Sphingosine 1-phosphate signaling is involved in skeletal muscle regeneration

Daniela Danieli-Betto,1,2 Samantha Peron,1 Elena Germinario,1,2 Marika Zanin,1 Guglielmo Sorci,2,3 Susanna Franzoso,4 Dorianna Sandonà,4 and Romeo Betto2,5

1Department of Human Anatomy and Physiology, University of Padova, Padua; 2Interuniversity Institute of Myology; 3Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Perugia; and 4Department of Biomedical Sciences, University of Padova, Padua; and 5Institute of Neurosciences, Consiglio Nazionale delle Ricerche, Padua, Italy

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Sphingosine 1-phosphate (SIP) signaling is involved in skeletal muscle regeneration. Am J Physiol Cell Physiol 298: C550–C558, 2010. First published December 30, 2009; doi:10.1152/ajpcell.00072.2009.—Sphingosine 1-phosphate (SIP) is a bioactive lysolipid known to control cell growth that was recently shown to act as a trophic factor for skeletal muscle, reducing the progress of denervation atrophy. The aim of this work was to investigate whether SIP is involved in skeletal muscle fiber recovery (regeneration) after myotoxic injury induced by bupivacaine. The postnatal ability of skeletal muscle to grow and regenerate is dependent on resident stem cells called satellite cells. Immunofluorescence analysis demonstrated that SIP-specific receptors SIP1 and SIP3 are expressed by quiescent satellite cells. Soleus muscles undergoing regeneration following injury induced by intramuscular injection of bupivacaine exhibited enhanced expression of SIP1 receptor, while SIP3 expression progressively decreased to adult levels. SIP2 receptor was absent in quiescent cells but was transiently expressed in the early regenerating phases only. Administration of SIP (50 μM) at the moment of myotoxic injury caused a significant increase of the mean cross-sectional area of regenerating fibers in both rat and mouse. In separate experiments designed to test the trophic effects of SIP, neutralization of endogenous circulating SIP by intraperitoneal administration of anti-SIP antibody attenuated fiber growth. Use of selective modulators of SIP receptors indicated that SIP1 receptor negatively and SIP3 receptor positively modulate the early phases of regeneration, whereas SIP2 receptor appears to be less important. The present results show that SIP signaling participates in the regenerative processes of skeletal muscle.

SIP receptor-dependent signaling has been demonstrated in skeletal muscle cells. The mRNAs of SIP1–SIP3 receptors are detectable in the myogenic C2C12 cell line derived from mouse satellite cells, with SIP1 expression being the highest (25, 36). The relative expression of SIP receptors changes during myogenic differentiation of C2C12 cells, particularly that of SIP2, which progressively diminishes during differentiation and becomes almost absent by the time myotubes are formed (22). Consistently in rat adult skeletal muscle RT-PCR and Western blot data demonstrated the expression of SIP1 and SIP3 receptors localized at the cell surface and at the nuclear membrane, while SIP2 receptor was almost undetectable (47). Recently, we demonstrated (47) that extracellular SIP counteracts atrophy development of denervated rat soleus muscle, suggesting a trophic function of SIP in skeletal muscle by the activation of SIP receptors and downstream signaling pathways.

A remarkable property of skeletal muscle is its capacity to regenerate, a process that repairs damaged muscle in response to injury or attempts to mitigate degenerative muscle disease processes. Muscle regeneration is dependent on satellite cells, a population of quiescent myogenic cells localized beneath the basal lamina that surrounds each muscle fiber. During muscle regeneration, satellite cells are activated, proliferate, migrate, differentiate, and fuse to form new myofibers (46). Hepatocyte growth factor (HGF) and its receptor, c-Met, likely are key players in satellite cell activation, although evidence shows that additional factors might activate satellite cells, possibly as redundant mitogens (3, 7).

On activation, satellite cell-derived myoblasts proliferate, migrate, and differentiate rapidly after injury to regenerate a large number of myotubes within a 3- to 4-day period (1, 7). A portion of proliferating myoblasts exits the cell cycle to reconstitute the quiescent satellite cell pool as part of a self-renewal process (7, 46). The myogenic regulatory factors MyoD, Myf5, myogenic regulatory factor 4 (MRF4), and myogenin are sequentially expressed and play distinct roles during the regenerative process (40). Mitogenic stimuli are thought to accompany the process of regeneration; however, the signaling pathways controlling satellite cell activation and proliferation during this process are not yet fully elucidated.

Growing evidence indicates that SIP signaling is involved in muscle regeneration and myogenic differentiation of muscle cells (2). It was initially demonstrated that SIP stimulates many signaling pathways in myogenic C2C12 cells, including activation of phospholipase D (21, 25) and RhoA (23). SIP also stimulates the expression of connexin 43 (41) and is responsible for Ca2+-dependent actin filament remodeling (11, 12).
SIP and muscle regeneration

SIP and muscle regeneration

12), events most relevant for the fusion process of myoblasts into myotubes. In addition, it was shown that SIP exerts an antiproliferative role in promoting differentiation of C2C12 myoblasts, through the activation of SIP2 receptor (8, 9). SIP was recently shown to act on SIP1 receptors in stimulating the proliferation of C2C12 reserve cells, a subpopulation of C2C12 cells comparable to quiescent satellite cells (36). These workers also showed that the mitogenic action of SIP in C2C12 reserve cells was also seen in primary satellite cells. The addition of SIP promoted the increased number of satellite cells entering the cell cycle, whereas inhibition of SIP production caused the reduction of satellite cell activation in culture and slowed the in vivo regeneration of injured muscle (32). Also consistent with a role of SIP in regeneration was the finding that the plasma membranes of quiescent satellite cells are particularly rich in sphingomyelin, which on activation is hydrolyzed to eventually result in the generation of SIP by sphingomyelinase and Sph kinase (32). Notably, blocking sphingomyelinase and Sph kinase reduced the number of dividing satellite cells in response to mitogenic stimuli (32). Thus it appears evident that SIP plays an important role as a regulator of satellite cells and muscle regeneration.

In the present work we investigated in vivo the involvement of SIP in muscle fiber regeneration. We localized SIP1 and SIP3, but not SIP2, receptors on the surfaces of quiescent satellite cells from adult rat soleus muscle. Moreover, the expression levels of SIP1 and SIP3 receptors underwent opposite directions during the first week of regeneration. The possible role of SIP in muscle regeneration was tested by a single injection of SIP given at the initiation of bupivacaine-induced tissue damage, a stimulus for the regeneration response. This protocol was utilized to ascertain the action of the bioactive lipid in the initial phases of regeneration, i.e., on activation and proliferation of satellite cells. The exogenous addition of SIP resulted in a significant trophic effect in regenerating rat and mouse soleus muscle fibers, whereas reduction of the circulating lipid produced the opposite effect. The use of selective pharmacological modulators of various SIP receptors demonstrated their variant roles during regeneration.

MATERIALS AND METHODS

Surgical procedures and treatments. All procedures were performed in accordance with Italian laws. The study and the experimental protocols were approved by the Ethics Committee of the Medical Faculty of the University of Padova and by the Italian Health Ministry.

All surgical procedures were performed in a single session in 2- to 3-mo-old male Wistar rats, anesthetized by an intraperitoneal injection of tiletamine and zolazepam (7 mg/kg each) and xylazine (14 mg/kg). The monoclonal murine antibody to SIP (44) was administered intraperitoneally in six animals (10 mg/kg body wt) 1 day before the same day, and 2 days after myotoxic drug injury. As controls, regeneration was induced in five mice to which a PBS solution (vehicle) was administered intraperitoneally. In a separate set of experiments, the bilateral regeneration of soleus was induced with notexin together with 50 μM SIP. Since regeneration in mice is slower than in rats, mice were euthanized 4 days after injury by neck dislocation, and soleus muscle of both legs was removed and frozen in liquid nitrogen-cooled isopentane.

Preparation of rat satellite cells and C2C12 myoblasts. Leg muscles from 3-day-old C57BL/6J mice were enzymatically dissociated to isolate a pure population of myoblasts, as previously described (34). Primary myoblasts were characterized by immunofluorescence with a polyclonal anti-c-Met antibody (1:50, Santa Cruz Biotechnology) after fixation with cold methanol for 7 min at −20°C; >97% cells were c-Met positive.

Mouse C2C12 skeletal muscle cells were cultivated in DMEM (Sigma) growth medium supplemented with 10% fetal calf serum (Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded at a density of ~10,000 cells/ml in an humidified atmosphere of 5% CO2 at 37°C and harvested 3 days later.

Antibodies. The rabbit polyclonal antibody specific for SIP1 receptor, previously characterized (33), was utilized for Western blot at a dilution of 1:2,000 in 2% BSA in Tris-buffered saline (TBS) and for immunofluorescence after 1:200 dilution in PBS, incubated in both overnight at 4°C. The monoclonal antibody specific for Pax7 (Devel-опmental Studies Hybridoma Bank) was utilized for immunofluorescence and diluted 1:10 in 5% fetal bovine serum and 0.2% Bovinas serum. The monoclonal antibody specific for myogenin (F5D, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) was diluted 1:50 in PBS and incubated for 1 h at room temperature. The monoclonal anti-SIP2 antibody (AP01198PU-N) was obtained from Acris (Herford, Germany) and was utilized for Western blots at a 1:500 dilution in 2% BSA in TBS and incubated overnight at 4°C.

The monoclonal antibody specific for α-actinin (EA-53, Sigma, St. Louis, MO) was used in Western blots at a dilution of 1:1,000 in 5% low-fat milk, 0.2% Tween 20 in TBS and incubated for 2 h at room temperature. The monoclonal antibody specific for MyoD (58A, Dako, Glostrup, Denmark) was diluted 1:400 in 5% low-fat milk, 0.05% Tween-20 in PBS and incubated overnight at 4°C. The monoclonal antibody specific for myogenin (F5D, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) was diluted 1:100 in 10% low-fat milk, 0.1% Tween 20 in TBS and incubated overnight at 4°C. The monoclonal antibody specific for Pax7 (Developmental Studies Hybridoma Bank) was utilized for immunofluorescence analysis and diluted 1:10 in 5% fetal bovine serum and 0.2% Triton X-100 in PBS and incubated overnight at 4°C. The polyclonal antibody specific for laminin (L9393, Sigma) was utilized for immunofluorescence and diluted 1:250 in 5% fetal bovine serum and incubated for 1 h at 37°C. The monoclonal antibody specific for the
αS-subunit of dihydropyridine (DHP) receptor (MAB 427, Chemicon, Temecula, CA) was diluted 1:250 in PBS and incubated overnight at 4°C.

Western blot analysis. Western immunoblotting analysis was performed on muscle fragments, satellite cells, C2C12 myoblasts, and rat soleus muscle membranes dissolved in SDS-PAGE buffer supplemented with Complete Protease Inhibitor Cocktail (Roche). Proteins were separated by SDS-PAGE on 10% gels and blotted onto nitrocellulose filters as previously described (47). Nitrocellulose filters were blocked 2 h in 1% BSA and 10% low-fat milk, in TBS, before being probed with the antibodies specific for S1P receptors; blocking solution for the other primary antibodies were the same used to dilute each antibody. The secondary antibody utilized for Western blot of S1P receptors was an anti-rabbit peroxidase-conjugated antibody (Chemicon) diluted 1:5,000 in 2% low-fat milk, 0.1% Tween 20 in TBS and incubated for 1 h. The secondary antibody utilized for MyoD, myogenin, and α-actinin was an anti-mouse peroxidase-conjugated antibody (Dako) diluted 1:10,000 in the same buffer as the cognate primary antibody and incubated for 1 h. Blots were developed for visualization with the ECL Plus detection system (Amersham).

Morphological analysis and immunolocalization of S1P receptors. Muscles were frozen in liquid nitrogen in a slightly stretched position. Serial cross sections (8 μm thick) were cut in a cryostat microtome set at −24 ± 2°C (Slee Pearson). Hematoxylin and eosin staining was performed on transverse muscle sections to examine the general morphology, and anti-laminin immunofluorescence (see above) was carried out to determine the cross-sectional area (CSA) of individual fibers. Morphometric analysis was performed on digital photographs of muscle fibers analyzed by ImageJ software (National Institutes of Health). More than 300 fibers per muscle were evaluated. Immunofluorescence staining was performed as previously described (13, 47). Briefly, muscle sections were incubated with selected antibodies under the conditions described above. Immunofluorescence double staining was performed by incubating the muscle cryostat sections first with the antibody specific for the S1P1 and S1P3 receptors, followed by the anti-Pax7 antibody (Developmental Studies Hybridoma Bank) to identify satellite cells. To mark the T-tubule membranes, each section was incubated with the antibody specific for the αS-subunit of DHP receptor. The secondary antibodies utilized were an anti-mouse TRITC-conjugated (Dako; 1:100 in 5% fetal bovine serum and 0.2% Triton X-100) and an anti-rabbit TRITC-conjugated (1:100, diluted in 2% rat serum in PBS; Dako) incubated for 1 h at room temperature. Nuclei were stained with 5% Hoechst.

To localize S1P2 receptor protein expression, muscle cryostat sections were incubated with the specific antibody as described above; the secondary antibody used was an anti-rabbit horseradish peroxidase (HRP)-conjugated (1:50 in 1% rat serum in PBS; Chemicon) incubated for 1 h at room temperature. The reaction

Fig. 1. Expression of sphingosine 1-phosphate (S1P) S1P1, S1P2, and S1P3 receptors in satellite cells of adult soleus muscle. A: immunofluorescence staining of soleus muscle sections with antibodies specific for S1P1 and S1P3 receptors confirms that both receptors are expressed in the sarcolemma of muscle fibers (47). In addition, an intense staining was produced by both antibodies in some nuclei (identified by Hoechst staining). Staining with Pax7 identified these nuclei as satellite cells. Merging of receptor and Pax7 staining confirms the colocalization of the 2 proteins in satellite cells. B: peroxidase staining of adult soleus muscle sections with antibodies specific for S1P1 and S1P2 confirms that S1P1 receptor is localized in the sarcolemma and in the wall of muscle vessels (47). In contrast, S1P2 receptor was not evident in the sarcolemma, whereas it is present in muscle vessels. Bar, 25 μm.
was visualized by diaminobenzidine (DAB). To confirm the localization of the satellite cells, double staining was performed in serial cryostat sections by incubating first with the antibody specific for Pax7 and then with the second antibody specific for laminin. The secondary antibody utilized was an anti-rabbit FITC (Sigma) diluted 1:80 (in 5% fetal bovine serum and 1:50 rat serum in PBS) and incubated for 1 h at 37°C. Muscle sections were examined in either Leica RD100 or Nikon Eclipse 80i fluorescence microscopes equipped with a digital camera.

**Statistical analysis.** All values are means ± SE. All data originate from experimental values of one muscle and its contralateral muscle from the same animal. Comparisons of mean values were performed after analysis of variance (ANOVA) and the Tukey post hoc test. For the comparisons of muscle fiber CSA, >300 fibers from each muscle were evaluated. The mean CSA values from individual muscles were then pooled, and the resulting means were compared with those of the contralateral muscle. After ANOVA, the significance of the results was determined by Student’s t-test. Differences were considered significant at the P < 0.05 level.

**RESULTS**

Expression and localization of S1P receptors in satellite cells. As we recently demonstrated (47), the immunofluorescence staining of soleus muscle sections with antibodies specific for S1P1 and S1P3 revealed that both receptors are expressed at muscle fiber sarcolemma (Fig. 1A). Here we show that both antibodies intensely decorated cells that also expressed Pax7, a specific marker of satellite cells. This evidence indicates that both receptors are expressed in quiescent satellite cells of adult soleus muscle. Figure 1A also shows that the

![Fig. 2. Localization of S1P receptors in regenerating soleus muscle. A: transverse cryostat sections from 3-, 4-, and 7-day regenerating (Reg) and adult soleus muscle stained with antibody specific for S1P1 and S1P3 receptors. S1P receptors have a diffuse intracellular localization in the 3- and 4-day regenerating muscle. However, sarcolemma and nuclei membranes also appear to be decorated by the antibodies. In 7-day regenerant muscle, sarcolemma staining become more definite, whereas the nuclear staining was still evident. Bar, 50 μm. B: longitudinal cryostat sections from 3-day regenerating soleus muscle showing the colocalization of receptors with the dihydropyridine receptor (DHPR), at the T-tubule membrane level. C: peroxidase staining of regenerating soleus stained with antibodies specific for S1P1 and S1P2 receptors. S1P2 receptor was evident in the wall of muscle vessels only, whereas S1P1 receptor confirms its localization in regenerating fibers. Inset, staining due to the secondary antibody alone. Bar, 20 μm.](http://ajpcell.physiology.org/)

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S1P1 receptor is localized in satellite cells both at the cell surface and intracellularly, where it colocalizes with the transcription factor Pax7. In contrast to S1P1, S1P3 seems to be located in the sarcolemma only. S1P2 receptor was localized neither at the sarcolemma of muscle fibers nor in quiescent satellite cells. S1P2 receptor was expressed largely in smooth muscle cells of arteries, along with S1P1 receptor (Fig. 1B).

SIP receptor expression in regenerating soleus muscle. The presence of S1P1 and S1P3 receptors in quiescent satellite cells prompted us to examine their expression in soleus muscle during regeneration. At variance with adult muscle, in which S1P1 and S1P3 receptors localize in the sarcolemma surface membrane (47), 3-day regenerating fibers showed a diffuse intracellular distribution of both receptors (Fig. 2A). The intracellular expression progressively decreased. By 7 days after degeneration, both receptors were expressed by sarcolemma and nucleus membranes. Since we previously demonstrated (47) that S1P1 and S1P3 receptors are also expressed in the T-tubule membranes of adult muscle fibers, we evaluated this possible localization in regenerating fibers. In longitudinal sections, both receptors colocalized with the DHP receptors, at the T-tubule membrane level (Fig. 2B). Immunohistochemistry analysis, however, failed to detect S1P2 receptor in 3-day regenerating myotubes, whereas it was still evident in the wall of arteries (Fig. 2C).

The expression level of S1P receptors was then measured by Western blot in rat adult and 3-, 4-, and 7-day regenerating soleus muscles and in 2-day-old rat hindlimb muscle homogenates. In muscles allowed to regenerate for 3 days, S1P1 receptor expression was lower than in the adult and similar to that of neonatal muscles. S1P1 expression progressively increased, reaching the adult level 7 days after degeneration (Fig. 3, left). Conversely, S1P3 receptor expression progressively decreased in 3-day regenerating muscle, falling from high levels of expression seen in the neonatal muscles to those of adult (Fig. 3, right).

The reported expression and action of S1P2 receptor in C2C12 myoblasts (8, 9, 22) prompted us to investigate its possible expression in activated satellite cells. Consistent with immunofluorescence data, Western blot analysis confirmed that S1P1 and S1P3, but not S1P2, receptors are evident in lysates of rat adult soleus muscle; by contrast, our data show that all three S1P receptors are expressed in proliferating satellite cells isolated from mouse neonatal muscles (Fig. 4A). Therefore, we examined the expression of S1P2 receptor during regeneration also by Western blot. First, our results show that S1P2 receptor expression is evident in neonatal rat hindlimb muscles, especially in muscles where satellite cells are intensely active (46). Moreover, S1P2 receptor was also evident in 3-day regenerating soleus muscles and shown to progressively decrease by the 4th and 7th days. Expression of S1P2 was undetectable in adult soleus muscle lysates as well as being absent from total membrane fractions isolated from adult rat soleus muscles (Fig. 4B).

Effects of exogenous SIP on soleus regeneration. The presence of S1P-specific receptors in satellite cells suggested a role for the bioactive sphingolipid during regeneration of skeletal muscle. We investigated the effect of exogenous S1P on muscle fiber regeneration by directly injecting the lipid into soleus muscle coincident to treatment with the myotoxic drug bupivacaine. With this protocol, the bioactive lipid was expected to influence the initial phases of degeneration/regeneration, i.e., in the hours immediately after the myotoxic injury. Thus the effects observed after 3 days of regeneration should be interpreted as late consequences of the initial application of S1P. Besides S1P, we also investigated the effects of the S1P precursor Sph and those of the S1P-related lipid SPC, low-affinity ligand of S1P receptors (29).

Treatment with all three lipids stimulated the growth of regenerating fibers, as demonstrated by the larger CSA of regenerating fibers compared with that of muscles treated with vehicle alone (Fig. 5A). The mean muscle fiber CSA of the 3-day regenerating fibers were significantly higher with S1P (+24%), Sph (+26.9%), and SPC (+15.9%) treatment compared with the contralateral untreated muscles (Fig. 5B). Treatment with the three lipids did not affect the overall expression level of both S1P1 and S1P3 receptors (data not shown).

To investigate whether the larger muscle fiber CSA produced by S1P treatment could be associated with changes of myogenic transcription factors, we measured MyoD and myo-
antibody is a murine IgG1, regeneration experiments were delivered systemically (44). Since the anti-S1P monoclonal and substantially reduces the level of the circulating lipid when clonal antibody that recognizes and neutralizes bioactive S1P regeneration. This was obtained by means of a specific mono-
untreated muscles exhibited similar proportions of embryonic and neonatal MyHC isoforms (Fig. 5D). In addition, we evaluated whether the apparently anticipated progression of regeneration was associated with changes in myosin heavy chain (MyHC) isoform profile. S1P treatment did not modify the composition of MyHC isoforms of 3-day regenerating muscles in that both S1P-treated and untreated muscles exhibited similar proportions of embryonic MyHC isoform; n, neonatal MyHC isoform.

Next, we examined the effects of S1P deprivation on muscle regeneration. This was obtained by means of a specific monoclonal antibody that recognizes and neutralizes bioactive S1P and substantially reduces the level of the circulating lipid when delivered systemically (44). Since the anti-S1P monoclonal antibody is a murine IgG1, regeneration experiments were carried out in mice in order to obviate the rat-against-mouse antibody reaction. We first tested in mouse soleus whether addition of exogenous S1P could produce the same effects in mice as seen in regenerating rat soleus muscles. Figure 5, E and F, show that administration of S1P induced an increase (+16.6%) of mean fiber CSA in the 4-day regenerating mouse soleus muscles. By contrast, systemic administration of the anti-S1P monoclonal antibody attenuated the growth of fibers as measured by CSA (Fig. 5F). As previously shown (47), treatment with the anti-S1P monoclonal antibody did not affect mouse body weight [−4.8% in mice treated with vehicle (8 mice) compared with −3.8% in mice treated with the antibody (6 mice)].

Role of S1P receptors in rat soleus regeneration. S1P receptor expression data indicate that all three S1P receptors, S1P1, S1P2, and S1P3, are expressed during the early phases of regeneration. The dynamic expression of individual receptors (Figs. 3 and 4) also provides an indication of their putative roles. The initial high level of S1P3 receptor suggests it might particularly intervene during differentiation. The apparently transient expression of S1P2 receptor suggests it might have a role at some stage in the proliferation/differentiation of satellite cells. Thus, to identify which of the S1P receptors was mediating growth of regenerating soleus fibers, we employed modulators of the
receptors added coincident with the initiation of muscle degeneration.

The S1P-mediated stimulation of regenerating muscle fiber growth by the endogenous lipid was significantly inhibited (−7.7%) by SEW-2871 (250 μM), a selective S1P1 agonist (39) (Fig. 6). This result suggests that activation of S1P1 receptor might negatively control the early phases of regeneration. We then tested the effects of VPC-23019, an antagonist selective for S1P1 over S1P3 receptor by 10-fold (6, 36). At low levels (50 μM), VPC-23019 produced a strong increase (+27.3%) of regenerating muscle fiber CSA. At higher levels (250 μM), the increase of muscle fiber CSA was significantly attenuated (Fig. 6). Since our data indicate a transient expression of S1P2 receptor, we treated the regenerating muscle with JTE-013 (250 μM), a potent and selective S1P2 receptor antagonist (45). The presence of S1P2 receptor inhibitor was, however, without evident effects on the growth of soleus muscle regenerating fibers (Fig. 6).

**DISCUSSION**

The present work demonstrates that S1P1 and S1P3 receptors are expressed in quiescent satellite cells of adult rat soleus muscle. During the first week of soleus muscle regeneration, the relative expression of S1P receptors changed. S1P1 progressively increased from a level similar to that of neonatal muscle to the adult level. Conversely, S1P2 was high in the regenerating muscle, as it is in neonatal muscles, and progressively decreased during regeneration to the adult level. S1P2 receptor was transiently expressed during the early phases of regeneration only. Our data show that the exogenous addition of S1P stimulated the growth of regenerating myofibers, whereas reduction of the circulating lipid by neutralization with an anti-SIP monoclonal antibody produced the opposite effect. Moreover, the use of selective modulators of S1P receptors was evidence for their involvement during regeneration, with apparently opposite actions for S1P1 versus S1P2.

Important for a direct action of S1P in skeletal muscle is that constitutive high levels of S1P are present in body fluids. The main sources of blood S1P are erythrocytes and platelets; in plasma the lipid is transported associated with albumin and high-density lipoproteins (42). In addition, S1P produced intracellularly can be liberated into the extracellular milieu through the specific transporter ABCC1 (31) and may exert autocrine/paracrine actions through activation of S1P receptors; S1P is also liberated at sites of tissue injury and stimulates cellular repair responses (26). The membrane-associated Spk is responsible for S1P synthesis can be exported in the extracellular milieu, thus contributing to the local production of the bioactive lipid (43), as was shown in myogenic C2C12 cells (21). Activity of Spk, and thus S1P production, can be stimulated by several factors, including HGF (10), a known activator of myogenesis (3).

In a previous study, we demonstrated (47) that skeletal muscle expresses S1P1 and S1P3 receptors, and that extracellular S1P exerts a trophic actions in denervated muscle. We have also shown that S1P has a protective effect during muscle fatigue development (5). Recently, it has been shown that S1P exerts a protective role in cardiomyocyte damage by stimulating both S1P2 and S1P3 receptors (26). Moreover, since S1P1 has a major role in angiogenesis (19), S1P could facilitate regeneration by improving revascularization of the injured muscle.

Here we report that the expression of S1P receptors changes during the first week of regeneration. These modifications clearly suggest the involvement of S1P signaling in the molecular events controlling the early stages of muscle regrowth of injured muscles. Moreover, our results show that the cellular localization of S1P receptors varies. In quiescent satellite cells, S1P1 and S1P3 receptors are localized in the cell membrane; however, S1P2, but not S1P3, displays an intracellular/nuclear localization. It has been reported that S1P stimulation of S1P1 may lead to internalization of the receptor to perinuclear vesicles, in a mechanism probably utilized to fine-tuning S1P signal (18). Thus dual localization of S1P1 receptor in quiescent cells might be the result of its dynamic trafficking from the cell membrane to the nuclear membrane. In the initial regeneration phases, S1P1 and S1P3 receptors have an intracellular localization that is gradually reduced in favor of sarcolemma localization. In regenerating myofibers, S1P1 and S1P3 receptors appear to be localized in the T-tubule membranes, which are invaginations of sarcolemma. This suggests that both receptors are not segregated intracellularly but instead remain exposed to the extracellular action of S1P. The transition of S1P receptor localization to the surface in the early regeneration phases could be related to the central position of nuclei in the growing myotubes/myofibers. With the progress of myofiber maturation, and translocation of nuclei in the subsarcolemmal area, as in adult fibers, the receptors are mainly expressed in the sarcolemmal cell membrane.

The effects of varied S1P levels available to the regenerating soleus muscle demonstrated that the bioactive lipid plays a major role in skeletal muscle regeneration. In fact, experiments adding exogenous S1P coincident with the initiation of myotoxic injury stimulated the growth of regenerating fibers. Importantly, the same trophic action of S1P was demonstrated both in rat and in mouse regenerating muscles. The opposite maneuver of lowering circulating S1P by a specific monoclonal antibody attenuated the growth of regenerating fibers compared with the untreated muscle. It is important to report that lessening the circulating lipid did not affect fiber CSA of uninjured muscles (not shown).
Myogenic regulator factors MyoD, Myf5, myogenin, and MRF4 play a critical role in defining the progression steps of myogenesis, being expressed in a coordinated and sequential manner during activation/proliferation (MyoD and Myf5) and differentiation (myogenin and MRF4) of muscle progenitor cells (3, 40). Our results show that the S1P-mediated enlargement of regenerating myofibers was associated with an increased expression of the myogenic factors MyoD and myogenin. In general, myofiber differentiation is accompanied by the appearance of adult MyHC isoforms, replacing the embryonic isoforms transiently expressed in the initial stages of regeneration (28). Analysis of MyHC isoforms in the S1P-treated 3-day regenerating muscles shows the presence of embryonic and neonatal MyHC isoforms as in the untreated muscle, whereas adult MyHC isoforms were not evident, suggesting that the S1P-mediated myofiber growth is not associated with an anticipated differentiation of regenerating fibers.

It is interesting to note that Sph and SPC also exerted trophic actions on growing myotubes. The Sph effect is apparently paradoxical as it is generally known as a negative regulator of cell development (20, 42). However, Sph promyogenic action can be explained by its conversion to S1P by Sph kinase, which is present in muscular cell membranes (21, 24). Sph kinase activity and S1P formation were found to be enhanced in myoblasts that became confluent as well as in differentiating cells (24). SPC is a sphingomyelin derivative produced by sphingomyelin deacylase, an enzyme also expressed in skeletal muscle (37). SPC is physiologically present in serum at concentrations compatible with a biological action (17) and has been implicated in a number of biological processes, including fibroblast proliferation, angiogenesis, and cell migration (29). Importantly, SPC is a low-affinity agonist of S1P receptors (29). Consistent with this finding, our results show that SPC stimulates the growth of regenerating myofibers less efficiently than S1P.

By using specific modulators of S1P receptors, we have investigated the specific involvement of the receptors expressed in the regenerating soleus. It is important to note that these drugs were administered at the moment of myotoxic injury and that quiescent satellite cells express S1P1 and S1P3 receptors. On the other hand, S1P2 receptor expression was absent in quiescent but then became evident in proliferating satellite cells later during the proliferation/differentiation phases. S1P1 and S1P3 receptor activation likely occurs transiently in the degenerating area without the participation of S1P2 receptors. The lack of a role for the S1P2 receptor is evidenced by the lack of effects of JTE-013 on fiber CSA of regenerating soleus.

The selective initial stimulation of S1P1 receptor by SEW-2871 resulted in a significant reduction of mean fiber CSA, compared with the untreated muscles. This suggests that the direct activation of S1P1 receptor negatively controls the early phases of regeneration of myofibers. It is however, possible that SEW-2871 might induce an acute internalization of S1P1 reducing the overall signal through the receptor (14), a possibility that would indicate a positive role of the receptor. Administration of VPC-23019, antagonist of S1P1 and S1P3, produced a substantial increase of regenerating fiber CSA. Since VPC-23019 is reported to display higher affinity to S1P1 than to S1P3 (6, 36), it is possible that only S1P1 was completely blocked, confirming the negative action of this receptor. Thus we also evaluated the effects of higher VPC-23019 levels, showing that the increase of muscle fiber CSA was smaller than that produced at low doses. This result suggests that high doses of VPC-23019 completely inhibit S1P1 and more efficiently also S1P3 receptor. Treatments with these modulators thus seem to indicate that S1P1 negatively and S1P3 positively control the growth of regenerating fibers. This result apparently differs with the role of S1P1 in C2C12 reserve cells, a small population of C2C12 cells resembling quiescent satellite cells, in which the S1P1 agonist SEW-2871 stimulated proliferation (36). However, in C2C12 reserve cells, S1P exerted a promyogenic action, because it was not able to stimulate cell proliferation (36) as it did in satellite cells (32). The diverse responses to S1P in C2C12 reserve cells and in satellite cells is probably related to different functional coupling of S1P1 receptor. Interestingly, activation of S1P1 is reported to exert a negative inotropic action in the heart, whereas that of S1P3 mediates the hypertrophy in cardiomyocytes, although in vitro and in vivo data show some conflicting results (27).

The putative positive role of S1P3 is also suggested by our results showing that SPC stimulated the growth of regenerating fibers, considering that this lipid was reported to specifically activate S1P3 (35). Moreover, as S1P1 is involved in PLC-dependent Ca2+ mobilization (15, 42), stimulation of this receptor might also represent a condition to favor the fusion of myoblasts into myotubes. Consistent with this possibility is the observation that S1P stimulates the expression of connexin 43 (41) and modulates Ca2+ homeostasis resulting in actin filament remodeling (11, 12). Future analyses of S1P receptor downstream signaling pathways and on the effects of knockdown of individual receptors’ expression would elucidate their specific role during regeneration.

In conclusion, the present work indicates the bioactive lipid S1P as a novel myogenic factor and the involvement of sphingolipid pathways in muscle growth.

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DISCLOSURES

The authors state that there are no conflicts of interest.

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