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Angiotensin II-induced pro-fibrotic effects require p38MAPK activity and transforming growth factor beta 1 expression in skeletal muscle cells

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ABSTRACT

Fibrotic disorders are typically characterised by excessive connective tissue and extracellular matrix (ECM) deposition that preclude the normal healing of different tissues. Several skeletal muscle dystrophies are characterised by extensive fibrosis. Among the factors involved in skeletal muscle fibrosis is angiotensin II (Ang-II), a key protein of the renin-angiotensin system (RAS). We previously demonstrated that myoblasts responded to Ang-II by increasing the ECM protein levels mediated by AT-1 receptors, implicating an Ang-II-induced reactive oxygen species (ROS) by a NAD(P)H oxidase-dependent mechanism.

In this paper, we show that in myoblasts, Ang-II induced the increase of transforming growth factor beta 1 (TGF-β1) and connective tissue growth factor (CTGF) expression through its AT-1 receptor. This effect is dependent of the NAD(P)H oxidase (NOX)-induced ROS, as indicated by a decrease of the expression of both pro-fibrotic factors when the ROS production was inhibited via the NOX inhibitor apocynin. The increase in pro-fibrotic factors levels was paralleled by enhanced p38MAPK and ERK1/2 phosphorylation in response to Ang-II. However, only the p38MAPK activity was critical for the Ang-II-induced fibrotic effects, as indicated by the decrease in the Ang-II-induced TGF-β1 and CTGF expression and fibronectin levels by SB-203580, an inhibitor of the p38MAPK, but not by U0126, an inhibitor of ERK1/2 phosphorylation. Furthermore, we showed that the Ang-II-dependent p38MAPK activation, but not the ERK1/2 phosphorylation, was necessary for the NOX-derived ROS. In addition, we demonstrated that TGF-β1 expression was required for the Ang-II-induced pro-fibrotic effects evaluated by using SB-431542, an inhibitor of TGF-βRI kinase activity, and by knocking down TGF-β1 levels by shRNA technique.

These results strongly suggest that the fibrotic response to Ang-II is mediated by the AT-1 receptor and requires the p38MAPK phosphorylation, NOX-induced ROS, and TGF-β1 expression increase mediated by Ang-II in skeletal muscle cells.

1. Introduction

Angiotensin II (Ang-II), the main effector of the renin angiotensin system (RAS), is involved primarily in the regulation of blood pressure. However, Ang-II has been associated with the genesis and progression of a fibrotic disorder in several tissues (Bataller et al., 2005; Brecher, 1996; Guo et al., 2001). Thus, an increase in Ang-II levels has been linked to the pathogenesis of fibrotic disorders in tissues such as the liver, cardiac muscle, and kidney (Bataller and Brenner, 2005; Brecher, 1996; Guo et al., 2001). Ang-II is a potent inducer of extracellular matrix (ECM) proteins such as type III collagen and fibronectin through the angiotensin II receptor type 1 (AT-1

Abbreviations: Ang-II, angiotensin-II; AT-1, angiotensin-II receptor type 1; ARB, angiotensin II receptor type I blocker; CTGF, connective tissue growth factor; DMD, Duchenne muscular dystrophy; ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; NAC, N-acetyl cystein; NOX, NAD(P)H oxidase; ROS, reactive oxygen species; TGF-β, transforming growth factor type beta.

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receptor) in several tissues and cell types, among them skeletal muscle cells (Seccia et al., 2003; Tarif and Bakris, 1997; Lakshmanan et al., 2011). One of the mechanisms by which Ang-II induces and maintains fibrosis is an increase of pro-fibrotic factor expression, as in the transforming growth factor type beta 1 (TGF-β1) and the connective tissue growth factor (CTGF) (Che et al., 2008; Iwanciw et al., 2003; Morales et al., 2011; Kupfahl et al., 2000; Cohn et al., 2007).

There is evidence that Ang-II activates the MAPK members, among them p38MAPK (de Boer et al., 2004; Kiribayashi et al., 2005), which is essential for the regulation of many cellular processes, including inflammation, cell differentiation, cell growth, and cell death. p38MAPK mediates the signals that are relevant to the development of fibrosis associated with a dysfunction in the heart, smooth muscle, retina, and kidney (Ma et al., 2009; Parsons et al., 2007; Clark et al., 2007).

Skeletal muscle fibrosis is developed in several skeletal muscle diseases such as Duchenne muscular dystrophy (DMD) and in its murine model, *mdx* mice (Mezzano et al., 2007; Fadic et al., 2006). Interestingly, pro-fibrotic factors CTGF and TGF-β1 have been involved in the fibrotic phenotype of the murine model of DMD (Zhou et al., 2006; Vial et al., 2008). Components of the RAS axis such as the angiotensin I-converting enzyme (ACE) and AT-1 receptor increase in the dystrophic skeletal muscle (Sun et al., 2009). This fact, in addition to evidence that shows the regulatory effect of the AT-1 receptor blocker (ARB) on TGF-β1 and CTGF activity, suggests that the local classical RAS axis is activated in the dystrophic skeletal muscle and contributes to the fibrotic phenotype of this disease (Cabello-Verrugio et al., 2012; Morales et al., 2011). Therefore, it is important to evaluate the mechanism through which Ang-II modulates skeletal muscle fibrosis. Previously, we demonstrated that Ang-II induced skeletal muscle fibrosis via the AT-1 receptor by a NAD(P)H oxidase (NOX)-derived reactive oxygen species (ROS)-dependent mechanism (Cabello-Verrugio et al., 2011a). In addition, it has recently been reported that NOX protein levels and activity are increased in the *mdx* muscle, which are a major source of ROS production in dystrophic muscles (Whitehead et al., 2010).

In the present study, we delved deeper into the mechanism involved in Ang-II-induced skeletal muscle fibrosis. We demonstrated in myoblasts that Ang-II induced an increase in the expression of pro-fibrotic factors TGF-β1 and CTGF through its AT-1 receptor by a mechanism dependent on the NOX-derived ROS generation. Ang-II also activated p38 and ERK1/2 MAPK through the AT-1 receptor, but only the p38MAPK activity was critical for the Ang-II-dependent pro-fibrotic effects that require NOX-derived ROS production. Interestingly, the Ang-II-dependent fibronectin and CTGF increase is dependent on the TGF-β1 induction mediated by Ang-II. Thus, our results expand and complement the insight into the mechanisms involved in Ang-II-induced fibrosis in skeletal muscle cells.

2. Materials and methods

2.1. Cell cultures

The skeletal muscle cell line C_2C_{12}, obtained from an adult mouse leg (American Type Culture Collection) was grown as described previously (Cabello-Verrugio and Brandan, 2007). The cells were serum-starved for 18 h and then subjected to different treatments. For the Ang-II treatment, the myoblasts were incubated for 48 h with Ang-II (500 nM) (Sigma, USA). For treatment with the AT-1 and AT-2 receptor blockers, the myoblasts were pre-incubated for 1 h with losartan (10 μM) and PD-123319 (10 μM) (both from Tocris Bioscience, USA), respectively, and subsequently incubated with Ang-II, as indicated in the figures. For treatment with reducing agents, the cells were pre-incubated for 1 h with N-acetyl cysteine (NAC) (10 mM, Sigma, USA) in the presence or absence of Ang-II (500 nM) for 48 h. For the NAD(P)H oxidase inhibition, the cells were pre-incubated for 1 h with the inhibitor apocynin (1 mM, Sigma, USA) and subsequently incubated with Ang-II (500 nM) for 48 h. For the inhibition of p38MAPK, the activity cells were incubated with SB-203580 (5 μM) (Alomone, Israel); for the inhibition of ERK1/2, phosphorylation cells were incubated with U0126 (5 μM, an inhibitor of MEK) (Alomone, Israel); and for the inhibition of TGF-β, signalling cells were incubated with SB-431542 (10 μM, an inhibitor of TGF-β receptor I kinase activity) (Tocris Bioscience, USA).

2.2. Short hairpin RNA transfection

The short hairpin RNA (shRNA) specific for the TGF-β1 mouse (Addgene plasmid 14973) was obtained from Addgene (Thomas and Massague, 2005). The TGF-β1 or control shRNA were transfected using the LipofectAMINE 2000 method (Invitrogen, USA). Following transfection, the FBS was added to the medium, and cells were cultured for 24 h. After that, treatment with Ang-II and/or inhibitors was performed for the lengths of time described above, and the extracts were obtained for the RT-qPCR, ELISA, or immunoblot analysis.

2.3. Measurement of intracellular ROS production by flow cytometry

The treated C_2C_{12} cells were harvested with trypsin/EDTA, washed twice in ice-cold PBS, resuspended, and loaded with one of two cell permeate dyes—dichlorodihydrofluorescein (DCF, 5 μM) or dihydroethidium (DHE, 10 μM) (both from Invitrogen, USA)—for ROS determination for 15–30 min in the dark at room temperature. They were then analysed immediately using a flow cytometry system (FACSCanto, BD Biosciences, USA). A minimum of 10,000 cells were analyzed per sample. The cellular intensity of the dyes was analyzed using FACSDiva software v4.1.1 (BD Biosciences, USA).

2.4. RNA isolation, reverse transcription, and quantitative real-time PCR

The myoblasts were serum-starved for 18 h and then incubated for different times with Ang-II (500 nM). The total RNA was isolated from the cell cultures at the times indicated in the figures using Trizol (Invitrogen, USA), according to the manufacturer's instructions. The total RNA (1 μg) was reverse-transcribed to cDNA using random hexamers and superscript reverse transcriptase (Invitrogen, USA). Taqman quantitative real-time PCR reactions were performed twice on a Stratagene MX 3005 Termocyler (Agilent Technology, USA), using predesigned primer sets for the TGF-β1 and CTGF mice as well as the GAPDH housekeeping gene (Taqman Assays-on-Demand, Applied Biosystems, USA). The mRNA expression was quantified using the comparative ΔCt method ($2^{-\Delta\Delta Ct}$), using GAPDH as the reference gene. The mRNA levels are expressed relative to the mean expression in the control group. The values correspond to the mean of the ΔCt value ± standard deviation (SD) of the three independent experiments.

2.5. Immunoblot analysis

For the cell immunoblot analyses, the protein extracts from the myoblasts were prepared in 50 mM Tris–HCl, pH 7.4, 0.1 M NaCl, and 0.5% Triton X-100 with a cocktail of protease inhibitors, 1 mM PMSF, and a cocktail of phosphatase inhibitors. The aliquots were subjected to the SDS-PAGE in 10% polyacrylamide gels, electrophoretically transferred onto PDVF membranes (Millipore,

USA), and probed with rabbit anti-fibronectin, mouse anti-tubulin (Sigma–Aldrich, USA), goat anti-CTGF (Santa Cruz, USA), rabbit anti-total ERK1/2, rabbit anti-phospho ERK1/2, rabbit anti-total p38MAPK, and rabbit anti-phospho p38MAPK (Cell signaling, USA). All immunoreactions were visualised by enhanced chemiluminescence (Thermo Scientific, USA).

2.6. Enzyme-linked immunosorbent assay

A TGF-β1 ELISA assay was performed to determine the TGF-β1 levels secreted to the medium in C_2C_{12} that was exposed to Ang-II under different conditions, following the manufacturer's protocol (TGF-β1 EIA kit, Enzo Life Science, USA). The results were normalised by protein amount and were expressed as percentage of Ang-II-induced TGF-β1 levels.

2.7. Protein determination

The proteins were determined in aliquots of cell extracts using the bicinchoninic acid protein assay kit (Thermo Scientific, USA), with BSA as the standard.

2.8. Statistics

Statistical significance was evaluated using one-way analysis of variance (ANOVA) with a post hoc Bonferroni multiple comparison test (Sigma Stat 3.5 Software). A difference was considered statistically significant at a P-value <0.05.

3. Results

3.1. The angiotensin II-induced expression of pro-fibrotic factors is AT-1 receptor dependent in skeletal muscle cells

Since angiotensin II (Ang-II) contributes to a fibrotic response in skeletal muscle cells (Cabello-Verrugio et al., 2011a), we decided to study the effect of Ang-II on the expression of pro-fibrotic factors such as TGF-β1 and CTGF. To evaluate the kinetic of the Ang-II-dependent expression of these factors, the C_2C_{12} cells were incubated with Ang-II, and their expression was evaluated by RT-qPCR assays. Fig. 1A shows an early increase in the Ang-II-dependent TGF-β1 expression, reaching a peak after 1 h of Ang-II treatment and decreasing to basal levels 12 h after the Ang-II treatment. Fig. 1B shows that the induction of the Ang-II-dependent CTGF expression started in 3 h, showing a peak 6 h after the Ang-II treatment. Since the Ang-II-induced fibrosis is mediated by its transmembrane receptor AT-1 in skeletal muscle cells (Cabello-Verrugio et al., 2011a), we evaluated its participation in the Ang-II-dependent expression of pro-fibrotic factors in skeletal muscle cells. Fig. 1C and D, respectively, show that the increase in the TGF-β1 and CTGF expression induced by Ang-II in the C_2C_{12} myoblasts was prevented when the cells were pre-incubated with the Ang-II receptor type 1 blocker (ARB) losartan, but no effect was observed when the cells were pre-incubated with the blocker receptor of the Ang-II receptor type 2 PD-123319. Similar results were obtained when the TGF-β1 and CTGF protein levels were determined by ELISA and immunoblot assays, respectively (Supplementary Fig. 1).

3.2. These results indicate that Ang-II increases TGF-β1 and CTGF expression through an AT-1 receptor-dependent mechanism

Pro-fibrotic factor expression induced by angiotensin II is dependent on NAD(P)H oxidase-induced reactive oxygen species (ROS) in skeletal muscle cells.

Since we previously demonstrated that the Ang-II-dependent NOX-induced ROS participates in the generation and development of fibrosis in skeletal muscle cells, we evaluated whether the increase of the TGF-β1 and CTGF expression induced by Ang-II is also mediated by this mechanism. The TGF-β1 and CTGF mRNA levels were determined in cells treated with Ang-II and pre-incubated in the presence or absence of apocynin, an inhibitor of the NOX activity (Becerra et al., 2011; Nunez-Villena et al., 2011; Simon and Fernandez, 2009). Fig. 2 shows that the inhibition of the NOX activity by apocynin completely prevented TGF-β1 (Fig. 2A) and CTGF (Fig. 2B) expression in response to Ang-II. In addition, apocynin decreased the Ang-II dependent TGF-β1 (Fig. 2C) and CTGF (Fig. 2D) protein levels.

We previously demonstrated that NOX produced an increase of intracellular ROS induced by Ang-II (Cabello-Verrugio et al., 2011a). To assay the role of the NOX-induced ROS under treatment with Ang-II on the pro-fibrotic factors' expression, we incubated the cells with Ang-II in the absence or presence of the antioxidant N-acetyl cysteine (NAC). Fig. 2A and B shows that the NAC decreased the TGF-β1 and CTGF expression induced by Ang-II, respectively. Similar results were obtained for the Ang-II-induced TGF-β1 and CTGF protein levels (Fig. 2C and D).

Together, these results suggest that Ang-II induces TGF-β1 and CTGF expression via a mechanism involving the NOX-induced ROS.

3.3. Angiotensin II induces p38 and ERK1/2 MAPK phosphorylation through the AT-1 receptor in skeletal muscle cells

Evidence indicates that Ang-II induces MAPK phosphorylation (Kiribayashi et al., 2005; Li et al., 2011; Lakshmanan et al., 2012). We studied the effect of Ang-II on the MAPKs' phosphorylation in skeletal muscle cells. Fig. 3A shows that the kinetic of the p38MAPK phosphorylation increased, with a first peak between 2.5 and 7.5 min, reaching basal levels in 10 min. A second peak of the p38MAPK phosphorylation was observed at 12 and 18 h after the Ang-II incubation. When the ERK1/2 phosphorylation was evaluated, an early activation was observed with a peak between 2.5 and 7.5 min and a decrease to basal levels at later times. Interestingly, both the Ang-II-induced p38MAPK phosphorylation (5 min and 18 h) and ERK1/2 phosphorylation (5 min) were prevented when the cells were pre-incubated with ARB losartan (Fig. 3C and D, respectively), indicating that the p38MAPK and ERK1/2 phosphorylation induced by Ang-II is mediated by the AT-1 receptor.

3.4. Angiotensin II-induced p38MAPK phosphorylation is dependent on the NAD(P)H oxidase-induced ROS in skeletal muscle cells

Since Ang-II activates the production of the NOX-induced ROS and p38MAPK, we evaluated the relationship between these two signalling pathways.

First, we treated the C_2C_{12} myoblasts with Ang-II in the absence or presence of SB-203580, an inhibitor of p38MAPK activity; subsequently, the intracellular ROS production in response to Ang-II was determined by flow cytometry using two different ROS-sensitive fluorescent probes, the DCF and DHE. Fig. 4A and B shows that the increase in the NOX-induced ROS production mediated by Ang-II does not change when the cells are incubated with the p38MAPK activity inhibitor. In the same figures, we observed that the ROS decreased to basal levels, as shown by the control cells when we treated the cells with Ang-II in the presence of an inhibitor of NOX activity (apocynin), such as we have previously demonstrated (Cabello-Verrugio et al., 2011a). Interestingly, we observed that the cells treated with Ang-II, together with the inhibitor of the MEK activity UO-126, which avoid the ERK1/2 phosphorylation, did not change the ROS production, confirming that the ERK1/2 activity is

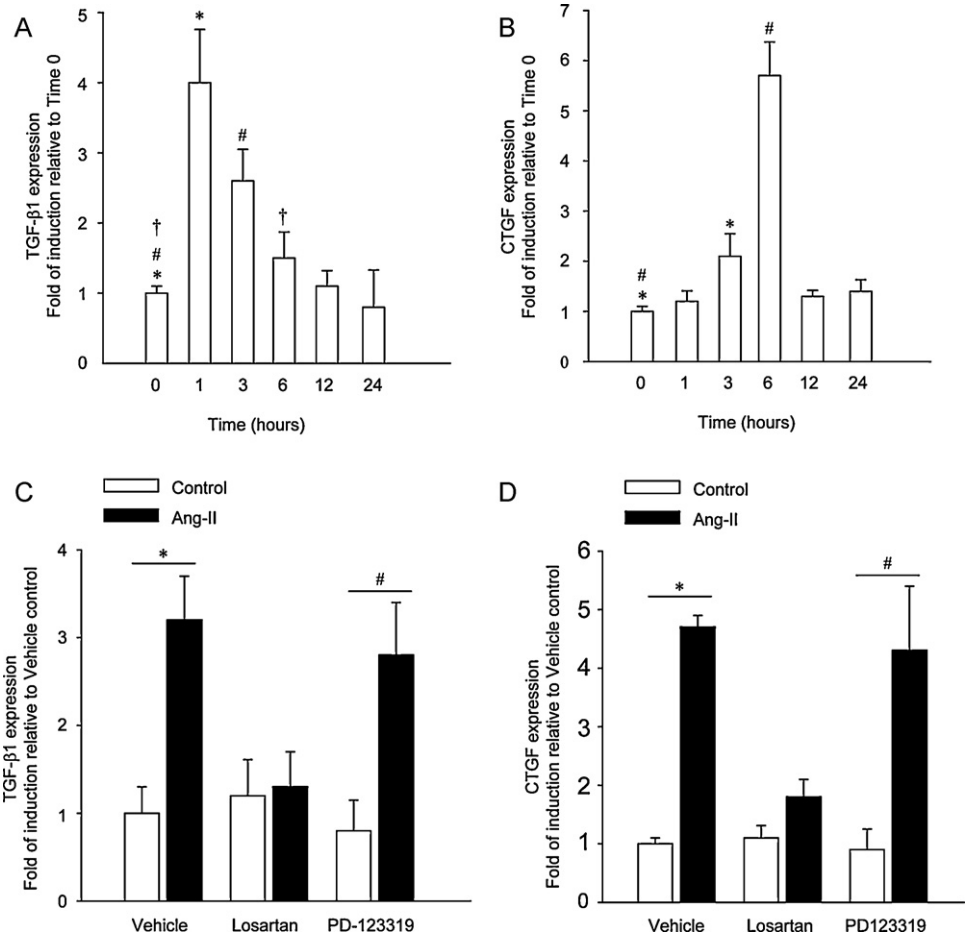

Fig. 1. Angiotensin II induces TGF-β1 and CTGF expression via an AT-1-dependent mechanism in skeletal muscle cells. The C_2C_{12} myoblasts were incubated with Ang-II (500 nM) for the times indicated in the figure. TGF-β1 (A) and CTGF (B) expression was determined by the RT-qPCR as explained in Section 2. The values correspond to the mean of the ΔCt value ± standard deviation of the three independent experiments. ($^{*,\#,\dagger}P < 0.05$). The C_2C_{12} cells were pre-incubated with the vehicle, losartan (an ARB, 10 μM) or PD-123319 (an AT-2 blocker, 10 μM), and then incubated with Ang-II (500 nM) for 1 h for TGF-β1 expression (C) and 6 h for CTGF expression (D). TGF-β1 and CTGF expression was determined by the RT-qPCR as explained in Section 2. The values correspond to the mean of the ΔCt value ± standard deviation of the three independent experiments ($^{*,\#}P < 0.05$).

not related to the Ang-II induced pro-fibrotic effects depending on the NOX-induced ROS.

To test whether the NOX-induced ROS are necessary to phosphorylate and activate the p38MAPK in response to Ang-II, we incubated the cells with Ang-II in the absence or presence of apocynin, and we detected the p38MAPK phosphorylated levels through a Western blot analysis. Fig. 4C shows that the inhibition of the NOX-induced ROS, when the cells were incubated with apocynin, decreased the p38MAPK phosphorylation induced by Ang-II at 5 min and 18 h. The quantitative analysis shown in Fig. 4D indicates that the inhibition of the NOX-induced ROS generation using apocynin decreased the Ang-II-dependent p38MAPK phosphorylation in the skeletal muscle cells. In addition, Ang-II-induced ERK1/2 phosphorylation was decreased by apocynin, indicating that it was dependent on the NOX activity (Fig. 4E and F).

These results suggest that Ang-II-induced p38MAPK phosphorylation requires NOX-induced ROS.

3.5. Angiotensin II-induced p38MAPK activity, but not ERK1/2 activity, is required for the induction of the pro-fibrotic factor and fibronectin expression in skeletal muscle cells

Since the Ang-II-dependent p38MAPK and ERK1/2 phosphorylation is mediated by the same receptor that participates in the pro-fibrotic effects of Ang-II, and both required the NOX activity, we tested whether or not the p38MAPK and ERK1/2 activities are required for the expression of pro-fibrotic factors TGF-β1 and CTGF as well as fibronectin mediated by Ang-II. To do so, the cells were incubated with the inhibitors of the p38MAPK (SB-203580) and ERK1/2 (UO-126) activities. Fig. 5A and B shows that the p38MAPK activity, but not the ERK1/2 activity, is involved in the expression of the TGF-β1 and CTGF mediated by Ang-II. Similar results were obtained for TGF-β1 and CTGF protein levels induced by Ang-II (Fig. 5C and D). In accordance with this observation, the induction of fibronectin in response to Ang-II was decreased by the p38MAPK inhibitor, but not for the ERK1/2 activity inhibitor, as shown in Fig. 5E. Fig. 5F shows the quantitative analysis of the experiments shown in Fig. 5E.

Since it has been recently demonstrated that SB-202109, another p38MAPK inhibitor, induces the phosphorylation of p38MAPK when used for a long period of time (Wouwer et al., 2012), we evaluated the effect of SB-203580 on p38MAPK phosphorylation. To test this possibility, C_2C_{12} cells were exposed to SB-203580 for different times, up to 48 h. Western blot analysis showed that this inhibitor does not activate p38MAPK phosphorylation (Supplementary Fig. 2).

Together, the results indicate that p38MAPK activity is required for an increase in the expression of pro-fibrotic factors and fibronectin induced by Ang-II.

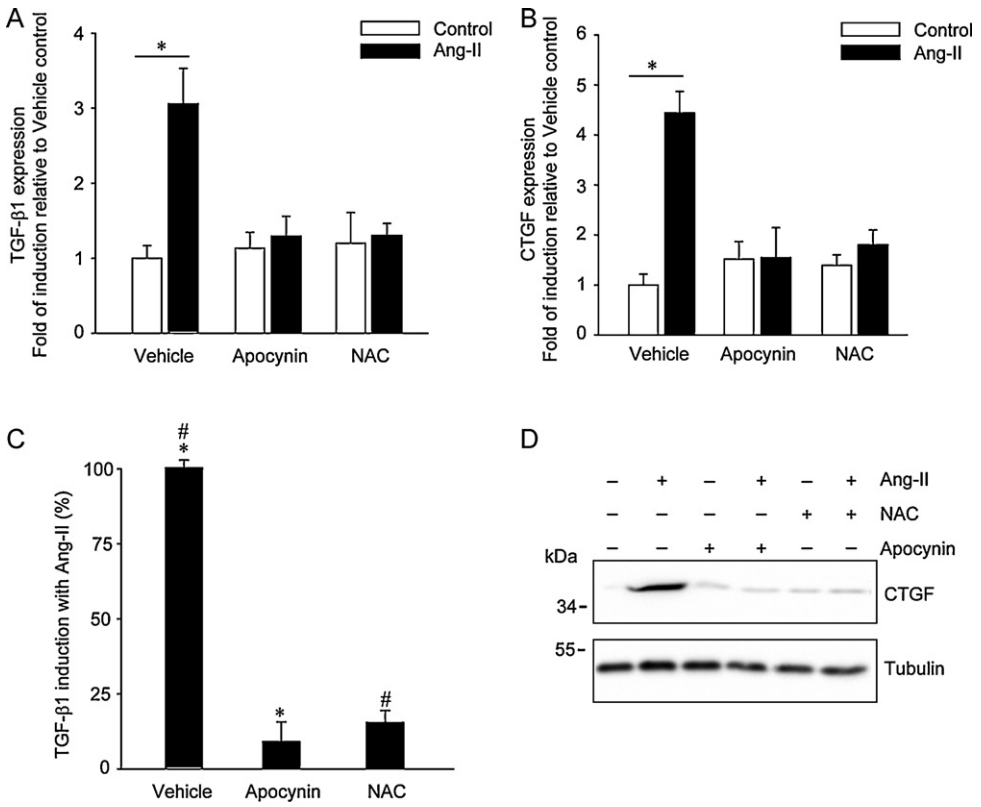

Fig. 2. Angiotensin II-induced TGF-β1 and CTGF expression requires NOX-induced ROS in skeletal muscle cells. The C_2C_{12} cells were pre-incubated with the vehicle, N-acetyl cysteine (NAC, 10 mM) or apocynin (a NOX inhibitor, 1 mM), and then incubated with Ang-II (500 nM) for 1 h for TGF-β1 expression (A) and 6 h for CTGF expression (B). TGF-β1 and CTGF expression was determined by the RT-qPCR as explained in Section 2. The values correspond to the mean of the ΔCt value ± standard deviation of the three independent experiments (*$P < 0.05$). (C) Detection by ELISA of TGF-β1 protein levels into the medium of C_2C_{12} exposed to Ang-II (500 nM) for 48 h in the absence (vehicle) or presence of apocynin (1 mM) or NAC (10 mM). The values are expressed as a percentage of the TGF-β1 levels secreted by C_2C_{12} cells incubated with Ang-II and the vehicle and are representative of three independent experiments (*,#$P < 0.05$). (D) CTGF protein levels evaluated by Western blot analysis in an extract of a C_2C_{12} cell incubated with Ang-II (500 nM) for 48 h in the absence (vehicle) or presence of apocynin (1 mM) or NAC (10 mM). The levels of tubulin are shown as loading control. The images are representative of the three independent experiments. The molecular weight standards are indicated in kilo Daltons (kDa).

3.6. Angiotensin II-induced TGF-β1 expression is required for late p38MAPK phosphorylation and pro-fibrotic effects mediated by angiotensin II in skeletal muscle cells

Since Ang-II induces TGF-β1 expression, we evaluated if the pro-fibrotic effects mediated by Ang-II are dependent on the TGF-β1 induction. For that, the C_2C_{12} cells were incubated with Ang-II in the absence or presence of an inhibitor of the TGF-β receptor I (TGF-βRI) kinase activity SB-431542 to inhibit the TGF-β signalling (Cabello-Verrugio et al., 2011b), and the p38MAPK phosphorylation was evaluated by Western blot analysis. Fig. 6A shows that the inhibition of the TGF-β1 signalling by SB-431542 did not have any effect on the early p38MAPK phosphorylation induced by Ang-II at 5 min, but completely inhibited its phosphorylation at 18 h.

To corroborate these results, we decreased the TGF-β1 levels using a specific shRNA for TGF-β1. The Ang-II-dependent TGF-β1 expression (Supplementary Fig. 3A) and protein levels (Supplementary Fig. 3B) were significantly decreased by transfection of the specific shRNA for TGF-β1 compared to cells transfected with the control shRNA. Under these conditions, we determined the p38MAPK phosphorylation mediated by Ang-II. Fig. 6B shows that the Ang-II-induced p38MAPK phosphorylation is inhibited in cells transfected with the shRNA for TGF-β1 at 18 h after the Ang-II incubation. However, it did not have any effect on its phosphorylation at 5 min.

Further, we evaluated the participation of the TGF-β1 signalling on the Ang-II-induced fibronectin and CTGF levels. Fig. 6C shows that the Ang-II-induced fibronectin and CTGF protein levels decreased in cells treated with the inhibitor of TGF-βRI kinase activity (SB-431542). Fig. 6D shows the observation that the decrease of the TGF-β1 levels by a specific shRNA for TGF-β1 diminished the Ang-II-induced fibronectin and the CTGF protein levels compared with cells transfected with a control shRNA.

Together, these results suggest that the pro-fibrotic effects mediated by Ang-II require the increase of TGF-β1 expression and its signalling.

4. Discussion

In this report, we show that the expression of CTGF and TGF-β1, two potent pro-fibrotic factors involved in skeletal muscle fibrosis, is induced by Ang-II in skeletal muscle cells by an AT-1 receptor-dependent mechanism involving p38MAPK NOX-derived ROS and TGF-β1 expression and signalling (Fig. 7). Moreover, we demonstrated that p38MAPK is critical not only to CTGF and TGF-β1 expression increases, but also to ECM protein induction mediated by Ang-II (Fig. 7). Interestingly, we have demonstrated that the pro-fibrotic effects induced by Ang-II are dependent on TGF-β1 expression and signalling, supported by the decrease of CTGF and fibronectin expression when an inhibitor of TGF-βRI kinase activity and a specific shRNA for TGF-β1 was used (Fig. 7).

The participation of Ang-II in the fibrotic phenotype in skeletal muscle was previously demonstrated by our group

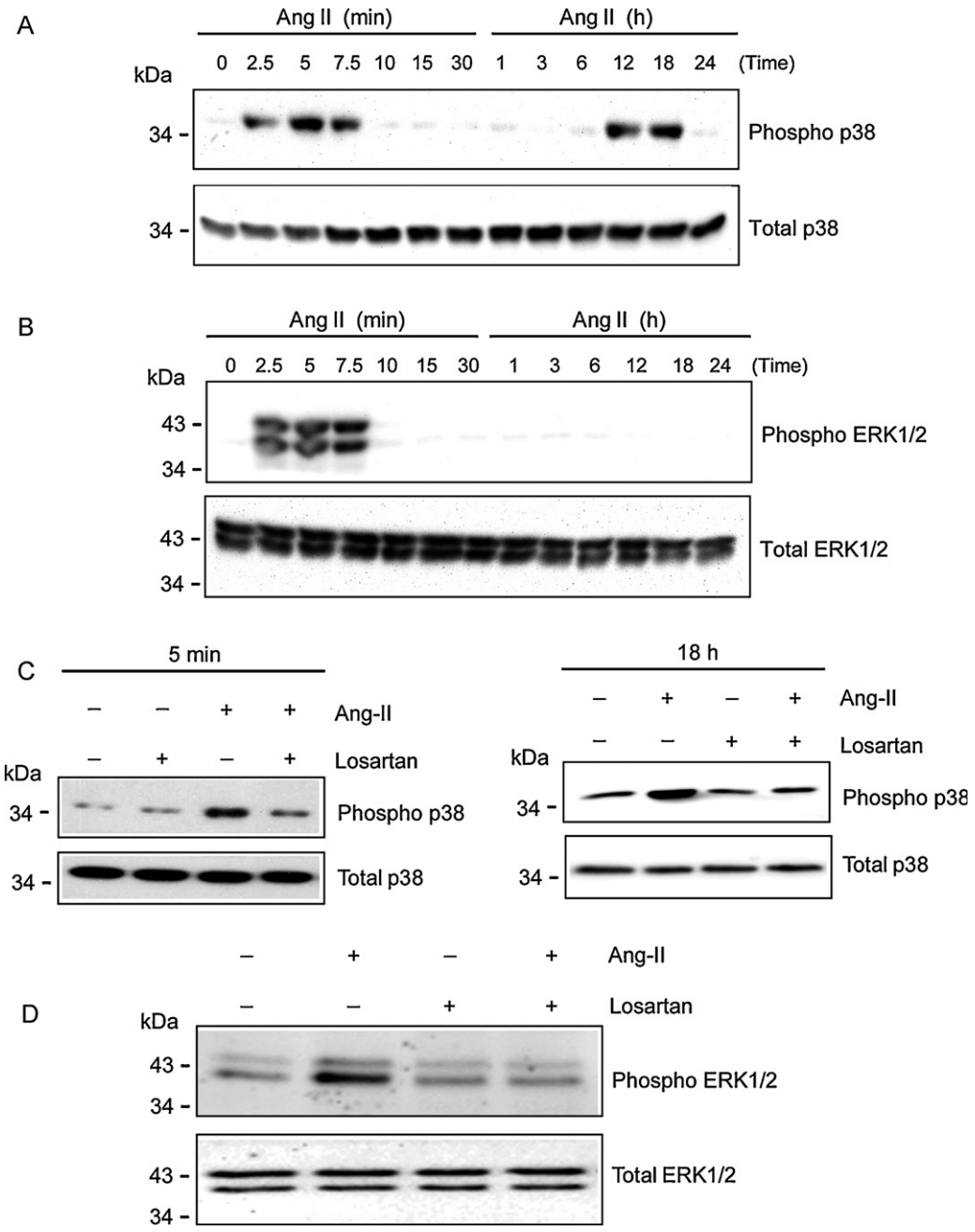

Fig. 3. Angiotensin II induces the phosphorylation of p38 and ERK-1/2 MAPK via the AT-1 receptor in skeletal muscle cells. (A) The C_2C_{12} myoblasts were incubated with Ang-II (500 nM). After the times indicated in the figure, the obtained extracts were separated by SDS-PAGE, and levels of the p38MAPK phosphorylated and p38MAPK total were evaluated by Western blot analysis. (B) The C_2C_{12} cells were treated as described in (A). ERK1/2-phosphorylated and ERK1/2 total protein levels were evaluated by Western blot analysis. To evaluate the participation of the AT-1 receptor in the activation of the MAPK mediated by Ang-II, the cells were pre-incubated with losartan (an AT-1 blocker, 10 μM) and then incubated with Ang-II (500 nM). The protein levels of the p38MAPK phosphorylated, p38MAPK total (C), ERK1/2 phosphorylated, and ERK1/2 total (D) were evaluated by Western blot. In A–D, the images are representative of the two independent experiments. The molecular weight standards are indicated in kilodaltons (kDa).

(Cabello-Verrugio et al., 2011a). However, the relationship between Ang-II and the expression of TGF-β1 and CTGF with the mechanism involved has not been studied in skeletal muscle fibrosis (Cabello-Verrugio et al., 2012; Morales et al., 2011; Cabello-Verrugio and Brandan, 2007).

Ang-II increases CTGF and TGF-β1 expression by several different mechanisms depending on cell types. Ang-II was found to stimulate TGF-β1 expression in rat heart endothelial cells blocked by losartan in a dose- and time-dependent manner (Chua et al., 1994). In human mesangial cells (HMCs), the induction of ECM proteins (fibronectin) and TGF-β production by Ang-II is mediated by the p38MAPK activation (Bolick et al., 2003). In cardiac myofibroblasts, the TGF-β1 production and secretion can be modulated by the specific Ang-II receptor blockers, suggesting the participation of Ang-II as the inducer of this fibrotic growth factor (Campbell and Katwa, 1997). On the other hand, CTGF has been demonstrated to be induced by Ang-II in an AT-1-dependent manner (Ahmed et al., 2004; Cabello-Verrugio et al., 2011a; Iwamoto et al., 2010). Moreover, pathways dependent on calcineurin-, PPARγ-, PKC-, or serum- and glucocorticoid-inducible kinase SGK1 have been described to

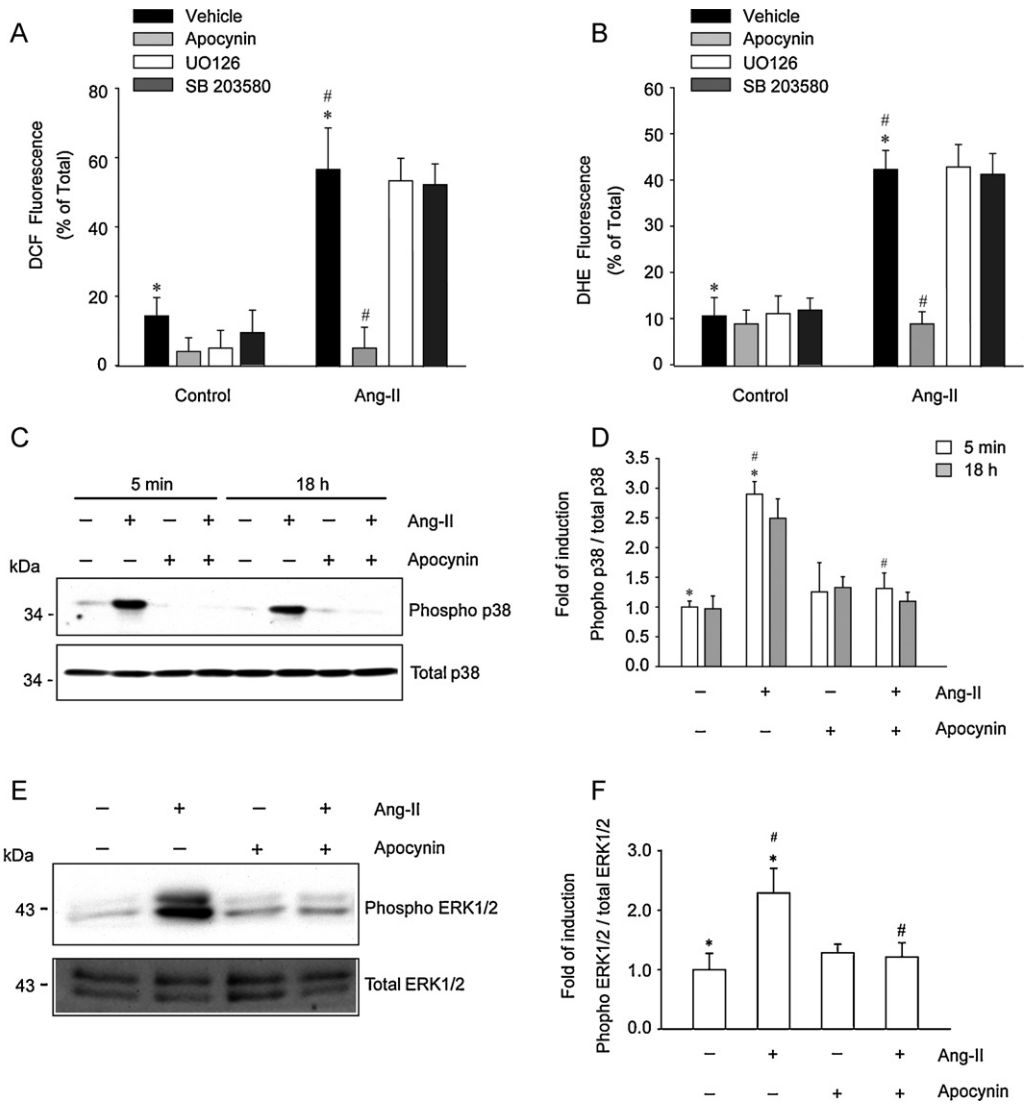

Fig. 4. p38MAPK phosphorylation induced by angiotensin II is dependent on NOX activity in skeletal muscle cells. C_2C_{12} cells were exposed to the vehicle alone—apocynin (an inhibitor of NOX, 1 mM), SB-203580 (an inhibitor of p38MAPK activity, 5 μM), or UO126 (an inhibitor of MEK, 5 μM)—in the absence (control) or presence of Ang-II (500 nM); DCF (A) or DHF (B) fluorescence from the three independent experiments was measured. The bars represent the percentage (mean ± standard deviation) ($^{*,\#}P < 0.05$). (C) The myoblasts were pre-incubated with the vehicle or apocynin (1 mM) and then were incubated with the Ang-II (500 nM) for 5 min or 18 h. The protein levels of the p38MAPK phosphorylated and p38MAPK total were evaluated by Western blot. The molecular weights are indicated in kilodaltons (kDa). (D) Quantification of three independent experiments representative of C ($^{*,\#}P < 0.05$). (E) The myoblasts were treated as described in C, and the incubation with Ang-II was for 5 min. The protein levels of the ERK1/2 phosphorylated and ERK1/2 total were evaluated by Western blot. The molecular weight standards are indicated in kilodaltons (kDa). (F) Quantification of three independent experiments representative of E ($^{*,\#}P < 0.05$).

participate in Ang-II-dependent CTGF induction (Finckenberg et al., 2003; Gao et al., 2007; He et al., 2005; Hussain et al., 2008). In cardiac fibroblasts, the Ang-II-induced TGF-β1 expression is mediated by the ERK-1/2 MAPK-dependent mechanism, and the CTGF expression is mediated by the activation of p38MAPK (Li et al., 2011). In our model, we previously demonstrated that CTGF expression is modulated by TGF-β1, lysophosphatidic acid (LPA) through the TGF-β receptor transactivation, and JNK activity (Cabello-Verrugio et al., 2011b). In this paper, our results suggest that the p38MAPK activation induced by Ang-II in skeletal muscle cells is required for the induction of TGF-β1 and CTGF expression. However, the participation of the ERK-1/2 activity induced by Ang-II in other processes occurring in skeletal muscle cells requires further experimental evidence.

Interestingly, there is evidence that Ang-II can cause a rapid activation of Smad signalling independent of the TGF-β and late effects which are Smad-dependent and TGF-β-mediated (Carvajal et al., 2008). In our model, we have observed that Ang-II does not phosphorylate directly to the Smad 2/3 (data not shown). However, the participation of TGF-β1 on the pro-fibrotic effects induced by Ang-II were determined. TGF-β1 expression was required for the Ang-II-induced p38MAPK phosphorylation observed in a second peak after incubation of Ang-II (12–18 h). Ang II-induced fibronectin and CTGF increase was dependent on the stimulation of TGF-β1 expression, as demonstrated using either an inhibitor of TGF-βRI kinase activity or short hairpin RNA (shRNA) for TGF-β1. A similar requirement of TGF-β1 was observed in the effect of Ang-II on PPARγ activity in aortic smooth muscle cells (Subramanian et al., 2012).

Ang-II is not only a fibrotic factor but also a pro-oxidant cytokine in several tissues, including the skeletal muscle (Cabello-Verrugio et al., 2011a; Cozzoli et al., 2011). Interestingly, the

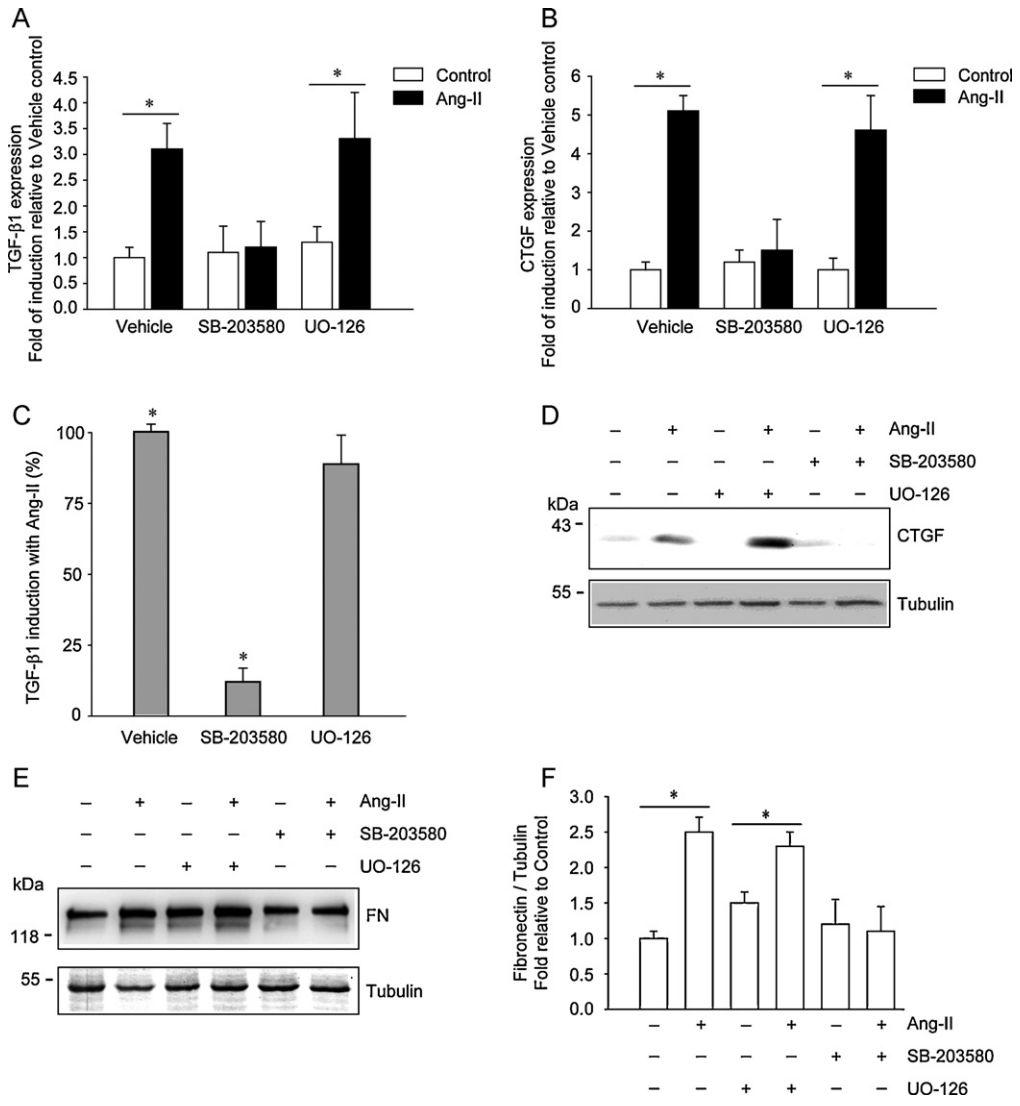

Fig. 5. p38MAPK, but not ERK1/2 activity, is required for angiotensin II-induced pro-fibrotic factor expression and fibrosis in skeletal muscle cells. The C_2C_{12} cells were pre-incubated with the vehicle, SB-203580 (an inhibitor of p38MAPK activity, 5 μM) or UO-126 (an inhibitor of MEK, 5 μM), and then incubated with Ang-II (500 nM) for 1 h for the TGF-β1 expression (A) and 6 h for CTGF expression (B). TGF-β1 and CTGF expression was determined by the RT-qPCR as explained in Section 2. The values correspond to the mean of the ΔCt value ± standard deviation of the three independent experiments (*$P < 0.05$). (C) Detection by ELISA of TGF-β1 protein levels into the medium of C_2C_{12} pre-incubated with the vehicle, SB-203580 (an inhibitor of p38MAPK activity, 5 μM) or UO-126 (an inhibitor of MEK, 5 μM), and then incubated with Ang-II (500 nM) for 48 h. The values are expressed as a percentage of the TGF-β1 levels secreted by C_2C_{12} cells incubated with Ang-II and the vehicle and are representative of three independent experiments (*$P < 0.05$). (D) The myoblasts were pre-incubated with the vehicle, SB-203580 (an inhibitor of p38MAPK activity, 5 μM) or UO-126 (an inhibitor of MEK, 5 μM), and then incubated with Ang-II (500 nM) for 48 h. The levels of CTGF were determined by Western blot. The levels of tubulin are shown as a loading control. The molecular weight standards are indicated in kilodaltons (kDa). The images are representative of the three independent experiments. (E) The myoblasts were pre-incubated with the vehicle, SB-203580 (an inhibitor of p38MAPK activity, 5 μM) or UO-126 (an inhibitor of MEK, 5 μM), and then incubated with Ang-II (500 nM) for 48 h. The levels of fibronectin (FN) were determined by Western blot. The levels of tubulin are shown as a loading control. The molecular weight standards are indicated in kilodaltons (kDa). The images are representative of the three independent experiments that are quantified and shown in graph (F) (*$P < 0.05$).

pro-fibrotic effects of Ang-II in the skeletal muscle are dependent on ROS production. The NOX, one of the sources of ROS, mediates the actions of Ang-II and plays a critical role in fibrogenesis (Bataller et al., 2003). In our previous report and in this paper, the experiments with an antioxidant such as NAC, or the use of NOX inhibitors such as apocynin, strongly suggest the participation of NOX-induced ROS as critical fibrotic mediators (Cabello-Verrugio et al., 2011a). This is in agreement with the decrease of the Ang-II-dependent TGF-β1 expression, ECM proteins, and p38MAPK signalling observed by treatment with anti-oxidant molecules (Bolick et al., 2003). In the context of skeletal muscle fibrosis, the identification of novel genes that participate in the fibrosis induced by Ang-II in a redox-sensitive manner could be further studied through microarray analysis.

To summarise, in this report, we show that Ang-II induces the expression of TGF-β1 and CTGF in the skeletal muscle through its AT-1 receptor by a mechanism involving p38MAPK and NOX-derived ROS. Moreover, we observed that p38MAPK and TGF-β1 induction are also critical to increase ECM protein levels mediated by Ang-II. These results strongly reinforce the importance of Ang-II in the fibrotic phenotype in the skeletal muscle and contribute to understanding the mechanism involved in its effects on skeletal muscle fibrosis.

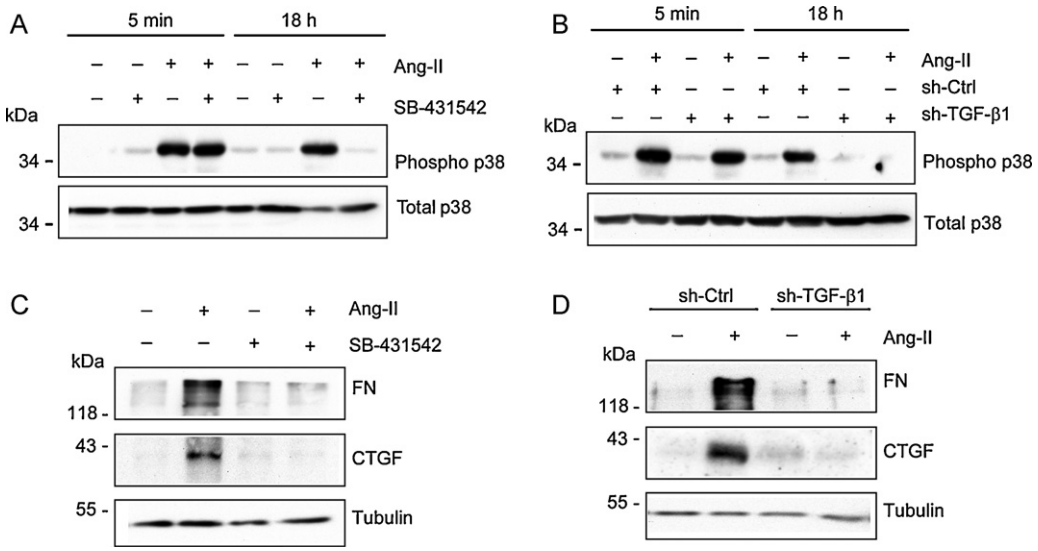

Fig. 6. Angiotensin II-induced TGF-β1 expression and signalling is required for the pro-fibrotic effects mediated by angiotensin II in skeletal muscle cells. (A) C_2C_{12} cells were exposed to the vehicle or to SB-431542 (an inhibitor of TGF-β receptor I kinase activity, 10 μM) in the absence (control) or presence of Ang-II (500 nM) for the times indicated in the figure. The protein levels of the p38MAPK phosphorylated and p38MAPK total were evaluated by Western blot. (B) C_2C_{12} myoblasts were transfected with a shRNA control or specific shRNA for TGF-β1. The transfected myoblasts were incubated in the absence (control) or presence of Ang-II (500 nM) for the times indicated in the figure. The protein levels of the p38MAPK phosphorylated and p38MAPK total were evaluated by Western blot. (C) C_2C_{12} cells were pre-incubated with the vehicle or SB-431542 (10 μM) in the absence (control) or presence of Ang-II (500 nM). The fibronectin (FN), CTGF, and tubulin protein levels were evaluated by Western blot. (D) The transfected myoblasts, as described in B, were incubated in the absence (control) or presence of Ang-II (500 nM). The fibronectin (FN), CTGF, and tubulin protein levels were evaluated by Western blot. For A–D, the molecular weight standards are indicated in kilodaltons (kDa).

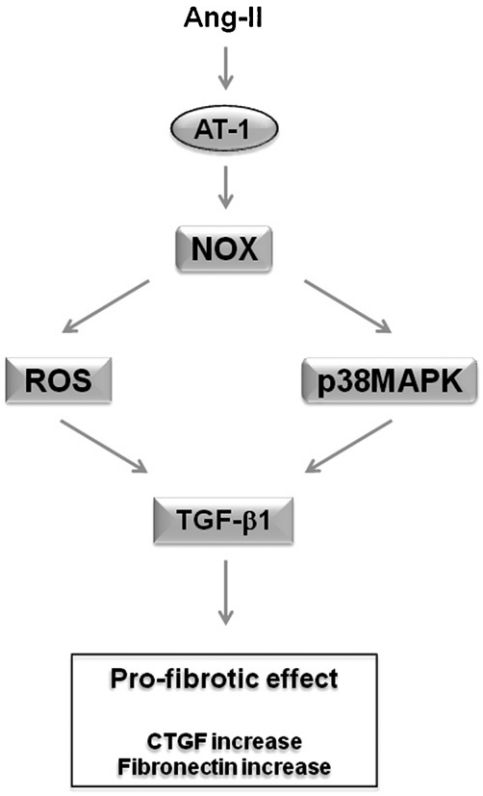

Fig. 7. Scheme for the signalling pathways involved in the angiotensin II-induced pro-fibrotic effects in skeletal muscle cells. When C_2C_{12} myoblasts are incubated with Ang-II, the AT-1 receptor is activated. Downstream, the AT-1-dependent NAD(P)H oxidase (NOX) activation (Cabello-Verrugio et al., 2011a) is required for the Ang-II-induced reactive oxygen species (ROS) production and p38MAPK phosphorylation. These events are required for the TGF-β1 expression, which is essential for the CTGF and fibronectin increase mediated by Ang-II.

Conflict of interest statement

The authors confirm that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2012.07.028.

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