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THE SPHINGOSINE KINASE ACTIVATOR K6PC-5 STIMULATES C2C12 MYOBLAST DIFFERENTIATION

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Previously, K6PC-5, a synthetic derivative of ceramide, was demonstrated to activate sphingosine kinase (SK)-1 in keratinocytes. In this study its potential biological effect in mouse myoblasts was examined. The obtained results show that K6PC-5 promotes myogenic differentiation by enhancing myogenic marker expression, differentiation index and fusion index. Interestingly, its biological action was prevented by pharmacological inhibition of SK or $S1P_2$ receptor, in full agreement with their recognized role in myoblast differentiation. This is the first evidence that pharmacological activation of SK accelerates myogenesis and suggests that this new therapeutic strategy could be possibly employed in skeletal muscle disorders where muscle regeneration is deficient.

The bioactive lipid sphingosine 1-phosphate (S1P) and its endogenous metabolism play a pivotal role in the control of biological processes such as cell proliferation, motility and survival (1-3). We previously highlighted a novel role for S1P in differentiation of cultured myoblasts, demonstrating that via ligation to its receptor $S1P_2$, it enhances the expression of skeletal muscle marker proteins (4). Moreover, we showed that the expression of sphingosine kinase-1 (SK1), responsible for S1P formation within the cells, was enhanced in confluent myoblasts and at early stage of differentiation into myotubes. Interestingly, the blockade of SK1 impaired cell growth arrest and differentiation, whereas its overexpression increased the pace of myogenesis *in vitro* (5). In agreement, stimulation of S1P formation induced by tumor necrosis factor (TNF)-α at low concentration was identified as a molecular mechanism whereby the cytokine exerts its pro-myogenic effect in cultured myoblasts (6). Importantly, S1P was identified to act as a trophic factor for adult skeletal muscle (7) and was also found implicated in skeletal muscle regeneration *in vivo* (8), reinforcing the notion that SK1/S1P axis is critical in many different biological processes that characterize skeletal muscle phenotype.

Recently, synthesized novel ceramide derivatives have been shown to mimic the pro-differentiating effect of S1P in epidermal keratinocytes (9). Interestingly, one of these compounds, which contains a ketone group, two hydroxy groups, two short alkyl groups, and an amide linkage with a chemical name of *N*-(1,3-dihydroxyisopropyl)-2-hexyl-3-oxo-decanamide (K6PC-5) was further

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demonstrated to act as direct activator of SK1. Stimulation with K6PC-5 resulted in terminal differentiation of keratinocytes triggering specific signaling pathways such as intracellular calcium increase, suggesting that it could represent a novel approach for the treatment of skin disorders (10). Additional *in vivo* studies also confirmed the pro-differentiating effects of K6PC-5 in a murine model, mainly through the simulation of S1P signaling (11).

In view of the important role played by SK1/S1P axis in skeletal muscle cell biology and its positive effect on cell differentiation (4;12), in this study the effect of K6PC-5 in mouse myoblasts was investigated.

The results obtained demonstrate that this ceramide derivative is capable of enhancing myogenic differentiation, supporting the view that its employment could be of help also in a number of pathological conditions in which skeletal muscle repair is impaired.

MATERIALS AND METHODS

Materials

Biochemicals, cell culture reagents, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), protease inhibitor cocktail, monoclonal anti-skeletal fast myosin heavy chain (MyHC) (clone MY-32) and bovine serum albumin (BSA) were from Sigma (St. Louis, MO, USA). Mouse skeletal muscle C2C12 cells were obtained from the American Type Culture Collection, (Manassas, VA, USA). SKI-2 (2-(*p*-hydroxyanilino)-4-(*p*11chlorophenyl)thiazole), D-*erythro*-S1P and propidium iodide (PI) were from Calbiochem (San Diego, CA, USA). Monoclonal anti-caveolin-3 was from Transduction Laboratories (Lexington, KY, USA). The specific antagonist of $S1P_2$, JTE 013 (13) was from Tocris Cookson Limited (Bristol, UK). K6PC-5 was synthesized as previously described (9). Antibodies against myogenin (F5D), β-actin (C-11), pan-Akt (H-136), phospho-pan-Akt (Ser 473), anti-mouse, anti-rabbit, and anti-goat immunoglobulin G1 conjugated to horseradish peroxidase and Blotto (non-fat dried milk) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Phospho-ERK1/2 and pan-ERK1/2, phospho-p38 and pan-p38 antibodies were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Uppsala, Sweden). CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay was from Promega (Madison, WI, USA). Fluorescein-conjugated horse anti-mouse secondary antibody was obtained from Vector (Burlingame, CA, USA).

Cell culture

C2C12 mouse myoblasts were grown in DMEM supplemented with 10% FCS. For proliferation experiments, cells were seeded in 96-well plates and used when ~40% confluent. For differentiation experiments, cells were seeded in 60-mm-diameter dishes and, when confluent, were shifted to DMEM without serum containing 1 mg/ml BSA. When appropriate, the specific inhibitor of SK (SKI-2, 10 μM) (14) or the specific $S1P_2$ antagonist JTE 013 (1 μM) were administered to the cells 30 min before agonist addition.

Cell proliferation

Cell proliferation was measured in serum-starved cells challenged with different concentrations of K6PC5 by MTS-dye reduction assay, according to Promega CellTiter 96® Non-Radioactive Cell Proliferation Assay's instructions, as previously described (4).

Western blot analysis

Total cell extracts were prepared and subjected to SDS-PAGE and Western blot analysis, as previously described (4).

Cell immunofluorescence

Cells were seeded on microscope slides, pre-coated with 2% gelatine, and then treated or not with K6PC-5 or S1P. After 72 h cells were fixed in 2% paraformaldehyde in PBS for 20 min and permeabilized in 0.1% Triton X-100-PBS for 30 min. Cells were then blocked in 3% BSA for 1 h and incubated with anti-MyHC antibody for 2 h and fluorescein-conjugated anti-mouse secondary antibody for 1 h. To stain nuclei, the specimen was incubated with 50 μg/ml propidium iodide in PBS for 15 min. Images were obtained using a Leica SP5 laser scanning confocal microscope with 40X objective. To quantify the differentiation and fusion of C2C12 cells after treatments, we calculated the differentiation index as the percentage of MyHC-positive cells above total nuclei and the fusion index as the average number of nuclei in MyHC-positive cells with at least three nuclei above total number of nuclei, respectively.

RESULTS

To establish the biological effect exerted by K6PC-5 in mouse myoblasts, it was initially examined whether this ceramide analogue could

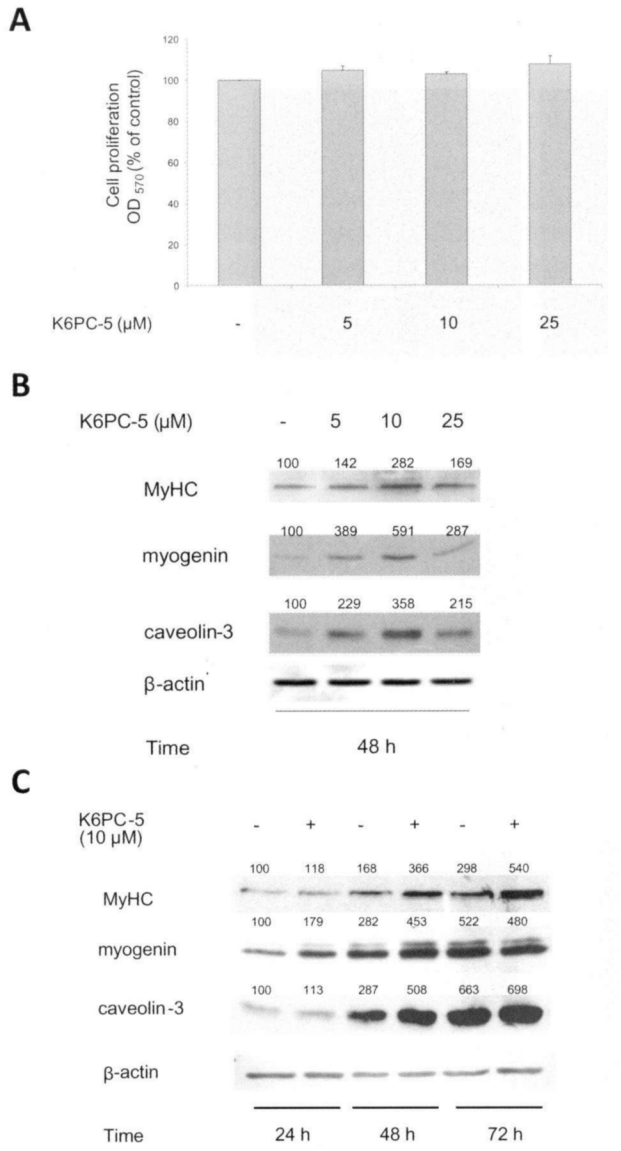

Fig. 1. *Effect of K6PC-5 on C2C12 myoblast proliferation and differentiation. **A)** Cell proliferation of serum-starved C2C12 myoblasts challenged for 48 h with K6PC-5 at the indicated concentrations, was measured by MTS-dye reduction assay. Data are means ± SEM of 3 independent experiments performed in quadruplicate. Confluent myoblasts were incubated for 48 h with (+) or without (-) the indicated concentrations of K6PC-5 (**B**) or for the indicated period of time with (+) or without (-) 10 μM K6PC-5 (**C**). Cell extracts were immunoblotted using specific antibodies. Equally loaded protein was checked by expression of the nonmuscle-specific β isoform of actin. A blot representative of 3 independent experiments with analogous results is shown. Band intensity is reported as percentage relative to the intensity of the band corresponding to the unchallenged control set as 100.*

influence cell proliferation. For this purpose, C2C12 cells, approximately 40% confluent, were treated with different concentrations of K6PC-5 and subjected to MTS-dye reduction assay to evaluate cell number. The data presented in Fig. 1A show that K6PC-5 in the concentration range 5-25 μM did not affect myoblast growth. Similar results were obtained when cell proliferation was evaluated by measuring radioactive thymidine incorporation into DNA (data not shown). The potential action of K6PC-5 was then studied on myoblast differentiation into myotube. To this end, cellular content of skeletal muscle marker proteins such as MyHC, myogenin and caveolin-3, normally absent in immature myoblasts but strongly expressed in differentiated cells, was determined by Western blot analysis. As illustrated in Fig. 1B, K6PC-5 at all the tested concentration (5-25 μM) was capable of enhancing the expression of the three myogenic markers after 48 h of incubation in sub-confluent, serum-starved myoblasts, 10 μM K6PC-5 being the most efficacious concentration, showing approximately three-fold increase of MyHC and caveolin-3 and five-fold increase of myogenin. Based on these results, all the subsequent experiments were performed employing 10 μM K6PC-5. Since myogenic differentiation is a time-dependent process and the chosen protein markers have a different time-course of expression, the transcription factor myogenin being the most precocious and the structural protein caveolin-3 being the latest, we then examined the effect of K6PC-5 on myogenic differentiation at different time-points. Results presented in Fig. 1C show that, in agreement with the release of autocrine differentiation factors (15-16), the marker expression in unchallenged myoblasts was enhanced in a time-dependent manner; moreover, myoblast treatment with 10 μM K6PC-5 increased the expression of the early marker myogenin at 24 and 48 h, that of the late marker caveolin-3 at 48 and 72 h and that of intermediate marker MyHC at all the examined time-points.

To provide further evidence that K6PC-5 exerts a myogenic action, we investigated whether it affects differentiation and fusion of myoblasts. Indeed, after 72 h treatment, differentiation index was augmented by 50% and fusion index increased up to two-fold compared with untreated cells (Fig. 2).

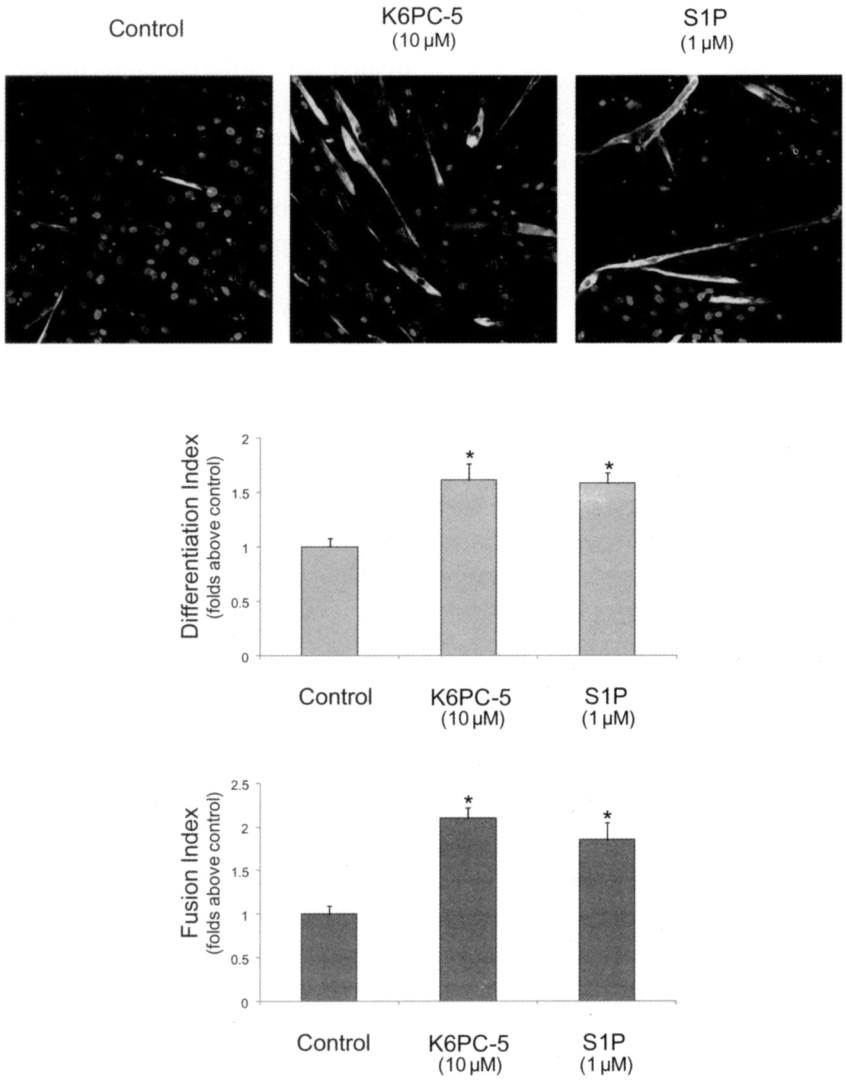

Fig. 2. *Effect of K6PC-5 on C2C12 myoblast differentiation and fusion. Representative immunofluorescences of myoblasts treated for 72 h with 10 μM K6PC-5 or 1 μM S1P are shown. Nuclei were stained with PI, myotubes also with an anti-MyHC antibody. Fusion index and differentiation index were calculated as described in the Methods section. Data are means ± SEM of 4 independent experiments. The effect of K6PC-5 or S1P was statistically significant by Student's t test (*p<0.05).*

Interestingly, in the same figure it is shown that 1 μM S1P, known inducer of myogenic marker expression (4), as expected, exerted a stimulatory effect on differentiation index and fusion index which was superimposable to that elicited by K6PC-5.

With the aim of gaining insight into the mechanism by which K6PC-5 induces its biological effect, the phosphorylation status of key signaling protein kinases such as p38 MAPK, ERK1/2 and Akt was subsequently evaluated. Western analysis of the phosphorylated forms of these protein kinases indicates that the ceramide derivative was responsible for an early and transient activation of p38 MAPK and ERK1/2 which was not detectable after 30 min of incubation (Fig. 3). In contrast, 10 μM K6PC-5 did not influence the phosphorylation

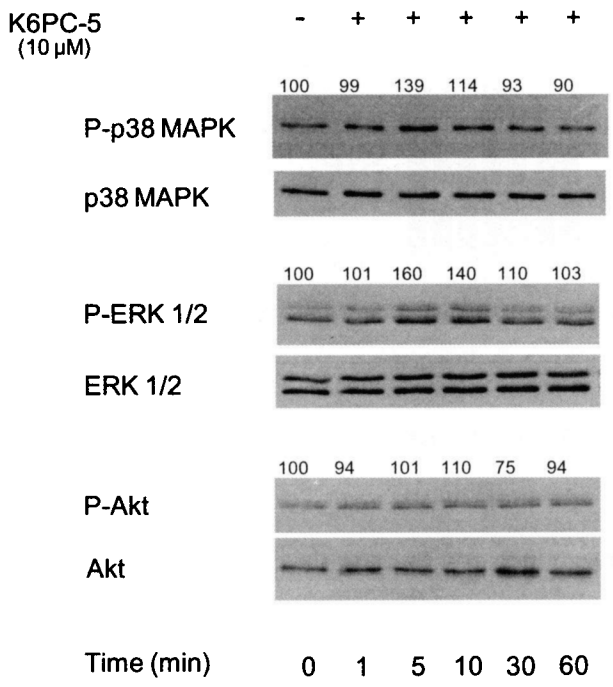

Fig. 3. *K6PC-5 activates p38 MAPK and ERK1/ERK2 in C2C12 myoblasts. Serum-starved confluent myoblasts were incubated with (+) or without (-) 10 μM K6PC-5 for the indicated times. Cell lysates were immunoblotted using specific antibodies. A blot representative of 3 independent experiments is presented. Band intensity corresponding to phosphorylated protein was normalized to its total content and reported as a percentage relative to control (time 0, no addition) set as 100.*

extent of Akt.

Finally, to investigate whether the mechanism by which K6PC-5 induces its pro-myogenic effect involves the regulation of myoblast S1P metabolism, the effect of the ceramide derivative on the myogenic marker expression was examined in myoblasts in which SK was inhibited by prior incubation with the specific inhibitor SKI-2 (10 μM), or $S1P_2$ receptor was blocked by the selective antagonist JTE013 (1 μM). In both these experimental conditions the positive effect of K6PC-5 on the expression of myogenin, MyHC and caveolin-3 was abrogated (Fig. 4) in favour of the hypothesis that it elicits the biological response via stimulation of SK, leading to increase of cellular S1P that, after being released outside the cell, triggers cell differentiation by $S1P_2$ engagement (Fig. 5).

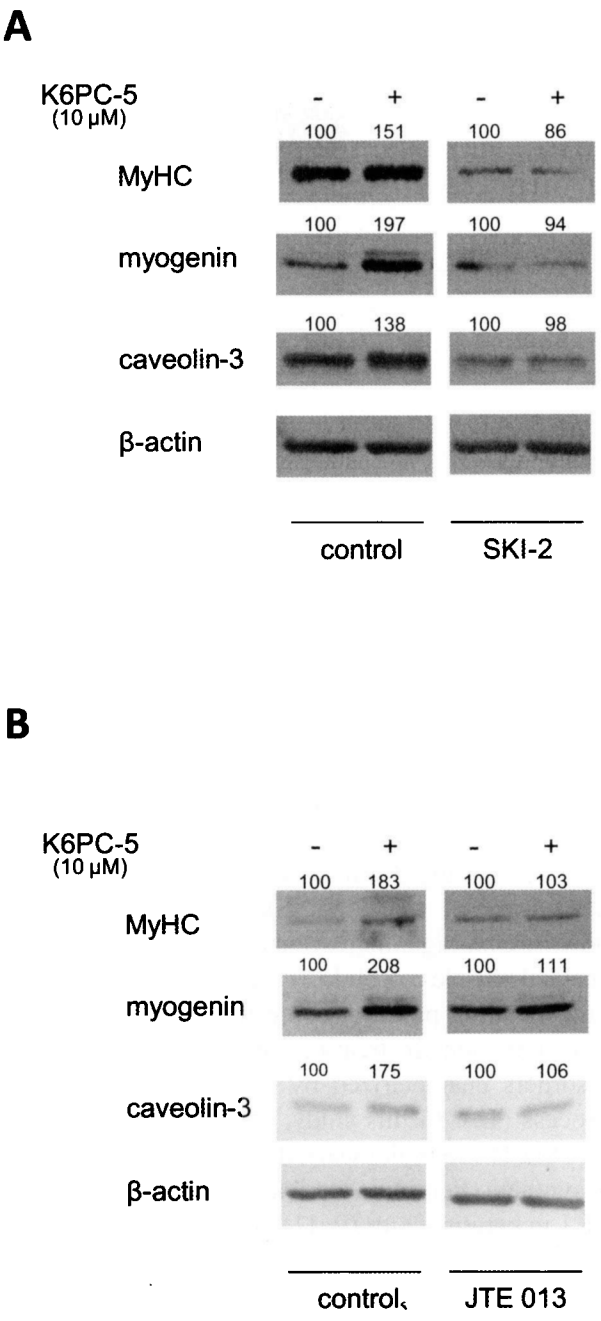

Fig. 4. *Involvement of SK and $S1P_2$ in the action mechanism of K6PC-5. Confluent myoblasts were pre-incubated for 30 min with (**A**) SK specific inhibitor (10 μM SKI-2) or (**B**) $S1P_2$ specific antagonist (1 μM JTE 013) before being challenged with 10 μM K6PC-5 for 48 h. Cell lysates were analyzed as described in the legend to Fig. 1. Blots representative of at least 3 independent experiments are shown.*

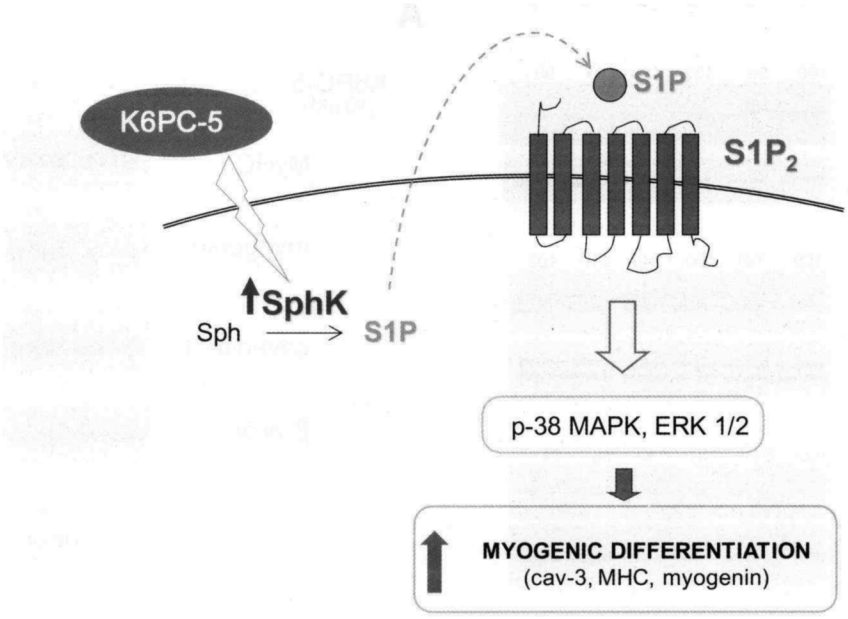

Fig. 5. *Working model of the mechanism by which K6PC-5 elicits myoblast differentiation.*

DISCUSSION

Studies carried out in recent years have clearly demonstrated that the SK/S1P axis and the downstream signaling events play a critical role in skeletal muscle cell biology, suggesting that targeting this signaling pathway could represent a promising new approach for treating multiple skeletal muscle disorders characterized by an altered regeneration process (12). In this study, we examined in mouse myoblasts the biological effect exerted by K6PC-5, a synthetic ceramide derivative known to activate SK1 in keratinocytes (9, 11), demonstrating that also in this cell type it activates SK, since its biological activity was fully abolished by cell treatment with SKI-2, a selective inhibitor of SK. More importantly, here we provide evidence that the pharmacological action of this compound is capable of fully mimicking the positive effect exerted by exogenous S1P on myoblast differentiation, since it enhanced the expression of known myogenic markers such as myogenin, MyHC and caveolin-3, similarly to that previously shown in response to S1P treatment (4) and, equally to S1P, significantly augmented two key parameters such as differentiation index and fusion index. In further agreement with S1P action in myoblasts, K6PC-5 did not influence *per se* cell proliferation. Moreover, here a new clue is added to the mechanism by which K6PC-5 brings about its effect. Indeed, $S1P_2$ receptor antagonism performed with the specific compound JTE013 prevented its biological action, thus demonstrating that S1P formed upon stimulation of SK by K6PC-5, subsequently to its release outside the cell, was able to trigger myoblast differentiation via interaction with $S1P_2$, in complete analogy to what was observed upon delivery of S1P to mouse myoblasts (4). Although this finding could appear simply confirmatory, this issue is not trivial since the intracellularly-formed S1P can elicit its biological response in a complex manner, acting either as intracellular mediator capable of directly modulating activity/functionality of key proteins such as histone deacetylases (17) or, subsequently to its export in the extracellular environment, as ligand of a family of five specific G-protein coupled receptors named S1P receptors (18). It has been previously shown that intracellular S1P generated in myoblasts after serum deprivation or in response to low concentration of TNFα induced myoblast differentiation by ligation to $S1P_2$ (5, 6), in full agreement with the mechanism by which exogenous S1P acts. At variance, intracellular S1P

formed in myoblasts in response to PDGF, acts through a similar inside-out mechanism but engages exclusively $S1P_1$, thus mediating inhibition of cell proliferation and not cell differentiation (19). This different outcome appears to be attributable to the regulation by PDGF of a discrete pool of SK1 that can favour the export of S1P outside cell membrane in close proximity of $S1P_1$, which although highly expressed in these cells is unresponsive to the selective agonist SEW2871, probably due to its peculiar membrane topology (20). Therefore, the pro-myogenic effect described here of K6PC-5 exerted via $S1P_2$ transactivation indicates that the activation of SK by the compound involves the same pool of enzyme that accounts for the physiological process of myoblast differentiation initiated by serum deprivation, that enables the engagement of $S1P_2$, essential for this biological response.

In this study, the signaling pathways triggered by K6PC-5 were also investigated. Interestingly, p38 MAPK and ERK1/2 were found to be transiently activated by this compound. Indeed, p38 MAPK activation by S1P was reported crucial for its pro-myogenic effect, while ERK1/2 was described to be involved in the cell cycle arrest caused by the bioactive lipid (4), therefore the present findings are fully coherent with a signaling process initiated by K6PC-5-induced S1P formation. However, K6PC-5 treatment, differently from exogenous S1P (4), did not provoke Akt activation. Although this signaling pathway was found marginally implicated in the pro-myogenic effect of S1P, this discrepancy could be explained taking into account that Akt is only partially coupled to $S1P_2$ in these cells (4), whereas the signaling of S1P formed in response to K6PC-5 is very likely strictly $S1P_2$-dependent, being the biological response fully abolished by blockade of $S1P_2$.

The present disclosure of an activator of SK1 as inducer of myoblast differentiation supports the notion that the signaling axis of SK/S1P is crucial for the accomplishment of mature phenotype of skeletal muscle cells. Moreover, it represents the first evidence that its pharmacological modulation can be pursued to accelerate this biological process. These promising results obtained in isolated cells *in vitro* solicit further investigation of this issue in regenerating skeletal muscle *in vivo*. Indeed, S1P formation inside cells is an ubiquitous event and by virtue of interacting with different receptors, this lipid is endowed with multiple properties, also opposite in different cells (21, 22). For this reason it will be important to examine the consequences on tissue repair of broad SK activation at the site of muscle lesion caused by pharmacological activators, since it could not only positively influence satellite cells, by promoting their differentiation, but also affect the functioning of various types of inflammatory cells that are known to actively participate in skeletal muscle healing.

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