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Angiotensin II receptor type 1 blockade decreases CTGF/CCN2-mediated damage and fibrosis in normal and dystrophic skeletal muscles

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Abstract

Connective tissue growth factor (CTGF/CCN-2) is mainly involved in the induction of extracellular matrix (ECM) proteins. The levels of CTGF correlate with the degree and severity of fibrosis in many tissues, including dystrophic skeletal muscle. The CTGF overexpression in tibialis anterior skeletal muscle using an adenoviral vector reproduced many of the features observed in dystrophic muscles including muscle damage and regeneration, fibrotic response and decrease in the skeletal muscle strength. The renin–angiotensin system is involved in the genesis and progression of fibrotic diseases through its main fibrotic components angiotensin-II and its transducer receptor AT-1. The use of AT-1 receptor blockers (ARB) has been shown to decrease fibrosis. In this paper, we show the effect of AT-1 receptor blockade on CTGF-dependent biological activity in skeletal muscle cells as well as the response to CTGF overexpression in normal skeletal muscle. Our results show that in myoblasts ARB decreased CTGF-mediated increase of ECM protein levels, extracellular signal regulated kinases 1/2 (ERK-1/2) phosphorylation and stress fibres formation. In tibialis anterior muscle overexpressing CTGF using an adenovirus, ARB treatment decreased CTGF-mediated increase of ECM molecules, α-SMA and ERK-1/2 phosphorylation levels. Quite remarkable, ARB was able to prevent the loss of contractile force of tibialis anterior muscles overexpressing CTGF. Finally, we show that ARB decreased the levels of fibrotic proteins, CTGF and ERK-1/2 phosphorylation augmented in a dystrophic skeletal muscle from mdx mice. We propose that ARB is a novel pharmacological tool that can be used to decrease the fibrosis induced by CTGF in skeletal muscle associated with muscular dystrophies.

Keywords: CTGF • skeletal muscle fibrosis • angiotensin II receptor type I blocker (ARB) • muscular dystrophies

Introduction

CTGF/CCN2 is a modular cysteine-rich protein involved in the regulation of several cellular functions being proposed as one of the main inducers of damage and fibrosis [1, 2]. CTGF is characterized by augmenting the ECM protein production [3]. The level of CTGF correlates with the degree and severity of fibrosis in tissues such as skin [4], lung [5], liver [6], kidney [7] and dystrophic skeletal muscle [8]. Moreover, we have recently shown that skeletal muscle cells respond to CTGF stimulation by inducing the synthesis of ECM molecules and stress fibre formation through a mechanism involving ERK-1/2 phosphorylation [9].

CTGF is expressed at low levels in normal tissue but its overexpression induces deleterious effects such as tissue damage and fibrosis [10, 11]. We overexpressed CTGF in tibialis anterior skeletal muscle using an adenoviral vector and we can reproduce many of features observed in skeletal muscular dystrophies, including skeletal muscle damage and regeneration,

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strong fibrotic response and a drop in the skeletal muscle strength [12].

The main components of rennin–angiotensin system (RAS) are angiotensin II, its transmembrane angiotensin II receptor type I (AT-1) and type II (AT-2) and the angiotensin-converting enzyme (ACE). Besides blood pressure regulation, RAS components have been described to participate in fibrotic disease in cardiac, lung and kidney tissues [13, 14]. ARB or ACE inhibitors have been used to decrease or prevent the appearance of fibrosis in several tissues [15, 16]. In skeletal muscle, myoblasts express ACE and AT-1 [17]. Studies *in vivo* have shown that the use of ARB losartan partially improved skeletal muscle features in several diseases [18].

The purpose of this study was to assess the role of AT-1 receptor blocking on CTGF-dependent biological activity. Here we show that ARB decreased the biological response of skeletal muscle cells to CTGF, including a decrease in ECM protein levels, ERK-1/2 phosphorylation and stress fibres formation. The studies *in vivo* showed that ARB decreased fibrotic response induced by CTGF overexpression in tibialis anterior muscles as shown by diminished levels of ECM molecules, α-SMA and ERK-1/2 phosphorylation. Moreover, ARB restored the net force of tibialis anterior muscles overexpressing CTGF. Accordingly with these results, ARB decreased the fibrosis associated to dystrophic skeletal muscle from *mdx* mice, which present higher CTGF levels than normal muscles.

Thus, we propose that ARB is a potential novel pharmacological tool that can be used to decrease fibrotic response to CTGF in skeletal muscle fibrosis.

Material and methods

Cell cultures

The skeletal muscle cell line C2C12 (American Type Culture Collection), was grown as described previously [19]. Myoblasts were pre-incubated with AT-1 receptor blocker ZD-7155 or Losartan (both from Tocris, Ellisville, MO, USA) and subsequently incubated with recombinant CTGF [9].

Recombinant adenovirus preparation

The adenoviruses encoding wild-type murine CTGF (Ad-mCTGF) and the control virus with a GFP transgene (Ad-GFP) were prepared and produced as previously described [12].

Animals and experimental skeletal muscle adenoviral infection

Twelve-weeks old male mice of C57BL/10 ScSn strain were studied. The animals were kept at room temperature with a 24-hr night–day cycle and fed with pellets and water *ad libitum*. Experimental infection of tibialis anterior muscles was performed by adenovirus injection (2×10^{11} viral particles of Ad-GFP or 2×10^{11} particles of Ad-mCTGF) in mice under ketamine/xylazine anaesthesia [80/12 mg/kg body-weight (BW), intraperitoneally (i.p.); Ref. 20]. Briefly, 40 μl of a phosphate-buffered saline (PBS) solution containing either the adenovirus or vehicle were injected along the whole length of the tibialis anterior. Two experimental groups were designed: those treated with vehicle (water) or losartan (90 mg/kg/day) [18] during 10 days prior and 5 days post-adenoviral infection. Blood pressure of the mice was measured as previously described [21]. At the end of the experiment, tibialis anterior muscles were dissected and removed under anaesthesia at day 5 post-infection, and then the animals were killed. Tissues were rapidly frozen and stored at -80°C until processing.

Male *mdx* mice (8-weeks old) were treated with vehicle (water) or losartan (90 mg/kg/day) during 6 months. After this treatment, the animals were killed and processed as described. All protocols were conducted in strict accordance and with the formal approval of the Animal Ethics Committee of the P. Universidad Católica de Chile.

Immunoblot analysis

Protein extracts from myoblasts were prepared in 50 mM Tris-HCl, pH 7.4, 0.1M NaCl, 0.5% Triton X-100 with a cocktail of protease inhibitors and 1 mM PMSF. For skeletal muscle extracts, tibialis anterior muscles were homogenized in Tris-EDTA buffer with a cocktail of protease inhibitors and 1 mM PMSF. Proteins were subjected to SDS-PAGE, transferred onto PDVF membranes (Schleicher & Schuell, St. Louis, MO, USA) and probed with goat anti-CTGF (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-fibronectin (1:5000), mouse anti-α-SMA (1:1000) and mouse anti-tubulin (1:10000; Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-collagen III (Rockland, Gilbertsville, PA, USA) and mouse anti-GAPDH (1:10000) (Chemicon, Temecula, CA, USA). All immunoreactions were visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

Immunofluorescence microscopy and stress fibre detection

Stress fibre formation and localization in C2C12 myoblasts were analysed by detection of fluorescent phalloidin as previously described [22]. For immunofluorescence analysis, transverse sections of tibialis anterior were fixed, blocked and incubated with the rabbit anti-fibronectin (1:100), rabbit anti-collagen type III (1:100), rabbit anti-collagen type I (1:100) and rabbit anti-phospho-Smad-3 (Cell Signaling, Danvers, MA, USA). As secondary antibody, rhodamine-conjugated goat-anti-rabbit IgG was used. After nuclear staining with Hoechst 33258, the cover slips were mounted using Fluoromont (Dako, Carpinteria, CA, USA) and viewed under a Nikon Diaphot inverted microscope, equipped for epifluorescence.

Skeletal muscle histology and Sirius red staining

Architecture and histology were detected by haematoxylin and eosin stain in transverse sections of tibialis anterior skeletal muscle. Total collagen content was detected by staining with Sirius red and quantification was performed by ImageJ software [23].

Contractile properties

After 5 days post-infection with adenovirus, mice were anaesthetized, tibialis anterior muscles was removed and the muscle contractile properties were measured as previously described [24, 25]. Briefly, optimum muscle length (Lo) and stimulation voltage were determined from micromanipulation of muscle length to produce maximum isometric twitch force. Maximum isometric tetanic force (Po) was determined from the plateau of the frequency–force relationship after successive stimulations at 1–200 Hz for 450 msec., with 2-min. rests between stimuli. After determination of isometric contractile properties, muscles were subjected to a three repeated tetanic stimulation protocol. Muscles at Lo were maximally stimulated for 450 msec. once every 5 sec. After functional testing, muscles were removed from the bath, trimmed of their tendons and any adhering non-muscle tissue, blotted once on filter paper, and weighed. Muscle mass and Lo were used to calculate specific net force [force normalized per total muscle fibre cross-sectional area (CSA), mN/mm^2].

Protein determination

Proteins were determined in aliquots of cell extracts using the Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL, USA), using BSA as the standard.

Statistics

The statistical significance of the differences between the means of the experimental groups was evaluated using one-way ANOVA with a *post hoc* Bonferroni multiple-comparison test (Sigma Stat Versión 3.5; Jandel Scientific Software, San Rafael, CA, USA). A difference was considered statistically significant at $P < 0.05$.

Results

CTGF-dependent pro-fibrotic activity is inhibited by blocking AT-1 receptor

Because CTGF is one of the main mediators involved in the pathogenesis of several fibrogenic diseases, including skeletal muscle dystrophies, it is important to find strategies and molecules that inhibit its pro-fibrotic activity. Thus, we evaluated the relevance of ARB use as possible modulators of CTGF biological activity in C2C12 myoblasts. Western blot analysis in Figure 1A shows that the increase in protein levels of fibronectin in response to CTGF was strongly inhibited by the ARB ZD-7155, reaching a maximal effect at 10 μM. Figure 1B shows the results of quantitative analysis of these experiments. Figure 1C shows that CTGF-dependent ERK-1/2 phosphorylation [9] was decreased by ZD-7155 in a dose-dependent manner. Figure 1D shows the results of quantitative analysis of these experiments. Similar results were found when the ARB losartan was used instead ZD-7155 (Fig. 1E). Figure 1F shows that the increase in stress fibres in response to CTGF decreased in a dose-dependent manner in response to ZD-7155, with a maximal inhibitory effect seen at 10 μM. All these results suggest that CTGF requires active AT-1 receptors to exert its biological responses.

ARB decreases CTGF-induced fibrosis in normal skeletal muscle

We decided to determine the effect of AT-1 receptor blocking on the fibrosis induced by CTGF overexpression in tibialis anterior muscles infected with an adenoviral vector encoding murine CTGF. Figure 2A shows that ARB losartan decreased the damage observed in tibialis anterior muscles infected with Ad-mCTGF, as evaluated by haematoxylin and eosin staining, diminishing the necrotic areas observed. Figure 2B shows that losartan strongly decreased the amount of collagen induced by CTGF overexpression in tibialis anterior muscles as evaluated by Sirius red staining. Figure 2C shows the results of quantitative analysis of experiments depicted in Figure 2B. Then, the effect of ARB on CTGF-induced ECM molecules in tibialis anterior muscles was evaluated. Immunofluorescence analysis in Figure 3 shows an important increase in the levels of fibronectin, collagen types I and III in tibialis anterior infected with Ad-mCTGF, which were strongly decreased by treatment with ARB losartan. Figure 4A shows by Western blot analysis that the increase of fibronectin, collagen type III and α-SMA protein levels observed in tibialis anterior muscles overexpressing CTGF compared to control-infected muscles is substantially diminished by the treatment with losartan. In addition, we observed that CTGF protein levels were sustained in tibialis anterior infected with Ad-mCTGF independently of treatment with losartan or vehicle. Figure 4B shows the results of quantitative analysis of these experiments. Together, all these results strongly suggest that active AT-1 receptors are required to mediate the pro-fibrotic effect induced by CTGF in a model of skeletal muscle fibrosis *in vivo*.

AT-1 receptor blockade decreases CTGF-dependent skeletal muscle fibrosis by a mechanism that involves diminished ERK-1/2 phosphorylation

We have previously described that CTGF induces fibrosis in skeletal muscle cells by a mechanism involving ERK-1/2 phosphorylation [9]. Thus, we evaluated the role of ERK-1/2 in the fibrosis induced by CTGF overexpression in tibialis anterior muscles. Figure 5A shows that ERK-1/2 phosphorylated protein levels increased in tibialis anterior muscles overexpressing CTGF compared to those in control infected muscles. Furthermore, treatment with losartan decreased ERK-1/2 phosphorylation in tibialis anterior muscles infected with Ad-mCTGF compared to mice that were treated with the vehicle alone. Figure 5B shows

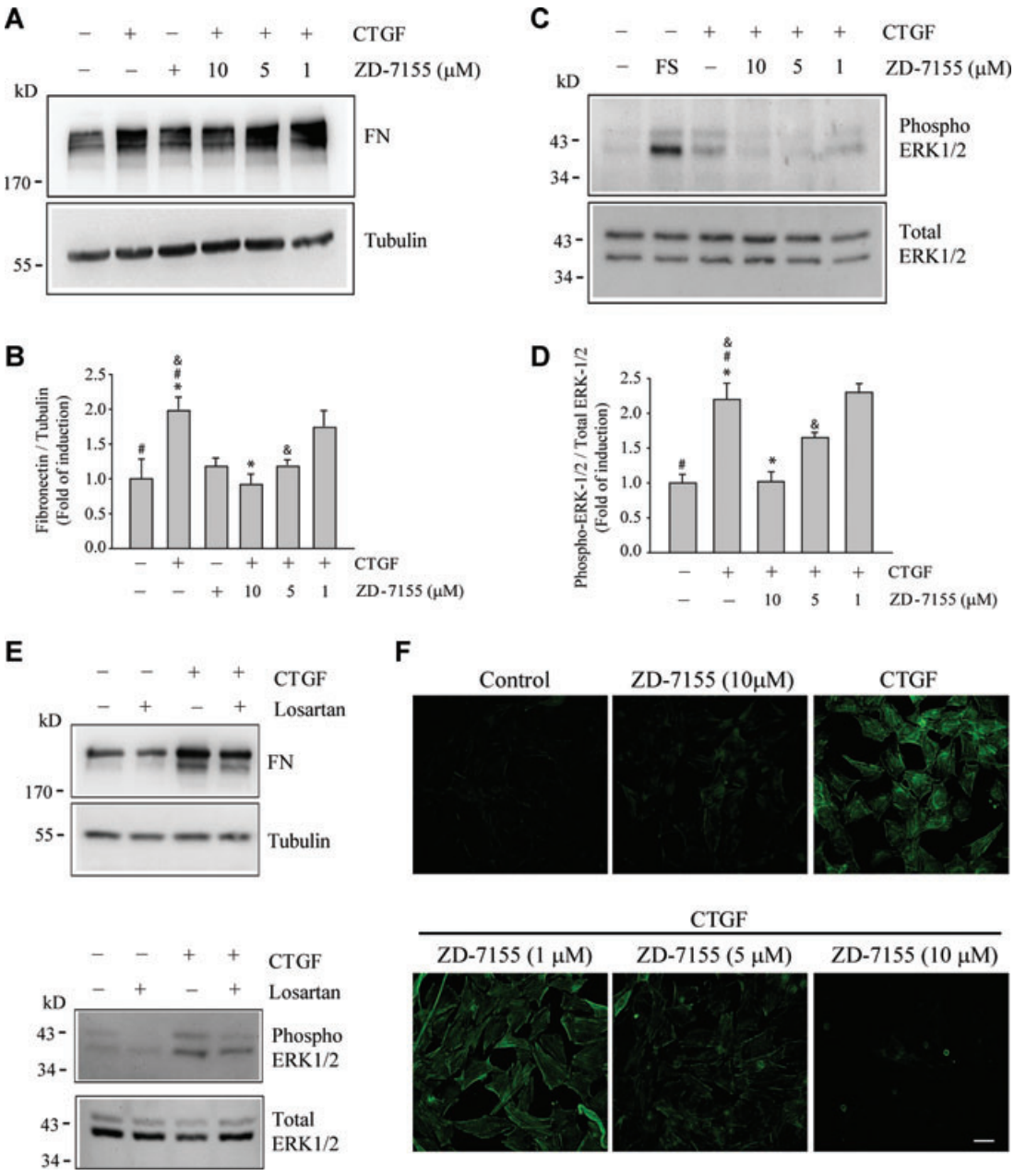

Fig. 1 Blocking of AT-1 receptor decreases CTGF-dependent pro-fibrotic activity in skeletal muscle cells. (**A**) C2C12 myoblasts were pre-incubated for 1 hr with increasing concentrations of the ARB ZD-7155. Then, cells were incubated with 80 ng/ml of recombinant CTGF for 48 hrs. Fibronectin (FN) protein levels were analysed by Western blot in cell extracts. Tubulin levels are shown as loading control. Molecular weights are shown in kD. (**B**) Relative levels of FN normalized to tubulin. The graphic shown corresponds to densitometric analysis of Western blots shown in (**A**). Values correspond to the mean ± standard deviation of three independent experiments ($^{*,\#,\&}P < 0.05$). (**C**) C2C12 cells treated as described in (**A**). Western blot for phospho-ERK-1/2 and total ERK-1/2 levels are shown. FS: foetal serum bovine. Molecular weights are shown in kD. (**D**) The graphic shown corresponds to densitometric analysis of Western blots shown in (**C**). Values correspond to the mean ± standard deviation of three independent experiments ($^{*,\#,\&}P < 0.05$). (**E**) C2C12 cells were pre-incubated with losartan (10 μM) and incubated with 80 ng/ml of recombinant CTGF. Levels of fibronectin (FN) and phospho-ERK-1/2 were determined as in (**A**) and (**C**), respectively. Densitometric analysis of Western blots was performed [Fibronectin, fold of induction relative to control: 2.3 ± 0.5 (CTGF), 1.2 ± 0.3 (CTGF + Losartan), $P < 0.05$; Phospho-ERK-1/2, fold of induction relative to control: 1.9 ± 0.5 (CTGF), 1.1 ± 0.2 (CTGF + Losartan), $P < 0.05$]. (**F**) C2C12 myoblasts were treated as in (**A**) and processed for direct fluorescence using phalloidin coupled to fluorescein. Nuclei were labelled with Hoechst 33258. Bar corresponds to 50 μm.

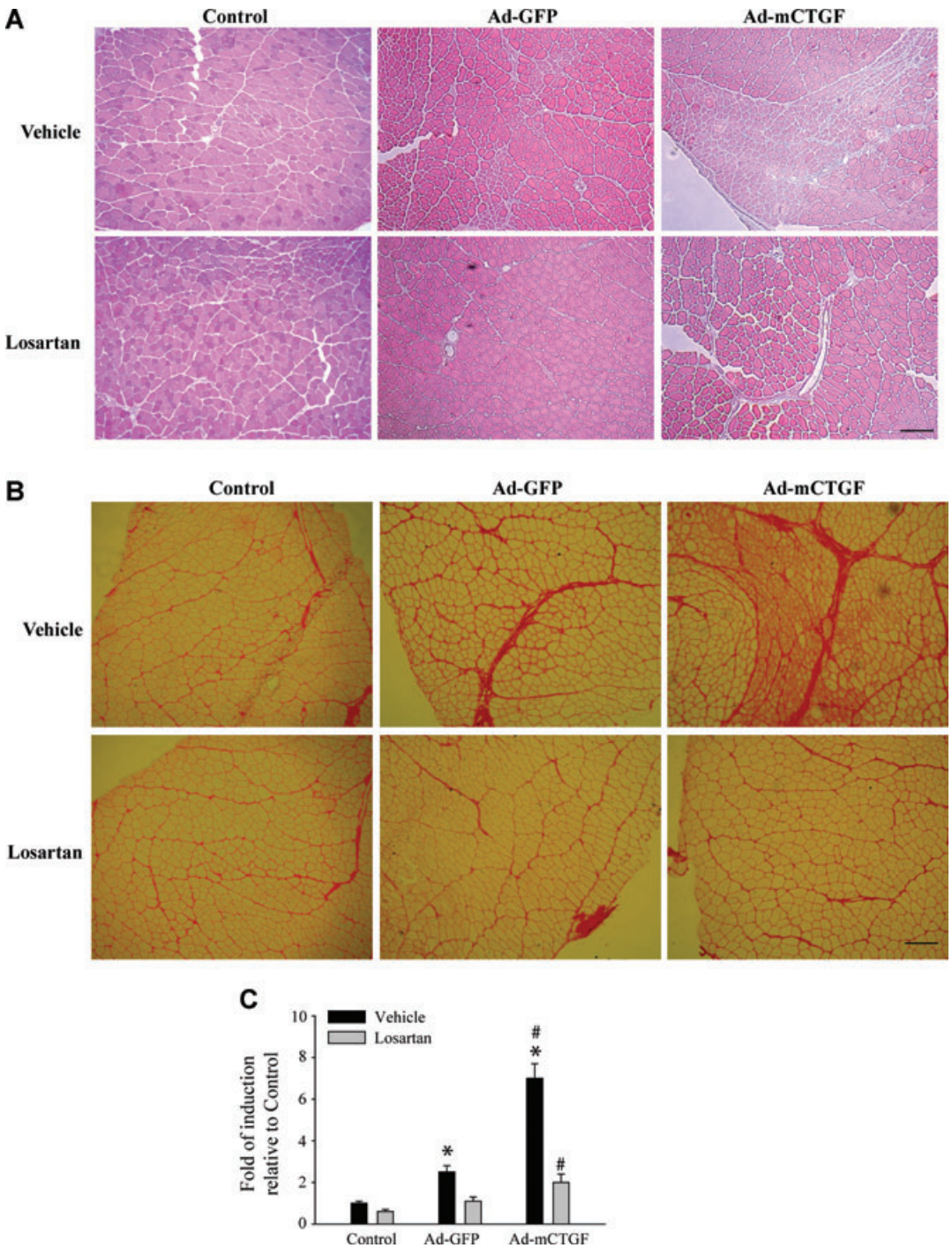

Fig. 2 Angiotensin II receptor type 1 blockade decreases CTGF-induced damage and collagen content in tibialis anterior muscle. Tibialis anterior (TA) of C57BL/10 mice systemically treated with vehicle or the ARB losartan were infected with control adenovirus (Ad-GFP) or with an adenovirus to overexpress CTGF (Ad-mCTGF). Three independent experiments were performed using two mice for each experimental condition. (**A**) Cryosections of TA muscles infected for 5 days were stained with haematoxylin and eosin. (**B**) Total content of collagen evaluated by Sirius red (SR) staining in cryosections of TA muscles infected for five days. Bar corresponds to 200 μm. (**C**) Quantification of total content of collagen stained by Sirius red (SR) using ImageJ software. Values correspond to the mean ± standard deviation ($^{*,\#}P < 0.05$).

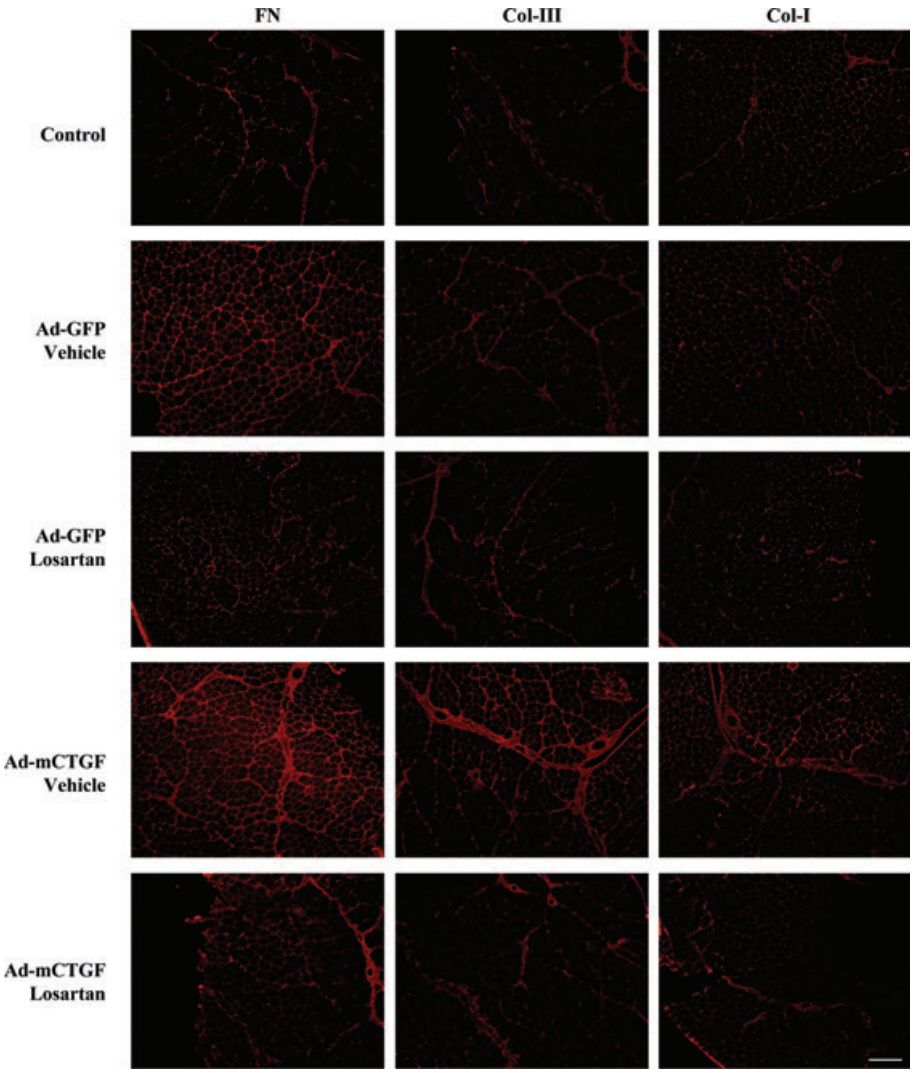

Fig. 3 CTGF-induced ECM accumulation is decreased by blockade Angiotensin II receptor type 1 in skeletal muscle. Tibialis anterior (TA) of C57BL/10 mice systemically treated with vehicle or the ARB losartan were infected with control adenovirus (Ad-GFP) or with an adenovirus to overexpress CTGF (Ad-mCTGF). Levels of fibronectin (FN), collagen I (Col I) and collagen III (Col III) were detected by indirect immunofluorescence analysis in cryosections of TA muscles infected for five days. Bar corresponds to 200 μm. The images are representatives from three independent experiments performed using two mice for each experimental condition.

quantitative analyses of the inhibition of ERK-1/2 phosphorylation in response to CTGF by losartan. Together, these results suggest that ERK-1/2 phosphorylation is a target for losartan action, which might explain its anti-fibrotic properties against CTGF observed *in vivo*.

Induction of TGF-β activity is consequence of the adenoviral infection, but not related to CTGF overexpression

It has been suggested that tissue growth factor-β (TGF-β) response is involved in increased dystrophic skeletal muscle [18]. We decided to evaluate if induction of phospho-Smad-3 occurs under adenoviral overexpression in tibialis anterior muscles. Figure 6A shows that both adenoviruses (Ad-GFP and Ad-mCTGF) strongly induce the induction of phospho-Smad-3 compared to the muscles that only received the vehicle. Interestingly, mice that were pre-treated with losartan showed a strong diminished response to both adenovirus infections. Figure 6B shows the results of quantitative analysis of these experiments. These results suggest that the induction of TGF-β signalling is related to the use of the adenovirus rather to the specific expression of CTGF.

Decrease of strength in skeletal muscle overexpressing CTGF is prevented by ARB

We decided to evaluate the maximum isometric force of tibialis anterior muscle overexpressing CTGF. Figure 7A shows the curve of net force generated for tibialis anterior muscles stimulated with

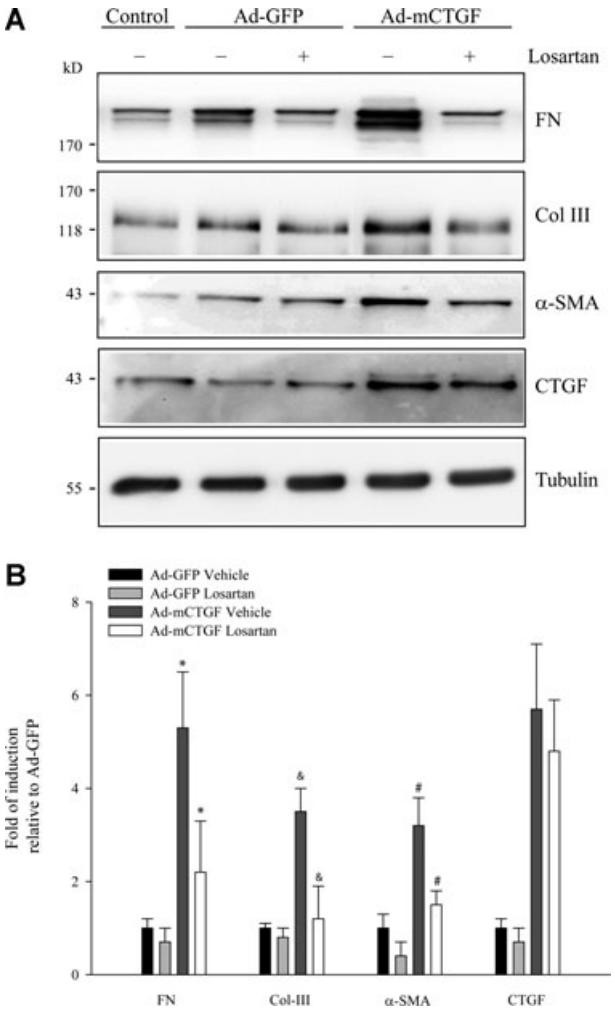

Fig. 4 Tibialis anterior muscle fibrosis induced by CTGF is prevented by blocking Angiotensin II Receptor Type 1. (**A**) Protein levels of fibronectin (FN), collagen III (Col III), α-SMA and CTGF were detected by Western blot analysis in extracts obtained from TA muscles of C57BL/10 mice without treatment (Control) or systemically treated with vehicle or losartan five days post-infection with control (Ad-GFP) or CTGF-overexpressing (Ad-mCTGF) adenoviruses. Tubulin levels are shown as loading control. Molecular weights are shown in kD. (**B**) Quantification of data corresponding to densitometric analysis from Western blot. Values correspond to the mean ± standard deviation of three independent experiments performed using two mice for each experimental condition ($^{*,\#,\&}P < 0.05$).

frequencies ranging from 1 to 200 Hz. Under these conditions, muscles infected with Ad-mCTGF at day 5 produced a minor net force, about 50% or less, compared to tibialis anterior muscles infected with Ad-GFP in all the ranges of stimulatory frequency evaluated. By contrast, muscles overexpressing CTGF from mice that were treated with losartan showed a curve of net force totally comparable with that of control muscles infected with Ad-GFP or injected with PBS. In control muscles, losartan not produced significant effects on net force. Figure 7B shows that maximal isometric tetanic force in tibialis anterior muscles overexpressing CTGF decreased by 35% compared to control muscle infected with Ad-GFP or injected with PBS. Treatment of mice with losartan allowed a significant rescue of maximal isometric tetanic force under conditions of CTGF overexpression, reaching similar values shown by control tibialis anterior muscles (infected with Ad-GFP or injected with PBS). These results strongly suggest that CTGF overexpression decreases skeletal muscle contractile properties evaluated by maximum isometric force, which can be prevented by blocking the AT-1 receptor.

Losartan diminishes amount of fibrotic proteins, the fibrotic factor CTGF and ERK-1/2 phosphorylation in a dystrophic skeletal muscle

Finally, we decided to evaluate if losartan also diminishes levels of fibrotic proteins, the pro-fibrotic factor CTGF and phospho-ERK in the *mdx* mouse model of skeletal muscular dystrophy. *Mdx* mice were treated with losartan for 6 months and fibronectin, α-SMA and CTGF levels were determined by Western blot analysis. Figure 8A shows that in *mdx* tibialis anterior an important increase in the amount of fibronectin, α-SMA and CTGF compared to the levels observed in wild-type mice. These levels were considerably reduced by losartan treatment. Furthermore, as shown above, losartan strongly diminished the increased level of phospho-ERK observed in the *mdx* tibialis anterior. Figure 8B shows that losartan decreases fibrosis in *mdx* TA anterior diminishing muscle damage, collagen amount and fibronectin levels. These results suggest that the anti-fibrotic effect of ARB has a strong effect in the dystrophic skeletal muscle whereas the fibrotic response, inducers and mediators are strongly induced.

Discussion

In this paper, we have shown a novel function of ARB as modulators of CTGF pro-fibrotic activity. The *in vitro* evidence strongly suggests that induction of fibrotic features mediated by CTGF, such as an increase in ECM protein levels and stress fibre formation, was prevented by ARB in a dose-dependent manner. Indeed, ARB was able to decrease ERK-1/2 phosphorylation induced by CTGF. These data are strongly supported by experiments *in vivo* in which we observed that ARB treatment of mice decreased the CTGF-mediated deleterious effects, such as skeletal muscle damage, induction of ECM proteins and increase in ERK-1/2 phosphorylation. These effects of ARB *in vivo* were accompanied by a prevention of the decrease mediated by CTGF in the maximum isometric specific force generated by tibialis anterior muscles.

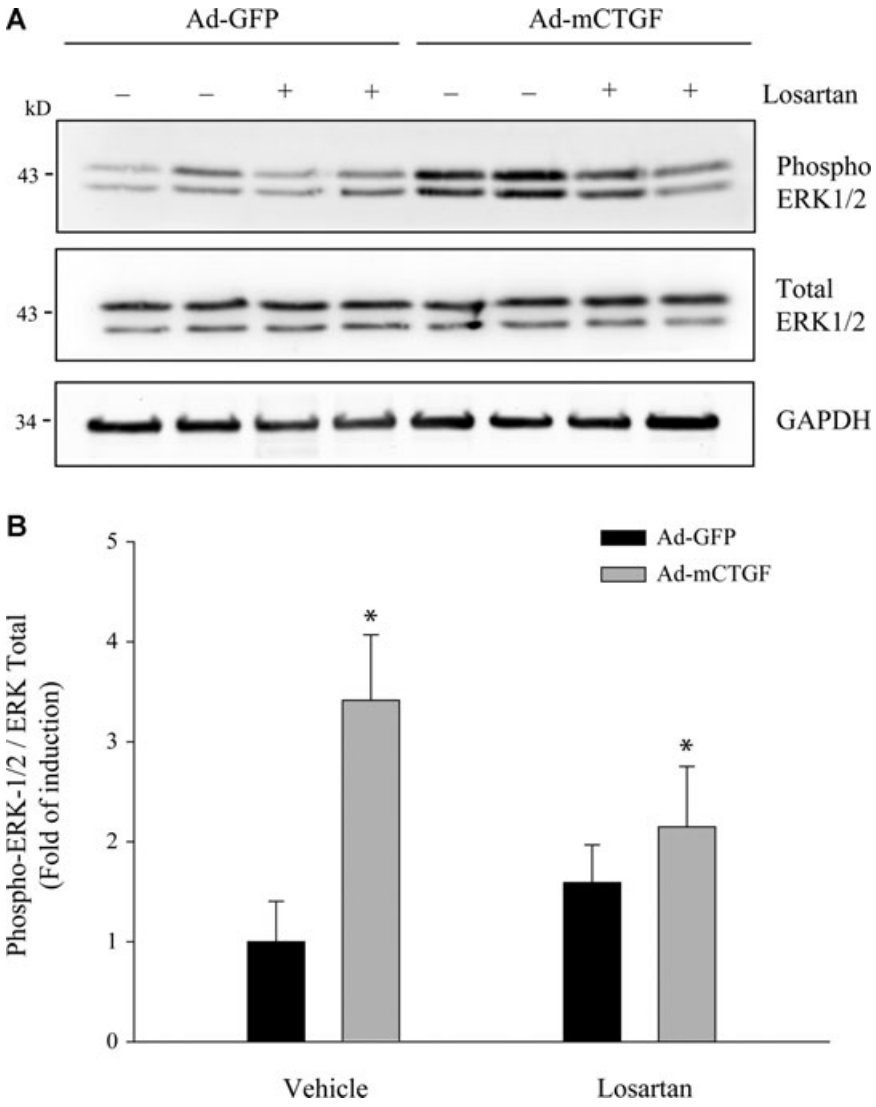

Fig. 5 CTGF-dependent skeletal muscle fibrosis is decreased by blockade AT-1 receptor through a mechanism that involves diminished ERK-1/2 phosphorylation. (**A**) Protein levels of phospho-ERK-1/2 and total ERK-1/2 were detected by Western blot analysis in extracts obtained from TA muscles of C57BL/10 mice systemically treated with vehicle or losartan 5 days post-infection with control (Ad-GFP) or CTGF-overexpressing (Ad-mCTGF) adenoviruses. GAPDH levels are shown as loading control. Molecular weights are shown in kD. (**B**) The graphic shown corresponds to densitometric analysis of Western blots shown in (**A**). Values correspond to the mean ± standard deviation of three independent experiments performed using two mice for each experimental condition ($^*P < 0.05$).

The remarkable fact that ARB decreases CTGF-dependent biological activity opens the question regarding the mechanisms involved in the requirement of AT-1 receptor for the effects induced by CTGF. The possibility that hypotension was involved in the reduction of fibrosis by ARB losartan was discarded because blood pressure was determined in mice treated with losartan without any significant drop compared to mice treated with vehicle (data not shown). This result agrees with previous ones [26]. The AT-1 receptor typically can signal through G-protein–dependent pathways [27]. However, there is evidence that AT-1 receptor can also signal by transactivation of tyrosine kinase receptors [28]. Because CTGF has been described to interact and activate tyrosine kinase receptors [29, 30], this could be a general mechanism through AT-1 receptor would be required for CTGF-dependent signalling and thus explain the effect of ARB on CTGF-mediated effects. Other possibility is that the treatment with ARB of tibialis anterior muscles overexpressing CTGF increases the angiotensin-II levels which are generally thought to stimulate AT-2 receptors that exert opposite effects to AT-1 receptor [31]. Among these effects are the anti-fibrotic actions mediated by AT-2 receptor stimulation which are opposite to CTGF- and AT-1–mediated activity [32]. However, the functions and involvement of AT-2 receptors on CTGF-mediated effects are unknown and requires further research.

The decrease of CTGF-induced ERK-1/2 phosphorylation mediated by ARB can be explained because the activated AT-1 receptors can couple to an array of intracellular signal transduction pathways including the mitogen-activated protein kinases (MAPK) among them ERK-1/2 [33]. Interestingly, CTGF have been described to activate MAPKs in several fibrotic diseases [34, 35], including ERK-1/2 in skeletal muscle fibrosis [9].

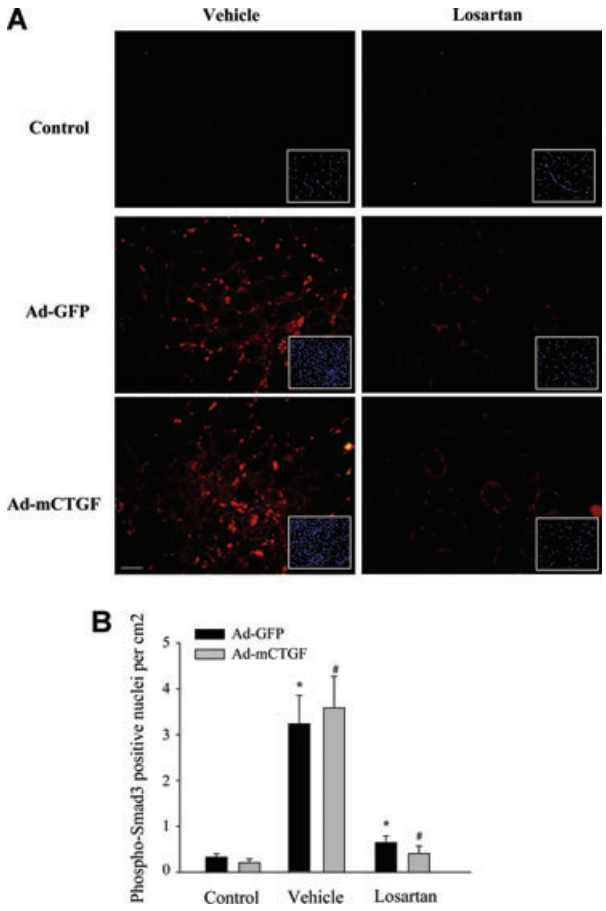

Fig. 6 Induction of TGF-β activity is consequence of the adenoviral infection, but not related to CTGF overexpression. (**A**) Detection of phospho-Smad-3 through IFI analysis in cryosections of tibialis anterior (TA) of C57BL/10 mice systemically treated with vehicle or ARB losartan for 5 days post-infection with control (Ad-GFP) or overexpressing CTGF (Ad-mCTGF). The pictures correspond to 40× (bar corresponds to 200 μm). The insert shown nuclei labelled with Hoechst 33258 (Bar correspond to 50 μm). (**B**) Phospho-Smad-3 positive nuclei quantification of cryosections of TA of C57BL/10 mice treated as in (**A**). Values correspond to the mean ± standard deviation from three independent experiments using two mice for each experimental condition ($^{*,\#}P < 0.05$).

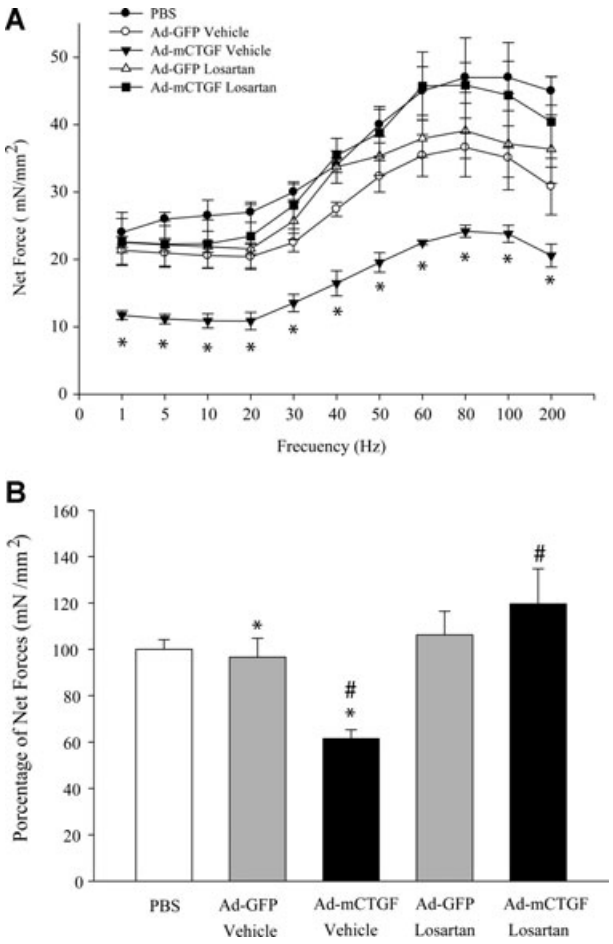

Fig. 7 CTGF-mediated decrease in isometric force of skeletal muscle is prevented by blocking AT-1 receptor. Tibialis anterior (TA) from *mdx* mice injected with PBS or infected with Ad-GFP or Ad-mCTGF for five days and systemically treated with vehicle or losartan were compared for (**A**) isometric specific force (mN/mm^2)-stimulation frequency (Hz) relationship and (**B**) tetanic-specific force measured at 80 Hz. In (**B**), the values are represented as percentage of specific isometric force generated by control muscle. In (**A**) and (**B**), the values corresponds to the mean ± standard deviation from three independent experiments using two mice for each experimental condition ($^{*,\#}P < 0.05$).

Multiple mechanisms that link AT-1 receptors to ERK-1/2 activation have been reported including the release of cytokine and growth factor ligands from cells, direct interaction between AT-1 receptors and upstream activators and scaffolds and the transactivation of tyrosine kinase receptors [33]. Further experiments must be performed to evaluate which is the mechanism whereby ARB decreases the CTGF-mediated ERK-1/2 phosphorylation. In addition, other mechanism by which ARB has been described to modulate skeletal muscle fibrosis is the decrease of TGF-β signalling [18, 36]. Elevated levels of phospho-Smad-3 were found after infecting the muscles with the Ad-GFP or Ad-mCTGF, suggesting that TGF-β signalling increase is a response to the infection procedure rather than to the specific overexpression of CTGF. Interestingly, the increase in phospho-Smad-3 was strongly diminished by ARB pre-treatment in accordance with studies previous [18, 36]. These results suggest that the effects mediated by CTGF *in vivo* are independent of TGF-β signalling. This experimental evidence reinforces the fact that ERK-1/2 signalling is the main pathway involved in the skeletal muscle fibrosis induced by CTGF.

Our results demonstrate that ARB decreased skeletal muscle fibrosis induced by CTGF. We have previously described that

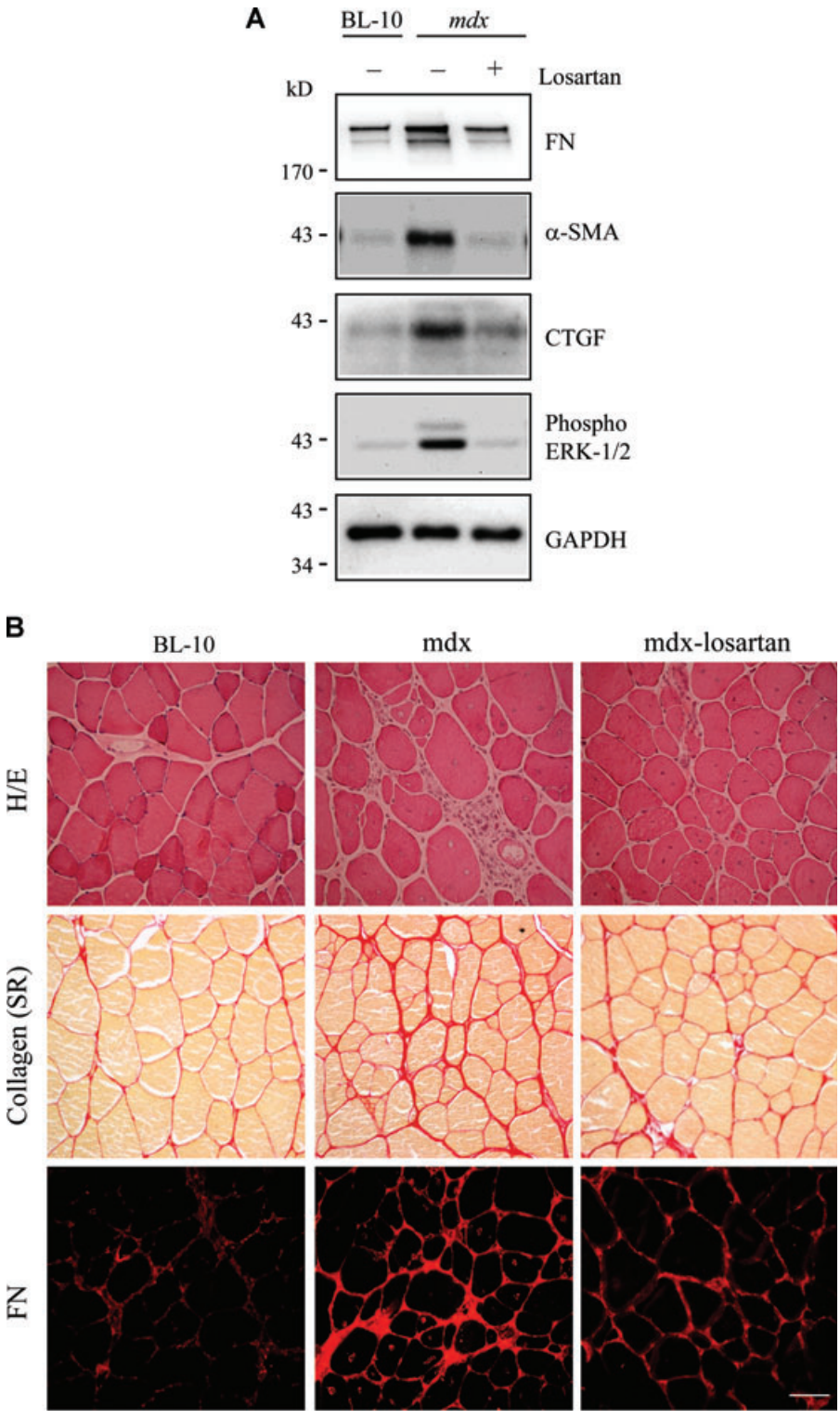

Fig. 8 Blockade of AT-1 receptor diminishes amount of fibrotic proteins, CTGF and ERK-1/2 phosphorylation in a dystrophic skeletal muscle. (**A**) Detection of fibronectin (FN), α-SMA, CTGF and ERK-1/2 phosphorylated levels were determinate by Western blot in TA muscles from normal (BL-10) and dystrophic mice (*mdx*) treated for six months with vehicle (water) or losartan as indicated in Material and methods. GAPDH levels are shown as loading control. Molecular weights are shown in kD. (**B**) Cryosections of TA muscles from normal (BL-10) and dystrophic mice (*mdx*) treated for six months with vehicle (water) or losartan as indicated in Material and methods were analysed for haematoxylin and eosin, Sirius red staining and fibronectin (FN) detected by indirect immunofluorescence analysis. Bar corresponds to 200 μm.

fibroblasts and myoblasts exposed to CTGF contribute to fibrotic state and ECM accumulation in skeletal muscle [9, 37]. Thus, ARB could be affecting the fibrotic response to CTGF of myoblasts and fibroblasts residents in the skeletal muscle explaining the prevention mediated by ARB of phenotipical changes induced by CTGF, such as stress fibres formation in myoblasts, the reduction of the induction in the levels of the typical marker of activated fibroblasts α-SMA, and the decrease of ECM protein levels.

It has been suggested that CTGF by itself might induce an inflammatory response [38, 39], which could precede the deleterious effect seen in skeletal muscle overexpressing CTGF. The experimental approach used in these experiments indicated that ARB inhibits the CTGF-mediated effect, although the levels of CTGF are still elevated, irrespective of the presence of ARB. Moreover, experimental evidence suggests AT-1 blocking has anti-inflammatory effects in different biological models [40]. Therefore, it cannot be rule out that ARB is affecting an inflammatory response triggered by CTGF. Thus, the potential role for CTGF as an inflammatory inducer and ARB as an anti-inflammatory agent requires further research.

Among the functions described for ARB in skeletal muscle is the modulation of injury-induced regeneration and fibrotic responses in several muscle diseases [18, 41]. We have described that levels of embryonic myosin are augmented in muscle overexpressing CTGF [12]. This increase was prevented by treatment of mice with ARB (data not shown), likely to be a reflection of increased skeletal muscle regeneration as a consequence of CTGF-induced damage. The fact that ARB might decrease fibrosis and improve regeneration [18] is especially relevant because skeletal muscle fibrosis not only causes muscle dysfunction and impairs muscle regeneration but also reduces both gene and stem cell delivery, and engraftment efficiency [42]. Our results strongly suggest that ARB, a group of clinically safe drugs administrated in treatment for hypertension, can be used as an effective anti-fibrotic strategy in addition to gene and cell therapies to optimally treat skeletal muscle diseases such as dystrophies. In addition, treatment of mice with ARB resulted in an improvement in skeletal muscle contractile properties decreased by CTGF overexpression. Other treatments, such as suramin, interferon-γ or halofuginone all shown to be anti-fibrotic molecules, also increase skeletal muscle strength [43–45].

Quite remarkable are the results using ARB in a model skeletal muscular dystrophy. Our results indicate that the elevated levels of fibrotic proteins, the pro-fibrotic factor CTGF and phosphor-ERK-1/2 observed in tibialis anterior of *mdx* mice can be strongly reduced by losartan treatment, opening an attractive therapeutic possibility of the use of quite well-characterized ARB in the treatment of dystrophic skeletal muscles.

To summarize, here we show a novel function of ARB inhibiting CTGF-induced damage, fibrosis and decrease in skeletal muscle force. These observations are extended to a dystrophic skeletal muscle model. Together, these results highlight the importance of ARB as a potential anti-fibrotic agent, especially inhibiting CTGF activity in skeletal muscle diseases such as dystrophies.

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Authors' contributions

G.M. and C.C.V. were responsible for the experimental design, carrying out the experiments, analysing and interpreting the data, and they were involved in drafting the manuscript. E.B. was responsible for the experimental design and he was involved in drafting the manuscript for publication. D.C. collaborated in the electrophysiological experiments and interpreting the data. C.V. was involved in blood pressure measurements. C.C.V. was responsible for the experimental design, conceived all the experiments and he was involved in analysing the data and preparing it for publication.

Conflict of interest

The authors confirm that there are no conflicts of interest.

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