygen availability to fulfill the ever-growing energy demand [42, 43]. In the early differentiation, oxidative phosphorylation contributes to only about one-third of the total ATP, whereas, as differentiation proceeds, mitochondrial respiration takes the lead and contributes to over 60% of the total ATP [44, 45]. Progressively higher intracellular lactate levels and LDH activity up to 4 days in hypoxia-exposed C2C12 cells indicate a delayed differentiation in comparison to normoxic cells where this effect is only seen up to 2 days (Fig. 5b, c). In the hypoxia-exposed group, where delayed differentiation was evident, an enormous ATP deficit developed apparently because of the reduced efficiency of anaerobic respiration involving the LDH and HK enzymes; however, the role of restrained activities of aerobic energy-producing enzymes could also not be ruled out [46]. It was noteworthy that S1P pretreatment prior to hypoxia exposure obliterated this hypoxia-induced energy deficit potentially via reinstating the aerobic enzyme activities, upregulating anaerobic enzyme activities and improving the glucose utilization [15].
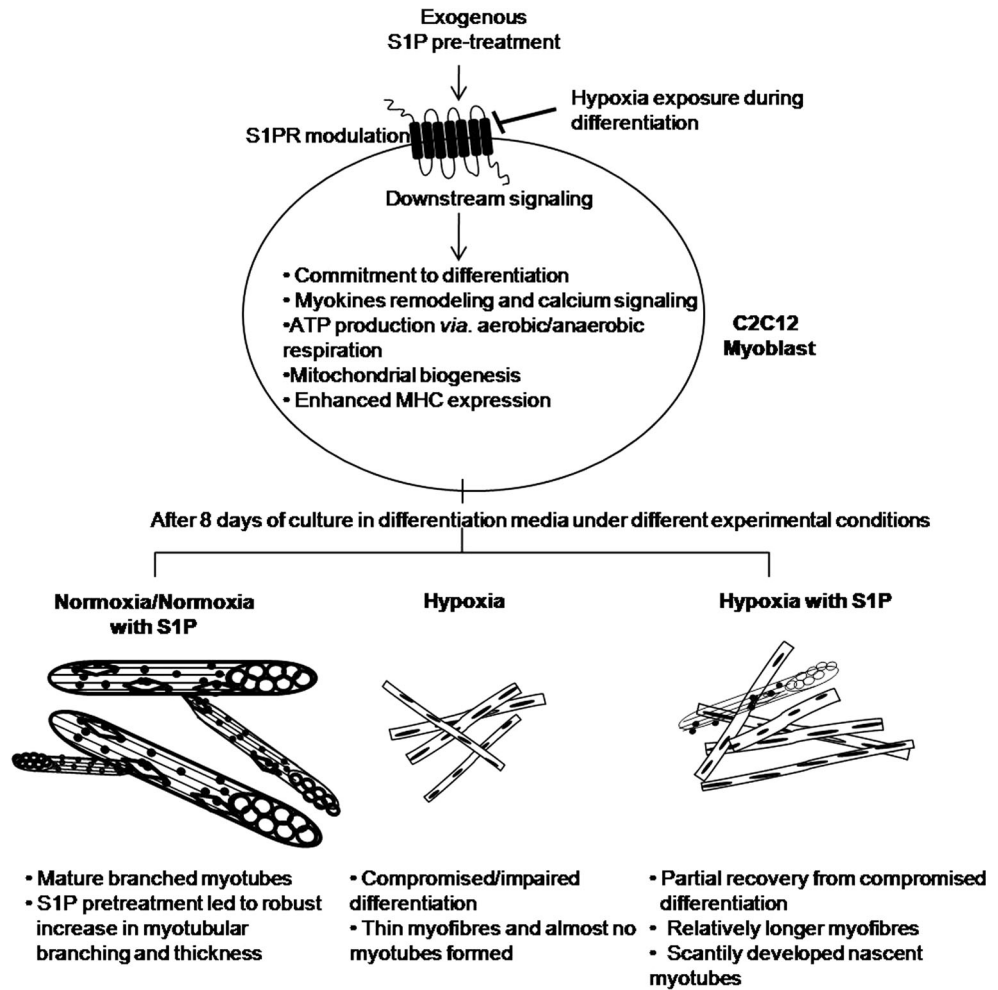
Further, the observed temporal increase in the intracellular glucose availability, in the normoxia group, appears to fuel the rising metabolic activity, while this increase was enormous in the S1P pretreated group and surpassed the control groups at all time points, thus appreciably facilitating the energy production as differentiation proceeded. In hypoxia, we speculate manifestation of a "Pasteur effect" an acute phase hypoxia survival strategy to compensate for the unmet energy needs, attributed to HIF-1α boosting the expression of glucose transporter GLUT-1 enhancing ATP generation [47, 48]. Acute hypoxia (up to 2D) led to higher intracellular glucose availability and S1P pre-conditioning owing to its HIF-1α-stabilizing properties improving it further [14, 15]. It is important to mention here that despite improved glucose, hypoxia-exposed cells could not match the glucose utilization efficiency of normoxic cells because of the reduced mitochondrial density with compromised Kreb's cycle activity (Figs. 5e, 6a) culminating in higher ATP levels in normoxic cells compared with hypoxic cells. In our previous study, we showed that preconditioning with S1P prior to hypoxia facilitates the recovery from hypoxia-induced energy deficits via activation of Rac 1 GTPase and PKC leading to HIF-1α stabilization [16].

In C2C12 myoblast differentiation, metabolic shift is a key event accompanying withdrawal from the proliferation state and switching to terminally differentiate into myotubes. However, at this juncture, due to cessation of proliferation, the metabolic rate dips transiently prior to a robust mitochondrial biogenesis at later stages [49]. In the present study, we observed an abrupt fall in ATP levels at 4D in normoxia and 6D in hypoxia groups co-existing with a lower level of intracellular lactate, LDH and CS enzyme activity (Fig. 5) along with a temporal increase in mitochondrial density. Decreased aerobic enzyme activities along with a compromised mitochondrial mass during the late differentiation stage in the hypoxia control group could be a reason for the compromised/impaired differentiation leading to failure of mature myotube formation. Interestingly, S1P pretreatment of these cells prior to hypoxia exposure significantly improved the mitochondrial density as well as the glucose utilization, leading to enhancement of ATP turnover via oxidative phosphorylation as evidenced by the observed boost mediated by HK, CK and CS activities in this study group. These are potential underlying causes for the S1P-mediated upregulation of MHC expression and enhanced terminal differentiation of C2C12 cells leading to partial recovery from hypoxia-induced impaired differentiation and nascent myotube formation. In Hep G2 cells, exogenous S1P improved the mitochondrial function and mass via $S1PR_2$-mediated upregulation of PGC-1α [50]. It is also reported that in stressful conditions a higher $i[Ca^{2+}]$ pool regulates mitochondrial biogenesis in C2C12 myoblasts [51]. Since in the present study exogenous S1P pretreatment of C2C12 cells upregulated $S1PR_{2-3}$ m-RNA expression along with $i[Ca^{2+}]$ and mitochondrial density, we propose this as a plausible mechanism behind S1P-induced mitochondrial biogenesis (Fig. 7b, c).

However, hypoxia induces disruption of cellular myokines/calcium homeostasis and mitochondrial functions/mass that impairs the aerobic respiration leading to bioenergetic disturbances, one of the main causes of cell death and compromised early/terminal differentiation. In hypoxia increased extracellular LDH activity could potentially be a result of the burden of hypoxia-induced cell death as well. With S1P pretreatment, the cellular damage was controlled while a parallel increase in the viability was observed under both normoxia and hypoxia at all time points (Fig. 1d, e). It is notable here that S1P pretreatment promotes pErk and pAkt mediated survival in splenocyte culture during hypoxia exposure [14]. S1P's pro-myogenesis potential was further recapitulated at 6 and 8 days of differentiation by commitment/differentiation surrogate markers where upregulated $S1PR_{1-2-3}$ expression promoted mitochondrial mass, CK activity and MHC

Fig. 8 Pictorial representation of C2C12 myoblast cell differentiation up to 8 days under normoxia and hypoxia with/without S1P pretreatment. S1P pretreatment facilitated the C2C12 myoblast differentiation under both normoxia and hypoxia via remodulated S1PR mRNA expression, altered myokine secretion, increased intracellular calcium and improved energy generation via boosting the aerobic/anaerobic metabolism and mitochondrial mass. Under normoxia, all of these led to a robust increase in myotubular branching and thickness following S1P pretreatment. Hypoxia exposure, an extreme stress, led to derangement of the S1PR$_{1-3}$ expression and impaired differentiation as evident from disturbed surrogate markers of differentiation leading to formation of thin and short nascent myofibers. However, S1P pretreatment prior to hypoxia exposure showed partial recovery from hypoxia-compromised differentiation with relatively longer myofibers and at least scantily developed nascent myotubes

protein expression [23, 50]. Strikingly, under hypoxia these markers were compromised with derangement of the above mentioned receptors' mRNA expression [23]. A potential underlying basis of pro-myogenic efficacy, at least in part, could be the upregulated expressions of S1PR$_{1-3}$ (Fig. 8). Each of these receptors has been demonstrated in various in vitro and in vivo models to individually have positive benefits on muscle mass formation [35, 36, 40, 41]. Also S1P promotes protein synthesis in a S1PR$_1$-dependent manner, which ultimately influences muscle mass and fiber size [52]. It is notable here as well that S1PR$_1$ expression was significantly upregulated following S1P pretreatment under normoxia with a significantly larger number of thicker and branched mature myotubular structures. Though the magnitude of this effect was lower in hypoxia-exposed cells owing to the extremely stressful environment, the observed benefit of S1P in promoting C2C12 differentiation cannot be denied and is indeed worth further exploration.

In conclusion, the present study sets the path for in-depth future studies for assessment of S1P's potential as a prophylactic intervention for hypoxia-related myo-pathologies.

References

1. Giaccia AJ, Simon MC, Johnson R (2004) The biology of hypoxia: the role of oxygen sensing in development, normal function, and disease. Genes Dev. doi:10.1101/gad.1243304

2. Kikusato M, Toyomizu M (2015) Moderate dependence of reactive oxygen species production on membrane potential in avian muscle mitochondria oxidizing glycerol 3-phosphate. J Physiol Sci. doi:10.1007/s12576-015-0395-2

3. Murray Andrew J (2009) Metabolic adaptation of skeletal muscle to high altitude hypoxia: how new technologies could resolve the controversies. Genome Med. doi:10.1186/gm117

4. Grocott M, Montgomery H, Vercueil A (2007) High-altitude physiology and pathophysiology: implications and relevance for intensive care medicine. Crit Care. doi:10.1186/cc5142

5. Nakai N, Fujita R, Kawano F, Takahashi K, Ohira T, Shibaguchi T, Nakata K, Ohira Y (2014) Retardation of C2C12 myoblast cell proliferation by exposure to low-temperature atmospheric plasma. J Physiol Sci. doi:10.1007/s12576-014-0328-5

6. Karalaki M, Fili S, Philippou A, Koutsilieris M (2009) Muscle regeneration: cellular and molecular events. In Vivo. 23:79–96

7. Chaillou T, Koulmann N, Meunier A, Pugnière P, McCarthy JJ, Beaudry M, Bigard X (2014) Ambient hypoxia enhances the loss of muscle mass after extensive injury. Pflugers Arch. doi:10.1007/s00424-013-1336-7

8. Nimker C, Kaur G, Revo A, Chaudhary P, Bansal A (2015) Ethyl 3,4-dihydroxy benzoate, a unique preconditioning agent for alleviating hypoxia-mediated oxidative damage in L6 myoblasts cells. J Physiol Sci. doi:10.1007/s12576-014-0348-1

9. Di Carlo A, De Mori R, Martelli F, Pompilio G, Capogrossi MC, Germani A (2004) Hypoxia inhibits myogenic differentiation through accelerated MyoD degradation. J Biol Chem. doi:10.1074/jbc.M313931200

10. Hoppeler H, Kleinert E, Schlegel C, Claassen H, Howald H, Kayar SR, Cerretelli P (1990) Morphological adaptations of human skeletal muscle to chronic hypoxia. Int J Sports Med Suppl. doi:10.1055/s-2007-1024846

11. Attanasio S, Snell J (2009) Therapeutic angiogenesis in the management of critical limb ischemia: current concepts and review. Cardiol Rev. doi:10.1097/CRD.0b013e318199e9b7

12. Chalfant CE, Spiegel S (2005) Sphingosine 1-phosphate and ceramide 1-phosphate: expanding roles in cell signaling. J Cell Sci. doi:10.1242/jcs.02637

13. Donati C, Cencetti F, Bruni P (2013) Sphingosine 1-phosphate axis: a new leader actor in skeletal muscle biology. Front Physiol. doi:10.3389/fphys.2013.00338

14. Chawla S, Sahni C, Tulsawani R, Singh M, Saraswat D, Bansal A, Saxena S (2014) Exogenous sphingosine 1-phosphate protects murine splenocytes against hypoxia-induced injury. Lipids. doi:10.1007/s11745-013-3860-9

15. Chawla S, Rahar B, Singh M, Bansal A, Saraswat D, Saxena S (2014) Exogenous sphingosine-1-phosphate boosts acclimatization in rats exposed to acute hypobaric hypoxia: assessment of haematological and metabolic effects. PLoS One. doi:10.1371/journal.pone.0098025

16. Chawla S, Rahar B, Saxena S (2016) S1P prophylaxis mitigates acute hypobaric hypoxia-induced molecular, biochemical, and metabolic disturbances: a preclinical report. IUBMB Life. doi:10.1002/iub.1489

17. Burattini S, Ferri P, Battistelli M, Curci R, Luchetti F, Falcieri E (2004) C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization. Eur J Histochem 48:223–233

18. Donati C, Meacci E, Nuti F, Becciolini L, Farnararo M, Bruni P (2005) Sphingosine-1-phosphate regulates myogenic differentiation: a major role for S1P2 receptor. Faseb J. doi:10.1096/fj.04-1780fje

19. Bradford MM (1976) A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

20. Kornberg A (1955) Lactate dehydrogenase of muscle. In: Colowick SP, Kaplan NO (eds) Methods in enzymology, vol 1. Acad Press, New York, pp 441–443

21. Supowit SC, Harris BG (1976) A scarissuum hexokinase: purification and possible function in compartmentation of glucose 6-phosphate in muscle. Biochim Biophys Acta. doi:10.1016/0005-2744(76)90007-3

22. Shepherd D, Garland PB (1966) Citrate synthase from liver. Biochem Biophys Res Commun. doi:10.1016/0076-6879(69)13006-2

23. Miyake T, McDermott JC, Gramolili AO (2011) A method for the direct identification of differentiating muscle cells by a fluorescent mitochondrial dye. PLoS One. doi:10.1371/journal.pone.0028628

24. Rasband WS (1997–2015) ImageJ. U. S. National Institutes of Health, Bethesda. http://imagej.nih.gov/ij/ (1997–2015)

25. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3(6):1101–1108

26. Burattini S, Ferri P, Battistelli M, Curci R, Luchetti F, Falcieri E (2004) C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization. Eur J Histochem 48(3):223–233

27. Ceafalan LC, Popescu BO, Hinescu ME (2014) Cellular players in skeletal muscle regeneration. Biomed Res Int. doi:10.1155/2014/957014

28. Muñoz-Cánoves P, Scheele C, Pedersen BK, Serrano AL (2013) Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword? FEBS J. doi:10.1111/febs.12338

29. Li YP (2003) TNF-alpha is a mitogen in skeletal muscle. Am J Physiol Cell Physiol. doi:10.1152/ajpcell.00453.2002

30. Tolosa L, Morlá M, Iglesias A, Busquets X, Lladó J, Olmos G (2005) IFN-gamma prevents TNF-alpha-induced apoptosis in C2C12 myotubes through down-regulation of TNF-R2 and increased NF-kappaB activity. Cell Signal. doi:10.1016/j.cellsig.2005.02.001

31. Przybylski RJ, MacBride RG, Kirby AC (1989) Calcium regulation of skeletal myogenesis. I. Cell content critical to myotube formation. In Vitro Cell Dev Biol. doi:10.1007/BF02623667

32. Willkomm L, Schubert S, Jung R, Elsen M, Borde J, Gehlert S, Suhr F, Bloch W (2014) Lactate regulates myogenesis in C2C12 myoblasts in vitro. Stem Cell Res. doi:10.1016/j.scr.2014.03.004

33. Yun Z, Lin Q, Giaccia AJ (2005) Adaptive myogenesis under hypoxia. Mol Cell Biol. doi:10.1128/MCB.25.8.3040-3055.2005

34. Hara M, Tabata K, Suzuki T, Do MK, Mizunoya W, Nakamura M, Nishimura S, Tabata S, Ikeuchi Y, Sunagawa K, Anderson JE, Allen RE, Tatsumi R (2012) Calcium influx through a possible coupling of cation channels impacts skeletal muscle satellite cell activation in response to mechanical stretch. Am J Physiol Cell Physiol. doi:10.1152/ajpcell.00068.2012

35. Donati C, Nincheri P, Cencetti F, Rapizzi E, Farnararo M, Bruni P (2007) Tumor necrosis factor-alpha exerts pro-myogenic action in C2C12 myoblasts via sphingosine kinase/S1P2 signaling. FEBS Lett. doi:10.1016/j.febslet.2007.08.007

36. Rapizzi E, Donati C, Cencetti F, Nincheri P, Bruni P (2008) Sphingosine 1-phosphate differentially regulates proliferation of C2C12 reserve cells and myoblasts. Mol Cell Biochem. doi:10.1007/s11010-008-9780-y

37. Loh KC, Leong WI, Carlson ME, Oskouian B, Kumar A, Fyrst H, Zhang M, Proia RL, Hoffman EP, Saba JD (2012) Sphingosine-1-phosphate enhances satellite cell activation in dystrophic muscles through a S1PR2/STAT3 signaling pathway. PLoS One. doi:10.1371/journal.pone.0037218

38. Porter GA Jr, Makuck RF, Rivkees SA (2002) Reduction in intracellular calcium levels inhibits myoblast differentiation. J Biol Chem. doi:10.1074/jbc.M203961200

39. Kanatous SB, Mammen PP, Rosenberg PB, Martin CM, White MD, Dimaio JM, Huang G, Muallem S, Garry DJ (2009) Hypoxia reprograms calcium signaling and regulates myoglobin expression. Am J Physiol Cell Physiol. doi:10.1152/ajpcell.00428.2008

40. Meacci E, Cencetti F, Formigli L, Squecco R, Donati C, Tiribilli B, Quercioli F, ZecchiOrlandini S, Francini F, Bruni P (2002) Sphingosine 1-phosphate evokes calcium signals in C2C12 myoblasts via Edg3 and Edg5 receptors. Biochem J. doi:10.1042/bj3620349

41. Sassoli C, Formigli L, Bini F, Tani A, Squecco R, Battistini C, Zecchi-Orlandini S, Francini F, Meacci E (2011) Effects of S1P on skeletal muscle repair/regeneration during eccentric contraction. J Cell Mol Med. doi:10.1111/j.1582-4934.2010.01250.x

42. Koopman R, Ly CH, Ryall JG (2014) A metabolic link to skeletal muscle wasting and regeneration. Front Physiol. doi:10.3389/fphys.2014.00032

43. Li W, Hu ZF, Chen B, Ni GX (2013) Response of C2C12 myoblasts to hypoxia: the relative roles of glucose and oxygen in adaptive cellular metabolism. Biomed Res Int. doi:10.1155/2013/326346

44. Leary SC, Battersby BJ, Hansford RG, Moyes CD (1998) Interactions between bioenergetics and mitochondrial biogenesis. Biochim Biophys Acta. doi:10.1016/S0005-2728(98)00105-4

45. Yamada K (2016) Energetics of muscle contraction: further trials. J Physiol Sci. doi:10.1007/s12576-016-0470-3

46. Wheaton WW, Chandel NS (2011) Hypoxia. 2. Hypoxia regulates cellular metabolism. Am J Physiol Cell Physiol 300:C385–C393

47. Seagroves TN, Ryan HE, Lu H, Wouters BG, Knapp M, Thibault P, Laderoute K, Johnson RS (2001) Transcription factor HIF-1 is a necessary mediator of the Pasteur effect in mammalian cells. Mol Cell Biol. doi:10.1128/MCB.21.10.3436-3444.2001

48. Hamilton DL, Beall C, Jeromson S, Chevtzoff C, Cuthbertson DJ, Ashford ML (2014) Kv1.3 inhibitors have differential effects on glucose uptake and AMPK activity in skeletal muscle cell lines and mouse ex vivo skeletal muscle. J Physiol. doi:10.1007/s12576-013-0285-4

49. Wagatsuma A, Sakuma K (2013) Mitochondria as a potential regulator of myogenesis. Sci World. doi:10.1155/2013/593267

50. Shen Z, Liu C, Liu P, Zhao J, Xu W (2014) Sphingosine 1-phosphate (S1P) promotes mitochondrial biogenesis in Hep G2 cells by activating Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α). Cell Stress Chaperones. doi:10.1007/s12192-013-0480-5

51. Biswas G, Adebanjo OA, Freedman BD, Anandatheerthavarada HK, Vijayasarathy C, Zaidi M, Kotlikoff M, Avadhani NG (1999) Retrograde Ca^{2+} signaling in C2C12 skeletal myocytes in response to mitochondrial genetic and metabolic stress: a novel mode of inter-organelle crosstalk. EMBO J 18(3):522–533

52. Ieronimakis N, Pantoja M, Hays AL, Dosey TL, Qi J, Fischer KA, Hoofnagle AN, Sadilek M, Chamberlain JS, Ruohola-Baker H, Reyes M (2013) Increased sphingosine-1-phosphate improves muscle regeneration in acutely injured mdx mice. Skelet Muscle. doi:10.1186/2044-5040-3-20