Effects of S1P on skeletal muscle repair/regeneration during eccentric contraction

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Abstract

Skeletal muscle regeneration is severely compromised in the case of extended damage. The current challenge is to find factors capable of limiting muscle degeneration and/or potentiating the inherent regenerative program mediated by a specific type of myoblastic cells, the satellite cells. Recent studies from our groups and others have shown that the bioactive lipid, sphingosine 1-phosphate (S1P), promotes myoblast differentiation and exerts a trophic action on denervated skeletal muscle fibres. In the present study, we examined the effects of S1P on eccentric contraction (EC)-injured extensor digitorum longus muscle fibres and resident satellite cells. After EC, skeletal muscle showed evidence of structural and biochemical damage along with significant electrophysiological changes, *i.e.* reduced plasma membrane resistance and resting membrane potential and altered Na^+ and Ca^{2+} current amplitude and kinetics. Treatment with exogenous S1P attenuated the EC-induced tissue damage, protecting skeletal muscle fibre from apoptosis, preserving satellite cell viability and affecting extracellular matrix remodelling, through the up-regulation of matrix metalloproteinase 9 (MMP-9) expression. S1P also promoted satellite cell renewal and differentiation in the damaged muscle. Notably, EC was associated with the activation of sphingosine kinase 1 (SphK1) and with increased endogenous S1P synthesis, further stressing the relevance of S1P in skeletal muscle protection and repair/regeneration. In line with this, the treatment with a selective SphK1 inhibitor during EC, caused an exacerbation of the muscle damage and attenuated MMP-9 expression. Together, these findings are in favour for a role of S1P in skeletal muscle healing and offer new clues for the identification of novel therapeutic approaches to counteract skeletal muscle damage and disease.

Keywords: eccentric contraction ● muscle damage ● myogenesis ● satellite cells ● sphingosine 1-phosphate ● sphingosine kinase

Introduction

Several lines of evidence indicate that strenuous exercise, such as rigorous eccentric muscle contraction, may result in severe muscle wasting and functional deterioration [1, 2]. However, skeletal muscle tissue can regenerate *via* the activation of satellite cells [3, 4], a population of quiescent myogenic cells, localized between the basal lamina and plasma membrane of the muscle fibre, capable of proliferating and differentiating in response to muscle injury or disease [5]. These cells are characterized by the expression of the paired box transcription factor Pax7, which is required for their survival and functions upstream of the expression of the myogenic regulator factors (MRFs) Myf5, MyoD, Mrf4 and myogenin [6, 7]. During muscle repair/regeneration, a small subpopulation of satellite cells does not execute the myogenic program and reconstitute a compartment of quiescent 'stem cell' (self renew), able to respond to repeated muscle injury [8, 9]. It is generally assumed that the mere presence of satellite cells is not sufficient to ensure a rapid functional recovery of the injured muscle and that the release of appropriate factors and the establishment of suitable microenvironment are even more important in determining the effectiveness of the myogenic response [10–12]. However,

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in the case of extended damage, the propensity for an enhanced inflammatory response and the excessive fibroblast proliferation and connective tissue deposition may exceed the ability of stem cells to pursue the myogenic program and the muscle's regenerative potential [10, 12]. Hence, the identification of factors and therapeutic interventions aiming at enhancing muscle regeneration and attenuating or preventing muscle damage and fibrosis would be very useful in order to preserve muscle functionality. Along this line, sphingosine 1-phosphate (S1P), a bioactive lipid present in a physiologic concentration in the plasma, exerts multiple biological functions, including the maintenance of skeletal muscle homeostasis, acting through S1P receptors [13, 14]. The inhibition of sphingosine kinase (SphK), the enzyme responsible for S1P formation, significantly reduces the amount of regenerating centrally nucleated myofibres in cardiotoxin-damaged muscle [15, 16]. Moreover, the administration of exogenous S1P has been shown to cause a dramatic reduction of muscle tension decline during fatigue [17] and attenuate the progress of denervation-induced muscle atrophy [18] suggesting that this bioactive sphingolipid may act as a myogenic trophic factor. In this context, studies from our group have demonstrated that S1P is capable to stimulate C2C12 myoblast differentiation and have identified the underlying cellular and molecular mechanisms [19–24]. Finally, there is evidence to support a role for the sphingolipid in the stimulation of extracellular matrix remodelling and in the regulation of matrix metalloproteinase 9 (MMP-9) expression in cardiomyocytes and skeletal myoblasts [25–28]. Interestingly, changes in extracellular matrix composition are considered as crucial steps for the restoration of the tissue architecture during skeletal muscle regeneration [29].

Based on these considerations, in the present study we further investigated the beneficial effects of S1P on skeletal muscle repair/regeneration, focusing our attention on the ability of this bioactive factor to protect the tissue against eccentric contraction (EC)-induced damage and exert its action on satellite cell function.

Materials and methods

The detailed version of materials and methods is reported in the supplemental file.

Animals and muscle injury model

Extensor digitorum longus (EDL) muscles were removed from anesthetized young adult Swiss mice (25–30 g) and subjected to forced EC in isometric condition, in the absence or presence of 1 μM S1P (EC+ S1P) or of compound II, a SphK1 inhibitor (EC + iSphK).

Morphological analysis of EDL skeletal muscle

Muscle tissue samples were fixed in buffered glutaraldehyde, post-fixed in phosphate-buffered OsO_4 and routinely processed for light and transmission electron microscopy [30]. The severity of tissue damage was evaluated by counting the number of fibres showing key morphological signs of cell damage and evaluating the degree of injury in each fibre, using a semi-

Table 1 Scoring method of skeletal muscle fibre damage

Score		Description
0	Normal myocyte	
1	Slight	Mild intermyofibre oedema Mild intramyofibrillar oedema
2	Moderate	Mild intramyofibrillar oedema Cytoskeletal disarrangement Focal sarcomere contraction Mild nuclear chromatin clumping
3	Severe	Severe intramyofibrillar oedema Severe myofibrillar disarrangement Plasma membrane ruptures

quantitative histological score, as reported previously for similar purposes [31]. The criteria used are reported in Table 1.

Lactate dehydrogenase (LDH) assay

LDH was assayed by oxidation of NADH with pyruvate [32].

Western blotting

Protein aliquots (10–30 μg) from EDL muscle tissue and satellite cell-enriched fraction lysates were separated by a SDS-PAGE and immunoblotted as described previously [21]. Specific primary antibodies anti-Bax, anti-Cytc, anti-SphK1, anti-cyclin D1, anti-phospho p44/42 mitogen-activated protein (MAP) kinase (MAPK) (Thr202/Tyr204) [p-extracellular-signal-regulated kinase 1/2 (p-ERK1/2)], anti-catalytic domain of MMP-9, anti β-actin were used and signal detected by peroxidase-conjugated secondary IgG1 using Enhanced Chemiluminescence (ECL) reagent. Densitometric analysis of the bands was performed with Imaging and Analysis Software by Bio-Rad (Hercules, CA, USA). For Bax expression determination, mitochondria fraction was prepared from muscle tissue lysates by centrifugation at 15,000 $\times$ g for 10 min. and the precipitate used.

Caspase 3/7 activity

Caspase activity was measured in the lysates by determining the cleavage of the fluorogenic caspase 3/7 substrate acetyl Asp-Glu-Val-Asp 7-amino-4-methylcoumarin-derived caspase (Ac-DEVD-AMC) (50 mM final concentration, Ex/Em 354 nm/442 nm) in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 1 mM ethylenediaminetetraacetic acid, 10 mM dithiothreitol, 100 mM NaCl, 10% glycerol. The extent of Ac-DEVD-AMC was measured as the increase in relative fluorescence units (RFU) resulting from the release of free fluorescent AMC.

TUNEL assay

Nuclear DNA fragmentation was analysed by TUNEL (terminal deoxyribonucleotidyl transferase mediated dUTP nick end labelling) reaction in cryosectioned muscle samples. Detection of apoptosis was performed with a commercially available *In Situ* Cell Death Detection Kit according to the instructions provided by the manufacturer. TUNEL apoptotic index was then expressed as relative percentage of TUNEL[+] nuclei on the total number of haematoxylin-stained nuclei evaluated in parallel sections.

Electrophysiology

Resting membrane potential (RMP) was recorded in current-clamp condition by a microelectrode inserted into a single fibre of a EDL isolated muscle bathed in Ringer–Krebs solution. The passive properties of the fibres (membrane resistance R_m and capacitance, C_m), L-type Ca^{2+} current (I_{Ca}) and Na^+ current (I_{Na}) were evaluated using the double Vaseline-gap method, as described previously [33].

Isolation of satellite cell-enriched fractions from EDL muscle

A population of muscle-derived cells, mostly satellite cells, was purified by enzymatic dissociation and 40–70% Percoll gradient centrifugation from EDL muscles as described previously, with minor modifications [34]. For 3-[4,5-dimethyltiazol-2yl]-2,5 diphenyltetrazolium bromide (MTS) assay and S1P quantification, satellite cell-enriched fractions were cultured on collagen-coated dishes in growth medium for 24 hrs.

Cell viability by MTS assay

3-[4,5-dimethyltiazol-2yl]-2,5 diphenyltetrazolium bromide (5 mg/ml) was added to the satellite cell-enriched fractions plated and incubated for 24 hrs after isolation. Cell viability was determined by measuring the absorbance at 490 nm with a microplate spectrophotometer and expressed as percent of cell viability (OD treated/OD control) setting the control (undamaged muscle-derived satellite cells) as 100.

SphK activity

SphK activity was determined in control and EC-injured muscle by *in vitro* assay essentially as described previously [21]. Briefly, equal amounts of protein (20–60 µg) from muscle, were incubated in SphK1 and SphK2 specific assay buffer [35] in the presence of 20 µmol/l sphingosine dissolved in 5% Triton X-100 and 200 mmol/l ATP ([^{32}P]ATP (3µCi/assay).

S1P quantification in satellite cell-enriched fractions

Sphingolipids were quantified in satellite cell-enriched fractions from control and damaged muscles incubated with serum-free medium and 2 µCi of [^{3}H]sphingosine for 24 hrs at 37°C, essentially as reported previously [21, 36].

Confocal immunofluorescence

Cryostat EDL muscle sections (8 µm) were fixed in paraformaldehyde vapours, incubated with the following antibodies: mouse monoclonal anti-Pax7, rabbit polyclonal anti-MyoD and mouse monoclonal anti-myogenin and then immunorevealed with specific Alexa Fluor 488- or Alexa Fluor 568- conjugated IgG. Observations were performed under a confocal Leica TCS SP5 (5 SpectroPhotometer channels) microscope (Leica Microsystems, Mannheim, Germany) as reported previously [22].

Culture of satellite cells isolated from single myofibres

Living single myofibres of EDL muscles were isolated and individually cultured as described previously [37]. After 48 hrs culture, the myofibres were removed and the derived satellite cells were cultured in growth medium until reaching confluence and then shifted in differentiation medium [DM: DMEM, 2% horse serum (HS)]. Cells sprouting from each single myofibre were counted 24 hrs after the fibre removal. Myotubes were evaluated after 4 days satellite cell culture in DM.

Determination of tissue fibrosis by Van Gieson staining

Cryostat EDL muscle sections (8 µm) were fixed in paraformaldehyde vapours and stained with Van Gieson method for collagen [38].

Results

Exogenous S1P exerts protective effects during EC- induced muscle damage

We first evaluated the effects of EC on EDL skeletal muscle by morphological, biochemical and electrophysiological analyses. At light microscopic examination, skeletal muscle fibres subjected to EC showed extensive and severe morphological alterations (histological score of 2.7 ± .0.1, Table 1; Fig. 1A and B). At the ultrastructural level, the majority of the injured fibres displayed variable degree of myofibrillar disarrangement and Z-disk disruption, dilated cistaerne and tubules of sarcoplasmic reticulum, and swollen mitochondria with shortening and disappearance of cristae (Fig. 1C and D). Biochemical analysis revealed that LDH activity was significantly decreased of approximately 35% in EC-injured muscle compared to control (8.9 ± 0.9 *versus* 13.7 ± 1.2; mean ± S.E.M.; $P < 0.05$, $n = 3$). Moreover, we demonstrated a significant increase in the association of the pro-apoptotic protein, Bax, with mitochondrial fractions, and in the release of respiratory chain protein, Cytc, into the cytosolic fractions obtained from EC-damaged muscle compared to control (Fig. 2A). However, EC damage provoked only a slightly increase in caspase 3/7 activity (Fig. 2B), and a certain positivity to the TUNEL reaction. In particular, positive nuclei were found both in the myofibres and in cells located in the close vicinity, and represented an approximately 2% of the total nuclei (Fig. 2C). The electrophysiological assessment of the effects of EC performed on single muscle fibres showed the occurrence of significant changes in the sarcolemnic functionality (reduced plasma membrane resistance (R_mC_m) and resting membrane depolarization, Table 2), and in the myofibre excitability (reduced Na^+ current amplitude (I_{Na}) and altered kinetic, Fig. 3A and C; Table 2). EC also affected the excitation-contraction coupling, reducing L-type-mediated Ca^{2+} current (I_{Ca}) and altering the channel kinetics (Fig. 3B and D; Table 2).

When EC was performed in the presence of 1 µM S1P (EC + S1P), the observed structural and ultrastructural muscle alterations appeared strongly attenuated (Fig. 1). In fact, S1P caused a significant reduction in the number of damaged fibres, with an average decrease ranging from 20% to 30% and decreased the severity of myofibre damage, as shown by the significantly lower histological score (1.8 ± 0.2, Table 1) as compared to that of EC-injured muscle (Fig. 1A and B). By transmission electron microscopy (TEM) analyses, most of the muscle fibres exhibited moderate myofibrillar disarrangements and mitochondria alterations (Fig. 1C and D). Moreover, the treatment with S1P of EC muscle was capable of strongly attenuating the expression levels of
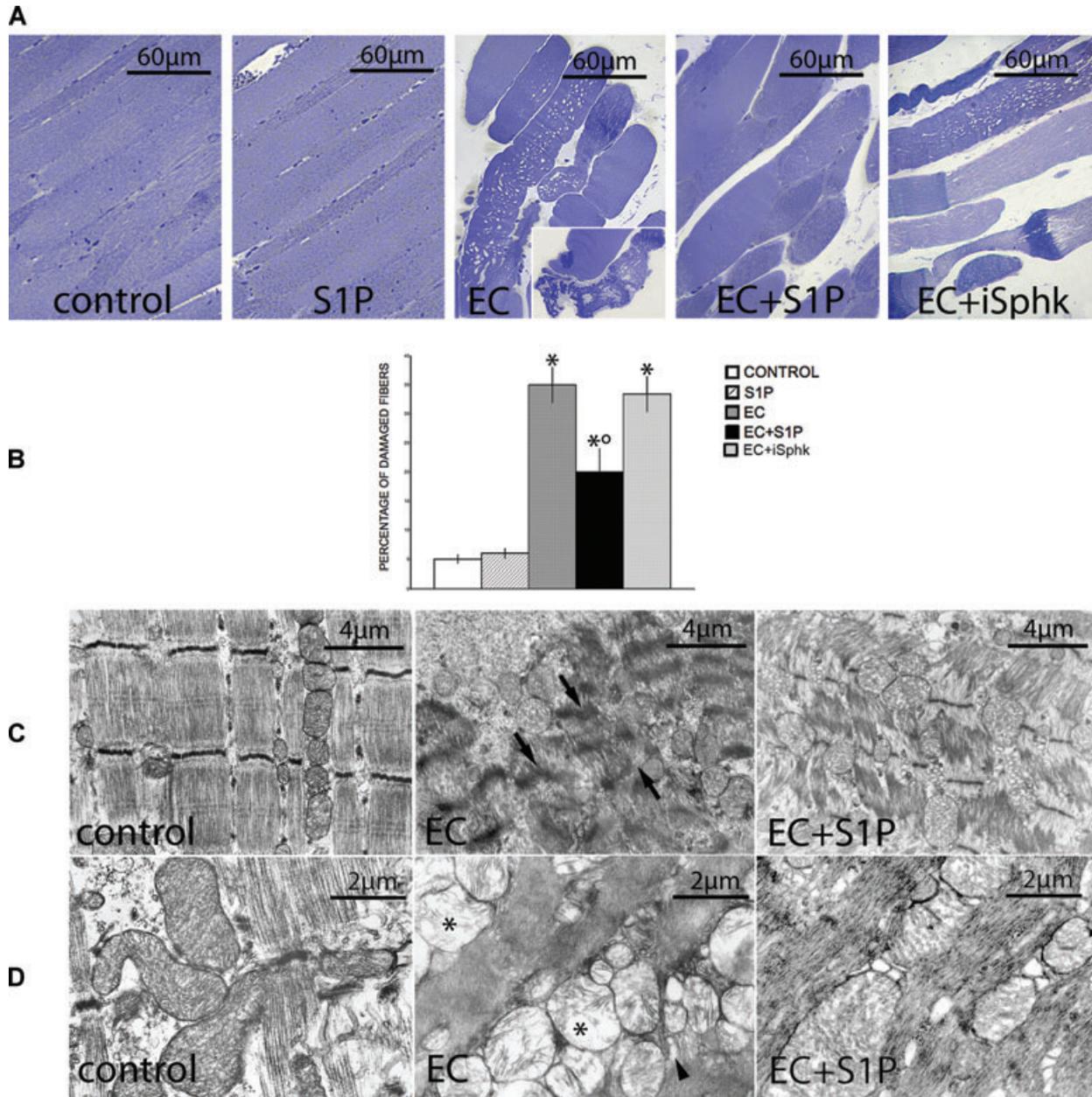
Fig. 1 Protective effect of S1P against EC-induced structural and ultrastructural damage in EDL muscle fibre. (**A**) Light microscopy. Semi-thin sections from control, S1P-treated, EC-, EC + S1P- and EC + SphK1 inhibitor, compound II (iSphK)-injured skeletal muscles. EC-injured fibres showed substantial damage and some displayed signs of necrosis (inset). These structural abnormalities are attenuated in EC + S1P muscle. Note that inhibition of the endogenous S1P production by iSphK does not confer much protection against EC damage. (**B**) Quantitative analysis of the number of damaged fibres expressed as percentage on the total fibre number (mean ± S.E.M., One-way ANOVA *$P < 0.05$ *versus* specific control; °$P < 0.05$ *versus* EC; $n = 3$). (**C–D**) Transmission electron microscopy. EC-damaged fibres display severe sarcomere disorganization and Z-disc disruption (arrows), together with swollen mitochondria with loss of organized cristae (asterisks) and dilated sarcoplasmic tubules (arrowheads). In the presence of S1P, these ultrastructural alterations appear strongly attenuated.

the assayed apoptotic markers (Fig. 2A) and caspase 3/7 activation (Fig. 2B), reducing the percentage of apoptotic nuclei (approximately of 0.8.%; Fig. 2C) and improving the examined electrophysiological parameters (Fig. 3, Table 2). The treatment with S1P of undamaged EDL muscle did not cause any morphological, biochemical and electrophysiological modifications (Figs 1–3).
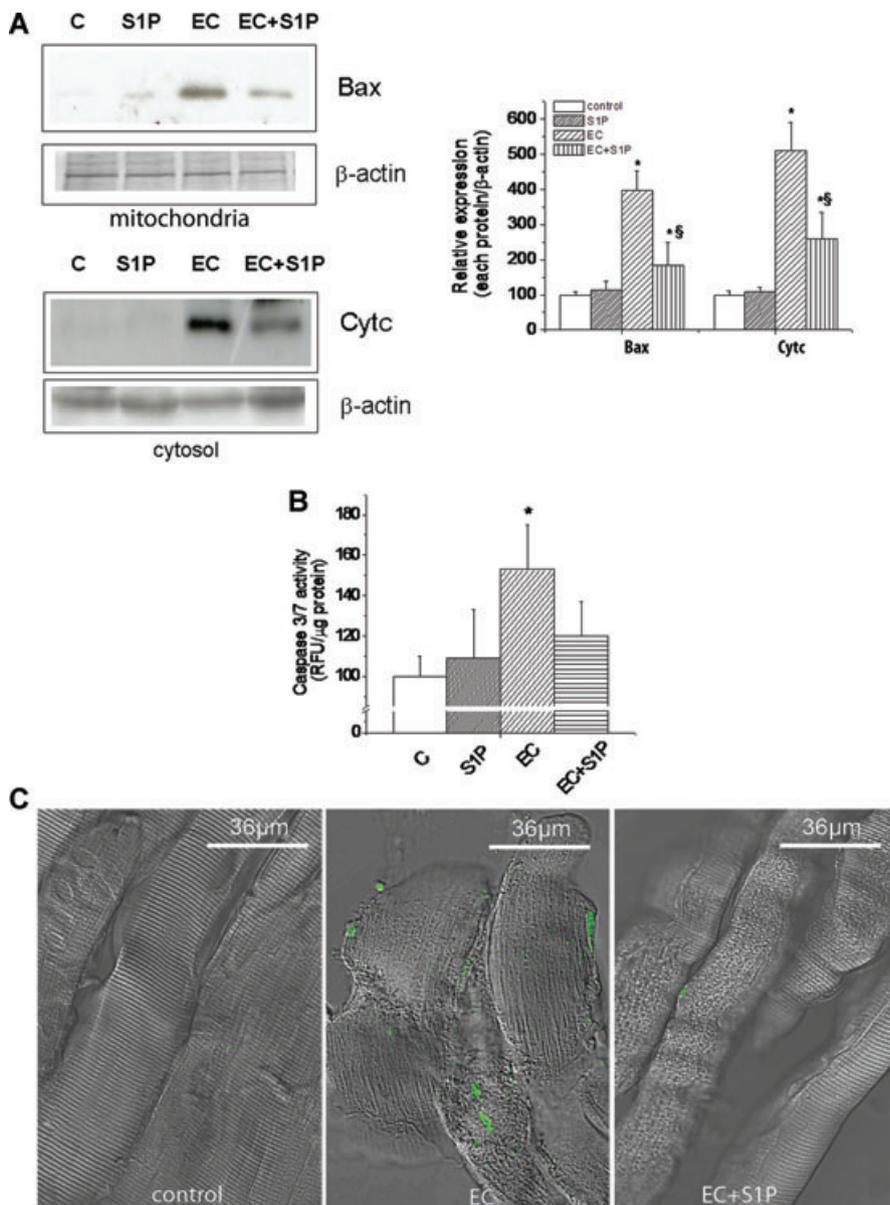
Fig. 2 Effects of S1P treatment on EC-induced apoptosis in EDL muscle. (**A**) Western blotting analysis of pro-apoptotic protein, Bax and Cytochrome c (Cytc) expression. Protein aliquots (10–25 μg) corresponding to the 15,000 × g fraction (mitochondria) and the cytosol obtained from control (C), S1P-treated, EC- and EC + S1P-injured muscle fibres were immunodetected by specific antibodies and revealed by enhanced chemiluminescence. The relative percentage of band intensity to control set as 100 (mean ± S.E.M.) and normalized to gel staining or β-actin is shown in the graphs. Student's t-test, *$P < 0.05$ versus specific control; $n = 3$; §$P < 0.05$ versus EC, $n = 3$. (**B**) Determination of caspase 3/7 activity. The caspase 3/7 assay was performed in lysates obtained from the muscle samples as indicated in (**A**). RFU resulting from the release of free fluorescent caspase 3/7 substrate (Ac-DEVD-AMC) are reported as mean ± S.E.M. (Student's t-test, *$P < 0.05$ versus specific control). (**C**) TUNEL assay. Cryostat sections from control, EC- and EC + S1P- injured muscles. Sparse apoptotic nuclei showing positive TUNEL labelling (green) are detected within the muscle fibres and the surrounding cells in EC injured muscles. Only scanty TUNEL^{+} cells can be seen in EC + S1P-injured muscles, whereas no apoptotic cells are detected in control muscles.

EC also affected satellite cell functionality; in fact, cell viability, evaluated performing MTS assay in satellite cell-enriched fractions declined drastically in the injured muscle (Fig. 4), suggesting that satellite cell metabolic activity could be impaired by muscle damage, as also previously reported [39–42]. Interestingly, satellite cell viability resulted significantly higher in EC + S1P muscle with respect to EC, thus extending the protective action of the sphingolipid in damaged skeletal muscle to the resident muscle stem cells (Fig. 4).

All together, the above findings, indicated that EC caused muscle damage, consisting in a prevalence of necrotic over apoptotic cell death, and suggested that S1P exerted beneficial effects, protecting both the myofibres and adjacent satellite cells in these conditions.

EC induces endogenous SphK1 activation and S1P formation

We next searched whether EC caused the activation of the endogenous S1P synthesis in the injured muscle fibres, by evaluating the activity of two known SphK isoforms (SphK1 and SphK2), responsible for the production of the bioactive lipid in skeletal muscle [43]. As shown in Fig. 5A, the activity of SphK1, but not SphK2 isoform, measured by specific in vitro assay, was significantly higher in EC-injured muscle lysates as compared to control; however, enhanced SphK1 activity was not paralleled by the up-regulation of SphK1 protein expression (Fig. 5B), as judged by Western

Table 2 Specific plasma membrane resistance (R_mC_m), RMP, Boltzmann parameters of activation and inactivation of I_{Na} and I_{Ca} in control, S1P treated, EC, EC + S1P and EC + iSphK fibres

Parameters	Control	1S1P	EC	EC + S1P	EC + iSphK
RmCm ($\Omega.\mu F$)	8900 ± 700	9200 ± 650	6240 ± 500***	7750 ± 500**	5700 ± 480§
RMP (mV)	−75 ± 7	−75 ± 6	−55 ± 6**	−73 ± 7	−50 ± 6
I_{Na}					
I_{Na} (nA/pF)	3.4 ± 0.2	3.5 ± 0.2	2.0 ± 0.2**	2.7 ± 0.3*	1.7 ± 0.2§
G_m/C_m (mS/μF)	157 ± 18	162 ± 17	77 ± 9 ***	114 ± 13**	68 ± 8§
Va (mV)	−22±0.6	−21 ± 0.7	−17±1.1**	−20±0.8	−16±1.2
ka (mV)	6.0 ± 0.5	6.0 ± 0.5	7.8 ± 0.7**	7.0 ± 0.6*	7.6 ± 0.6
Vr (mV)	53 ± 4	54 ± 5	43 ± 4**	49 ± 5	38 ± 4§
Vh (mV)	−60 ± 5	−63 ± 5	−72 ± 5**	−64 ± 6	−76 ± 6
kh (mV)	6.6 ± 0.6	6.6 ± 0.5	8.5 ± 0.7**	7.8 ± 0.6*	8.6 ± 0.6
Tp (msec.)	0.5 ± 0.1	0.5 ± 0.2	0.9 ± 0.1**	0.6 ± 0.1	1.3 ± 0.2§
I_{Ca}					
I_{Ca} (pA/pF)	4.1 ± 2	4.4 ± 2	0.8 ± 0.07***	3.1 ± 1**	0.4 ± 0.08§
Gm/Cm (μS/μF)	150 ± 16	155 ± 17	33 ± 7***	100 ± 18**	26 ± 6§
Va (mV)	−2.1 ± 1	−11.2 ± 3***	12.2 ± 3***	3.0 ± 1*	13 ± 2
ka (mV)	7.2 ± 0.4	7.1 ± 0.5	8.0 ± 0.4**	7.4 ± 0.4	7.9 ± 0.4
Vr (mV)	76 ± 5	73 ± 6	67 ± 5**	74 ± 6	60 ± 5§
Vh (mV)	−53 ± 4	−48 ± 3**	−63 ± 5**	−56 ± 5	−65 ± 5
Kh (mV)	6.5 ± 0.5	6.4	8.3 ± 0.5**	7.3 ± 0.6*	8.4 ± 0.6
Tp (msec.)	150 ± 32	140 ± 35	250 ± 45**	160 ± 34	299 ± 46§

In control fibres the presence of S1P shifts the voltage dependence values of Va and Vh parameters of I_{Ca}. EC injured fibres show significant changes of R_mC_m and RMP indexes of a leaky plasma-membrane as compared to controls. The increased intracellular $[Na^+]$ and $[Ca^{2+}]$ is confirmed by the negative voltage shift of Vr of both I_{Na} and I_{Ca}. Moreover, EC injured fibres show a reduction of the maximal I_{Na} and I_{Ca} peaks and related Gm; both are index of reduced channel functionality. The voltage dependence of I_{Na} and I_{Ca} activation and inactivation (Boltzmann functions) indicate significant changes of Va, ka, Vh and kh. Finally, the current kinetics appear altered: the maximal I_{Na} and I_{Ca} show an increased time to peak (Tp). All these alterations are strongly reduced in EC + S1P fibres. By contrast, in the presence of iSphK (EC + iSphK fibres), some electrophysiological parameters worsen compared with EC. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ *versus* control. $^{\S}P < 0.05$ *versus* EC. Data in each experimental condition were from 24 to 28 fibres (16 EDL; 14 mice).

analysis. The levels of $[^3H]$S1P formed in the $[^3H]$sphingosine-labelled satellite cell-enriched fractions from EC-injured muscle were also increased (Fig. 5C), confirming the ability of EC to activate intracellular SphK1/S1P axis. Interestingly, the addition of exogenous S1P to damaged fibres further increased SphK1 activity (Fig. 5A), providing the first evidence that the activation of S1P receptor-mediated signalling could augment the endogenous S1P synthesis during EC and suggesting the intriguing hypothesis that the treatment with the sphingolipid could amplify the physiological role played by endogenous S1P in muscle protection. Consisting with these findings, we observed an exacerbation of some electrophysiological parameters (R_mC_m, V_r and G_m/C_m for both I_{Na} and I_{Ca}, Table 2) when EC was performed in the presence of compound II, a specific SphK1 inhibitor (iSphK). However, no detectable morphological differences could be observed between EC and EC + iSphK (Fig. 1A and B; histological score 2.5 ± 0.2, Table 1).

Exogenous S1P promotes satellite cell renewal and differentiation during EC muscle damage

Next, we analysed the effects of S1P on the resident satellite cells, in an attempt to evaluate the contribution of the bioactive lipid to EDL muscle repair/regeneration after damage. It has been previously reported that upon activation, quiescent Pax7^{+} satellite cells rapidly express MyoD and proliferate as Pax7^{+}/MyoD[+] co-expressing cells, before adopting divergent fates and giving rise to two cell populations: one which down-regulates Pax7, maintains MyoD expression (Pax7^{−}/MyoD[+]) and starts the myogenic program, and the other which down-regulates MyoD and maintains Pax7 (Pax7^{+}/MyoD[−]) contributing to satellite cell reservoir [4, 9]. On this basis, confocal immunofluorescence analysis revealed that EC *per se* caused a significant increase of Pax7^{+}/MyoD[+] activated satellite cells (Fig. 6A). Notably, in the presence of S1P, the pool of
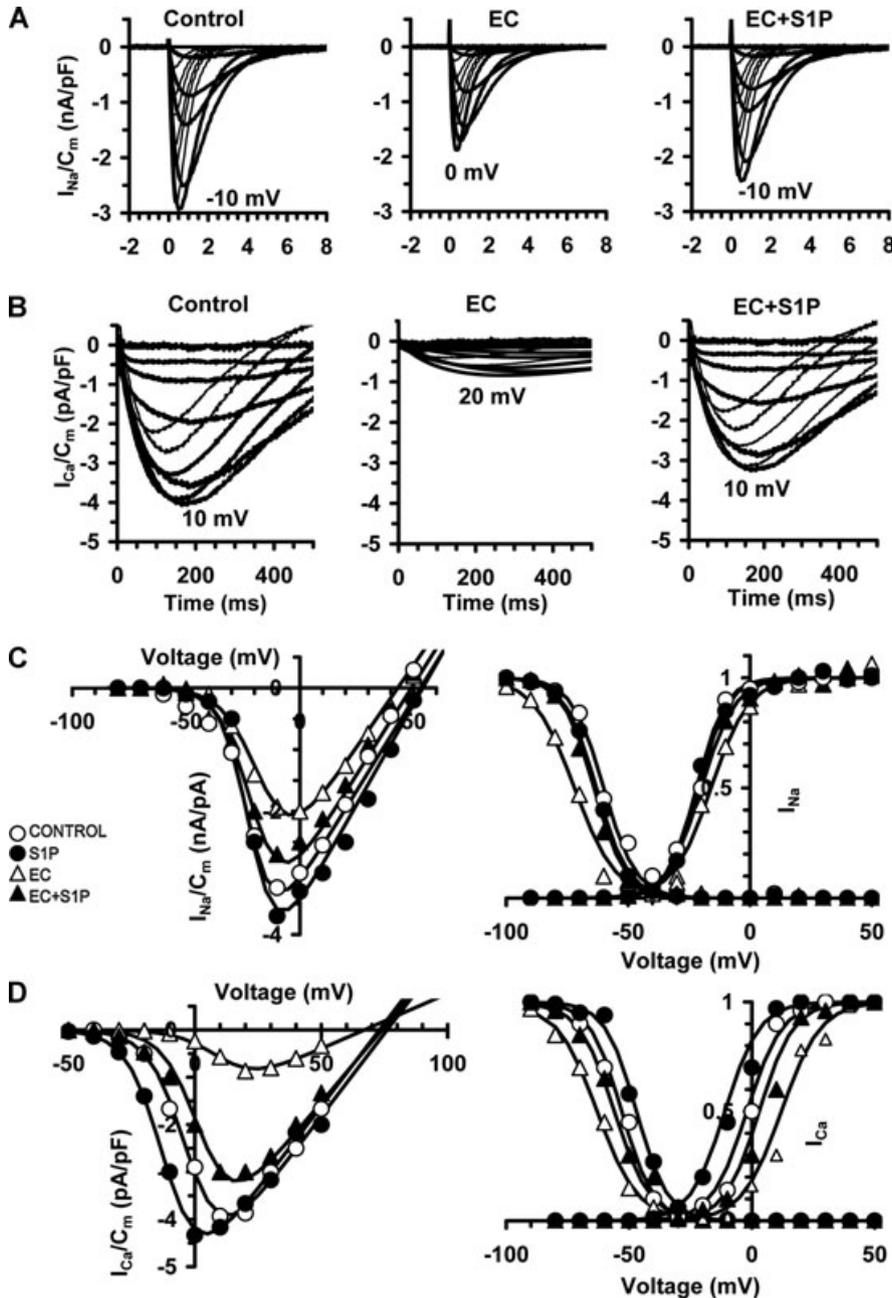
Fig. 3 Protective effect of S1P against EC-induced electrophysiological changes in EDL muscle. Electrophysiological analyses of the functional properties of sarcolemma of control, S1P-treated, EC- and EC + S1P-injured muscle fibres evaluated by the double-Vaseline gap method in voltage-clamp. (**A–B**) Representative normalized Na^+ current (I_{Na}) and L-type Ca^{2+} current (I_{Ca}) traces. (**C–D**) Activation and inactivation Boltzmann curves for I_{Na} and I_{Ca} (Current to peak-V plots). RMP data and Boltzmann parameters are in Table 2.

activated $Pax7^+/MyoD^+$ cells appeared significantly reduced, while the number of quiescent $Pax7^+/MyoD^-$, committed progenitor $Pax7^-/MyoD^+$ and terminally differentiated myogenin$^+$ cells were markedly increased (Fig. 6A), suggesting the ability of S1P to positively influence the self-renewal and differentiation potential of satellite cells after EC. As judged by the biochemical analysis, the expression of cyclin D1 and p-ERK1/2 were significantly increased in satellite cell-enriched fractions obtained from EC + S1P muscle compared to EC, underscoring the ability of the bioac-

tive lipid to activate signalling pathways leading to satellite cell proliferation during EC (Fig. 6B).

Satellite cell activation and viability was also investigated in the cells sprouting from cultured isolated myofibres (Fig. 7). It was found that the number of viable muscle satellite cells derived from EC and EC + S1P muscle fibres was significantly higher (approximately 1.5- and 2-fold, respectively) compared to control (Fig. 7A and B). In agreement, the sprouting myoblasts from EC and EC + S1P-damaged fibres exhibited a higher tendency (approximately
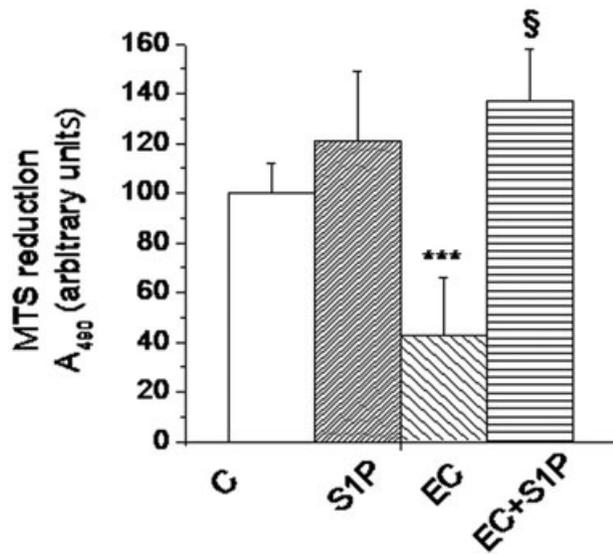
Fig. 4 Effects of S1P on viability of satellite cell-enriched fractions from EC-injured EDL muscle. Cell viability was evaluated by MTS assay in satellite cell-enriched fractions 24 hrs after isolation from muscle in the indicated experimental conditions. Relative percentage to control set as 100 (mean ± S.E.M.) is shown. Student's t-test, ***P < 0.01 *versus* control, *n* = 4; §P < 0.01 *versus* EC, *n* = 4.

2- and 3-fold, respectively) to fuse into multinucleated myotubes than those from control (Fig. 7C–F).

In the absence of the muscle damage, the addition of S1P to the isolated EDL muscle did not affect the proliferating progeny nor modify the number of committed satellite cells, indicating that the exogenous S1P *per se,* without any stress-mediated signals, was unable to affect satellite cell functions (Figs 6 and 7).

S1P affects extracellular matrix remodelling in EC-damaged muscle fibres

We finally evaluated the effects of S1P treatment on the remodelling of the extracellular matrix proteins. Staining of EDL muscle sections with Van Gieson, which stains collagen, revealed considerable reduced fibrosis in EC + S1P compared with EC (Fig. 8A). We also showed that the treatment with S1P up-regulated (approximately 2-fold) MMP-9 expression in EC-injured muscle (Fig. 8B). Interestingly, EC treatment by itself increased MMP-9
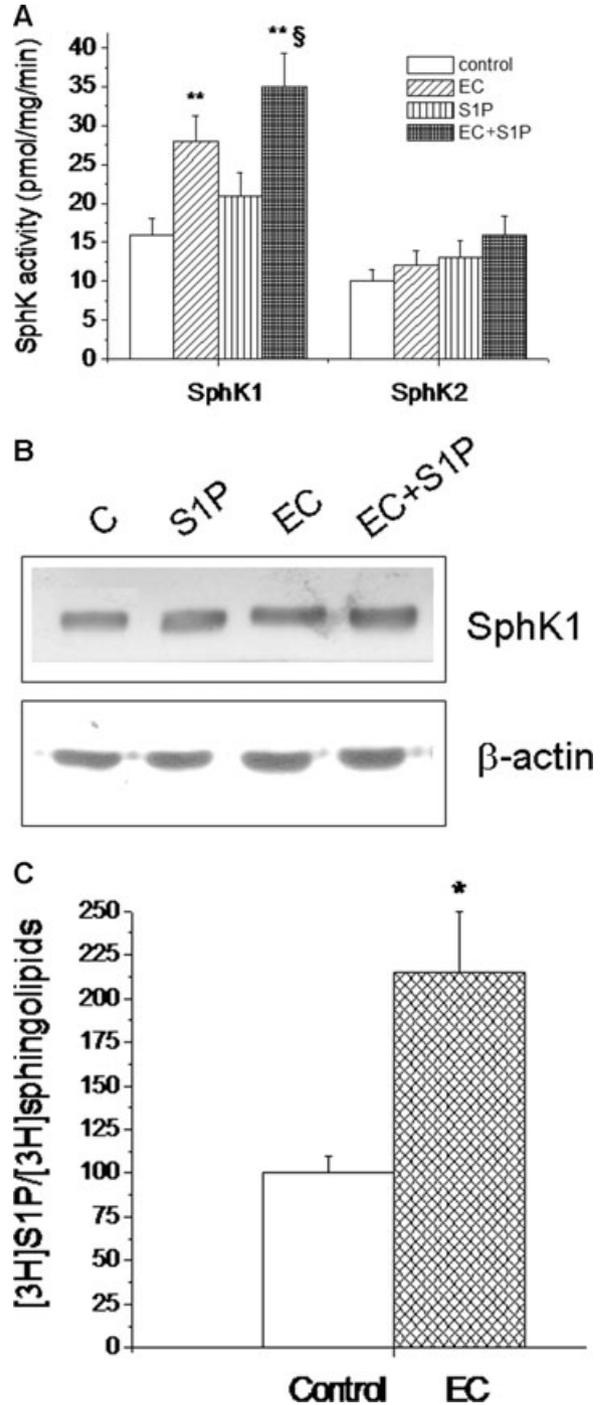

Fig. 5 Effect of EC on SphK activity, enzyme expression and S1P formation in EDL muscle. (**A**) Tissue lysates (40–70 μg) from control, S1P-treated, EC- and EC + S1P-injured muscles were analysed for SphK1 and SphK2 activity. Data are mean ± S.E.M. (Student's t-test, **P < 0.05 *versus* control, *n* = 3; §P < 0.05 *versus* EC, *n* = 3). (**B**) SphK1 expression. Protein aliquots (20 μg) of lysates from muscle samples as indicated in (**A**) were immunoblotted and detected by ECL. The relative percentage of band intensity to control arbitrarily normalized to 100 set (mean) is shown. S.E.M. (not shown) is less than 15%. C: control. (**C**) S1P formation in satellite cell-enriched fractions from Percoll gradient obtained from control and EC-injured fibres. [³H]S1P formation was determined in 2 μCi [³H]sphingosine-labelled cells after 24 hrs incubation. Lipids were extracted by adding chloroform-methanol, and samples from TLC separation evaluated for the radioactivity corresponding to [³H]S1P. Data are means ± S.E.M. (Student's t-test; *P < 0.05; *n* = 3).
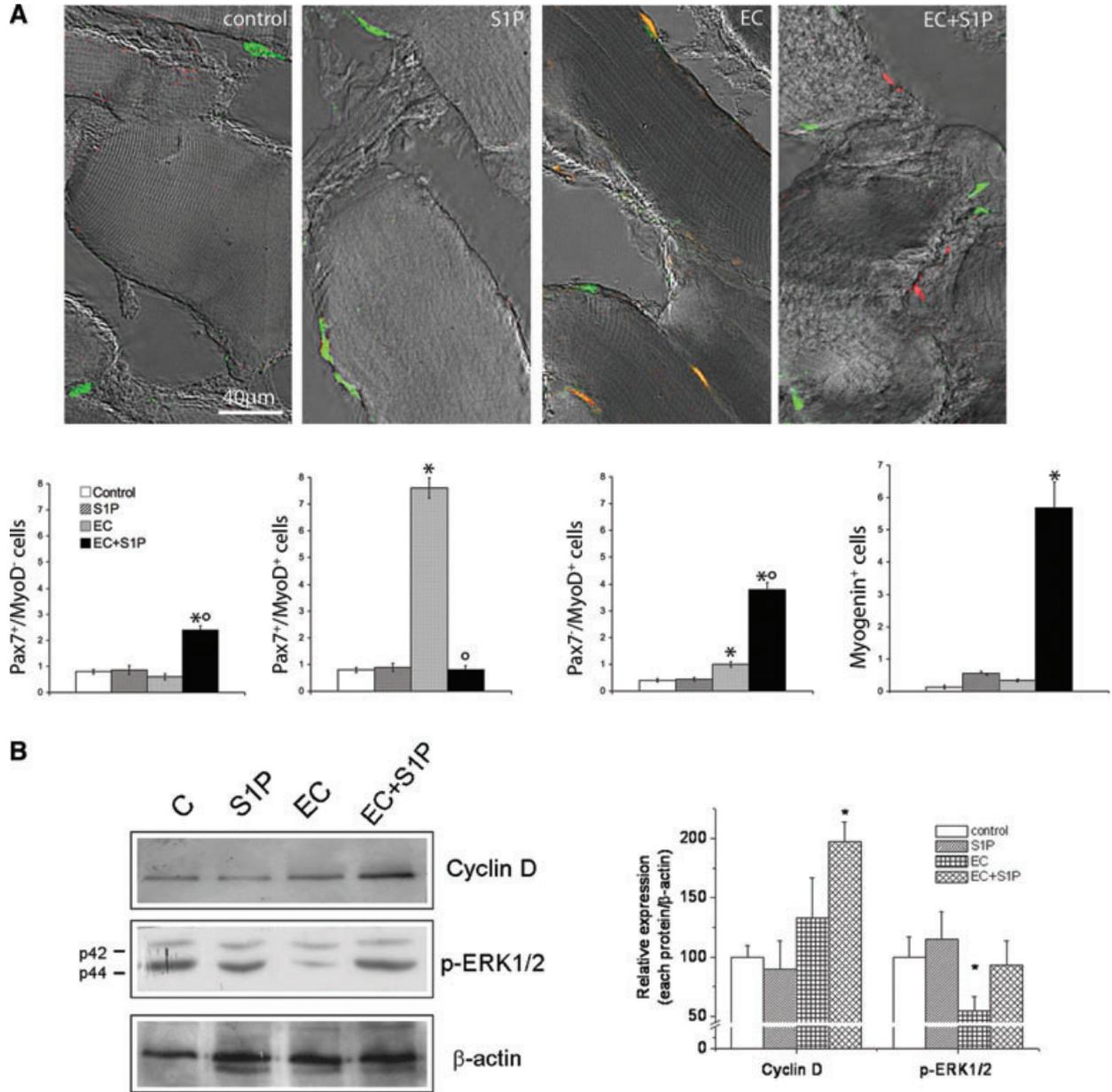
Fig. 6 Effect of S1P on satellite cell renewal and differentiation. (**A**) Superimposed DIC and confocal immunofluorescence images of myofibres from control, S1P-treated, EC- and EC + S1P-injured EDL muscle immunostained for Pax7 (green) and MyoD (red). Cells co-expressing both the markers are stained in orange. In the histogram quantitative analysis of the number of Pax7$^+$/MyoD$^-$, Pax7$^+$/MyoD$^+$ and Pax7$^-$/MyoD$^+$ and myogenin$^+$ satellite cells. Data are mean ± S.E.M. (One-way ANOVA. *$P < 0.05$ *versus* control; °$P < 0.05$ *versus* EC; $n = 3$). (**B**) Western Blotting analysis of cyclin D1 and phospho p44/42 MAPK (p-ERK1/2) expression in satellite cells-enriched fractions. Relative percentage of band intensity to control set as 100 (mean ± S.E.M.) is shown in the graph. (Student's t-test, *$P < 0.05$, $n = 3$).

expression of approximately 2-fold above the control and this increase appeared significantly reduced when muscle fibres were incubated in the presence of SphK1 inhibitor (Fig. 8C), supporting the role of endogenous S1P and the activation of SphK1/S1P axis by EC in counteracting muscle tissue fibrosis.

Discussion

S1P is a well-described bioactive lipid mediator which has been shown to mediate a wide variety of fundamental biological processes, including cell growth, survival, migration and differentiation.
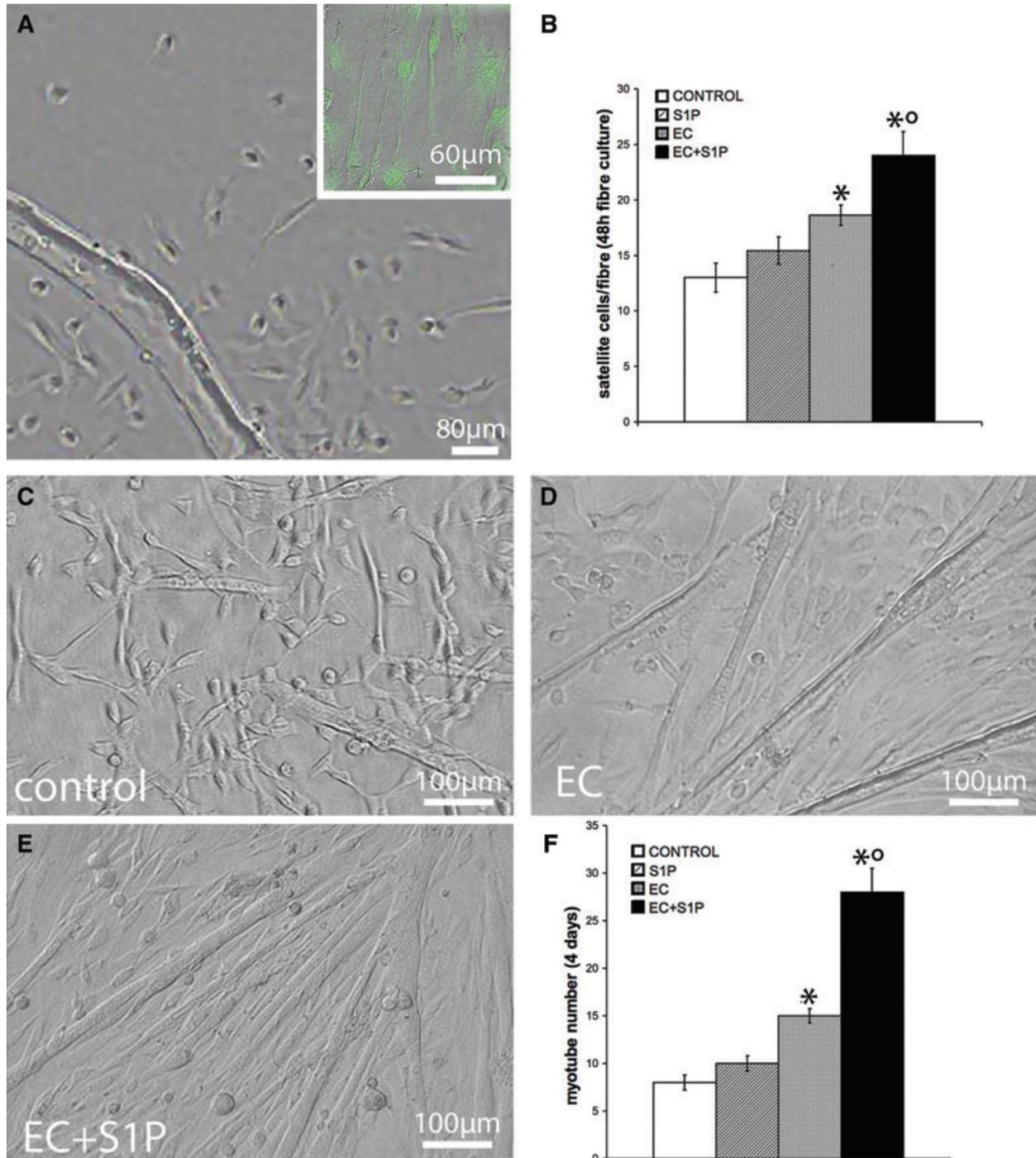
Fig. 7 Proliferation and differentiation of satellite cell derived from single muscle fibres. Satellite cells were isolated from single fibres of control, S1P-treated, EC- and EC + S1P-injured EDL muscle and analysed for cell proliferation and differentiation. (**A**) Representative reverse-phase contrast microscopic image of a single fibre cultured for 48 hrs and sprouting satellite cells positive for Pax7 (inset). (**B**) Quantitative analyses of the number of sprouting cells evaluated 24 hrs after the fibre removal (One-way ANOVA, *$P < 0.05$ *versus* control; °$P < 0.05$ *versus* EC; $n = 3$, 24 fibres in each experiment). (**C–E**) Representative reverse-phase contrast images of myotube formation. (**F**) Quantitative analyses of myotubes counted in the same samples of (**B**). *$P < 0.05$ *versus* control; °$P < 0.05$ *versus* EC; (One-way ANOVA; $n = 3$).
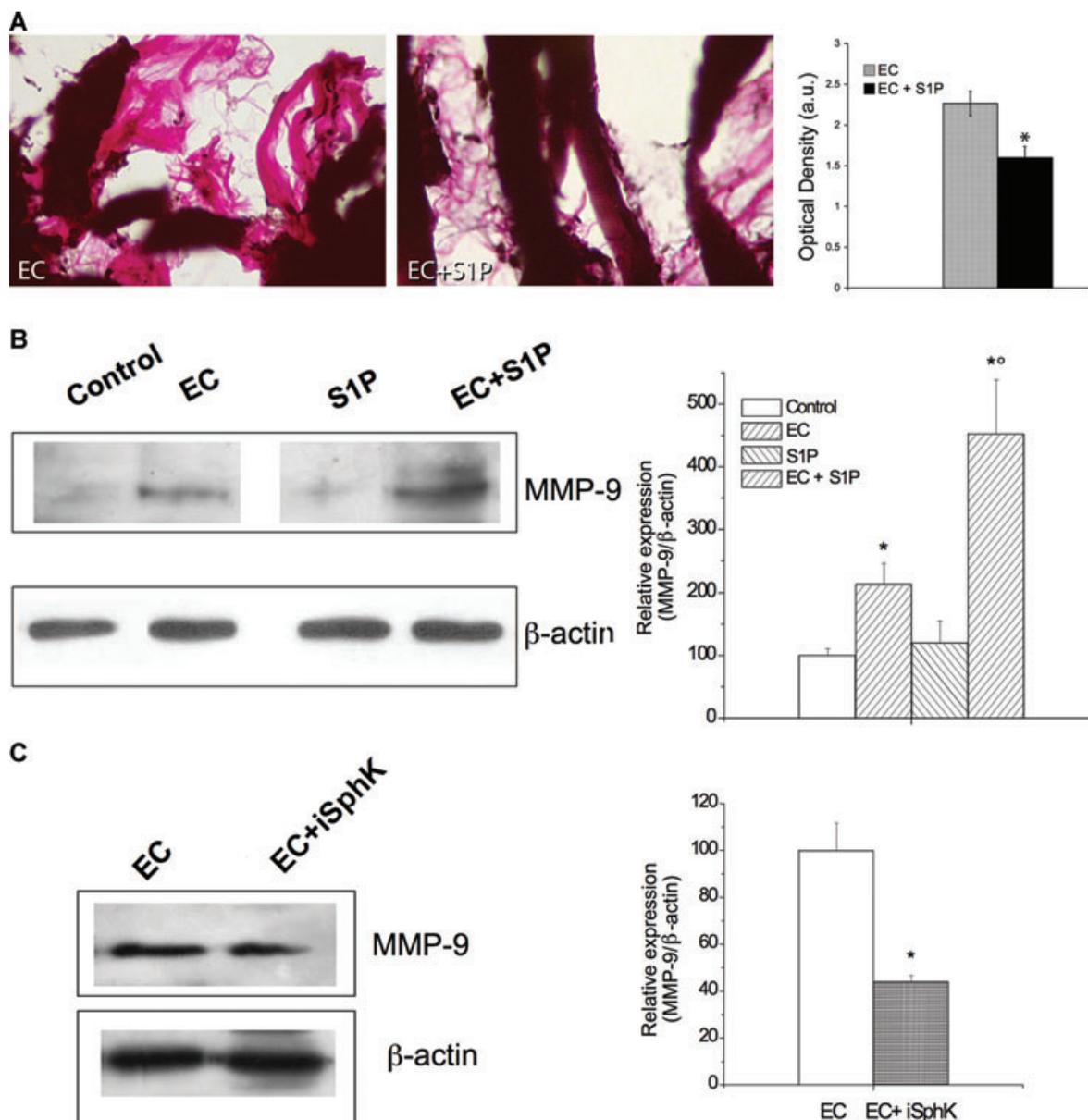
Fig. 8 Effect of S1P on extracellular matrix remodeling and MMP-9 expression. (**A**) Light microscopy. Van Gieson stained muscle sections revealing collagen fibres (pink). In the histogram densitometric analysis of collagen staining intensity (Student's t-test; *$P < 0.05$, $n = 3$). (**B**) Western blot analysis of MMP-9 protein expression in control, S1P-treated, EC- and EC + S1P-damaged muscle fibres. (**C**) MMP-9 protein expression in EC-treated cells in the absence and presence of SphK1 inhibitor (iSphK). Protein aliquots (30 μg) of tissue lysates were immunoblotted and detected by ECL. Band intensity was determined by densitometry and relative percentage to control arbitrarily normalized to 100 is shown in the graphs. (Student's t-test; *$P < 0.05$ *versus* specific control, $n = 3$; °$P < 0.05$ *versus* EC).

It is considered a physiological factor which is released in the extracellular milieu by activated platelets and by many cell types through a member of the ATP-binding cassette (ABC) transporter family [44, 45]. Membrane-associated SphK1, the enzyme responsible for S1P production, can act as an exoenzyme, as shown in myogenic C2C12 cells [43], and contribute to the autocrine/ paracrine action of the sphingoid molecule *via* the activation of specific S1P receptors [13, 14, 28].

Recent studies are beginning to highlight the importance of S1P in skeletal muscle homeostasis and disease, exerting a protective role against muscle atrophy and promoting myoblast differentiation and satellite cell activation [15, 16, 19–23, 46]. In

this contest, the data reported here contribute to expand the list of the beneficial effects of S1P on muscle tissue, unrevealing a major role for this factor in the prevention and attenuation of EC-induced morphological, biochemical and electrophysiological skeletal muscle damage. We also found a strong up-regulation of intracellular S1P synthesis after EC, as demonstrated by the rise in SphK1 activity and S1P formation in skeletal muscle fibres and satellite cells, respectively, further supporting the protective role of S1P against skeletal muscle injury. Interestingly, S1P was able to augment SphK1 activity in response to damage, suggesting that the activation of endogenous SphK1/S1P axis could represent potential mechanisms of S1P muscle protection. Consistent with this assumption, some electrical parameters appeared exacerbated when endogenous S1P formation was prevented during EC. In view of our previous reports, it is also likely that S1P may afford cytoprotection of muscle cells through its ability to promote cortical F-actin organization and plasma membrane stiffening [47, 48] which could enhance the resistance in these cells to the stress caused by EC. This assumption is consistent with the data of other groups showing that eccentric exercise elicits a cytoskeletal remodelling response in human skeletal muscle in the attempt to limit the extent of the mechanical stress-induced damage [49, 50]. Moreover, as predicted by the tensegrity cell model [51], it also possible that actin cytoskeleton may transmit signals to the nucleus of the muscle cells through the activation of peculiar mechanical Ca^{2+} channels and biochemical processes leading to cell survival and protection [20, 22, 23, 52].

We also found that EC caused a significant reduction in satellite cell viability, in line with the growing evidence in the literature showing a loss of satellite cell population following extensive muscle damage. Interestingly, S1P preserved satellite cell viability. Although, we did not investigate the molecular aspects of satellite cell protection by S1P, it may be suggested that increased satellite cell viability is related to the ability of the sphingolipid to limit the damaged tissue, thus contributing to the recreation of a less prohibitive environment for satellite cell functions.

Satellite cell renewal and differentiation was also potentiated by the bioactive lipid.

Indeed, we reported here that the number of activated $Pax7^+/MyoD^+$ co-expressing cells significantly enhanced after damaged and that S1P forced this pool to enter cell cycle and subsequently adopt divergent fates; in fact, most of them differentiated, down-regulating Pax7 and maintaining MyoD expression ($Pax7^-/MyoD^+$) and expressing myogenin, while others regained a phenotype characteristic of quiescent satellite cells ($Pax7^+/MyoD^-$). Consisting with these data, we found that number of viable satellite cells sprouting from EC + S1P-injured muscles together with their tendency to form myotubes was increased as compared with those from EC muscles, in perfect agreement with the known myogenic action of S1P [15]. The observed inability of S1P *per se* to affect satellite cells of uninjured tissue is in line with the recent findings showing that the specific S1P receptor isoform, $S1P_2$, is rapidly up-regulated in skeletal muscle undergoing regeneration, but it is absent in quiescent cells [46].

It is generally accepted that dismantling the extracellular matrix is essential for myoblast migration, differentiation and fusion *in vivo* [53]. Unfortunately, these events may be hindered in case of severe muscle wasting by the excessive deposition of collagen fibres in the form of a fibrotic scar which limits vascular perfusion of the injury site [54]. In such a view, it was particularly interesting to find out that S1P decreased fibrosis and up-regulated MMP-9 expression in the EC-injured muscle, inducing an additive effect in promoting collagen remodelling beyond the EC effect. This event was strongly reduced by incubation with SphK1 inhibitor, suggesting a critical contribution of SphK1/S1P axis in counteracting tissue fibrosis.

In conclusion, the results of the present study provide evidence that S1P is capable of protecting skeletal myofibres against muscle damage and influencing muscle repair/regeneration. The findings concerning the activation of SphK1/S1P axis in response to muscle damage further reinforce the importance of the endogenous bioactive sphingolipid in the promotion of skeletal muscle repair/regeneration and provide clues for the identification of novel targets for improving muscle healing after injury.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting Information

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