Activation of the ATX/LPA/LPARs axis induces a fibrotic response in skeletal muscle

Adriana Córdova-Casanova [a,b], Meilyn Cruz-Soca [a,b], Jerold Chun [c], Juan Carlos Casar [d] and Enrique Brandan [a,b,e]

a - Centro de Envejecimiento y Regeneración, *CARE Chile UC, Santiago, Chile*
b - Departamento de Biología Celular y Molecular, Facultad de Ciencias Biológicas, *Pontificia Universidad Católica de Chile, Libertador Bernardo O'Higgins 340 Code, Santiago 8331150, Chile*
c - Sanford Burnham Prebys Medical Discovery Institute, *La Jolla, CA, United States*
d - Departamento de Neurología, *Pontificia Universidad Católica de Chile, Santiago, Chile*
e - Centro Científico y Tecnológico de Excelencia Ciencia & Vida, *Santiago, Chile*

Corresponding to Enrique Brandan: *Departamento de Biología Celular y Molecular, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Libertador Bernardo O'Higgins 340 Code, Santiago 8331150, Chile.*
ebrandan@bio.puc.cl.
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Abstract

Several common chronic diseases, muscular dystrophies (MDs), and aging lead to progressive fibrous connective tissue (fibrosis) accumulation in skeletal muscle. Cumulative past evidence points to the role of signaling lipids such as lysophosphatidic acid (LPA) and its receptors (LPARs) in different models of fibrosis. However, the potential contribution of these molecules to the fibrotic process in skeletal muscle has not been explored. Here, we show the expression of ATX/LPA/LPARs axis components in skeletal muscle, which suggests their potential relevance for the biology of this tissue. We investigated if the skeletal muscle responds to the stimulus of intramuscular (IM) LPA injections, finding an early induction of the pro-fibrotic factor connective tissue growth factor/Cellular Communication Network factor 2 (CCN2) and extracellular matrix (ECM) proteins. Also, we found that LPA induces an increase in the number of fibro/adipogenic progenitors (FAPs), which are the primary cellular source of myofibroblasts. These effects were for the most part prevented by the inhibitor Ki16425, which inhibits the LPA receptors LPA_1 and LPA_3, as well as in the LPA_1-KO mice. We also evaluated the *in vivo* activation of extracellular signal-regulated kinases (ERK 1/2), AKT, c-Jun N-terminal kinase (JNK), and Yes-asocciated protein 1 (YAP) in response to LPA. Our results show that LPA induces ERK 1/2 phosphorylation in WT muscle, but not in LPA_1-KO mice. Treatment with the ERK 1/2 inhibitor U0126 prevented the induction of fibronectin in response to LPA, suggesting that this pathway is involved in LPA-induced fibrosis. Altogether, these results demonstrate that ATX/LPA/LPARs constitute a pro-fibrotic axis and suggest a possible role in muscular diseases.

Introduction

Fibrosis is commonly described as the accumulation of extracellular matrix (ECM) in a tissue due to an imbalance between its synthesis and degradation rates [1,2]. This pathological condition is associated with many chronic diseases affecting various organs and tissues, including the skeletal muscle. Muscular dystrophies (MDs) are a group of degenerative muscular disorders characterized by the progressive development of fibrosis, a feature which by itself affects the cellular microenvironment and physiology [3,4]. Fibrosis contributes to the loss of muscle strength in MDs and other diseases, and it is currently well established that fibrosis reduction can improve muscle function [5–8]. Therefore, exploring the molecules and mechanisms underlying the development of fibrosis may

contribute to finding relevant therapeutic targets for this group of diseases.

Throughout the years, the study of fibrosis was focused on numerous proteins signaling factors such as transforming growth factor type β (TGF-β), platelet-derived growth factor (PDGF), connective tissue growth factor/Cellular Communication Network factor 2 (CCN2), and interleukins [9−13]. However, it has been proposed that signaling lipids could also participate in the establishment and maintenance of fibrosis in different organs, such as skin, liver, heart, and lung [14−21]. Lysophosphatidic acid (LPA) is among the best-studied lipid factors involved in fibrosis. Structurally, LPA is composed of a glycerol backbone, a phosphate head group, and a fatty acid chain that varies in length, position, and degree of unsaturation, representing different LPA species such as oleoyl LPA 18:1 one of the best studied [22]. LPA is mainly synthesized by the secreted lysophopholipase D enzyme autotaxin (ATX) that removes the choline group from lysophosphatidylcholine (LPC); LPA is degraded by different lipid phosphate phosphatases (LPPs 1-3), enzymes that generate the non-signaling lipid monoacylglycerol by removing its phosphate group [22,23]. LPA signals through 6 protein G-coupled receptors, called LPA_1 to LPA_6 [24−28]. Depending on their functional relevance, these receptors are differentially expressed in tissues, and their expression pattern may change under pathological conditions [29−31]. All these components are grouped in the ATX/LPA/LPARs axis. LPA activates many signaling pathways, involving molecules like AKT, ERK 1/2, and JNK [32−34], leading to cellular responses such as proliferation, differentiation, and survival [32]. Interestingly, the ATX/LPA/LPARs axis has been linked to the development of fibrosis in different organs (kidney, lung, and skin) since the pharmacological inhibition or genetic ablation of different LPA receptors results in a decrease in ECM accumulation in models of induced fibrosis [21,35,36]. We hypothesized that the ATX/LPA/LPARs axis might be involved in the inflammatory and fibrotic response observed in different skeletal muscle diseases [37].

The myofiber, a large multinuclear cell, is the functional contractile unit of mature skeletal muscle. Like in all tissues, other cell types are required to support the main functional ones. Satellite cells, endothelial cells, fibroblasts, and fibro/adipogenic progenitors (FAPs), among others, belong to this category [38,39]. FAPs are CD31−, CD45−, Sca1+, PDGFRα+ cells [40,41] that play a relevant role in developing fibrosis in MDs, muscle damage, denervation, and muscle regeneration [12,42−44]. Their name describes their ability to differentiate into adipogenic or myofibroblast lineages, being the latter their predominant progeny in fibrotic tissues. Myofibroblasts are the main ECM producing cells, so inhibiting their proliferation and/or differentiation could be critical for fibrosis prevention. To elucidate the mechanisms or molecules that determine myofibroblast number (survival, proliferation, cell death) in the tissue is a necessary step towards this aim.

Here we show that the ATX/LPA/LPARs axis components are present in different skeletal muscles at the mRNA and protein levels. LPA_1 and LPA_6 are the most expressed LPA receptors in all analyzed muscle types. We also report that skeletal muscle responds to LPA by inducing the expression of canonical proteins of the fibrotic response, such as CCN2, fibronectin, and collagens and the early phosphorylation ERK. Interestingly, we found that LPA triggers an increase in the number of FAPs and promotes their differentiation into myofibroblasts rather than into adipogenic cells. On the other hand, the use of the LPA_1 and LPA_3 inhibitor Ki16425, and the genetic absence of LPA_1 in a KO model [45,46], prevent partially the fibrotic response induced by LPA in skeletal muscle. These results suggest that LPARs or at least LPA_1 could be part of the pathophysiology of fibrosis in some diseases affecting skeletal muscle.

Results

Components of the ATX/LPA/LPARs axis are expressed in skeletal muscle

We explored if the skeletal muscle expresses ATX/LPA/LPARs axis components and if this axis respond to LPA by inducing a fibrotic response. As shown in Fig. 1A, LPA is synthesized from LPC by the ATX enzyme, signals through 6 receptors, and is degraded by LPPs. Our RT-qPCR results show that mRNAs for all these enzymes are present in skeletal muscle, being more abundant in the diaphragm (DIA) (Fig. 1B). We also studied the presence of LPARs mRNA using the same technique. A similar expression pattern was observed for LPARs mRNA in the three muscles analyzed, with LPA_1 and LPA_6 as the most abundant (Fig. 1C, qPCR products are shown in supplementary Fig. 1A). When we analyzed the expression of these receptors in other tissues such as the liver a different pattern was observed, as previously described [47] (Supplementary Fig. 1B). In the liver, higher levels of LPA_6 than LPA_1 mRNA were observed [47], while in the muscle, LPA_6 mRNA levels were similar to LPA_1 levels, and even lower in the DIA. The LPA_3-LPA_5 receptors show significant lower levels than LPA_1. Unfortunately, we were unable to detect LPA_1 protein levels through immunoblotting because of the difficulty in finding specific antibodies. LPA_3 and LPA_6 were detected in immunoblots

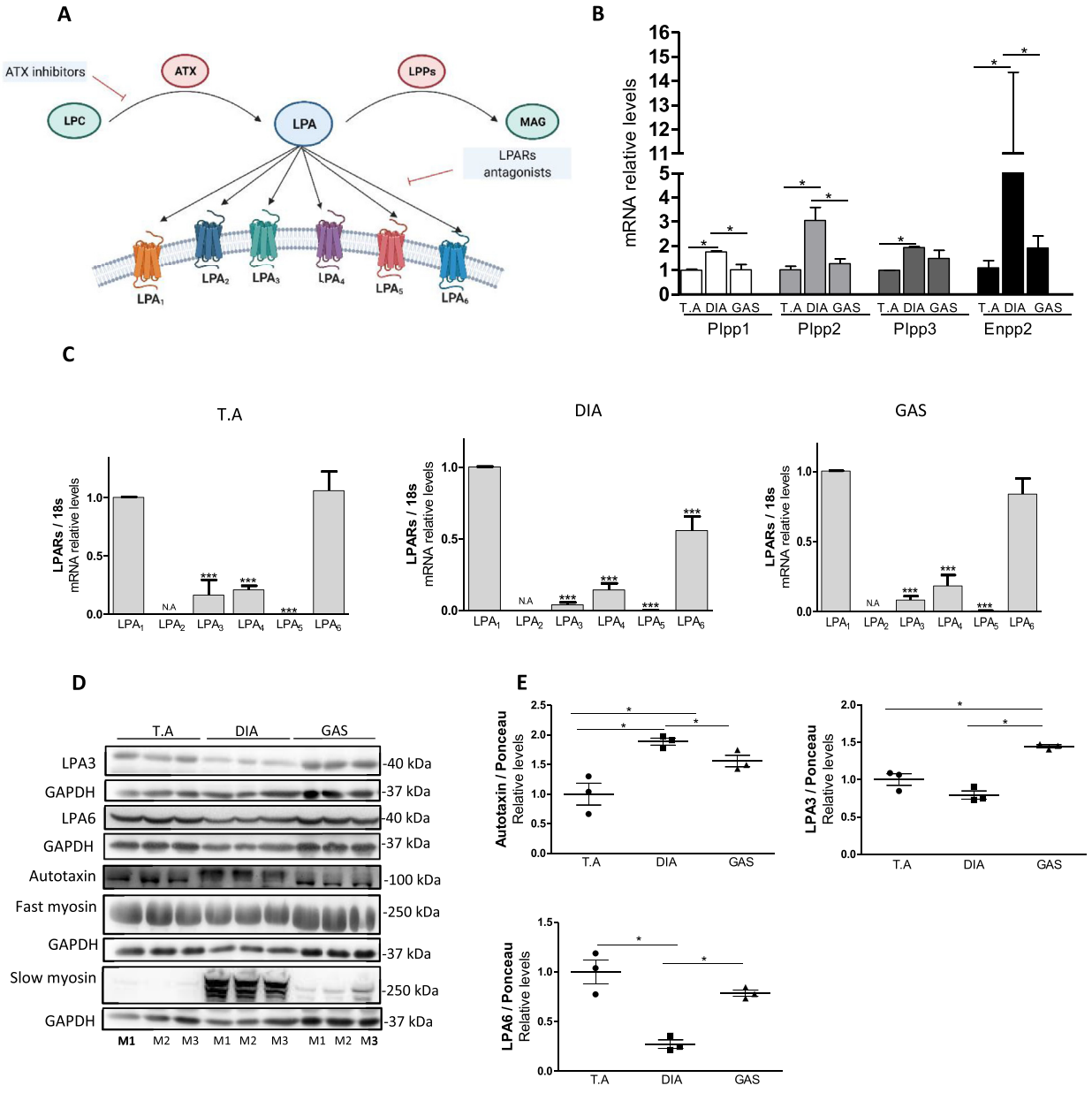


Fig. 1. Presence of ATX/LPA/LPARs axis components in the skeletal muscle. (A) Lysophosphatidylcholine (LPC) is converted to LPA by autotaxin (ATX). LPA signals through 6 receptors called LPA_1 to LPA_6. LPA is degraded by different lipid phosphate phosphatases (LPPs). The diagram was created with BioRender.com. (B) Plpp 1-3 (LPPs 1-3) and Enpp2 (ATX) mRNA levels were analysed by RT-qPCR in WT tibialis anterior (T.A), diaphragm (DIA), and gastrocnemius (GAS) muscles. (C) $LPA_{1\text{-}6}$ mRNA levels were analysed by RT-qPCR in T.A, DIA, and GAS muscles from WT mice, LPA_2 mRNA was not analyzed (N.A). LPARs levels were normalized to LPA_1 expression. 18s was used as reference gene. Statistical comparisons were made against LPA_1 (D) ATX, fast and slow myosin, LPA_3, LPA_6, and GAPDH protein levels were analysed by immunoblot in T.A, DIA and GAS muscles. GAPDH was used as the loading control. (E) Quantification of protein levels in D. M refers to mouse. $^{***}P < 0{,}001$, $^{*}P < 0.05$ by one-way ANOVA with Tukey's post-test; $n = 3$.

and their protein levels were higher in tibialis anterior (TA) and gastrocnemius (GAS) than in DIA (Fig. 1D and E); The protein expression pattern of ATX seemed to have a different distribution with higher expression in the DIA (Fig. 1D and E). We detect slow and fast myosin as a control of the muscles studied. The presence of ATX//LPA/LPARs axis components in skeletal muscle raises questions about their possible functional role in this tissue.

LPA induces the expression of CCN2 in skeletal muscle

We evaluated the levels of CCN2, a hallmark of fibrosis. T.A were injected with LPA and the muscles were extracted 4 or 24 h later (Fig. 2A). After LPA treatment, we analyzed CCN2 mRNA levels and found a statistically significant increase at 4 (Fig. 2B) and 24 h (Fig. 2C). This result led us to evaluate CCN2 protein levels revealing that muscles showed a dose-dependent increase in CCN2 4 h after LPA injection (Fig. 2D and E). The levels of CCN2 were also evaluated by indirect immunofluorescence (IIF) in T.A cross-sections 4 h after LPA injection. We observed a notorious increase in the signal corresponding to CCN2 in the interstitial space of the muscles injected with LPA (Fig. 2F). These results indicate that the pro-fibrotic factor CCN2 increases in skeletal muscle in response to LPA injection. To confirm our results, we injected T.A with different doses of LPARs agonist 2S-OMPT. Like LPA, 2S-OMPT induced an increase in protein levels of CCN2 (Supplementary Fig. 2A, B and C)

The skeletal muscle responds to LPA by inducing ECM components

After finding that LPA increases the pro-fibrotic factor CCN2 we evaluated the expression of ECM molecules such as collagen type 3, fibronectin and periostin. We found increased mRNA levels for periostin and fibronectin (Fig. 3A) at 4 or at 24 h of treatment with LPA. Fibronectin protein levels were analyzed by immunoblot and a significant increase was detected 4 h after LPA injection, which was maintained for at least 24 h after treatment (Fig. 3B and C). We used IIF and Sirius red staining on tissue sections to evaluate the fibronectin and total collagen content, respectively. We found that LPA injection increases fibronectin (Fig. 3D) and total collagen accumulation (Fig. 3E), evidencing an incipient fibrotic process. In order to confirm that the LPA pro-fibrotic effect is not due to muscular cells death, we analized the protein levels of active caspase 3 to detect apoptosis. We did not find statistical differences between BSA, and LPA treated muscles at 4 h, however, we detect a strong diminution after 24 h of LPA injection (Supplementary Fig. 3A, B), this is in accordance with previous report that shown the pro-survival effect of LPA [48–50]. We also quantified myonecrosis, by analyzing IgG uptake by myofibers (loss of sarcolemma integrity). We observed that BSA and LPA induce myonecrosis, but we did not find significant differences between both conditions (Supplementary Fig. 3C). It is possible that this damage to sarcolemma is due to the intramuscular injection (IM) or to the use of BSA as vehicle of LPA. We conclude that LPA injection into skeletal muscle induces the expression of ECM components.

Pharmacological inhibition of $LPA_{1/3}$ prevents the fibrotic response to LPA in skeletal muscle

To elucidate if the fibrotic response of skeletal muscle to LPA is mediated by LPARs we treated mice intraperitoneal (I.P) with Ki16425, a widely used inhibitor of LPA_1 and LPA_3, daily for three days before the intramuscular (IM) T.A injection with LPA (Fig. 4A). We extracted the muscles 4 h later and analyzed the levels of the fibrotic markers. As shown in Fig. 4B, C and D, the use of Ki16425 prevents the induction of CCN2 and fibronectin response to LPA. We evaluated tisular IgG levels as an inflammation marker in response to LPA injections and Ki16425. We observed that LPA increased skeletal muscle IgG content and Ki16425 blocked this effect (Fig. 4B and E). IIF analyses confirmed the inhibition of the CCN2 and fibronectin induction response on the histological level (Fig. 4F and G). These results indicate that LPA favors an early pro-fibrotic response in skeletal muscle, signaling through LPA_1 and/or LPA_3.

Role of LPA_1 in the fibrotic response induced by LPA

Considering that: (i) LPA_1 is one of the most studied LPA receptors in the development of fibrosis in organs such as kidney, lung, and skin [21,35,36]; (ii) LPA_1 is one of the most abundantly expressed LPA receptors in skeletal muscle (Fig. 1C); and (iii) LPA_1 is targeted by Ki16425 (Fig. 4), we challenged LPA_1-KO mice with intramuscular LPA treatment and evaluated the fibrotic response after 4 h. We first confirmed the absence of LPA_1 mRNA in the skeletal muscle of LPA_1-KO mice (Fig. 5A). The expression of other LPA receptors showed a high variability among the studied LPA_1-KO mice, and despite a possible upward trend in their expression, no statistically significant difference was detected as compared to WT (Supplementary Fig. 4).

We evaluated the response to LPA injection on CCN2 and fibronectin mRNA levels. We found that the genetic ablation of LPA_1 seems to have prevented the LPA-induced increase of CCN2 without affecting fibronectin mRNA levels (Fig. 5B and C). We found a decrement in fibronectin and CCN2 protein levels in the LPA-treated muscles from LPA_1-KO mice as compared to LPA-treated WT mice (Fig. 5D and E). We analyzed the content and distribution of CCN2 and fibronectin in LPA-treated skeletal muscle sections by IIF with similar results: a decreased response to LPA was observed in LPA_1-KO mice compared to WT mice (Fig. 5F, G). These results suggest a role for LPA_1 in establishing muscle fibrosis induced by LPA.

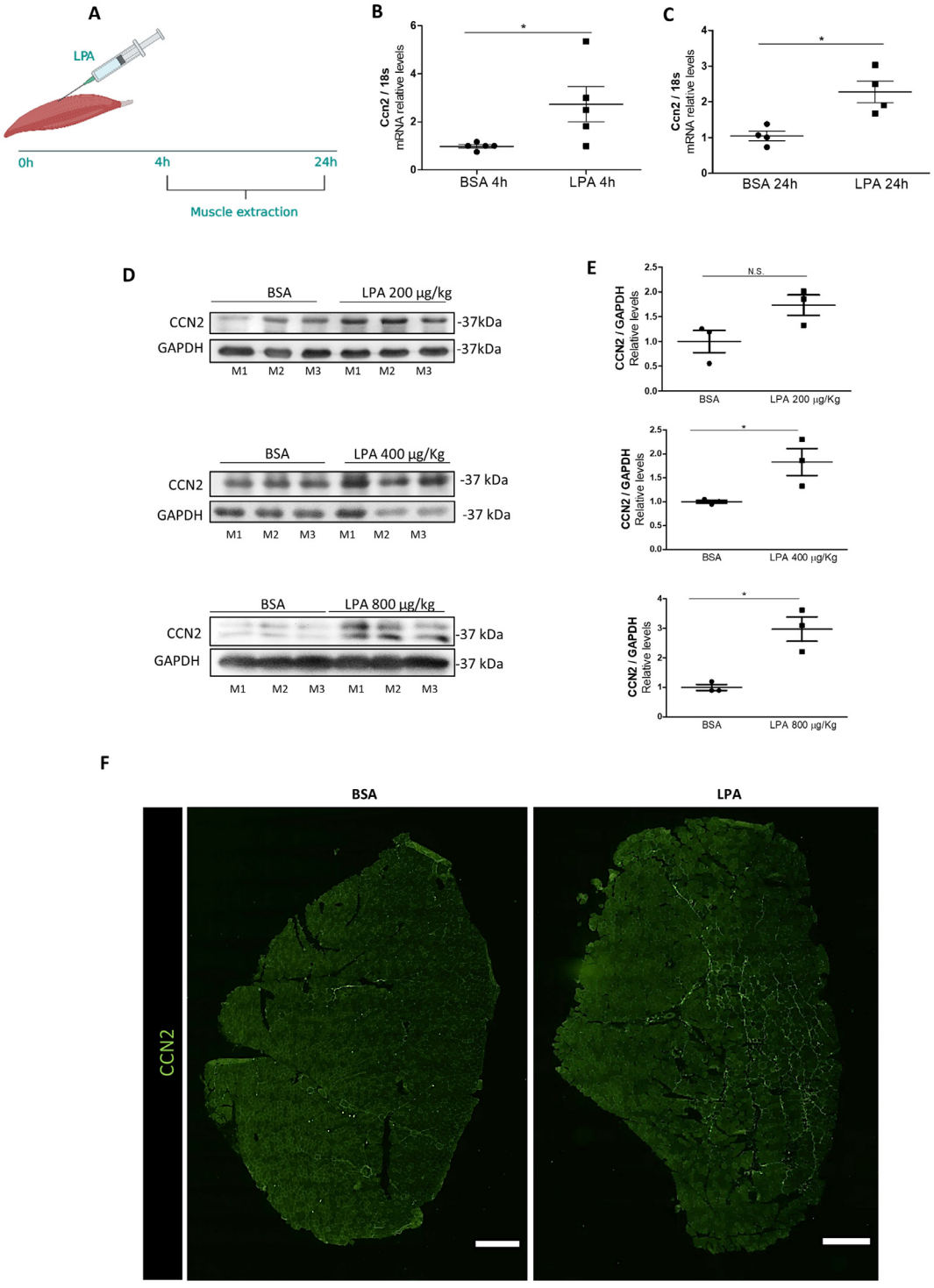


Fig. 2. LPA induces CCN2 expression in skeletal muscle. (A) Experimental design diagram created with BioRender.com. Briefly, LPA was injected in T.A and the muscles were extracted at 4 or 24 h later. (B) Gene expression of Ccn2 at 4 and (C) 24 h after LPA injection. (D) CCN2 protein levels 4 h after 200, 400 and 800 µg/Kg of IM LPA injection. (E) Quantification of D. (F) Representative CCN2 immunofluorescence of complete transversal section of LPA and BSA injected T.A muscles. Scale bar, 500 µm. M refers to mouse. N.S not significant, * $P < 0.05$ by two-tailed Student's *t*-test. $n = 3$.

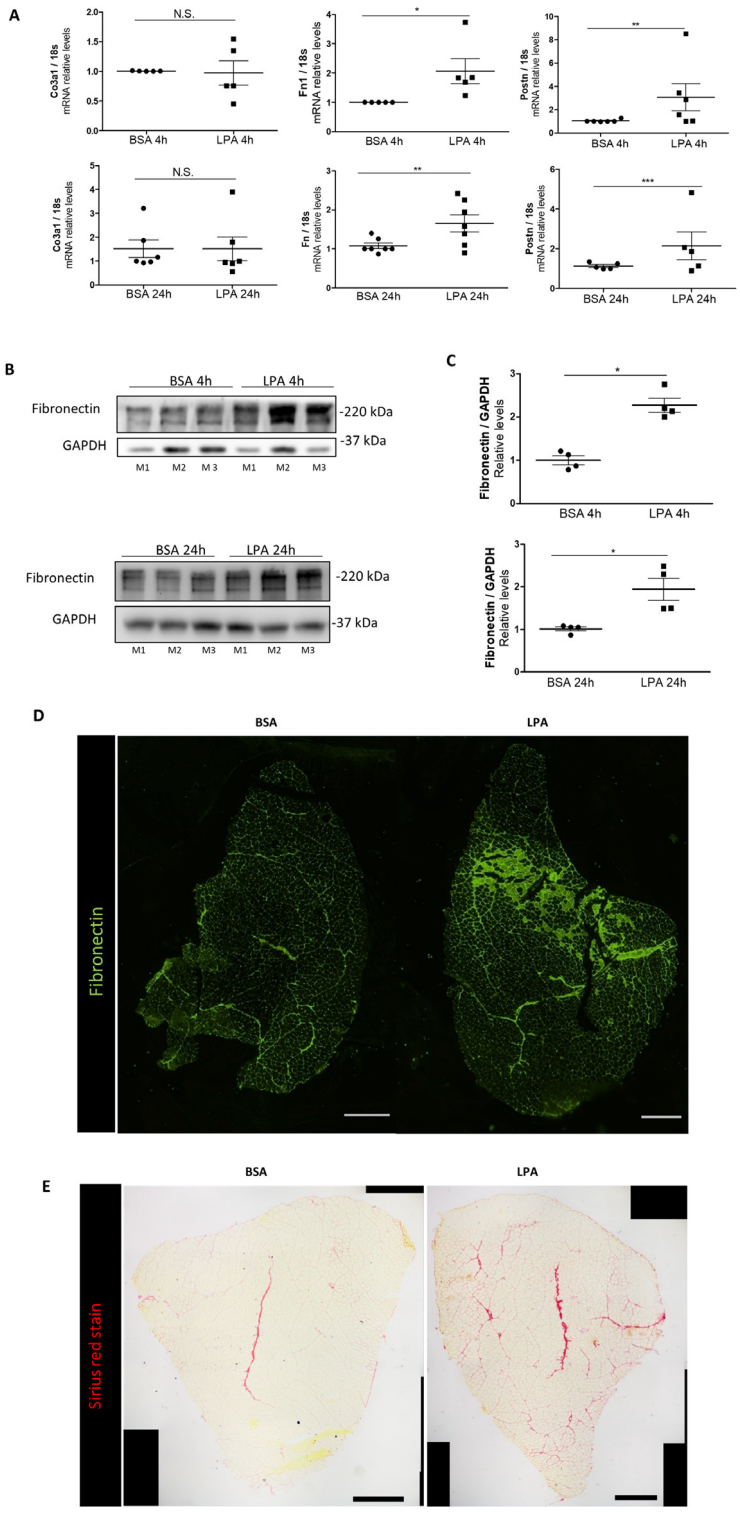


Fig. 3. The skeletal muscle induces ECM accumulation in response to LPA. (A) mRNA expression levels of Col 3a1 (collagen 3), Fn1 (fibronectin), and Postn (periostin) in T.A muscle injected with BSA or LPA 400 μg/Kg for 4 h or 24 h, obtained by RT-qPCR analysis. B) Fibronectin protein levels were analyzed by immunoblot in T.A from mice injected with BSA or LPA (400 μg/Kg IM) 4 h (top) and 24 h (bottom) before analysis. GAPDH was used as the loading control. (C) Quantification of fibronectin protein levels. (D) Representative epifluorescence images of fibronectin in T.A muscle 4 h after injection with BSA or LPA 400 μg/Kg. Scale bar, 500 μm. (E) Representative images of Sirius red staining in T.A muscle 24 h after being injected with BSA or LPA 400 μg/Kg. Scale bar, 500 μm. M refers to mouse. $^{**}P < 0.01$, $^{*}P < 0.05$, N.S not significant, by two-tailed Student's *t*-test. $n = 3$.

LPA induces ERK phosphorylation through LPA_1

To explore some of the early signaling pathways involved in the skeletal muscle fibrotic response mediated by LPA, the phosphorylation of well-studied proteins involved in LPA signaling and the induction of CCN2 was assessed in WT and LPA_1-KO mouse skeletal muscles early after LPA treatment. We studied the levels of pERK, pJNK, pAKT, and pYAP [33,34,51,52] in T.A muscles obtained 10 min after LPA injection. We found a significant increase in ERK phosphorylation in WT skeletal muscle, but we did not find statistically significant changes in pAKT, pJNK or pYAP (Fig. 6A and B). In the absence of LPA_1 the phosphorylation of ERK was partially prevented (Fig. 6A and B). This result led us to evaluate the effect of the ERK pathway inhibitor, U0126 in the LPA response. mRNA and protein levels of previously analyzed genes were studied at a 4 h time-point in LPA-injected WT skeletal muscle from mice previously treated with U0126 or vehicle (I.P) for three days. The inhibition of this pathway prevented the increase of fibronectin but no CCN2 protein levels by LPA (Fig. 7A, B and C). IIF studies of CCN2 and fibronectin content in T.A cross-sections corroborate the pattern of response observed by immunoblot (Fig. 7D, E). These results indicate that the levels of phosphorylated ERK increase early in response to LPA in skeletal muscle and that the inhibition of the ERK pathway partially prevents the fibrotic response induced by LPA injection.

LPA increases the number of FAPs in skeletal muscle, but inhibition of LPA_1 and LPA_3 does not prevent it

FAPs correspond to a PDGFRα-expressing mesenchymal progenitor cell population in skeletal muscle that is critical in the fibrotic process [42]. To investigate whether the skeletal muscle can respond to LPA by increasing the number of FAPs, we injected T.A with LPA or BSA and analyzed PDGFRα protein levels (the most widely used marker of FAPs) after 4 and 24 h. We found no significant difference after 4 h of LPA IM injection (Fig. 8A and C), but PDGFRα protein levels were increased after 24 h (Fig. 8B and D). To study whether the elevated PDGFRα protein levels were a consequence of an increase in the number of FAPs, we used the $PDGFR\alpha^{H2BEGFP}$ knock-in reporter

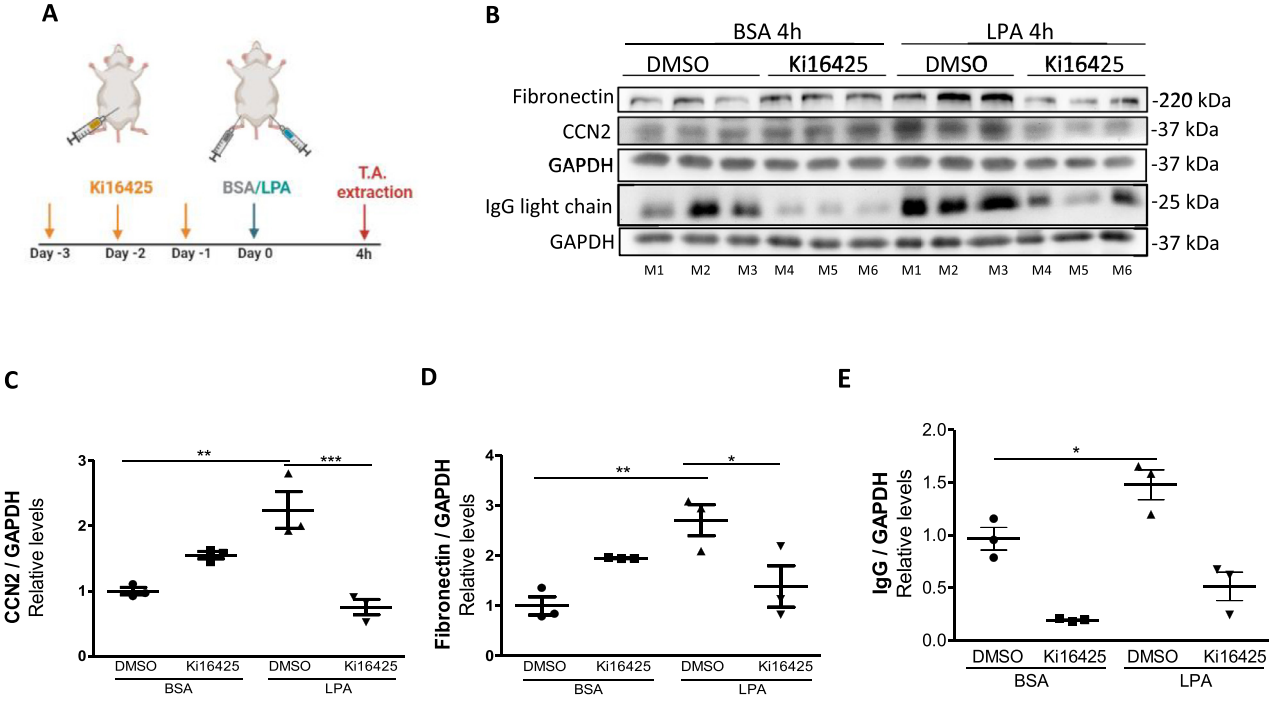
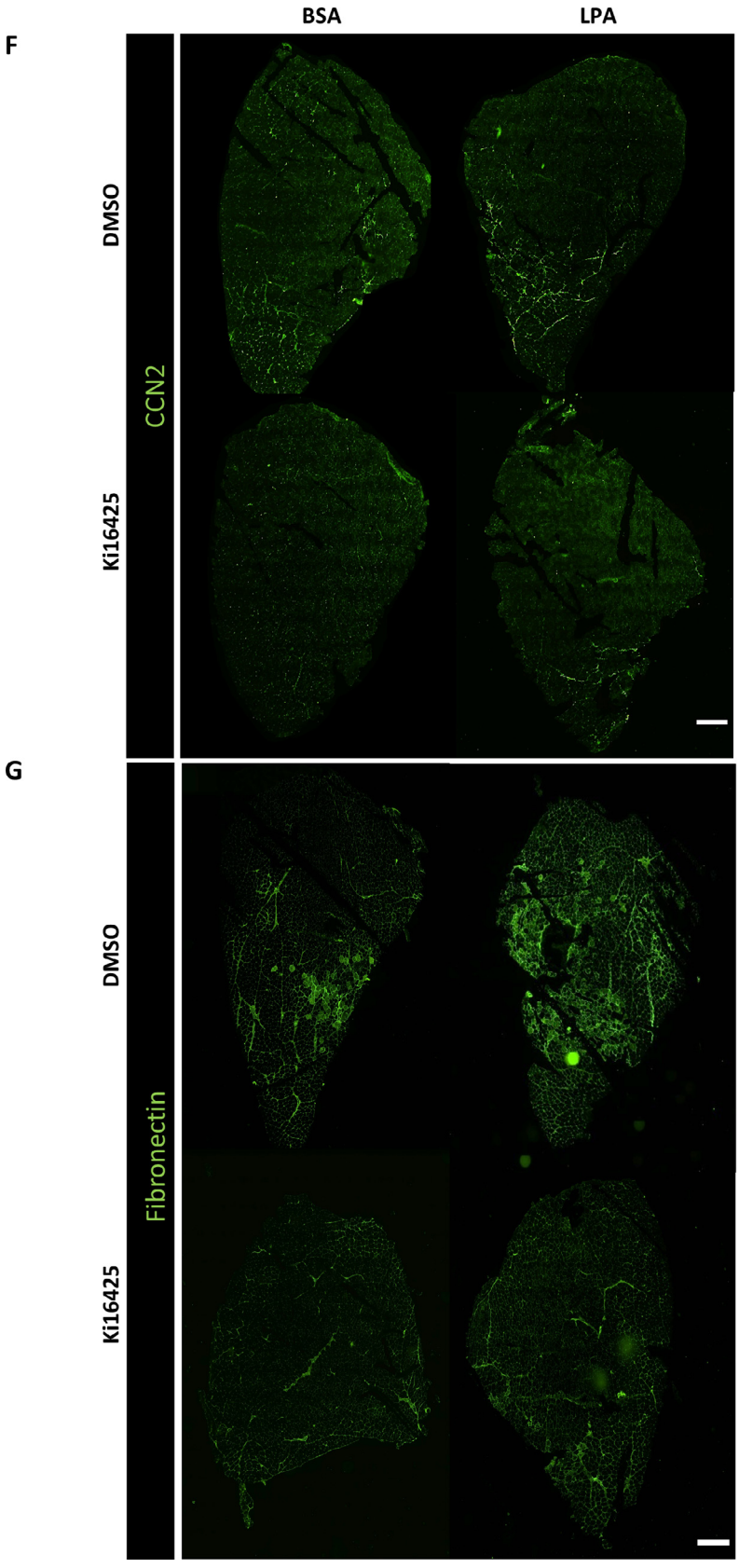


Fig. 4. Pharmacological inhibition of $LPA_{1/3}$ prevents the skeletal muscle fibrotic response to LPA. (A) Treatment plan, male mice were treated with Ki16425 5 mg/Kg or vehicle (DMSO) daily for 3 days and 40 min before BSA and LPA injection (for 4 h). (B) Fibronectin, CCN2, IgG protein levels were analyzed by immunoblot. GAPDH was used as the loading control. (C, D and E) Quantification of protein expression. (F) Representative epifluorescence images of CCN2 and (G) Fibronectin in T.A muscle. Scale bar 500 μm. M refers to mouse. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ by one-way ANOVA with Tukey's post-test; $n = 3$.



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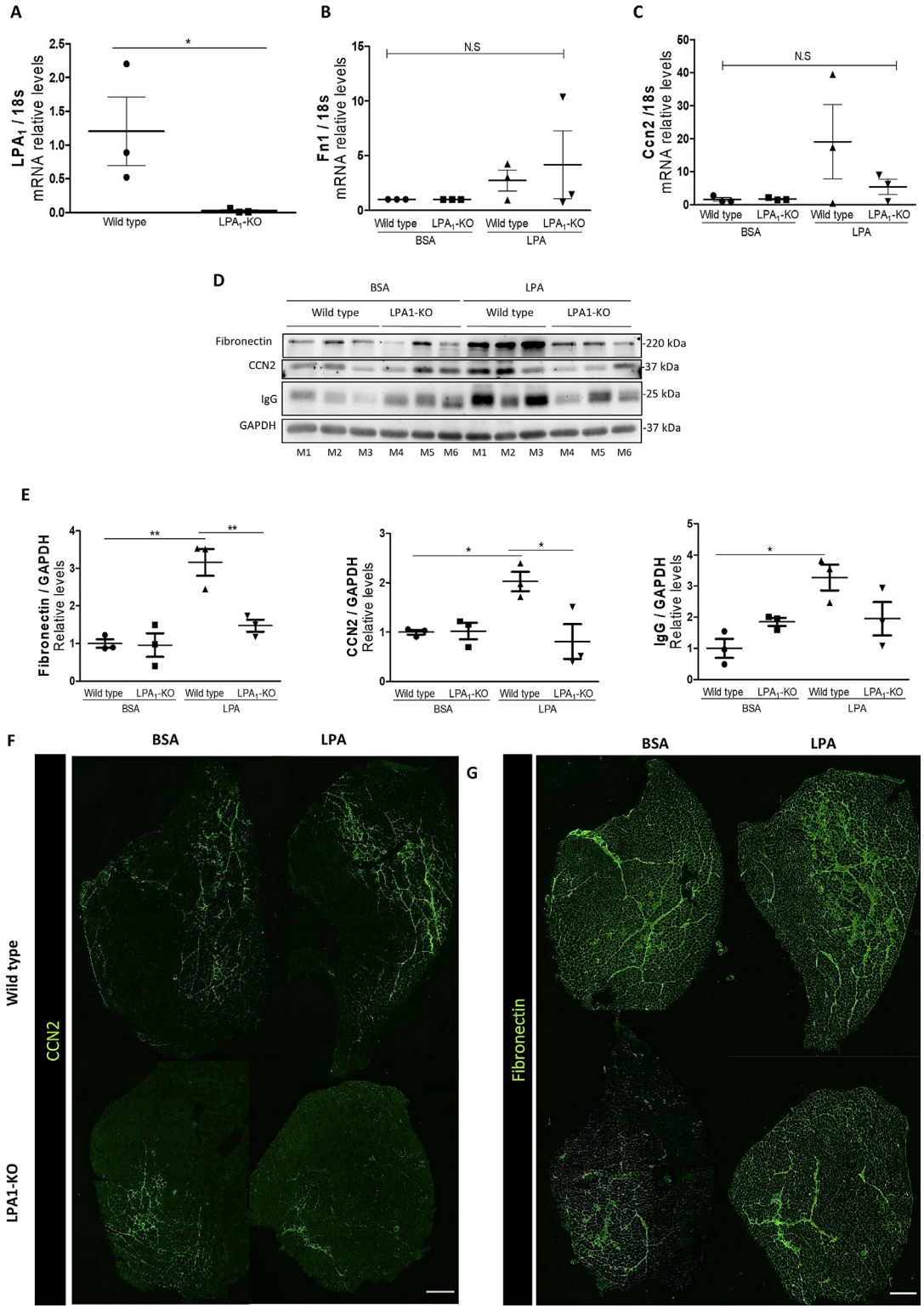

Fig. 5. LPA_1 is one of the main mediators of the LPA-induced fibrotic response in skeletal muscle. (A) LPA_1 mRNA levels in T.A from WT and LPA_1-KO mice. (B–E) Fibronectin and CCN2 were analyzed by RT-qPCR and immunoblot to determine their mRNA (B and C) and protein (D and E) levels in T.A from WT and LPA_1-KO male mice 4 h after been injected with BSA or LPA 400 μg/Kg IM. GAPDH was used as the loading control. (F and G) Representative epifluorescence images of CCN2 and fibronectin IIF in whole reconstructed cross-sections of BSA and LPA-injected T.A. Scale bar, 500 μm. M refers to mouse. $^{**}P < 0.01$, $^{*}P < 0.05$, N.S not significant by one-way ANOVA with Tukey's post-test; $n = 3$.

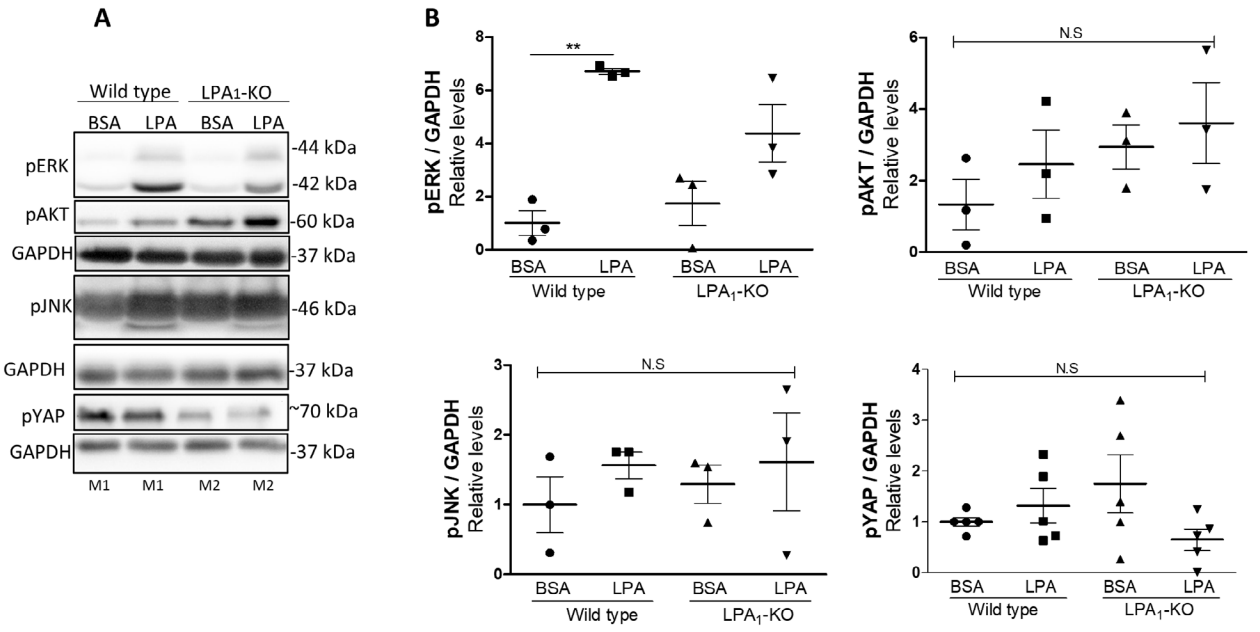

Fig. 6. LPA induces ERK 1/2 phosphorylation through LPA1 (A) pERK, pAKT, pJNK, pYAP and GAPDH levels were analyzed by immunoblot in T.A from WT and LPA_1-KO mice 10 min after being injected with BSA or LPA 400 μg/Kg IM. GAPDH was used as the loading control. (B) Quantification of phosphorylated proteins. *M* refers to mouse. $^{**}P < 0.01$, N.S not significant by one-way ANOVA with Tukey's post-test; $n = 3$-4.

mice [53,54]. In this experimental model, FAPs are recognized by the expression of a nuclear fusion protein H2B-eGFP controlled by the promoter of the PDGFRα gene. 24 h after LPA intramuscular treatment, immunoblot analyses showed a significant increase in PDGFRα and eGFP protein levels (Fig. 8E, F and G). The number of eGFP positive nuclei was quantified in tissue sections (Fig. 8H and I), the results indicated that LPA promotes an increase in the number of FAPs. To evaluate if the inhibition of LPARs prevented the increase in the number of FAPs in response to LPA, we treated $PDGFR\alpha^{H2BEGFP}$ knock-in reporter mice with Ki16425 for three days before they were IM injected with LPA. The increase in the FAPs number was not prevented by Ki16425, suggesting the participation of other LPAR subtypes.

We found that $PDGFR\alpha^{H2BEGFP}$ knock-in reporter mice showed a significant rise in total nuclei number (Fig. 8J), determined by Hoescht staining. The total number of nuclei in LPA-treated muscles exceeds the number of green nuclei (FAPs) (Fig. 8K), indicating that LPA injection increases the number of other cell types in the muscle.

Discussion

Previous studies have reported evidence that LPA is necessary for proper function of resident muscle cells. In 2008, Xu et al. demonstrated that LPA induces an increase of intracellular Ca^{+2} and cell proliferation in the myoblast cell line C2C12 [55]. In 2018, D'Souza et al. determined that LPA impairs skeletal muscle insulin signaling and mitochondrial function [56]. Ray et al. showed that ablation of ATX expression or its pharmacological inhibition affects muscle regeneration induced by LPA [57]. These studies suggest that LPA is a relevant agent in the physiological regulation of muscle cells and tissue. Here, we explored a possible role of LPA as a fibrosis-inducing agent in skeletal muscle. We determined the expression of several components of the ATX/LPA/LPA axis and evaluated the effect of local skeletal muscle administration of LPA on the fibrotic response and the number of FAPs, critical cells in the process of ECM synthesis. We also determined the potential participation of different LPAR subtypes and the role of early phosphorylation pathways in the LPA-mediated fibrotic response.

Our laboratory has previously demonstrated that the number of FAPs is augmented in three models of increased skeletal muscle fibrosis: denervated muscle, a model of repetitive damage, and in the *mdx*, a mouse model of Duchenne muscular dystrophy (DMD) [42]. In the present study, we found that LPA injection in adult skeletal muscle elevated the protein levels of PDGFRα, a marker expressed by FAPs. We found that the number of eGFP-positive cells in the $PDGFR\alpha^{H2BEGFP}$ knock-in reporter mice increased 24 h after treatment, supporting the idea that LPA induces a fibrotic phenotype by augmenting FAPs number. However, LPA induces a higher increase in total nuclei number than the one

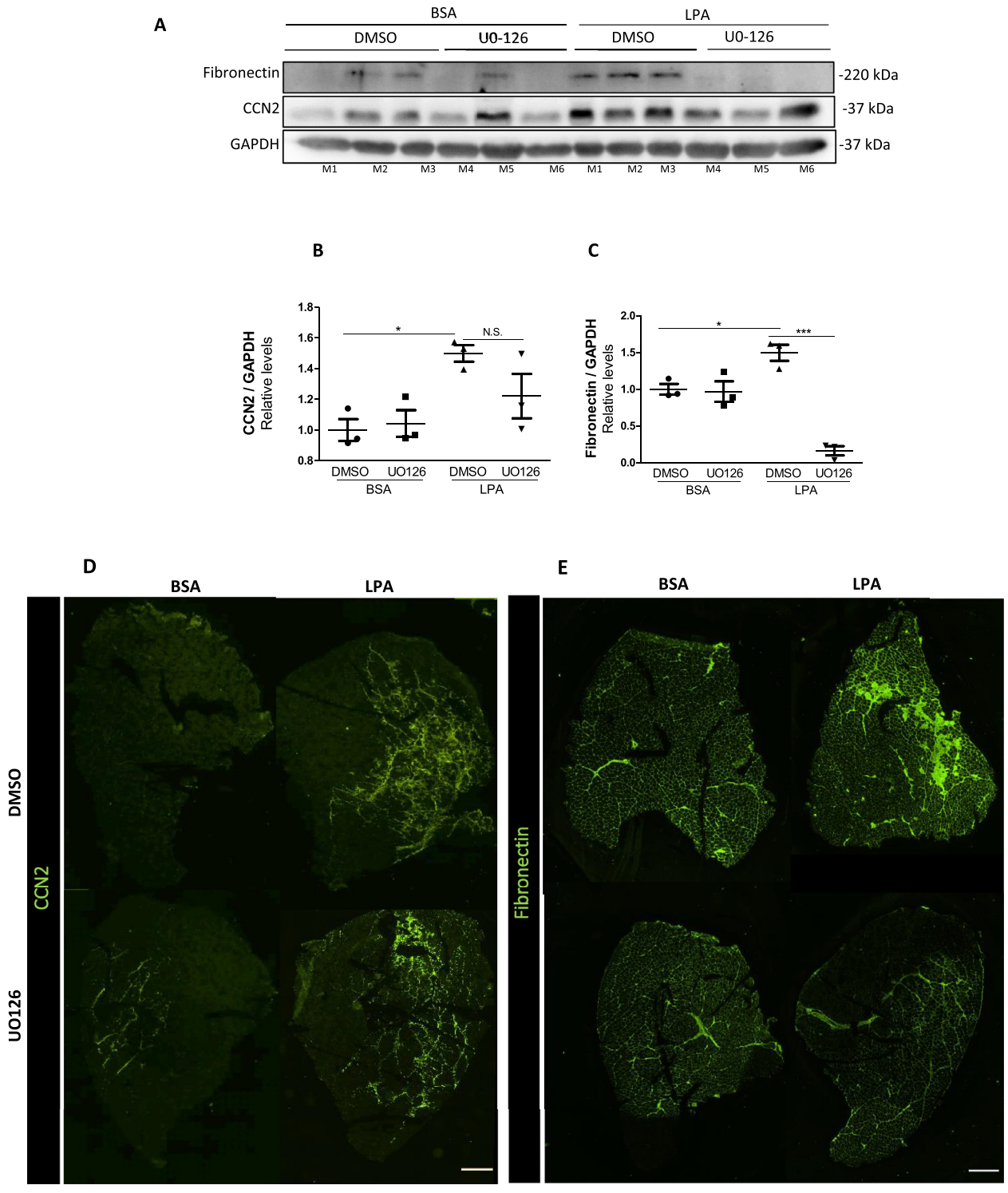

Fig. 7. Inhibition of ERK 1/2 prevents LPA-induced fibrotic responses in skeletal muscle. (A) Fibronectin and CCN2 protein levels were analyzed by immunoblot in T.A from mice treated with U0126 for 3 days and 4 h after being injected with BSA or LPA 400 μg/Kg IM. GAPDH was used as the loading control. (B, C) Quantification of protein expression. (E) CCN2 immunofluorescence reconstruction from epifluorescence images. Scale bar, 500 μm. (F) Fibronectin immunofluorescence reconstruction from epifluorescence images. Scale bar, 500 μm. *M* refers to mouse. **P* < 0.05, N.S not significant by one-way ANOVA with Tukey's post-test; *n* = 3.

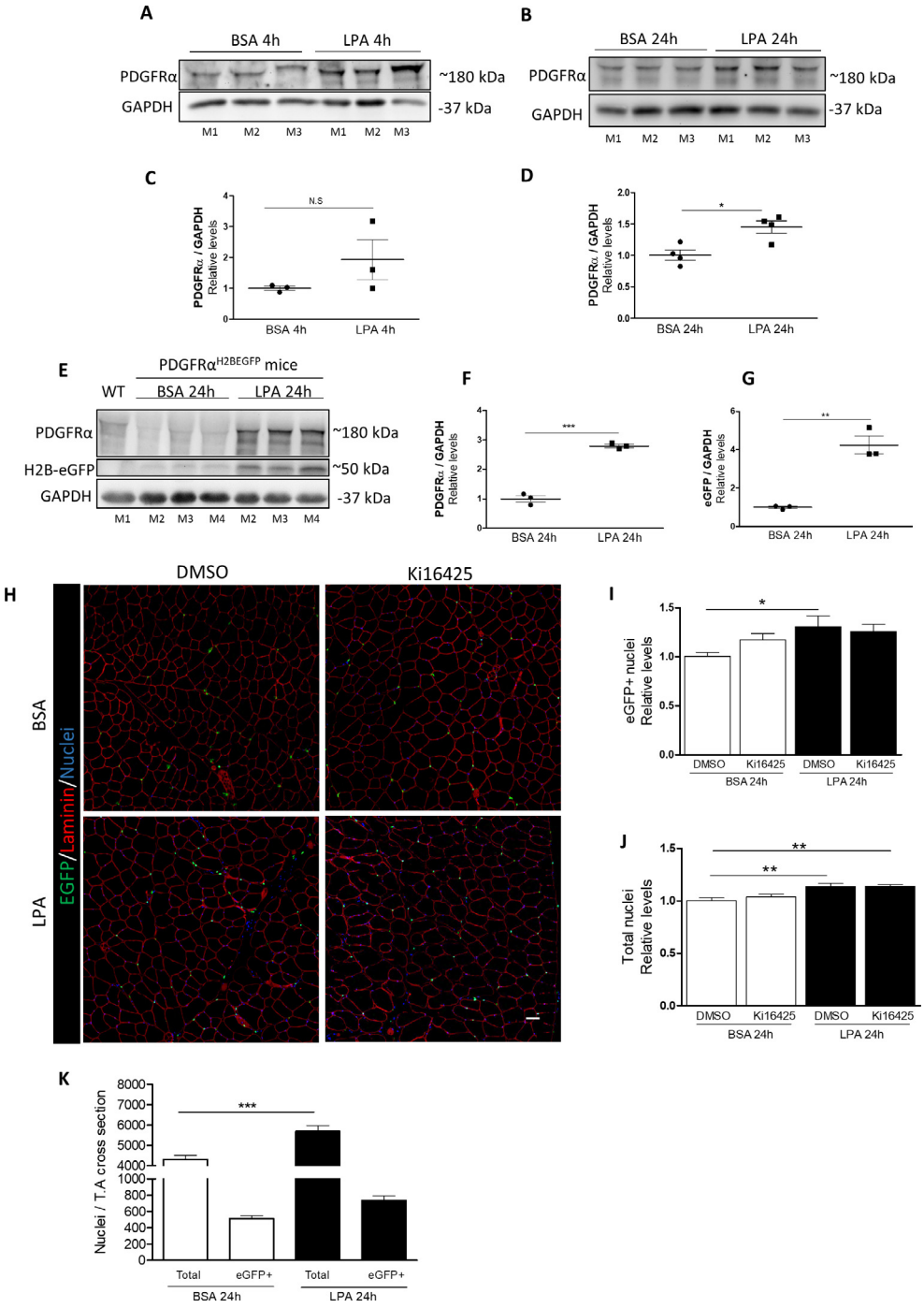

Fig. 8. LPA increases the number of FAPs in skeletal muscle. (A, B) PDGFRα levels in T.A were analyzed by immunoblot from WT mice at 4 h (A) or 24 h (B) after being injected with BSA or LPA 400 μg/Kg IM. GAPDH was used as the loading control. *n* = 3. (C) Quantification of A. D) Quantification of B. E) PDGFRα and H2B-eGFP protein levels analyzed by immunoblot in T.A from PDGFRα^{H2BEGFP} knock-in reporter mice 24 h after being injected with BSA or LPA 400 μg/Kg IM, GAPDH was used as the loading control. *n* = 3. (F and G) Quantification of E. H) GFP-positive nuclei, laminin and Hoechst in tissue sections from the T.A of PDGFRα^{H2BEGFP} knock-in reporter mice treated with Ki16425 and LPA observed with an epifluorescence microscope, *n* = 5. Scale bar, 100 μm. (I, J) Quantification of eGFP-positive nuclei and total nuclei in reconstructed whole muscle cross-sections subjected to IIF, respectively. (K) Summary graph showing total and eGFP positive nuclei in reconstructions of T.A cross-sections. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, N.S not significant by *t*-test (to compare 2 groups) or one-way ANOVA with Tukey's post-test (to evaluate more than two experimental groups).

observed in FAPs (Fig. 8K), indicating that LPA may also increase the number of other resident or infiltrating cell types in muscle. Consistent with this, we observed an increase in the level of an inflammatory marker, IgG, after LPA injection (Fig. 4B, E). It has been shown that LPA induces chemokines like interleukin-8 in bronchial epithelial and squamous cells [58–60]. In C2C12 myoblasts, LPA promotes the expression and secretion of the monocyte chemoattractant protein-1 MCP-1 [61], a critical chemokine that promotes the infiltration of monocytes/macrophages in target tissues [62]. In accordance, Davies et al. have shown that I.P LPA treatment triggers the infiltration of mononuclear cells in rotator cuff muscle [63]. Taken together, these results suggest that LPA injection can mediate the recruitment of circulating cells to the muscle, as it is observed in acute and chronic injury conditions such as those present in DMD muscles [64], consistent with prior studies on promotion of LPA-mediated cell motility [65]. Other mechanisms that are not mutually exclusive include increased LPA receptor-mediated cell survivial [32,66–68], and proliferation of resident cells, as has been reported in cancer studies [69,70]. These processes could explain the increase in the total number of nuclei found after treatment with LPA.

LPA is a lipid factor that could be triggering a wide response in the skeletal muscle, but in order to study the effect of LPA as a fibrosis promoting factor, we decided to explore the pro-fibrotic factor CCN2, which is overexpressed in several chronic diseases [71]. We had previously demonstrated that CCN2 induces a fibrotic response in wild-type mice, remodeling healthy muscle into a protein-rich ECM environment [72], while the reduced expression or inhibition of this protein decreases muscle fibrosis in the *mdx* mouse [5]; in an ALS animal model [8,73]; and after muscle denervation [6,43]. We found that LPA increased CCN2 mRNA and protein levels, especially in the area injected with LPA, in accordance with the fact that CCN2 levels correlate with the severity of fibrosis [74–76]. We also found increased fibronectin, collagen, periostin and IgG levels implicating LPA as an inductor of fibrosis in the skeletal muscle.

We explored if the fibrotic response to LPA could be prevented by interfering its signaling pathways with the well-known cognate LPA_1 and LPA_3 inhibitor, Ki16425. We found that mice systemically treated with this inhibitor showed a decreased response to LPA when injected into the muscle. Considering that LPA_1 mRNA levels are much higher than LPA_3 levels in muscle, we studied the effect of LPA injection in LPA_1-KO mice. Our results suggest that the absence of LPA_1 in these mice reduces the fibrotic responses to LPA. The possible involvement of other LPAR subtypes in this response, however, cannot be excluded. It is tempting to speculate that LPA_6 could also be participating in the process because the levels of LPA_6 mRNA in the T.A muscle are like LPA_1. The few reports available about LPARs in skeletal muscle have explored mainly LPA_1 but no other receptors. Consequently, it will be important to consider the contribution of LPA_6 to the biology of skeletal muscle in future research.

We also studied the pathways that could be potentially involved in the fibrotic response. Since LPA signaling triggers the activation of signaling pathways such as ERK1/2, AKT, JNK, and YAP in different cell types [77–80], we studied the effect of LPA on the phosphorylation of these proteins in muscle. We found that LPA seems to induce increased phosphorylation in the four pathways after 10 min of LPA treatment, but only ERK 1/2 reached statistical significance. This effect was not observed in the LPA_1-KO mice. The ERK 1/2 signaling pathway mediates extracellular signals triggering growth and pro-differentiation cell responses [81]. Therefore, we speculate that the ERK 1/2 pathway could mediate the increase in the number of FAPs observed in muscles treated with LPA.

Our work focused on FAPs, cells capable of differentiating into myofibroblasts, the primary type of ECM producing cell associated with skeletal MDs. Our results show that DIA presents higher levels of PDGFRα in comparison to T.A and GAS (Supplementary Fig. 5A and B). LPA induces vinculin mRNA (Supplementary Fig. 5C) and, possibly, vimentin and αSMA mRNA levels (as a non-statistically significant upward trend was observed for the latters; supplementary Fig. 5D and E). This could support the idea that LPA also promotes FAPs differentiation into the myofibroblast phenotype. FAPs, whose number, as mentioned above, is elevated in different fibrotic skeletal muscle models [42], can also differentiate towards an adipogenic phenotype [82–84]. Our results show that LPA injection in skeletal muscle induces a transitory augmentation in the adipocyte marker PPARγ after 4 h of treatment (Supplementary Fig. 6A), but we observed a decrease at 24 h in the protein levels of PPARγ and in the mRNA levels of adipoq, another cognate adipocyte marker (Supplementary Fig. 6B and C). LPA_1 activation has been reported to downregulate PPARγ2 in differentiating adipocytes [85], supporting that LPA could be acting as a pro-fibrotic factor rather than a pro-adipogenic factor in skeletal muscle.

Different clinical trials have explored the role of ATX/LPA/LPARs as a therapeutic target for human fibrotic diseases, mainly in idiopathic pulmonary fibrosis and systemic sclerosis (NCT02738801, NCT03798366, NCT01766817, NCT04308681. Our findings confirm the presence of different components of the ATX/LPA/LPAR axis in mouse skeletal muscle and demonstrate that this tissue responds to local increases of LPA triggering a fibrotic phenotype (Fig. 9). This is the first study on the potential

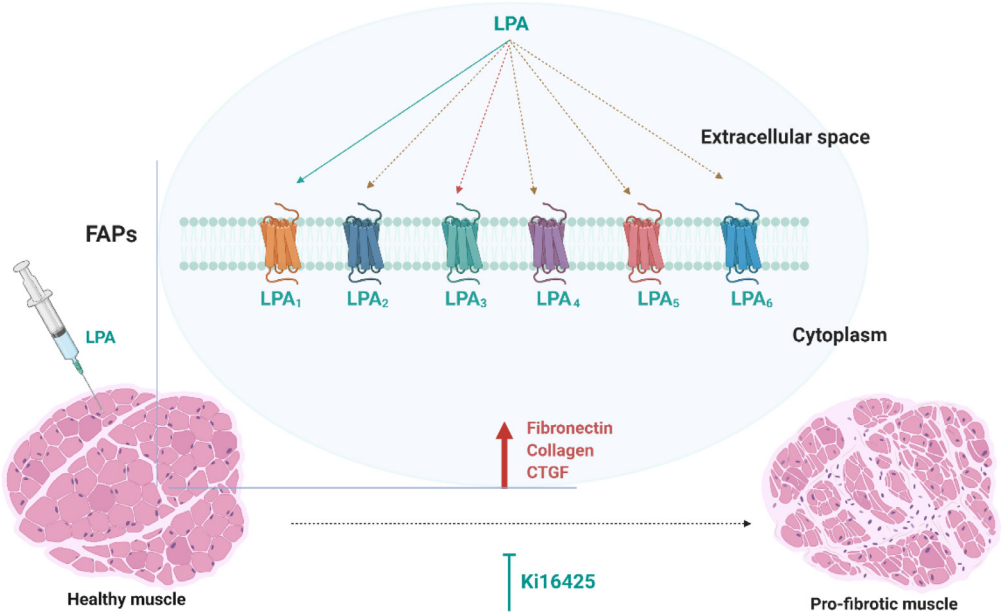

Fig. 9. Schematic view of the skeletal muscle response to LPA. Treatment with LPA induces an increase in the number of FAPs, CCN2 and ECM molecules, such as fibronectin and collagen. The pharmacological inhibition of $LPA_{1/3}$ with Ki16425 and the absence of the LPA_1 gene prevents some fibrotic effect. LPA pro fibrotic effect may be due to the increase in FAPs number and their differentiation into a myofibroblast-like phenotype. These results support the idea that LPA could be involved in the establishment and development of fibrosis in skeletal muscle. The diagram was created with BioRender.com.

contribution of LPA to the development of fibrosis in skeletal muscle, a critical aspect in the pathogenesis of diseases such as DMD. Future research may benefit from considering the use of LPA axis inhibitors in models of muscle fibrosis.

Experimental procedures

Animal experiments

All animal experiments were performed following the protocols approved by the Animal Ethics Committee, Pontificia Universidad Católica de Chile (Protocols 180810006, 180821022, and 180820006). Male mice were maintained in a 12 h light-dark cycle with a regular diet and water access. C57BL/10, C57BL/6, and $Pdgfra^{tm11(EGFP)Sor}$ mice (referred to as $PDGFR\alpha^{H2BEGFP}$) [53,86] were obtained from The Jackson Laboratory. LPA_1-KO mice were also used [87]. After their treatments, mice were sacrificed under isoflurane overdose by cervical dislocation at the ages indicated in each figure.

Treatment with Ki16425 and U0126

Ki16425 (Cayman Chemical, Ann Arbor, MI) and U0126 (Cell Signaling Technology, Danvers, MA, USA) were administered to 3-month-old wild-type male mice by I.P injection in 5 mg/Kg/day or 10 mg/Kg/day doses, respectively, for three days before and also 40 min before LPA (Sigma-Aldrich, St. Louis, MO) treatment.

LPA intramuscular injection

Groups of male mice were studied at the ages indicated in each figure. LPA IM injections were administered in mice anesthetized with 2.5 % isoflurane gas in oxygen. The LPA (400 μg/kg in a volume of 50 μl) injection was administered in the T.A muscle. The contralateral T.A was injected with BSA and used as a vehicle-injected control. Once the experiment ended, the animals were sacrificed. The T.A was removed, snap-frozen in chilled isopentane and cut into 20 μm transversal sections. Alternate sections were stored in two separate tubes for protein and RNA extraction. Five μm cross-sections representative of 6 different areas of each T.A were also obtained for histological staining and IIF.

Protein extraction, SDS-polyacrylamide gel electrophoresis and immunoblot analyses

Whole-muscle extracts were obtained by homogenization of the tissue or its sections in Tris-EDTA buffer pH 7.4 with 1mM phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na_3VO_4), and sodium fluoride (NAF) using an Ultraturrax T25

(Labortechnik). Then, a second buffer containing 20% glycerol, 4% SDS and 0.125M Tris pH 6.8 was added to the homogenates and mixed with a micropipette. Muscle homogenates were incubated at 55 °C for 20 min and centrifuged for 10 min at 14,000 rpm to pellet insoluble material. Protein concentration was determined using the BCA Assay kit (Pierce, Rockford, IL, USA). 50 μg of protein extracts were subjected to SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% nonfat milk in TBS-Tween (50 mM Tris-Cl, pH 7.6; 150 mM NaCl, 0,1% Tween 20) and probed with the following antibodies at 4 °C overnight: anti-fibronectin (Sigma-Aldrich, St. Louis, MO, USA), anti-PDGFRα (R&D Systems, Minneapolis, MN, USA), anti-CTGF/CCN2 (Santa Cruz, USA), active caspase 3, fast myosin and slow myosin (Abcam, Cambridge, UK), ATX and LPA_3 (Cayman Chemical, Ann Arbor, MI), LPA_6 (Abcepta, San Diego, CA, USA), pERK, pJNK, pAKT (Cell Signaling, Danvers, MA, USA), anti-pYAP (Cell Signaling, Danvers, MA, USA) and, anti-GAPDH (Proteintech, Rosemount, IL, USA). Then, the primary antibodies were detected with horseradish-peroxidase-conjugated secondary antibodies. All immunoreactions were visualized by enhanced chemoluminescence (Pierce, Rockford, IL, USA) using a ChemiDoc-It HR 410 imaging system (Upland, Calif., USA). Densitometric analysis and quantification were performed using the ImageJ software (NIH, USA).

RNA isolation, reverse transcription and qPCRs

Total RNA was isolated from T.A, DIA, and GAS muscles using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis was performed using random primers and M-MLV reverse transcriptase (Invitrogen, CA, USA). Quantitative real-time polymerase chain reactions (qPCR) were performed using an Eco Real-Time PCR System (Illumina, CA, USA). A list of primers used in this work is given in Supplementary Table 1. mRNA expression was quantified using the comparative dCt method (2-ddCT), using 18S as the reference gene. The mRNA levels were expressed relative to the mean expression in the control condition.

Indirect immunofluorescence and microscopy

Frozen muscles were sectioned into 5 μm slices, fixed for 30 min in 4% paraformaldehyde, and washed in phosphate-buffered saline (PBS). Permeabilized in 1% Triton X-100 in PBS. Tissue sections were blocked for 60 min in 1% BSA in PBS, incubated overnight at 4 °C with primary antibodies: rabbit anti-fibronectin (1:200; Sigma-Aldrich, St. Louis, MO, USA), CCN2 (1:50; Cell Signaling, Danvers, MA, USA), Laminin (1:200; Sigma-Aldrich, St. Louis, MO, USA). Samples were then washed in PBS, incubated for 1 h at room temperature with a secondary antibody Alexa-Fluor-488 donkey anti-rabbit IgG (H+L) (Invitrogen, CA, USA) and washed in PBS. Then, the samples were incubated with Hoechst 33342 (2 mg/ml diluted in PBS) for 10 min and mounted with fluorescent mounting medium (DAKO). Images were acquired by a blind operator with a Nikon Ti2-E inverted microscope at the Unidad de Microscopía Avanzada (UMA) Facility, Pontificia Universidad Católica de Chile. eGFP positive and total nuclei were performed using the ImageJ software (version 1.46r, NIH, USA).

Statistical analyses

Data and statistical analyses were performed using the Prism5 software (Graph Pad Software, CA, USA). Data are presented as Mean ± SEM. When only 2 groups were compared, an unpaired *T*-test (two tailed) was performed (BSA-vehicle vs. LPA-treated muscle). One-way ANOVA was used to evaluate more than two experimental groups. Tukey's post-test was performed to compare differences between groups. A difference was considered statistically significant with *p*-values: $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; $****p \leq 0.0001$.

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Declaration of competing interests

The authors declare no competing or financial interests.

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Supplementary materials

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List of abbreviations:

ATX, autotaxin; BSA, bovine serum albumin; CTGF or CCN2, connective tissue growth factor/Cellular communication network factor 2; DIA, diaphragm; DMD, Duchenne muscular dystrophy; ECM, extracellular matrix; ERK, extracellular signal-regulated kinases; FAPs, fibro/adipogenic progenitors; GAS, gastrocnemius; IIF, indirect immunofluorescence; I.M, intramuscular; I.P, intraperitoneal; JNK, c-Jun N-terminal kinase; KO, knockout; LPPs, lipid phosphate phosphatases; LPA, lysophosphatidic acid; LPAR, lysophosphatidic acid receptor; MDs, muscular dystrophies; PKB or AKT, protein kinase B; T.A, tibialis anterior; WT, wild type; YAP, yes-associate protein

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