RESEARCH ARTICLE

Ablation of S1P3 receptor protects mouse soleus from age-related drop in muscle mass, force, and regenerative capacity

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Bondi M, Germinario E, Pirazzini M, Zanetti G, Cencetti F, Donati C, Gorza L, Betto R, Bruni P, Danieli-Betto D. Ablation of S1P3 receptor protects mouse soleus from age-related drop in muscle mass, force, and regenerative capacity. Am J Physiol Cell Physiol 313:C54–C67, 2017. First published April 26, 2017; doi:10.1152/ajpcell.00027.2017. —We investigated the effects of S1P3 deficiency on the age-related atrophy, decline in force, and regenerative capacity of soleus muscle from 23-mo-old male (old) mice. Compared with muscle from 5-mo-old (adult) mice, soleus mass and muscle fiber cross-sectional area (CSA) in old wild-type mice were reduced by ~26% and 24%, respectively. By contrast, the mass and fiber CSA of soleus muscle in old S1P3-null mice were comparable to those of adult muscle. Moreover, in soleus muscle of wild-type mice, twitch and tetanic tensions diminished from adulthood to old age. A slowing of contractile properties was also observed in soleus from old wild-type mice. In S1P3-null mice, neither force nor the contractile properties of soleus changed during aging. We also evaluated the regenerative capacity of soleus in old S1P3-null mice by stimulating muscle regeneration through myotoxic injury. After 10 days of regeneration, the mean fiber CSA of soleus in old wild-type mice was significantly smaller (~28%) compared with that of regenerated muscle in adult mice. On the contrary, the mean fiber CSA of regenerated soleus in old S1P3-null mice was similar to that of muscle in adult mice. We conclude that in the absence of S1P3, soleus muscle is protected from the decrease in muscle mass and force, and the attenuation of regenerative capacity, all of which are typical characteristics of aging.

aging; sarcopenia; soleus muscle; regeneration; S1P3 receptor

AGING OF SKELETAL MUSCLE is associated with the gradual loss of skeletal muscle mass and strength, a condition known as sarcopenia. Multiple phenomena, not totally understood, contribute to the development of sarcopenia, including intrinsic factors such as hormonal imbalance, inflammation, denervation, oxidative stress, mitochondrial dysfunction, and impaired satellite cell (SC) function; and extrinsic factors such as reduced physical activity and inadequate nutrition (48).

The reduction of muscle mass in old humans was demonstrated to be secondary to the loss of a-motoneurons and motor units, and to an incomplete reinnervation of previously denervated muscle fibers, evidence confirmed in rat-aging models (36, 37). Diversely, little or no neuronal death was evidenced in aged mice (14). On the other hand, oxidative stress and decreased release of trophic factors seem to influence neuromuscular junction (NMJ) integrity and contribute to denervation of the oldest muscle fibers (11, 14, 15). Large evidence was also accumulated demonstrating that altered intracellular and/or metabolic conditions lead to dysfunctional mitochondria; these conditions include impaired mitochondrial energetics, decreased rate of synthesis of mitochondrial proteins, increased mitochondrial-mediated apoptosis, and decreased autophagy, and therefore mitophagy (45). Therefore, defective autophagy increases sarcopenia by causing NMJ degeneration, and accumulation of large mitochondria and carbonylated proteins (11). Importantly, the latter seems to contribute to the reduced specific tension generated by old muscle fibers. In fact, the decrease in muscle force-generating capacity with age appears to be larger than the reduced muscle mass (1). The age-dependent fast-to-slow phenotype transformation could contribute to the reduction in muscle force (1), as fast fibers are stronger than slow fibers (9).

Aging is also characterized by a gradual decline in the regenerative efficiency of skeletal muscle (4), though recent evidence indicates that the process appears just delayed and completely fulfilled (2, 58). Whether this is due to extrinsic changes in the environment and/or to cell-intrinsic mechanisms associated with aging is still debated. Adult muscle regeneration involves activation of SCs, a stem cell population located between the basal lamina and plasma membrane. Activated SCs undergo proliferation and generate myoblasts, which fuse to each other or to injured myofibers to promote repair and regeneration. There was no significant difference in SC number between young and old muscle (18), but it is reported that with age, the systemic environment is less effective in maintaining the myogenic fate of muscle stem cells and facilitates the conversion to fibrogenic fate (12).

The bioactive sphingolipid sphingosine 1-phosphate (S1P) exerts important functions in almost all tissues and organs, and particularly in vascular and immune systems (46, 60). S1P acts by autocrine/paracrine mechanisms as a ligand of five distinct S1P receptors, (S1P1–5) coupled to multiple heterotrimeric G proteins that activate distinct signaling pathways (46, 60). Three S1P receptor subtypes (S1P1, S1P2, and S1P3) are expressed in adult skeletal muscle. S1P1 and S1P3 receptors are localized both in the cell and nuclear membranes of adult fibers (67). In addition, S1P1 was also detected in the neuromuscular junction (67). S1P1 and S1P3 are expressed by quiescent satellite cells of...
adult muscle, whereas S1P2 is only transiently expressed in activated SCs (21, 26, 33).

Studies of the role of S1P signaling in skeletal muscle are carried out in skeletal muscle cellular models, in in vivo experiments by modulating S1P signaling, and in transgenic mice lacking individual S1P receptors, demonstrating on the whole that S1P has pleiotropic effects on muscle functions (22). Our previous results indicate that exogenous S1P administration makes extensor digitorum longus (EDL) muscle more resistant to fatigue (20). Moreover, exogenous application of S1P counteracts the reduction in rat muscle mass caused by denervation, whereas neutralization of the extracellular lipid with a specific anti-S1P monoclonal antibody accelerated the atrophy caused by denervation in mice (67). It was then demonstrated that S1P stimulates quiescent SCs to enter the cell cycle, and muscle regeneration is compromised when S1P biosynthesis is inhibited (47). In addition, exogenous administration of S1P at the moment of myotoxic injury favors the growth of regenerating fibers in both rat and mouse (21). Moreover, reduced S1P catabolism improves muscle regeneration in mdx mice (33, 41). The role played by single S1P receptors in skeletal muscle is only partly revealed, and divergent evidence is reported. Previous results suggest that S1P3 may positively control muscle trophism, because its expression dramatically drops in denervated rat soleus muscle (67). However, in S1P3-null mice, denervation atrophy is reduced in the EDL muscle (27). In vivo pharmacological data suggest that S1P1 negatively regulates the growth of regenerating rat soleus fibers, whereas S1P3 seems to have an opposite, positive action (21). However, it has been recently demonstrated in vivo that in the absence of S1P3, acute regeneration is enhanced both after a single cycle and repeated cycles of muscle injury in mouse tibialis anterior muscle (26). Moreover, it was suggested that signaling through S1P3 may contribute to control the quiescence of SCs (26), whereas signaling that operates through S1P2 may then drive S1P-mediated proliferation and/or differentiation in myogenic C2C12 cells (41). Finally, the lack of S1P2 resulted in a delay of regenerating muscle fiber growth (29, 41).

In the present work, we explored the effects produced by the absence of S1P3 on the characteristics of aged soleus muscle using S1P3-null mice. The absence of S1P1 receptor in old soleus muscle seems to favor the preservation of muscle mass, muscle force, NMJ integrity, and regenerating capacity.

MATERIALS AND METHODS

The Italian Health Ministry approved all animal experimental protocols. C57BL/6j (wild type) and S1P3-null mice, generated by Jerold Chun (Scripps Institute, La Jolla, CA) (34) and kindly provided by Dr. Bodo Levkau, University of Essen, Essen, Germany (the genotype of S1P3-null mouse was confirmed by PCR), were housed under a 12:12-h light-dark cycle in an air-conditioned room with ad libitum access to a standard chow and water diet. Male mice aged 5 and 25 mo were used. Throughout the experimental work, a total of 22 adult and 18 old, wild-type mice, and 24 adult and 20 old, S1P-null mice were used.

Muscle contractile properties of old soleus muscle. The experiments were performed in vitro in a vertical muscle apparatus (300B; Aurora Scientific, Aurora, ON, Canada) containing a Ringer solution of the following composition: 120 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 3.15 mM MgCl2, 1.3 mM Na2HPO4, 25 mM NaHCO3, 11 mM glucose, and 30 μM d-tubocurarine, pH 7.2–7.4, 30°C, bubbled with 95% O2/5% CO2. Muscles were stretched to the optimal length (i.e., the length that allowed maximal tension development in response to a single pulse) and electrically stimulated via two parallel electrodes with supramaximal pulses (0.5 ms duration) delivered by a Grass S44 electronic stimulator through a stimulus isolation unit (Grass SIU5). Muscle response was recorded through an isometric force transducer (Grass FT03) connected to an AT-MIO 16AD acquisition card (National Instruments). Data were analyzed using LabView software (National Instruments) (27). Time to peak of the twitch, half relaxation time of the twitch and of the tetanus, and the maximum rate of rise of the tension were measured. Twitch and tetanic tensions were normalized to the muscle wet weight (specific tension, N/g). Force-frequency curve was determined by stimulating soleus muscle at 1, 20, 30, 40, 60, 80, 100, 120, and 150 Hz. Muscles were weighed at the end of each experiment.

Histological and immunofluorescence analysis. Muscles were isolated and quickly frozen in liquid nitrogen in a slightly stretched position. Serial cross sections (8 μm thick) were cut in a cryostat microtome (Slee, London, UK) set at −24 ± 1°C. Hematoxylin & eosin and succinate dehydrogenase (SDH) staining were performed on transverse muscle sections to examine the general morphology and overall mitochondrial content (27). To evaluate the extension of fibrosis, muscle cryostat sections were subjected to PicroSirius Red staining (31). Laminin staining was carried out to determine the cross-sectional area (CSA) of individual fibers (21, 67). Muscle sections were incubated for 1 h at 37°C with the polyclonal antibody specific for laminin (L9393; Sigma, St. Louis, MO) diluted 1:150 in 5% fetal bovine serum. Laminin was revealed with an anti-rabbit Alexa Fluor 488 (goat anti-rabbit IgG; Invitrogen, Carlsbad, CA) diluted 1:200 in PBS incubated for 1 h at 37°C. Nuclei were evidenced with DAPI staining. Muscle sections were examined in a Leica RD100 fluorescence microscope equipped with a digital camera. Muscle fiber CSA was measured on digital photographs using ImageJ software (National Institutes of Health, Bethesda, MD). Type 1 and type 2A fibers were identified, respectively, with BA-F8 (diluted 1:100) and SC-71 (diluted 1:10) monoclonal antibodies (University of Iowa, Developmental Studies Hybridoma Bank). The secondary antibodies were Alexa Fluor goat anti-mouse IgG2b conjugate for type 1 and IgG1 conjugate for type 2A, diluted 1:200.

Neuromuscular junction immunohistochemistry. Immediately after isolation, soleus muscle was fixed in 4% (wt/vol) paraformaldehyde (PFA) in PBS for 30 min at room temperature. Samples were then quenched in 50 mM NH4Cl in PBS (NH4Cl was used to quench residual active aldehydes after PFA fixation), permeabilized and saturated for 2 h in blocking solution (15% vol/vol goat serum, 2% wt/vol BSA, 0.25% wt/vol gelatin, and 0.2% wt/vol glycerin in PBS), containing 0.5% Triton X-100. Bundles of 5 to 6 muscle fibers were dissected and incubated for at least 48 h with primary antibodies specific for the vesicle-associated membrane protein 1/synaptobrevin-1 (VAMP; rabbit polyclonal, diluted 1:200 in PBS, catalog no. A11008; Invitrogen by Thermo Scientific, Waltham, MA) supplemented with Alexa 488 (goat anti-rabbit IgG; Molecular Probes by Thermo Scientific, Waltham, MA) to counterstain presynaptic nicto-tonic acetylcholine receptors. Images were collected with a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a ×100 HCX PL APO NA 1.4 objective. Laser excitation line, power intensity, and emission range were chosen according to each fluorophore in different samples to minimize bleed-through.

Surgical procedures. Surgical procedures were performed while the mice were under general anesthesia via intraperitoneal injection of tiletamine and zolazepam (7 mg/kg, Virbac, Carros) and xylazine (14 mg/kg, Bayer). C57BL/6j and S1P-null mice aged 5 and 23 mo were...
used. Acute degeneration was induced in the left soleus muscles by injecting 0.075 ml of the myotoxic drug notexin (0.5%, Sigma) as previously published (29). Shortly thereafter, soleus muscle was exposed by making a small, lateral incision and then gently detaching it from the covering muscles with the tip of a surgical scissor. Notexin was injected by inserting the tip of the needle longitudinally into the muscle until it slightly swelled up, to avoid leaking out of the toxin and the injury of surrounding muscles. Mice were euthanized 10 days after degeneration by neck dislocation, and the soleus muscle of both legs was removed.

Western blotting and SDS-PAGE analysis. Western immunoblotting was performed on muscle fragments dissolved in SDS-PAGE buffer supplemented with Complete protease (04693132001; Roche, Basel, Switzerland) and phosphatase (P0044 and P5726; Sigma) inhibitor cocktail, diluted as suggested. Muscle lysates (25–30 μg each) were electrophoresed on 10% SDS-PAGE gels. Electroblotting was performed as previously described (67). Nitrocellulose filters were probed with the selected primary antibody incubated overnight at 4°C at the conditions described below.

The following rabbit antibodies were used: phospho[Ser173]-Akt, 1:1,000 in 5% BSA and 0.1% Tween-20 in TBS (catalog no. 4060; Cell Signaling Technology); Akt, 1:500 in 5% BSA and 0.1% Tween-20 in TBS (9272; Cell Signaling Technology); phospho-[Ser473]-AKT, 1:500 in 5% BSA and 0.1% Tween-20 in TBS (5364; Cell Signaling Technology); phospho[Ser422/423]-Smad3, 1:1,000 in 5% BSA and 0.1% Tween-20 in TBS (Ab52903; Abcam); GAPDH, 1:5,000 in 2% BSA, 0.2% Tween-20 in TBS (GT10100118; GenTex); LC-3, 1:1,000 in 5% low-fat milk, 0.1% Tween-20 in TBS (L7543; Sigma); P62, 1:1,000 in 5% low-fat milk, 0.1% Tween-20 in TBS (P0067; Sigma); PGC-1α, 1:1,000 in 5% low-fat milk, 0.1% Tween-20 in TBS (ab54481; Abcam); MuRF-1, 1:500 in 5% low-fat milk, 0.04% Tween-20 in TBS (MP3401; ECMBioscience); Tom20, 1:500 in 5% BSA, 0.05% Tween-20 in TBS (sc11415; Santa Cruz Biotechnology); and Bcl2, 1:1,000 in 5% BSA, 0.1% Tween-20 in TBS (2876S; Cell Signaling Technology). The secondary antibody was an anti-rabbit peroxidase-conjugated antibody (P0447; Dako) diluted 1:5,000 in the same buffer as the cognate primary antibody and incubated for 1 h. A goat polyclonal antibody anti-SERCA2 (1:1,000 in 5% low-fat milk, 0.05% Tween-20 in TBS) (sc8094; Santa Cruz Biotechnology) was used. The secondary antibody was an anti-goat peroxidase-conjugated antibody (A5420; Sigma) diluted 1:10,000 in the same buffer as the cognate primary antibody and incubated for 1 h. Moreover, the following mouse monoclonal antibodies were used: Smd3, 1:2,500 in 5% BSA and 0.1% Tween-20 in TBS (Ab75512, Abcam); SERCA1 (D1G8), 1:3,000 in 1% BSA in TBS, generated as described (28); myogenin, 1:100 in 10% low-fat milk, 0.1% Tween-20 in TBS (F5D; Developmental Studies Hybridoma Bank); MyoD1, 1:150 in 5% low-fat milk, 0.05% Tween-20 in TBS (M3512; Dako); and embryonic MyHC isoform (1:500, in 2% BSA, 0.2% Tween-20 in TBS) (F1.652; Developmental Studies Hybridoma Bank). The secondary antibody was an anti-mouse peroxidase-conjugated antibody (P0447; Dako) at 1:10,000 dilution (in the same buffer as the cognate primary antibody), incubated for 1 h. Visualization of reaction bands was performed either via tetramethylbenzidine staining or enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK). Signal intensities were evaluated by densitometry. Analysis of oxidatively modified proteins was performed by detecting carboxylated proteins using an OxyBlot Protein Oxidation Detection Kit (Millipore, Billerica, MA).

Analysis of MyHC isoforms was performed by the SDS-PAGE method previously described (26). Small muscle fragments were weighed, ground with a ceramic pestle in liquid nitrogen, and extracted at 2 mg/ml in SDS-PAGE sample buffer (62.5 mM Tris-HCl pH 6.8, 2.3% SDS, 5% mercaptoethanol, and 10% glycerol). Muscle protein samples (10 μg each) were electrophoresed on 8% SDS-PAGE slab gels. MyHC protein bands were revealed with Coomassie brilliant blue staining. MyHC isoform percentage composition was determined by densitometry of gels by using a Bio-Rad Imaging Densitometer (GS-670).

Real-time PCR. Total RNA of soleus from old (age, 23 mo) S1P3-null and wild-type mice was purified by TRI-Reagent, 1 μg of which was used for reverse transcription, using the SuperScript IV first-strand synthesis system, according to the manufacturer’s instructions (Life Technologies, Carlsbad, CA). Quantification of S1P receptors (S1P1, S1P2, and S1P3) and CTGF mRNA level was performed via real-time PCR employing TaqMan Gene Expression Assays (S1P1, Mm00514644_m1; S1P2, Mm01177794_m1; S1P3, Mm00515669_m1; and CTGF, Mm01192933_g1). Each measurement was carried out in triplicate using an automated ABI Prism 7500 Sequence Detector System (Life Technologies) as described previously (23) by simultaneous amplification of the target sequence together with the housekeeping gene β-actin, whose expression was highly stable across all the groups. Results were analyzed using ABI Prism Sequence Detection Systems software, version 1.7 (Life Technologies), the 2−ΔΔCt method was applied as a comparative method of quantification (40), and data were normalized to β-actin expression.

Statistical analysis. All values are expressed as means ± SE. The mean CSA values from individual muscles were pooled and the resulting mean was compared. More than 600 fibers from each muscle were measured. Data were analyzed with one-way-between-subjects ANOVA followed by a Newman-Keuls post hoc test. For real-time PCR experiments, results are expressed as means ± SE of fold changes according to the 2−ΔΔCt method, with 18S rRNA in each specimen used for housekeeping and the S1P receptor in wild-type soleus as a calibrator. Statistical significance of real-time PCR data was assessed with two-way ANOVA followed by a Bonferroni post hoc test. Differences were considered significant at P < 0.05.

RESULTS

Aging of S1P3-null soleus. Skeletal muscle aging is characterized by the loss of muscle mass and strength, generally termed sarcopenia. We evaluated the effects produced by the absence of S1P3 receptor on aging of soleus muscle by using adult (5 mo old) and old (23 mo old) wild-type and S1P3-null mice (34).

First, the body mass of wild-type mice was similar (±2%) in adult and old age mice, whereas that of S1P3-null mice was substantially increased by ~14% (Fig. 1A). Soleus muscle mass of old wild-type mice was largely smaller (~26%) than that of adult mice (Fig. 1B), confirming the atrophy observed in old skeletal muscle (1). Consistently, the ratio between soleus mass and body weight was markedly decreased in wild-type mice (Fig. 1C). Soleus muscle mass of S1P3-null mice decreased only slightly during aging (Fig. 1B). As a consequence, the ratio between soleus mass and body weight also decreased in S1P3-null mice. Preservation of muscle mass during aging in S1P3-null mice is also observed in EDL (10.7 ± 0.6 mg, n = 8, vs. 9.9 ± 0.9 mg, n = 7) and in tibialis anterior, the mass of which increased compared with that of adult animals (30.8 ± 2.9 mg, n = 8, vs. 37.9 ± 3.3 mg, n = 3, P < 0.03). On the other hand, laminin staining evidenced that old wild-type soleus fibers were clearly smaller than those of adult wild-type mice and adult and old S1P3-null mice (Fig. 1D), confirming the loss of mass of old wild-type soleus. Analysis of the mean CSA of soleus fibers shows evidence that old wild-type soleus fibers are 24% smaller than adult soleus fibers, whereas the mean CSA of old S1P3-null fibers is similar to that of adult animals (Fig. 1E). Fiber CSA distribution (Fig. 1F) demonstrates that old wild-type soleus contains a higher number of smaller fibers than old S1P3-null mice. Finally, the
total number of fibers was comparable in adult and old wild-type and S1P3-null soleus (916 ± 30, n = 6, and 899 ± 31, n = 6, in adult and old wild-type, respectively; 906 ± 24, n = 5, and 845 ± 35, n = 7, in adult and old S1P3-null, respectively).

Histological analysis of adult and old soleus muscles from wild-type and S1P3-null mice did not reveal overt morphological or pathological signs, however, some interesting differences emerged. The extent of collagen/fibrosis in old S1P3-null mice was significantly lower (P < 0.05) compared with that in wild-type animals (8.3 ± 0.4%, n = 6, and 12.5 ± 1.8%, n = 6, respectively) (Fig. 1G), whereas adult muscles did not show any difference (not shown). Consistent with the lower amount of fibrosis in old S1P3-null soleus, mRNA expression of CTGF (CCN2), a molecular marker and a mediator of fibrosis, was significantly (P < 0.05) lower in old S1P3-null soleus (0.57 ± 0.09) compared with that of wild-type soleus (1.00 ± 0.10). In addition, we carried out an analysis of phosphorylated Smad3, a downstream effector of TGF-β, which through S1P3, regulates muscle fibrosis (13). The P-Smad3-to-Smad3 ratio did not change during aging either in wild-type soleus (0.98 ± 0.38, n = 5, and 1.60 ± 0.48, n = 4, adult and old, respectively) or in S1P3-null soleus (1.19 ± 0.38, n = 5, and 1.14 ± 0.30, n = 5, adult and old, respectively). Finally, the intensity of SDH staining did not show evident differences between old S1P3-null and wild-type soleus fibers, with both muscles exhibiting in many fibers the presence of subsarcolemmal mitochondria aggregates (Fig. 1H).

Old wild-type and S1P3-null soleus display a similar low number of fibers with central nuclei (likely a sign of regeneration) (3.7 ± 0.4%, n = 5 and 5.9 ± 1.3%, n = 7, respectively) and did not show angular fibers (a sign of denervation). Despite the apparent absence of denervation, myogenin protein expression level, usually high in denervated muscles (26, 61), was fivefold higher in old wild-type soleus compared with adult wild-type soleus (2.5 ± 0.59 vs. 0.59 ± 0.08; n = 4, P < 0.02). Myogenin was unchanged in old S1P3-null soleus compared with that of adult soleus (1.26 ± 0.44 vs. 1.26 ± 0.25; n = 4).
A hallmark of aging atrophy is the progressive deterioration of NMJ integrity, which also accounts for the reduced performance of aged muscles. Figure 2, A and D, shows NMJs from a young soleus muscle stained either for VAMP1 or syntaxin 1A1B, respectively, two markers of the motor axon terminal, and for the ionotropic acetylcholine receptors, for the postsynaptic apparatus on the muscle fiber. Notably, the latter appears like a continuous and sinuous wavy line, assuming the classical pretzel-like shape that is perfectly matched by the presynaptic terminals. In agreement with previous reports (14, 15, 62), the morphology of the aged NMJ is characterized by a significant loss of the pretzel-like structure, which becomes highly fragmented and accompanied by a more complex branching of the presynaptic elements (Fig. 2, B and E). Remarkably, this alteration is significantly restrained in the age-matched S1P3-null NMJ, which still displays a continuous and curvy architecture, more similar to that displayed by adult mice, though in some cases slightly less elaborated (Fig. 2, C and F).

Aging of skeletal muscle is known to be associated with a progressive decline in muscle function (1). To evaluate whether the absence of S1P3 may affect age-related changes in soleus performance, we compared the contractile properties of adult and old wild-type and S1P3-null soleus. The absolute twitch and tetanic tensions of wild-type soleus were largely lower (−40.2 and −41%, respectively) in old compared with adult mice. On the contrary, no difference in absolute tension was observed between old and adult soleus of S1P3-null mice (Fig. 3, A and B). Moreover, the specific twitch and tetanic tensions of old S1P3-null soleus were higher compared with adult and old wild-type muscles (Fig. 3, A and B). Old wild-type soleus showed a significant lengthening of twitch contraction time, which was not evident in old S1P3-null soleus. The maximal rate of tetanus rise was dramatically reduced (−55%) in old wild-type soleus compared with adult muscle, but unchanged in old S1P3-null soleus. The half-relaxation time of the twitch is similar in the four muscles, whereas the half-relaxation time of the tetanus increased in old wild-type soleus only (Fig. 3, A and B).

The smaller tension developed by the twitch and tetanus of old wild-type soleus is also confirmed by the force-frequency curves, in which the absolute tension developed at each stimulation frequency tested was clearly smaller in old wild-type soleus than adult wild-type soleus (Fig. 3C). Therefore, old S1P3-null soleus displays a similar absolute force-frequency curve (Fig. 3C) and a larger specific force-frequency curve to that of adult muscle at all stimulation frequencies (Fig. 3D).

Thus, aging produces a slowing of contractile properties in wild-type but not in S1P3-null soleus. To evaluate whether the lengthening of contraction and relaxation times are associated with a reduced reaccumulation of myoplasmic calcium by the sarcoplasmic reticulum calcium pump (SERCA), we measured the expression level of fast and slow SERCA isoforms. In the soleus of old S1P3-null mice the expression level of the fast SERCA1 isoform was lower than that in wild-type muscles (Fig. 4A). On the contrary, expression levels of the slow SERCA2 isoform were similar in adult and old soleus muscles (Fig. 4B).

The slowing of contractile properties could also be the consequence of fast-to-slow changes in the proportion of myosin isoforms. To evaluate possible changes in muscle phenotype we examined the composition of MyHC isoforms in adult and old soleus muscles. First, S1P3-null adult soleus has a slower phenotype compared with that of wild-type muscle, showing a higher content of type 1 MyHC isoform and a lower content of type 2A and 2X isoforms (Table 1). Aging of wild-type soleus is characterized by a significant increase in type 1 fibers to the detriment of type 2A and 2X MyHC isoforms (Table 1), whereas no changes were observed when adult and old S1P3-null soleus were compared.
Moreover, because the number of hybrid fibers (i.e., fibers expressing two or even more MyHC isoforms) has been reported during aging (52), we analyzed the fiber type composition of old soleus muscles. Results indicate that both old wild-type and S1P3-null soleus possess a very low number of hybrid fibers (i.e., fibers expressing both type 1 and type 2A MyHC) (4.0 ± 1.4 vs. 5.0 ± 2.1% for old wild-type and S1P3-null soleus, respectively).

Oxidative stress contributes, at least in part, to the progressive loss of muscle mass and function, leading to accumulation of oxidatively modified (carbonylated) proteins, including regulatory and contractile proteins (38, 49, 51). We investigated whether the extent of carbonylated proteins could explain the higher specific tension of old S1P3-null soleus muscle. The results show that the amount of total carbonylated proteins was significantly lower in old S1P3-null muscle compared with adult S1P3 muscles, but not compared with adult or old wild-type muscles (Fig. 4C).

We next examined the consequences of the lack of S1P3 on the expression of proteins involved in aging-related atrophy. PGC-1α is a key regulator of mitochondria biogenesis (37) that could protect the S1P3-null soleus muscle during aging. However, the expression level of PGC-1α did not change during aging either in wild-type soleus or in S1P3-null soleus (Fig. 5A), which is in agreement with the similar SDH staining intensity (Fig. 1H) and the amount of Tom20, a component of the translocase receptor complex of the mitochondrial outer membrane, and an index of mitochondria content (Fig. 5B). The expression level of MusRF1, the E3 ligase involved in the proteolytic events of muscle atrophy (5) was comparable both in wild-type and S1P3-null muscles (Fig. 5C). Moreover, the expression level of whole Akt and activated Akt (P-Akt), as well as the ratio between P-Akt and Akt, whose increase is related to muscle growth (6), did not change with age in wild-type or in S1P3-null soleus. However, levels of P-Akt in adult muscle and that of whole Akt in old S1P3-null muscles were higher than those in cognate wild-type muscles (Fig. 5D).

We also examined the level of phosphorylated S6, the ribosomal protein marker of mammalian target of rapamycin (mTOR) and protein synthesis activation. No difference in the level of P-S6 was observed in the four conditions (Fig. 5E). Next, we measured the amount of p62, a protein involved in the recognition of autophagy substrates, and the LC3-II/LC3-I ratio, which is important in autophagosome formation (7).
During aging, the expression level of p62 protein did not change in wild-type soleus, whereas it was significantly decreased in S1P3-null muscle (Fig. 5F). Conversely, the LC3-II/LC3-I ratio did not change during aging either in wild-type or S1P3-null soleus (Fig. 5G). Finally, to evaluate possible apoptotic events, we measured the expression level of Bcl-2, which, however, did not change (Fig. 5H).

We recently reported that expression of S1P1 and S1P2 transcripts, which are physiologically expressed in skeletal muscle, increases in the fast-twitch EDL muscle of S1P3-null mice (27). Thus, we examined whether the absence of S1P3 affects the expression level of the other S1P receptors in the slow-twitch soleus muscle. Using real-time PCR, we quantitated the transcript level of S1P1 and S1P2 receptors in soleus muscle. The data show that S1P3 deficiency does not modify the expression level of S1P1 or S1P2 receptors either in adult or in old soleus muscle (Fig. 6).

**Table 1. Myosin heavy chain composition of soleus muscle from wild-type and S1P3-null soleus muscle**

<table>
<thead>
<tr>
<th>MyHC Isomorph</th>
<th>Wild Type (n = 8)</th>
<th>S1P3-Null (n = 6)</th>
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<tbody>
<tr>
<td>Type 1</td>
<td>40.3 ± 1.7</td>
<td>48.6 ± 3.2*</td>
</tr>
<tr>
<td>Type 2A</td>
<td>37.8 ± 1.6</td>
<td>33.6 ± 2.6</td>
</tr>
<tr>
<td>Type 2X</td>
<td>20.2 ± 1.0</td>
<td>15.3 ± 1.1*</td>
</tr>
<tr>
<td>Type 2B</td>
<td>1.7 ± 1.7</td>
<td>4.9 ± 1.7</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = no. of muscles examined). MyHC, myosin heavy chain. *P < 0.05 vs. adult wild type.

**Regeneration of old S1P3-null soleus**. We next examined the role of the S1P3 receptor on the regenerative capacity of soleus muscle during aging. Regeneration of soleus muscle was induced by myotoxic injury (21, 29) to both adult and old soleus muscles from wild-type and S1P3-null mice. Only muscles almost entirely regenerated were examined. Regenerating fibers were identified by the presence of centrally located nuclei after DAPI staining (Fig. 7A).

After 10 days of regeneration, adult wild-type soleus fibers had a mean CSA similar to that of age-matched S1P3-null soleus (Fig. 7, A and B), indicating that regeneration progressed similarly in the two muscles and that the absence of the receptor does not seem to influence the regenerative process of adult soleus muscle.

The mean CSA of old wild-type soleus after 10 days of regeneration was significantly smaller (−28%) compared with that of adult regenerated muscle (Figs. 7, A and B), suggesting that regeneration of old wild-type soleus either proceeds more slowly or is impaired compared with that of adult muscle. On the contrary, regeneration of old S1P3-null soleus was apparently as vigorous as in adult muscle, with a mean fiber CSA similar to that of adult muscle, and consequently, significantly larger (+27%) compared with that of old wild-type muscle (Fig. 7, A and B). Analysis of CSA distribution demonstrates that compared with old wild-type soleus, the larger mean CSA of S1P3-null muscle derives from the presence of larger fibers and the absence/reduction of smaller ones (Fig. 7C).

The extent of fibrotic areas, as evidenced by PicroSirius Red staining, was not statistically different in old S1P3-null soleus...
Fig. 5. Protein expression in adult and old WT and S1P3-null soleus muscle. A: PGC-1α, peroxisome proliferator-activated receptor-γ co-activator 1α. B: Tom20, a component of the translocase receptor complex of mitochondrial outer membrane, an index of mitochondria content. C: MuRF1, E3 ligase involved in the proteolytic events of muscle atrophy. D: Akt, P-Akt, and P-Akt/Akt ratio. E: P-S6, phosphorylated ribosomal protein S6, a marker of mTOR and protein synthesis activation. F: p62, a protein involved in recognition of autophagy substrates. G: LC3 II/I ratio, an index of autophagosome formation. H: Bcl-2, an antiapoptotic factor. Top: all plots are examples of the relative Western blots carried out as described in MATERIALS AND METHODS. GAPDH was used as an internal control. Number of soleus muscles examined: adult WT (n = 7), old WT (n = 5), adult S1P3-null (n = 9), and old S1P3-null (n = 8). *P < 0.05.
(12.8 ± 1.4%, n = 5) compared with wild-type regenerating soleus (18.6 ± 2.5%, n = 4) (Fig. 7D).

Finally, we measured the myogenic transcription factors usually involved in muscle regeneration. The expression level of MyoD, a marker of cell proliferation (44), was significantly lower in old S1P3-null soleus compared with adult transgenic muscle, whereas no difference was evident in wild-type muscles (Fig. 8A). MyoD levels in old regenerating S1P3-null soleus were also lower than those of old wild-type regenerating muscle. The level of myogenin, a muscle differentiation marker (44), was larger in old wild-type soleus than in the adult muscle. Moreover, the level of myogenin was lower in old S1P3-null soleus than in old wild-type soleus and was unchanged compared with that of adult transgenic muscle (Fig. 8B). Finally, the expression level of embryonic myosin, which was used to evaluate the progression of differentiation, was similar in the four regenerating muscles (Fig. 8C).

**DISCUSSION**

In this work, we evaluated whether and how S1P3 receptor ablation influences the effects produced by age on soleus muscle. The study compared the morphological and functional characteristics, as well as the regenerative capacity of adult and old soleus muscle of wild-type and S1P3-null mice. Some significant differences emerged in old animals: 1) old S1P3-null mice were heavier than age-matched wild-type mice; 2) soleus muscles of S1P3-null mice were protected from the age-related changes; 3) regeneration capacity was preserved in old S1P3-null soleus muscle. The study compared the morphological and functional characteristics, as well as the regenerative capacity of adult and old soleus muscle of wild-type and S1P3-null mice. Some significant differences emerged in old animals: 1) old S1P3-null mice were heavier than age-matched wild-type mice; 2) soleus muscles of S1P3-null mice were protected from the age-related changes; 3) regeneration capacity was preserved in old S1P3-null soleus muscle.
age-related drop in muscle mass; 3) the soleus muscles of old S1P3-null mice were stronger and faster than those of wild-type mice; 4) NMJs of old S1P3-null mice are less deteriorated than those of wild-type mice; and 5) the regenerative capacity of old S1P3-null soleus is as efficient as it is in adulthood and is more efficient than it is in old wild-type soleus.

First, it is worth mentioning that the absence of S1P3 appears to have different effects in fast and slow muscles. We recently characterized 3-mo-old S1P3-null EDL (27). A common response to the absence of S1P3 is a higher content of type 1 fibers that, only in soleus, is compensated by a decrease in type 2A and 2X fibers. Vice versa, the expression level of S1P1 and S1P2 in S1P3-null EDL muscle is higher than in wild-type EDL muscle but it did not differ in soleus. Moreover, contractile properties of EDL were affected by the absence of S1P3 (for example, lower twitch and tetanic tensions) but were mostly unaffected in soleus (27). Thus, the absence of S1P3 seems to produce some fiber type-dependent differences.

Adult wild-type and S1P3-null mice exhibited similar body weight, soleus muscle mass, and force. Differently, old S1P3-null mice were heavier than old wild-type mice. Wild-type mice usually grow up to approximately 16–18 mo of age and then slowly lose their body mass (62). The larger body mass of old S1P3-null mice was heavier than old wild-type mice, so that the muscle-to-body mass ratio was decreased as others (55, 64), in wild-type and S1P3-null old soleus muscle, both downstream of Akt, did not change in transgenic soleus. Nevertheless, it is not surprising that the absence of S1P3 may perturb Akt signaling. In fact, P-Akt and total Akt increase after denervation in S1P3-null EDL (27). Moreover, it is known that S1P1 and S1P2 have opposite actions on cell proliferation through the stimulation and inhibition, respectively, of signaling pathways, including Akt (60).

Fig. 8. Expression of muscle regeneration protein markers. A: MyoD expression level in 10-day regenerating adult (n = 5) and old (n = 5) WT and adult (n = 4) and old (n = 5) S1P3-null soleus muscle. The expression level refers to that of GAPDH. B: myogenin expression level in 10-day regenerating adult (n = 5) and old (n = 6) WT and adult (n = 5) and old (n = 6) S1P3-null soleus muscle. C: embryonic myosin in 10-day regenerating adult (n = 5) and old (n = 5) WT and adult (n = 4) and old (n = 5) S1P3-null soleus muscle. Top: all plots are examples of the relative Western blots carried out as described in MATERIALS AND METHODS. The expression level refers to that of GAPDH. *P < 0.05, **P < 0.025.

Diminished protein synthesis in acute atrophy is associated with downregulation of Akt/mTOR signaling (3, 6, 7, 43), although even this correlation was not observed in the age-related atrophy of wild-type soleus (56, 64). In agreement, here we found that the level of total Akt and activated P-Akt did not change during aging either in wild-type or in S1P3-null soleus. However, the expression levels of Akt and P-Akt were higher in adult and old S1P3-null soleus than in wild-type soleus, a condition that could contribute to the maintenance of muscle mass in aged transgenic soleus. However, MuRF1 and P-S6, both downstream of Akt, did not change in transgenic soleus. Nevertheless, it is not surprising that the absence of S1P3 may perturb Akt signaling. In fact, P-Akt and total Akt increase after denervation in S1P3-null EDL (27). Moreover, it is known that S1P1 and S1P2 have opposite actions on cell proliferation through the stimulation and inhibition, respectively, of signaling pathways, including Akt (60).

The UPS and the lysosomal-autophagy system are coordinately activated in atrophying muscles (7, 43). We measured the amount of p62, a protein involved in the recognition of autophagy substrates (for example, mitochondria), and the LC3-II/LC3-I ratio, to evaluate the extent of autophagy. At variance with recent reports (11, 65), but in agreement with others (55, 64), in wild-type and S1P3-null old soleus muscle, the LC3-II/LC3-I ratio did not change during aging compared with the ratio in adult-age muscle. During aging, the level of p62 did not change in wild-type soleus, whereas it significantly decreased in old S1P3-null muscle. The reduced level of p62 protein could indicate a reduced autophagy in old S1P3-null soleus, and therefore, a reduced atrophy. However, reduced or defective autophagy are reported to compromise mitochondrial homeostasis (11, 45) and mitochondrial-mediated apoptosis in
old muscles (65), eventually worsening sarcopenia (25). Instead, our results show that mitochondrial homeostasis seems preserved in old S1P3-null soleus, as indicated by SDH staining, constitutive Tom20 protein, and PGC-1α levels that were comparable to those of adult-age muscle. Finally, the expression level of the antiapoptotic Bcl-2, reported to increase in fast but not in slow old rat muscles (53), was similar in adult and old wild-type and S1P3-null soleus.

Another possible element contributing to the reduced sarcopenia in the knockout mice could be the better preservation of the architecture of their neuromuscular junctions. Indeed, it is long known that motor nerve terminals and their interactions with myofibers are greatly altered during aging (14, 15, 62).

Even though we cannot establish whether reduced NMJ deterioration is a cause or an effect of the reduced muscle mass loss in S1P3-null mice, it is, however, conceivable that the improved maintenance of acetylcholine receptor clusters in these animals may result in a more efficient and reliable stimulation of muscle fibers, which is trophic by itself. Obviously, this may in turn trigger a positive feedback toward nerve endings through the release of neurotrophic factors from the muscle fiber, thus leading to an overall endurance of the neuromuscular system. Vice versa, NMJ deterioration in old wild-type soleus could contribute to the progressive atrophy of the muscle, however, without the appearance of small and angular fibers and/or the reduction in total number of muscle fibers, which are clear signs of denervation.

Old S1P3-null soleus was not only protected from age-related loss of mass but also from the loss of force. The literature (1) and our results show a significant drop in absolute force only in old wild-type soleus muscle, mainly attributable to the loss of muscle mass. By contrast, in old S1P3-null soleus, not only was the absolute force unchanged, but it is evident of a significant increase in specific force. The results also show a slowing of contractile properties in old compared with adult wild-type soleus, a change, however, that was not observed in old S1P3-null soleus. Different factors could modulate muscle force development and contraction speed: a greater relative number of slow-type fibers (19, 59), the oxidative damage to proteins involved in cross-bridge cycling (38), or alterations in excitation-contraction coupling (50). It is known that slow fibers have lower specific force than fast fibers (8, 9), so the fast-to-slow shift in old wild-type soleus could be responsible, at least in part, for the reduced force and contraction speed. However, compared with wild-type soleus, adult S1P3-null soleus displays a slower phenotype (more type 1 and fewer type 2A and 2X fibers) but not varied contractile properties, a characteristic that did not change during aging. Slowing of contractile properties could also be due to the changes in the total amount of SERCA isoforms. Because SERCA1 and SERCA2 isoforms have the same enzymatic activity (42), the speed of calcium reaccumulation into the sarcoplasmic reticulum is related to the total amount of SERCA isoforms in a muscle. Our data did not show overt differences in adult muscles, and SERCA1 content was significantly reduced only in old S1P3-null soleus. So, preservation of contractile properties in old S1P3-null soleus does not seem to be related either to a fiber type switch or to a higher content of SERCA isoforms. An additional possibility is that the absence of S1P3 could maintain a more favorable microenvironment in old animals compared with adult animals. Accordingly, myofibrilar protein carbonylation, an index of oxidative stress, was substantially decreased in old S1P3-null soleus. Intriguingly, reactive oxygen species were found to be involved in S1P3-mediated pathological cardiac remodeling in mice (61).

Morphologically, we did not observe a major difference between wild-type and S1P3-null soleus. However, we noted that old wild-type soleus had a higher fibrosis level than old S1P3-null soleus as confirmed by a lower level of CTGF (CCN2) mRNA in the transgenic muscle. Recent findings show that the concentration of collagen and of fibrosis in old mice is almost twice that in young mice (66). Because CTGF expression in adult tissues is a clinically relevant molecular marker of fibrosis (10), this result indicates that a less fibrotic phenotype is maintained in old soleus in the absence of S1P3 receptor. It is worth mentioning that the reduced expression of CTGF in S1P3-null relative lower extent of fibrosis in S1P3-null compared with wild-type old soleus are in agreement with recent data demonstrating a correlation between S1P3 and activation of Yes-associated protein (YAP) (16). YAP is a transcriptional regulator that acts through the TEA domain family (TEAD) transcription factor, a recognized early activator of CTGF expression (68). Therefore, we can hypothesize that the lower extent of skeletal muscle fibrosis in S1P3-null old soleus could be ascribed to decreased YAP/TEAD activation, which is responsible for CTGF expression. Moreover, S1P3 is the dominant signaling receptor upon TGF-β1 challenge and seems to be implicated in the transdifferentiation of myoblasts into myofibroblasts (13). In agreement, the structural analogue of S1P, FTY720 phosphate, has been reported to elicit myofibroblast differentiation of fibroblasts via S1P3, as its effect was abrogated in S1P3-null mice (35). Activation of Smad3 signaling is also important for myofibroblast transdifferentiation (35). However, we did not observe significant changes in the P-Smad3/Smad3 ratio either in old wild-type or in S1P3-null soleus. The absence of S1P3 seems to be beneficial in the age-related accumulation of collagen in old soleus, which is consistent with the finding that S1P3 ablation represses cardiac fibrosis (61).

Regeneration is less efficient or at least delayed in old muscles (4, 30). The lower regenerative capacity of aged muscle is associated with several factors, including the decrease of resident muscle stem cells and/or to a less favorable environment for stem cell activation and function (2, 12). Our data indicate that the absence of S1P3 facilitates the growth of regenerating fibers. Multiple findings support the notion that S1P3 may play a critical role in muscle regeneration. Indeed, quiescent SCs display very high levels of S1P3 compared with proliferating SCs (26), suggesting that the drop in receptor level promotes proliferation. As a confirmation, SCs isolated from young (6–8 wk of age) S1P3-null mice exhibited enhanced proliferative ability and more extensively differentiated into myotubes (26). Moreover, regeneration of tibialis anterior, the fast muscle of young S1P3-null mice, was transiently enhanced compared with that of wild-type mice (26).

During regeneration, MyoD is related to proliferation of SCs, whereas myogenin is related to muscle differentiation. And whereas in young muscles myogenin and MyoD mRNA levels return to baseline after regeneration, in old muscles they remain upregulated (44), suggesting a more prolonged need for myogenic factors in old muscles. Ten-day-old regenerating S1P3-null soleus contains less MyoD and myogenin proteins.
than old wild-type soleus. Therefore, the reduced levels of MyoD and myogenin in old S1P3-null regenerating muscle suggests a more efficient regeneration compared with that of wild-type muscle; however, it is not paralleled by a reduction in embryonic MyHC isoform. Because myogenin increases during muscle denervation (27, 63), the altered functional innervation observed in old wild-type muscle could contribute to the elevated myogenin expression of regenerating muscle.

Sarcopenia is a progressive, multifactorial impairment of skeletal muscle. Present results show that the absence of S1P3 protects soleus from the decrease in muscle mass and force, and attenuation of regenerative capacity, all of which are typical characteristics of aging. The lack of S1P3 seems to modulate the aging process without, apparently, affecting obvious molecular targets, most likely by modulating critical signaling pathways. During aging, the progressive adaptability of soleus muscle to the absence of S1P3 and related signals probably produces a condition that allows a better resistance to sarcopenia. In conclusion, our results identify S1P3 and its signaling as candidate targets for controlling the effects of aging on skeletal muscle. Because various novel molecules have been identified to modulate S1P signaling (60), our findings represent the basis for the development of treatments for sarcopenia.

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