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ROLE OF THE ATX / LPA / LPARS AXIS IN THE FIBROTIC RESPONSE OF SKELETAL MUSCLE

Tesis entregada a la Pontificia Universidad Católica de Chile en cumplimiento parcial de los requisitos para optar al Grado de Doctor en Ciencias Biológicas con Mención en Biología Celular y Molecular

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ACTA DEFENSA FINAL DE TESIS

Doña Adriana Paz Córdova Casanova, estudiante del Programa de Doctorado en Ciencias Biológicas Mención Biología Celular y Molecular, ha presentado la Defensa Pública de Tesis.

En consideración a los conocimientos generales sobre Biología Celular y Molecular, y la defensa de la Tesis Doctoral, titulada **"Role of the ATX / LPA / LPARS axis in the fibrotic response of skeletal muscle"**, el Comité de Tesis a resuelto calificar esta actividad académica como Aprobada y con nota …7.0.

Se firma la presente acta en la ciudad de Santiago, el día 11 de julio de 2022.

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These works are attached at the end of the thesis.

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ABBREVIATIONS

ATX: Autotaxin

BSA: Bovine serum albumin

CCN2: Connective tissue growth factor/Cellular Communication Network factor 2.

CTRAL: Contralateral

DEN: Denervated

DIA: Diaphragm

DMD: Duchenne muscular dystrophy

ECM: Extracellular matrix

ERK: Extracellular signal-regulated kinases

FAPs: Fibro/adipogenic progenitors

GAS: Gastrocnemius

IIF: Indirect immunofluorescence

IM: Intramuscular

IV: Intravenous

JNK: c-Jun N-terminal kinase

KO: Knockout

LGMD: Limb Girdle Muscular Dystrophy

LPPs: Lipid Phosphate Phosphatases

LPA: Lysophosphatidic acid

LPAR: Lysophosphatidic acid receptor

MDs: Muscular dystrophies

Sgcd: Delta-Sarcoglican

PKB or AKT: Protein kinase B

TA: Tibialis Anterior

WT: Wild type

RESUMEN

Fibrosis se define como la acumulación excesiva de componentes de la matriz extracelular (MEC), y es una característica de diversas enfermedades crónicas. En esta situación patológica se reemplaza el tejido normal por MEC y existe una pérdida progresiva de la funcionalidad del tejido u órgano involucrado. Dentro de las patologías que conducen al desarrollo de fibrosis en el músculo esquelético se encuentran las distrofias musculares. Actualmente numerosos trabajos indican que la reducción de la fibrosis del músculo esquelético se asocia a una ganancia de la función y fuerza lo cual cobra relevancia terapéutica.

A lo largo de los años se ha enfatizado en el estudio de factores proteicos como elementos gatillantes de fibrosis. No obstante, recientemente se ha correlacionado el desarrollo de fibrosis en distintos órganos/tejidos con la producción incrementada de ácido lisofosfatídico (LPA), un lisofosfolípido que señaliza a través de 6 receptores (LPARs, LPA_{1-6}). El LPA es sintetizado principalmente por la enzima autotaxina (ATX) y degradado por fosfatasas de fosfato de lípidos (LPP). El tratamiento con antagonistas específicos de LPA_1 y LPA_3 previene la inducción de fibrosis dermal, pulmonar y renal en distintos modelos murinos. En su conjunto, estos antecedentes sugieren que LPA y sus receptores participan activamente de procesos fibróticos en diversos órganos. Resultados preliminares de nuestro laboratorio indican que el LPA puede inducir la expresión de del Factor 2 de Red de Comunicación Celular, CCN2, también llamado Factor de Crecimiento del Tejido Conectivo (CTGF) en mioblastos. CCN2 es una proteína matricelular pro-fibrótica, la cual está aumentada en el músculo de pacientes con distrofia muscular de Duchenne (DMD) y en el ratón *mdx*, siendo este último el modelo más estudiado de la DMD. CCN2 también se

encuentra incrementado el musculo de ratones deficientes para Sgcd (Sgcd-KO), modelo de distrofia muscular de cinturas (LGMD) y en el modelo de denervación muscular, los cuales conllevan el desarrollo de fibrosis. Experimentos de ganancia de función indican que la sobreexpresión de CCN2 en músculo esquelético de ratones wild type (WT) determina un incremento en marcadores de fibrosis como fibronectina, decorina y α-SMA, junto a una pérdida en fuerza muscular. Por otra parte, experimentos de pérdida de función indican que la disminución de la fibrosis muscular en ratones *mdx* que presentan una deleción hemicigota para CCN2, (*mdx*/Ctgf+/-) y ratones *mdx* tratados con un anticuerpo neutralizante contra CCN2 (FG3019/Pamrevlumab) mejoran el fenotipo distrófico, la fuerza muscular y la función locomotora. Similares hallazgos en la disminución de la fibrosis por la inhibición de CCN2 se han observado en músculo de ratones denervados o en un modelo animal para esclerosis lateral amiotrófica (ALS).

Por los antecedentes mencionados, esta tesis se encuentra focalizada en el estudio del rol que cumple el LPA en la fibrosis del músculo esquelético y en particular, en la expresión de CCN2.

En esta tesis demostramos que existe expresión de los receptores de LPA en el músculo esquelético y en progenitores fibro/adipogénicos (FAPs), células capaces de dar origen a miofibroblastos y responsables en gran medida de la síntesis de MEC. Además, demostramos que tanto el LPA como el agonista específico de LPA_3, 2s-OMPT, inducen CCN2 en cultivo primario de progenitores FAPs y que la activación de AKT, ERK y JNK podrían estar participando en la inducción de este importante factor pro-fibrótico.

Además, se determinó que existen niveles alterados de los LPARs en músculos fibróticos, indicando un posible aumento de la sensibilidad y respuesta del músculo a LPA

en ese contexto patológico. Así mismo, demostramos que el músculo esquelético es capaz de responder al estímulo de LPA induciendo CCN2 y moléculas de la MEC como fibronectina y colágeno. Se determinó también que el LPA induce un aumento significativo en el número de FAPs en el músculo y se estudió la participación del eje ATX / LPA / LPARs, en la fibrosis inducida por denervación a través de experimentos de pérdida de función de los LPA_1 y LPA_3, utilizando antagonistas y un modelo deficiente para LPA_1 (LPA_1-KO), donde encontramos disminución de marcadores fibróticos a las 2 semanas de denervación muscular.

Los resultados de esta tesis nos permiten concluir que el músculo esquelético y los FAPs expresan LPARs y que estos son capaces de responder a su estímulo induciendo una respuesta fibrótica, Así mismo, demostramos que el eje ATX / LPA / LPARs participa directamente en la inducción de la fibrosis en el modelo de denervación muscular.

Nuestros resultados proveen información relevante para iniciar pruebas experimentales en modelos animales distróficos bloqueando el eje ATX / LPA / LPAR como un paso necesario en la búsqueda de nuevas herramientas terapéuticas en pacientes con distrofias musculares.

ABSTRACT

Fibrosis is defined as the excessive accumulation of extracellular matrix (ECM) components and is a feature of various chronic diseases. In this pathological situation, normal tissue is replaced by ECM and there is a progressive loss of the functionality of the organ involved. Muscular dystrophies are among the pathologies that lead to the development of fibrosis in the skeletal muscle. Currently, numerous studies indicate that the reduction of skeletal muscle fibrosis is associated with a gain in function and strength, which is of therapeutic relevance.

Over the years, emphasis has been placed on studying protein factors as elements that trigger fibrosis. However, the development of fibrosis in different organs has recently been correlated with the increased production of lysophosphatidic acid (LPA), a lysophospholipid that signals through 6 receptors (LPARs, LPA1-6), which is synthesized mainly by the enzyme autotaxin (ATX) and degraded by lipid phosphate phosphatases (LPP). Treatment with specific antagonists of LPA_1 and LPA_3 prevents the induction of dermal, pulmonary, and renal fibrosis in different murine models. These antecedents suggest that LPA and its receptors actively participate in fibrotic processes in various organs. Preliminary results from our laboratory indicate that LPA can induce in myoblasts the expression of Cellular Communication Network Factor 2,CCN2, knows also as Connective Tissue Growth factor (CTGF). CCN2 is a pro-fibrotic matricellular protein, which is increased in the muscle of patients with Duchenne muscular dystrophy (DMD) and in the *mdx* mouse, the latter being the most studied model of DMD. CCN2 is also increased in the muscle from mice deficient for Scgd (Scgd-KO), model of limb-girdle muscular dystrophy (LGMD) and in the denervated muscle, conditions that leads to the development of fibrosis. Gain-of-function

experiments indicate that overexpression of CCN2 in skeletal muscle of wild type (WT) mice determines an increase in fibrosis markers such as fibronectin, decorin, and α-SMA, together with a loss in muscle strength. On the other hand, loss-of-function experiments indicate that decreased muscle fibrosis in *mdx* mice displaying a hemizygous deletion for CCN2, (mdx / Ctgf +/-) and in *mdx* mice treated with a neutralizing antibody against CCN2 (FG3019 / Pamrevlumab) improve the dystrophic phenotype, muscle strength, and locomotor function. Similar findings in decreased fibrosis by CCN2 inhibition have been observed in muscle from denervated mice or in an animal model for amyotrophic lateral sclerosis (ALS).

Due to the aforementioned background, this thesis is focused on the study of the role played by LPA in skeletal muscle fibrosis and, in particular, in the expression of CCN2.

In this thesis we demonstrate that there is expression of LPA receptors in skeletal muscle and in fibro / adipogenic progenitors (FAPs), cells capable of giving rise to myofibroblasts and largely responsible for ECM synthesis. In addition, we show that both LPA and the specific agonist of LPA_3, 2s-OMPT, induce CCN2 in primary culture of FAPs progenitors and that the activation of AKT, ERK and JNK could be participating in the induction of this important pro-fibrotic factor.

In addition, it was determined that there are altered levels of LPARs in fibrotic muscles, indicating a possible increased sensitivity and response of the muscle to LPA in this pathological context. Likewise, we demonstrate that skeletal muscle can respond to LPA stimulation by inducing CCN2 and ECM molecules such as fibronectin and collagen. It was also determined that LPA induces a significant increase in the number of FAPs in the muscle and the participation of the ATX / LPA / LPARs axis in denervation-induced fibrosis was studied through LPA_1 and LPA_3 loss-of-function experiments, using antagonists and a

deficient model for LPA_1 (LPA_1-KO), where we found a decrease in fibrotic markers at 2 weeks of muscle denervation.

The results of this thesis allow us to conclude that skeletal muscle and FAPs express LPARs and that they can respond to their stimulus by inducing a fibrotic response. Likewise, we demonstrate that the ATX / LPA / LPARs axis participates directly in the induction of fibrosis in the muscle denervation model.

Our results provide relevant information to initiate experimental tests in dystrophic animal models blocking the ATX / LPA / LPAR axis as a necessary step in the search for new therapeutic tools in patients with muscular dystrophies.

I. INTRODUCTION

1. Skeletal muscle

Skeletal muscle represents 40% of the mass of the human body. This tissue is highly regulated, relevant in maintaining glucose homeostasis and participating in storage for substrates like amino acids and carbohydrates. Skeletal muscle also promotes thermogenesis and force generation, allowing body movement and communication; therefore, it is significant for daily human functions (Frontera & Ochala, 2015). The preservation of the integrity of skeletal muscle is essential to respond to physical stress and chronic illness; thus, the loss in quantity and quality of muscle worsens the prognosis of many diseases, increasing morbidity and mortality. Therefore sarcopenia, a syndrome characterized by progressive loss of skeletal muscle mass and strength as an organism ages, has been proposed as a predictor of cirrhosis-related complications and the prognosis of patients. (Zeng et al., 2021) (Marasco et al., 2021).

Skeletal muscle is composed of the functional cell unit called myofiber, a large multinuclear non-proliferating cell. Satellite cells are adult muscle stem cells located between the basal lamina and the myofiber sarcolemma (Mauro, 1961) (Mukund & Subramaniam, 2020). When damage or rupture of the myofiber occurs, these cells are activated, proliferate, and differentiate, allowing tissue repair. Skeletal muscle also contains supporting cells, like vascular endothelial cells, fibroblasts, resident macrophages, and fibro / adipogenic progenitors (FAPs) (Mukund & Subramaniam, 2020).

The architecture of skeletal muscle is primarily due to the presence and structure of the extracellular matrix (ECM), a three-dimensional arrangement that is found around cells in all

tissues and is essential for life. Under physiological conditions of growth and repair, ECM components provide mechanical support for new tissue formation (Bonnans et al., 2014). Additionally, the ECM participate in force transmission and signal transduction inside the cell, triggering cellular responses as diverse as proliferation, polarization, migration, differentiation, survival, and apoptosis through ECM receptors localized at the cell surface, such as integrins (Frantz et al., 2010)(Ingber, 2006)(Rozario & Desimone, 2011). In skeletal muscle, ECM is organized in three layers; the endomysium surrounds the myofiber, composed mainly of collagen type I, type III, type V, and fibronectin. The perimysium is located around a group of myofibers. It is composed of collagen type I and type III, fibronectin, and finally, the whole muscle is surrounded by the epimysium composed mainly of collagen type I (Mahdy, 2019).

2. Skeletal muscle fibrosis

Under certain pathophysiological conditions, particularly chronic diseases, the balance between ECM production and degradation can be disturbed, generating fibrosis, that is, an exacerbated increase in the deposition of some elements, especially interstitial collagens (I and III), but also other components of ECM like fibronectin, proteoglycans and basal lamina proteins, such as laminin, a condition that alters the cell's microenvironment and function (Mahdy, 2019)(Baiocchini et al., 2016).

Fibrosis appears after tissue injury stays and progresses if the damage is maintained with time. Contrary to acute damage, the development of fibrosis involves a permanent activation of fibroblasts and infiltration of immune cells (Diagram 1). Fibrosis determines the appearance of a scar-like phenotype and reduced tissue function. This process is associated with many chronic

diseases in different organs. Skeletal muscle develops fibrosis in different contexts like denervation, sarcopenia, severe damage, muscular dystrophies (MDs), and in amyotrophic lateral sclerosis (ALS) (Rebolledo et al., 2019)(Alcalde-Estévez et al., 2020)(Stilhano et al., 2017)(Klingler et al., 2012)(Gonzalez et al., 2017)(Barbe et al., 2020).

There are several pro-fibrotic factors that mediate fibrosis in skeletal muscle. The most studied are: Transforming Growth Factor-Beta (TGF-β1), Cellular Communication Network Factor 2 (CCN2), Platelet-Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), and Fibroblast Growth Factor (FGF) signaling pathways (Mann et al., 2011)(Laumonier & Menetrey, 2016). These protein factors induce fibrosis by stimulating fibroblasts to differentiate to a myofibroblast phenotype and produce ECM proteins.

Our laboratory has shown an improvement in the functionality of the skeletal muscle of mice previously when the fibrosis induced by pro-fibrotic stimuli is reduced (Acuña et al., 2014)(Cabrera et al., 2014)(Morales et al., 2013)(Gonzalez et al., 2018). Moreover, the reduction in fibrosis improves the efficiency of cell therapy in fibrotic muscle (Cabrera et al., 2014)(Morales et al., 2013).

As Rebolledo and Brandan explained, the loss of strength in fibrotic muscles is mainly due to two reasons: i) the ECM excess extends in the space previously occupied by contractile muscle fibers modifying the availability of different growth factors, and that could impact the muscle by reducing the mass and / or the number of myofibers and other resident cells. ii) the ECM act as a rigid physical barrier, impairing biological processes like neovascularization,

reinnervation, and myoblast migration in conditions of muscle injury, affecting muscle regeneration (Rebolledo et al., 2021).

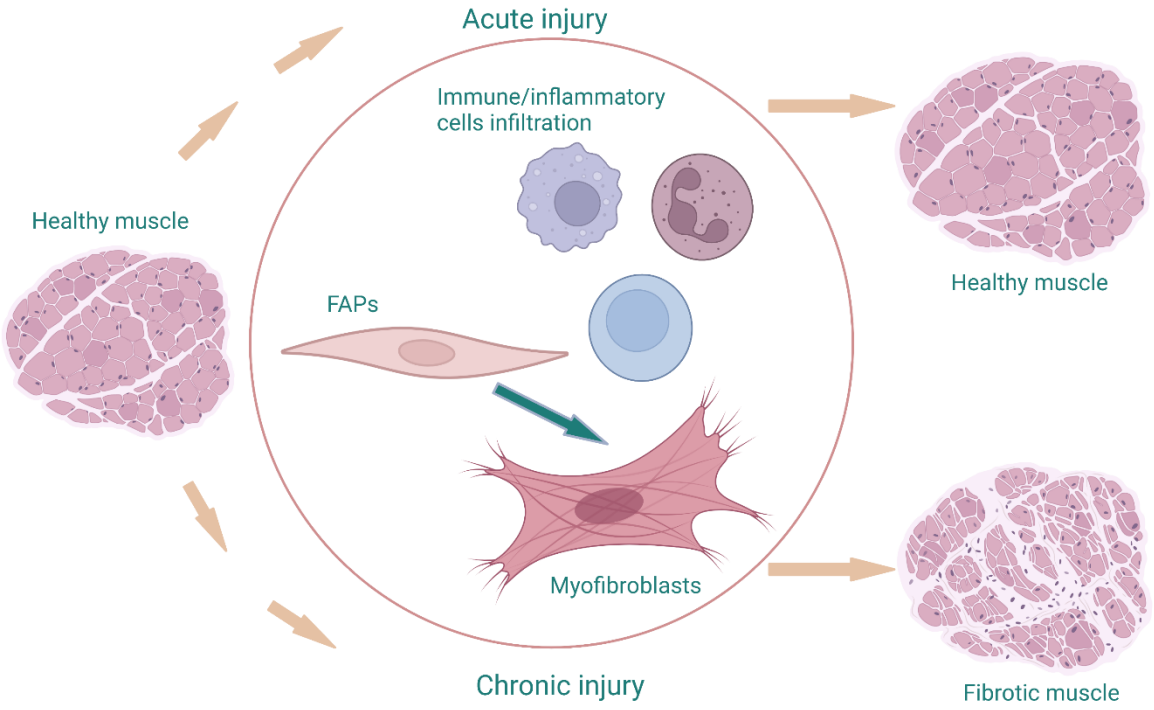


Diagram 1. Acute and chronic muscle injury. Acute injury to healthy muscle triggers a controlled (transient) inflammation and ECM deposition, creating a pro-regeneration environment. However, when the muscle is subjected to repetitive damage, a sustained inflammatory and fibrotic response are triggered over time, worsening muscle regeneration conditions. Diagram created with BioRender.com.

3. Muscular dystrophies

MDs are a heterogeneous group of inherited diseases characterized by progressive muscle weakness and degeneration that occurs in varying degrees, leading to reduced lifespan

of patients. The common characteristics of MDs are inflammation, fibrosis, and reduced tissue regeneration. Degenerating muscle fibers are replaced with fibrosis and adipose tissue (Guiraud et al., 2015)(Bertini et al., 2011), affecting normal muscle function.

Duchenne Muscular Dystrophy (DMD) and Limb-Girdle Muscular Dystrophy (LGMD) are two examples of MDs that differ in their genetic origin, affected muscles, and incidence, but as all the MDs present as a common factor in the development of muscular fibrosis. DMD is the most frequent and severe MD, causing a dramatic loss of muscle strength. DMD mainly affects males with a prevalence of 7,1 in 100.000 (Thompson & Straub, 2016) due to its X-linked recessive inheritance (Hoffman et al., 1987). The molecular defect in DMD is the complete absence of the protein dystrophin. On the other hand, LGMD is less common than DMD (Thompson & Straub, 2016) and presents autosomal dominant or recessive inheritance. This disease belongs to sarcoglycanopathies, a group of MDs affecting the expression of one of the sarcoglycan proteins, α-, β-, γ- and δ-SG (Angelini, 2020). LGMD also triggers the onset of fibrosis and loss of muscle strength.

Dystrophin and sarcoglycan are proteins of the dystrophin-associated glycoprotein complex (DGC). This complex, under physiological conditions, anchors the ECM to the actin cytoskeleton, providing stability to the muscle fiber against the mechanical stress of contraction (Gumerson & Michele, 2011). The absence of members of DGC in DMDs and LGMD makes myofibers more susceptible to muscular dystrophy characteristics (Ganote & Armstrong, 2002) like cell death, inflammation, and fibrosis as primary compensatory and repair mechanisms.

Fibrosis contributes to the loss of muscle strength in these MDs. Currently, numerous studies indicate that reducing fibrosis may represent a viable therapeutic approach for DMD (Morales

et al., 2013)(Fernández-Simón et al., 2022)(Rebolledo et al., 2021). Therefore, to discover possible therapeutic targets that prevent or reverse the development of fibrosis in this group of diseases, **it is necessary to know the molecular factors and cellular components that can promote and maintain it.**

4. Denervation as a skeletal muscle fibrosis model

Skeletal muscle denervation could be defined as the loss of communication between motor neurons and skeletal muscle. Consequently, patients with skeletal muscle denervation present weakness and atrophy in muscular groups innervated by a single peripheral nerve or root. Denervation occurs in conditions like ALS (Pasinelli & Brown, 2006), alcoholic and diabetic neuropathy (Julian et al., 2019)(Tack et al., 2002), myasthenia gravis (Thanvi & Lo, 2004), aging (Carlson, 2004), or directly by transection of nerves (Rebolledo et al., 2019).

Skeletal muscle responds to denervation through inflammation, fibrosis, and severe atrophy. After denervation, an increase in inflammatory factors such as IL-6 and TNFα that induce protein breakdown is observed (Wu et al., 2019). One proteolytic pathway involved in denervation atrophy is the ubiquitin-proteasome system which participates in the degradation of contractile proteins reducing the skeletal muscle fiber size (Baumann et al., 2016).

Our laboratory has demonstrated that fibrotic proteins such as fibronectin and collagen are induced two days after denervation and are maintained at least for two weeks (Rebolledo et al., 2019). Moreover, CCN2, a pro-fibrotic cytokine, seems to be involved in this response since the CCN2 blocking antibody FG-3019 reduces the fibrotic phenotype in denervated animals

(Rebolledo et al., 2019). Still, **the molecular mechanisms and pathways participating in CCN2 induction in denervation are unknown.**

5. Cellular Communication Network Factor 2

CCN2 belongs to the CCN family of proteins, which comprises a group of six secreted modular proteins that specifically associate with the ECM (Bork, 1993) and participate in various cellular processes such as cell adhesion, mitogenesis, migration, differentiation, angiogenesis, chondrogenesis, tumorigenesis, and wound healing (Brigstock, 1999)(Lau & Lam, 1999)(Perbal, 2001).

CCN2 is composed of 4 domains, the first is homologous to IGF-1 binding proteins, the second domain is homologous to the type C repeat of von Willebrand factor, the third domain is homologous to the type 1 repeat of thrombospondin, and the fourth domain (C-terminal domain) allows its binding to heparan sulfate proteoglycans (HSPG) (Diagram 2)(Takigawa, 2018). CCN2 expression is induced by various pro-fibrotic cytokines such as TGF-β and lysophosphatidic acid (LPA)(Kiwanuka et al., 2017)(Vial et al., 2008). CCN2 induces myofibroblast formation by transdifferentiating from other cells, including stellate cells (Paradis et al., 2002), and induces stress fibers formation in C2C12 myoblasts (Vial et al., 2011). In addition, CCN2 activates myofibroblasts and stimulates ECM protein synthesis, causing tissue remodeling and fibrosis. Interestedly, CCN2 induces the expression of cytokines such as TGF-β and VEGF (Yang et al., 2010) (Liu et al., 2014), which induces the expression of CCN2. Thus, positive feedback pathways for CCN2 expression may contribute to the progressive nature of fibrosis.

It has been shown that there is an increase in CCN2 levels in the muscle of DMD patients and fibrotic mouse models such as muscle denervation and the *mdx* mouse, the latter being the most studied model of DMD (Sun et al., 2008) since *mdx* mice present a point mutation in exon 23 of the gene encoding dystrophin, creating a premature termination codon and leading to the absence of this protein (Sicinski et al., 1986)(Willmann et al., 2009). CCN2 it is also increased in fibrotic skeletal muscle in an animal model for ALS (Gonzalez et al., 2018). The overexpression of CCN2 by adenovirus in skeletal muscle of WT mice determines an increase in fibrosis markers such as fibronectin, decorin and α-SMA, in addition to a reduction in isometric contractile force (Morales et al., 2011). Thus, there is a decrease in muscle fibrosis in *mdx* mice that present a hemizygous deletion for CCN2, (*mdx*/Ctgf+/-) and also in *mdx* mice treated with a CCN2 neutralizing antibody (FG3019)(Morales et al., 2013). In 2020, Barbe et al showed that established skeletal muscle fibrosis induced by overuse injury could be improved by blocking CCN2 (Barbe et al., 2020). Also, it has even been seen that CCN2 inhibition improves muscle phenotype, reducing fibrosis and atrophy associated with a model of ALS disease (Gonzalez et al., 2018). These works lead to three clinical trials using FG3019; an open-label, single-arm phase 2 clinical trial with FG3019 in non-ambulatory DMD patients and two phase 3 clinical trial using FG3019 or placebo in combination with systemic corticosteroids, in subjects with non-ambulatory DMD (NCT04371666) and Ambulatory DMD (NCT04632940).

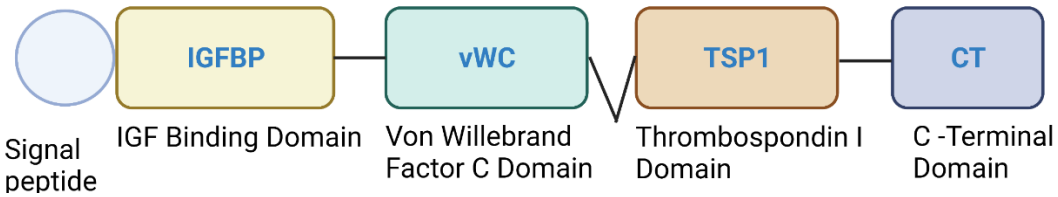


Diagram 2. CCN2 modular domains. The image shows the domains of CCN2 molecule. Adapted from (Zaykov & Chaqour, 2021). Created with BioRender.com.

6. Fibro / adipogenic progenitors

Different cell types are required to support the main functional ones in all tissues. In skeletal muscle, satellite cells, vascular endothelial cells, fibroblasts, and FAPs, among others, belong to this category. The main ECM-producing cells correspond to myofibroblasts, which are characterized by the expression smooth muscle actin (α-SMA), generating contractile microfilament bundles in the cytoplasm (stress fibers) that give them contractile properties (Sandbo & Dulin, 2011). Myofibroblasts are involved in the lung (Hardie et al., 2009), liver (Xu et al., 2014), heart (Van Den Borne et al., 2010), and skeletal muscle fibrosis (Serrano et al., 2011). The number of myofibroblasts is augmented in muscles of DMD patients and the *mdx* mice and an ALS murine model (Contreras et al., 2016)(Hori et al., 2011). Myofibroblast derives mainly from FAPs, cells CD31−, CD45−, Sca1+, PDGFRα+, that are also increased in fibrotic models such as *mdx* mice, the hSOD1^{G93A} mouse, and muscle denervation model (Contreras et al., 2016)(Uezumi et al., 2014)(Gonzalez et al., 2017). Their name describes their ability to differentiate into adipogenic or myofibroblast lineages, being the latter their predominant progeny in fibrotic tissues. As myofibroblasts are the main ECM

producing cells, inhibiting their proliferation and/or differentiation could be critical for fibrosis prevention, so **elucidating the mechanisms or molecules that determine myofibroblast number (survival, proliferation, cell death) in the tissue is a critical step towards this aim.**

7. Lysophosphatidic acid

Throughout the years, the study of fibrosis was focused on protein signaling factors and interleukins, and cytokines (Borthwick et al., 2013). However, it has been proposed that lipids could also participate in the establishment and maintenance of fibrosis in different organs, such as the liver, kidney and lung (Wobser et al., 2009)(Du et al., 2019)(Suryadevara et al., 2020). Lysophosphatidic acid (LPA 1-or 2-acyl-sn-glycerol 3-phosphate), a small phospholipid (430–480 Da), is the best-studied lipid factor 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 (Geraldo et al., 2021). Extracellular LPA is mainly synthesized by the secreted lysophospholipase D enzyme ATX which removes the choline group from lysophosphatidylcholine (LPC). ATX is the main source of circulating LPA; heterozygotes *Enpp2* mutant mice have half of plasmatic LPA, and ATX activity analyzed by hydrolysis of the fluorogenic substrate FS-3 was 50% decreased compared to WT mice (Fotopoulou et al., 2010). LPA has been found in many tissues and fluids (Yung et al. 2014) but adipose tissue has been proposed as the primary source of LPA in the blood (Ferry et al., 2003)(Dusaulcy et al., 2011), where is found in an albumin-bound form.

As a regulatory mechanism, LPA upregulation decreases ATX synthesis (Benesch et al., 2015). LPA is degraded by different lipid phosphate phosphatases (LPPs 1-3), which generate the non-signaling lipid monoacylglycerol (MAG) by removing its phosphate group from LPA. Therefore, LPP1-deficient mice have high plasma LPA levels (Tomsig et al., 2009).

8. LPA receptors

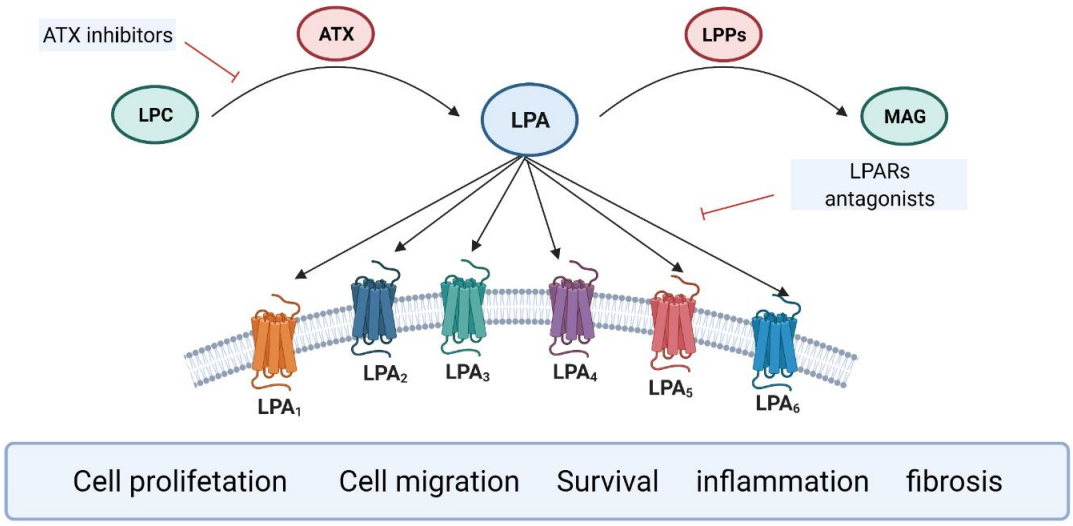
LPARs are six G-protein coupled receptors (GPCRs), called LPA_1 - LPA_6. All of them are rhodopsin-like GPCRs. LPA_1, LPA_2, LPA_3, belong to the family of endothelial differentiation genes (Edg); the other three are structurally distant receptors, LPA_4, LPA_5, LPA_6, which are associated with the purinergic family of receptors (Geraldo et al., 2021)(Yung et al., 2014).

LPA participates in different cellular processes through its receptors, triggering multiple downstream pathways involved in differentiation, cell survival, intracellular calcium transients, proliferation, and other cell responses in physiological and pathological conditions (Diagram 3)(Valdés-Rives et al., 2017). Depending on their functional relevance, these receptors are differentially expressed in tissues, and their expression pattern may change under pathological conditions (Brown et al., 2017)(Enooku et al., 2016). LPA activates many signaling pathways, involving molecules like AKT, ERK 1/2, and JNK, leading to different cellular responses.

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 LPARs results in a decrease in ECM accumulation in models of induced fibrosis (Pradère et al., 2007)(Tager et al., 2008)(Ohashi & Yamamoto, 2015). We

recently hypothesized that the ATX / LPA / LPARs axis might be involved in the inflammatory and fibrotic response observed in skeletal muscle diseases (Gallardo et al., 2021).

Diagram 3. LPA / LPARs axis and biological responses. LPC is converted to LPA by the enzyme ATX. LPA signals through 6 receptors called LPA_1 to LPA_6. LPA is degraded by



different LPPs. The main cell functions for LPA are cell proliferation, migration, and survival. LPA also participates in inflammation and fibrosis. The diagram was created with BioRender.com.

Currently, numerous pharmacological compounds targeting the ATX / LPA / LPARs axis has been developed (Diagram 4). Many of the agonists and antagonists of this molecular pathway have been tested only under *in vitro* conditions exploring responses such as intracellular calcium mobilization and growth-factor-like outcomes in different cells. (Luquain et al., 2003)(Mills and Moolenaar, 2003)(Moolenaar et al., 2004). However, some **agonists** have been administrated directly on animals; for example, in 2019, Kano et al., reported that the IV

injection of LPA 18:1 (the most studied LPA) trigger a transient blood pressure increase through different LPA receptors, mainly LPA_4 in mice (Kano et al., 2019). In addition, the use of LPA_3 agonist, 1-oleoyl-2-methyl-sn-glycerol-3-phosphorothioate (2s-OMPT), has been reported to promote erythropoiesis in Zebrafish (Lin et al., 2016).

Regarding LPA receptor **antagonist**, the most used in research are Ki16425 and AM095, which inhibits LPA_1 / LPA_3 and LPA_1, respectively. Ki16425 has been shown that can reduce the severity of abdominal inflammation and organ damage in peritoneal endotoxin exposure in mice (J. Zhao et al., 2015). On the other hand, AM095 attenuates diabetic nephropathy in mice (Lee et al., 2019) and reduces a demyelinating lesion in a model of intraspinal injection of LPA in mice (Santos-Nogueira et al., 2015). Indicating that the LPARs pharmacological inhibition has been used in different models with high effectiveness.

Until now, several clinical trials testing the effect of inhibitors of ATX / LPA / LPARs axis have been developed mainly on idiopathic pulmonary fibrosis and systemic sclerosis (NCT03798366, NCT01766817, NCT04308681). Unfortunately, **the effect of the LPAR antagonist on skeletal muscle fibrosis has not yet been studied in animal models of muscle fibrosis or in humans with muscular dystrophies.**

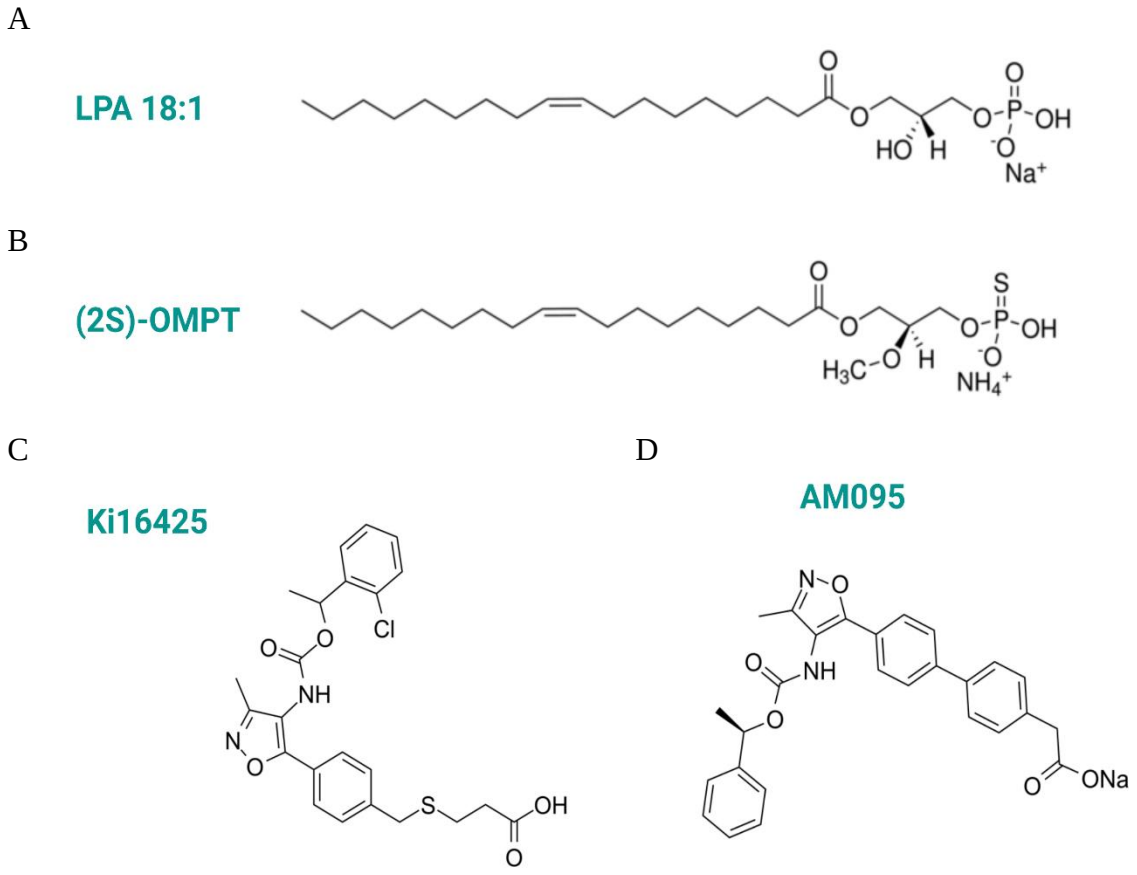


Diagram 4. Chemical structure of agonists and antagonists of LPARs. A) Chemical structure of LPA 18:1, the most widely used LPA in scientific research and one of the most abundant in human plasma. B) Chemical structure of (2S)-OMPT, LPA_3 agonist. C) Chemical structure of Ki16425, an inhibitor of LPA_1 and LPA_3. D) Chemical structure, AM095, LPA_1 inhibitor. Created with BioRender.com.

9. ATX / LPA / LPARs axis in skeletal muscle fibrosis

LPA has shown a pro-fibrotic role in different organs and diseases. Patients with idiopathic pulmonary fibrosis present increased LPA levels in exhaled breath condensate and bronchoalveolar lavage fluid (BAL) (Montesi et al., 2014)(Tager et al., 2008). LPA and ATX

are also augmented liver from patients infected with hepatitis C virus, which present liver fibrosis in advanced stages of the pathology (Watanabe et al., 2007). Similarly, there are high levels of LPA in culture medium from fibrotic kidney explants (Pradère et al., 2007) and in BAL from the bleomycin-induced lung fibrosis model (Black et al., 2016). These data suggest that there is local LPA secretion in the fibrotic organ.

Studies that inhibit the function or that modulate the presence / absence of LPARs in fibrotic models show a role for the ATX / LPA / LPARs axis. The primary LPARs that appear to be involved in fibrosis are LPA_1 and LPA_3. LPA_1 mRNA is increased in the kidney of WT mice after unilateral ureteral obstruction UUO (a model of renal induced fibrosis), but the LPA_1 KO mice show decreased fibrosis markers such as collagen III. The LPA_1 and LPA_3 pharmacological inhibitor Ki16425 triggered a similar response (Pradère et al., 2007).

Similarly, the lung fibrosis model induced by radiation increases LPA_1 and LPA_3 mRNA. The LPA_1 / LPA_3 inhibitor VPC12249, reduces collagen deposition and avoids the induction of pro-fibrotic factors like CCN2 and TGF-β in the lung (Gan et al., 2011). The positive effects of blocking LPARs in lung fibrosis were also observed in the bleomycin-induced model (Ohashi & Yamamoto, 2015).

In 2021 Gento-Caro showed that in the ALS murine model $SOD1^{G93A}$, the treatment with AM095, an LPA_1 inhibitor, showed motor skills improvement evaluated by grip strength, rotarod, and runtime (Gento-Caro et al., 2021). These results are relevant because the animal model $SOD1^{G93A}$ and ALS disease present a severe fibrotic phenotype in skeletal muscle (Gonzalez et al., 2017). Davies et al. shown that intraperitoneal LPA injections have a deleterious effect in atrophy and fibrosis triggered by tendon nerve injury in rotator cuff muscle

(Davies et al., 2017). All these data support the use of pharmacological inhibitors of the ATX / LPA / LPARs axis as a therapeutic tool in fibrotic disorders, as addressed in numerous clinical trials, mainly in idiopathic pulmonary fibrosis and scleroderma. **It would be interesting to explore if those inhibitors can be valuable tools for treating skeletal muscle fibrosis in pathologies such as MDs.**

The effect of LPA in fibrosis could be mediated through the differentiation of resident cells towards a myofibroblast-like type since in a model of peritoneal fibrosis, the absence of LPA_1 prevents the accumulation of myofibroblast (Sakai et al., 2013). FAPs are the main source of the myofibroblast in skeletal muscle and are increased under fibrotic conditions such as the *mdx* mice, denervated muscles and ALS (Contreras et al., 2016). It is essential to study if LPA is involved in differentiating FAPs towards a myofibroblast phenotype in muscles from these animals. LPA could also be mediating its pro-fibrotic effects by inducing other responses in FAPs, like proliferation, migration, or even diminishing apoptosis, as has been shown previously in different cell types in cancer studies (Stähle et al., 2003)(Yamada et al., 2008).

LPA pathway could be related to TGF-β signaling since in Tenon's fibroblasts, the contraction induced by TGF-β is blocked by Ki16425, the antagonist LPA_1 and LPA_3, and downregulates the SMAD 2 / 3 proteins, which are canonical mediators in TGF-β pathway (Wen et al., 2019). Ki16425 also avoids the migration and proliferation of these fibroblasts.

Interestedly, our laboratory has previously demonstrated that C2C12 myoblasts can respond to LPA, by inducing CCN2 expression (Vial et al., 2008) (Riquelme-Guzmán et al., 2018) through a mechanism that requires the activation of the TGF-β receptor I (Cabello-Verrugio et al., 2011). Preliminary experiments have shown that inhibition of LPA_1 and LPA_3

(using Ki16425) prevents the induction of CCN2 by TGF-β, indicating possible crosstalk between both pro-fibrotic pathways.

LPA and TGF-β require integrins to induce CCN2 in myoblasts (Riquelme-Guzmán et al., 2018), findings deeply explored in Ph.D. Thesis, Meilyn Cruz-Soca, 2022, indicates that the main integrins participating are αvb3 and αvb5; thus, the ECM / integrin interaction is a new factor in LPA signaling. The interaction of LPA / TGF-β / integrin has also been proposed by Xu et al. based on results that demonstrated that LPA induces αvβ6-mediated TGF-β activation through RhoA and Rho kinase in epithelial cells (Xu et al., 2009).

Taken together, these data suggest that the **ATX / LPA / LPARs axis could be part of a novel pro-fibrotic pathway in skeletal muscle diseases causing muscular fibrosis with solid pharmacological projections.**

II. HYPOTHESIS

The ATX / LPA / LPARs axis promotes a fibrotic response in skeletal muscle mainly through fibro / adipoagenic progenitors

III. GENERAL OBJECTIVE

To determine if the ATX / LPA / LPARs axis is present in skeletal muscle and promotes a fibrotic response through fibro / adipogenic progenitors

IV. SPECIFIC OBJECTIVES

1. To characterize the ATX / LPA / LPARs axis expression in skeletal muscle from murine models of muscular fibrosis and controls

2. To study the fibrotic response of skeletal muscle to LPA, fibro / adipoagenic progenitors participation and the involvement of the ATX / LPA / LPARs axis in the denervation-induced fibrotic model

3. To identify the LPARs and the possible signaling pathways involved in the induction of CCN2 in muscular fibro / adipoagenic progenitors

V. MATERIALS AND METHODS

1. Materials

1. 1 Biological material

1.1.1 Animals

All animal experiments were performed following the protocols approved by the Animal Ethics Committee, Pontificia Universidad Católica de Chile (Protocols 180810006, 180821022, and 180820006). C57BL/10, C57BL/6, and *Pdgfra*$^{tm11(EGFP)Sor}$ mice in background C57BL/6 (PDGFRα^{H2BEGFP}) were obtained from The Jackson Laboratory, USA. LPA_1-KO in background BALB/c mice were kindly donated by Dr. Jerold Chun (Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, United States.)

All the animals were kept in the animal facility of the Faculty of Biological Sciences of the Pontificia Universidad Católica de Chile, in ventilated racks with filtered air ensuring a free environment of pathogens. Mice had free access to water and a standard diet that allowed adequate reproduction and growth. The animals were subjected to a light regime corresponding to alternate cycles of 12 hours (day/night) and controlled temperature.

1.1.2 Cell lines

The following cell lines were used:

C2C12 cells: commercial cell line, established by Yaffe and Saxel (Yaffe & Saxel, 1977) from regenerating skeletal muscle of an adult C3H mouse.

10 T1/2 cells: clonal cell line established by Reznikoff et al. (Reznikoff et al., 1973). These cells are derived from 14-17 days C3H mouse embryos.

1.1.3 FAPs primary culture

Skeletal muscle FAPs were obtained from 2-3 months male mice C57BL/6 or BALB/c following the protocol described in the methods section.

1.1.4 Antibodies

Table 1: List of antibodies, host, applications, and dilution in which they were used. WB: Western blot, IIF: indirect immunofluorescence.

Antibody	**Host**	**Dilution**	**Origin**
Autotaxin	Rabbit	WB: 1:500	Cayman Chemical, MI, USA)
CCN2	Rabbit	WB: 1:1000	Abcam, (Cambridge, UK)
CCN2	Goat	WB: 1:500	Santa Cruz (CA, USA)
CCN2	Rabbit	IIF: 1:50	Cell Signaling, (MA, USA),
Fibronectin	Rabbit	WB: 1:1000 IIF: 1:200	Sigma-Aldrich (MO, USA)
Fast myosin	Rabbit	WB: 1:1000	Abcam, (Cambridge, UK)
GAPDH	Rabbit	WB: 1:2000	Proteintech (IL, USA)
LPA_3	Rabbit	WB: 1:1000	Cayman Chemical (MI, USA)
LPA_6	Rabbit	WB: 1:1000	ABCEPTA (CA,USA)
Slow myosin			Abcam, (Cambridge, UK)
PDGFRα	Goat	WB: 1:1000 IIF: 1:100	R&D Systems, (MN, USA
Tubulin	Mouse	WB: 1:1000	
pERK	Rabbit	WB: 1:1000	Cell Signaling, (MA, USA)
pAKT	Rabbit	WB: 1:1000	Cell Signaling, (MA, USA)
pJNK	Rabbit	WB: 1:1000	Cell Signaling, (MA, USA)
pYAP	Rabbit	WB: 1:1000	Cell Signaling, (MA, USA)
α-SMA	Mouse	IIF: 1:100	Sigma-Aldrich (MO, USA)

Tubulin	Rat	IIF: 1:100	Abcam, (Cambridge, UK)

2. Reagents

2.1 General reagents

The following reagents were obtained from Merck (Darmstadt, Germany): analytical grade solvents ethanol, isopropanol, methanol, and chloroform. From Thermo scientific (IL, USA): Proteinase K, PVDF membranes, super signal west femto maximum sensitivity substrate, super signal west dura extended duration substrate, micro–BCA Protein assay kit, PVDF membranes, ammonium persulfate (PSA), protein ladder and 1Kb DNA ladder. From Life technologies (CA, USA), TRIzol reagent. From PhytoTech (Kansas, USA): Tris base. From Sigma-Aldrich (MO, USA): Fatty acid-free bovine serum albumin (BSA), collagenase/dispase, Triton x-100, PMSF, Ponceau S. From USBiological (Swampscott, MA, USA): Sodium dodecyl sulfate (SDS). From Winkler (Santiago, Chile): Bovine serum albumin (BSA), Tween 20, methanol, glicine, sodium chloride, Acrilamide, Bis-Acrilamide.

2.2 Cell culture mediums and materials

The following cell culture materials were obtained from Corning (USA): Cell strainer 70 µM, 35 mm, 60 mm, 150 mm plates. 15 mL and 50 mL polypropylene conical tubes were obtained from Falcon (St. Louis, MO, USA). 0.22 µm syringe filters were purchased from EDLAB. From Gibco, Life Technologies (NY, USA): Dulbecco's Modified Eagle Medium (DMEM), Trypsin-EDTA 10X. From Hyclone laboratories (UT, USA): fetal bovine serum.

2.3 LPAR agonists

LPA (Oleoyl-L-α-lysophosphatidic acid sodium salt) purchased from Sigma-Aldrich (St. Louis, MO, USA). (2s)-OPMT (1-oleoyl-2-O- methyl-rac-glycerophosphothionate), an agonist of $LPAR_3$, was purchased from Cayman Chemical (Ann Arbor, MI, USA).

2.4 Inhibitors

To inhibit LPA signaling, we used the $LPAR_1$ and $LPAR_3$ antagonist Ki16425 (Cayman Chemical, Ann Arbor, MI) and the $LPAR_1$ antagonist, AM095 (Sigma-Aldrich, MO, USA). To inhibit kinases, we used PI3K/AKT inhibitor LY294002 (Merck-Calbiochem, Darmstadt, Germany), ERK1/2 inhibitor, U0126 (Cell Signaling Technology, Danvers, MA, USA), and the JNK inhibitor VIII (Merck-Calbiochem, Darmstadt, Germany).

3 Methods

3.1 DNA extraction and LPA_1 mice genotyping

The genomic DNA used as a template for the PCR reaction was obtained from mice ears. The tissue was digested in lysis buffer (100 mM Tris-HCI pH 8.0, 5 mM EDTA pH 8.0, 0.2% SDS, 200 mM NaCl, 0,1 mg /ml Proteinase K) at 55 °C in a dry thermoregulated bath overnight. After the incubation, the samples were centrifuged for 10 min at 14.000 g, the supernatant was collected, and a volume of isopropanol was added to precipitate the genomic DNA. Samples were centrifugated at 14,000 g x 10 min at 4°C. The precipitate was washed with 1 ml of 75% ethanol. The supernatant was removed, and the genomic DNA obtained was dried for at least 1 hour and then resuspended in 15 µl of nuclease-free water. The samples obtained were stored at -20°C.

The PCR reaction was carried out in a final volume of 25 µl containing: 100-200 ng of genomic DNA, buffer for taq DNA polymerase 1X, 25 mM $MgCl_2$, 0.8 mM dNTPs (dATP, dCTP, dGTP, dTTP), 0.8 uM primers mix, 1.25 U Taq DNA polymerase, H_2O nucleases free. The amplification program used was: 40 cycles of: 94°C, 30s; 59°C, 45s; and 72° C, 50s in Eppendorf mastercycler gradient thermal cycler. The obtained PCR product was visualized on a 2% agarose gel on UV light.

3.2 *In vivo* treatment with Ki16425 and U0126

Ki16425 and U0-126 were administered to 3-month-old WT male mice by intraperitoneal injection in 5 mg/Kg/day or 10 mg/Kg/day doses, respectively, for three days before LPA treatment.

For the intraperitoneal injection, the mice were taken facing up, and the head was at a 40° angle to the horizontal position. The right lower quadrant of the abdomen was injected with a 30G syringe.

3.3 LPA intramuscular injection

Groups of male mice were studied at the ages indicated in each figure. LPA (Oleoyl-L-α-lysophosphatidic acid sodium salt) was dissolved in phosphate-buffered saline (PBS) 1 mg/ml fatty acid-free BSA. Intramuscular injections were administered in mice anesthetized with 2.5 % isoflurane gas in oxygen. The animals were kept at 37°C in a thermoregulated plate. LPA (400 µg/kg in a volume of 50 µl) injection was administered in the tibialis anterior (TA) muscle.

The contralateral TA was injected with BSA and used as a vehicle-injected control. Once the experiment ended, the animals were sacrificed. The TA 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 TA were also obtained for histological staining and indirect immunofluorescence (IIF).

3.4 Muscle denervation

The muscle denervation model was used for fibrosis induction in 5 to 6 months WT mice (Rebolledo et al., 2019). Prior to the surgery, aseptic techniques were performed such as cleaning the area with saline and later with 2% chlorhexidine. The surgery consists of exposing the sciatic nerve through an incision at the hip of the mouse. Once the nerve is exposed, 3-5 mm of the sciatic nerve will be unilaterally transected only in the right hind limb to restrict its regeneration. Surgery was sutured with surgical staples. Mice were medicated with Tramadol in a dose consistent with their weight for 3 days from the time of surgery. For this thesis, two denervation time-points were considered: four days and 2 weeks.

3.5 Post-mortem muscle extraction

The animals were sacrificed with an overdose of anesthesia with isoflurane: first, induction was generated using an isoflurane vaporizer through continuous inhalation and then cervical dislocation. Once the animal's death is verified, through indicators of loss of reflexes, 70% ethanol will be applied in the area to be incised. Using clean and disinfected surgical material with 70% ethanol, an incision was made in the skin and the muscles of the posterior

extremities of the animal exposed, and the TA and gastrocnemius (GAS) muscles were extracted. Additionally, in most cases, the diaphragm (DIA) of the animal was also removed. The muscles that were used for IIF and those used for Sirius red staining were removed and immediately frozen in isopentane to remove water molecules and thus prevent tissue destruction due to the freezing process. The muscles were kept at -80 °C until processed.

3.6 C2C12 and 10T1/2 maintenance

The C2C12 and 10T1/2 cell cultures were maintained in DMEM culture medium supplemented with 10% FBS and 1% antibiotic-antimycotic at 37°C and 8% $C0_2$ for C2C12 or 5% for 10T1/2 (Contreras et al., 2018)(Contreras, et al., 2019). The culture medium was changed periodically (every two days). When the cells reached 80 to 90 % confluence were trypsinized and reseeded to continue their growth.

3.7 FAPS primary culture

FAPs were isolated by the differential adhesion method described by our laboratory (Contreras et al., 2019). The animals used are the C57Bl/6ScSc mouse aged 2 to 3 months. The euthanasia of the animals was made by sedation with isoflurane using an anesthesia chamber. The anesthetic depth was performed by evaluating the foot reflex, once this has been verified, the cervical dislocation was performed with the help of a metal clamp. The TA, GAS, DIA, triceps, and pectoralis muscles was removed and plated with DMEM 10% FBS. The muscles were mechanically disaggregated with a scalpel blade for 5 minutes, the homogenate was transferred to a 50 mL conical tube with DMEM supplemented with 10% FBS and collagenase/dispase 25 mg/g of tissue. The tube was placed in a thermoregulated bath at 37°C

with agitation for 40-50 min. The disaggregated tissue extract was filtered through a 70 µm filter, and the homogenate was centrifuged at 2,200 g for 5 min. The resulting pellet was resuspended in DMEM medium supplemented with 10% FBS and seeded in 150 mm plates. After an hour and a half in the 5% CO2 incubator, a change of medium was made. After approximately 5 days, with a change of maintenance medium every two days, a confluent plate with around 1,2 x10^6 cells were obtained; an amount that allows seeding about 20 plates of 35 mm at a confluence of 5,000 cells/cm2 for experiments, and a plate of 150 mm for maintenance. The cells were used until the second passage to prevent differentiation to myofibroblast phenotype.

3.8 *In vitro* FAPs treatments

The concentration and time to use LPA or 2s-OMPT in FAPs treatment were defined by performing concentration and time curves respectively. All inhibitors (Ki16425, AM095, U0126, JNK inhibitor VIII, LY294002) were added to the cell plate 40 min before LPA or 2s-OMPT treatment. The vehicle of all inhibitors used in this thesis was DMSO.

3.9 Protein extraction in cell lysates

Protein extracted were obtained from cells using RIPA 1X (Tris-HCI 50 mM pH 7,4, NP-40 1%, Na Deoxycholate 0,25%, NaCI 150 mM, EDTA 1mM, EGTA 2 mM) supplemented with protease and phosphatase inhibitors: 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM sodium orthovanadate (Na_3VO_4), and 1mM sodium fluoride (NAF). The samples were sonicated for 10 to 15 seconds and centrifugated at 12,000 g for 10 minutes.

3.10 Protein extraction in muscle homogenates

Whole-muscle extracts were obtained by homogenization of the tissue or its sections in Tris-EDTA buffer pH 7.4 with 1mM PMSF, Na_3VO_4, and 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 12,000 g to pellet insoluble material. The samples obtained are stored at -20°C until their use.

3.11 Protein detection in cell lysates and muscle homogenates

Protein concentration was determined by the bicinchoninic acid assay (Mallia et al., 1985) using the BCA Assay kit y (Thermo Scientific, Il, USA). The reaction was carried out in a 96-well ELISA plate, according to the manufacturer's instructions and the absorbance was measured in a plate reader (Synergy H1, hybrid reader, BioTek). The values were interpolated on a calibration curve prepared from a solution of BSA of known concentration.

3.12 SDS-polyacrylamide gel electrophoresis and immunoblot analyses

20 ug (of protein extract from cell culture) or 50 µg (of protein extracts from muscle homogenates) were subjected to SDS-PAGE and transferred to PVDF membranes. 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 primary antibodies (Table 1) at 4°C overnight. Primary antibodies were detected with horseradish-peroxidase-conjugated secondary antibodies. All immunoreactions were visualized by enhanced chemoluminescence using a ChemiDoc-It HR

410 imaging system (Upland, Calif., USA). Densitometric analysis and quantification were performed using the ImageJ software (NIH, USA).

3.13 RNA isolation, reverse transcription, and qPCRs.

Total RNA was isolated from TA, DIA and GAS muscles and FAPs primary culture 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) or Quant studio 3 (ThermoFisher scientific (IL, USA). A list of primers used in this work is given in Table 2. mRNA expression was quantified using the comparative dCt method (2-ddCT), using 18S as the reference gene. For the LPAR expression analysis studies, the intra-assay primers efficiency was determined. The efficiency value used in the qPCR data analysis was derived from the observed amplification data. The amplification efficiency was calculated from the standard curve slope, a plot of experimental C_t values versus the log-concentration of a serial two-fold dilution of cDNA (dilutions ranging from 1:4 to 1:64). The results were expressed as times of change according to the dCt method, replacing the amplification efficiency equals 2 for the efficiency obtained for each primer.

The mRNA levels were expressed relative to the mean expression of the control condition.

Table 2. List of primers used in qPCR reactions.

	Forward sequence 5'-3'	Reverse sequence 5'- 3'
Lpar1	CTG CCT CTA CTT CCA GCC CTG TAA	TGC TCA CTG TGT TCC ATT CTG TGG
Lpar2	GCT GGT TAT TGC AGC CAT CG	ACT CCA CGC TGG CTG TAT ATG
Lpar3	CCA CTT TCC CTT CTA CTA CCT GCT	GACGGT CAA CGT TTT CGA CAC C
Lpar4	GTC AAC AAT GCG ACC ACC AC	AAG CAC CAC AGA AGA ACA AGA AAC A
Lpar5	ACT CCA CGC TGG CTG TAT ATG	GTA GCC AAA GGC CTG GTA TCC
Lpar6	GAT CAC TCT CTG CAT CGC TGT TTC	CCC TGA ACT TCA GAG AAC CTG GAG
*Plpp1 (*LPP1)	GGG AGA CTG GGC AAG ACT CTT	CAC TCG AGA AAG GCC CAC AT
*Plpp2 (*LPP2)	CGC GAT CCA ACT TCA ACA ACT	CAG CCC CGA ACA GAA AGG T
*Plpp3 (*LPP3)	CCA TCC TGG CGA TCA TTA CAG	AAA GGA AGC ATC CCA CTT GCT
*Enpp2 (*Autotaxin)	GAC CCT AAA GCC ATT ATT GCT AA	GGG AAG GTG CTG TTT CAT GT
*Ctgf (*CCN2)	CAG GCT GGA GAA GCA GAG TCG T	CTG GTG CAG CCA GAA AGC TCA A
Col3A1(Collagen 3)	AGC ACC TGT TTC TCC CTT T	CTG GTA TGA AAG GAC ACA GAG
*Fn1 (*Fbronectin)	AGA TTG GCG ACA AGT GGA GG	AGG TTT GCA GGT CCA TTC CC
Postn (Periostin)	ATC AGG GGT CGG GAT CAG G	TTC CCG CAG ATA GCA CCT TG
Vcl (Vinculin)	CCT CAG GAG CCT GAC TTC C	AGC CAG CTC ATC AGT TAG TCG
*Vim (*Vimentin)	TGT CCA AAT CGA TGT GGA TGT TTC	TTG TAC CAT TCT TCT GCC TCC TG
Acta2 (αSMA)	TCC CTG GAG AAG AGC TAC GA	CTT CTG CAT CCT GTC AGC AA
Adipoq (Adiponectin)	GGA ACT TGT GCA GGT TGG AT	TCT CCA GGA GTG CCA TGT CT
18s	TGA CGG AAG GGC ACC ACC AG	CAC CAC CAC CCA CGG ATT CG

3.14 Indirect immunofluorescence

For IFF, 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 1hour in 1% BSA in PBS, incubated overnight at 4°C with primary antibodies (Table 1). Samples were then washed three times 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).

3.15 Sirius red stain

To detect interstitial collagens, Sirius red staining was made. Skeletal muscle was processed obtaining cryosections of 5 μm thickness, which were fixed in 100% ethanol for 30 minutes at -20°C, washed for 3 minutes in distilled water, and incubated in saturated aqueous picric acid for 60 minutes at 50°C. Subsequently, the samples were washed for 3 minutes in distilled water, incubated for 2 minutes in 0.2% aqueous PMA and washed 2 times for 3 minutes in distilled water. Next, the samples were incubated in 1% picrosirius red in saturated aqueous picric acid for 5 minutes, washed in 0.001N HCL for 2 minutes and in 70% ethanol for 5 minutes. Finally, the samples were dehydrated in an alcohol battery, from 70% to 100% ethanol, dipped in xylol and mounted with entellan.

3.16 Microscopy

Images were acquired by a blinder user with a Nikon Ti2-E inverted microscope at the Unidad de Microscopía Avanzada (UMA) Facility, Pontificia Universidad Católica de Chile. All the immunofluorescence reconstructions of TA sections quantification and counting of eGFP positive and total nuclei were performed using the ImageJ software (version 1.46r, NIH, USA). FAPs immunofluorescence representative images were acquired in Nikon Eclipse Ti inverted microscope located in the laboratory of mitochondrial communication and function directed by Dr. Verónica Eisner.

3.17 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: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

VI. RESULTS

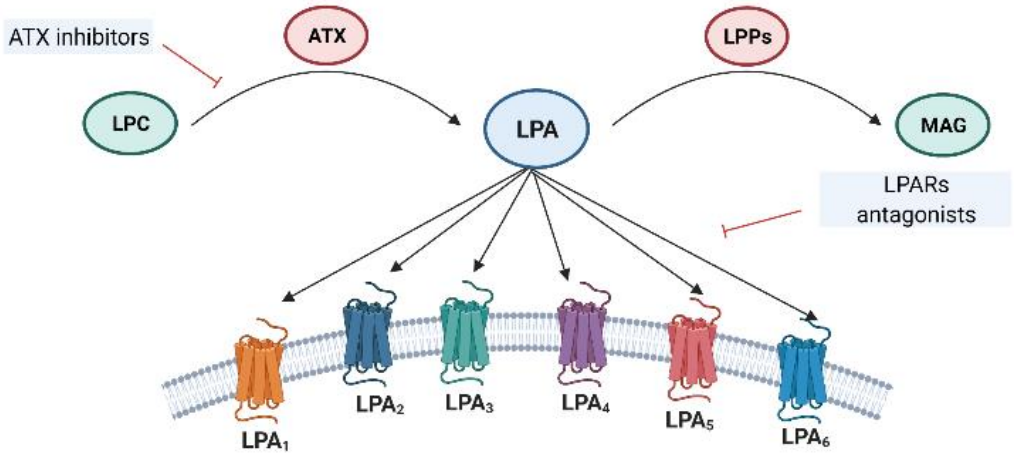
1. Specific objective 1

To characterize the ATX / LPA / LPARs axis expression in skeletal muscle from murine models of muscular fibrosis and controls

1.1. Skeletal muscle expresses components of the ATX / LPA / LPARs axis

To understand whether the LPA / LPARs / ATX axis has a role in establishing and maintaining fibrosis in skeletal muscle, we must first determine if skeletal muscle expresses components of the axis at the mRNA and protein levels. The enzyme ATX synthesizes LPA from LPC and it is degraded by LPPs (Fig. 1A). To elucidate if enzymes that participate in the production and degradation of LPA are present in skeletal muscle, we evaluated their expression by RT-qPCR. Our results show that mRNAs ATX and LPPs 1-3 are present in the three analyzed muscles, being more abundant in the DIA than in TA or GAS (Fig. 1B).

A



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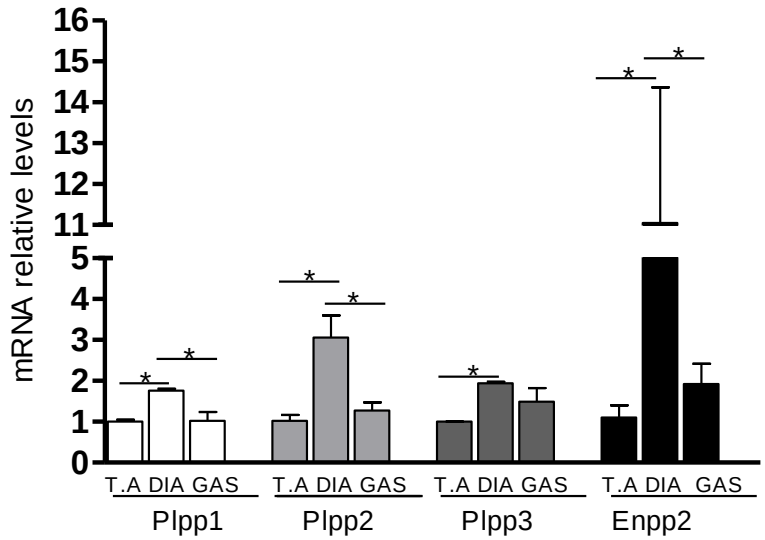
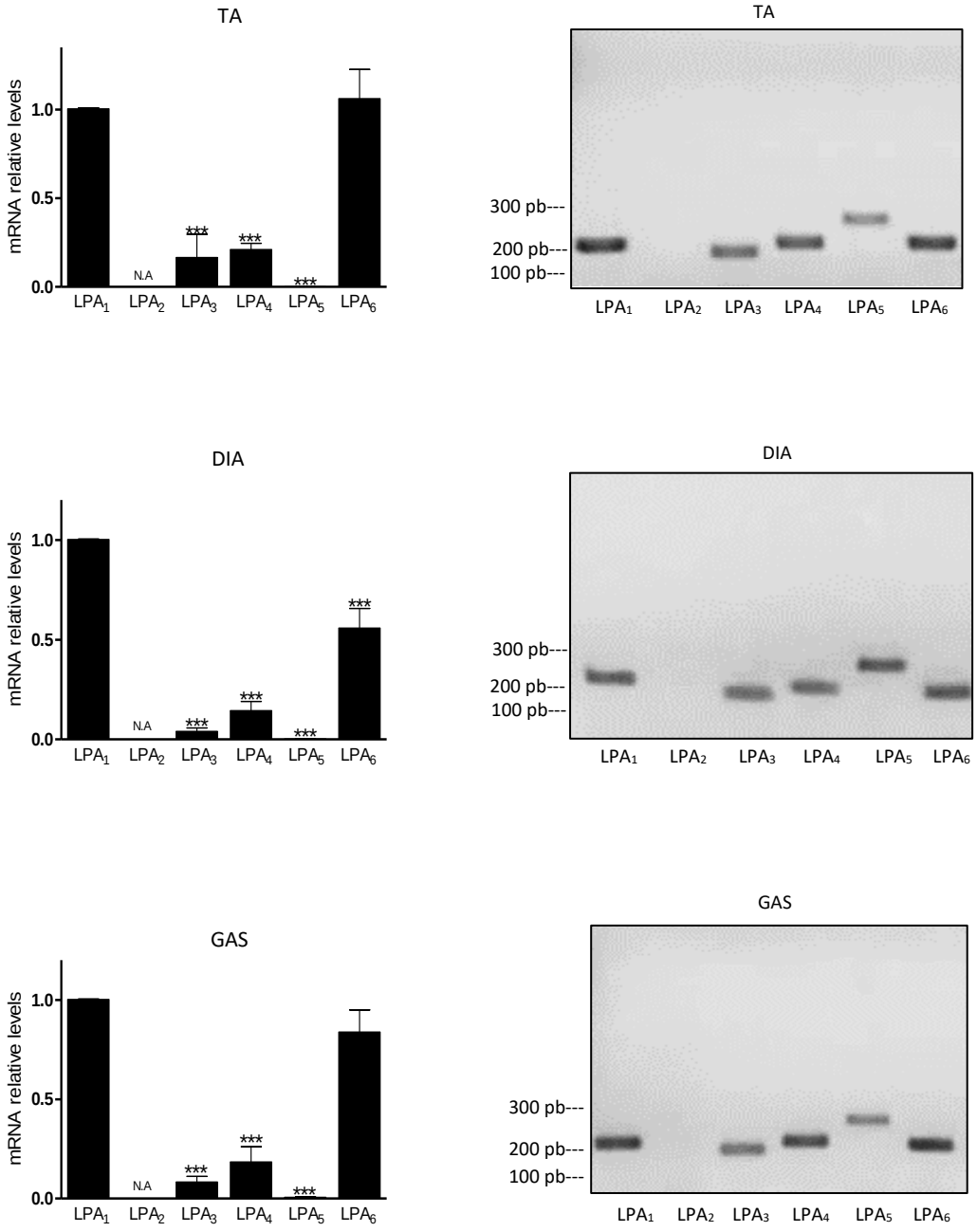


Figure 1. Skeletal muscle expresses LPA synthesis and degradation enzymes. A) LPC is converted to LPA by ATX. LPA signals through 6 receptors called LPA_1 to LPA_6. LPA is degraded by different LPP 1-3. The diagram was created with BioRender.com. B) Plpp 1-3 (LPPs 1-3) and Enpp2 (ATX) mRNA levels were analyzed by RT-qPCR in WT TA, DIA, and GAS muscles. 18s was used as a reference gene. The expression of the different genes are relative to T.A levels. The values correspond to the mean ± SEM. *P < 0.05 by one-way ANOVA with Tukey post-test. n = 3.

There are six G protein-coupled receptors for LPA (Fig. 1A). We studied the expression of LPARs mRNA in skeletal muscle using RT-qPCR and western blot. We observed a similar expression pattern in LPARs mRNA between the three analyzed muscles (TA, DIA, GAS), being the LPA_1 and LPA_6 the most abundant (Fig. 2 A). To control the used method, we decided to analyze the expression of these receptors in liver tissue; our results were similar to those previously described (Simo et al., 2014). In the liver, higher levels of LPA_6 than LPA_1 mRNA were observed (Supplementary Fig. 1). In muscle, conversely, LPA_6 mRNA levels were like LPA_1 levels. The LPA_3-LPA_5 receptors are expressed significantly lower than LPA_1 (Fig. 2A).

Unfortunately, we cannot present immunoblot for all LPARs because of the difficulty of finding specific antibodies, as reported previously (Suckau et al., 2019). LPA_3 and LPA_6 were detected in immunoblots, and their protein levels were higher in TA and GAS than in the DIA (Fig. 2B, C, and D). ATX protein levels showed higher expression in the DIA than TA and GAS (Fig. 2B and E). As control of the muscle types analyzed, we show slow and fast myosin immunoblots (Fig. 2B). **These results indicate the presence of the ATX / LPA / LPARs axis components in skeletal muscle and open many questions about its function on this tissue.**

A


TA
mRNA relative levels
1.0
0.5
0.0
N.A
***
LPA1 LPA2 LPA3 LPA4 LPA5 LPA6
TA
300 pb---
200 pb---
100 pb---
LPA1 LPA2 LPA3 LPA4 LPA5 LPA6
DIA
mRNA relative levels
1.0
0.5
0.0
N.A
***
LPA1 LPA2 LPA3 LPA4 LPA5 LPA6
DIA
300 pb---
200 pb---
100 pb---
LPA1 LPA2 LPA3 LPA4 LPA5 LPA6
GAS
mRNA relative levels
1.0
0.5
0.0
N.A
***
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GAS
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LPA1 LPA2 LPA3 LPA4 LPA5 LPA6

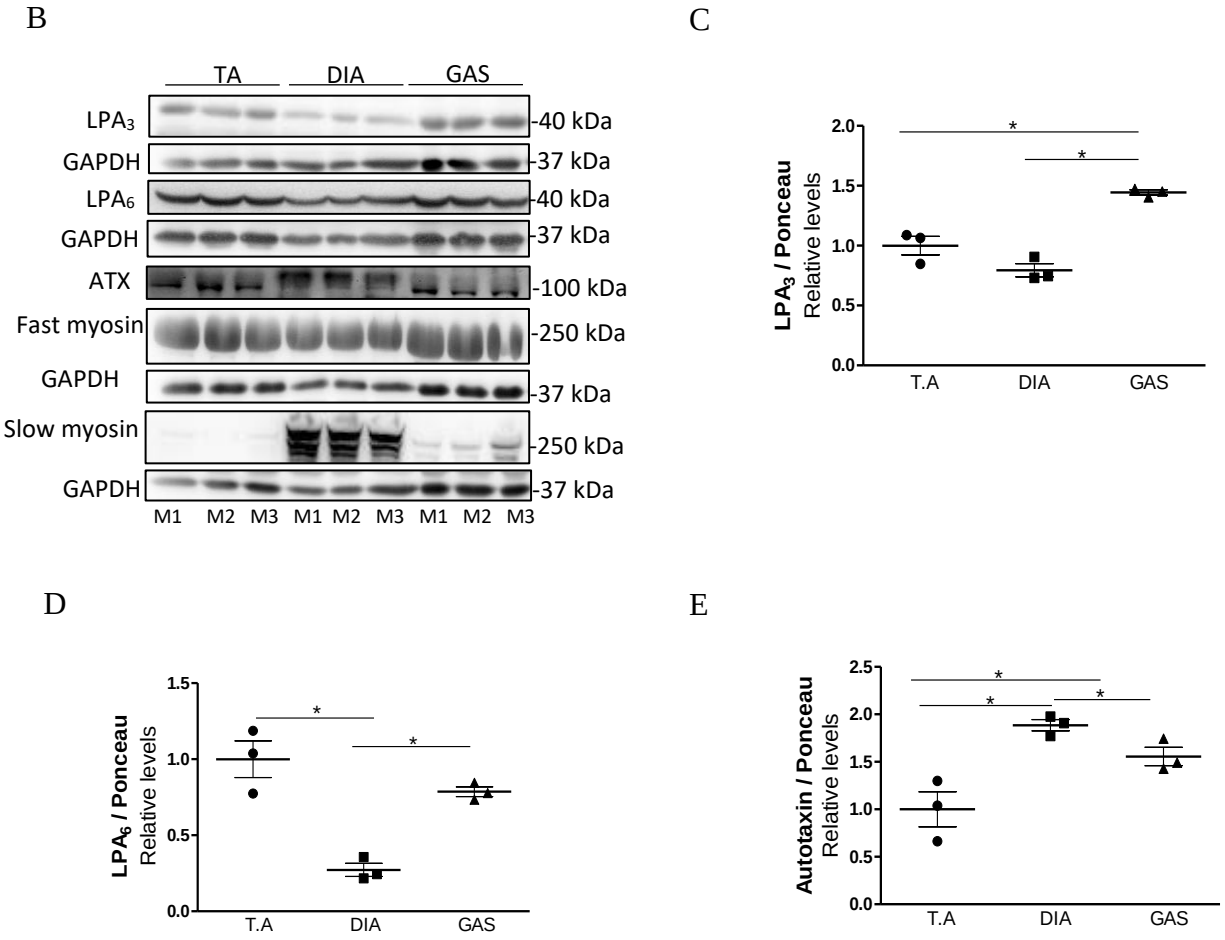


Figure 2. Skeletal muscle expresses LPARs. A) LPA_{1-6} expression was analyzed by RT-qPCR in TA, DIA, and GAS muscles from 6 months WT mice, LPA_2 mRNA was not analyzed (N.A). The expression of LPARs is relative to LPA_1. 18s was used as a reference gene. Gene products are shown. B) ATX, fast and slow myosin, LPA_3, LPA_6, and GAPDH protein levels were detected by immunoblot in TA, DIA, and GAS muscles. GAPDH was used as a loading control. C, D, and E) Quantification of protein levels. M1 to M3 indicate each processed mouse. The values correspond to the mean ± SEM. *P<0.05, ***P<0,001 by one-way ANOVA with Tukey's post-test. n=3.

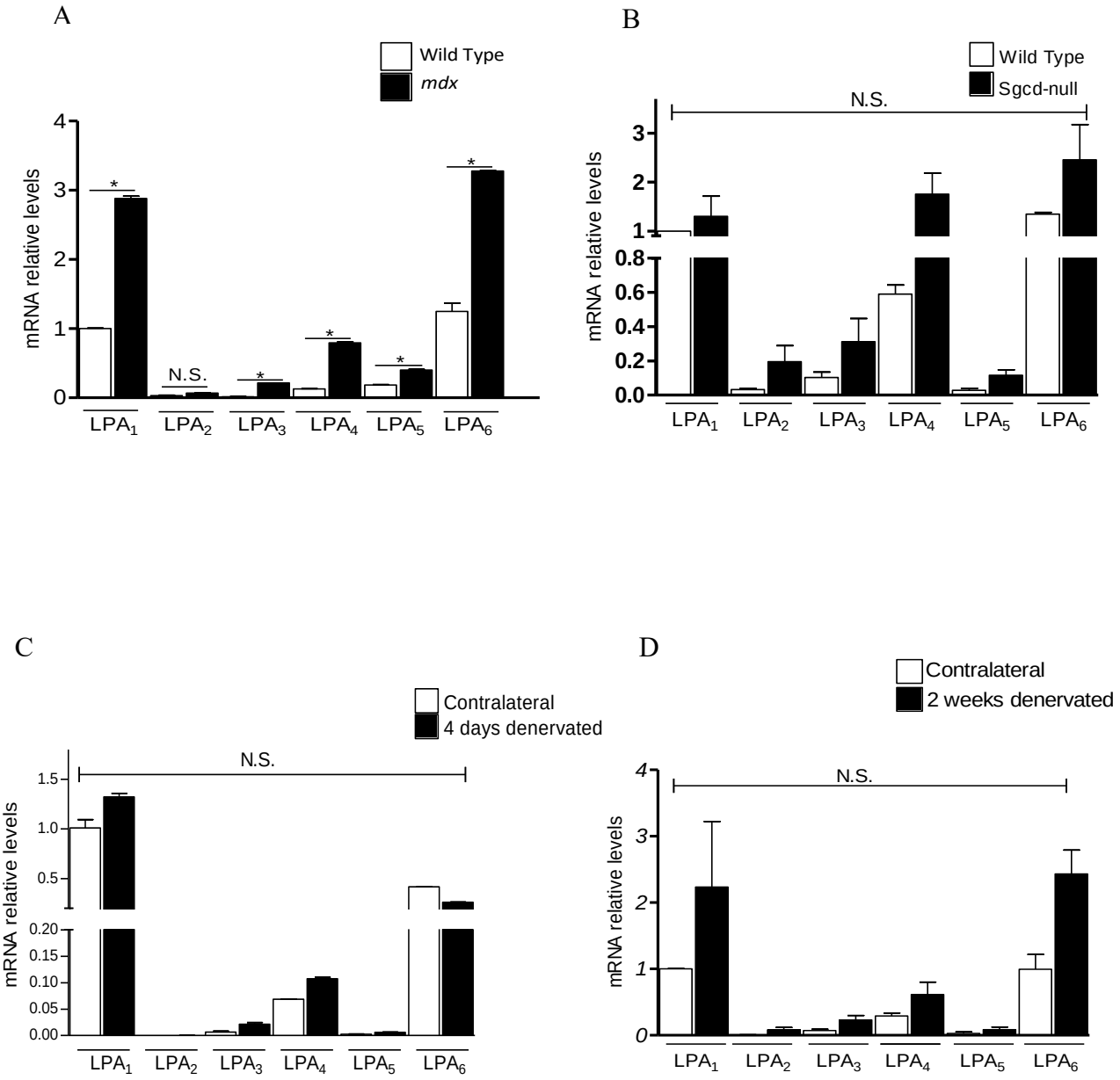
1.2 Fibrotic skeletal muscle models express ATX / LPA / LPARs axis components differentially

To begin the study of the ATX / LPA / LPARs axis in skeletal muscle fibrosis, we decided to analyze the levels in the components of this axis in muscles under normal conditions (controls) and in fibrotic muscle. First, we studied the MDs murine models, *mdx,* (for DMD) and *Sgcd*-KO (for LGMD). We analyzed the levels of the six LPARs in DIA by RT-qPCR from 2 months WT and *Sgcd*-KO mice. Our results did not show statistically significant changes compared to control mice (Fig. 3A). Due to the above, we decided to evaluate the levels of LPARs in DIA of 9-month-old *mdx* mice, since these animals present more severe and consolidated fibrosis. We found that LPA_1, LPA_3, LPA_4, LPA_5, and LPA_6 were increased in DIA from *mdx* mice (Fig. 3B).

Muscle denervation is a well-established model of fibrosis. An induced model allows studying the beginning and maintenance of the fibrotic process because it appears more synchronously than the *mdx* and *Sgcd*-KO model. We studied the mRNA levels of LPARs in denervated GAS muscles and their control (contralateral gastrocnemius muscle) after denervation. We chose two time points: 4 days and 2 weeks after denervation. No changes were observed at 4 days, but two weeks after denervation, we detected a trend to increase in LPA_6 levels, but it is not statistically significant with n = 3 (Fig. 3C, D).

We also evaluated LPA_3 and ATX by immunoblot; after 4 days (Fig. 3E, F) and 2 weeks (Fig. 3G, H) of denervation, we did not find significant changes in the protein levels of these components of the ATX / LPA / LPARs axis. Unfortunately, we could not assess the levels of the six LPARs due to the mentioned difficulty in obtaining specific LPARs antibodies. **These**

results indicate that consolidated fibrotic muscle present deregulated mRNA levels of LPARs, suggesting a role of the ATX / LPA / LPARs axis in muscle fibrosis.



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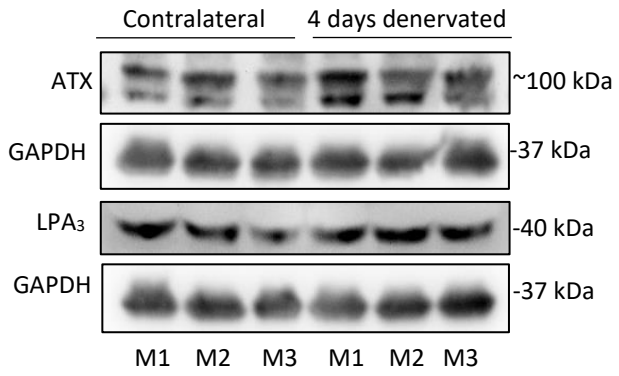
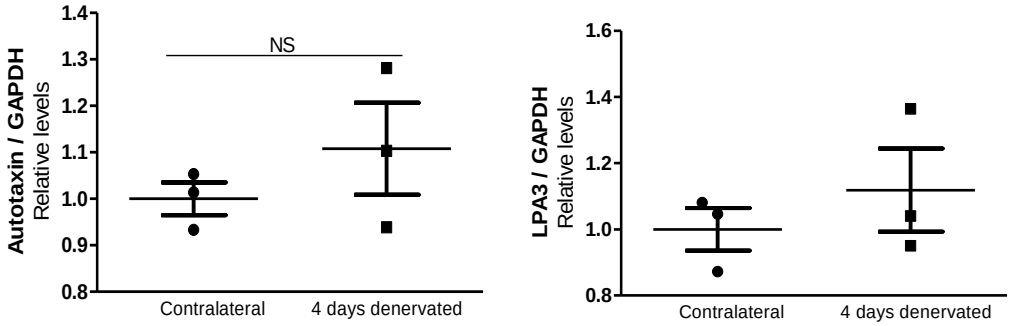
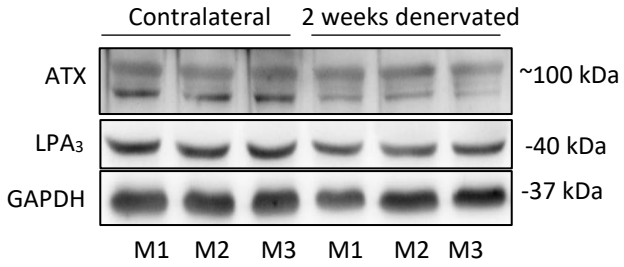

Contralateral
4 days denervated
ATX
~100 kDa
GAPDH
-37 kDa
LPA3
-40 kDa
GAPDH
-37 kDa
M1 M2 M3 M1 M2 M3

F


Autotaxin / GAPDH
Relative levels
NS
1.4
1.3
1.2
1.1
1.0
0.9
0.8
Contralateral
4 days denervated
LPA3 / GAPDH
Relative levels
1.6
1.4
1.2
1.0
0.8
Contralateral
4 days denervated

G


Contralateral
2 weeks denervated
ATX
~100 kDa
LPA3
-40 kDa
GAPDH
-37 kDa
M1 M2 M3 M1 M2 M3

H

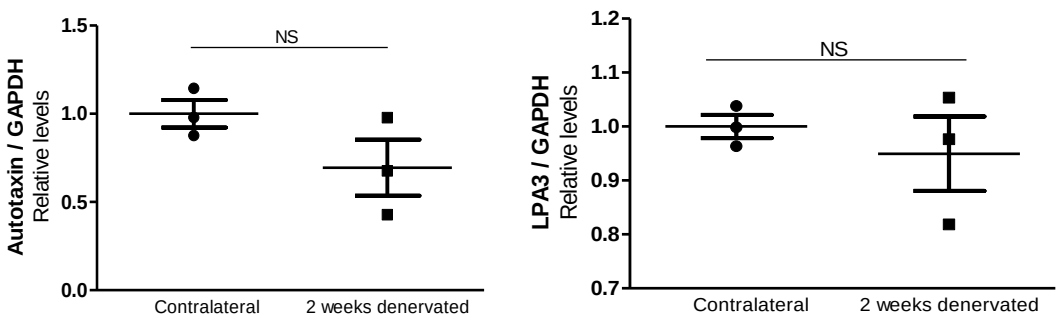

Autotaxin / GAPDH
Relative levels
NS
1.5
1.0
0.5
0.0
Contralateral
2 weeks denervated
LPA3 / GAPDH
Relative levels
NS
1.2
1.1
1.0
0.9
0.8
0.7
Contralateral
2 weeks denervated

Figure 3. LPARs are dysregulated in fibrotic skeletal muscles. A) LPA $_{1\text{-}6}$ expression in 2 months C56/BL6 WT and *Sgcd*-KO DIA. n= 2. B) LPA $_{1\text{-}6}$ expression in 9-10 months C57/BL10 WT and *mdx* DIA. n= 3. C) LPA $_{1\text{-}6}$ expression in contralateral and 4 days denervated GAS. n= 3. D) LPA $_{1\text{-}6}$ expression in contralateral and 2 weeks denervated GAS. n= 3. Al the graphics were normalized to 18s and relative to the expression of LPA_1 of control. E) ATX, LPA3 protein expression analyzed by immunoblot, GAPDH was used as a loading control. F) Quantification of E. n=3 G) ATX, LPA3 protein expression analyzed by immunoblot, GAPDH was used as a loading control. H) Quantification of G. n=3. M refers to the mouse. The values correspond to the mean ± SEM. *P<0.05 by two-tailed Student's t-test.

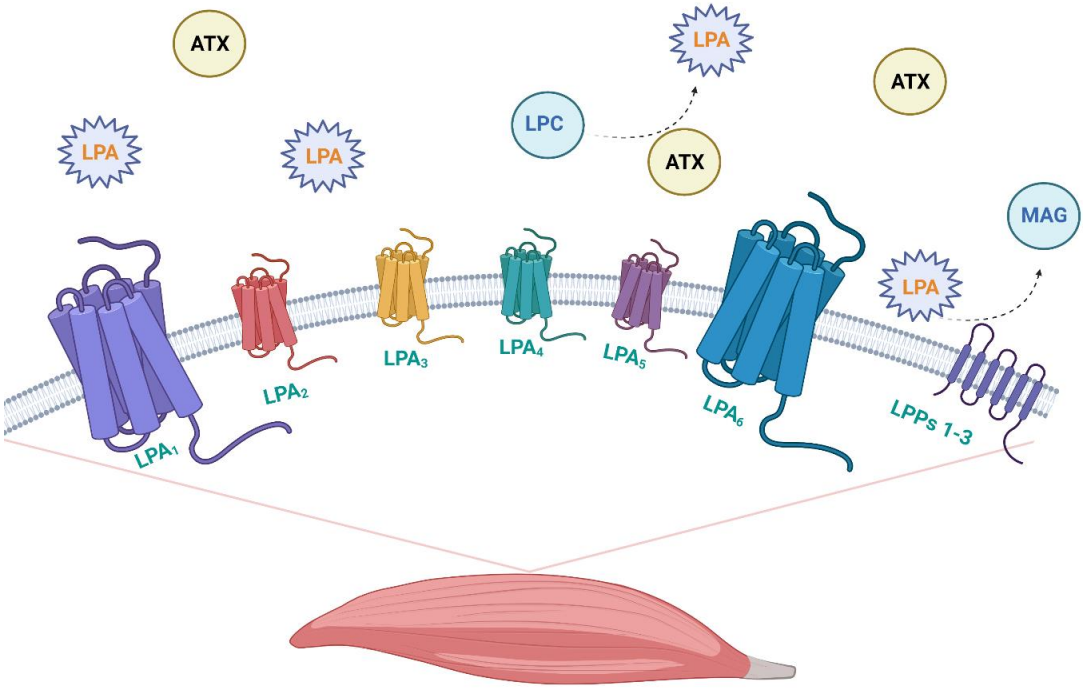


Diagram 5. Schematic view of ATX / LPA / LPARs axis presence in skeletal muscle. Our results indicate e the presence of components of the ATX / LPA / LPARs axis in skeletal muscle. There is expression of LPARs at least at the mRNA level, with LPA_1 and LPA_6 being the most abundant. We also verified the presence of the main enzyme involved in the synthesis of LPA, ATX at the mRNA and protein levels. Regarding the LPA degradation pathways (LPPs 1-3), we confirmed the presence of the mRNA of the three LPPs in all the muscles analyzed. The diagram was created with BioRender.com.

1.3 Summary of results objective 1

1. Skeletal muscle expresses mRNA of enzymes that participates in LPA synthesis and degradation.
2. There are LPARs mRNA presence in skeletal muscle from WT mice, being LPA_1 and LPA_6 the more abundant.
3. We were able to verify the presence of ATX, LPA3 and LPA6 proteins in WT skeletal muscle.
4. LPARs expression is dysregulated in *mdx* consolidated fibrotic diaphragm.

These results are illustrated in diagram 5.

2. Specific objective 2

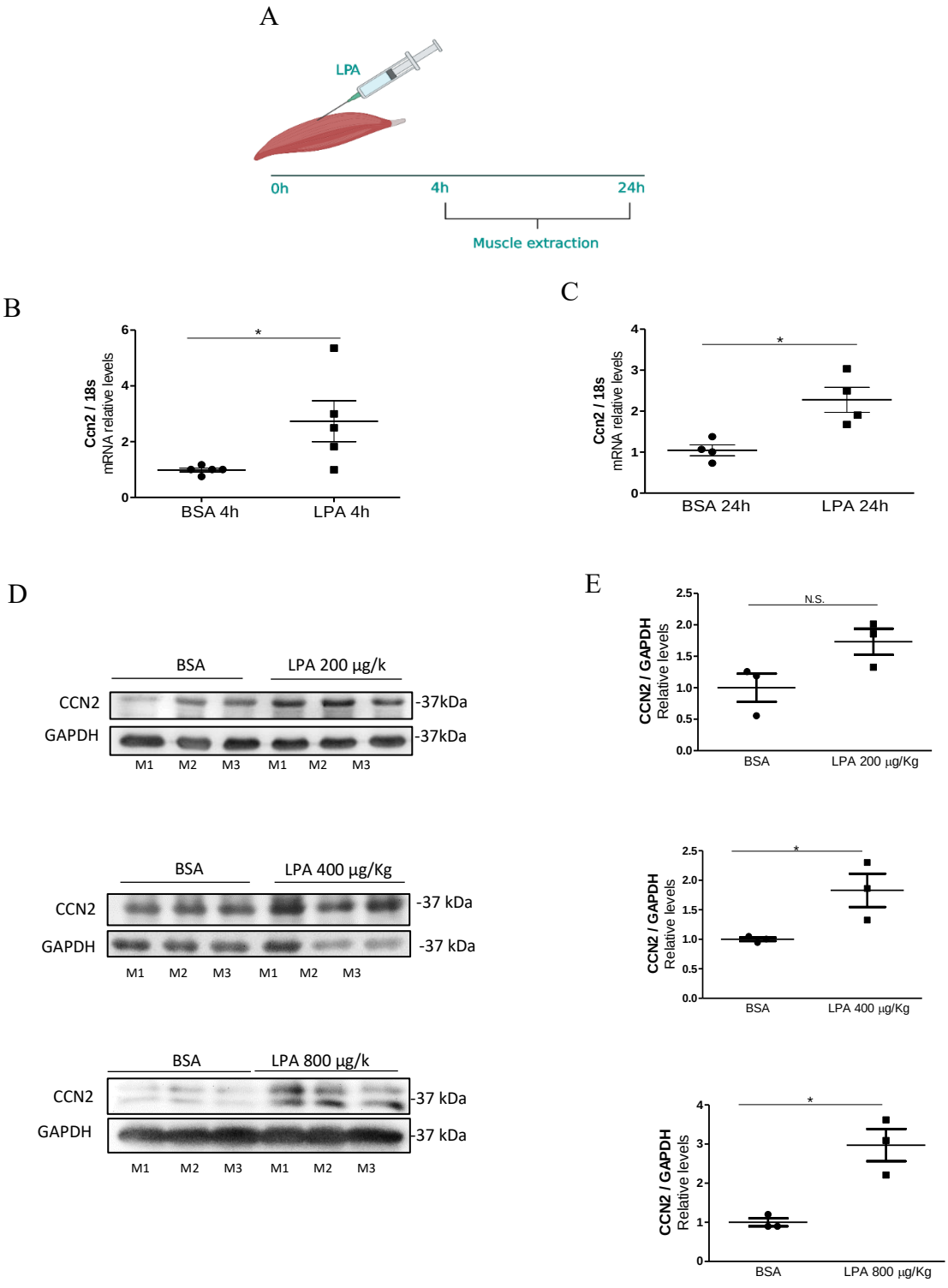
To study the fibrotic response of skeletal muscle to LPA, fibro / adipoagenic progenitors participation and the involvement of the ATX / LPA / LPARs axis in the denervation-induced fibrotic model

2.1 Skeletal muscle responds to LPA by inducing the expression of CCN2

Considering the results shown in the first objective of this thesis that demonstrate presence of components of the ATX / LPA / LPARs axis, we evaluated whether the skeletal muscle could respond to LPA by inducing fibrosis markers.

CCN2 is a hallmark and a driver of fibrosis (Morales et al., 2013)(Gonzalez et al., 2018) (Rebolledo et al., 2021). We decided to evaluate its mRNA and protein levels after LPA IM injection. We injected the TA, an accessible muscle located in the anterior of the leg, involved in the dorsiflexion of the foot. TA was injected with LPA and the contralateral TA from the same animal was injected with the vehicle, BSA. The muscles were extracted 4 h or 24 h later, as shown in the diagram of Fig. 4A. After LPA treatment, we cut the entire muscle into 20 µM sections and separated sequentially into three fractions, one for mRNA extraction (RT-qPCR analysis), one for protein extraction (WB analysis), and the third fraction for microscopy (IIF analysis). We found a statistically significant increase in CCN2 mRNA after 4 h (Fig. 4B) and 24 h (Fig. 4C). By immunoblot we also found a significative protein augment in CCN2, this effect was dose dependent at 4 h of treatment (Fig. 4D, E). Our results also were confirmed by IIF in TA cross-sections (Fig. 4F). We observed an increase in CCN2 in the interstitial space of the muscles injected with LPA. To confirm that the muscle can respond to LPARs agonist, we

IM injected the LPA_3 agonist, 2s-OMPT. We found that the skeletal muscle responds to 2s-OMPT inducing CCN2 protein level after 4 h of treatment (Supplementary Fig. 2). **Our results demonstrated that skeletal muscle respond to LPA by inducing the pro-fibrotic factor CCN2.**



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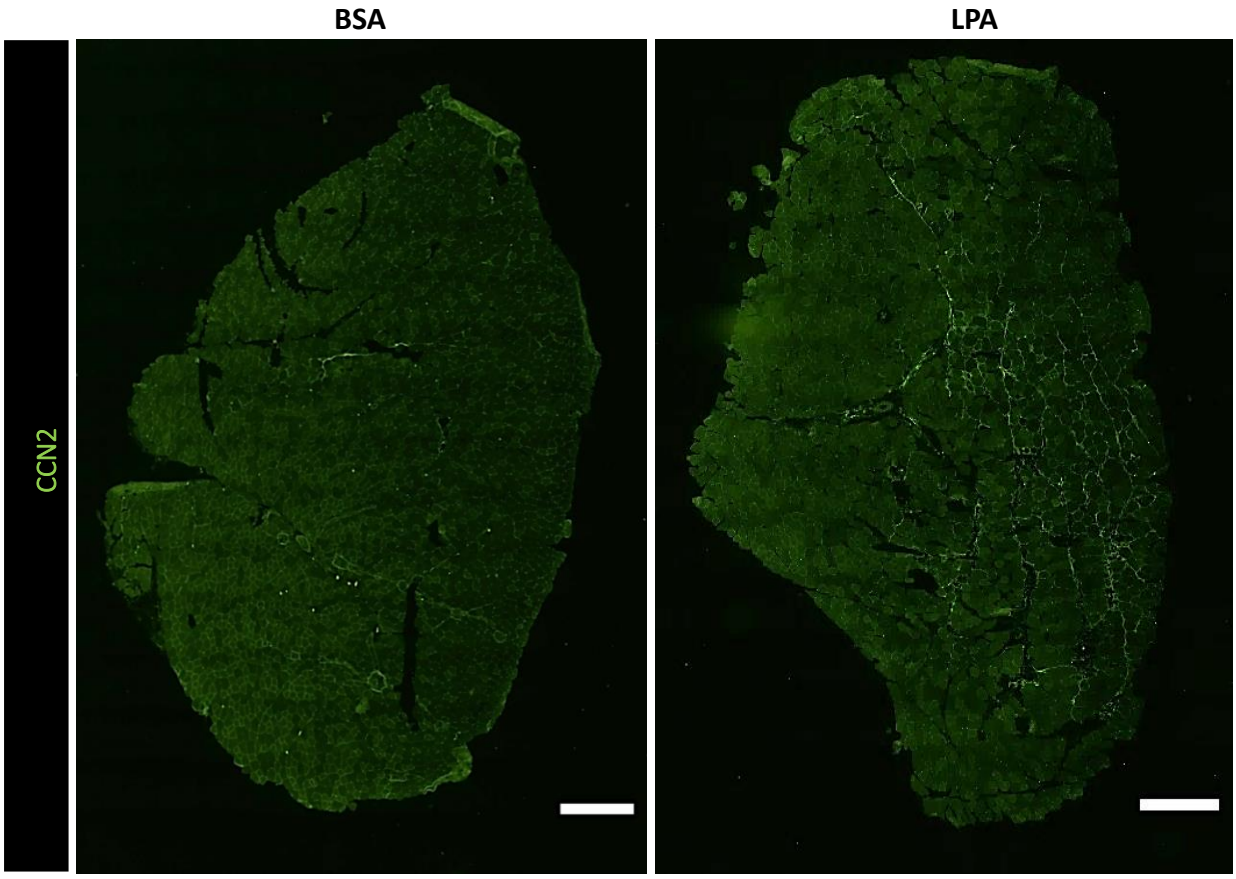


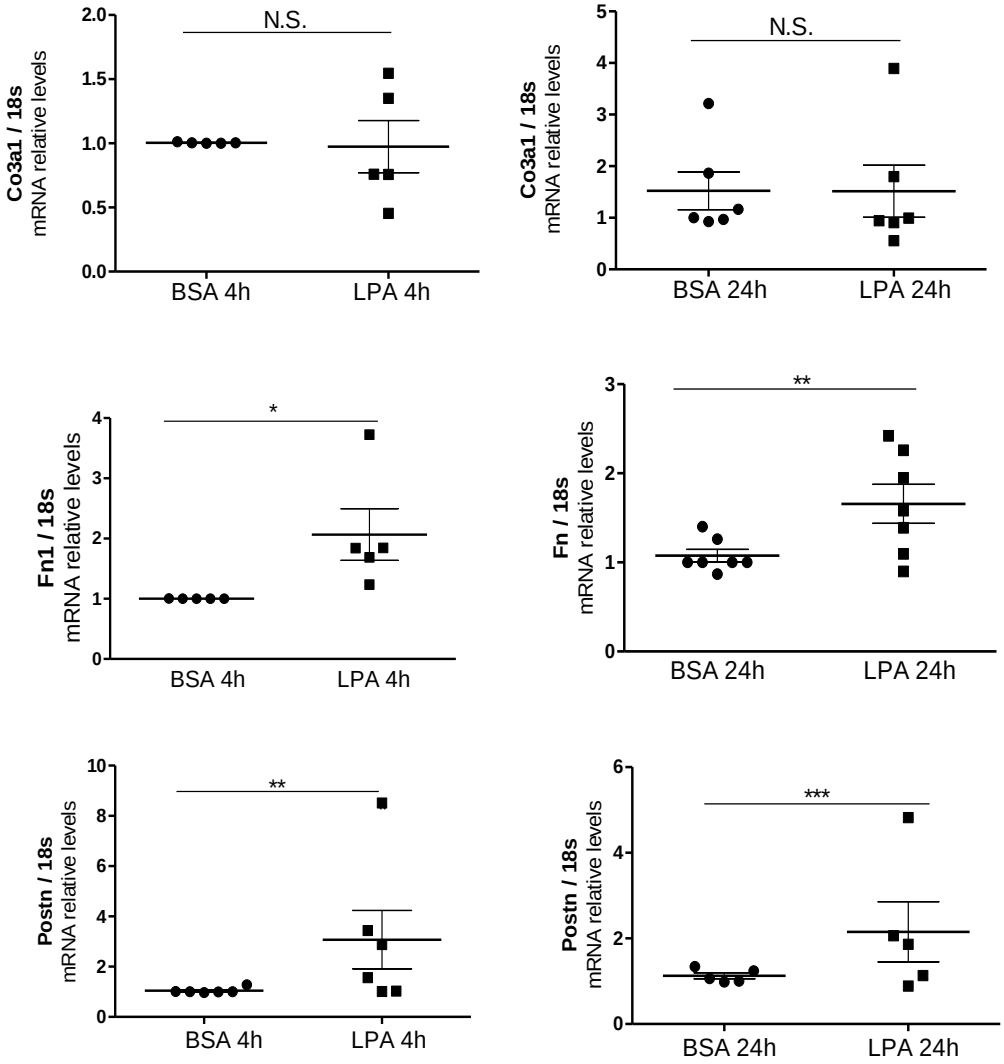
Figure 4. Skeletal muscle responds to LPA by inducing the expression of CCN2. A) Experimental design diagram created with BioRender.com. BSA or LPA was injected in TA and the muscles were extracted at 4 h or 24 h later (BSA was injected in the contralateral TA). B) mRNA expression of Ctgf (Ccn2) at 4 (n=5) and C) 24 h (n=4) after LPA injection. D) CCN2 protein levels 4 h after 200 µg/Kg, 400 µg/Kg, and 800 µg/Kg of IM LPA. GAPDH was used as the loading control. n=3. E) Quantification of D. F) CCN2 IIF of transversal section of injected TA with BSA (vehicle) or LPA. Scale bar, 500 µm. H). M refers to a mouse. The values correspond to the mean ± SEM. *P<0.05 by two-tailed Student's t-test.

2.2 LPA induces the expression of ECM components

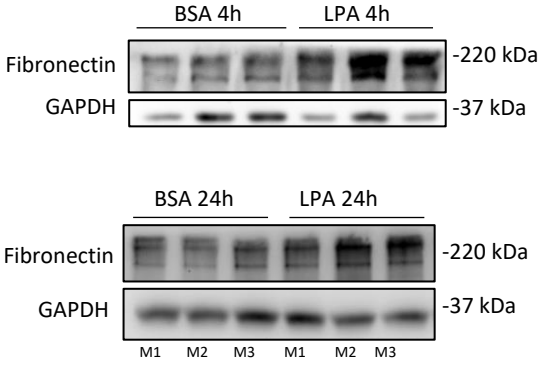
Next, we evaluated if LPA induces a whole fibrotic response. ECM mRNA components such as collagen type 3, periostin, and fibronectin were analyzed. We found increased expression in fibronectin and periostin mRNA levels (Fig. 5A) after 4 h and 24 h of treatment with LPA. Fibronectin was analyzed by immunoblot, and a significant increase was detected in protein levels 4 h and 24 h after LPA injection (Fig. 5B and C). We also evaluated fibronectin by IIF and Sirius red staining on tissue sections for assessing total collagen content. We found that LPA injection increases fibronectin (Fig. 5D) and total collagen accumulation in muscle (Fig. 5E), showing the onset of a fibrotic phenotype. **Our results indicate that LPA injection in skeletal muscle induces the expression of ECM molecules.**

However, to confirm that the LPA pro-fibrotic effect is not due to death of muscular cells, we studied the protein levels of active caspase 3 to detect apoptosis. We did not find statistical differences between BSA and LPA treated muscles after 4 h, however, we detected a strong diminution after 24 h of LPA injection (Supplementary Fig. 3A, B), this is in accordance with previous reports that showed the pro-survival effect of LPA (Weiner & Chun, 1999)(Kostic et al., 2015). 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 is due to the intramuscular injection or to the use of BSA as vehicle of LPA.

A


Co3a1 / 18s
mRNA relative levels
N.S.
BSA 4h
LPA 4h
Co3a1 / 18s
mRNA relative levels
N.S.
BSA 24h
LPA 24h
Fn1 / 18s
mRNA relative levels
*
BSA 4h
LPA 4h
Fn / 18s
mRNA relative levels
**
BSA 24h
LPA 24h
Postn / 18s
mRNA relative levels
**
BSA 4h
LPA 4h
Postn / 18s
mRNA relative levels
***
BSA 24h
LPA 24h

B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LPA 4h
Fibronectin
GAPDH
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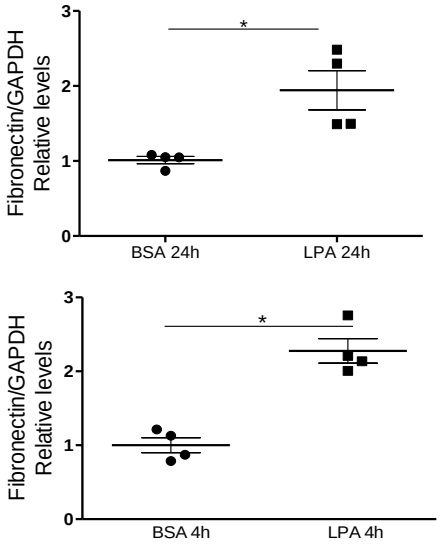
BSA 24h
LPA 24h
Fibronectin
GAPDH
-220 kDa
-37 kDa
M1 M2 M3 M1 M2 M3

C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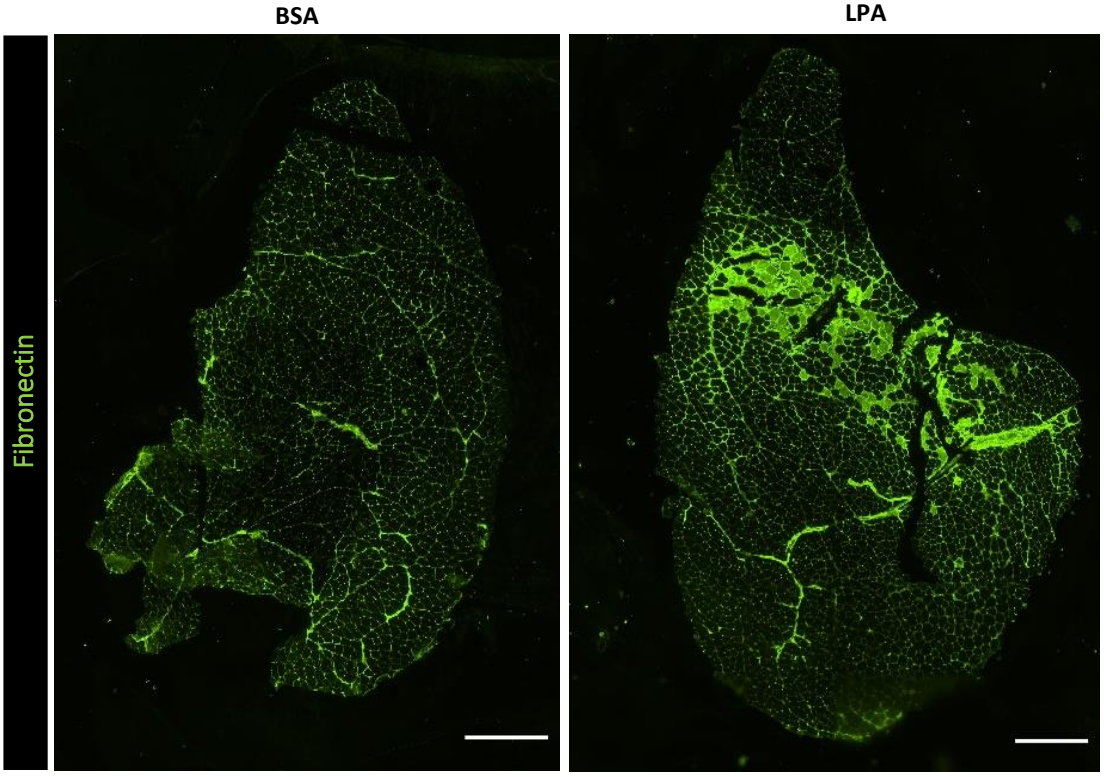
Relative levels
*
BSA 24h
LPA 24h
Fibronectin/GAPDH
Relative levels
*
BSA 4h
LPA 4h

D


BSA
LPA
Fibronectin

E

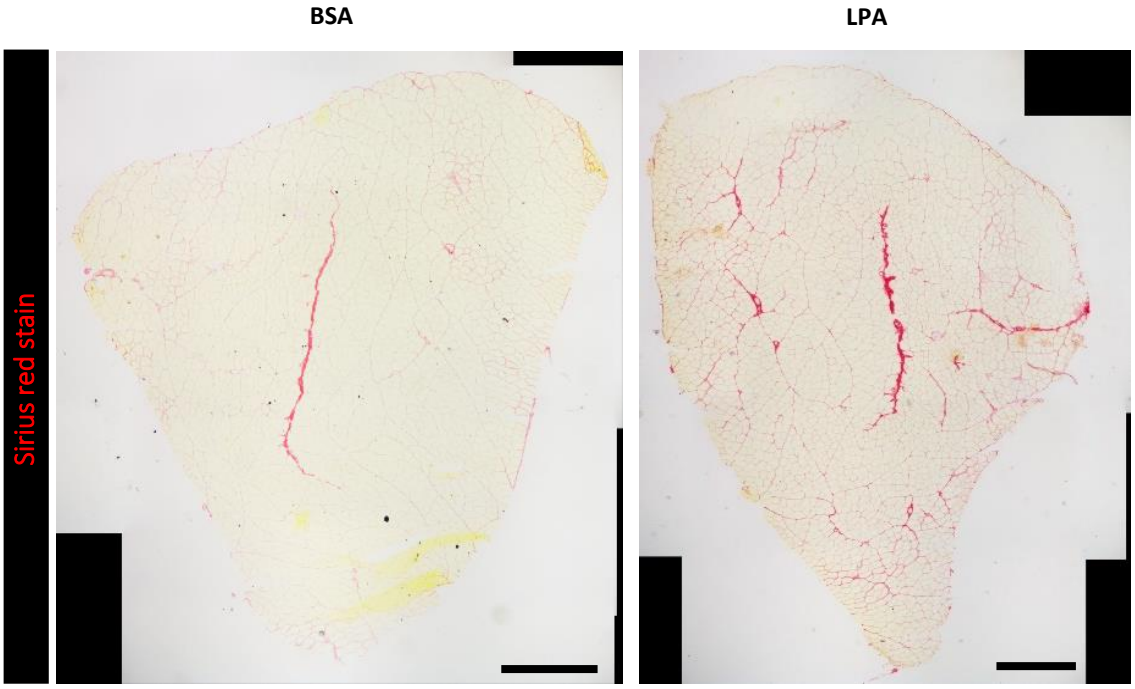
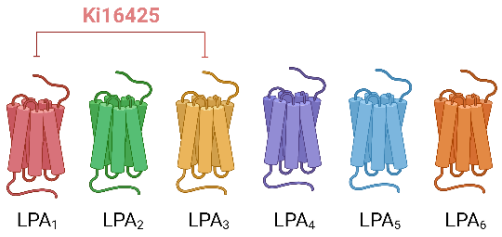


Figure 5. The skeletal muscle induces ECM molecules in response to LPA. A) TA muscle was injected with BSA or LPA 400 µg/Kg (BSA was injected in the contralateral TA). The mRNA levels of Col 3a1 (collagen 3), Fn1 (fibronectin), and Postn (Periostin) analyzed by using RT-qPCR. n=5-6. B) Fibronectin was analyzed by immunoblot in TA from mice injected with BSA or LPA (400 µg/Kg IM) 4 h (top) and 24 h (bottom). GAPDH was used as the loading control. n=4. C) Quantification of B. D) Representative epifluorescence images of fibronectin in TA cross-section muscle 4 h after injection with BSA or LPA 400 µg/Kg. Scale bar, 500 µm. E) Representative images of Sirius red staining in TA muscle 24 h after injection with BSA or LPA 400 µg/Kg. Scale bar, 500 µm. M refers to a mouse. The values correspond to the mean ± SEM. **P<0.01, *P<0.05, N.S not significant, by two-tailed Student's t-test. n=3.

2.3 Inhibition of $LPA_{1/3}$ prevents the fibrotic response to LPA

We were interested in studying the ATX / LPA / LPARs axis in the instauration and maintenance of muscular fibrosis; in this line, we demonstrated that LPA induces fibrotic markers like CCN2, collagens and fibronectin. Considering that LPA_1 is the most expressed LPA receptor in skeletal muscle, the next step was to establish if blocking the $LPA_{1/3}$ with the specific inhibitor Ki16425 (Fig. 6A) prevents the response to LPA. The treatment scheme is shown in Fig. 6B. We found that Ki16425 prevented the induction of fibronectin, IgG (an inflammatory marker), and CCN2 induced by LPA (Fig. 6C and D). We corroborated our immunoblot results by performing IIF of CCN2 and fibronectin (Fig. 6E and F). **These results indicate that $LPA_{1/3}$ are probably the receptors that mediate the pro-fibrotic response of LPA in skeletal muscle.**

A



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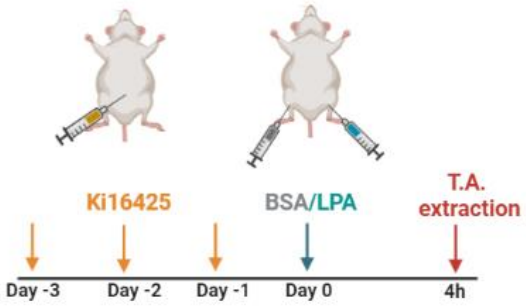

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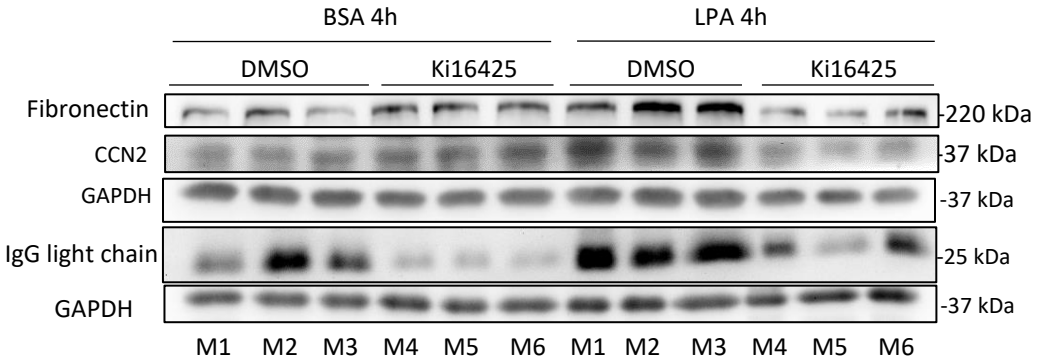
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DMSO
Ki16425
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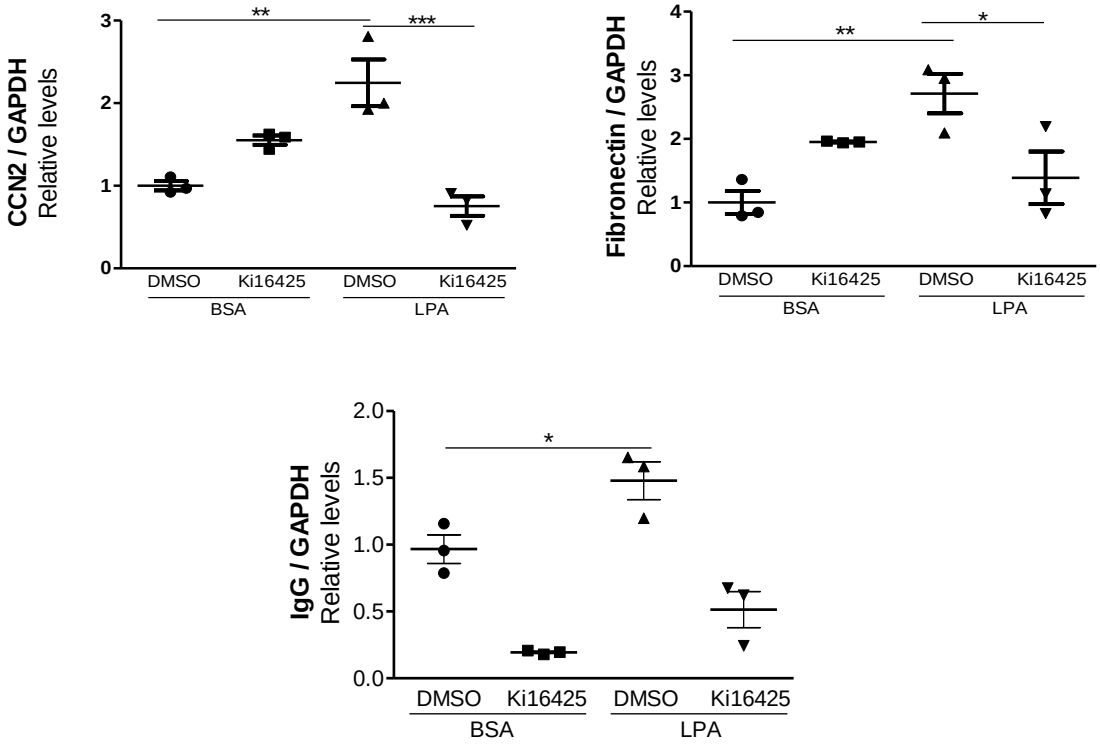
D


CCN2 / GAPDH
Relative levels
**
***
DMSO
Ki16425
DMSO
Ki16425
BSA
LPA
Fibronectin / GAPDH
Relative levels
**
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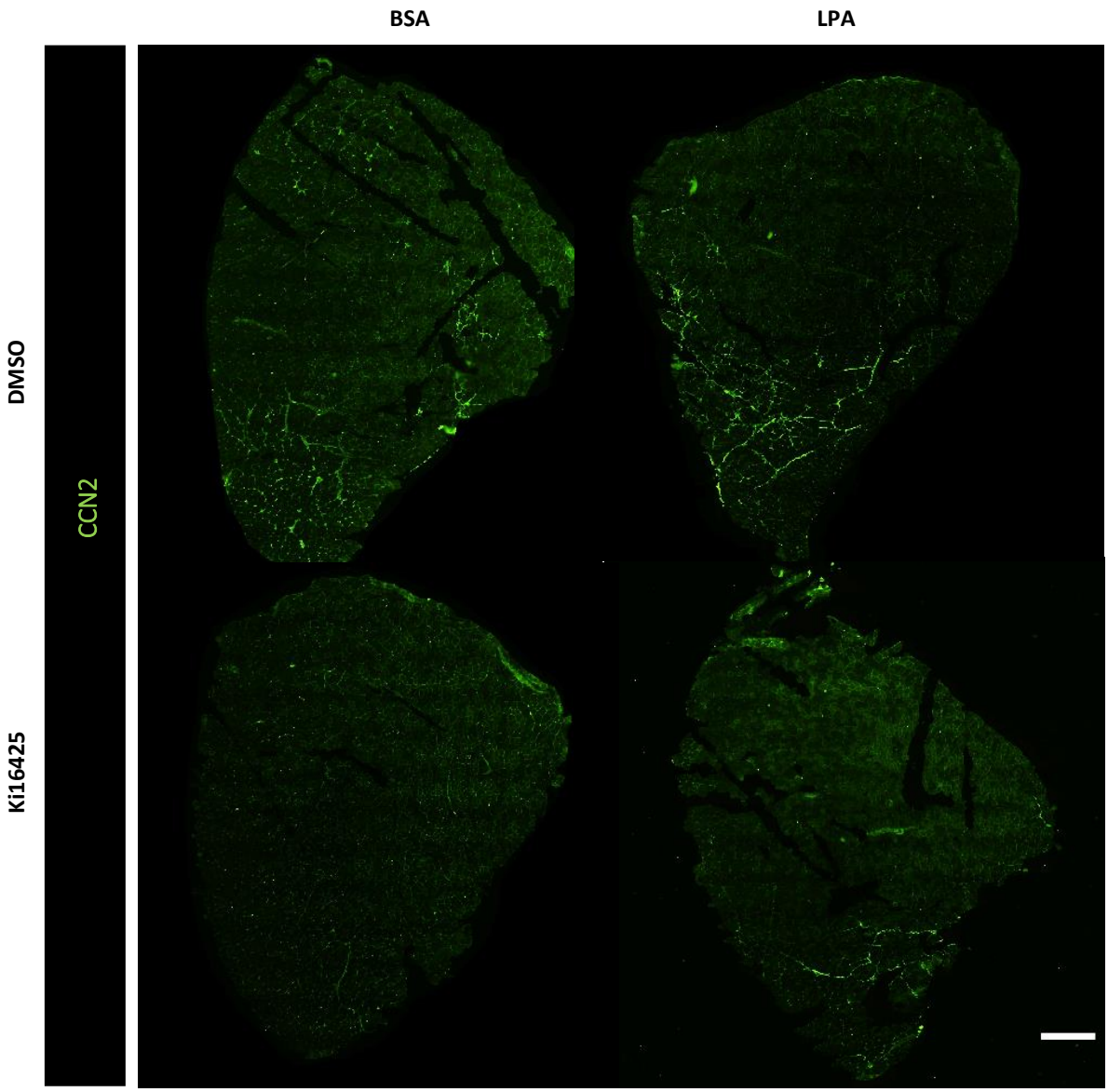
DMSO
Ki16425
DMSO
Ki16425
BSA
LPA
IgG / GAPDH
Relative levels
*
DMSO
Ki16425
DMSO
Ki16425
BSA
LPA

E



F

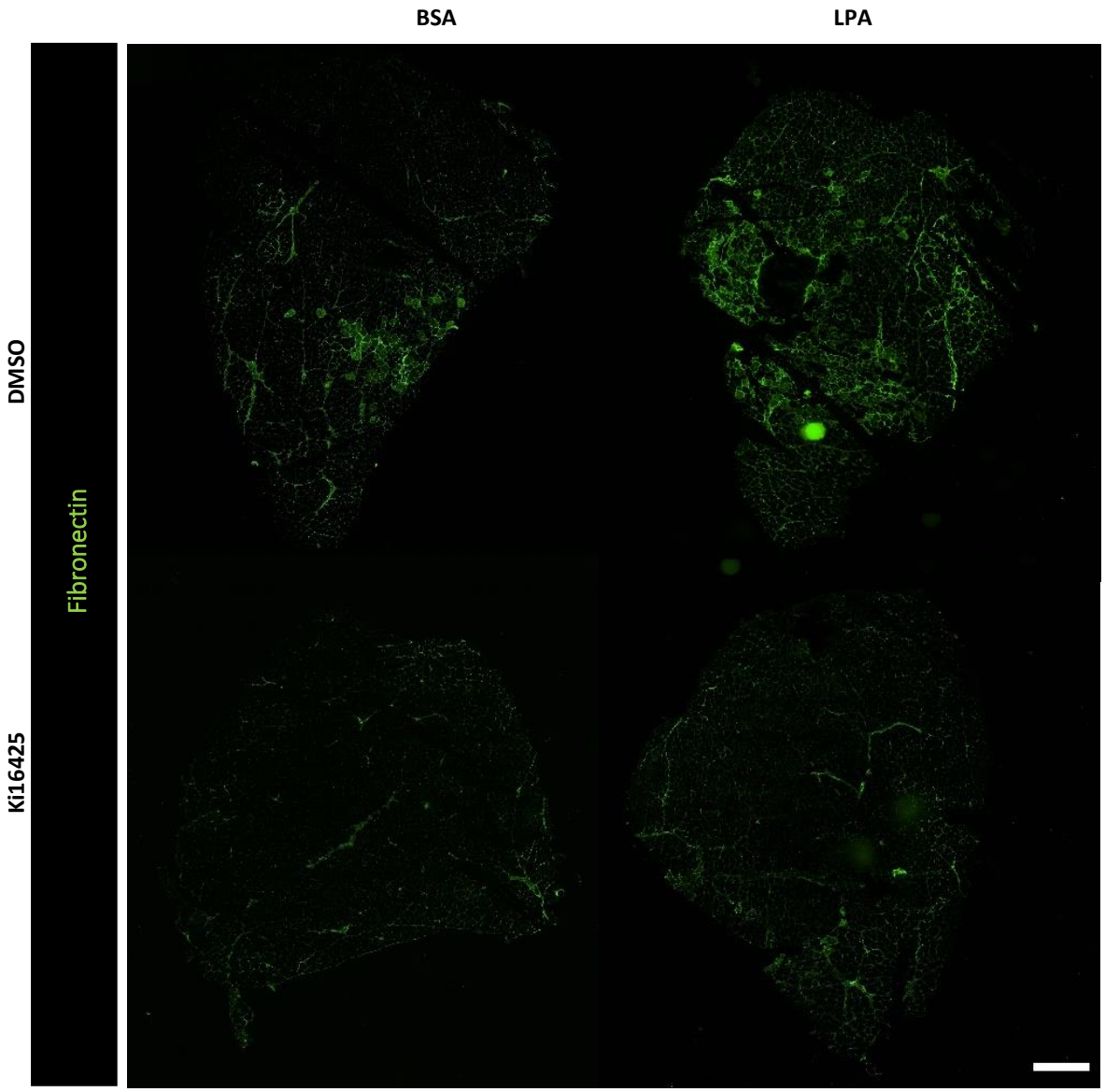


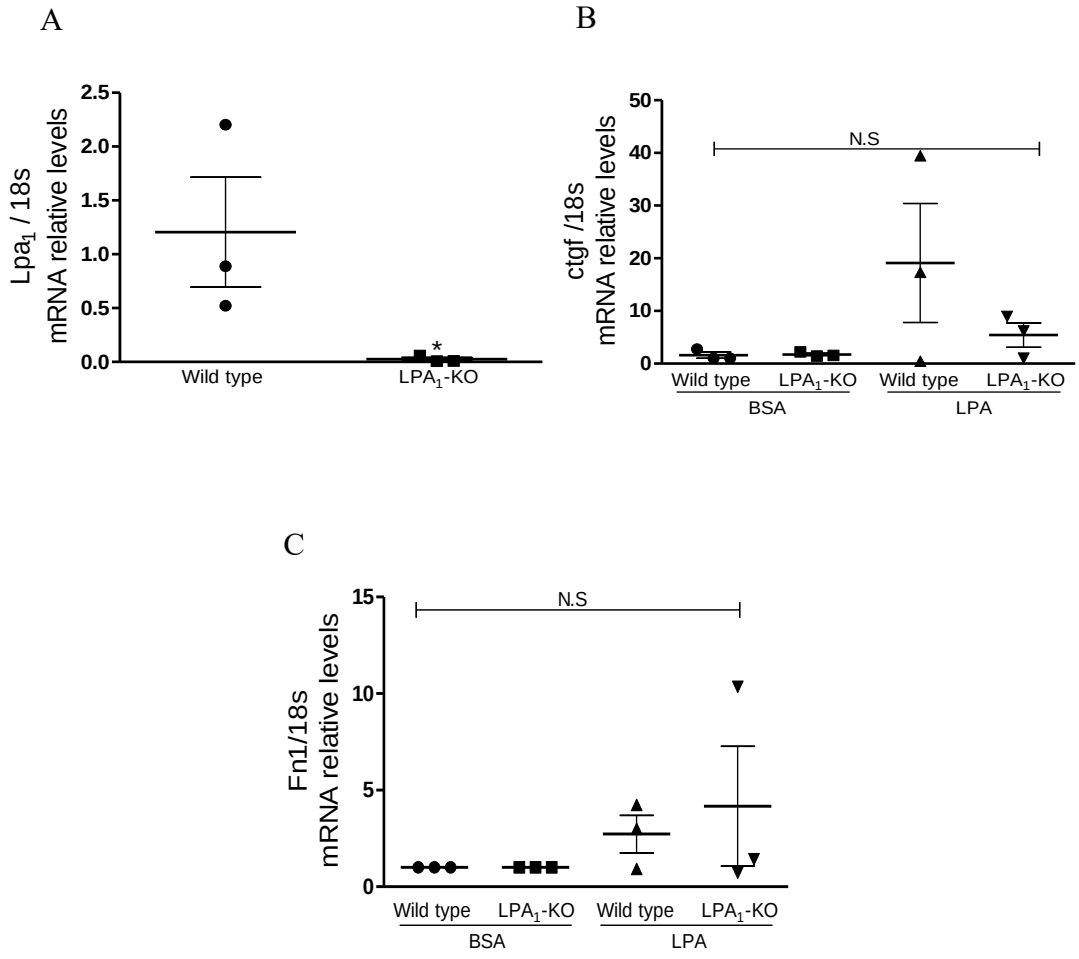
Figure 6. Inhibition of $LPA_{1/3}$ prevents the fibrotic response to LPA. A) Diagram of LPARs inhibited by Ki16425. B) Treatment scheme, male mice were injected with Ki16425 5 mg/Kg or vehicle (DMSO) daily for 3 days and injected with BSA or LPA for 4 h (BSA was injected in the contralateral TA). C) Fibronectin, IgG, CCN2 and GAPDH protein levels were analyzed by immunoblot. GAPDH was used as the loading control. D) Quantification of C. E) Representative epifluorescence images of CCN2 and F) Fibronectin in TA muscle. Scale bar

500 µm. M refers to the mouse. The values correspond to the mean ± SEM. **P<0.01, *P<0.05 by one-way ANOVA with Tukey's post-test. n=3.

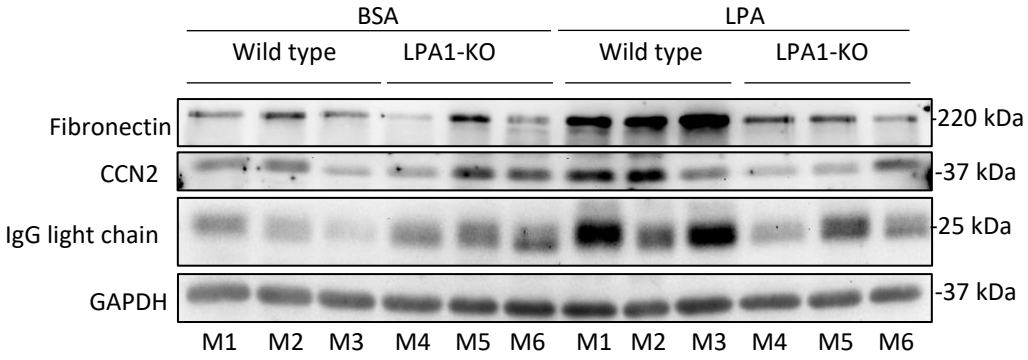
2.4 LPA_1 is in part responsible of the fibrotic response induced by LPA

As we have shown previously, LPA_1 is one of the most expressed LPARs in skeletal muscle (Fig. 2A) and the use of the $LPA_{1/3}$ inhibitor (Ki16425) avoids the LPA-induced fibrotic response (Fig. 6), we hypothesized that LPA is mounting a fibrotic response through LPA_1. We decided to treat BALB/c LPA_1-KO (LPA_1-KO) mice with IM LPA injection and explore the fibrotic response after 4 h of treatment. First, we confirmed the absence of LPA_1 mRNA in the skeletal muscle of LPA_1-KO mice (Fig. 7A). The other LPARs showed high variability in mRNA levels among the studied LPA_1-KO mice (Supplementary Fig. 4). Despite a possible upward trend in their expression, no statistically significant difference was detected compared to WT mice.

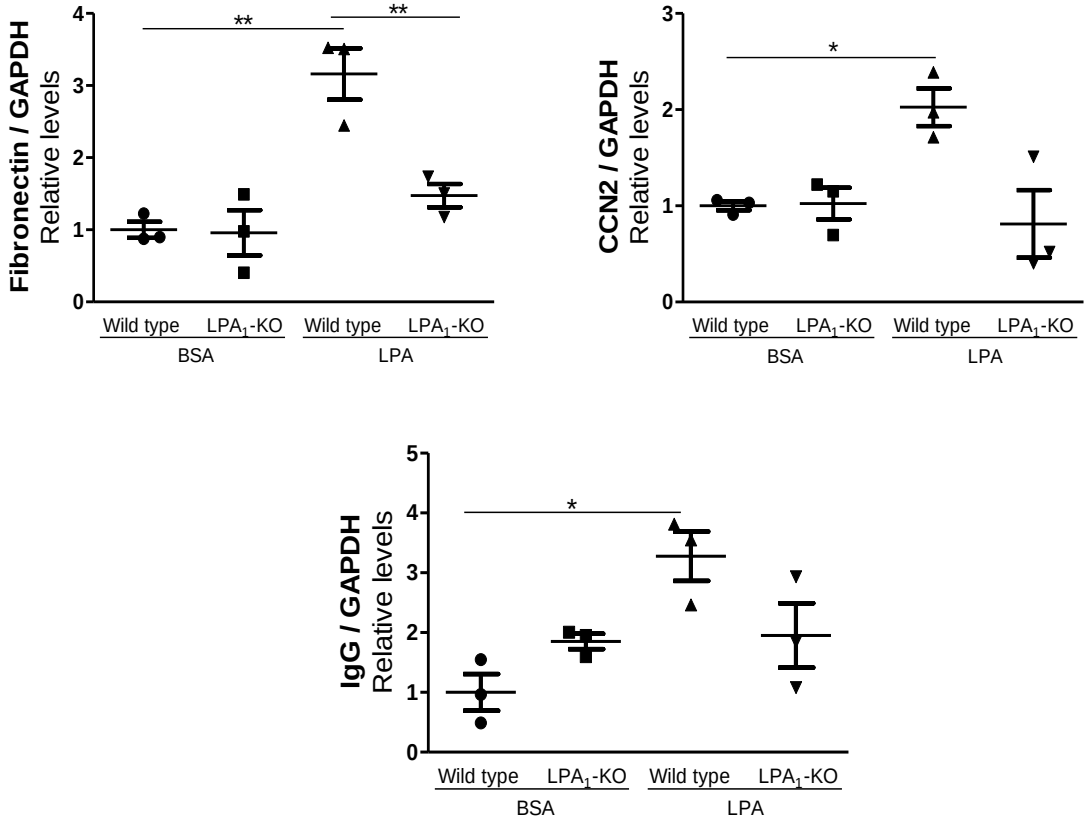
We did not find statistical differences in CCN2 and fibronectin mRNA levels (Fig. 7B and C) in BALB/c WT mice or LPA_1-KO mice. By immunoblot, we found reduced fibronectin and CCN2 protein levels in the LPA-treated muscles from LPA_1-KO mice as compared to LPA-treated WT mice (Fig. 7D and E). We analyzed the content 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. 7F, 7G.). **The results at the protein level suggest a role for LPA_1 in establishing muscle fibrosis induced by LPA.**


A
B
C
Lpa1 / 18s
mRNA relative levels
2.5
2.0
1.5
1.0
0.5
0.0
Wild type
LPA1-KO
*
ctgf /18s
mRNA relative levels
50
40
30
20
10
0
N.S
Wild type
LPA1-KO
Wild type
LPA1-KO
BSA
LPA
Fn1/18s
mRNA relative levels
15
10
5
0
N.S
Wild type
LPA1-KO
Wild type
LPA1-KO
BSA
LPA

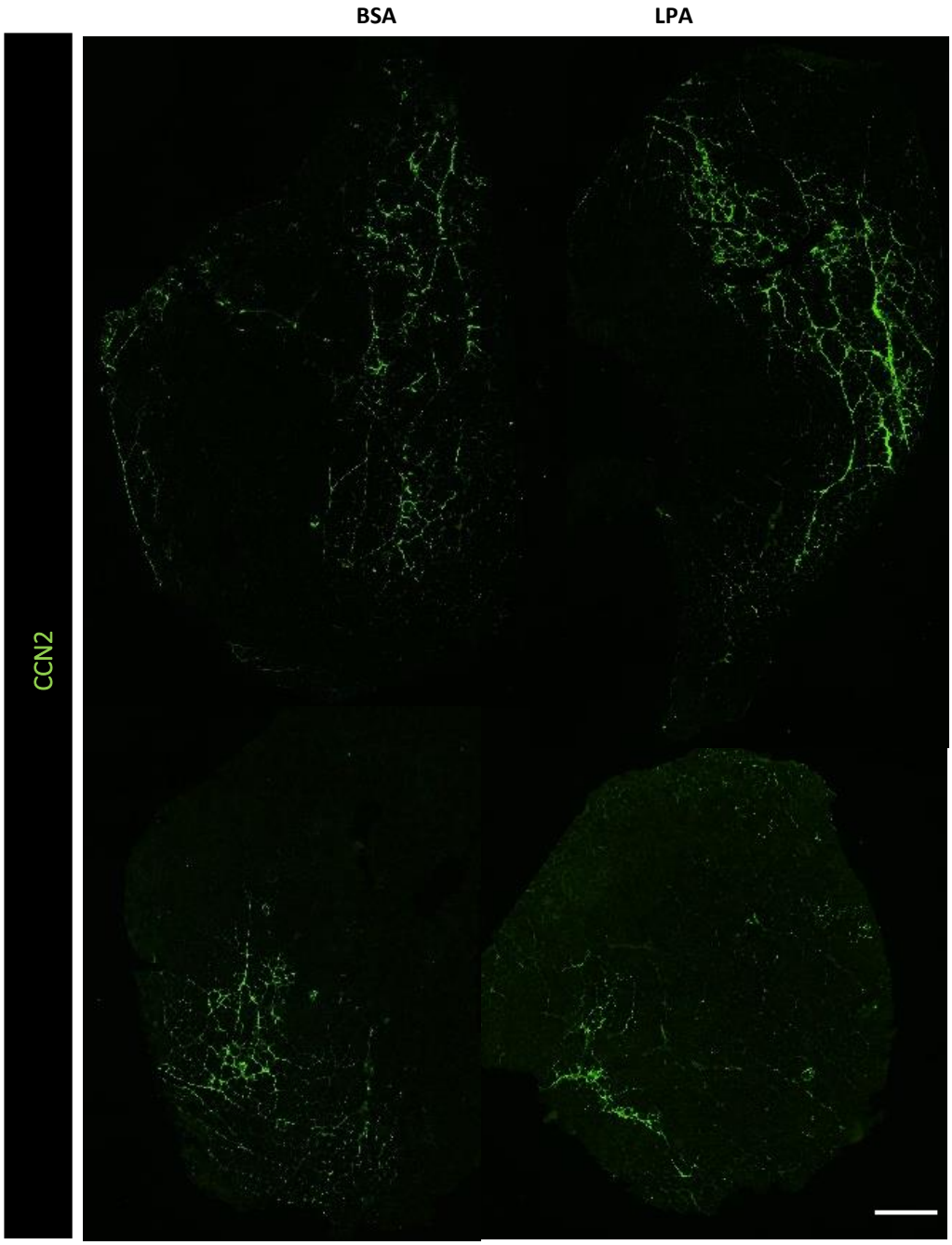
D


BSA
LPA
Wild type
LPA1-KO
Wild type
LPA1-KO
Fibronectin
CCN2
IgG light chain
GAPDH
220 kDa
37 kDa
25 kDa
37 kDa
M1 M2 M3 M4 M5 M6 M1 M2 M3 M4 M5 M6

E


Fibronectin / GAPDH
Relative levels
**
**
Wild type
LPA1-KO
Wild type
LPA1-KO
BSA
LPA
CCN2 / GAPDH
Relative levels
*
IgG / GAPDH
Relative levels
*

F



G

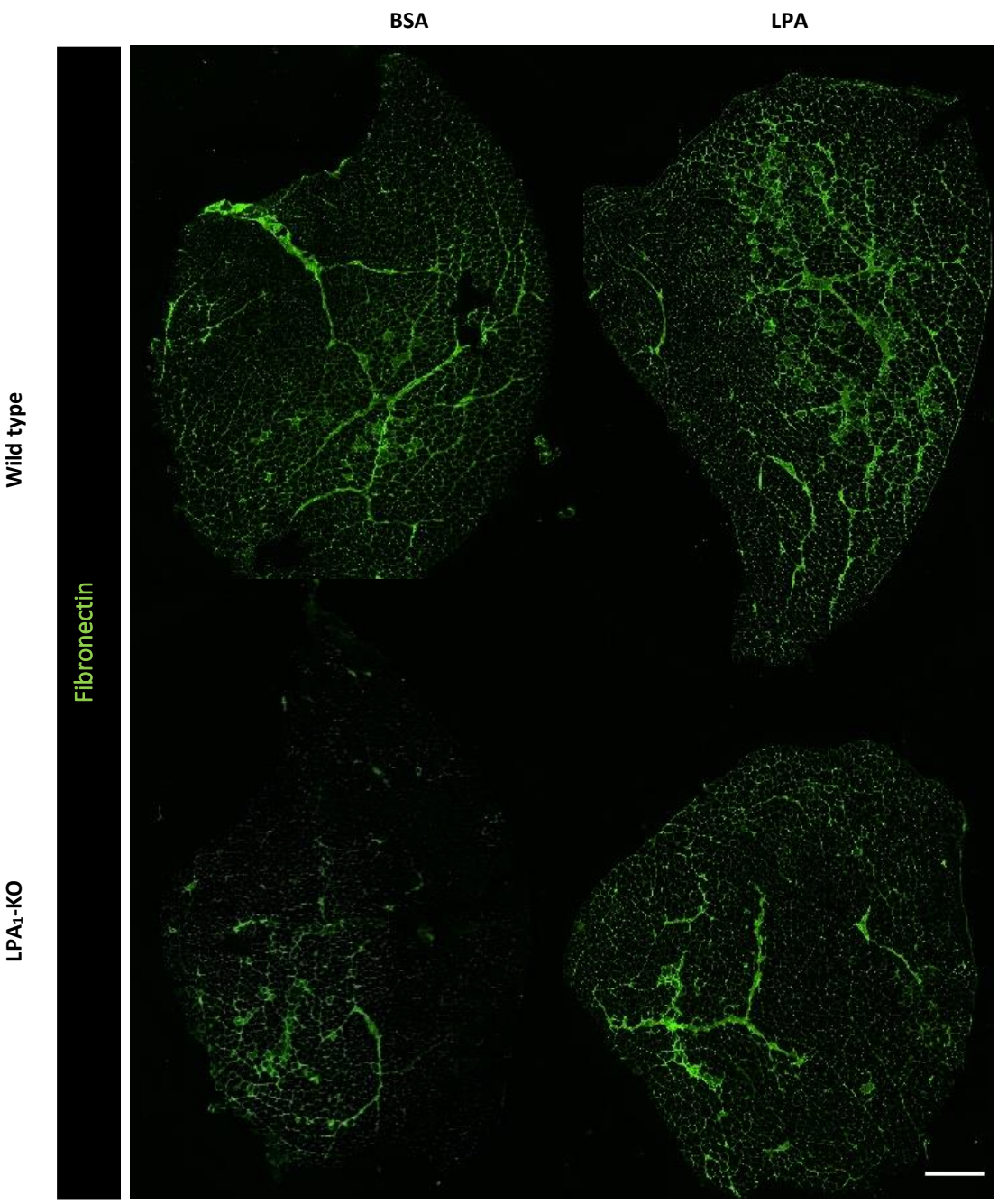


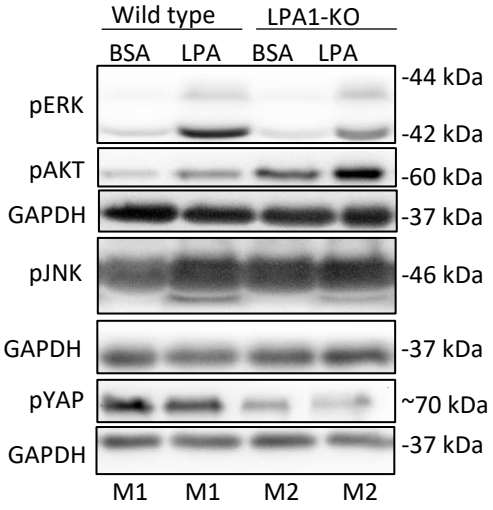
Figure 7. LPA_1 is the main LPA receptor for the LPA-induced fibrotic response in skeletal muscle. A) LPA_1 mRNA levels in TA from WT and LPA_1-KO mice. B-E) CCN2 and fibronectin were analyzed by RT-qPCR and immunoblot to determine their mRNA (B and C) and protein (D and E) levels in TA from WT and LPA_1-KO male mice 4 h after being injected with BSA or LPA 400 µg/Kg IM (BSA was injected in the contralateral TA). GAPDH was used as the loading control. F and G) Representative epifluorescence images of CCN2 and fibronectin IIF in whole reconstructed cross-sections of LPA-injected TA muscles, scale bar, 500 µm. M refers to the mouse. The values correspond to the mean ± SEM. **P<0.01, *P<0.05, N.S not significant by one-way ANOVA with Tukey's post-test. n=3.

2.5 LPA induces ERK phosphorylation through LPA_1

Our results indicate that LPA_1 is the LPAR, or one of the principals that mediates the fibrotic response induced by LPA agonists; however, it is important to elucidate the transduction pathways of this response within the cell. For this purpose, we injected TA muscles with LPA for 10 minutes to assess the phosphorylation of signaling molecules that have been seen activated by LPA (Zhou et al., 2018)(Budnik et al., 2003)(Sasaki et al., 1998). We explore the AKT, ERK, JNK, and YAP pathways. We found a statistically significant increase in ERK phosphorylation in WT muscle, but we did not find significant changes in pAKT, pJNK or pYAP (Fig. 8A and B). In LPA_1-KO mice, the phosphorylation of ERK was partially prevented (Fig. 8A and B).

To explore whether the inhibition of ERK 1/2 prevents the induction of the fibrotic response of LPA, we treated mice with 10 mg/Kg with the inhibitor U0126 for 3 days, and on the fourth day, we injected LPA IM for 4 hours. We found that ERK ½ inhibition prevents fibronectin induction by LPA, but it did not prevent the CCN2 induction, as shown by immunoblot (Fig. 9A, B) and by IIF (Fig. 9C and D). **These results indicate that CCN2 and fibronectin induction depend on different cell pathways upon LPA activation.**

A


Wild type
LPA1-KO
BSA
LPA
BSA
LPA
pERK
-44 kDa
-42 kDa
pAKT
-60 kDa
GAPDH
-37 kDa
pJNK
-46 kDa
GAPDH
-37 kDa
pYAP
~70 kDa
GAPDH
-37 kDa
M1
M1
M2
M2

B

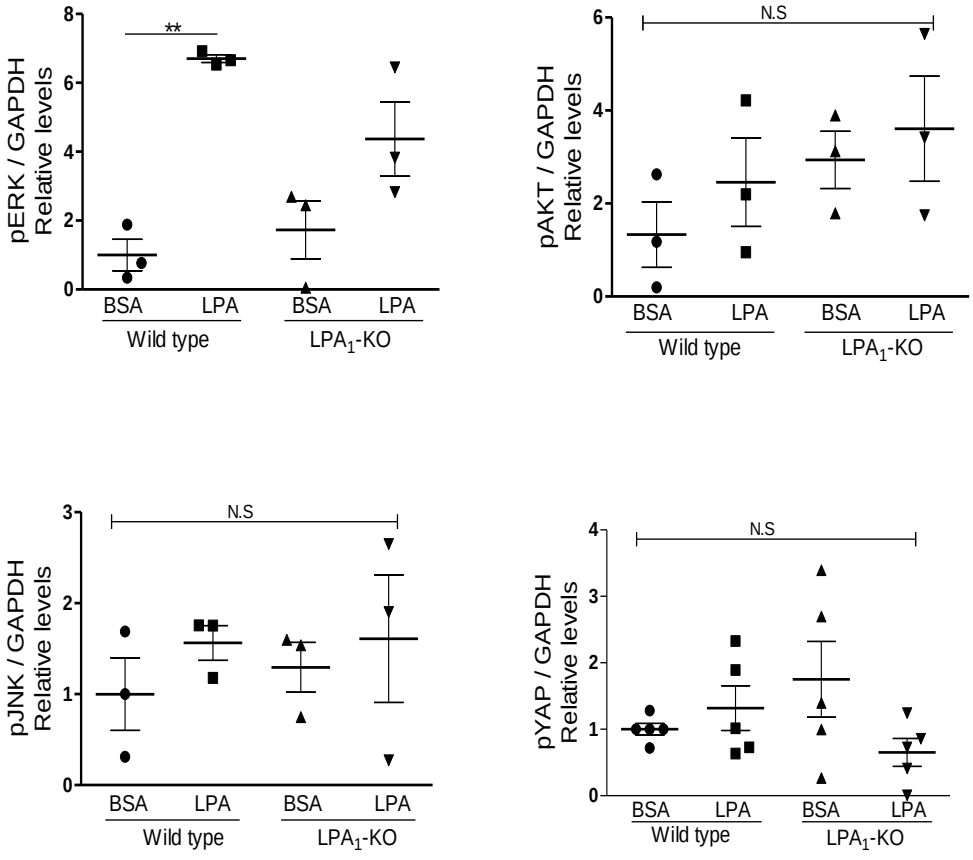
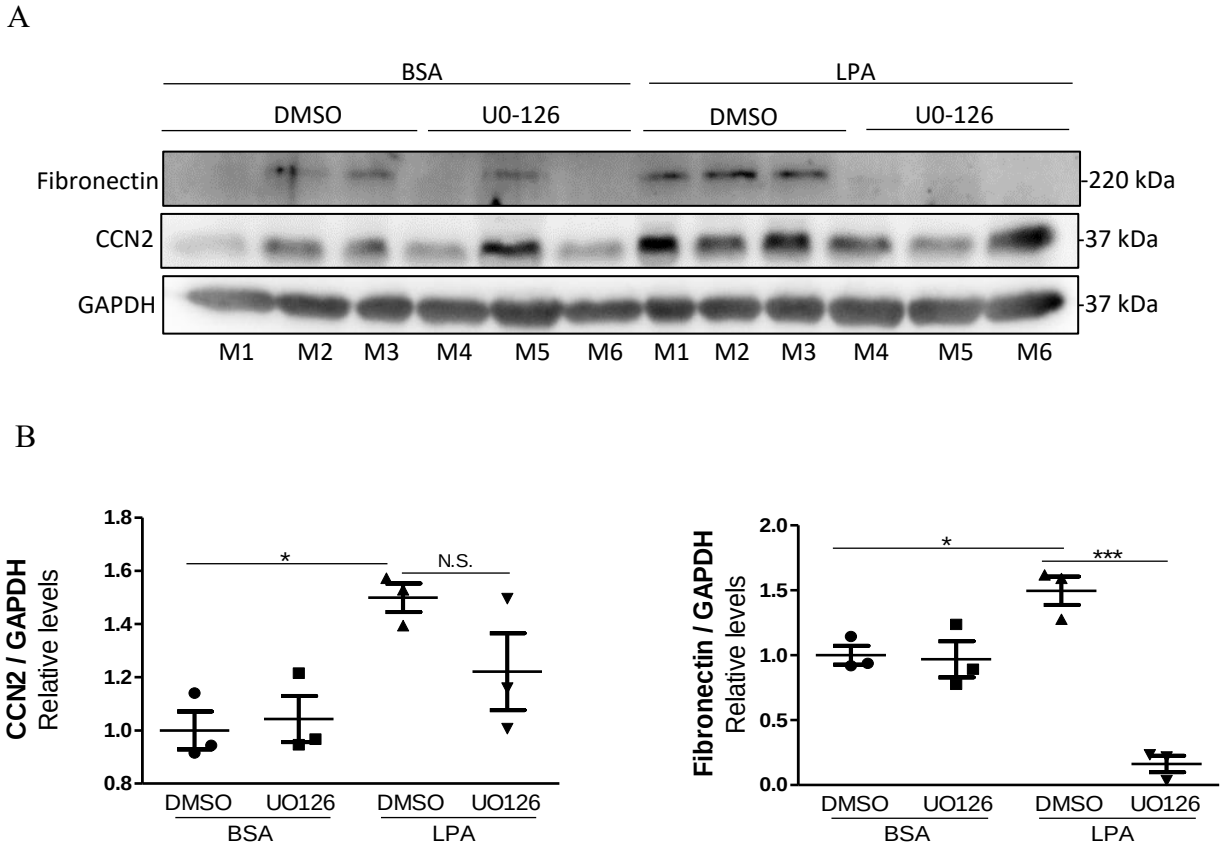
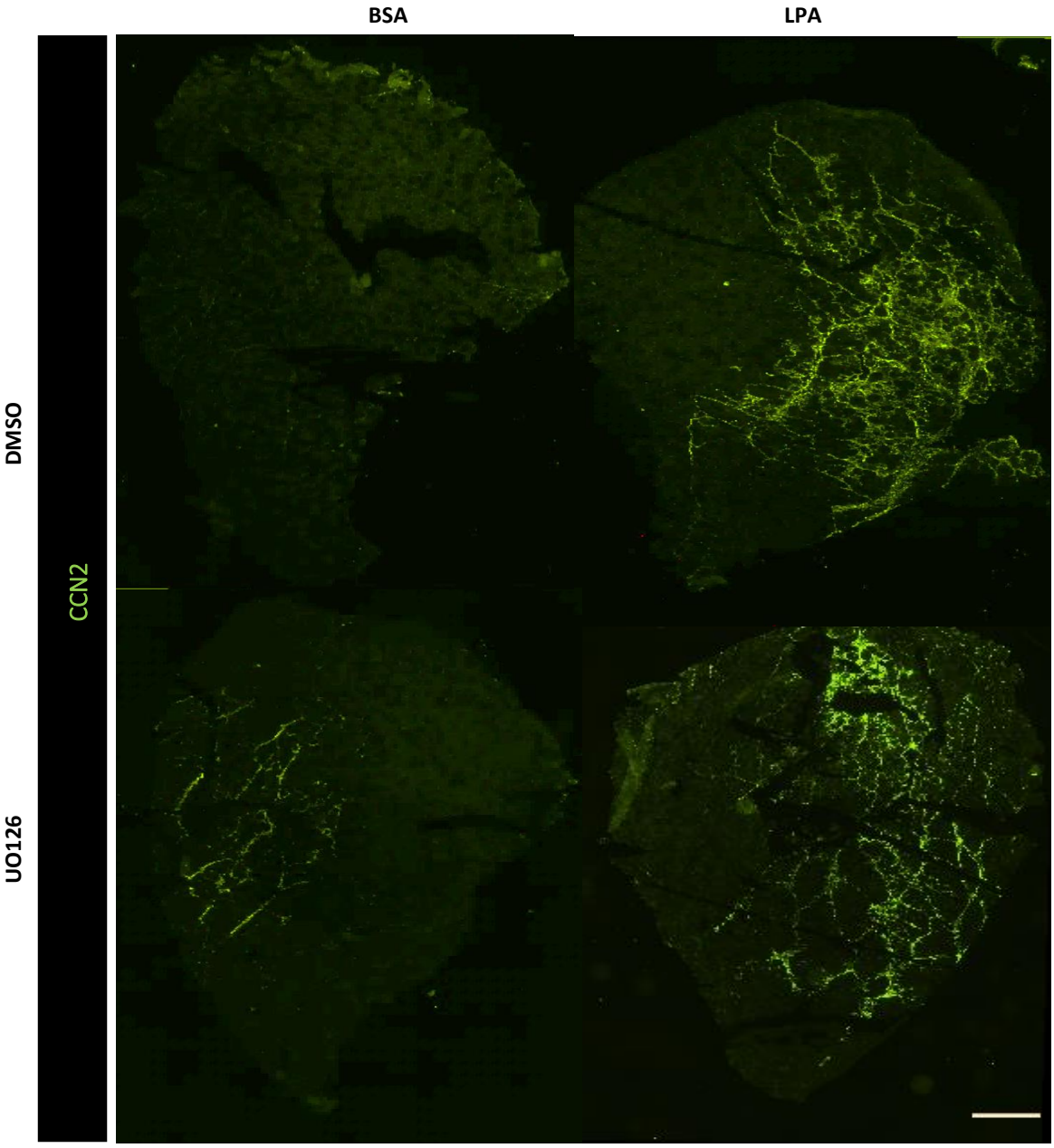

pERK / GAPDH
Relative levels
**
BSA
LPA
BSA
LPA
Wild type
LPA1-KO
pAKT / GAPDH
Relative levels
N.S
pJNK / GAPDH
Relative levels
N.S
pYAP / GAPDH
Relative levels
N.S

Figure 8. LPA induces ERK 1/2 phosphorylation through LPA_1 A) pERK, pAKT, pJNK, pYAP and GAPDH levels were analyzed by immunoblot in TA from WT and LPA_1-KO mice 10 minutes after being injected with BSA or LPA 400 µg/Kg IM (BSA was injected in the contralateral TA). GAPDH was used as the loading control. B) Quantification of phosphorylated proteins. M refers to the mouse. The values correspond to the mean ± SEM. **P<0.01, N.S not significant by one-way ANOVA with Tukey's post-test. n=3-4.



C



D

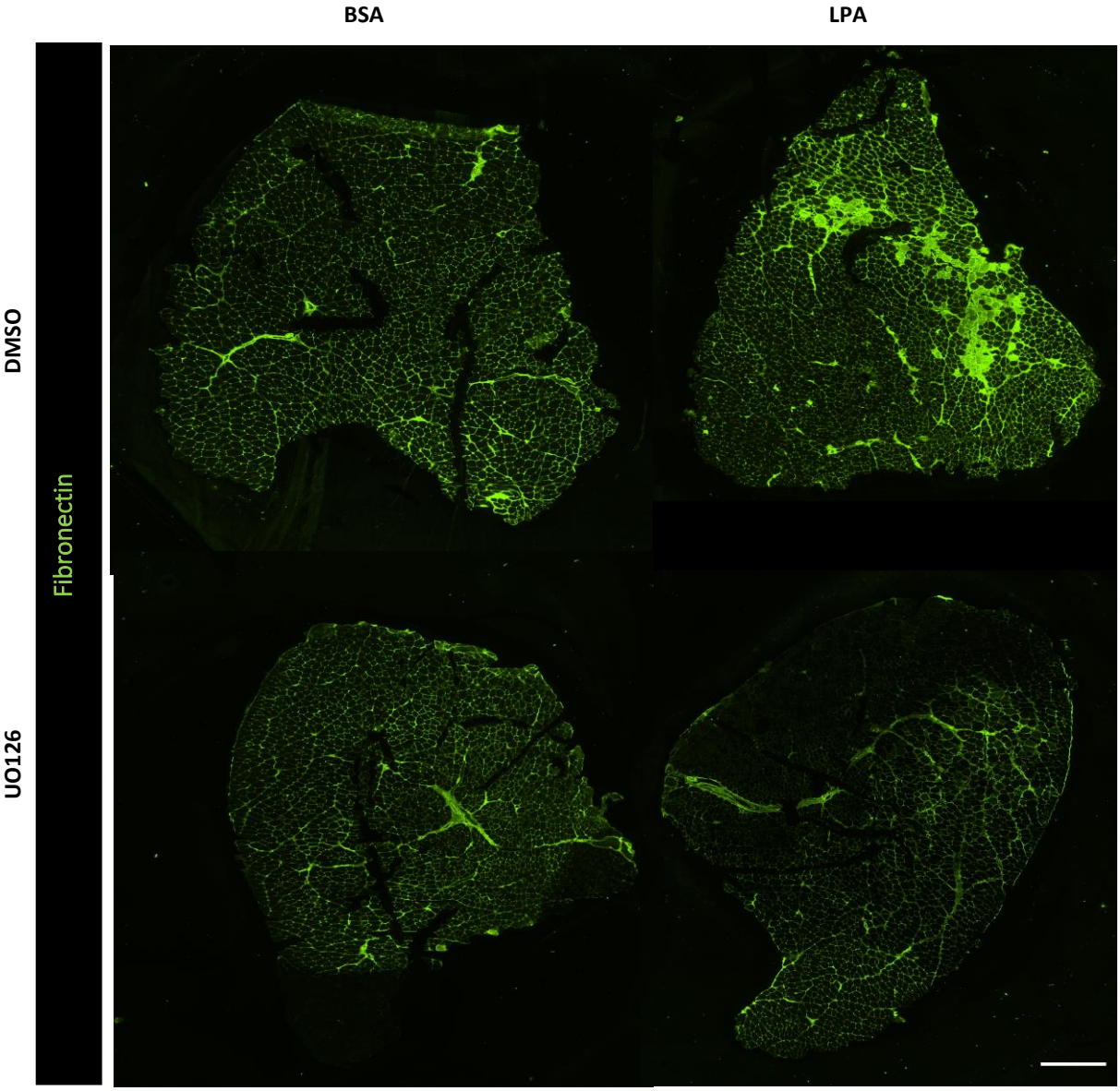


Figure 9. Inhibition of ERK 1/2 prevents LPA-induced fibrotic responses in skeletal muscle. A) Fibronectin and CCN2 protein levels were analyzed by immunoblot in TA from mice treated with U0126 for 3 days, and then injected 4 h with BSA or LPA 400 µg/Kg IM (BSA was injected in the contralateral TA). GAPDH was used as the loading control. B) Quantification of protein expression. C) CCN2 immunofluorescence reconstruction from epifluorescence images. Scale bar, 500 µm. D) Fibronectin immunofluorescence reconstruction from epifluorescence images. Scale bar, 500 µm. M refers to the mouse. The values correspond to the mean ± SEM. *P<0.05, ***P<0.01 by one-way ANOVA with Tukey's post-test. n=3.

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

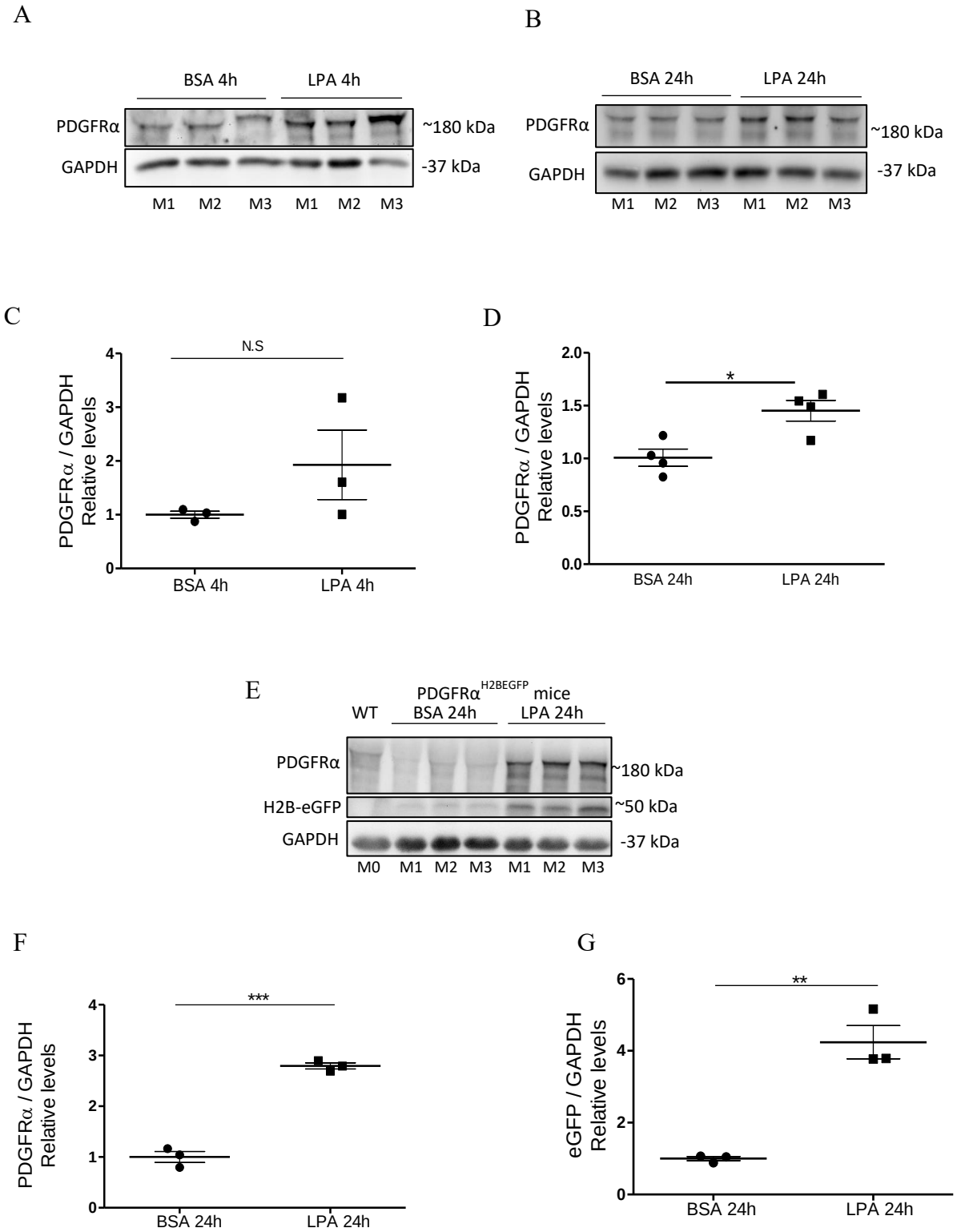
Since LPA increases CCN2 and fibrosis, we wandered for the cells responsible for the fibrotic response. FAPs are mesenchymal progenitor cells in adult skeletal muscle critical in the fibrotic process since they can differentiate into myofibroblast or adipocytes, depending on their tissular context. Our results show that the three different analyzed muscles (TA, DIA and GAS) express the FAPs marker, PDGFRα (Supplementary Fig. 5A and B), indicating the basal presence of these cells in the muscle. We decided to explore if skeletal muscle can respond to LPA by increasing the number of FAPs; first, we injected TA muscles with LPA or BSA and analyzed PDGFRα protein levels, a well cognate marker of FAPs, After 4 h of LPA intramuscular injection, we did not detect significant differences in PDGFRα protein levels between BSA and LPA-treated muscles (Fig. 10 A and C). In contrast, after 24 h treatment, PDGFRα protein levels were increased (Fig. 10 B and D). Then, we study whether the elevated PDGFRα protein levels induced by LPA resulted from an increase in the number of FAPs or just an increase in its expression. We treated the $PDGFR\alpha^{H2BEGFP}$ knock-in reporter mice, where FAPs can be visualized by the expression of a nuclear fusion protein H2B-EGFP controlled by the promoter of the PDGFRα gene. 24 h after LPA intramuscular treatment, we observed a significant increase in PDGFRα and eGFP protein levels in this mouse (Fig. 10E, F, and G). We quantified the number of eGFP-positive nuclei in TA muscle sections (Fig. 10H and I), and the results shown that LPA triggers an increase in the number of muscular 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 the IM injection with LPA. The increase in the FAPs number was not prevented by Ki16425 (Fig. 10H

and I), **suggesting the participation of other LPARs subtypes in FAPs number induction by LPA.**

We also found that injection of LPA in the PDGFRα^{H2BEGFP} knock-in reporter mice significantly increased total nuclei number (Fig. 10J), determined by Hoescht staining. We also observed that the total number of nuclei in LPA-treated muscles exceeds the number of GFP nuclei (FAPs) (Fig. 10K), **suggesting that LPA injection increases the number of other cell types in the muscle.**

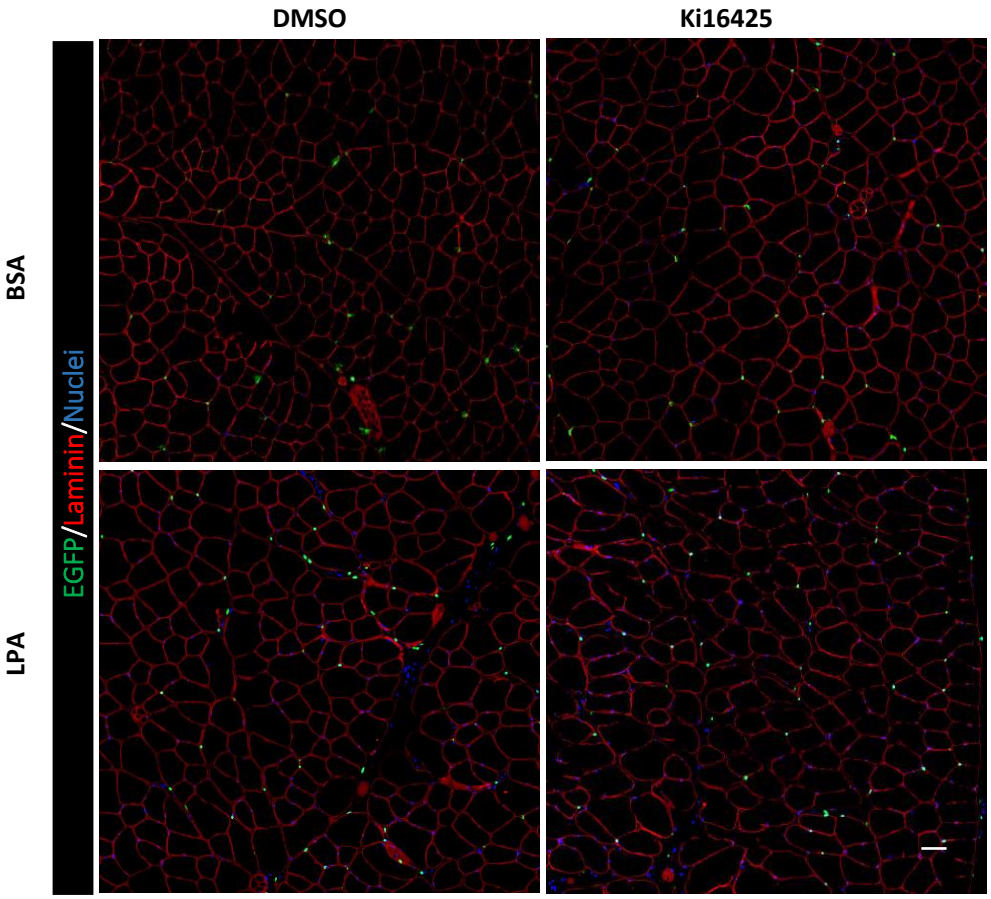
FAPs have the potential to differentiate into adipocytes and myofibroblast phenotypes. We wondered if LPA could have an effect by promoting the myofibroblast phenotype. To answer that, we analyzed the Vcl (Vinculin), Vim (Vimentin) and Acta2 (αSMA) mRNA levels by RT-qPCR in TA treated with BSA or LPA for 4 h or 24 h, we observed an upward trend, but it did not find statistically significant differences (Supplementary Fig. 5C, D and E). On the other hand, we also explore if LPA IM treatment induces FAPs differentiation to adipocytes. We studied the levels of the adipocyte marker PPARγ by immunoblot in LPA-treated TA, we found a transitory increase in PPARγ at 4 h but it did not remain increased until 24 h after LPA treatment. We used adipocyte cell culture protein lysate as a control (Supplementary Fig. 6 A, B). We also analyzed the level of Adipoq mRNA by RT-qPCR in TA treated with BSA or LPA for 4 h, we did not find statistically significant differences (Supplementary Fig. 6 C), indicating that LPA does not induce adypogenesis in skeletal muscle. In summary, these results suggest that **LPA triggers a pro-fibrotic response by inducing ECM components like CCN2 and fibronectin, and FAPs number in skeletal muscle. The LPARs participating in these effects**

are mainly LPA_1, but we cannot exclude a role for other LPARs in these responses (Fig.

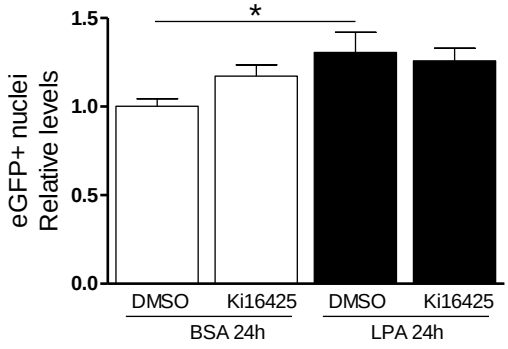


11).

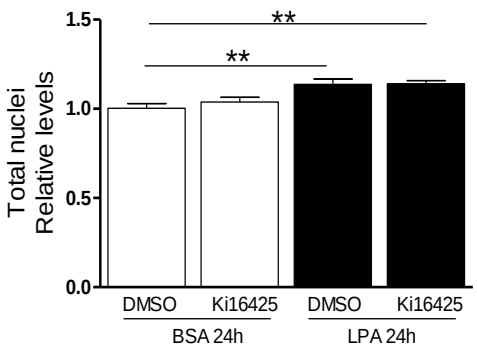
H


DMSO
Ki16425
BSA
LPA
EGFP/Laminin/Nuclei

I


eGFP+ nuclei
Relative levels
1.5
1.0
0.5
0.0
*
DMSO
Ki16425
DMSO
Ki16425
BSA 24h
LPA 24h

J


Total nuclei
Relative levels
1.5
1.0
0.5
0.0
**
**
DMSO
Ki16425
DMSO
Ki16425
BSA 24h
LPA 24h

K

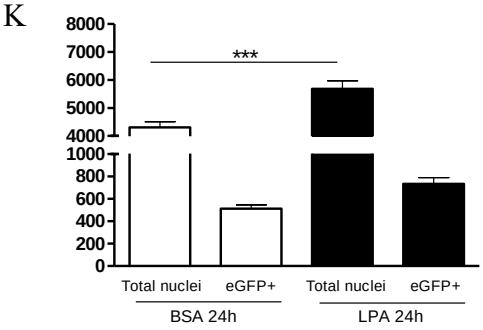

8000
7000
6000
5000
4000
1000
800
600
400
200
0
***
Total nuclei
eGFP+
Total nuclei
eGFP+
BSA 24h
LPA 24h

Figure 10. LPA increases the number of FAPs in skeletal muscle. A, B) PDGFRα levels in TA were analyzed by inmunoblot from WT mice at 4 h (A) or 24 h (B) after being injected with BSA or LPA 400 µg/Kg IM (BSA was injected in the contralateral TA). 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 TA 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 TA 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 GFP-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 TA cross-sections. M refers to a mouse. The values correspond to the mean ± SEM. *P<0.05, **P<0.01, ***P<0.01, N.S not significant by one-way ANOVA with Tukey's post-test. Figure in collaboration with Meilyn Cruz.

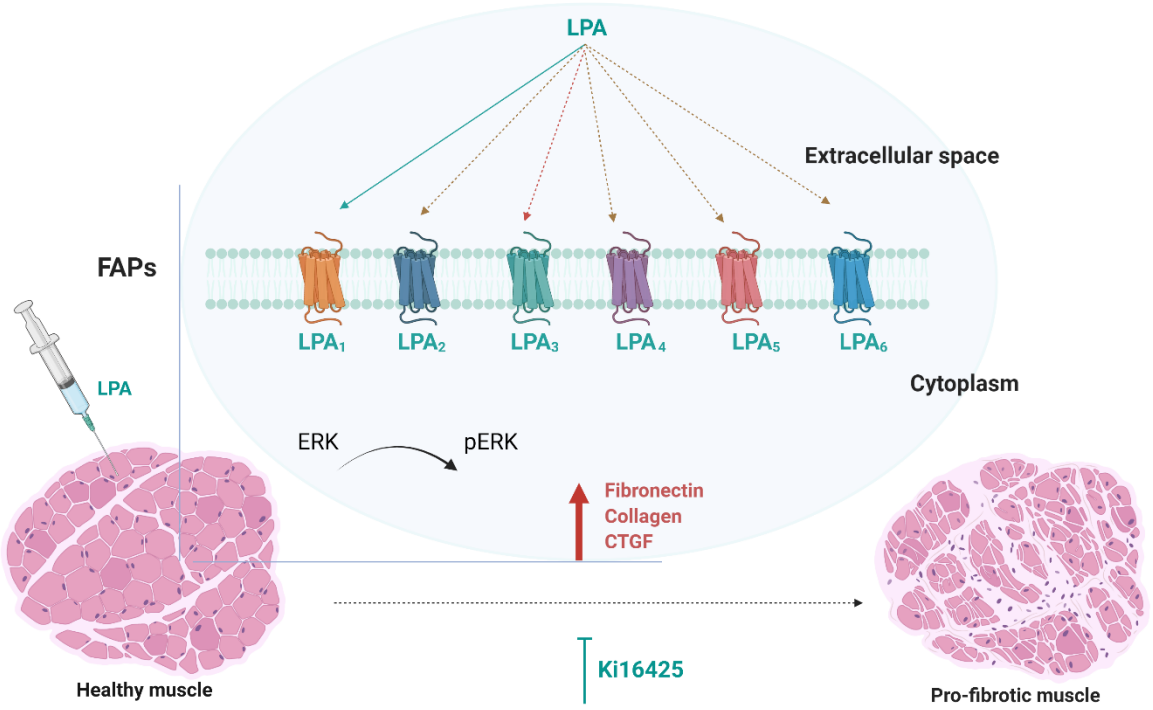


Diagram 6. Schematic view of the skeletal muscle response to LPA. Treatment with LPA induces an increase in the number of FAPs, CCN2 and ECM molecules, such as fibronectin and collagen. The inhibition of $LPA_{1/3}$ with Ki16425 and the absence of the LPA_1 gene prevents the fibrotic effect. The inhibition of ERK pathway in LPA-stimulated muscle has a partial effect (only avoiding the induction of fibronectin protein levels). These results support the idea that LPA could be involved in establishing and developing fibrosis in some pathologies affecting skeletal muscle. The diagram was created with BioRender.com.

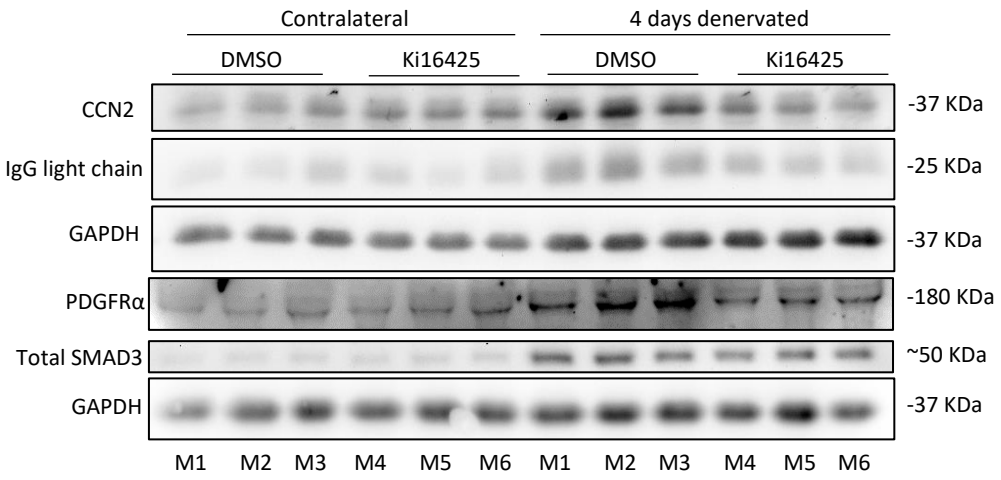
2.7 ATX / LPA / LPARs axis in denervation induced fibrosis

Considering that the muscle denervation surgery induces fibrosis mediated by CCN2 (Rebolledo et al., 2019) and that this model allows studying the induction of fibrosis synchronously in a defined and relatively short time, we decided to block the ATX / LPA / LPARs axis in the muscle denervation model by two approaches, firstly, by inhibiting LPA_1 and LPA_3 with the pharmacological antagonist, Ki16425 in denervated WT mice, and secondly, by denervating LPA1-KO mice. In both approaches, we analyzed the fibrotic response.

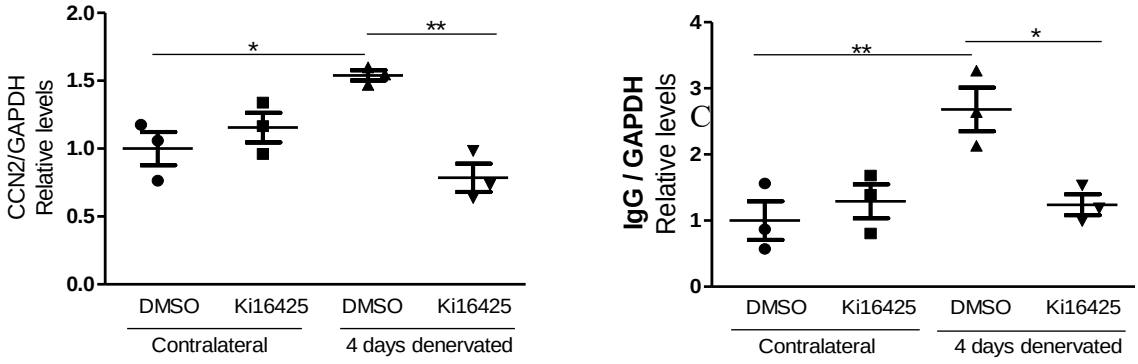
Muscle denervation by transecting the sciatic nerve confirmed that early denervation at 4 days induces CCN2 expression, as shown previously (Liu et al., 2015)(Rebolledo et al., 2019). We found that the blockade of LPA_1 and LPA_3 by using Ki16425 this response was avoided (Fig. 11A, B). The protein levels of IgG, as a marker of inflammatory response were analyzed using immunoblot, detecting that Ki16425 blunted the LPA-induced increase (Fig. 11A, C).

These results suggest that ATX / LPA / LPARs axis would participate in inducing CCN2 in denervated muscle. Since our previous results showed that IM injections of LPA in WT mice increased PDGFRα levels and the number of FAPs, we evaluated this marker of FAPs in our denervation model. We found that PDGFRα levels, determined by WB are increased 4 days after denervation, as has been reported previously (Contreras et al., 2016), Ki16425 prevents this increase (Fig 11A, D), suggesting that in the denervation model, the increase in FAPs could be mediated by LPA_1 and/or LPA_3. As a control for denervation surgery at 4 days, we used the increase of total SMAD3 protein levels. **These results indicate that the induction of CCN2, IgG and PDGFRα in early denervation is mediated by LPA_1 and /or LPA_3.**

A


Contralateral
4 days denervated
DMSO
Ki16425
DMSO
Ki16425
CCN2
IgG light chain
GAPDH
PDGFRα
Total SMAD3
GAPDH
-37 KDa
-25 KDa
-37 KDa
-180 KDa
~50 KDa
-37 KDa
M1 M2 M3 M4 M5 M6 M1 M2 M3 M4 M5 M6

B


CCN2/GAPDH
Relative levels
2.0
1.5
1.0
0.5
0.0
*
**
DMSO
Ki16425
DMSO
Ki16425
Contralateral
4 days denervated

C

IgG / GAPDH
Relative levels
4
3
2
1
0
**
*
DMSO
Ki16425
DMSO
Ki16425
Contralateral
4 days denervated

D

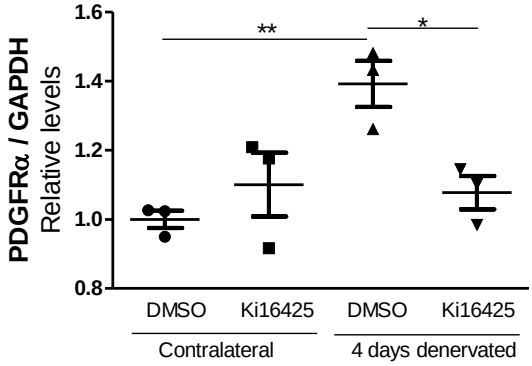

PDGFRα / GAPDH
Relative levels
1.6
1.4
1.2
1.0
0.8
**
*
DMSO
Ki16425
DMSO
Ki16425
Contralateral
4 days denervated

Figure 11. LPA_1 and LPA_3 inhibition avoid CCN2 and PDGFRα induction after muscle denervation. A) CCN2, IgG light chain and PDGFRα were analyzed by immunoblot to determine their protein levels in GST from C57BL/6 WT male mice treated with Ki16425 and denervated for 4 days. GAPDH was used as the loading control. B, C and D) Quantification of A. M refers to the mouse. For each mouse, we analyzed the denervated and the contralateral muscle. The values correspond to the mean ± SEM. **P<0.01, *P<0.05 by one-way ANOVA with Tukey's post-test. n=3.

2.8 LPA_1 is mediating the fibrotic response induced by denervation

Considering that LPA_1 is the most expressed LPAR in skeletal muscle and the results obtained in the previous experiment in denervated mice treated with Ki16425, we wonder if LPA_1 is the primary receptor by which LPA mediates its fibrotic response in denervation. We denervated WT and LPA_1-KO mice and evaluated fibrosis markers such as CCN2, PDGFRα, and fibronectin after 2 weeks, when the fibrotic response is consolidated (Rebolledo et al., 2019). Interestingly, the absence of LPA_1 strongly prevented the induction of CCN2 (Fig. 12A, B), fibronectin (Fig. 12A, C), and PDGFRα (Fig. 12A, D). **These results show that LPA and its LPA_1 receptor are relevant in denervation-induced fibrosis.** It is important to elucidate in future research the role of LPA / LPA_1 in the pathophysiology of denervation, considering studying the three main elements: inflammation, atrophy, and fibrosis to obtain the big picture of the muscle denervation phenomena.

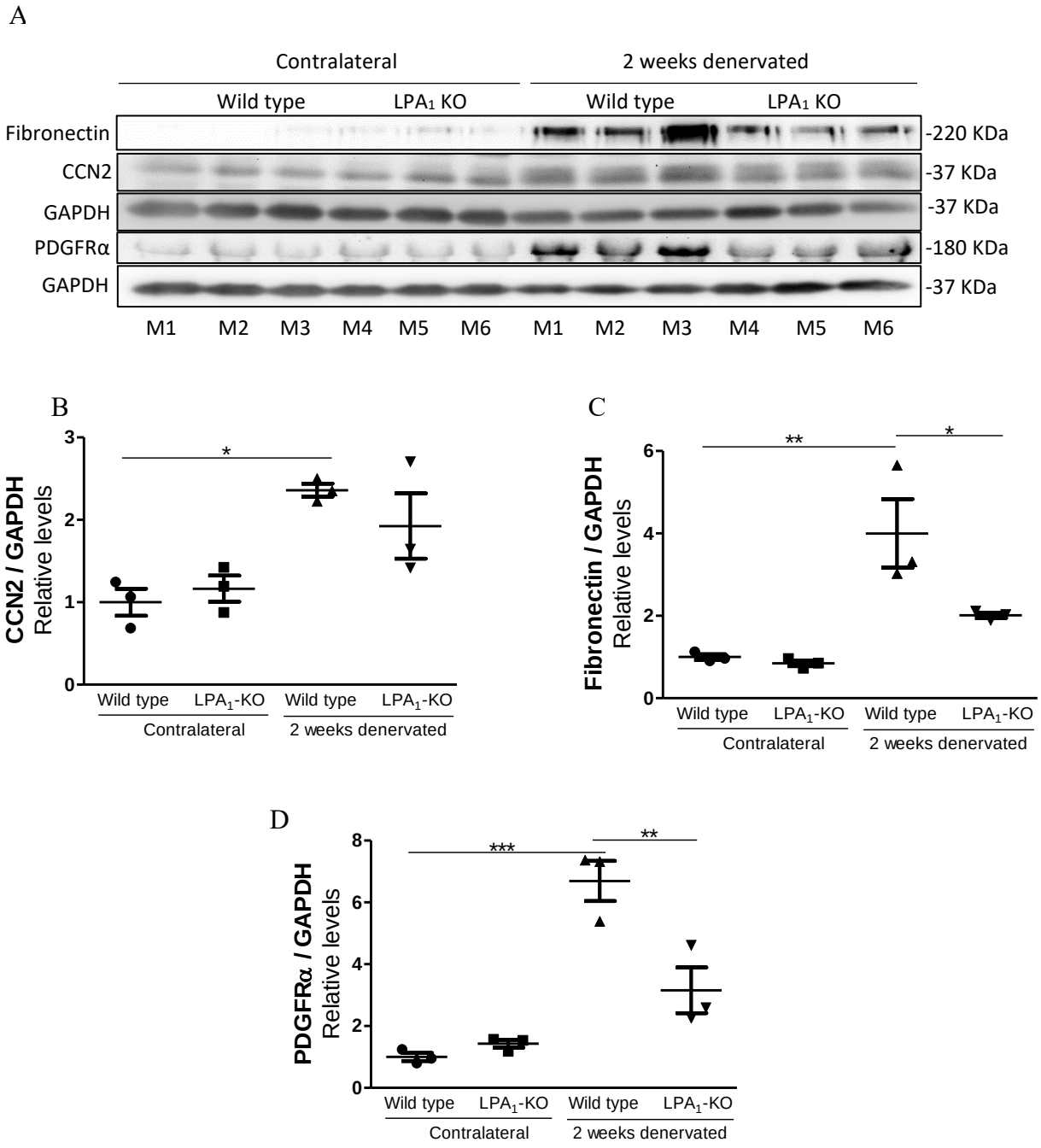


Figure 12. The absence of LPA$_1$ reduces the fibrotic response after 2 weeks of denervation. Fibronectin, CCN2 and PDGFRα were analyzed by immunoblot to determine their protein levels in GST from BALB/c WT and LPA$_1$-KO mice denervated for 2 weeks. GAPDH was used as the loading control. B, C and D) Quantification of A. M refers to the mouse. For each mouse, we analyzed the denervated and the contralateral muscle. The values correspond to the mean ± SEM. ***P<0,001, **P<0.01, *P<0.05 by one-way ANOVA with Tukey's post-test. n=3.

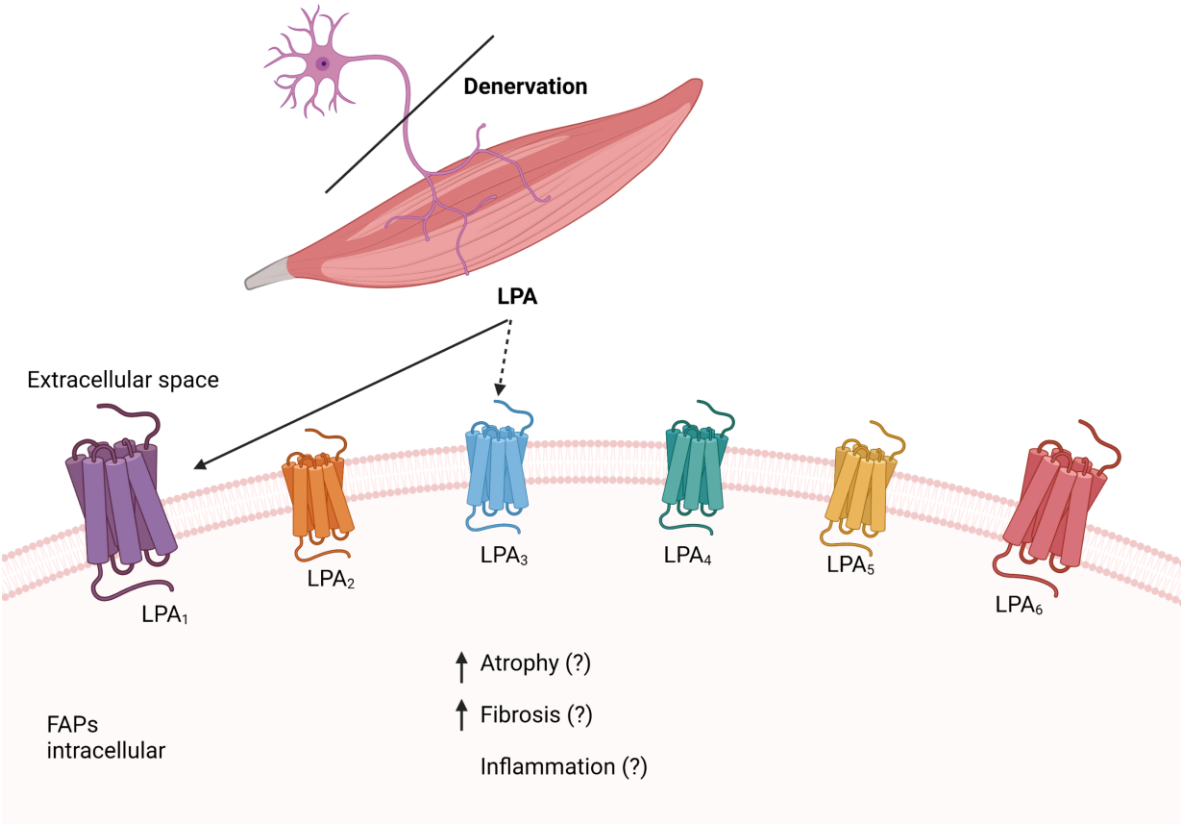


Diagram 7. Schematic view of ATX / LPA / LPARs axis participation in the skeletal muscle denervation response. Muscle denervation induces an increase in CCN2, fibronectin and PDGFRα protein levels. The inhibition of $LPA_{1/3}$ with Ki16425 and the absence of the LPA_1 gene prevent the fibrotic effect after denervation. These results support the hypothesis that LPA could be involved in establishing and developing fibrosis in some pathologies affecting skeletal muscle. The diagram was created with BioRender.com.

2.9 Summary of results objective 2

1. Skeletal muscle responds to LPA by inducing CCN2, fibronectin, collagen, and PDGFRα protein levels.
2. Inhibition or absence of LPA_1 prevents the LPA fibrotic response in skeletal muscle.
3. LPA participates in muscular fibrosis induced by denervation through LPA_1.

These results are illustrated in diagram 6 and 7.

3. Specific objective 3

To identify the LPARs and the possible signaling pathways involved in the induction of CCN2 in muscular fibro / adipoagenic progenitors

3.1. FAPs primary culture

FAPs are PDGFRα positive mesenchymal cells resident in skeletal muscle and they are capable of differentiate into myofibroblasts. These cells produce ECM in physiological conditions (wound healing) and pathological conditions (fibrosis) (Bochaton-Piallat et al., 2016). Since IM LPA stimulus induces an increase in the number of FAPs in skeletal muscle (Fig. 10 H, I), we evaluated if these cells express the ATX / LPA / LPARs axis components and respond to LPA by inducing the pro-fibrotic cytokine CCN2. To answer these questions, we decided to carry out primary culture of muscular FAPs from 2-3 months WT male mice in C57BL6 background. As we pointed out in the Materials and Methods section, FAPs cell culture standardization was previously reported by Contreras et al (Contreras, et al., 2019). In the representative IIF of FAPs primary culture shown in Fig. 13A we can observe that most of the cells express PDGFRα, and there is a low degree of differentiation toward a myofibroblast phenotype since few cells express αSMA and there is no lack PDGFRα expression (Fig. 13A).

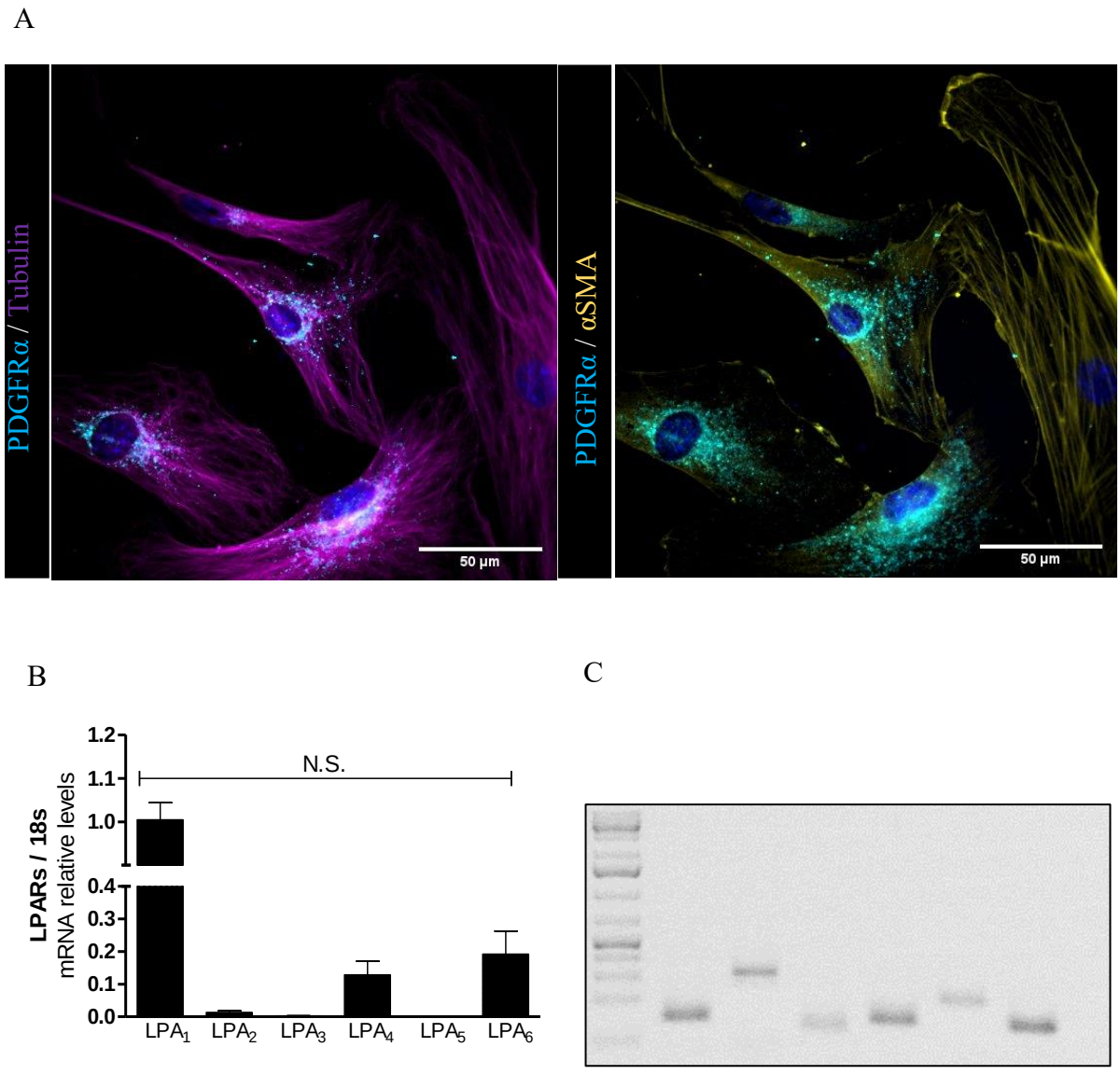


Figure 13. FAPs express LPARs. A) Representative IIF image of FAPs cell culture. In cyan the FAPS marker, PDGRα. In purple tubulin. In yellow αSMA and blue, Hoechst. Scale bar: 50 µm. B) LPARs expression profile analyzed by RT-qPCR. C) qPCR products. The values correspond to the mean ± SEM. ***P<0.01 by one-way ANOVA with Tukey's post-test. n=3.

3.2. FAPs express LPARs and respond to LPA by inducing CCN2

To determine whether FAPs express LPARs and thus can respond to the agonists, we analyzed the mRNA levels of the six LPARs by RT-qPCR. The LPARs profile in FAPs (Fig. 13B, C) is similar to the found in skeletal muscle (Fig. 2A). However, the expression of LPA_6 seems lower and LPA_4 higher in FAPs than in skeletal muscle. A comparative RT-qPCR between skeletal muscle and FAPs LPARs is needed to complete this conclusion.

Many cell types have been shown to respond to LPA by inducing CCN2, including myoblasts and C2C12 myotubes (Vial et al., 2008)(Cabello-Verrugio et al., 2011). However, it has not yet been described that FAPs respond to LPA, to what extent they compare other cell types of residents in skeletal muscle. We decided to treat myoblasts, myotubes, 10 T1/2 fibroblasts, and FAPs with the same concentration of LPA (30 µM) for 3 hours. Our results indicate that muscular FAPs respond more strongly to LPA by inducing CCN2 than other cell types (Fig. 14A, B). **This observation validated our idea of working with FAPs as an *in vitro* model to demonstrate the pro-fibrotic potential of LPA.**

In Fig. 15A, we show an LPA-inducing CCN2 kinetic. We observed a statistically significant increase at 3 h after the stimulus. Based on this result, we decided to treat our cells for that time. We also observed that the optimal concentration of LPA to reach statistical changes is 30 µM (Fig. 15B). Therefore, the treatment time and concentration of LPA chosen for the development of the following experiments was LPA 30 µM for 3 h.

Additionally, we wonder if FAPs respond to other LPAR agonists, such as 2s-OMPT, a synthetic molecule marketed as an LPA_3 agonist. Surprisingly, considering the low levels

observed of mRNA LPA_3 in FAPs, we found strong induction of CCN2, much greater than the effect of LPA in those cells. Like LPA, 2s-OMPT showed that the optimal time (statistically significant) to induce CCN2 was 3 h (Fig. 15C), but at a much lower concentration (2 µM) (Fig. 15D) than that used in LPA treatments (30 µM). **These results indicate that FAPs express LPARs and are greater responders to both LPAR agonists (LPA and 2s-OMPT) by inducing the levels of the CCN2.**

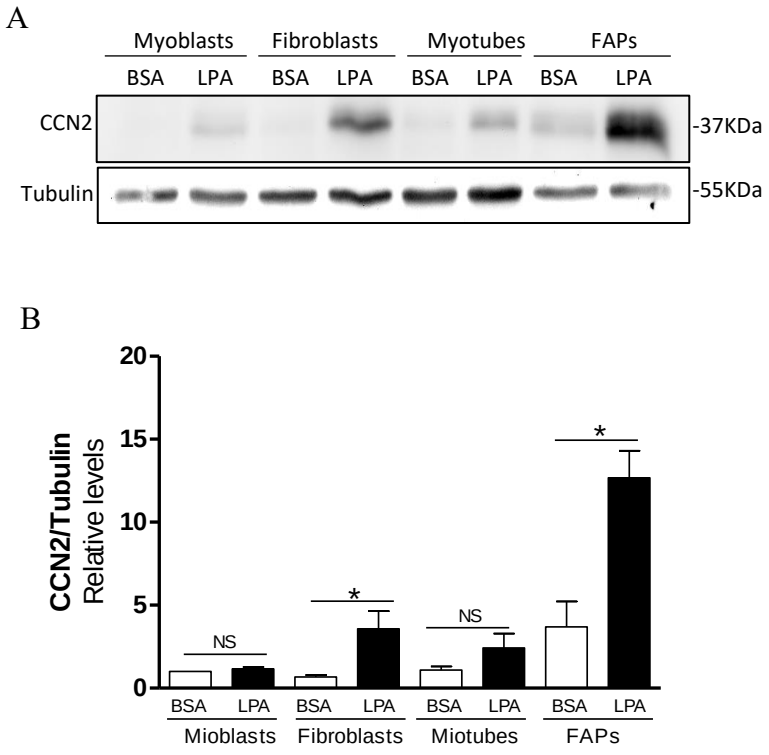


Figure 14. FAPs respond strongly to LPA stimulation. A) Immunoblot for CCN2 in C2C12 myoblasts and myotubes differentiated up to day 6, 10T1/2 fibroblasts cell line and muscular primary culture of FAPs treated with LPA 30 µM for three h, tubulin was used as a loading control. B) Quantification of A. The values correspond to the mean ± SEM. *P<0.05 by one-way ANOVA with Tukey's post-test. n=3.

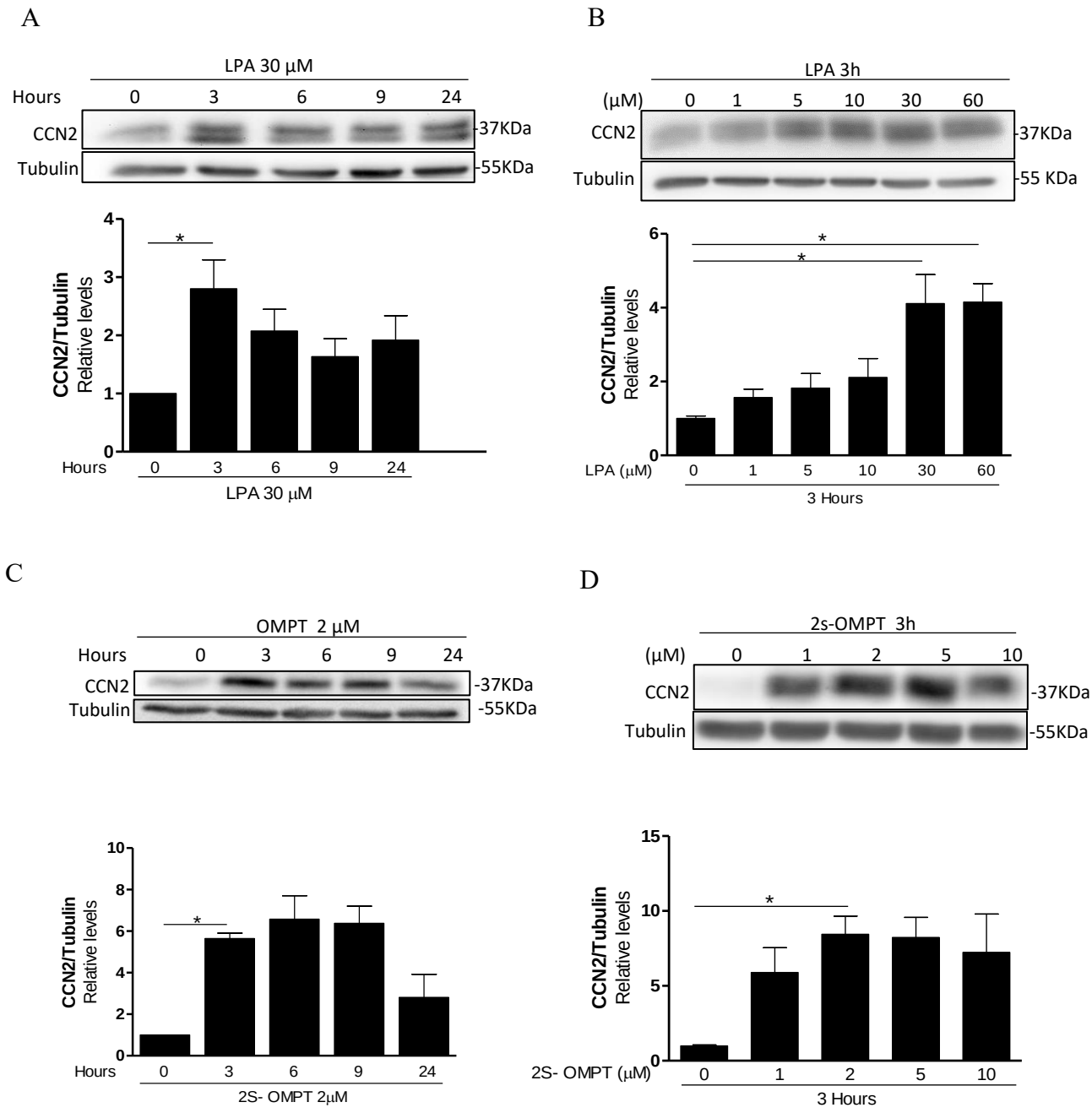

A
LPA 30 µM
Hours 0 3 6 9 24
CCN2
Tubulin
37KDa
55KDa
CCN2/Tubulin Relative levels
Hours 0 3 6 9 24
LPA 30 µM
B
LPA 3h
(µM) 0 1 5 10 30 60
CCN2
Tubulin
37KDa
55 KDa
CCN2/Tubulin Relative levels
LPA (µM) 0 1 5 10 30 60
3 Hours
C
OMPT 2 µM
Hours 0 3 6 9 24
CCN2
Tubulin
37KDa
55KDa
CCN2/Tubulin Relative levels
Hours 0 3 6 9 24
2S- OMPT 2µM
D
2s-OMPT 3h
(µM) 0 1 2 5 10
CCN2
Tubulin
37KDa
55KDa
CCN2/Tubulin Relative levels
2S- OMPT (µM) 0 1 2 5 10
3 Hours

Figure 15. FAPs respond to LPA and 2s-OMPT by inducing CCN2. Immunoblot for CCN2 in C2C12 C57BL/6 WT muscular FAPs treated with A and B) LPA or C and D) 2s-OMPT in different times and concentrations. Tubulin was used as a loading control. Quantification of experiments are shown under immunoblots. The values correspond to the mean ± SEM. *P<0.05 by one-way ANOVA with Tukey's post-test. n=3-4.

3.3. Inhibition of LPARs prevents induction of CCN2 by LPARs agonist

After confirming that FAPs respond to LPA agonists, we wondered if this response can be inhibited using **LPAR antagonists**. To determine the LPAR involved in the induction of CCN2 in FAPs, we decided to use the LPA_1 and LPA_3 antagonist, Ki16425. To validate its use, we tested different concentrations of Ki16425 (from 0 to 20 µM) in the presence of the LPA stimulus (Fig. 16A). Our results indicate that 10 µM is the proper concentration to inhibit the CCN2 induction, so we decided to use 10 µM for the following experiments with Ki16425.

As we have shown previously, 2s-OMPT is a potent inductor of CCN2 in FAPs; therefore, to confirm that LPA_1 / LPA_3 mediates this response, we treated FAPs with 2s-OMPT in the presence of Ki16425. Our results show that inhibition of LPA_1 and LPA_3 can inhibit the CCN2 increased levels induced by 2s-OMPT (Fig. 16B). In Fig. 16C, we show an immunoblot that summarizes the magnitudes in which LPA and 2s-OMPT cause CCN2 increase and the effect of Ki16425 avoiding this induction.

In order to confirm our results that propose LPA_1 as the main mediator of LPA-induced CCN2 levels, we decided to use a new LPA_1 inhibitor, AM095 (Supplementary Fig. 7). The results indicate that AM095 inhibits the CCN2 increase by LPA or 2s-OMPT. Similar to the results obtained with Ki16425, suggesting that probably 2s-OMPT and LPA at the concentration used in this thesis, are signaling through LPA_1 to induce CCN2.

Finally, we wondered if LPA modifies CCN2 levels in a transcriptional manner by analyzing the mRNA levels of CCN2 using RT-qPCR and, interestedly, we found that CCN2 mRNA showed the same response compared to its protein levels 3 h after the LPA stimulus (Fig. 16D). These results indicate that CCN2 is an early response to LPA in a process that involves the transcriptional machinery. Still, these results cannot exclude that the increase of CCN2 by LPA could also be due to a blockade or delay in the CCN2 protein degradation. Future experiments can deepen this hypothesis. **Our results indicate that the main LPAR mediating CCN2 transcriptional induction by LPA in FAPs is LPA_1.**

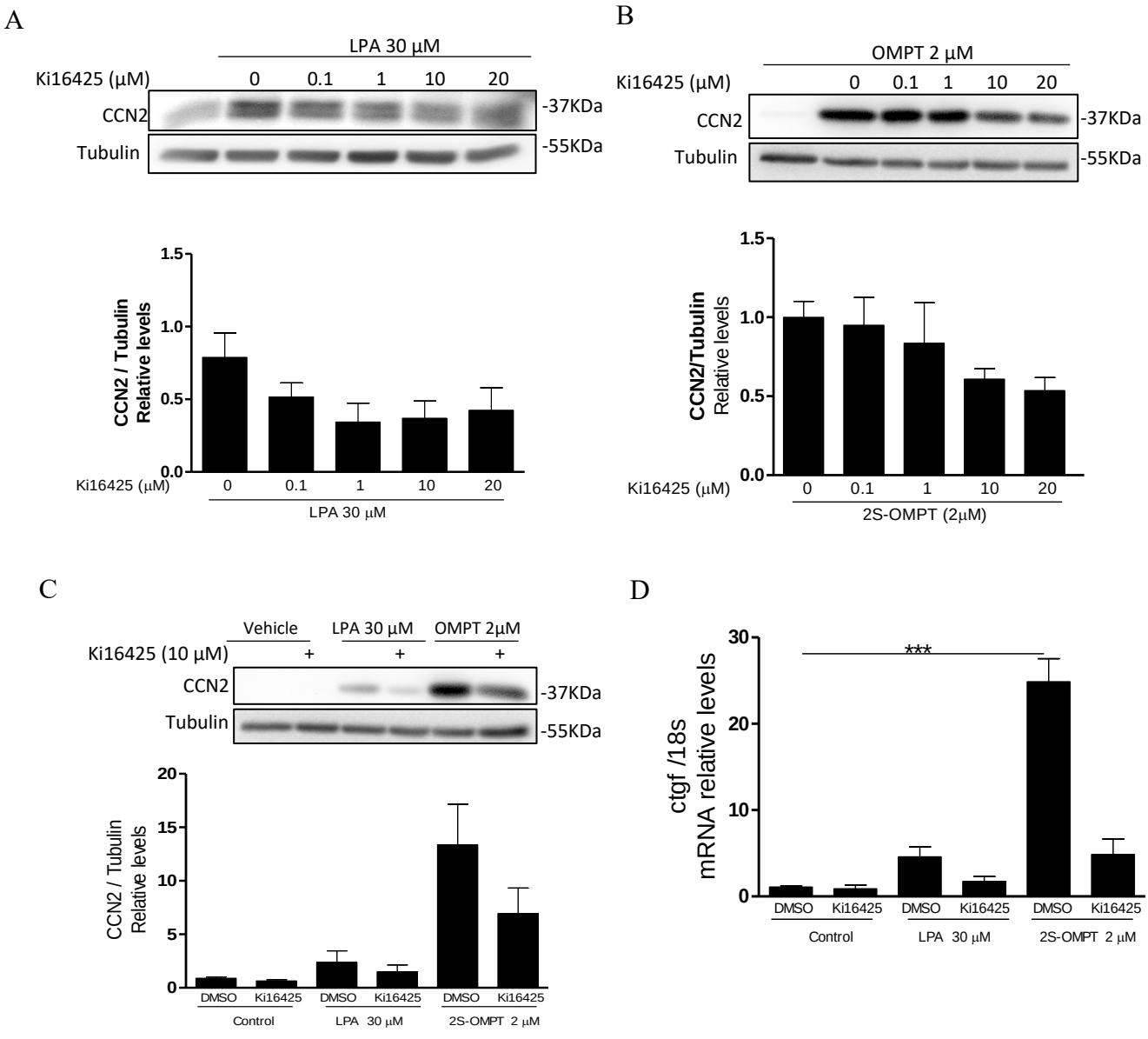


Figure 16. Inhibition of LPARs prevents induction of CCN2 by LPARs agonist. A) CCN2 protein levels analyzed by immunoblot blot in C57BL/6 WT FAPs treated with LPA 30 μM or B) 2s-OMPT 2 μM for 3 h in the presence or absence of different concentrations of Ki16425. C) Comparatives immunoblot with definitive concentration of LPA, 2s-OMPT and Ki16425 used in this thesis. D) CCN2 mRNA levels analyzed by RT-qPCR. Values correspond to the mean ± SEM. *P<0.05, N.S not significant by one-way ANOVA with Tukey's post-test. n=4.

3.4 Genetic ablation of LPA_1 prevents induction of CCN2 by LPARs agonists

LPA_1 is the most highly expressed receptor in skeletal muscle and FAPs at the transcriptional level. Although Ki16425 and AM095, both LPA_1 inhibitors, blocked the CCN2-inducing response by LPA and 2s-OMPT, we wanted to specifically confirm the identity of the LPAR that mediates the induction of CCN2 to contribute to the knowledge of the signaling pathway underlying the induction of this potent pro-fibrotic factor.

For this purpose, we performed the primary culture of FAPs from the muscles of LPA_1-KO mice and their control WT mice, both in BALB/c background. In Fig 17, we show the LPARs mRNA levels of BALB/c WT and LPA_1-KO FAPs. We confirm the absence of LPA_1 mRNA in LPA_1-KO FAPs, but we did not observe differences in other LPARs comparing WT and LPA_1-KO FAPs (Fig. 17).

Our results indicate that LPA induces CCN2 in BALB/c WT FAPs in a sustained manner over time since the induction of CCN2 does not decay 24 hours after stimulation, unlike what was observed in WT FAPs of background C57BL6 mice (Fig. 18A, B). Regarding the LPA_1-KO FAPs response, we found that the ability to induce CCN2 under the stimulation of LPA is much lower since WT mice can induce CCN2 with an average of 4-fold of change. In contrast, FAPs LPA_1-KO can only induce CCN2 by an average of 2-fold of change at 3 h of treatment (Fig. 18A, B).

As we showed previously, 2s-OMPT, an LPA_3 agonist, strongly induces CCN2 in FAPs; however, it is difficult to think that this agonist is signaling through LPA_3 since the FAPs express very lower levels of this LPAR at leat at mRNA level. We hypothesize that 2s-OMPT is signaling through to LPA_1 to induce CCN2 in FAPs. To corroborate this idea, we challenged LPA_1-KO FAPs with 2 µM 2s-OMPT and evaluated the induction of CCN2. Our results showed that the response of FAPs LPA_1-KO was lower than in FAPs WT. However, possibly due to the data dispersion, we could not observe significant differences with the experimental number used (n=3) (Fig 19A, B).

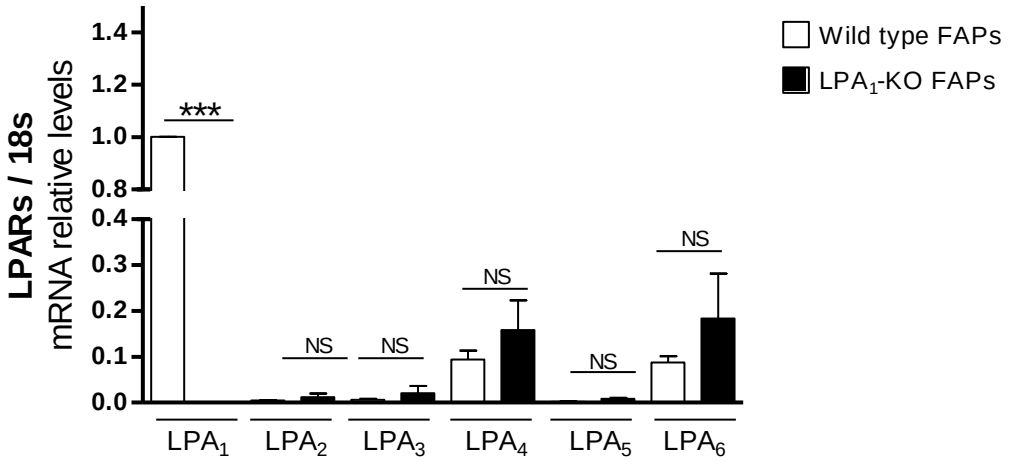


Figure 17. LPARs expression in FAPs WT y LPA1-KO. LPARs mRNA profile analyzed by RT-qPCR. in BALB/c WT and LPA_1-KO FAPs. The values correspond to the mean ± SEM. ***P<0.01, N.S not significant by one-way ANOVA with Tukey's post-test. n=3.

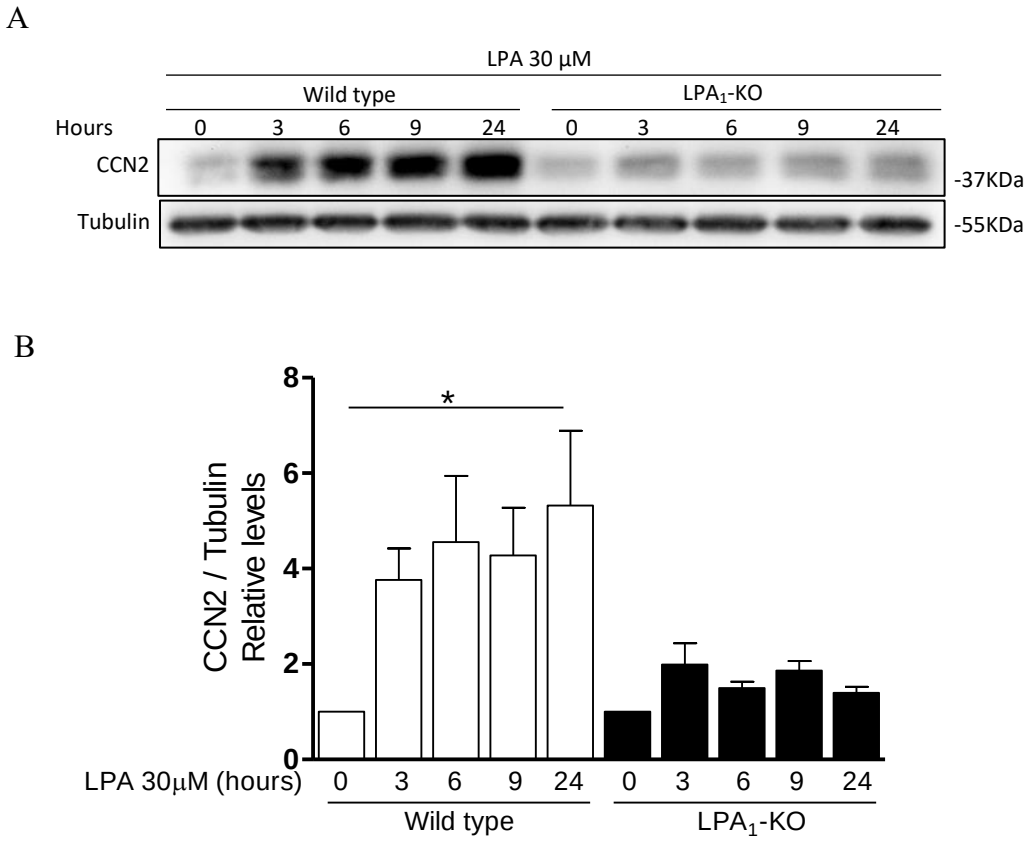


Figure 18. LPA$_1$ absence avoids the induction of CCN2 by LPA in FAPs. A) CCN2 protein levels analyzed by immunoblot blot in BALB/c WT and LPA$_1$-KO FAPs treated with LPA 30 µM for different hours. B) quantification of A. Values corresponds to the mean ± SEM. *P<0.05, N.S not significant by one-way ANOVA with Tukey's post-test. n=4

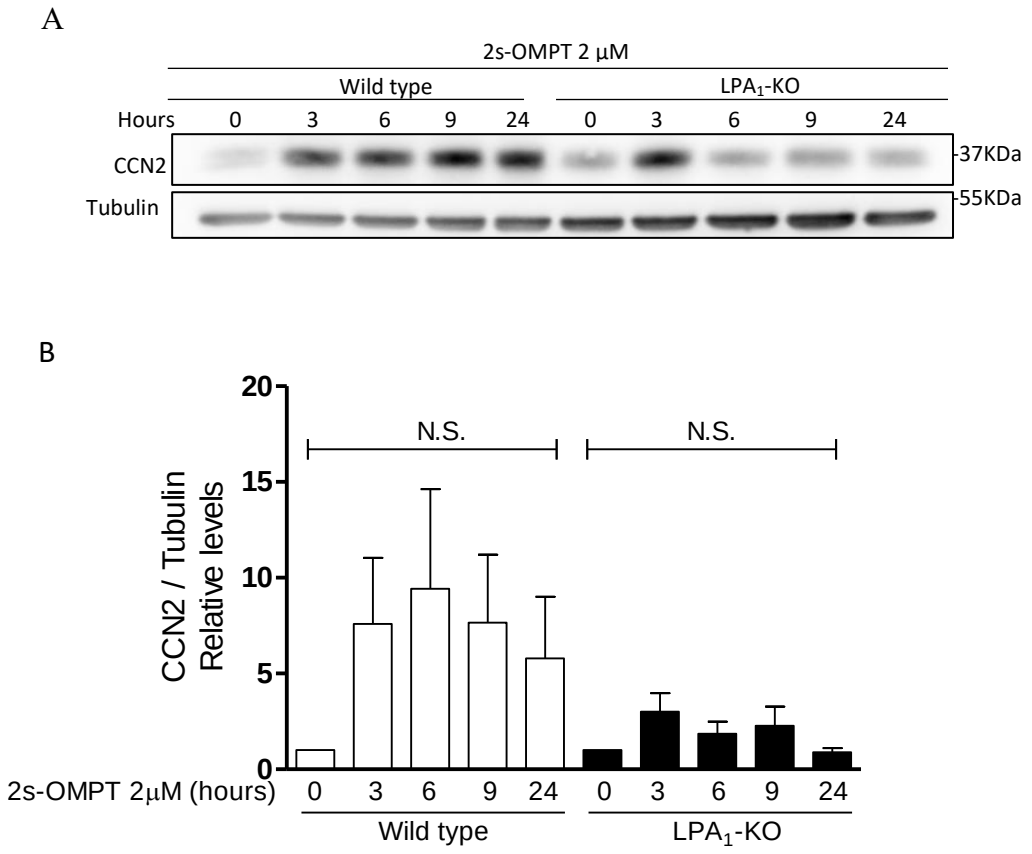
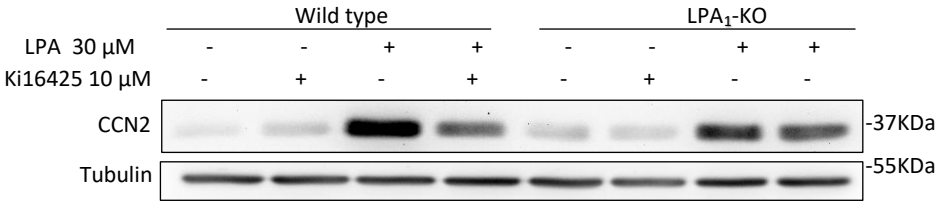


Figure 19. LPA$_1$ absence seems to avoid the induction of CCN2 by 2s-OMPT in FAPs. A) CCN2 protein levels analyzed by immunoblot blot in BALB/c WT and LPA$_1$-KO FAPs treated with 2s-OMPT 2 µM for different hours. B) quantification of A. Values corresponds to the mean ± SEM. N.S not significant by one-way ANOVA with Tukey's post-test. n=3.

Finally, we take advantage of LPA_1-KO FAPs to demonstrate that Ki16425 usage in FAPs primarily prevents LPA signaling via LPA_1. We treated WT and LPA_1-KO FAPs with LPA in the presence of Ki16425. Our results showed that, as we had already seen in Fig. 18, WT FAPs respond more strongly to LPA by inducing CCN2 than LPA_1-KO FAPs (Fig. 20 A, B). We were able to show that the LPA_1-KO FAPs present the same CCN2 levels as the LPA_1-KO FAPs treated with Ki16425 (Fig. 20 A, B), **demonstrating that Ki16425 in FAPs is inhibiting the CCN2 induction throught LPA_1. It is important to highlight that in LPA_1-KO FAPs treated with LPA in the presence of Ki16425, CCN2 levels do not reach basal levels (control situation), indicating that more LPARs are mediating the response to LPA in the protein induction of CCN2.** Considering the LPARs expression pattern in FAPs, we could propose LPA_6 as a new LPAR to study in this mechanism.

A



B

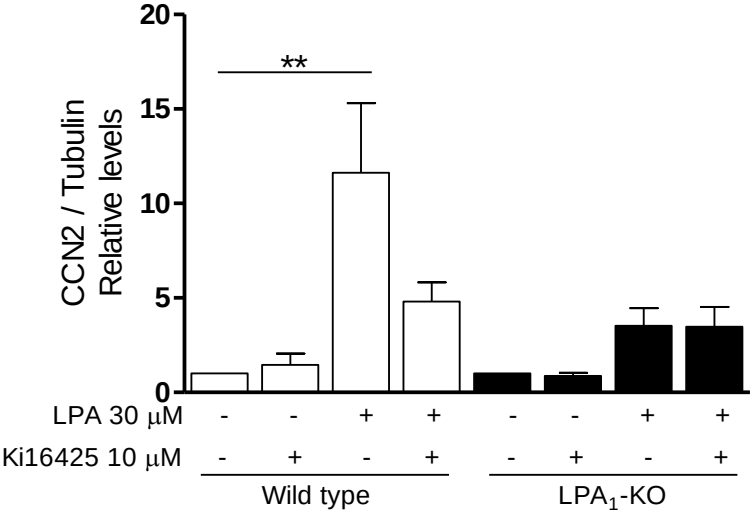


Figure 20. LPA_1 is the main LPA receptor involved in CCN2 induction by LPA in FAPs. A) CCN2 protein levels were analyzed by immunoblot blot in BALB/c WT and LPA_1-KO FAPs treated with LPA 30 μM for 3 hours in the presence and absence of $LPA_{1,3}$ inhibitor, Ki16425. B) quantification of A. Values corresponds to the mean ± SEM. **P<0.01 by one-way ANOVA with Tukey's post-test. n=3.

3.5 LPA and 2s-OMPT induces early ERK, AKT and JNK phosphorylation

Having identified LPA_1 as the main LPAR involved in the induction of CCN2 by LPA in FAPs, we decided to explore possible signaling pathways that: i) had been reported participating in CCN2 induction and ii) that are reported in the LPA signaling in other cell types.

AKT, ERK and JNK pathways are activated by LPA in different cells and contexts (Zhou et al., 2018)(Budnik et al., 2003)(Sasaki et al., 1998). so we decided to study if these pathways are participating in the induction of CNN2 by LPA. First, we wanted to know if LPA can trigger the activation of these molecules through its phosphorylation. Our results indicate that LPA promotes early ERK phosphorylation at 5 minutes after the LPA stimulus (Fig. 21A, B and C), we also observe a strong upward trend in the pAKT and pJNK levels but these results did not show statistical differences with the experimental number used, n=3. We also studied the effect of the LPAR agonist 2s-OMPT in the activation of these molecules and we found the same response that LPA (Fig. 22A, B, and C). **These results showed that these pathways are activated under LPA or 2s-OMPT stimulus and they are good candidates for studying their requirement in the CCN2 induction.**

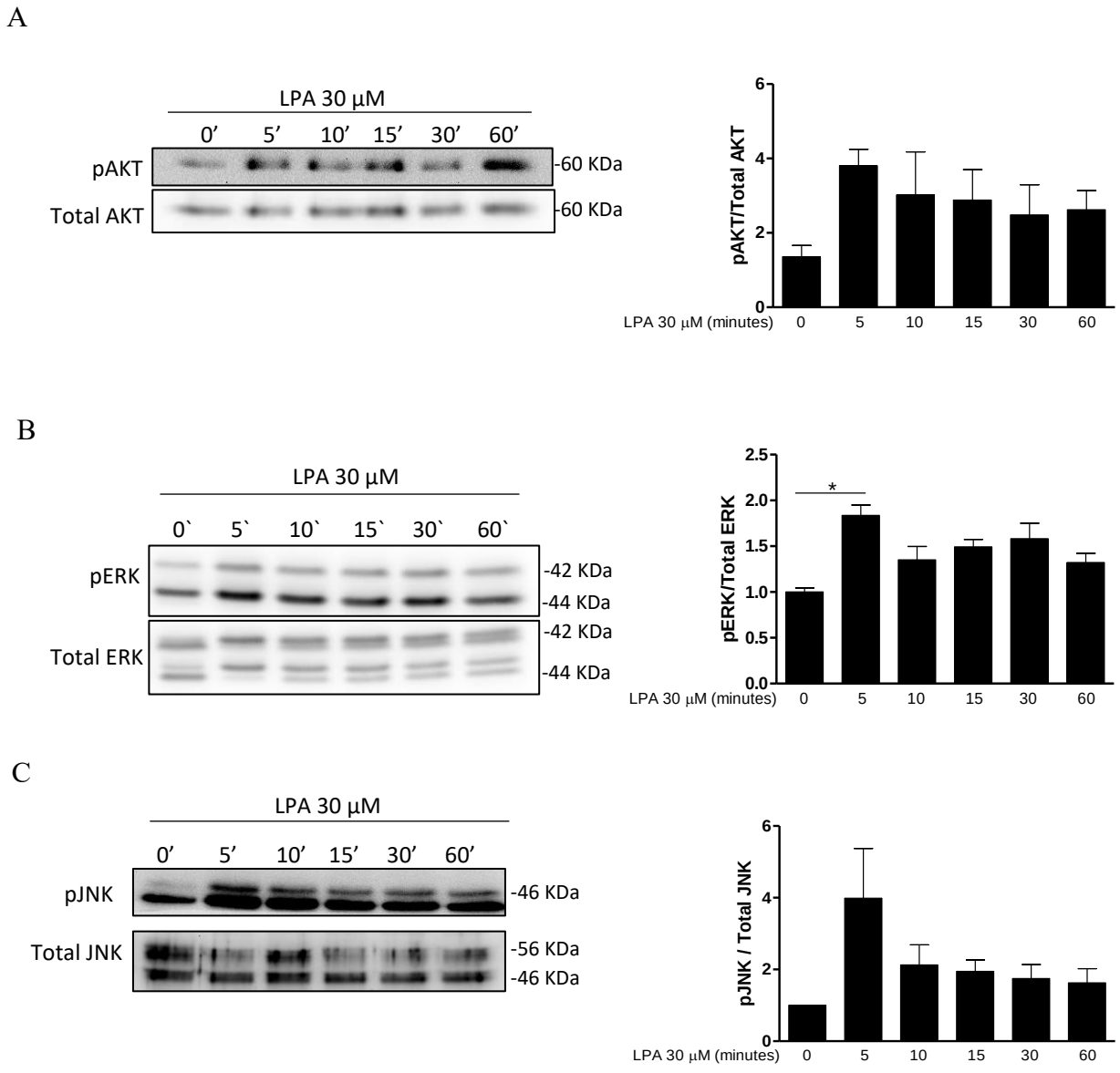


Figure 21. LPA triggers AKT, ERK and JNK phosphorylation. pAKT, pERK and pJNK, total AKT, total ERK and total JNK protein levels analyzed by immunoblot blot in C57BL/6 WT FAPs treated with LPA 30 μM for different times (0 to 60 minutes). Quantifications are shown right to representative immunoblot. Values correspond to the mean ± SEM. *P<0.05 by one-way ANOVA with Tukey's post-test. n=3.

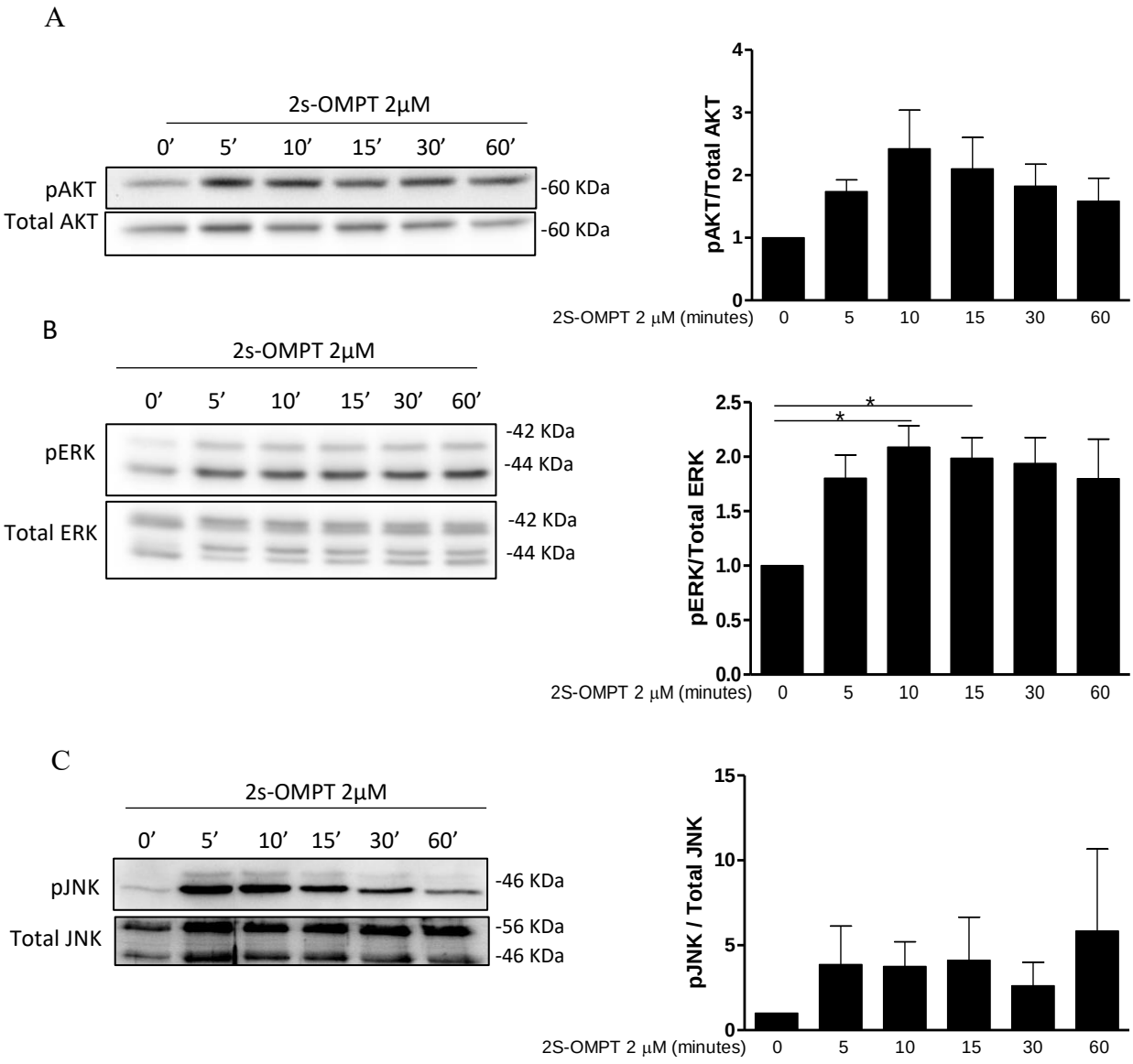
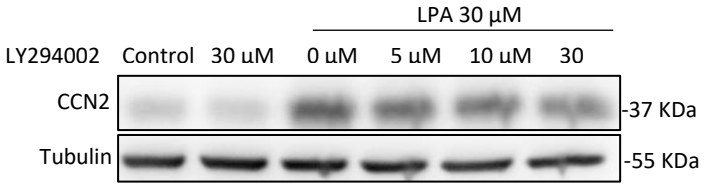


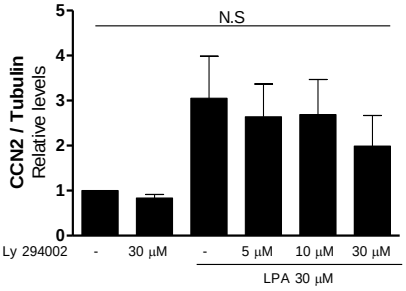
Figure 22. 2s-OMPT triggers AKT, ERK and JNK phosphorylation. pAKT, pERK and pJNK, total AKT, total ERK and total JNK protein levels were analyzed by immunoblot blot in C57BL/6 WT FAPs treated with 2s-OMPT 2 µM for different times (0 to 60 minutes). Quantifications are shown right to representative immunoblot. Values correspond to the mean ± SEM. *P<0.05 by one-way ANOVA with Tukey's post-test. n=3.

3.6 Inhibition of AKT, ERK and JNK partially prevents CCN2 induction

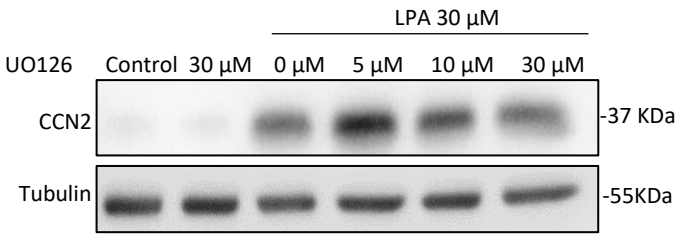
To study the CCN2 expressionthe CCN2 expression deeply by ATX / LPA / LPARS axis is relevant in developing therapies for MDs. To elucidate whether the induction of CCN2 by LPA requires the activation of common pathways like AKT, ERK, or JNK, we studied the effect of inhibitors of these signaling pathways in the presence of LPA. We treated FAPs with LPA in the presence of different inhibitors of these pathways; LY294002 to inhibit AKT, U0126 to inhibit ERK, and JNK inhibitor VIII to inhibit JNK phosphorylation. We found that inhibition with a high concentration (30 μM) of the combination of the three inhibitors (Inh. mixture) seems to strongly prevent the induction of CCN2 (Fig. 23) (statistical analyses do not reach significance at n=3 but show a clear trend). Considering the high concentration of these inhibitors to reach an effect in CCN2 levels, it is important to elucidate in future experiments if this is a specific response to the inhibition of these pathways or a secondary effect of the mixture of drugs for FAPs. Other pathways mediating CCN2 induction by LPA must be explored, being a good target to study the hippo/YAP/TAZ pathway, which has been involved in the transcriptional induction of CCN2 through TEAD family of transcription factors (B. Zhao et al., 2008)(Moon et al., 2020).

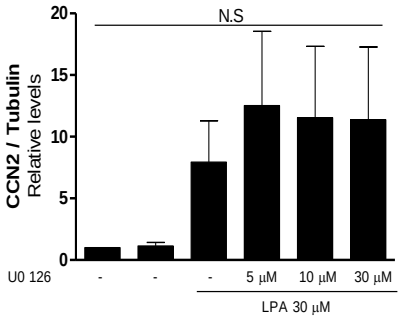
A


LPA 30 μM
LY294002 Control 30 μM 0 μM 5 μM 10 μM 30
CCN2
-37 KDa
Tubulin
-55 KDa

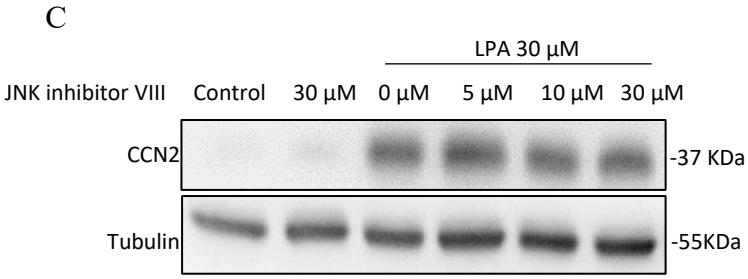

N.S
CCN2 / Tubulin
Relative levels
Ly 294002 - 30 μM - 5 μM 10 μM 30 μM
LPA 30 μM

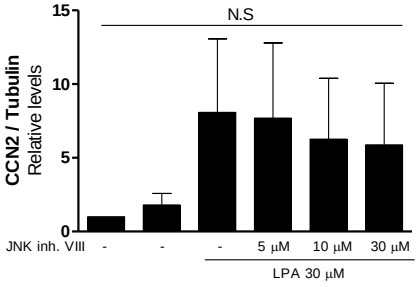
B


LPA 30 μM
UO126 Control 30 μM 0 μM 5 μM 10 μM 30 μM
CCN2
-37 KDa
Tubulin
-55KDa

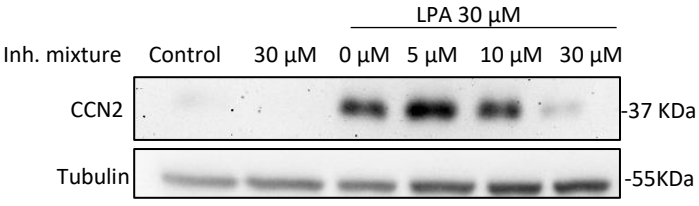

N.S
CCN2 / Tubulin
Relative levels
U0 126 - - - 5 μM 10 μM 30 μM
LPA 30 μM

C


LPA 30 μM
JNK inhibitor VIII Control 30 μM 0 μM 5 μM 10 μM 30 μM
CCN2
-37 KDa
Tubulin
-55KDa


N.S
CCN2 / Tubulin
Relative levels
JNK inh. VIII - - - 5 μM 10 μM 30 μM
LPA 30 μM

D


LPA 30 μM
Inh. mixture Control 30 μM 0 μM 5 μM 10 μM 30 μM
CCN2
-37 KDa
Tubulin
-55KDa

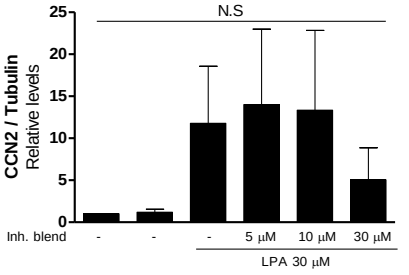

N.S
CCN2 / Tubulin
Relative levels
Inh. blend - - - 5 μM 10 μM 30 μM
LPA 30 μM

Figure 23. AKT, ERK and JNK pathways inhibition partially prevents the CCN2 induction by LPA. CCN2 protein levels in FAPS treated with LPA 30 µM in presence of different concentration of A) Ly294002, AKT inhibitor, B) U0126, ERK inhibitor, C) JNK inhibitor VIII, an inhibitor of JNK and D) with a mixture of the three mentioned inhibitors. GAPDH was used as a loading control. Quantifications are shown right to representative immunoblot. Values correspond to the mean ± SEM. n=3-4. N.S, not significant by one-way ANOVA with Tukey's post-test. n=3.

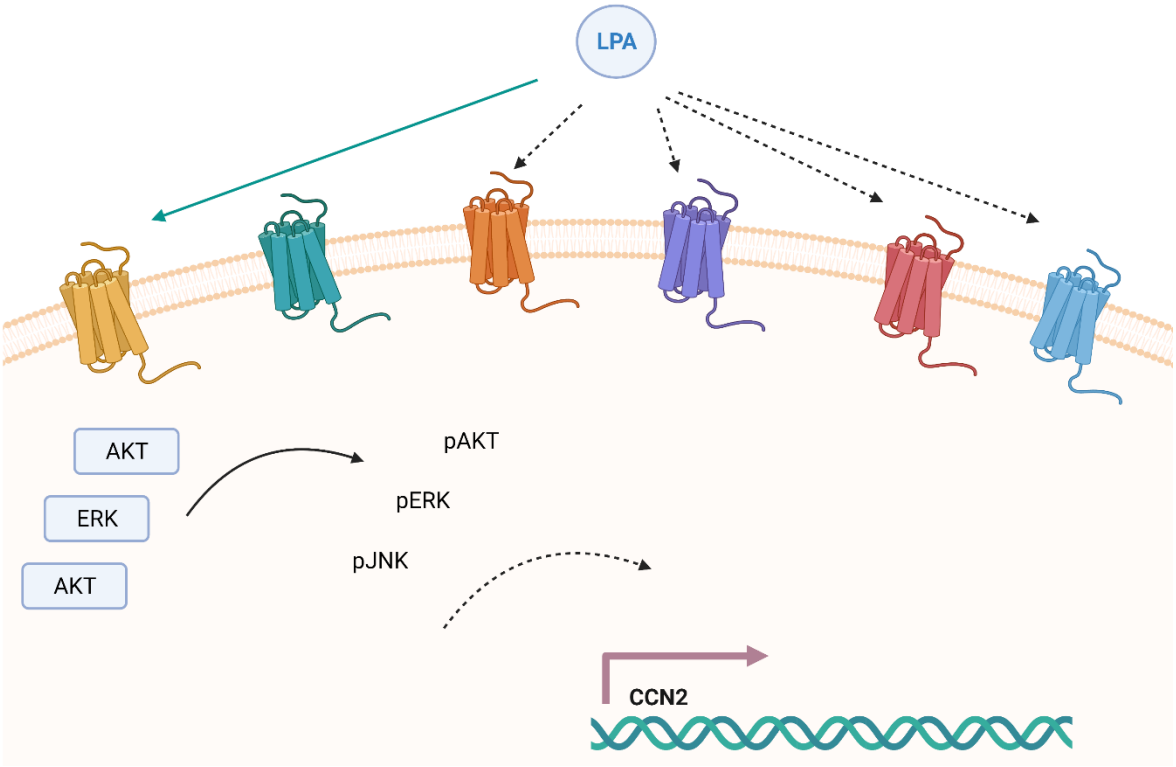


Diagram 8. Schematic view of LPA / LPARs axis participation in CCN2 induction in muscular FAPs. LPA signals through LPA_1 mediating the transcripcional induction of CCN2, but other LPARs could be involved. LPA triggers the AKT, ERK and JNK phosphorylation, possibly required for CCN2 induction. The diagram was created with BioRender.com.

3.7 Summary of results objective 3

1. Muscular FAPs express LPARs mRNA, being LPA_1 the most abundant.
2. *In vitro* FAPs respond strongly to LPA by inducing CCN2 compared to other representative cell types of skeletal muscle.
3. LPA and 2s-OMPT act through LPA_1, modulating the induction of CCN2 levels in FAPs.
4. LPA promotes early phosphorylation of AKT, ERK, and JNK, molecular pathways that could be partially required in CCN2 induction by LPA in FAPs.

These results are illustrated in diagram 8.

VII. DISCUSSION

1. Fibrosis: a key component in muscular dystrophies

DMD is the most frequent and severe of muscular dystrophies (Theadom et al., 2014)(Guiraud, Chen, et al., 2015); however, it currently has no cure. Many possible therapies have been explored for this disease, being the restoration of the dystrophin protein through cell therapy or viral vectors one of the most promising. However, the diagnosis of these patients is usually made when there is already fibrotic involvement in the muscle, which makes it difficult for therapeutic molecules to reach the target cells and hinders muscle regeneration. Due to this, it is essential to discover new targets that allow combating the development and maintenance of fibrosis as a complementary therapy to curative approaches, even more so when current treatments such as corticosteroids have detrimental multisystemic effects on patients like cushingoid features (Matthews et al., 2016). The efforts to find palliative but specific treatments are necessary. In this context, we decided to study the unexplored ATX / LPA / LPARs axis as a possible player in muscular fibrosis.

ATX / LPA / LPARs axis has been extensively studied in different biological contexts; however, until now, a few works have approached its study in skeletal muscle. Most of the scientific articles in the field of LPA in skeletal muscle address the effect that LPA can have on cell cultures of myoblast and myotubes, measuring different responses like Ca^{2+} increase, cell differentiation, cell proliferation, and induction of diverse protein factors (Xu et al., 2008)(Vial et al., 2008)(Cabello-Verrugio et al., 2011)(Riquelme et al., 2018)(Bernacchioni et al., 2018) but a few works directly study the effect of ATX / LPA / LPARs on skeletal muscle by

modulating its components (Ray et al., 2021). This thesis allowed us to elucidate that: i) the ATX / LPA / LPARs axis components are present in the skeletal muscle being LPA_1 and LPA_6 the most expressed LPARs in all analyzed muscle types (illustrated in diagram 5). ii) LPAR agonists can induce a fibrotic response in WT skeletal muscle mainly by LPA_1 signaling, (illustrated in diagram 6). iii) ATX / LPA / LPARs axis participates in denervation-induced fibrosis (illustrated in diagram 7). and iv) ATX / LPA / LPARs is relevant in the pro-fibrotic factor CCN2 induction in FAPs in response to LPA agonists (illustrated in diagram 8). **Our results suggest that the ATX / LPA / LPARs could participate in the development and maintenance of muscle fibrosis.**

2. ATX / LPA / LPARs axis is present in skeletal muscle

First, as it is shown in diagram 5, we find expression of LPARs in different skeletal muscles, in a similar pattern to the spinal cord (Santos-Nogueira et al., 2015) and lower esophageal sphincter (Feng et al., 2014) but a different pattern comparing to other tissues like the heart (Brown et al., 2017) and liver (Simo et al., 2014). However, these reports and our results studied the mRNA levels of LPARs, but the protein levels could show a different pattern of expression; moreover, the antibodies for LPARs used to be inespecific and unreliable, for that reason, we were able to determine only the protein levels of LPA_3 and LPA_6 on this thesis. Our results show that at mRNA level, LPA_1 and LPA_6 are the most expressed in the three analyzed muscles, suggesting a functional role in this tissue for at least these LPARs; confirming this idea, a recently published work demonstrates that LPA_1 is mediating skeletal muscle hypertrophy and regeneration (Ray et al., 2021). The other LPARs ($LPAR_3$-$LPAR_5$) are

expressed in lower levels, so we can hypothesize that its functional role in skeletal muscle could be less relevant for the whole tissue and expressed by a less abundant resident cell type or in low quantity.

Considering that our interest was to study the role that could play the ATX / LPA / LPARs in muscle fibrosis, we decided to explore if exist differential expression of components of this axis in fibrotic muscle in comparison to controls, as has been shown previously in pulmonary, dermal, and renal fibrotic models (Swaney et al., 2010)(Ledein et al., 2020)(Pradere et al., 2007). We studied the levels of mRNA LPARs in muscles after four days or 2 weeks of denervation as a model of muscular-induced fibrosis, we also analyzed LPARs on dystrophic muscles from Sgcd-KO and *mdx* mice, but we only found statistically significant differences in the LPARs RNA levels in the diaphragm of 9-10 months *mdx* mice, where the LPARs were upregulated. This could be explained because the *mdx* mice diaphragm is characterized by consolidated fibrosis, which could be a necessary condition to observe deregulation on LPARs, or maybe because other elements in the complexity of the pathology in older *mdx* muscle have an effect on the level of those receptors respect to other more "pure or incipient" fibrotic models such as the muscle denervation model or 2 months Sgcd-KO mice.

We found that ATX mRNA levels in muscle fibrosis models are lower than in control muscles; this could be due to high levels of LPA inducing a reduction in ATX transcripts as a compensatory effect (Benesch et al., 2015). On the other hand, the LPPs, a group of enzymes that remove phosphate groups from different lipids like LPA and S1P are also present in skeletal muscle. We concluded that ATX / LPA / LPARs axis is expressed in skeletal muscle and is

dysregulated in dystrophic muscle, but future research must directly explore the LPA levels in muscle in different fibrotic conditions.

3. Skeletal muscle fibrotic response to LPA

Although the study of fibrosis has been focused on protein factors, mainly due to the availability of tools for its research, over the last few years, the study of the role that lipids might play in the induction, maintenance, and modulation of fibrosis has been emphasized (Suryadevara et al., 2020). Phospholipids such as sphingosine-1-phosphate (S1P) have been linked to satellite cell cycle progression and myoblast differentiation (Fortier et al., 2013)(Squecco et al., 2006). Ieronimakis et al., showed that S1P modulates the skeletal muscle fibrosis in the *mdx* model since the augment of S1P levels improves the fibrotic phenotype indicating that S1P acts as an anti-fibrotic factor and suggesting that the S1P pathway could be part of an intrinsic anti-fibrotic mechanism present in the skeletal muscle (Ieronimakis et al., 2013).

As it is shown in a diagram 6, we were able to confirm that skeletal muscle responds to LPA by increasing known markers of fibrosis like CCN2, collagen, fibronectin, PDGFRα and FAPs number. Our results are in accordance with the published data (Vial et al., 2008)(Cabello-Verrugio et al., 2011)(Riquelme et al., 2018), showing that LPA induces CCN2 in muscular cells (C2C12 myoblast and myotubes). We observed that the inhibition of LPARs, specially LPA_1 and LPA_3, and the genetic absence of LPA_1 in a mouse deletion model avoids at least partially some of these responses. Our results using agonist and antagonist of LPARs and a genetic deletion model for LPA_1 allowed us to hypothesize that LPARs or at least LPA_1 could be part of the pathophysiology of fibrosis in diseases affecting skeletal muscle.

To explore this idea, we decided to use the denervation model for the induction of fibrosis for four main reasons: i) the synchronicity of the model. ii) the early induction of a fibrotic phenotype. iii) the possibility of intervening the ATX / LPA / LPARs axis using IP injections antagonists, and iv) the use of contralateral limb muscles as control of the experiment reduces inter-animal variability.

Denervation leads to many features in muscle like atrophy, inflammation (mainly cytokine expression) and fibrosis (Hanwei & Zhao, 2010)(Rebolledo et al., 2019), but the mechanisms behind these responses are controversial. It is known that the lost of the communication of nerves with the muscles leads to activation of catalytic pathways like the ubiquitin-proteasome pathway, associated with lower rates of protein synthesis (Glass, 2003) inducing atrophy. It is possible that some neuron-secreted factors from the nerve terminals in the neuromuscular junction are necessary to maintain the structure and size of the muscle, these factors would be lost after denervation. On the other hand, Tanaka et al, suggest that absence of stretc of muscle is responsible for fibrosis under denervation, since repetitive stretching of denervated muscle might suppress histological fibrosis (Tanaka et al., 2020). Intending to find new molecular pathways involved in the installation of fibrosis in denervated muscle and based on our results that show that LPA can induce a fibrotic response in WT muscle, we wondered if LPA and their corresponding LPARs are involved in denervation-induced fibrosis in skeletal muscle. We treated WT mice with the LPAR inhibitor, Ki16425, to inhibit LPA_1 and LPA_3, and performed denervation surgery. Our results indicate that LPA is involved in the fibrotic phenotype's instalation since the agonist approach reduced fibrotic markers induction. Furthermore, two weeks of denervated LPA_1-KO muscle indicated a strong inhibition in the fibrotic response compared to WT muscle. This concept is a remarkable preliminary antecedent

to studying the ATX / LPA / LPARs axis in animal models of MDs in the search for new therapeutic targets.

4. CCN2 induction by LPA in FAPs

CCN2 is a relevant factor in the induction and maintenance of skeletal muscle fibrosis (Morales et al., 2013)(Rebolledo et al., 2019)(Gonzalez et al., 2018). Finding new factors that strongly induce or regulate its expression is critical for skeletal MD research. FAPs are multipotent mesenchymal progenitors that can differentiate into myofibroblasts, adipocytes, chondrogenic, or osteogenic cells in different environments (Uezumi et al., 2010)(Uezumi et al., 2014)(Wosczyna et al., 2012)(Contreras et al., 2019). In this thesis, we show that FAPs expressed LPARs and respond to LPA by inducing the expression of the pro-fibrotic cytokine CCN2. Also, we demonstrate that FAPs are great responders to LPA compared to the response of myoblasts, myotubes and fibroblasts of the 10T1/2 line. This result, associated with the fact that LPA induces a FAPs increase in skeletal muscle (Fig. 10), led us to hypothesize that FAPs are possibly the primary cell that responds to LPA by inducing a pro-fibrotic phenotype in skeletal muscle. It would be interesting to determine the levels of LPARs between cell types in skeletal muscle to correlate the LPAR levels and the LPA response among different cells.

Our results show that LPA_1 is the main LPAR mediating CCN2 induction in FAPs. However, the pharmacological inhibition of LPA_6 also prevents the CCN2 induction by LPA (data non shown), confirming that other LPARs are required for CCN2 increase, as is suggested in the experiment shown in Fig. 18.

The treatment of FAPs with LPA and 2s-OMPT led to early AKT, ERK, and JNK activation. To determine if these pathways are required in the LPA to mediate CCN2 induction, we used

pharmacological inhibitors of AKT, ERK, and JNK. Still, high concentrations seem to attenuate LPA-mediated CCN2 expression. The results are agreed with previous work in C2C12 myoblasts, where CCN2 induced by LPA is mediated by JNK phosphorylation (Cabello-Verrugio et al., 2011).

5. Perspectives: ATX / LPA / LPARs axis as a possible target in muscular dystrophies

Why is it valid to study the ATX / LPA / LPARs in MDs? What cell type could be the source of LPA in dystrophic muscle? Different ideas could support the hypothesis that LPA could be participating in muscular dystrophies. First, some pathologies belonging to this group of diseases are characterized by the fragility of the sarcolemma caused by different genetic defects, leading to myonecrosis and subsequent lipid alterations. The *mdx* model show changes in the phospholipid composition of different muscles, including the levels of phosphatidylcholine (Valentine et al., 2022), which is a substrate of phospholipase D (PLD) to synthesize phosphatidic acid (PA), and this molecule could be finally processed by phospholipase 1A or 2A (PLA1A or PLA2A) to generate LPA (Yung et al., 2014). This suggest that LPA could be increased in dystrophic muscles because of metabolic alterations. This hypothesis must be tested by directly determining LPA levels and their variants using mass spectrometry. Another possible approach is to blunt some enzymes like PLD and PLA1A / PLA2A in MDs animal models. Secondly, another well-known source of LPA are platelets. These pieces of megakaryocytes contain ATX in their α-granules (Leblanc et al., 2014), which are maintained inside the platelets until their activation under damage, causing the exocytosis of the granules to contribute to blood clots generation (Flaumenhaft & Sharda, 2018). This idea

could be controversial with some studies that indicate that DMD patients present impaired platelet granule secretion (Schorling et al., 2021), but it still could be tested in future works. A third possible source of LPA in muscular dystrophies are adipocytes, the main cell type source of ATX and responsible for LPA circulating levels (Fotopoulou et al., 2010). As we mentioned previously DMD, shows high levels of adipogenesis in skeletal muscle, and, it has been reported that adipocytes are involved in the establishment of a fibrotic phenotype, through the proliferation and differentiation of colonic fibroblasts through ATX / LPA axis (Huang et al., 2022), this is interesting because FAPs could differentiate to adipocytes and myofibroblasts, so we can hypothesize that adipocytes secretes LPA inducing FAPs differentiation to myofibroblasts, however, that hypothesis must be tested. Finally, MDs are considered a multisystemic disease for some investigators since the mutations are present in all the cells of the patients, so the phenomena or damage in an organ could affect other tissues, triggering inter-organ crosstalk. Under this point of view, we can hypothesize that LPA in MDs could come from other remote tissues distinct from skeletal muscle in the context of muscular dystrophies.

Considering this scenario and from the results generated in this thesis, future experiments must be performed to explore the role and relevance of the ATX / LPA / LPARs axis in animal models of muscular dystrophies.

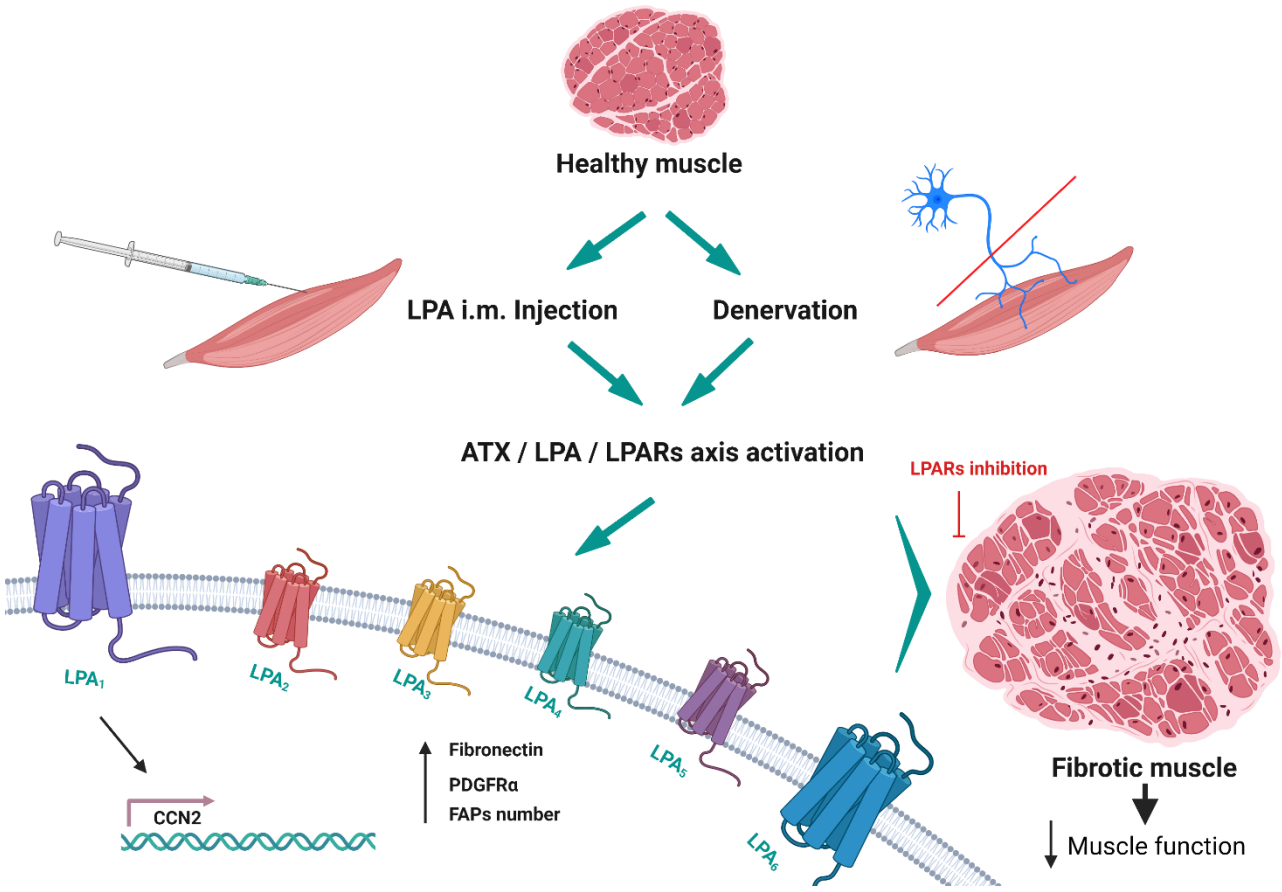


Diagram 9. Role of the ATX / LPA / LPARs axis in skeletal muscle fibrosis. We report that skeletal muscle responds to LPA by inducing the expression of canonical proteins of the fibrotic response, such as CCN2, fibronectin, and PDGFRα. Interestingly, we find that LPA triggers an increase in the number of FAPs. Most of these responses are mediated by LPA_1. We also determined that LPA_1 is participating in denervation-induced fibrosis. These results suggest that LPARs or at least LPA_1 could be part of the fibrosis pathophysiology in some diseases affecting skeletal muscle like muscular dystrophies.

VIII. CONCLUSIONS

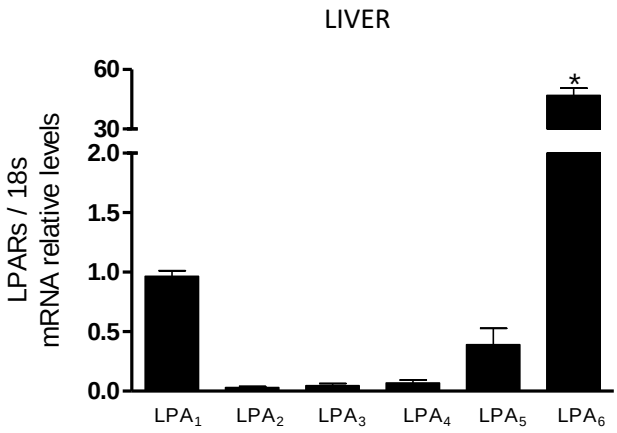
1. Skeletal muscle expresses mRNA of enzymes that participates in LPA synthesis and degradation.
2. There are LPARs mRNA in skeletal muscle from WT mice, LPA_1 and LPA_6 the more abundant.
3. LPARs expression is dysregulated in *mdx* fibrotic diaphragm.
4. Skeletal muscle responds to LPA by mounting a fibrotic response by inducing CCN2, fibronectin, collagen, and increasing FAPs number. The inhibition or absence of LPA_1 prevents most of it.
5. LPA participates in muscular fibrosis induced by denervation through LPA_1.
6. *In vitro* muscular FAPs express LPARs mRNA and respond strongly to LPA through LPA_1 by inducing CCN2 compared to other representative cell types of skeletal muscle.
7. LPA promotes early phosphorylation of AKT, ERK, and JNK, molecular pathways that could be partially required in CCN2 induction by LPA in FAPs.

These results are illustrated in diagram 9.

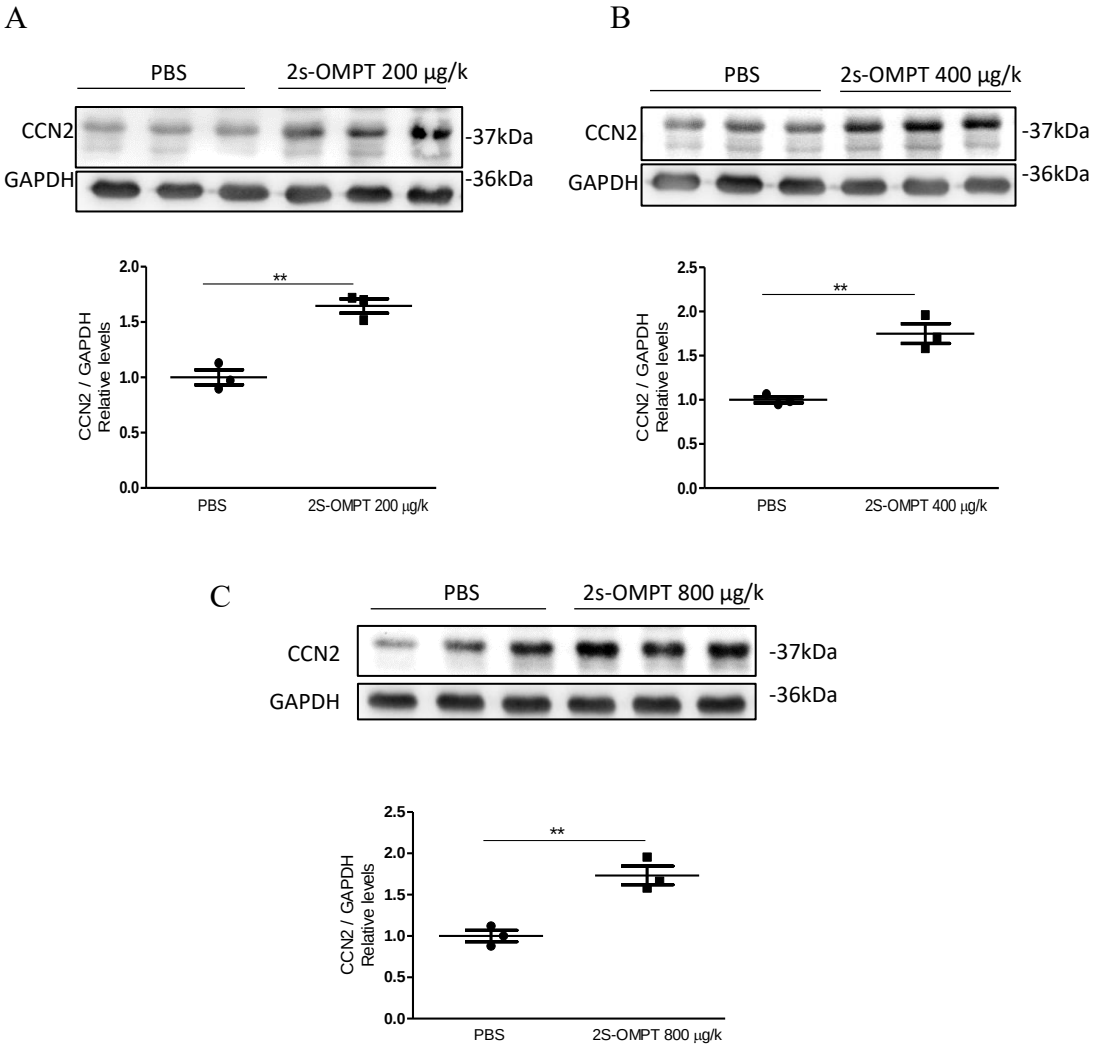
Part of the results of the three objectives have already been published in a paper atached to this thesis.

Córdova-Casanova A, Cruz-Soca M, Chun J, Casar JC, Brandan E. (2022). Activation of the ATX/LPA/LPARs axis induces a fibrotic response in skeletal muscle. *Matrix Biol*.109:121-139.

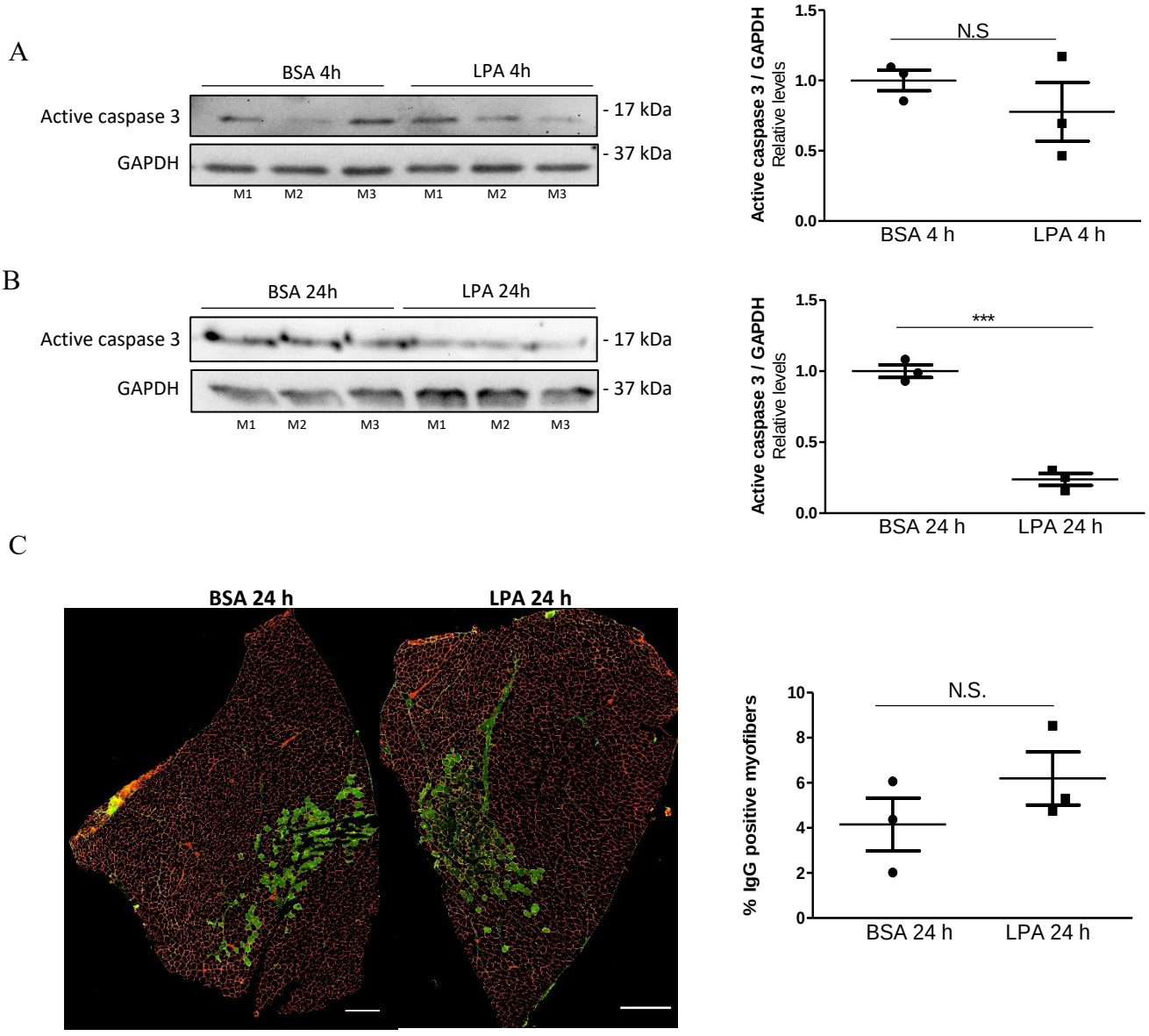
IX. SUPPLEMENTARY FIGURES



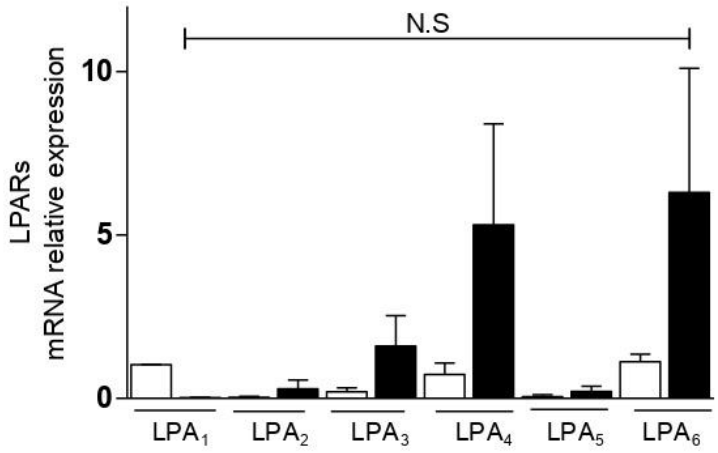
Supplementary figure 1. qPCR of LPAR subtypes in liver of C57BL6 mice. 18S was used as reference gene. *P<0.05 comparing to LPA_1 by one-way ANOVA with Tukey's post-test; n=3.



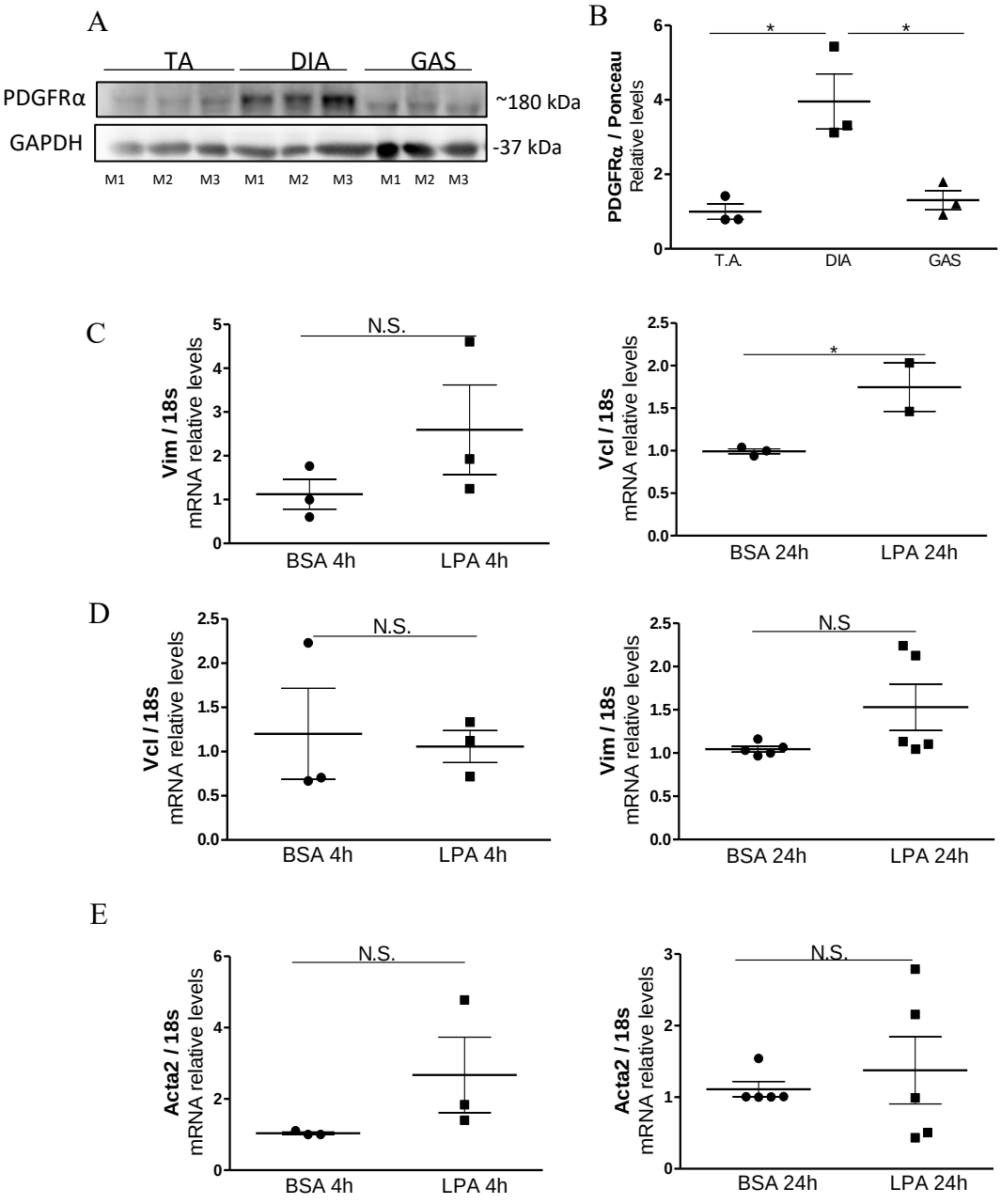
Supplementary figure 2. 2s-OMPT induces CCN2 expression in skeletal muscle. A, B, C) CCN2 and GAPDH protein levels and quantification of TA injected with 200, 400 and 800 µg/Kg of 2s-OMPT for 4 h. M refers to mouse. **P<0.01 by two-tailed Student's t-test. n=3.



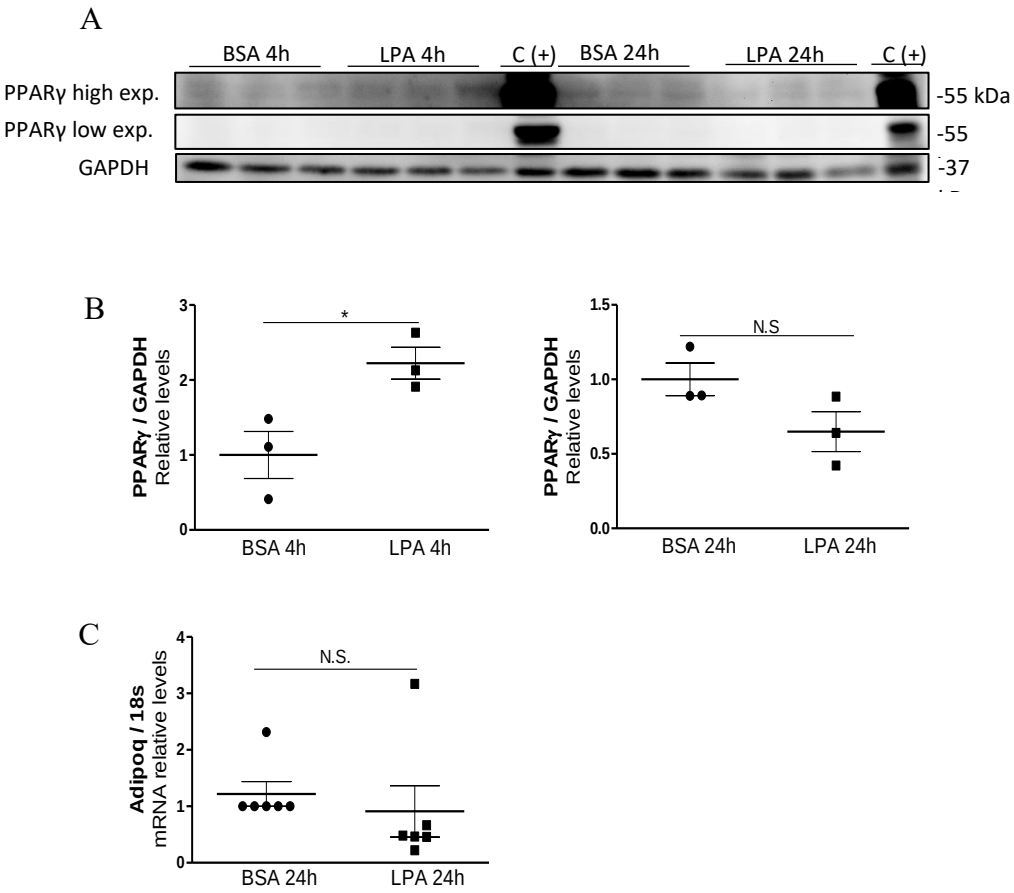
Supplementary figure 3. IM LPA injection does not induce cell death. A, B) Active caspase 3 and GAPDH protein levels obtained by immnunoblot and quantification. C) Left: representative IIF image, in red laminin, in green murin IgG. Right: quantification of % of IgG positive myofibers in TA injected with BSA or 400 µg/Kg of LPA for 24 h. (Result in collaboration with Meilyn Cruz) Scale bar 500 µm. M refers to mouse. N.S not significant, ***P<0.001 by two-tailed Student's t-test. n=3.



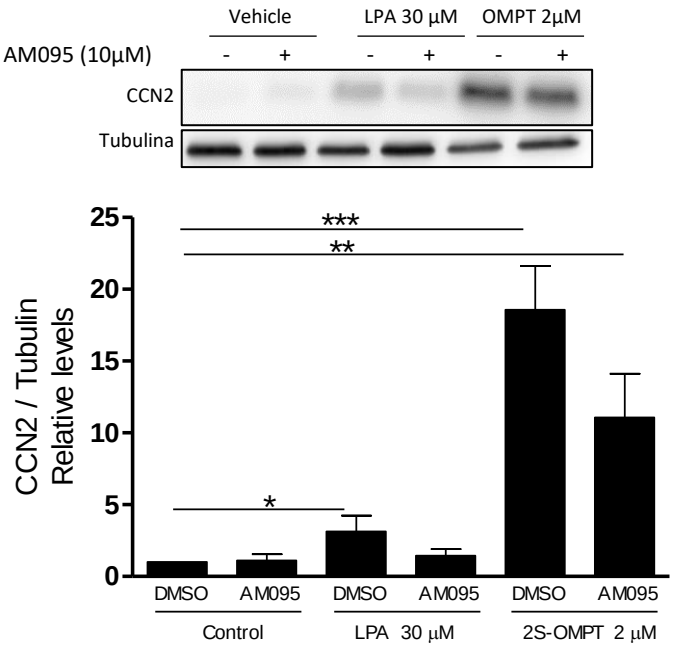
Supplementary figure 4. qPCR of LPAR subtypes in TA from WT (white bars) and LPA_1-KO mice (black bars). 18S was used as reference gene. N.S comparing all bars by one-way ANOVA with Tukey's post-test; n=3.



Supplementary figure 5. Effect of LPA in the myofibroblasts markers levels. A) PDGFRα protein levels were analyzed by immunoblot in TA, DIA and GAS from 6 months WT mice. GAPDH was used as the loading control. B) Quantification of PDGFRα protein expression. C) Vcl (Vinculin), Vim (Vimentin) and Acta2 (αSMA) mRNA were analyzed by RT-qPCR in TA. Treated with BSA or LPA. 18S was used as reference gene. *P<0.05, N.S not significant by one-way ANOVA with Tukey's post-test or two-tailed Student's t-test. N=3.



Supplementary figure 6. Effect of LPA in adipocytes markers levels. A) PPARγ protein levels were analyzed by immunoblot in LPA treated TA from WT mice. C (+), adipocyte cell culture control. GAPDH was used as the loading control. B) Quantification of PPARγ protein expression showed in A. C) Adipoq mRNA were analized by RT-qPCR in TA treated with BSA or LPA for 4 h. 18S was used as reference gene. *P<0.05, N.S not significant by two-tailed Student's t-test. n=3.



Supplementary figure 7. AM095 inhibit the LPA and 2s-OMPT response. Top, CCN2 protein levels analized by immunoblot blot in C57BL/6 WT FAPs trated with LPA 30 µM or 2s-OMPT 2 µM for 3 hours in presence or absence of AM095 10 µM. bottom, quantification of A. n=3. Values correspond to the mean ± SEM. *** P<0,001, **P<0,01, *P<0.05, N.S not significant by one-way ANOVA with Tukey's post-test.

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XI. ANNEX

The following annex includes the publications associates to this thesis

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REVIEW ARTICLE

The linkage between inflammation and fibrosis in muscular dystrophies: The axis autotaxin–lysophosphatidic acid as a new therapeutic target?

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Abstract

Muscular dystrophies (MDs) are a diverse group of severe disorders characterized by increased skeletal muscle feebleness. In many cases, respiratory and cardiac muscles are also compromised. Skeletal muscle inflammation and fibrosis are hallmarks of several skeletal muscle diseases, including MDs. Until now, several keys signaling pathways and factors that regulate inflammation and fibrosis have been identified. However, no curative treatments are available. Therefore, it is necessary to find new therapeutic targets to fight these diseases and improve muscle performance. Lysophosphatidic acid (LPA) is an active glycerophospholipid mainly synthesized by the secreted enzyme autotaxin (ATX), which activates six different G protein-coupled receptors named LPA_1 to LPA_6 (LPARs). In conjunction, they are part of the ATX/LPA/LPARs axis, involved in the inflammatory and fibrotic response in several organs-tissues. This review recapitulates the most relevant aspects of inflammation and fibrosis in MDs. It analyzes experimental evidence of the effects of the ATX/LPA/LPARs axis on inflammatory and fibrotic responses. Finally, we speculate about its potential role as a new therapeutic pharmacological target to treat these diseases.

Keywords Muscular dystrophies · Fibrosis · Inflammation · Lysophosphatidic acid · Autotaxin

Abbreviations

ATX	Autotaxin
CTGF or CCN2	Connective tissue growth factor
DMD	Duchenne muscular dystrophy
DGC	Dystrophin-associated glycoprotein complex
EC	Endothelial cells
ECM	Extracellular matrix
FAPs	Fibro/adipogenic progenitors
iNOS	Inducible nitric oxide synthetase
IFN	Interferon
IL	Interleukin
ICAM-1	Intracellular adhesion molecule 1
LPA	Lysophosphatidic acid
LPAR	Lysophosphatidic acid receptor
MP	Macrophages
MDs	Muscular dystrophies
RAS	Renin-angiotensin system
SC	Satellite cells
TGF-β	Transforming growth factor
TNF	Tumor necrosis factor

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Introduction

Under conditions of damage and healing, the skeletal muscle presents a formidable capability to regenerate. An acute inflammatory reaction and the deposition of extracellular matrix (ECM) occur as scaffolding responses to form new muscle fibers. Under chronic damage conditions, the muscle fibers are partially replaced by new ones, and persistent inflammation ensues. These events are followed by

overexpression of pro-fibrotic factors, transformation or activation of specialized resident ECM-producing cells, and exacerbated ECM accumulation. These characteristics define fibrosis, a hallmark of several pathological conditions such as skeletal muscle dystrophies, motor-neuron diseases, and models of denervation and myotrauma.

There have been several attempts to reduce inflammation and fibrosis in different animal models, which have been revised in excellent comprehensive reviews (Mahdy 2019; Mann et al. 2011; Smith and Barton 2018; Dort et al. 2019; Tidball 2011). Inflammation and fibrosis are common characteristics of several chronic diseases that develop in lung, liver, kidney, heart, and other organs (Wynn and Vannella 2016; Wynn 2011).

In the last few years, there has been increased interest in lysophosphatidic acid (LPA), an active glycerophospholipid signaling lipid; their signaling receptors (LPA_1 to LPA_6); and autotaxin (ATX), one of the enzymes responsible for its synthesis. This ATX/LPA/LPARs axis has been involved in inflammatory and fibrotic responses in several organs and tissues and as a result of these investigations, some molecules with pharmacological potential have been identified.

This paper reviews the characteristics of inflammation and fibrosis in skeletal muscle dystrophies and the role of the ATX/LPA/LPARs axis in these two responses. We also speculate about the therapeutic value of agonists/inhibitors of this axis for their potential use in muscular dystrophies.

Muscular dystrophies

Muscular dystrophies (MDs) are a heterogeneous group of inherited skeletal muscle diseases primarily characterized by progressive muscle weakness and degeneration, leading to reduced lifespan of the affected individuals. The most common and severe is Duchenne muscular dystrophy (DMD), an X-linked recessive type of MD (Hoffman et al. 1987). DMD affects 1 in 3500 live-born males and causes severe loss of muscle strength and learning disabilities, potentially leading to death due to respiratory or cardiac failure. The common characteristics of MDs are persistent inflammation, fibrosis (excessive accumulation of ECM), and reduced tissue regeneration capability. Histologically, degenerating muscle fibers are replaced with fibrofatty tissue (Mann et al. 2011), affecting muscle architecture and functionality.

DMD is caused by mutations in the 2.5 Mb *DMD* (loci) gene, which encodes a 427 kDa membrane-associated cytoplasmic protein called dystrophin that is essential for skeletal muscle cell-ECM linkage. For this reason, dystrophin is constantly expressed by differentiated myotubes and myofibers. Surprisingly, the skeletal muscle stem cells responsible for muscle regeneration also express dystrophin, which has a distinct role in this cell type (discussed below) (Dumont et al. 2015). Some mutations in the *DMD* sequence result in reading-frame shifting, causing the translation of a truncated protein and, consequently, loss of expression. However, many DMD mutations correlate with the severity of disease onset (Aartsma-Rus et al. 2006). Indeed, Becker MD, the DMD-linked mild-form of MD, is characterized by a mutation in the *DMD* gene which, unlike DMD, the skeletal muscle is able to express a truncated form of dystrophin.

Dystrophin is a member of the dystrophin-associated glycoprotein complex (DGC), formed by proteins of the sarcoglycan (α-, β-, γ- and δ-SG) and dystroglycan (α- and β-DG) complexes (Kanagawa and Toda 2006). In normal skeletal muscle, dystrophin mediates the attachment of the cytoskeleton of the muscle fibers to the endomysial basement membrane, the muscle connective tissue composed by ECM proteins such as laminin-2, perlecan and collagen IV. The direct interaction with the ECM, primarily with laminin-2, is mediated by members of the DG complex, where the extracellularly localized α-DG anchors the sarcolemma to the basement membrane through interaction with β-DG. This protein associates with dystrophin, which is anchored to the F-actin filaments of the cytoskeleton, thus creating the mechanical link. SG proteins also play a crucial role in stabilizing these interactions, mainly through SG-DG interaction. Therefore, the loss of SG proteins leads to sarcoglycanopathies (Blain and Straub 2011), other forms of autosomal recessive MDs, such as Limb-girdle MD, which cause disruption of the DGC. Skeletal muscles lacking dystrophin are more fragile and weaker than healthy ones, and they undergo contraction-induced skeletal fiber death (necrosis), which is the main pathophysiological DMD mechanism.

Muscle regeneration in muscular dystrophies

In normal skeletal muscle repair, quiescent muscle stem cells residing between the basement membrane and the sarcolemma, formally called satellite cells (SC; $Pax7^+$), are activated to regenerate and repair the damaged tissue. These stem cells proliferate upon activation, and some daughter cells are further committed into a myogenic differentiation program primarily controlled by transcription factors of the Muscle regulatory factors (MRF) family such as MyoD, myogenin and others. The newly formed myocytes fuse with preexisting damaged myotubes to facilitate growth and regeneration, with central nuclei as their histological hallmark (Bentzinger et al. 2013).

SCs are typically defined as stem cells that give rise to skeletal muscle cells. However, it is widely accepted that they have the potential to acquire different fates. For instance, SCs can achieve osteogenic, adipogenic, and fibromesenchymal differentiation depending on extrinsic signals

such as soluble factors and the presence of immune cells (Pessina et al. 2015; Madaro et al. 2019). Surprisingly, dystrophin can also regulate SC differentiation. Essentially, activated stem cells undergo asymmetric division to give rise to one self-renewal pool that maintains the stem cell population and another committed pool that further differentiates. Dystrophin is expressed in SCs and regulates polarity-dependent asymmetric divisions. The absence of dystrophin in SCs impairs these mechanisms, leading to a reduced number of asymmetric divisions and committed cell number, resulting in a reduced number of myogenin^{+} cells (Dumont et al. 2015).

In inherited MDs, the newly differentiated muscle cells lack components of the DGC that are later participants of the characteristic continuous degenerative/regenerative cycle. This phenomenon leads to exhaustion in the regenerative capacity of SC (Jejurikar and Kuzon 2003; Ribeiro et al. 2019), which, with the development of fibrosis, produces the muscle's degeneration.

Although fibrosis is detrimental for parenchyma function in all tissues, the controlled-acute deposition of ECM coupled with an inflammatory response is critical for proper skeletal muscle repair. However, dystrophic muscles illustrate the opposite side of the dysregulated process.

Inflammation in muscular dystrophies

Inflammation is a necessary response for the removal of damaged tissue and pathogens. Since skeletal muscle from MDs is continuously prone to degeneration, there is a persistent inflammatory response leading by immune cells and resident skeletal muscle cells (Tidball et al. 2018). Inherited MDs have no cure and glucocorticoids are nowadays the standard accepted treatment because they improve muscle strength (Shieh 2018). However, these drugs can have several adverse effects. Evidence of inflammation in DMD comes from pharmacological research using prednisolone-immunosuppressed *mdx* mice, which found reduced infiltrating inflammatory cell numbers and cell adhesion molecules in *mdx* muscles (Wehling-Henricks et al. 2004; Angelini 2007), showing that inflammation is an essential process in MDs.

Inflammation can be acute or chronic depending on its duration, types of cells implicated, and the response's resolution. Due to the constant regeneration/degeneration cycles in MDs, the response is chronic and accompanied by periodic activation of acute inflammation (Tidball et al. 2018). The main cell types that infiltrate *mdx* skeletal muscles are macrophages (MP) and lymphocytes, typical cells in chronic inflammation (McDouall et al. 1990; Porter et al. 2002). However, there are other immune cells that can participate in the pathology, such as neutrophils and eosinophils.

Relevancy of macrophages in acute and dystrophic inflammation

MPs are the most prominent inflammatory cells in *mdx* mice (Tidball et al. 2018; Wehling-Henricks et al. 2004). These cells are derived from circulating monocytes that differentiate into MPs when reaching the target tissue. These cells can be subclassified into two different phenotypes; classically activated M1-MP and alternatively activated M2-MP, corresponding to pro-inflammatory and anti-inflammatory MPs, respectively (Gordon 2003). In response to acute injury, the first cells to infiltrate the muscle are mostly leukocytes belonging to the myeloid lineage, mainly neutrophils and monocytes/MP. Pro-inflammatory monocytes (Ly6C^{high}) are recruited to the injured muscle and differentiate towards a pro-phagocytic inflammatory MP (M1 phenotype) to clean the damaged tissue by removing cellular debris. These cells then shift towards an anti-inflammatory phenotype (M2 phenotype) required for proper tissue regeneration (Arnold et al. 2007). However, in chronic injury, like in MDs, inflammatory cells persist in the tissue modifying the well-orchestrated microenvironment for muscle regeneration.

As mentioned before, M1-MPs, positive for CD68, are present in the initial/acute inflammatory response. They can be activated by inflammatory signals such as lipopolysaccharides from bacteria, and interferon-γ (IFN-γ) secreted from Th1 T cells and others. M1-MPs express inducible nitric oxide synthetase (iNOS). This enzyme uses arginine to produce citrulline and nitric oxide, triggering muscle fiber damage through nitric oxide-dependent cytotoxicity. As expected, iNOS null *mdx* mice show reduction in muscle fiber damage compared with *mdx* mice (Villalta et al. 2009). On the other hand, M2-MPs, positive for CD206 and CD163, and activated by IL-10 and IL-4, are implicated in myoblast proliferation, and anti-inflammatory and fibrotic responses (Wynn and Vannella 2016; Villalta et al. 2011a). M2-MPs secrete IL-10 and TGF-β (Hsieh et al. 2018). They also express arginase I, which uses arginine, the same substrate of iNOS, to produce proline, which is required for collagen synthesis (Wynn 2004). The metabolism of arginine in M2-MP promotes cardiac and muscle fibrosis in *mdx* mice (Wehling-Henricks et al. 2010), suggesting that the M1-MP is an essential cell type that mediates the acute inflammatory response, whereas the M2-MP phenotype contributes to fibrosis development.

It is hypothesized that skeletal muscle cells can also promote M1-MP activation by signaling toll-like receptors (TLR) and cytokines' release. TLR are membrane or cytosolic pattern recognition receptors activated by different molecules such as damage-associated molecular patterns including the high mobility group box 1 protein (HMGB1), a nuclear-resident protein released in response to cell damage. HMGB1 binds TLR4 on the membrane surface and activates

a signaling pathway that promotes the release of pro-inflammatory cytokines (Klune et al. 2008). Skeletal muscle from *mdx* mice expresses a wide range of TLR, including TLR4 (Henriques-Pons et al. 2014). HMGB1 is increased in *mdx* mice muscles, and TLR4-deficient *mdx* mice show reduced inflammation and skeletal muscle dystrophy, probably due to a shift of the MP phenotype towards an anti-inflammatory M2-biased MP (Giordano et al. 2015). The canonical adaptor Myeloid differentiation primary response 88 (MyD88) transduces the signal of the HMBG1/TLR pathway. In accordance with the role of TLR4, *mdx* mice show increased MyD88 expression. Moreover, satellite cell-specific deletion of MyD88 in *mdx* mice disrupts myogenesis, associated with the aggravation of the fibrotic phenotype and increased mRNA expression of CD206 and CD163, markers of M2 MP (Gallot et al. 2018).

As discussed above, different signals from distinct cells can regulate the MP phenotype and eventually contribute to muscle regeneration defects. However, increased abundance of MPs in *mdx* is still necessary. Madaro et al. showed that macrophage depletion worsens the dystrophic phenotype of *mdx* mice, increasing fibrosis and adipogenesis (Madaro et al. 2019). Mechanistically, MP depletion leads to a change in SC fate, developing a novel phenotype with adipogenic potential. These cells can form adipocytes in a dystrophic MP-depleted muscle context, reinforcing that SC fate can be regulated by its niche where MPs play a pivotal role (Madaro et al. 2019). However, MP depletion also increases the population of FAPs and neutrophils, which can regulate fibrosis and myogenesis (Arecco et al. 2016; Uezumi et al. 2014a).

Granulocytes degenerate the tissue in muscular dystrophies

Neutrophils are constituents of the granulocyte family of leukocytes that also includes basophils and eosinophils. Neutrophils are sensitive inflammatory cells that express myeloperoxidase (MPO), a cytolytic enzyme responsible for muscle cells damage in the early stages of DMD (Nguyen et al. 2005). Moreover, these cells can also interrupt skeletal muscle regeneration by disrupting myoblast myogenesis through the production of elastase, a serine protease augmented in *mdx* mice muscles (Arecco et al. 2016).

Eosinophils, another cell type that belongs to the granulocyte family, are incremented in muscles of *mdx* mice and DMD patients (Cai et al. 2000; Wehling-Henricks et al. 2008). There are strong indications that these cells can promote fibrosis. During acute injury, eosinophils (Siglec F^+ $CD11b^+$), which are an important source of IL-4 essential for muscle regeneration, are recruited to the muscle. IL-4 promotes the proliferation of FAPs. Interestingly, eosinophil-depleted mice show decrease proliferation of FAPs after muscle injury. A similar outcome is observed in IL-4/IL-13 null mice (Heredia et al. 2013). Whether IL-4-rich eosinophils are important participants in the development of fibrosis in chronic injury by increasing the rate of FAPs is still unknown. Moreover, IL-4 is a potent inducer of MP's M2 phenotype (Villalta et al. 2009). Therefore, eosinophil-secreted IL-4 could be an unexpected signal for MP polarization. Supporting this role albeit probably through a different mechanism, muscles from *mdx* mice that do not express the major basic protein 1 (MBP-1), a constituent of the cytotoxic granules of eosinophils, show lower hydroxyproline concentrations and weaker immunostaining for collagen I, III, and IV in muscle sections from heart, limbs, and diaphragm (Wehling-Henricks et al. 2008).

T lymphocytes are acquired immunity-specialized cells that mediate cellular immunity. The number of T cells is increased in DMD muscle biopsies with a predominance of $CD4^+$ over $CD8^+$ T cells (McDouall et al. 1990). These cells are critical in MDs since antibody-dependent depletion of both $CD4^+$ and $CD8^+$ T cells improves muscle histopathology in *mdx* mice (Spencer et al. 2001). In agreement with the mechanism of $CD8^+$-mediated cell lysis, *mdx* mice lacking perforin, a membrane pore-forming protein, show fewer apoptotic myonuclei and MP invaded-muscle fibers than *mdx* mice (Spencer et al. 1997). Moreover, immunodeficient *mdx* mice that lack the thymus (an essential organ for T cell maturation) have less collagen content in skeletal muscles compared with *mdx* mice (Morrison et al. 2000), confirming critical participation of both T cells subpopulations in MDs. Recently, regulatory T cells (T regs; $Foxp3^+$), a specialized $CD4^+$ lymphocyte, have been described as critical regulators of muscle regeneration. These cells accumulate in the muscle upon acute or chronic injury (*mdx* mice) and controls MPs phenotype dynamics through the regulation of cytokine production (discussed below in the cytokine section) (Villalta et al. 2014; Panduro et al. 2018). Importantly, depletion of T regs during acute injury increases inflammation and fibrosis. Moreover, T regs depletion in *mdx* worsens inflammation and myofiber damage, suggesting that these cells can be a valuable immune cell population for therapeutic approaches.

The infiltration of the skeletal muscle by several immune cells due to damage is a well-orchestrated process. The correct influx and efflux of myeloid and lymphoid cells into the muscle after an injury is essential for the proper establishment of a pro-regenerative microenvironment in the tissue. Nevertheless, persistent inflammation due to chronic maintenance of immune cells with pro-fibrotic, pro-myonecrotic, or anti-regenerative capabilities is very deleterious to the muscle architecture. Pharmacological and genetic modification of immune cells revealed their critical participation in MDs and in the establishment of fibrosis. Thus, efforts to find molecules with immune modulatory properties are required. Nevertheless, extracellular inflammatory

molecules (cytokines and chemokines) add another spatio-temporal regulation of inflammation in the skeletal muscle and are significantly involved in MDs.

Cytokines in muscular dystrophies

Interleukin-1β is the main pro-inflammatory cytokine involved in the acute response acting locally or systemically. IL-1β is increased in the serum on *mdx* mice (Mancio et al. 2017) and colocalizes with MPs in the diaphragm (Hnia et al. 2008), suggesting that this cell is an important source of this cytokine in MDs. In relation to myogenesis, IL-1β-stimulated myogenic cells isolated from non-dystrophic mice showed delayed differentiation. However, IL-1β was able to completely suppress myogenesis in myogenic cells isolated from dystrophic mice through the induction of Jagged1 expression, a Notch signaling pathway ligand (Nagata et al. 2017). This result suggests that a pro-inflammatory context commanded by IL-1β could be a critical factor in the loss of regenerative capacity of DMD muscles. It has been suggested that IL-1β can also indirectly regulate the fate of FAPs through MPs. The adipogenic differentiation of FAPs could be reduced by factors released by IL-1β-polarized MPs (Moratal et al. 2018).

The tumor necrosis factor family of cytokines are mediators of acute inflammation, acting on EC and neutrophils promoting its activation. In many other cell types, TNF induces apoptosis through TNF receptor 1 (TNFR1) death domain signaling. The most studied member of the TNF family, TNF-α, is extensively involved in skeletal muscle inflammation, and is expressed in myocytes, EC and leukocytes, among other cell types (Collins and Grounds 2001; Peterson et al. 1985). MP-secreted TNF-α is greatly relevant to control fibrosis establishment due to prevent FAPs accumulation by inducing its apoptosis (Lemos et al. 2015). Skeletal muscles from DMD patients have increased number of TNF-α-expressing myofibers that correlates with regenerating fibers (Tews and Goebel 1996; Kuru et al. 2003), supporting a role for this cytokine on muscle fiber inflammation. Moreover, the expression of TNF-α mRNA also increases in other muscle fibrosis models such as denervation and barium chloride-induced chronic damage (Contreras et al. 2016). Consistently, the pharmacological inhibition of TNF-α binding to its receptor in *mdx* mice decreases the percentage of necrotic muscle fiber (Hodgetts et al. 2006), suggesting TNF-α as a key myonecrotic factor.

Interleukin-6 is another pro-inflammatory cytokine released by the phagocytic system (MPs, dendritic cells) and other cells whose function overlaps with TNFα and IL-1β in inflammatory responses. In healthy skeletal muscle, IL-6 mRNA is barely detectable, whereas upon injury it is over-expressed, especially in myoblasts (Kurek et al. 1996). This high expression declines to similar levels as those found in the uninjured control muscles after one week. Therefore, IL-6 is implicated in the inflammatory response during skeletal muscle regeneration. Indeed, IL-6 functions as a pro-myogenic factor that regulates the proliferation of SCs and myoblasts (Okazaki et al. 1996; Serrano et al. 2008; Al-Shanti et al. 2008). The expression levels of muscle IL-6 are increased in DMD patients and correlate positively with age (Messina et al. 2011), suggesting a chronic role for this cytokine associated with the degenerative stage. Contrary to DMD patients, increased IL-6 levels in *mdx* mice decay around 24 weeks of age. IL-6 overexpression in the *mdx* mice exacerbates the dystrophic phenotype by increasing necrotic and regenerative fibers, as well as increasing the expression of inflammatory mediators such as TNFα and NF-κB associated with impaired muscle function (Pelosi et al. 2015). These effects are consistent with IL-6 sustaining the continuous cycle of degeneration/regeneration that governs MD.

Contrary to the classical view as a pro-inflammatory cytokine, IL-6 function in MDs is controversial, and it has also been postulated as an anti-inflammatory cytokine. Treatment of *mdx* mice with a monoclonal antibody against the IL-6 receptor, which blocks locally and systematically IL-6 signaling, enhances the number of infiltrated mononuclear cells and increases the expression of the intracellular adhesion molecule 1 (ICAM-1) in the gastrocnemius muscle. ICAM-1 expressed by EC is essential for leukocyte transmigration (Kostek et al. 2012). Nevertheless, the blockage of the IL-6 receptor does not improve muscle function and regeneration, probably due to the lack of IL-6-dependent cellular regulatory mechanisms, such as myogenesis, during regeneration (Okazaki et al. 1996; Serrano et al. 2008; Al-Shanti et al. 2008). In DMD, the depletion of SCs and the increased number of FAPs is the typical scenario. Overexpression of IL-6 in *mdx* mice causes increased activation of SCs, but of at the same time reduces the pool of SCs and increases the number of FAPs (Pelosi et al. 2015).

Interleukin-10 is a homodimer that exerts one of the most crucial anti-inflammatory activities. IL-10 is mainly released by Th2 cells and M2-MPs and promotes the deactivation of M1-MP, inhibiting the secretion of some pro-inflammatory cytokines such as TNFα and IL-12 (Howes et al. 2014). Accordingly, IL-10 induces activation of M2-MPs, while IL-10 mutant mice experience reduced regeneration capacity associated with increased number of necrotic fibers (Deng et al. 2012), confirming the importance of IL-10 in promoting skeletal muscle regeneration by regulating the MP subtype population. This mechanism is supported by previous reports showing that monocytes/MP recruited to muscles upon injury, which express M2-MPs-like cytokine profiles (IL-10 and TGFB1) and stimulated with M2-MPs-inducers cytokines (IL4 and IL-10), can promote SC differentiation (Arnold et al. 2007). In the context of MDs, IL-10 transcript

levels in *mdx* mice differ among different muscles. At four weeks of age IL-10 mRNA is overexpressed in hamstring muscles, while later on (12 weeks) it is overexpressed in quadriceps muscle (Villalta et al. 2009, 2011a). MPs isolated from *mdx* muscles respond to IL-10, increasing Arginase I expression and decreasing iNOS expression, and reducing myotube damage in co-culture assays. Notably, IL-10 deficient *mdx* mice show exacerbated muscle fiber damage and muscle function impairment associated with a reduced M2-MPs population compared to *mdx* mice (Villalta et al. 2011a). Thus, IL-10 expression and signaling are critical for driving skeletal muscle dystrophy progression.

Interferons are a group of cytokines classified into two types. Type I interferons (IFN-α secreted by MP and IFN-β secreted by fibroblasts) regulate cellular responses associated with the anti-viral state and class I major histocompatibility complex (MHCI). On the other hand, type II IFN-γ, released from Th1 T cells, is implicated in adaptive immunity through the activation of M1-MP. This cytokine is induced in a time-dependent manner during acute skeletal muscle injury, reaching its highest levels around 5 days post-injury. Its highest levels correlate with increased abundance of MPs, T cells, natural killer cells, and myoblasts within the muscle since it is expressed by all these cell types (Cheng et al. 2008). Interestingly, $Foxp3^{+}CD4^{+}$ regulatory T cells (Treg) are critical regulators of IFN-γ/MP dynamics. Injured muscle in mice depleted of Tregs presents elevated numbers of IFN-γ-expressing cells and enhanced IFN-γ response by MPs, associated with a more pro-inflammatory phenotype (Panduro et al. 2018). Therefore, Treg is a novel immune cell that regulates MP phenotype and muscle physiology through IFN-γ production.

IFN-γ plays a pivotal role in regulating skeletal muscle regeneration and fibrosis. Strategies using blocking antibodies against the IFN-receptor in skeletal muscle and the IFN-γ null mice showed that the skeletal muscle shows decreased regeneration, and the presence of collagen deposits following cardiotoxin-induced damage. The skeletal muscle also exhibits fewer MPs, iNOS expression, and myoblast differentiation (Cheng et al. 2008).

How is IFN-γ associated with fibrosis? IFN-γ-mediated activation of the IFN-receptor triggers a signaling pathway that involves the Janus kinase 1/Signal transducer that regulates gene expression. The mechanism by which blocking IFN-γ expression/signaling alters muscle regeneration and collagen deposition could be attributable to the capacity of IFN-γ to act as an antagonist of TGF-β1 signaling by inhibiting the formation of the TGF-β receptor-Smad3 complex (Ulloa et al. 1999). IFN-γ inhibits muscle-derived fibroblast growth and TGF-β1 effects after muscle injury. Interestingly, in TGF-β1-overexpressing myoblasts that acquire a myofibroblast-like phenotype, IFN-γ inhibits the expression of α-SMA and vimentin (Foster et al. 2003). In laceration-induced muscle injury, there is increased collagen deposition that can be attenuated by the administration of soluble IFN-γ, which also improves muscle regeneration and function (Foster et al. 2003).

Regarding MD, *mdx* mice show increased IFN-γ mRNA levels compared to wild type mice, and IFN-γ stimulation of MPs from *mdx* mice shows enhanced iNOS expression (Villalta et al. 2009). Moreover, 12-week-old IFN-γ null *mdx* mice, show less muscle fiber damage and improved function compared to *mdx* mice of the same age. This is probably due to reduced number of MI-MP and increased number of M2-MPs, which would facilitate myogenesis (Villalta et al. 2011b). These effects were not seen at the 4-weeks-stage, showing that the expression of IFN-γ is detrimental in late, but not in early MD stages.

Chemokines in muscular dystrophies

Other soluble factors that mediate inflammation responses are chemokines. These low molecular weight proteins are cytokines with chemoattractant properties, which recruit different population of cells to the tissues where are synthesized and secreted. Chemokines are classified based on the position of cysteine residues in their amino-terminal end in C, C–C, C–X–C, and C–X–3C families (Baggiolini 2001). Several chemokines and chemokine receptors are transiently overexpressed after acute skeletal muscle injury, such as monocyte chemoattractant protein 1 (MCP-1) or C–C motif chemokine ligand 2 (CCL2), macrophage inflammatory protein (MIP)-1α, MIP-1β, and some G protein-coupled receptors such as C–C motif chemokine receptor 2 (CCR2) and CCR5 (Warren et al. 2005).

The relevance of CCR2, the receptor for CCL2, CLL7, and CCL8, has been extensively studied. CCR2 mRNA and protein expression are transiently upregulated in skeletal muscle after injury. The CCR2 protein co-localizes with MP and myogenic precursor cell markers (Warren et al. 2004, 2005). Consistent with this localization, C2C12 myoblasts express CCR2 and respond to its ligand CCL2, which induces cell proliferation through a CCR2/$G_{\alpha i}$/ERK1/2-dependent mechanism (Yahiaoui et al. 2008). Moreover, CCL2 administration into cardiotoxin-injured tibialis anterior muscle in mice transiently decreases myogenin expression, a transcription factor that governs myoblast differentiation, suggesting that CCL2 can also regulate myoblast behavior. CCR2 null mice show decreased myofiber size and increased fibroblast markers, fat and collagen. Increased muscle fat deposition after cardiotoxin injury was also observed in mice lacking CCL2 expression (Martinez et al. 2010). These tissue characteristics are associated with impaired clearance of macrophages and neutrophils that persist in the muscle 14 days after injury (Warren et al. 2005; Martinez

et al. 2010). Functionally, these muscles also show lower strength recovery rates 14 days after injury (Warren et al. 2004), suggesting that CCR2 expression and signaling is an essential regulator of skeletal muscle repair.

MDs display a persistent abundance of inflammatory cells. Gene expression analysis showed the overexpression of distinct chemokines and chemokine receptors in *mdx* mice, including CCL2, CCL6, CLL7, CCL8, CCL9, CCR1, CCR2, and CCR5 (Porter et al. 2002, 2003). CXCL-14, which belongs to the C–X–C chemokine family, and its receptor CXCR4 are also upregulated in *mdx* mice and are specifically involved in lymphocyte recruitment. However, CCL7 and CCL8 are not overexpressed at the protein level (Porter et al. 2003).

Recently, increased transcript levels of CCL2, CCL7, CCL8, CCL12, and CCR2 were found in the diaphragm and tibialis anterior muscles from *mdx* mice (Mojumdar et al. 2014). This study also showed that the MP population is attenuated in the muscle of 6-week-old CCR2-deficient *mdx* mice but not in 12-week-old *mdx* mice, suggesting that more than one mechanism regulates MP recruitment and persistence during late stages of the pathology. Moreover, there are differences between the phenotypes of early and late MP populations. Indeed, at 6 weeks of age, the increment in the M1-MPs population within the muscle of *mdx* mice is reversed in CCR2-deficient *mdx* mice to levels like those present in WT mice. This was also associated with normalization in the expression of CD206 by MPs in CCR2-deficient *mdx* mice, which was decreased in *mdx* mice (Mojumdar et al. 2014). Whether this mechanism also regulates the MP population in the regenerative stage (12-week-old mice) is still unknown.

Histopathological analysis showed that myonecrosis and fibrosis (measured as hydroxyproline content) improved to WT levels in CCR2-deficient *mdx* mice compared to *mdx* mice. In addition, the administration of an antibody targeting CCR2 (fusokine) to *mdx* mice causes a decrease in the number of macrophages and in collagen abundance. Fusokine also increases fiber size like what is seen if CCR2-deficient *mdx* mice (Mojumdar et al. 2014). These data show the critical role of CCR2 expression and signaling in dystrophic muscle and strongly suggest that the recruitment of MPs and phenotype dynamics are its main mechanisms.

In conclusion, cytokines and chemokines play pivotal roles in regulating immune cell functions associated with muscular dystrophies. Cytokines can also act on resident muscle cells regulating their pro-regenerative functions. Several cytokines and chemokines are modulators of fibrosis and together with immune cells are essential participants of skeletal muscle diseases.

Fibrosis development in muscular dystrophies

The nature of inflammation, commanded by the type of damage, affects how the skeletal muscle architecture is recovered. The establishment of chronic inflammation determine a distinct microenvironment within the tissue modifying cell–cell communications and signaling pathways primarily controlled in acute inflammation. Effects such as constantly cytokine and chemokine's release, impaired immune cells persistency, ECM-producing cells propagation, and myogenic cells differentiation defects often conclude in tissue degeneration and fibrosis, a typical result of chronic diseases like DMD.

Fibrosis is characterized by the accumulation of ECM components such as collagen and fibronectin due to the imbalance between synthesis and degradation, which generates a scar-like phenotype and progressive muscle strength loss.

The cell type mainly responsible for the synthesis and deposition of ECM in skeletal muscle are myofibroblasts. They are α-SMA positive and are characterized by the presence of contractile microfilament bundles in the cytoplasm (stress fibers) that give them contractile properties (Sandbo and Dulin 2011). The number of myofibroblasts is increased in muscles from DMD patients and in the *mdx* mice (DMD mouse model) (Contreras et al. 2016; Hori et al. 2011). These cells derive mainly from fibro/adipogenic progenitors (FAPs), PDGFRα positive cells, that are also increased in muscle from DMD patients and in fibrotic models such as muscle denervation (Contreras et al. 2016, 2019; Uezumi et al. 2014b).

Major signaling pathways that regulate fibrosis

The biomolecular mechanisms underlying fibrosis include the participation of many factors and pathways. The crosstalk among them at different levels has been described, adding new evidence for a deleterious vicious cycle that promote an uncontrolled fibrogenic program.

Renin-Angiotensin System (RAS), signals through its classical and non-classical axes. The classical axis, which includes Angiotensin II and its type-1 receptor (AT-1), seems to have a pro-fibrotic effect since it induces fibronectin and collagen-III expression in the muscle cell line C2C12 (Cabello-Verrugio et al. 2011a). The expression of these ECM proteins has also been observed in skeletal muscle from mice treated with Angiotensin II (Morales et al. 2014), while pharmacological inhibition of the Angiotensin II synthesizing enzyme (angiotensin-converting enzyme, ACE) and AT-1 by enalapril and losartan

respectively, reduces ECM accumulation and improves muscle strength in *mdx* mice (Morales et al. 2013a; Cohn et al. 2007). On the other hand, RAS's non-classical axis, composed of Angiotensin 1–7 and its receptor Mas, seems to play a protective role in the muscle of *mdx* mouse since treatment with Angiotensin 1–7 triggers a decrease in fibronectin and collagen levels and increases skeletal muscle strength (Acuña et al. 2014). These opposite effects between the classical and non-classical RAS pathways have been observed in other biological processes such as the regulation of vascular tone. Therefore, their opposite effects on fibrosis is not unexpected.

Transforming growth factor type β (TGF-β) is another relevant molecule in fibrosis. It is involved in regulating many cellular processes such as differentiation, apoptosis, and proliferation under physiological conditions and also in fibrotic tissue (Massague 2012; Biernacka et al. 2011; Kim et al. 2018). In DMD and *mdx* muscles, TGF-β is upregulated (Bernasconi et al. 1999; Ishitobi et al. 2000) and contributes to ECM deposition mainly through its canonical pathway, which involves phosphorylation of SMAD proteins (Ismaeel et al. 2019). The pro-fibrotic effect of TGF-β in *mdx* and muscle regenerating animals is prevented by the use of a monoclonal antibody against TGF-β (Cohn et al. 2007; Andreetta et al. 2006) and proteoglycans such as decorin and biglycan (Kolb et al. 2001; Casar et al. 2004). Moreover, increased expression of ECM proteins, such as type I collagen, has been observed in wild type tibialis anterior muscle injected with TGF-β1 (Li et al. 2004). These results confirm the extraordinary ability of TGF-β to induce ECM even in a non-fibrotic context. This pro-fibrotic effect of TGF-β can be explained at least partially by this factor's ability to promote the differentiation of FAPs into myofibroblast-like cells in vitro (Uezumi et al. 2010).

TGF-β promotes the expression of the matricellular protein Cellular communication network factor 2/connective tissue growth factor (CCN2/CTGF) in different cell types (Chen et al. 2000; Cheng et al. 2015). Interestingly, the effect of TGF-β on CCN2 induction is enhanced in myotubes in vitro and in vivo in skeletal muscle fibers by the HIF1α-mediated hypoxia signaling pathway (Valle-Tenney et al. 2020). These observations are essential for two reasons: (1) hypoxia has been related to fibrosis in different organs (Darby and Hewitson 2016) and (2) CCN2 is a remarkable ECM remodeling factor and is augmented in DMD.

CCN2 expression is increased in *mdx* mice and in denervated muscles (Morales et al. 2013b, 2018; Rebolledo et al. 2019). The experimental overexpression of CCN2 in wild-type muscle using a sequence-containing adenovirus causes increased expression of collagens, fibronectin, and α-SMA, suggesting an expansion of the myofibroblast population. Furthermore, the return of CCN2 to basal levels reverses the CCN2 triggered phenotype (Morales et al. 2011). Moreover, reduced expression of CCN2 in the *mdx* mouse triggers decreased expression of fibrotic markers and the formation of necrotic regenerative foci (Morales et al. 2013b, 2018). Moreover, treatment with a neutralizing monoclonal antibody against CCN2 (FG3019 or Pamrevlumab) produces a similar effect in fibrotic markers (Morales et al. 2013b). In agreement with these results, blocking CCN2 with Pamrevlumab can reverse the fibrosis triggered by overused-induced muscle injury in rats (Barbe et al. 2020). Taken together, these data strongly suggest that CCN2 is involved in DMD's pathophysiology and supports the ongoing clinical trial that is testing the use of anti-CCN2 antibodies in DMD patients (NCT02606136, NCT04371666).

Summarizing, the RAS, TGF-β and CCN2 pathways are involved in the onset, maintenance, and progression of muscular fibrosis. Discovering tools that modulate these pathways can open treatment possibilities for chronic diseases such as DMD.

The ATX/LPA/LPARs axis

Lysophosphatidic acid (LPA, 1-or 2-acyl-sn-glycerol 3-phosphate) is a small (430–480 Da) cytokine-like, membrane-derived, bioactive glycerophospholipid composed of a glycerol backbone, a single saturated or unsaturated fatty acid chain, and a phosphate group.

LPA has a half-life of approximately 3 min in circulation (Tomsig et al. 2009), depending on its synthesis and degradation rates. LPA is produced in the intra- and extracellular environments. Intracellular synthesis is mediated by phospholipases A1 and A2 using cell membrane phosphatidic acid as substrate (Aoki et al. 2008). It can also be generated by acylation of glycerol 3-phosphate, a reaction catalyzed by glycerophophate acyltransferase, and by phosphorylation of monoacylglycerol through a monoacylglycerol kinase (Pages et al. 2001). Extracellular LPA is synthesized mainly from lysophosphatidylcholine and lysophosphatidylserine by ATX, a lysophospholipase D secreted enzyme, encoded by the *Enpp2* gene. ATX is the main source of circulating LPA since heterozygotes *Enpp2* mutant mice have a 50% reduction in LPA plasma levels compared to wild-type mice (D'Souza et al. 2018). LPA has been found in several tissues and biological fluids (Yung et al. 2014), but adipose tissue has been proposed as the primary source of LPA in circulation (Ferry et al. 2003; Dusaulcy et al. 2011).

As a regulatory mechanism, LPA accumulation decreases ATX synthesis (Benesch et al. 2015). LPA can be degraded to monoacylglycerol by lipid-phosphate phosphatases (LPP 1–3). Therefore, LPP1-deficient mice shown high plasma LPA levels (Tomsig et al. 2009).

LPA signals through six different G-protein coupled receptors (GPCRs), called LPA_1 through LPA_6. All of

them are rhodopsin-like GPCRs with seven transmembrane helices. LPA_1 through LPA_3, belong to the family of endothelial differentiation genes (Edg), while the other more structurally distant receptors, LPA_4 through LPA_6, are associated with the purinergic family of receptors (Yung et al. 2014). LPA regulates many cellular processes through its receptors, triggering multiple downstream molecular pathways, such as Rho/Rock, PI3K/Akt and PLC/IP3 to mediate cytoskeleton remodeling, cell survival, intracellular calcium transients, differentiation, and other cellular processes under physiological and pathophysiological conditions (Hemmings and Brindley 2020; Valdes-Rives and Gonzalez-Arenas 2017). The axis is summarized in Fig. 1.

The relative expression of the LPA receptors varies in different tissues and organs, depending on their role. Although in skeletal muscle the expression and distribution of LPA receptors are not fully known, there is some evidence that supports their presence in this tissue, being LPA_1 the most highly expressed (Anliker and Chun 2004; Jean-Baptiste et al. 2005; Zderic and Hamilton 2012). However, the roles that LPA and its receptors are playing in the skeletal muscle under physiological and pathological conditions are still unknown. Despite that, LPA signaling has been well characterized in inflammation and fibrosis, and is an possible new attractive participant in muscle biology.

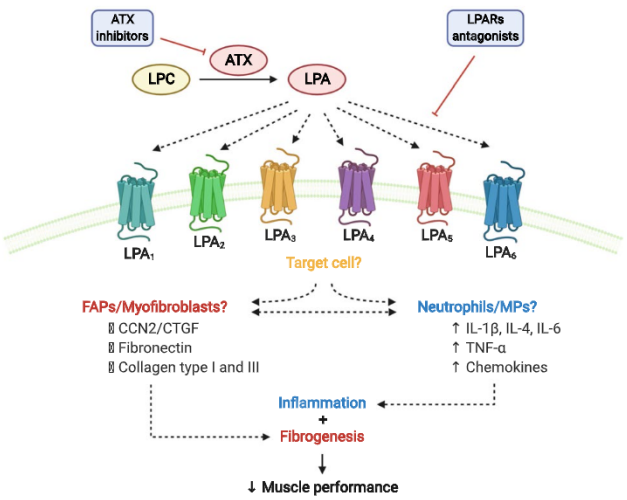

Fig. 1 Model for the role of ATX/LPA/LPARs axis in MDs. ATX synthesizes LPA from lysophosphatidylcholine (LPC). LPA activates G-protein-coupled receptors (LPA_{1-6}), triggering various cellular signaling in different cell types. LPA increases the expression of profibrotic factors such as CCN2, and ECM proteins such as fibronectin, type I and III collagen in FAPs and myofibroblast. In neutrophils and MPs, LPA signaling increases inflammatory cytokines such as IL-1β, IL-4, IL-6, TNF-α, and some chemokines. This condition may lead to the activation of inflammation and fibrotic responses, impairing muscle performance. Created with biorender.com

The LPA/LPARS/ATX axis in the inflammatory response

An increasing amount of evidence places LPA as a potent modulator of inflammation in different tissues such as lung, liver, and cancer-related-organs (reviewed in Valdes-Rives and Gonzalez-Arenas 2017; Gonzalez-Arenas et al. 2008). In the lungs, LPA induces neutrophil chemoattractant activity in epithelial cells, increasing neutrophil influx. Mechanistically, in response to LPA bronchial epithelial cells increase the expression of the IL-8 chemokine (neutrophil-specific C-X-C chemokine) through activity regulation of NF-κB and AP-1, classical transcription factors involved in cytokine regulation (Cummings et al. 2004; Saatian et al. 2006). This mechanism also requires G protein isoforms G_i and $G_{12/13,}$ and protein kinase C (PKC) isoform PKCδ. Inhibition of these transducers attenuates LPA-induced IL-8 expression and NF-κB activation (Cummings et al. 2004). However, more than one signaling pathway can converge in order to regulate cytokine expression. In this context, (Saatian et al. 2006) LPA could also regulate IL-8 secretion through two independent mechanisms involving p38/NF-κB and JNK/AP-1, where LPA_1 and LPA_3 are also required. This evidence supports the regulation of cytokine production by LPA through different molecular pathways (Fig. 1).

Non-resident immune cells are recruited from the bloodstream to the inflamed tissue through EC adhesion and communication. Using intravital microscopy, Kranig et al. (Kranig et al. 2019) showed that in *mdx* mice, there is augmented leukocyte extravasation and leukocyte expression of LFA-1 and Mac-1. These molecules are members of the β2-integrin family that interact with ICAM-1 in EC, a process necessary for leukocyte transmigration. Consistent with its role in inflammation, it is possible that LPA could regulate leukocyte recruitment since EC from human umbilical veins (HUVECs) increase ICAM-1 expression after LPA treatment. This response requires LPA_1 expression, which promotes EC-monocyte adhesion (Lee et al. 2004; Lin et al. 2007). *mdx* mice also show increased expression of cell adhesion molecules mRNAs such as endothelial VCAM-1 (Porter et al. 2002). Further studies on the modulation of adhesion molecules in skeletal muscles as a response to LPA are necessary.

As in bronchial epithelial cells, EC also responds to LPA, increasing the expression and secretion of IL-8 and MCP-1 through an LPA_1-, Gi-, Rho-, and NF-κB-dependent mechanism. Furthermore, LPA also induces the expression of IL-1β in these cells, mediated by LPA3. Notably, the induction of IL-8 and MCP-1 are dependent on the activation of IL-1R (Lin et al. 2006, 2007). Of note, the conditioned medium from these EC

cultures has chemoattractant activity, consistent with these chemokines' functions. Accordingly, the silencing of LPA_1 and LPA_3 inhibits LPA-induced chemoattractant activity. However, LPA_1 but not LPA_3 silencing impairs LPA-enhanced EC-monocyte adhesion, consistent with IL-8/MCP-1 and ICAM-1 expression pattern. These results show that LPA could be an essential element in promoting an inflammatory response by increasing chemokine and adhesion molecule expression and subsequent leukocyte recruitment.

LPA-regulated MCP-1 expression is not exclusive of EC. Human smooth muscle cells also increase MCP-1 expression in response to LPA in a Rac1- and ROS generation-dependent fashion (Kaneyuki et al. 2007). Moreover, IL-6, another critical cytokine involved in MDs is also regulated by LPA in smooth muscle cells. LPA increases IL-6 expression at the messenger and protein levels. This regulation also requires the LPA_1 receptor and a signaling pathway that involves the activation of the $G_{i/o}$ protein isoform, PKC, and p38 (Hao et al. 2010).

Regarding the skeletal muscle, MCP-1 expression increases in LPA-treated C2C12 myoblast. LPA is also involved in increasing myoblast proliferation (Tsukahara and Haniu 2012). These data suggest that LPA can mediate leukocyte recruitment by regulating chemokine secretion in different resident cells of the skeletal muscle, such as EC and smooth muscle cells, including the vasculature and myoblasts.

Monocyte differentiation and MP activation can be regulated by different signals (Shi and Pamer 2011). LPA is an inducer of $CD11b^+$ monocyte differentiation towards $F4/80^+$ MP. This mechanism depends on the Akt/mTOR signaling pathway and PPARγ as the downstream regulator (Ray and Rai 2017). Importantly, LPA can also promote human monocyte differentiation to macrophages by the same mechanism. It is not known if LPA regulates MP subtype specification. Nevertheless, there is some evidence suggesting MP phenotype modulation by LPA. LPA has been shown to induce the expression of IL-1β from MP by a mechanism that depends on G_i/Rho signaling and the generation of ROS (Chang et al. 2008), which is consistent with a pro-inflammatory phenotype. On the other hand, LPA increases the number of $CD86^+$ and $CD206^+$ microglia cells (the macrophages of the brain's immune system), referred to as pro-and anti-inflammatory markers respectively (Plastira et al. 2020). Thus, expression analysis of specific subtype markers in MP would help determine their role in the skeletal muscle in response to LPA and its potential participation in inflammation and fibrosis.

There is only one report that analyzes the role of LPA administration on inflammation of chronic injured skeletal muscle. Induced chronic injury of the rotator cuff muscles promotes an increment of the macrophage and neutrophil population associated with the onset of fibrosis and fat deposition. As expected, intraperitoneal administration of LPA enhances MP and neutrophil infiltration and worsens the fibrotic and adipose phenotype seen in damaged-muscle (Davies et al. 2017).

The modulation of the ATX/LPA/LPARs axis also regulates the inflammatory response in different organs. In a mouse model for Chron's disease, a chronic inflammatory bowel pathology, the symptoms can be attenuated with the administration of PF8380 (a pharmacological inhibitor of ATX). The expression of IL-4 (here postulated as a pro-fibrotic cytokine), IL-13, and TNF-α, as well as leucocyte infiltration in the ileal tissue, are increased in a Chron's disease-mice model and can be significantly attenuated in mice treated with PF-8380 (He et al. 2018), decreasing the cellular differentiation defects.

In a myocardial infarction model, injured mice show increased LPA levels that correlate positively with the number of inflammatory cells in plasma (Tripathi et al. 2020). The injured heart also showed increased expression and activity of ATX. The administration of PF8380 just after injury attenuated the augmented infiltration of neutrophils, pro-inflammatory monocytes, and MP. This inhibitor also decreased mRNA expression of several inflammatory cytokines and chemokines compared to vehicle-treated mice. As expected, the size of scarred tissue, measured through collagen staining, was also decreased in PF8380-treated mice after infarction challenge, improving the tissue's functional recovery (Tripathi et al. 2020). Also, the global or adipocyte-specific deletion of the ATX gene can attenuate the increased levels of inflammatory cytokines in adipose tissue and lipid accumulation in the liver in obesity associated with a high-fat diet (Brandon et al. 2019).

Finally, in the brain, administration of LPA can modulate the inflammatory context. In a mouse model of acute brain inflammation in mice (LPS administration), there were increased LPA levels and augmented expression of some LPARs in the brain. Furthermore, isolated microglia, showed increased expression of cytokines, such as IL-1b, IL-6, and TNF-a, as well as chemokines CCL5, CXCL2, and CXCL10 when treated with LPA (Plastira et al. 2020).

The ATX/LPA/LPARs axis in the development of fibrosis

Numerous studies propose a pro-fibrotic role for the ATX/LPA/LPARs axis in different organs and diseases. LPA is increased in bronchoalveolar lavage fluid (BAL) from the bleomycin-induced lung fibrosis model. In agreement with these results, LPA is increased in BAL and in exhaled breath condensate from patients with idiopathic pulmonary fibrosis (Tager et al. 2008; Montesi et al. 2014). LPA and ATX are

also upregulated in the liver from patients with HCV infection, which is accompanied by liver fibrosis in advanced stages of the disease (Watanabe et al. 2007). Similarly, there are increased levels of extracellular LPA in explants from fibrotic kidneys induced by a unilateral ureteral obstruction (UUO) (Pradere et al. 2007). These data strongly suggest that there is local LPA secretion and signaling in the fibrotic organ. Even though there are no reports of LPA levels in muscles from patients with skeletal muscle fibrosis-driving diseases, intraperitoneal LPA injections worsen atrophy and fibrosis induced by tendon nerve injury in rotator cuff muscle (Davies et al. 2017).

Studies that modulate the function or the presence/absence of LPA receptors yielded the most promising results that suggest a role for the ATX/LPA/LPARs axis in fibrosis pathogenesis (Fig. 1). There is increasing data suggesting that pharmacological blockage or genetic deletion of LPA receptors could prevent the development of induced fibrosis in different organs. The primary LPA receptors that appeared to be involved in fibrosis are LPA_1 and LPA_3. LPA1 transcripts are increased in the kidney after UUO, and LPA_1 KO mice show decreased fibrosis markers such as collagen III. The LPA_1 and LPA_3 inhibitor Ki16425 triggered a similar response (Pradere et al. 2007).

In the same way, lung fibrosis induced by radiation shows dramatic increases in LPA_1 and LPA_3 transcripts. Treatment with the LPA_1/LPA_3 inhibitor VPC12249, reduces collagen deposition and prevents the induction of pro-fibrotic cytokines such as CCN2 and TGF-β in the lung (Gan et al. 2011). The positive effects of inhibiting LPA receptors in lung fibrosis were also replicated in the bleomycin induced model (Ohashi and Yamamoto 2015). In the murine model SOD1-G93A that mimics amyotrophic lateral sclerosis (ALS), which causes skeletal muscle fibrosis, the treatment with AM095, an LPA1 inhibitor, causes motor skills improvement evaluated by grip strength, rotarod, and runtime (Gento-Caro et al. 2021). These results support the use of inhibitors of the ATX/LPA/LPARs axis in the treatment of fibrotic disorders, as addressed in numerous clinical trials, mainly in idiopathic pulmonary fibrosis and scleroderma. It would be interesting to know if those inhibitors can be useful tools in treating muscle fibrosis in diseases such as DMD.

The differentiation of resident cells towards a myofibroblast-like phenotype could be one of the roles of LPA in the development/maintenance of fibrosis (Fig. 1). A mouse peritoneal fibrosis model shows that the accumulation of αSMA positive cells is prevented in the absence of LPA_1 (Sakai et al. 2013). In skeletal muscle, one of the primary sources of myofibroblasts are FAPs, which are increased under fibrotic conditions such as those found in *mdx* and in denervated muscles (Contreras et al. 2016). It is necessary to investigate if LPA is involved in the differentiation of FAPs towards a myofibroblast phenotype in muscles from these animals. Furthermore, it is possible that LPA could be mediating pro-fibrotic effects by inducing other biological behaviors in FAPs, such as migration and proliferation, as has been shown in different cell types in cancer (Stähle et al. 2003; Yamada et al. 2008).

It seems that LPA is closely related to the TGF-β pathway since, in Tenon's fibroblasts, the contraction induced by TGF-β is inhibited by Ki16425, which also downregulates the SMAD 2/3 proteins (Wen et al. 2019). Similarly, Ki16425 prevents the migration and proliferation of these cells. It has been proposed that the synergistic fibrotic effect of LPA and TGF-β could also be explained by the participation of the Hippo/YAP/TAZ pathway (Zmajkovicova et al. 2020). This pathway includes several proteins, with the Yes-asocciated protein (YAP) and the transcriptional co-activator with PDZ-binding motif (TAZ) being the key final effectors. These proteins require translocation to the nucleus to exert their function as co-transcriptional activators. The Hippo signaling pathway is mediated by G-protein-coupled receptor (GPCR) ligands such as LPA (Cai and Xu 2013). Besides, the YAP/TAZ pathway is required for TGF-β/ Smad signaling since decreased YAP/TAZ levels induces Smad 7, a known negative regulator of the TGF- β pathway (Qin et al. 2018) (Fig. 2). In the same line, our laboratory has demonstrated that C2C12 myoblasts respond to LPA, increasing the expression of CCN2 through a mechanism that requires the activation of the TGF-β receptor I (Cabello-Verrugio et al. 2011b). Preliminary experiments using the same cell line have shown that inhibition of LPA receptors 1 and 3 (using Ki16425) prevents the induction of CCN2 by TGF-β, suggesting that there is crosstalk between both pathways. Accordingly, YAP binding to the CCN2 promoter results in its induction (Zhao et al. 2008). It would be interesting to determine if the induction of CCN2 by LPA and TGF- β is prevented by inhibiting the YAP/TAZ pathway using tools such as the inhibitor Verteporfin or siRNAs targeting components of this signaling pathway in myoblasts (Fig. 2). In a recent publication, Riquelme-Guzmán showed that LPA and TGF-β require integrin signaling for the induction of CCN2 in myoblasts, adding the ECM/integrin axis as a new factor in LPA signaling and supporting the idea of cross-talk between LPA and TGF-β pathways in muscle cells (Riquelme-Guzman et al. 2018). This functional interaction among LPA/TGF-β/integrin has also been suggested by Xu et al. based on results that demonstrated that LPA induces αvβ6-mediated TGF-β activation in epithelial cells through RhoA and Rho kinase (Xu et al. 2019).

Taken together, these data strongly suggest that the ATX/LPA/LPARs axis could be part of an unexplored pro-fibrotic program in skeletal muscle pathologies causing muscular fibrosis such as DMD and Limb-girdle MD.

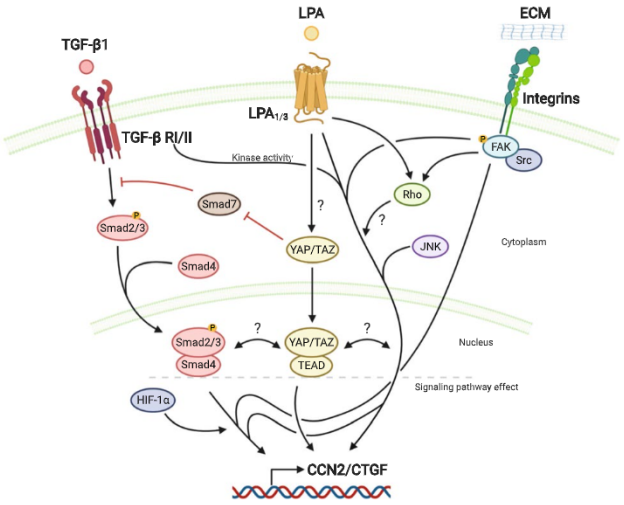

Fig. 2 The interplay between CCN2/CTGF regulatory signaling pathways. Different signaling pathways of varying nature can converge to regulate the pro-fibrotic factor CCN2. For instance, the serine/threonine kinase receptor activatied by TGF-β signaling promotes Smad2/3-dependent induction of CCN2, a response synergistically potentiated by low oxygen availability. Such receptor activity is also required for $LPA_{1/3}$-dependent LPA induction of CCN2. Adding more complexity, LPA-dependent signaling also requires JNK activation to induce CCN2, while Rho GTPase could be involved as well, being activated by LPA and the integrin/FAK axis. Furthermore, this axis also supports the effects of TGF-β and LPA. Hippo/YAP/TAZ signaling pathway emerges as the central sensor of those responses because it can be modulated by different extracellular cues and it can induce CCN2. This pathway can feedforward into TGF-β/Smad signaling by reducing Smad7 levels. Importantly, the YAP/TAZ pathway is activated by GPCRs such as those that are part of the LPA/LPARs axis. Whether LPA and TGF-B1, coupled with the integrin/FAK axis, converge with YAP/TAZ to regulate CCN2 is unknown, but would add a new spatial component for the control their pro-fibrogenic activity. Created with biorender.com

The ATX/LPA/LPARS axis as a novel therapeutic target in muscular dystrophies

According to the evidence summarized in this review, many investigations show promising effects of pharmacological inhibitors of the ATX/LPA/LPARs axis in murine models of kidney, skin, and lung fibrosis (Gan et al. 2011; Ohashi and Yamamoto 2015; Pradere et al. 2008; Castelino et al. 2011; Ninou et al. 2018). Moreover, the inhibition of ATX in a murine chronic colitis model improved inflammation, down-regulating IL-6, and STAT3 in the colon (Dong et al. 2019).

There is a recent report on the structure-based finding of new inhibitors of ATX, which seeks to improve their specificity and optimize possible treatments (Magkrioti et al. 2020). Until now, clinical trials testing the potential use of ATX and LPA_1 axis inhibitors in humans have been conducted predominantly on idiopathic pulmonary fibrosis and systemic sclerosis (NCT03798366, NCT01766817, NCT04308681).

The ATX/LPA/LPARs axis is emerging as an attractive target for the development of new treatments for inflammatory and fibrotic diseases, including muscular dystrophies. However, there are still no studies linking LPA with these pathologies. It is necessary to study the effect of ATX/LPA/LPARs inhibitors in murine models of MDs.

Concluding remarks

MDs are a wide range of skeletal muscle degenerative diseases with no cure or satisfactory treatment. DMD is the most lethal type, with a life expectancy of 30 years in affected patients. Skeletal muscle structure and function are impaired due to low regeneration capability, chronic inflammation, and fibrosis.

Skeletal muscle inflammation is a necessary response for normal regeneration after damage. However, when this response becomes chronic, characterized by the constant maintenance of immune cells and the overexpression of pro-fibrotic factors, it triggers fibrosis. The functional skeletal muscle tissue is replaced by disorganized fibrotic and adipogenic stromal tissue, which is deleterious for muscle performance.

Several lines of evidence demonstrated that inflammation plays a pivotal role in the induction of fibrosis. For instance, the imbalance and dysregulation of immune cell populations and the overexpression of a wide range of cytokines and chemokines are associated with fibrosis. Moreover, inflammatory cells and cytokines with anti-muscle regeneration properties are also overrepresented in dystrophic muscles. Therefore, inflammation can govern two major scenarios related to MDs.

For several years, research was focused on improving muscle function through the targeting of molecules with pro-inflammatory or pro-fibrotic functions. The finding of novel molecules with the capacity to control both inflammation and the fibrotic response would help develop new potential therapeutic drugs to combat MDs. Here, we propose the novel ATX/ LPA/LPARs axis as a new target. This axis can modulate the expression of cytokines and chemokines and the abundance of immune cells in tissues affected by chronic inflammation. It can influence the establishment of fibrosis, regulating the overexpression of pro-fibrotic factors in other organs, such as lung, liver, and kidney. Figure 3 summarizes the axis's components in MDs and the potential effect of the pathway on different target cells, resulting in increased inflammatory and fibrotic responses that affect muscle performance.

Many responses regulated by this axis are present in dystrophic muscles. However, there is little evidence of its function in this tissue. Despite this, there are already significant contributions (Tsukahara and Haniu 2012; Davies et al.

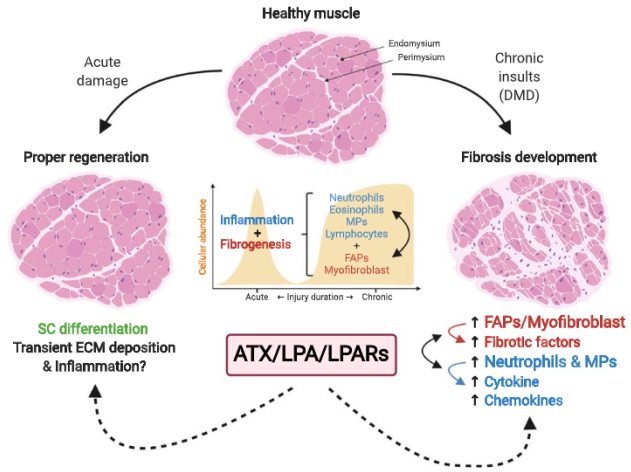

Fig. 3 Proposed functions for the ATX/LPA/LPARs axis in acute and chronic damage. Normal skeletal muscle regeneration is affected by the nature of the damage. Acute injuries are resolved by a well-orchestrated and transient increase of inflammatory cells and ECM-producing cells. The crosstalk between each cell population establishes a scaffolding network for proper muscle regeneration. On the other hand, as occurs in DMD patients, chronic insults result in persistent accumulation of both cell populations with enhanced capacities. We propose that the expression of ATX, and as a consequence an increase in LPA-mediated signaling, could be necessary for muscle regeneration by regulating SCs differentiation. Whether this axis is essential for acute ECM deposition and inflammation in skeletal muscle is unknown. Activation of LPA-mediated signaling induces some pathophysiological responses present in different organs and tissues in DMD. Some of them correspond to the induction of cytokines, chemokines, and fibrotic factors expression and the accumulation of myofibroblast, MPs, and neutrophils. If these effects are present in the skeletal muscle, this axis would be an attractive signaling pathway in future therapeutic considerations for MDs. Created with biorender.com

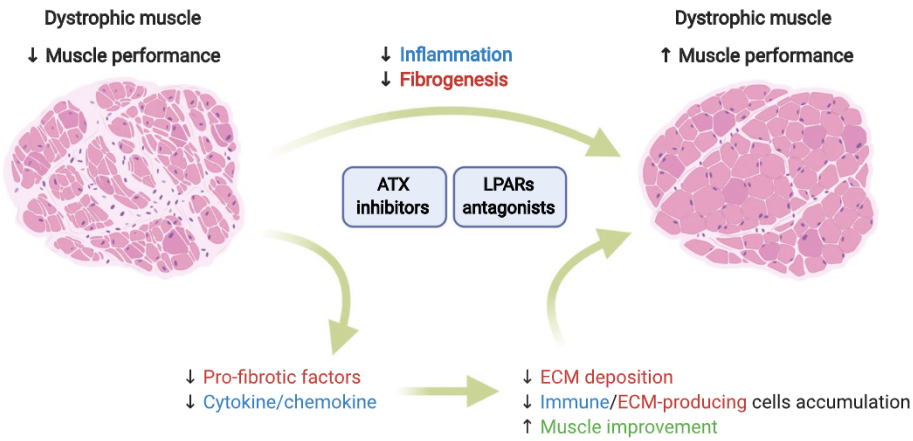

Fig. 4 The ATX/LPA/LPARs axis as a potential therapeutic target for MDs. Muscle degeneration due to inflammation and fibrosis is the most influencing issue on muscle performance in MDs. The regulation of inflammation and fibrogenesis is the most attractive way to fight MDs. LPA induces pro-fibrotic factors, cytokines, and chemokines. We propose that the use of LPARs antagonists or inhibitors of ATX activity could attenuate the generation of these molecules, decreasing ECM protein deposition and the continued presence of immune and ECM-producing cells, therefore improving muscle regeneration and performance. Created with biorender.com

2017; Cabello-Verrugio et al. 2011b; Riquelme-Guzman et al. 2018). Recently, Sah et al. demonstrated that ATX expression and function are required for the myogenic differentiation of C2C12 myoblasts. Furthermore, using injured muscles from mice, they found that ATX mRNA expression increases substantially on day four after injury, and that in vivo deletion of the ATX-encoding gene (Enpp2) negatively affects the regeneration process (Sah et al. 2020).

In conclusion, the ATX/LPA/LPARs axis is a pathway with promising roles in the pathophysiology of MDs. Figure 4 shows the ATX/LPA/LPARs axis as a potential therapeutic target in MDs. Its involvement in the modulation of inflammation and fibrosis makes it an attractive pharmacological target for several chronic diseases. Future research focusing on its role in the skeletal muscle may open new possibilities for understanding muscle homeostasis and disease.

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

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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

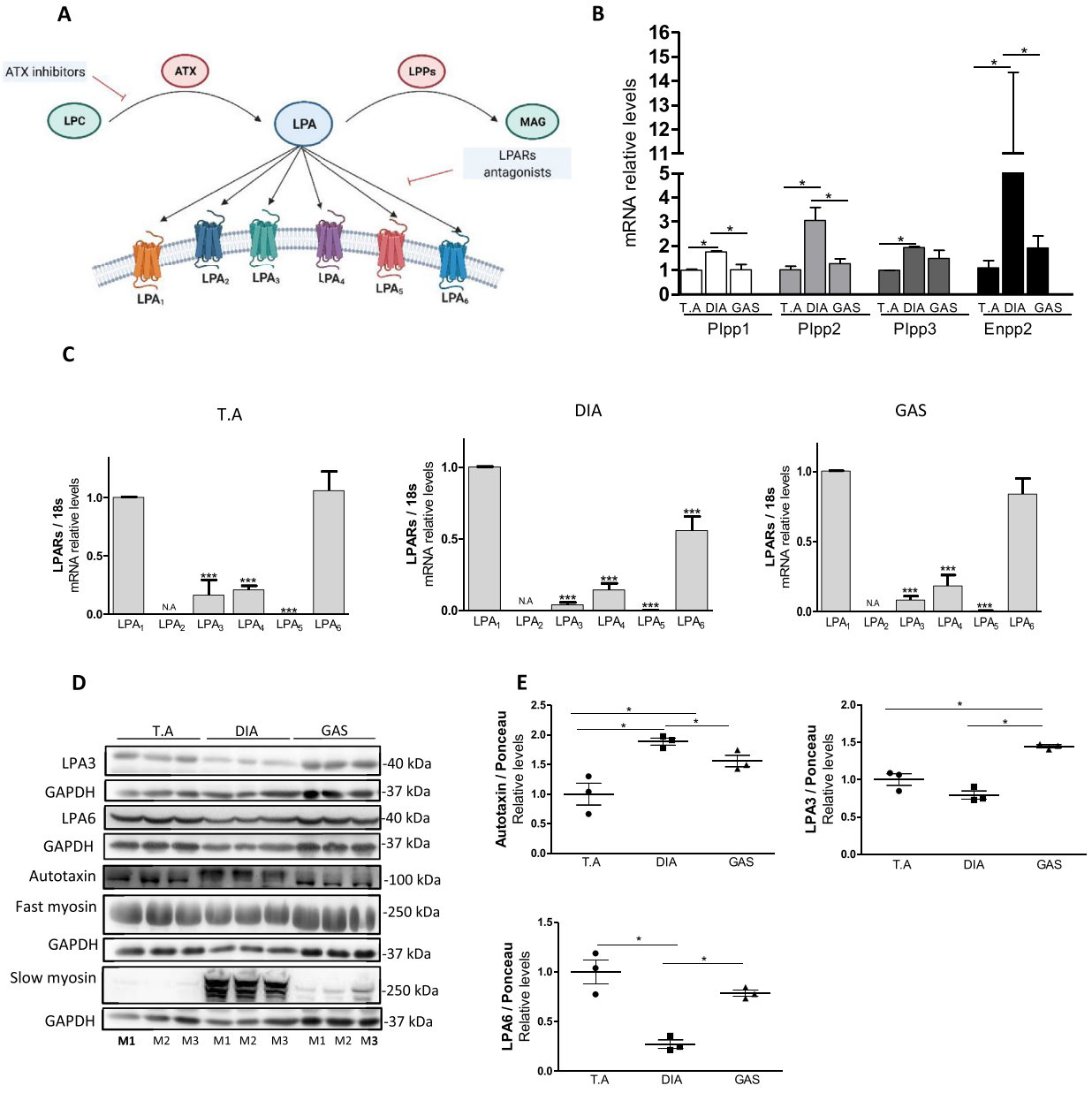


Fig. 1. Presence of ATX/LPA/LPARs axis components in the skeletal muscle. (A) Lysophosphatidylcholine (LPC) is converted to LPA by autotaxin (ATX). LPA signals through 6 receptors called LPA_1 to LPA_6. LPA is degraded by different lipid phosphate phosphatases (LPPs). The diagram was created with BioRender.com. (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.

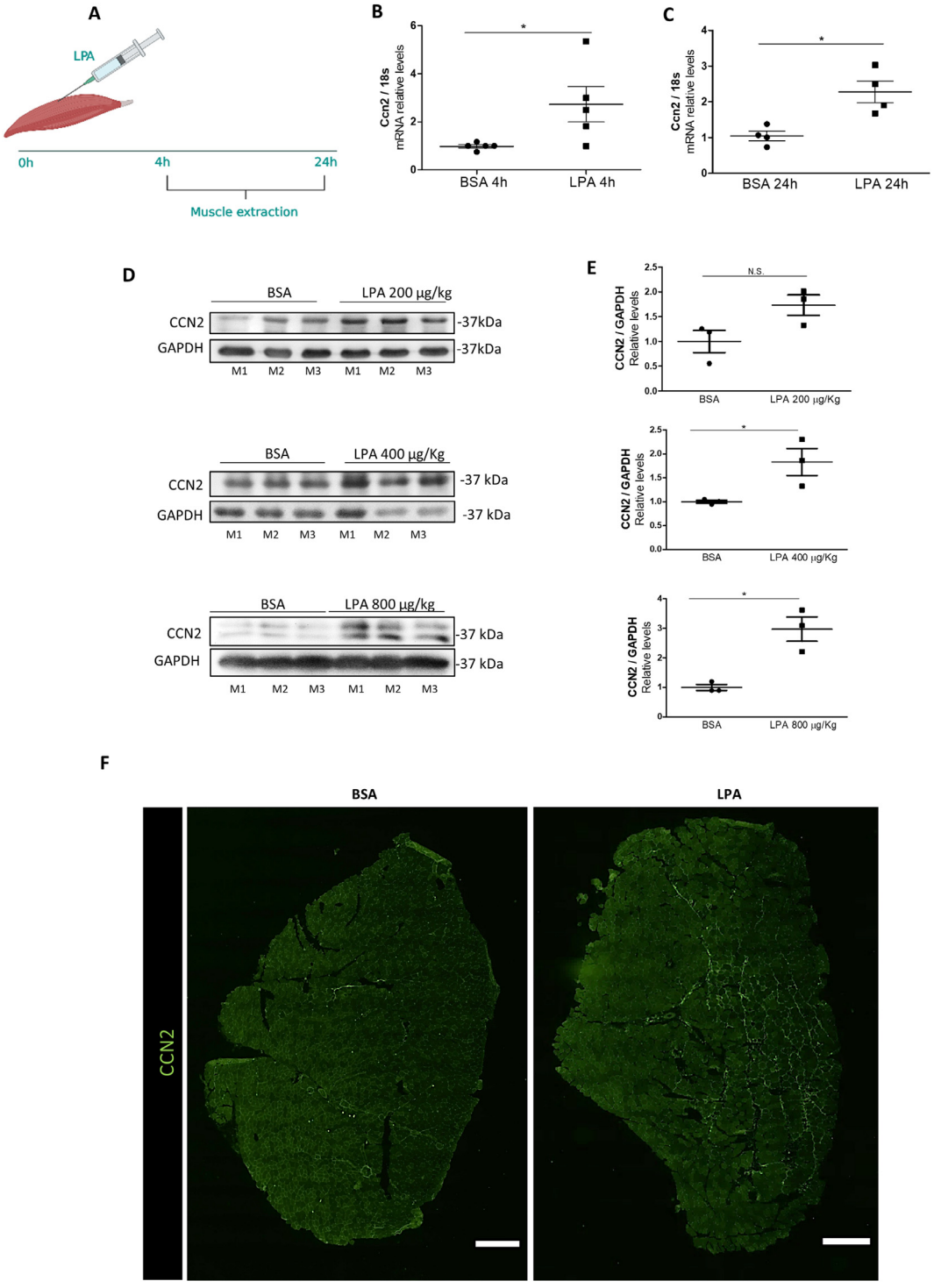


Fig. 2. LPA induces CCN2 expression in skeletal muscle. (A) Experimental design diagram created with BioRender.com. Briefly, LPA was injected in T.A and the muscles were extracted at 4 or 24 h later. (B) Gene expression of Ccn2 at 4 and (C) 24 h after LPA injection. (D) CCN2 protein levels 4 h after 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. $n = 3$.

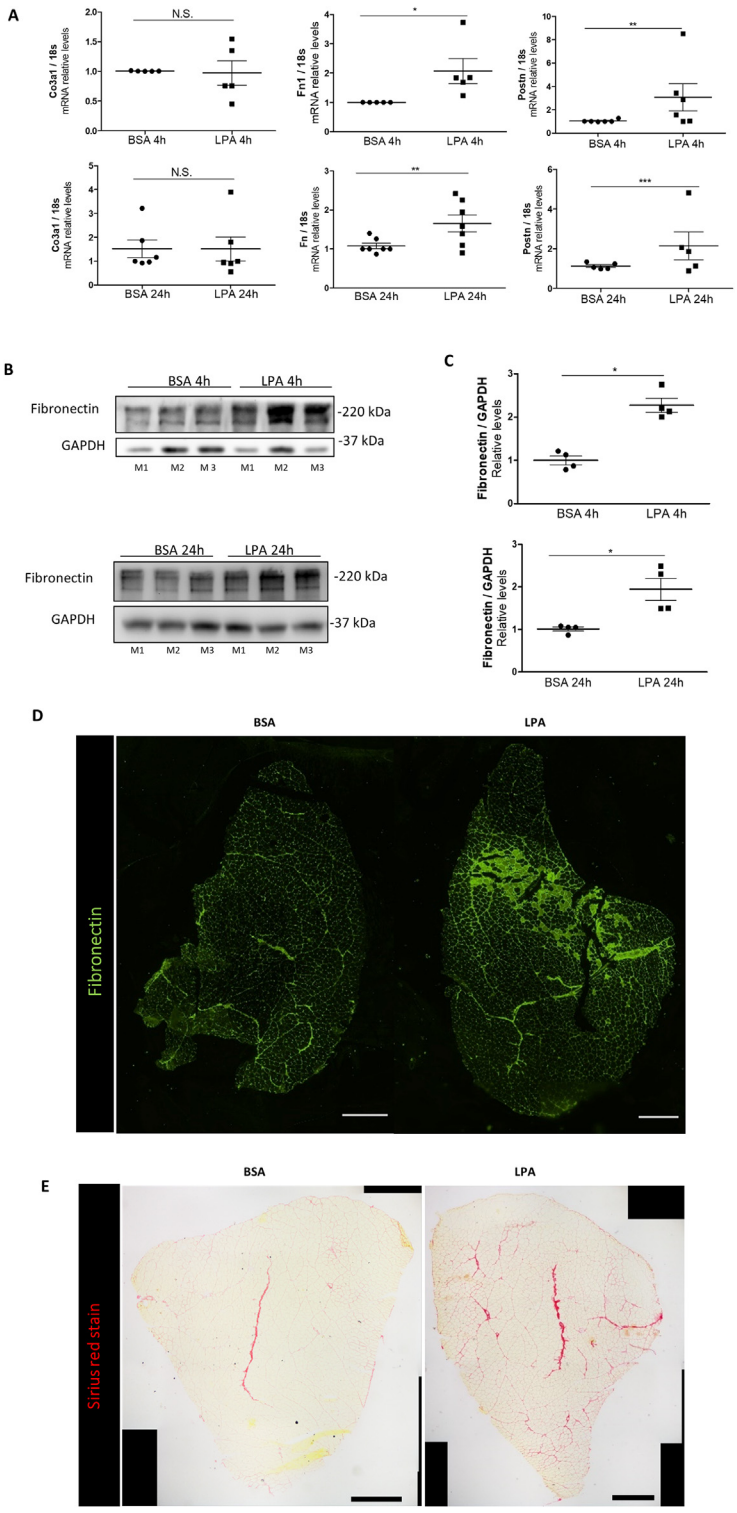


Fig. 3. The skeletal muscle induces ECM accumulation in response to LPA. (A) mRNA expression levels of Col 3a1 (collagen 3), Fn1 (fibronectin), and Postn (periostin) in T.A muscle injected with BSA or LPA 400 μg/Kg for 4 h or 24 h, obtained by RT-qPCR analysis. B) Fibronectin protein levels were analyzed 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

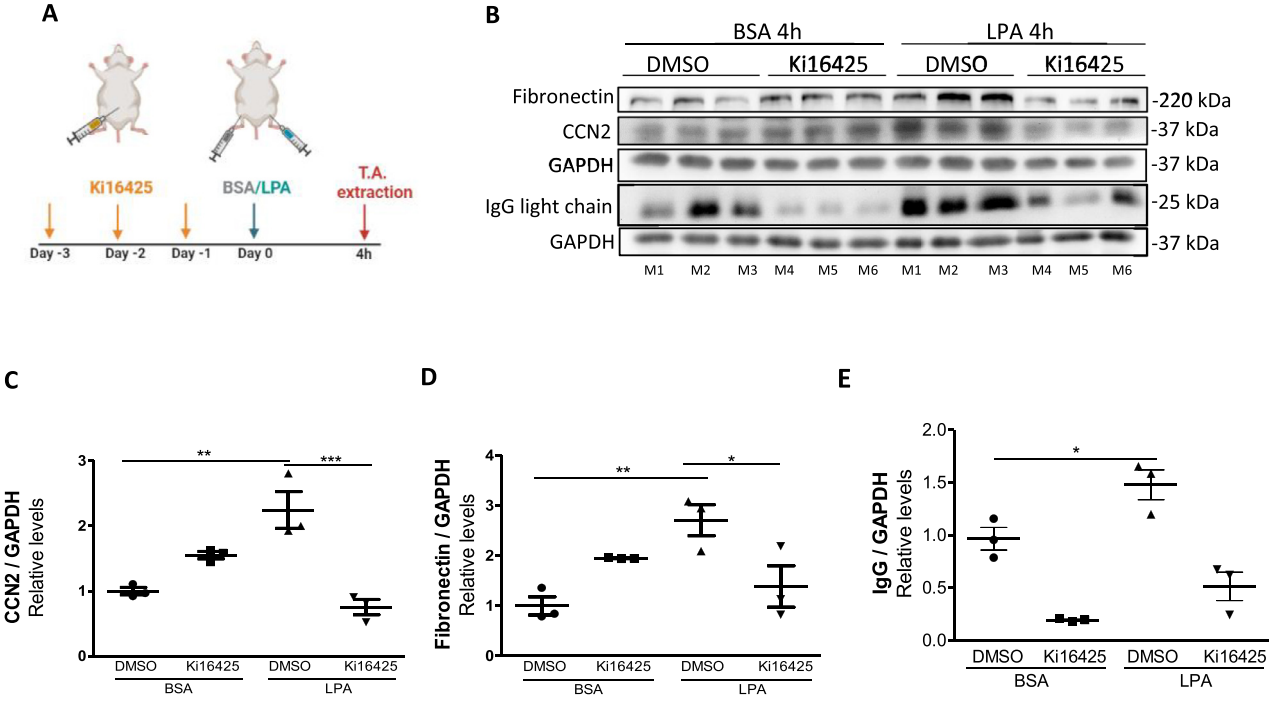
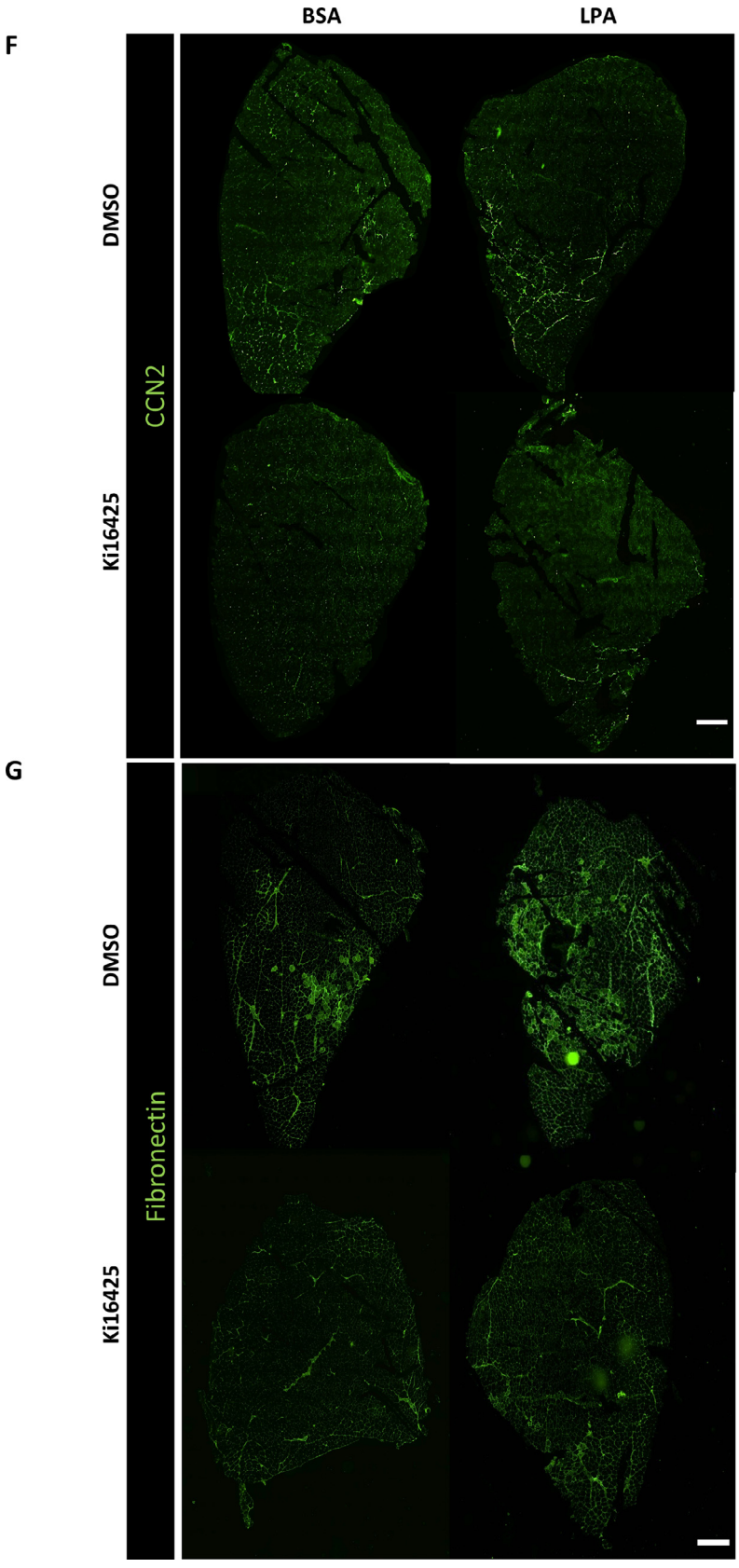


Fig. 4. Pharmacological inhibition of $LPA_{1/3}$ prevents the skeletal muscle fibrotic response to LPA. (A) Treatment plan, male mice were treated with Ki16425 5 mg/Kg or vehicle (DMSO) daily for 3 days and 40 min before BSA and LPA injection (for 4 h). (B) Fibronectin, CCN2, IgG protein levels were 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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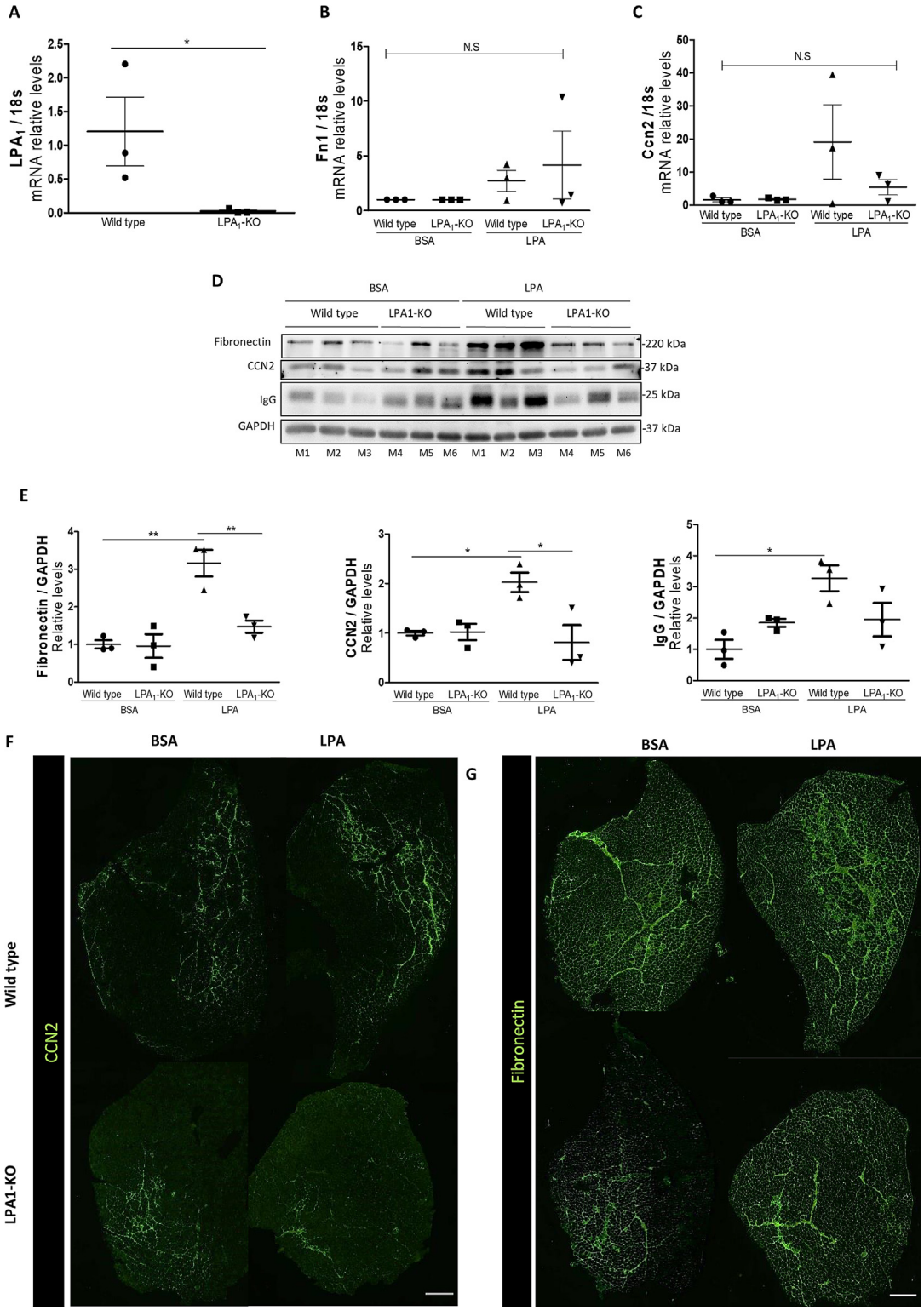

Fig. 5. LPA_1 is one of the main mediators of the LPA-induced fibrotic response in skeletal muscle. (A) LPA_1 mRNA levels in T.A from WT and LPA_1-KO mice. (B–E) Fibronectin and CCN2 were analyzed by RT-qPCR and immunoblot to determine their mRNA (B and C) and protein (D and E) levels in T.A from WT 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; $n = 3$.

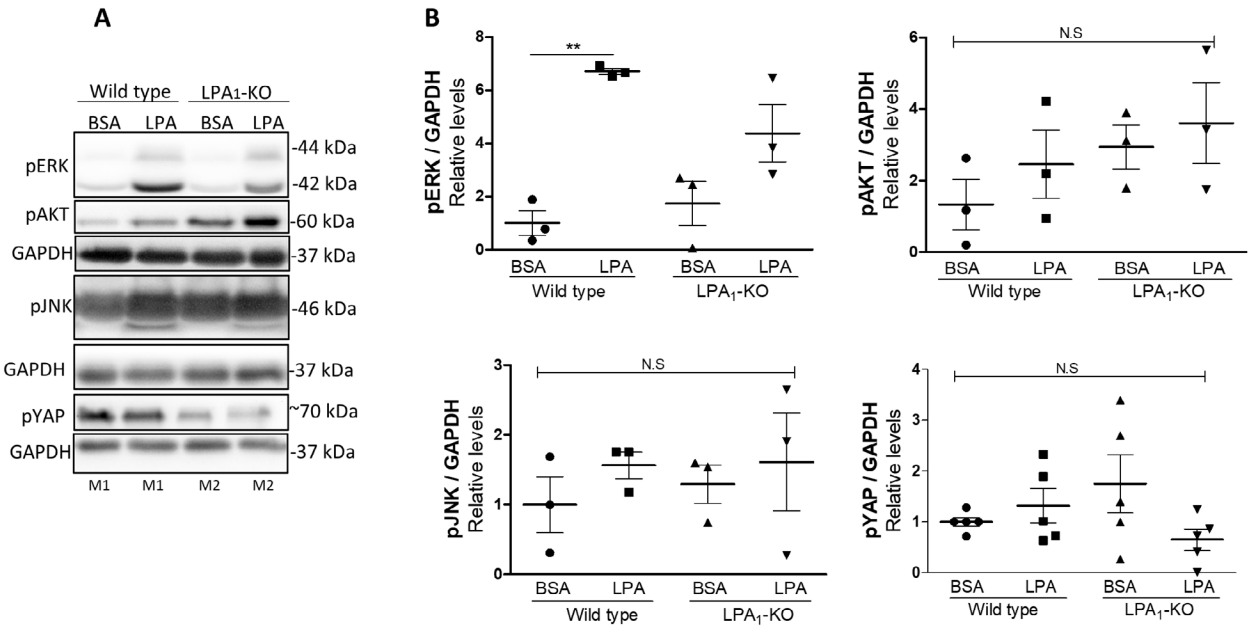

Fig. 6. LPA induces ERK 1/2 phosphorylation through LPA1 (A) pERK, pAKT, pJNK, pYAP and GAPDH levels were analyzed by immunoblot in T.A from WT and LPA_1-KO mice 10 min after being injected with BSA or LPA 400 μg/Kg IM. GAPDH was used as the loading control. (B) Quantification of phosphorylated proteins. *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

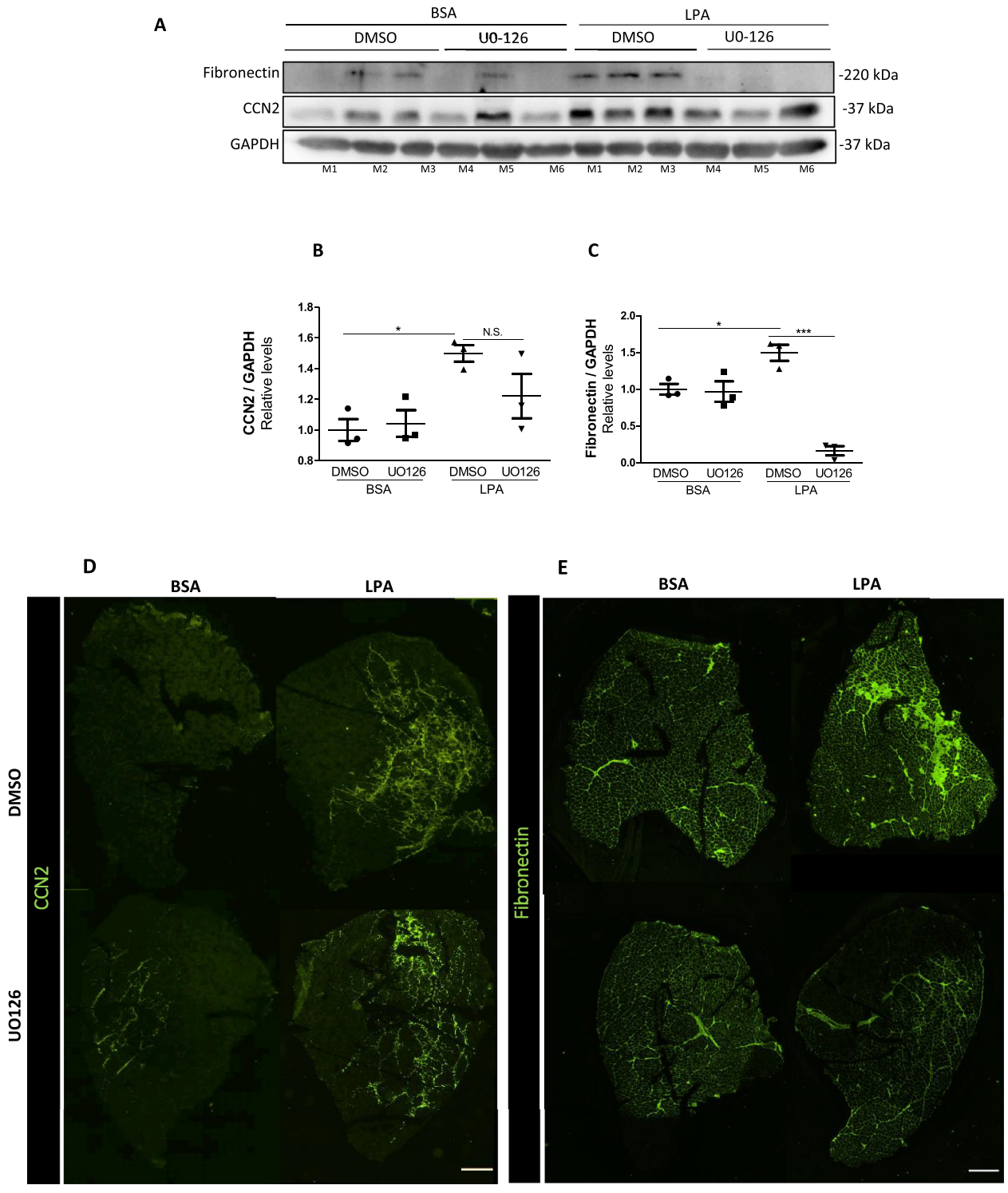

Fig. 7. Inhibition of ERK 1/2 prevents LPA-induced fibrotic responses in skeletal muscle. (A) Fibronectin and CCN2 protein levels were analyzed by immunoblot in T.A from mice treated with U0126 for 3 days and 4 h after being injected with BSA or LPA 400 μg/Kg IM. GAPDH was used as the loading control. (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.

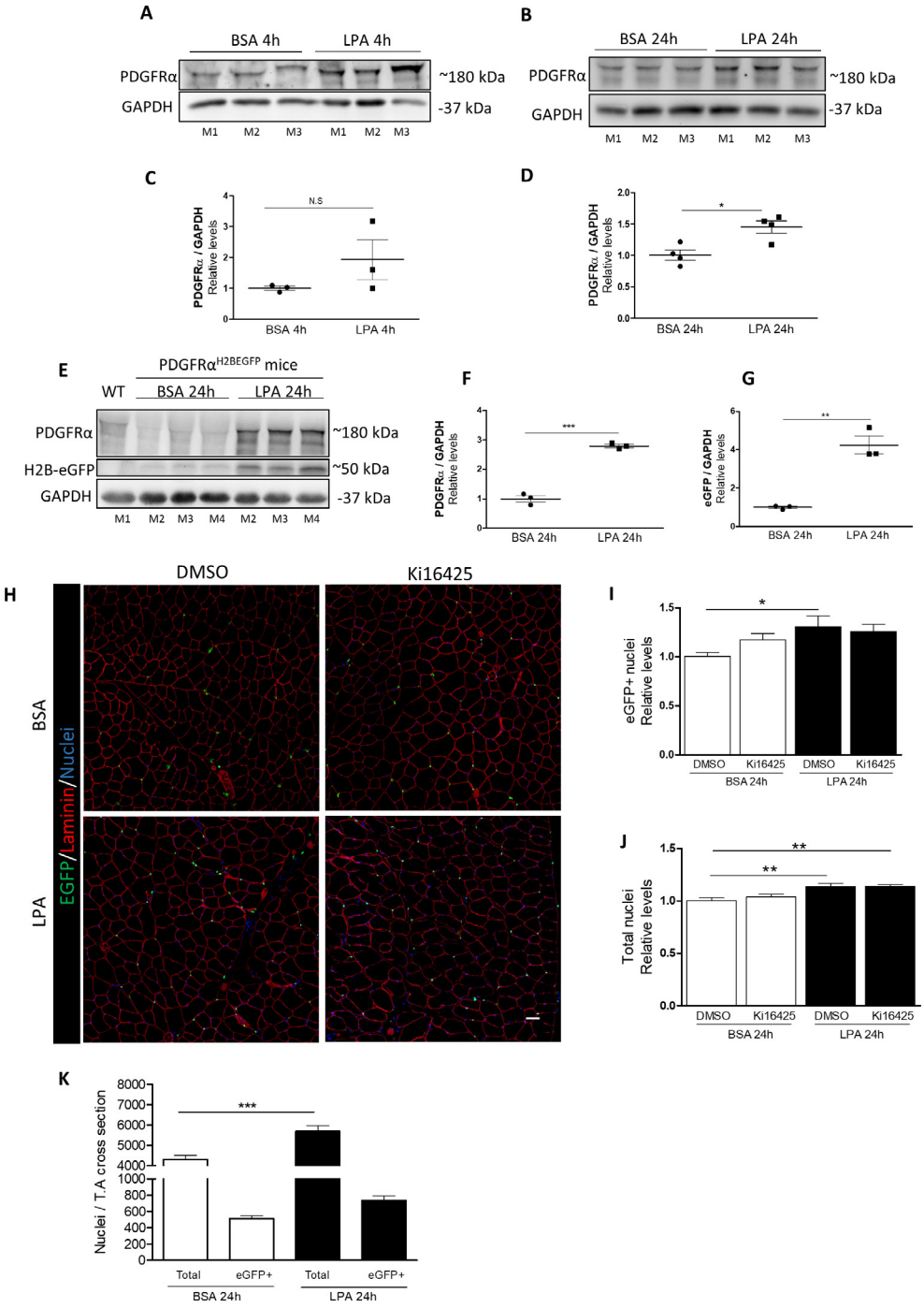

Fig. 8. LPA increases the number of FAPs in skeletal muscle. (A, B) PDGFRα levels in T.A were analyzed by immunoblot from WT mice at 4 h (A) or 24 h (B) after being injected with BSA or LPA 400 μg/Kg IM. GAPDH was used as the loading control. *n* = 3. (C) Quantification of A. D) Quantification of B. 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

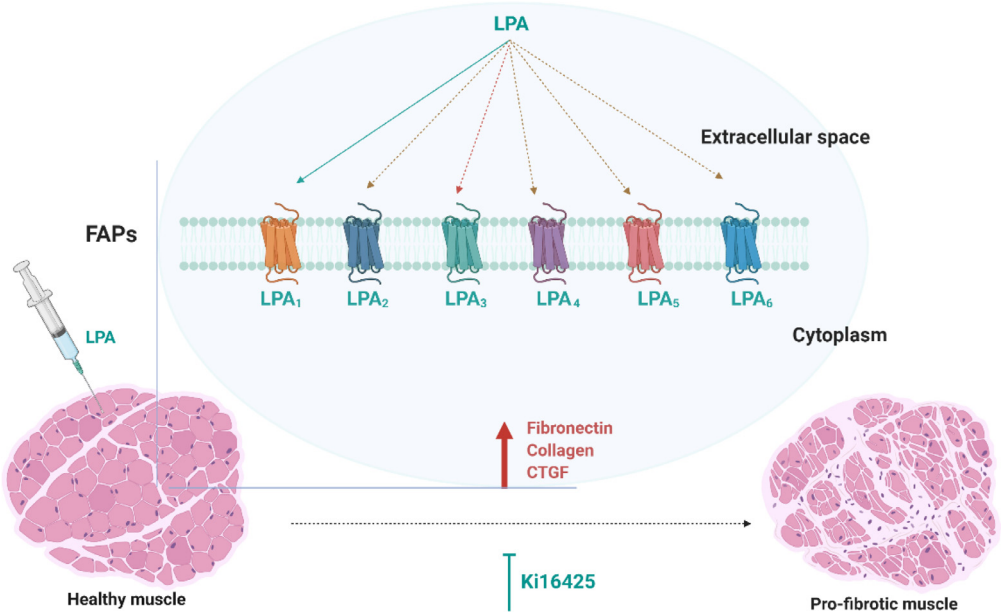

Fig. 9. Schematic view of the skeletal muscle response to LPA. Treatment with LPA induces an increase in the number of FAPs, CCN2 and ECM molecules, such as fibronectin and collagen. The pharmacological inhibition of $LPA_{1/3}$ with Ki16425 and the absence of the LPA_1 gene prevents some fibrotic effect. 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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Muscular dystrophies

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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