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Fibrotic response induced by angiotensin-II requires NAD(P)H oxidase-induced reactive oxygen species (ROS) in skeletal muscle cells

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ABSTRACT

Fibrotic disorders are typified by excessive connective tissue and extracellular matrix (ECM) deposition that precludes normal healing processes in different tissues. Angiotensin-II (Ang-II) is involved in the fibrotic response. Several muscular dystrophies are characterized by extensive fibrosis. However, the exact role of Ang-II in skeletal muscle fibrosis is unknown. Here we show that myoblasts responded to Ang-II by increasing protein levels of connective tissue growth factor (CTGF/CCN2), collagen-III and fibronectin. These Ang-II-induced pro-fibrotic effects were mediated by AT-1 receptors. Remarkably, Ang-II induced reactive oxygen species (ROS) via a NAD(P)H oxidase-dependent mechanism, as shown by inhibition of ROS production via the NAD(P)H oxidase inhibitors diphenylene iodonium (DPI) and apocynin. This increase in ROS is critical for Ang-II-induced fibrotic effects, as indicated by the decrease in Ang-II-induced CTGF and fibronectin levels by DPI and apocynin. We also show that Ang-II-induced ROS production and fibrosis require PKC activity as indicated by the generic PKC inhibitor chelerythrine.

These results strongly suggest that the fibrotic response induced by Ang-II is mediated by AT-1 receptor and requires NAD(P)H-induced ROS in skeletal muscle cells.

1. Introduction

Fibrotic disorders are typically characterized by excessive connective tissue and extracellular matrix (ECM) deposition that precludes normal healing in different tissues. A marked increase in angiotensin-II (Ang-II) levels has been linked to the pathogenesis of fibrotic disorders in tissues such as liver, cardiac muscle and kidney [1–3]. Ang-II is a potent inducer of ECM proteins such as collagen-III and fibronectin, and of CTGF expression through the AT-1 receptor [4–6].

Reactive oxygen species (ROS) have been implicated in the signal transduction of Ang-II-dependent cellular responses via activation of redox-sensitive signaling cascades [1,7]. One of the main sources of ROS is NAD(P)H oxidase (NOX), a multi-protein enzyme complex that uses NAD(P)H as a substrate to convert molecular oxygen to ROS [8]. One of the main activation mechanisms of NOX is the phosphorylation of the regulatory subunit $p47^{phox}$ by the Ca^{2+}-dependent protein kinase C (PKC) [9].

Different experimental evidence suggests the involvement of NAD(P)H oxidase-1 and NAD(P)H oxidase-2 in fibrotic disease in several tissues among them skeletal muscle [10]. Ang-II activates NAD(P)H oxidase in several cell types [11]. Furthermore, NAD(P)H oxidase activation and increased ROS production are implicated in Ang-II-induced effects such as vascular smooth muscle hypertrophy, hypertension and cardiac and liver fibrosis [12,13].

Several muscular dystrophies, among them Duchenne muscular dystrophy (DMD) and its murine model, *mdx* mice, are characterized by development fibrosis [14,15]. Among the cell types that contribute to fibrotic response in skeletal muscle are the myoblasts [16,17]. Indeed, ROS were postulated to contribute to the pathogenesis of DMD [18,19]. In addition, it has recently been reported that NAD(P)H oxidase protein levels and activity are increased in *mdx* muscle, being a major source of ROS production in dystrophic muscle [19].

Abbreviations: Ang-II, angiotensin-II; AT-1, angiotensin-II receptor type 1; ARB, angiotensin-II receptor type I blocker; CTGF/CCN2, connective tissue growth factor; DTT, dithiothreitol; DMD, Duchenne muscular dystrophy; ECM, extracellular matrix; NOX, NAD(P)H oxidase; ROS, reactive oxygen species; TGF-β, transforming growth factor type-β.

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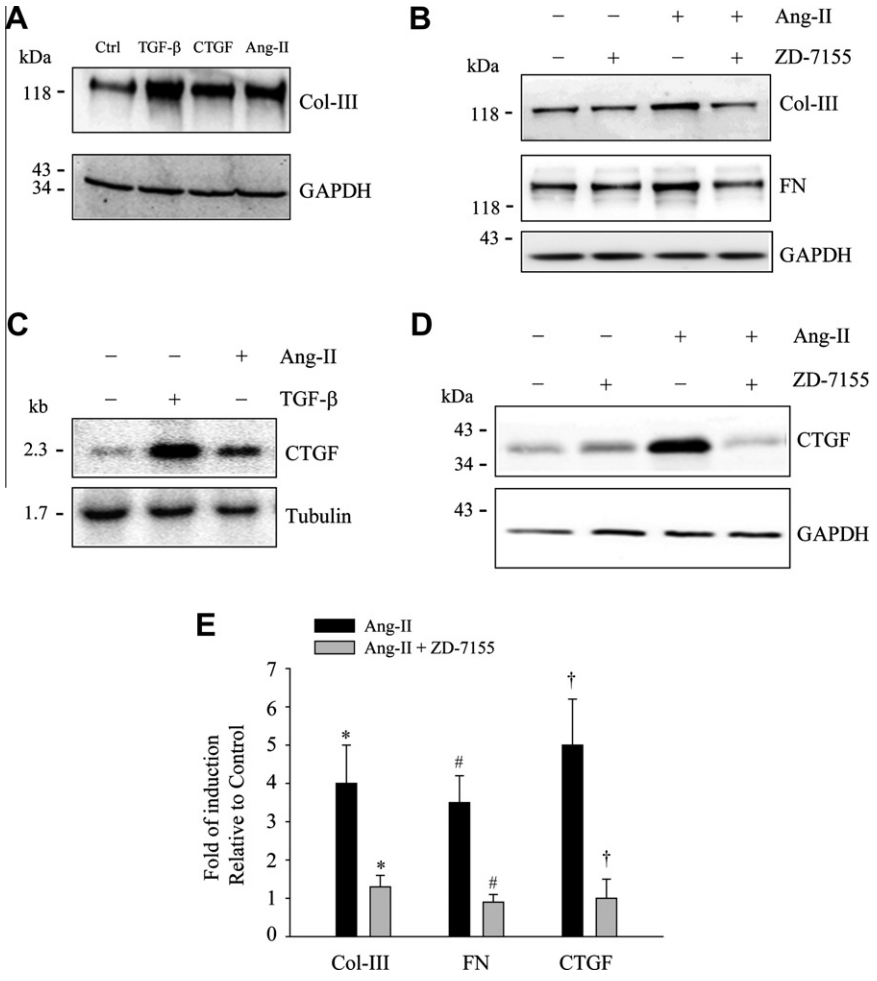

Fig. 1. Angiotensin-II induces fibrosis via an AT-1-dependent mechanism in skeletal muscle cells. (A) C_2C_{12} myoblasts were incubated with Ang-II (500 nM), TGF-β1 (10 ng/ml) or CTGF (80 ng/ml) for 48 h. The extracts obtained were separated by SDS–PAGE. The presence of collagen-III (Col-III) was evaluated by Western blot using anti-Col-III antibody. Levels of GAPDH are shown as a loading control. (B) C_2C_{12} cells were pre-incubated with ARB ZD-7155 (10 μM) and then incubated with Ang-II (500 nM) as in A. Levels of collagen-III (Col-III) and fibronectin (FN) were determined by Western blot. Levels of GAPDH are shown as a loading control. (C) Myoblasts were incubated with Ang-II (500 nM) or TGF-β1 (5 ng/ml) for 1 and 6 h respectively. Total RNA was isolated, blotted and probed with cDNA for mouse CTGF and tubulin as a loading control. The sizes of CTGF and tubulin mRNAs are shown in kilobases (kb). (D) C_2C_{12} cells were pre-treated with ZD-7155 (10 μM) and incubated with Ang-II as in (A). Levels of CTGF protein were determined by Western blot. Levels of GAPDH are shown as a loading control. (E) Quantitative analysis of experiments shown in (B and D). The graph shows the fold of induction relative to control treatment. Values correspond to the mean ± standard deviation from three independent experiments ($^{*, \#, \dagger}P < 0.05$). In A, B and D the molecular weight standards are indicated in kilodaltons (kDa).

In the present study, we therefore investigated the role of Ang-II in skeletal muscle fibrosis using C_2C_{12} myoblasts and the participation of ROS as a key mediator. We demonstrated that Ang-II induced an increase in ECM and CTGF protein levels through its AT-1 receptor. These pro-fibrotic effects of Ang-II were dependent on NAD(P)H oxidase-induced ROS generation. Indeed, the activation of NAD(P)H oxidase was a Ca^{2+}-dependent PKC-induced event. Thus, our results provide an insight into the fundamental mechanisms of 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 adult mouse leg (American Type Culture Collection), was grown as described previously [20]. Cells were serum-starved for 18 h and then subjected to different treatments. For pro-fibrotic cytokine treatment myoblasts were incubated for 48 h with TGF-β1 (10 ng/ml) (R&D System, USA), recombinant CTGF (80 ng/ml) [16], H_2O_2 (Merck, USA) or Ang-II (500 nM) (Sigma, USA). The following inhibitors were pre-incubated for 1 h prior to exposure to Ang-II: AT-1 and AT-2 receptor blockers ZD-7155 (5 μM) and PD-123319 (10 μM) (both from Tocris, USA) respectively, dithiothreitol (DTT) (0.5 mM, Invitrogen, USA), NAD(P)H oxidase inhibitors diphenylene iodonium (DPI) (0.1, 1 and 5 μM, Sigma, USA) or apocynin (1 mM, Sigma, USA), or PKC inhibitor chelerythrine (10 μM, Sigma, USA).

2.2. Measurement of intracellular ROS production and [Ca^{2+}] by FACS

Treated C_2C_{12} cells were harvested, resuspended, and loaded with the following cell permeant dyes: dichlorodihydrofluorescein (DCF, 5 μM) or dihydroethdium (DHE, 10 μM) for ROS determination, and Fura-3 (5 μM) for Ca^{2+} measurements (all from Invitrogen, USA) for 15–30 min at room temperature in the absence of light. They were then analyzed immediately by Fluorescence Activated Cell Sorting (FACS) 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).

2.3. RNA isolation and Northern blot analysis

Myoblasts were serum-starved for 18 h and then incubated for 1 h with Ang-II (500 nM) or for 6 h with TGF-β1 (10 ng/ml). Total RNA was isolated from cell cultures at the indicated times using Trizol (Invitrogen, USA). RNA samples (20 μg/lane) were electrophoresed, transferred and hybridized with probes for mouse tubulin and CTGF as described previously [21].

2.4. Immunoblot analysis

For cell immunoblot analyses, protein extracts from myoblasts were prepared as previously described [16,17] and subjected to SDS–PAGE, electrophoretically transferred onto PDVF membranes (Schleicher & Schuell, USA) and probed with goat anti-CTGF (Santa Cruz Biotechnology, USA), rabbit anti-fibronectin and mouse anti-tubulin (Sigma–Aldrich, USA), rabbit anti-collagen-III (Rockland, USA), and mouse anti-GAPDH (Chemicon, USA). All immunoreactions were visualized by enhanced chemiluminescence (Pierce, USA).

2.5. Protein determination

Proteins were determined in aliquots of cell extracts using the bicinchoninic acid protein assay kit (Pierce, USA) with BSA as the standard.

2.6. Statistics

The statistical significance of the differences between the means of the experimental groups 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. Angiotensin-II-induced fibrotic response is AT-1 receptor-dependent in skeletal muscle cells

We studied the effect of Ang-II as a possible inducer of fibrosis in skeletal muscle cells. Fig. 1A and B show an increase in the amount of collagen-III and fibronectin of C_2C_{12} cells in response to Ang-II. TGF-β1 and CTGF are showed as fibrotic factors increasing the levels of collagen-III and fibronectin [16,17]. Fig. 1B also shows that the increase in collagen-III and fibronectin induced by Ang-II in C_2C_{12} myoblasts was prevented in presence of the Ang-II receptor type 1 blocker (ARB) ZD-7155 suggesting that is mediated by AT-1 receptor. Fig. 1C and D show that Ang-II induces increased levels of CTGF mRNA and protein respectively. Fig. 1D also shows that Ang-II-induced CTGF levels are dependent on the AT-1 receptor. Fig. 1E shows the quantitative analysis of the ARB ZD-7155 effect on Ang-II-induced collagen-III, fibronectin and CTGF levels.

Together, these results suggest that Ang-II induces pro-fibrotic effects in skeletal muscle cells.

3.2. Angiotensin-II-induced intracellular production of reactive oxygen species (ROS) is AT-1 receptor-dependent and participates in the fibrosis of skeletal muscle cells

We tested whether skeletal muscle cells increase their intracellular ROS production in response to Ang-II. By means of flow

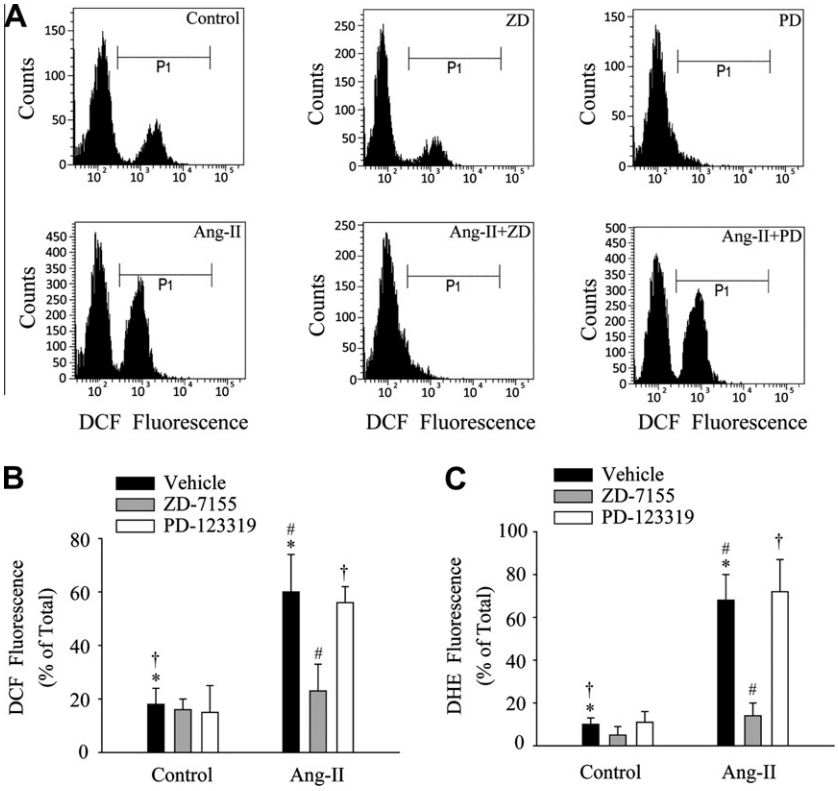

Fig. 2. Reactive oxygen species (ROS) are induced by angiotensin-II via the AT-1 receptor in skeletal muscle cells. (A) Representative DCF fluorescence histograms from FACS experiments of C_2C_{12} cells exposed to vehicle alone (left upper panel), ZD-7155 alone (middle upper panel), PD-123319 alone (right upper panel), Ang-II (left lower panel), Ang-II + ZD-7155 (middle lower panel), or Ang-II + PD-123319 (right lower panel). Data obtained from three to four independent experiments such as those depicted in the histograms above are summarized in (B and C) for DCF and DHE respectively. Bars represent the percentage (mean ± standard deviation) of cells within the P_1 population of DCF (B) or DHE (C) fluorescence. ($^{*, \#, \dagger}P < 0.05$).

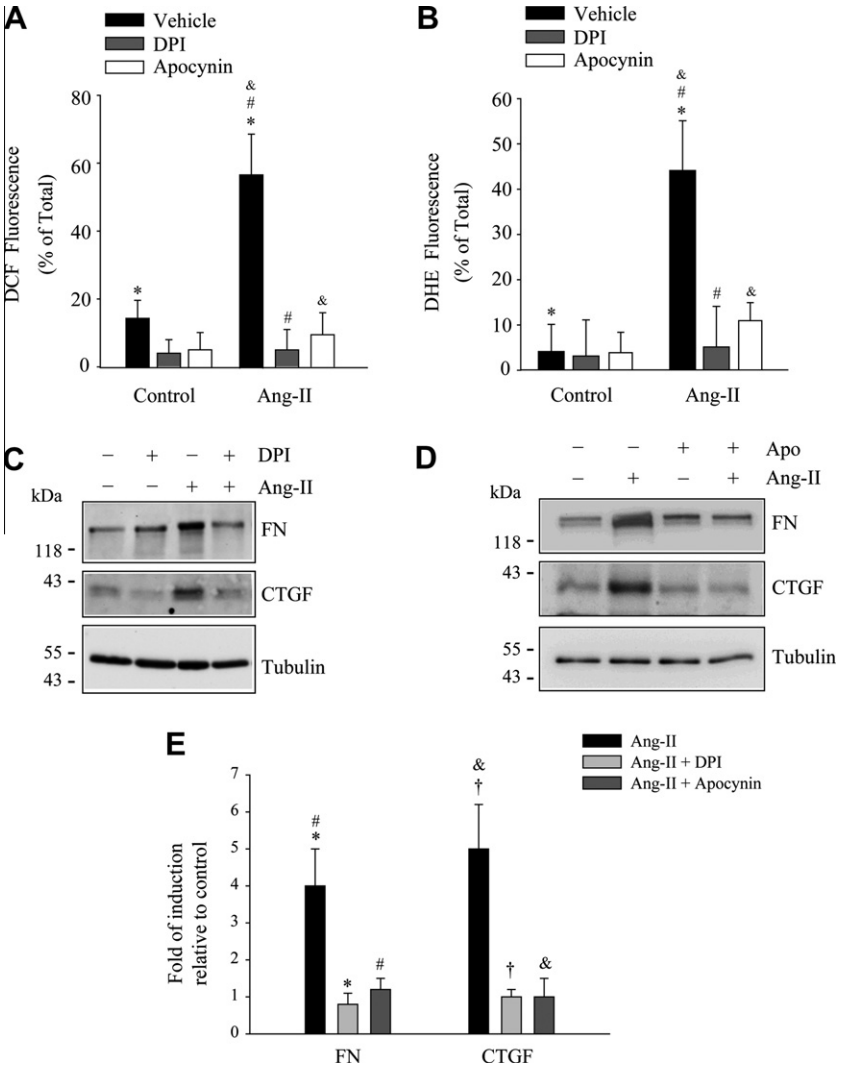

Fig. 3. Angiotensin-II-induced ROS production is mediated by NAD(P)H oxidase and participates in the fibrosis of skeletal muscle cells. C_2C_{12} cells were exposed to vehicle alone, DPI (5 μM) or apocynin (1 mM) in the absence (control) or presence of Ang-II (500 nM), and DCF (A) or DHF (B) fluorescence from three to four independent experiments was measured. Bars represent the percentage (mean ± standard deviation) ($^{*, \#, \&}P < 0.05$). Myoblasts were pre-incubated with DPI (5 μM) (C) or apocynin (1 mM) (D) and then incubated with Ang-II (500 nM). After 48 h extracts were obtained, separated by SDS–PAGE, blotted and probed with anti-fibronectin and anti-CTGF antibodies. Tubulin levels are shown as a loading control. The molecular weight standards are indicated in kilodaltons (kDa). (E) Quantitative analysis of experiments shown in (C and D). The graph represents the fold of induction relative to control treatment. Values correspond to the mean ± standard deviation of three independent experiments ($^{*, \#, \dagger, \&}P < 0.05$).

cytometry, we determined that intracellular ROS levels were increased by Ang-II evaluated using a ROS-sensitive fluorescent probe DCF in C_2C_{12} cells (Fig. 2A). This increase in Ang-II-induced ROS was completely decreased in presence of ARB ZD-7155 (Fig. 2A), whereas AT-2 blocker PD-123319 had no significant inhibitory effect (Fig. 2A). The quantitative analysis of these experiments is shown in Fig. 2B. Similar results were obtained with ROS-sensitive fluorescent probe DHE (Fig. 2C). These results suggest that Ang-II induces an increase in ROS production through an AT-1-dependent mechanism.

The Ang-II-induced ROS production participates in the generation and development of fibrosis in skeletal muscle cells since pre-incubation of cells with DTT prevented the Ang-II-dependent increase in ECM protein (collagen-III and fibronectin) and CTGF protein levels (Supplementary 1A). An exogenous source of ROS (H_2O_2) induced similar increase in levels of fibronectin and CTGF (Supplementary 1B).

The results suggest that Ang-II induces skeletal muscle fibrosis via a mechanism involving ROS generation through its AT-1 receptor.

3.3. Angiotensin-II-induced ROS production is mediated by the NAD(P)H oxidase and participates in the fibrosis of skeletal muscle cells

We evaluated the participation of NAD(P)H oxidase in the production of ROS induced by Ang-II in skeletal muscle cells. As depicted in Fig. 3A, two NAD(P)H oxidase inhibitors such as DPI or apocynin completely prevented the increase in Ang-II-induced ROS production measured as DCF fluorescence. Similar results were obtained using the DHE probe (Fig. 3B). When DPI was used in a lower concentration, the decrease in the ROS production also was observed (Supplementary 2A and B).

Then we evaluated the participation of ROS in Ang-II-induced pro-fibrotic effects. DPI (Fig. 3C) or apocynin (Fig. 3D) prevented the increase in Ang-II-induced fibronectin and CTGF protein levels in C_2C_{12} myoblasts. Fig. 3E shows the quantitative analysis of the experiments depicted in Fig. 3C and Fig. 3D. A low DPI concentration produced the same effect on fibronectin and CTGF levels induced by Ang-II (Supplementary 2C).

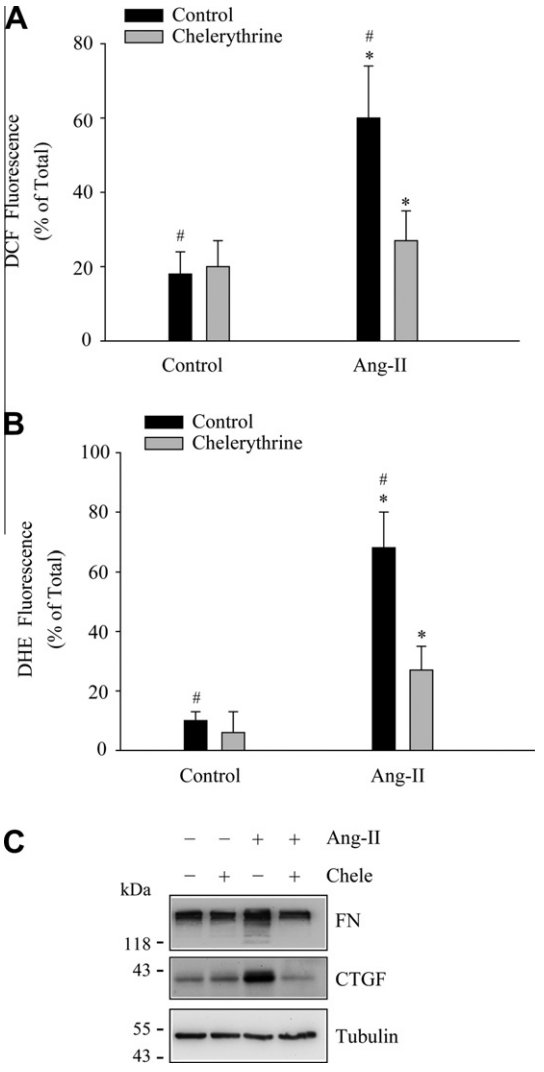

Fig. 4. Angiotensin-II-induced ROS production and fibrosis require PKC activity in skeletal muscle cells. C_2C_{12} cells were exposed to vehicle alone or the generic PKC inhibitor chelerythrine (10 μM) in the absence (control) or presence of Ang-II, and DCF (A) or DHE (B) fluorescence from three to four independent experiments was measured. Bars represent the percentage (mean ± standard deviation) ($^{*,\#}P < 0.05$). (C) Myoblasts were pre-incubated with chelerythrine (10 μM) and then incubated with Ang-II (500 nM). After 48 h extracts were obtained and fibronectin (FN) and CTGF protein levels were determined by Western blot analysis. Tubulin levels are shown as a loading control. The molecular weight standards are indicated in kilodaltons (kDa). The images are representative of two independent experiments.

These results strongly suggest that NAD(P)H oxidase-induced ROS is involved in the pro-fibrotic effects induced by Ang-II in skeletal muscle cells.

3.4. Protein kinase C is required for angiotensin-II-induced intracellular ROS production and fibrosis of skeletal muscle cells

We determined that inhibition of PKC activity by chelerythrine prevented Ang-II-induced ROS production in C_2C_{12} cells, as evaluated using DCF (Fig. 4A) and DHE (Fig. 4B) probes. Also, we show that inhibition of PKC reduced Ang-II-induced fibronectin and CTGF protein levels to basal levels in C_2C_{12} myoblast (Fig. 4C). These results suggest that PKC activity is essential for mediating Ang-II-induced ROS production and fibrosis in skeletal muscle cells.

Ang-II also increased levels of intracellular Ca^{2+} mediated by AT-1 receptor in C_2C_{12} cells (Supplementary 3A) which was not affected by DPI or apocynin, suggesting that this calcium increase occurs upstream of the NAD(P)H oxidase activity and ROS generation induced by Ang-II (Supplementary 3B).

Together, these results suggest that PKC activity is required for induction of fibrosis by Ang-II and required to activate NAD(P)H oxidase.

4. Discussion

In this paper we show that Ang-II produces pro-fibrotic effects in skeletal muscle cells through an AT-1-dependent mechanism involving the participation of ROS generated by NAD(P)H oxidase, which is activated by Ca^{2+}-dependent PKC.

Skeletal muscles from DMD or its murine model *mdx* mice develop fibrosis [22,23]. These dystrophic skeletal muscles have increased levels of angiotensin-II converting enzyme (ACE) and AT-1 receptor compared to normal muscle [24]. According our results *in vitro*, the RAS axis would be more active in DMD, producing more intramuscular Ang-II which could augment signaling through its fibrotic receptor AT-1, thus contributing to the fibrosis found in dystrophic skeletal muscle. Experimental evidence from Cohn et al. and our laboratory (data not published) suggests that AT-1 receptor blockade decreases skeletal muscle fibrosis and improves tissue regeneration [25]. However, the direct involvement of Ang-II in skeletal muscle fibrosis *in vivo* must be analyzed further.

Our study is the first evidence of the participation of Ang-II-induced ROS in the skeletal muscle fibrosis. Inhibition of the Ang-II-induced fibrotic effect using DTT demonstrates a redox effect of ROS. Moreover the participation of ROS in fibrotic skeletal muscle associated with DMD or *mdx* mice is strongly supported by the use of antioxidants as a therapeutic tool to decrease fibrosis [26,27]. However, the precise mechanism by which ROS produces its fibrotic effect is unknown. One possibility is that gene expression of the pro-fibrotic factor CTGF and ECM proteins can be induced in a ROS-dependent manner by Ang-II such as have been described in other models [28,29].

Our results show that Ang-II-induced ROS production appeared to be generated by NAD(P)H oxidase activation. The expression and activity of NAD(P)H oxidase in skeletal muscle have been reported previously [30–32]. The reduction in Ang-II-induced ROS generation produced by DPI and apocynin NAD(P)H oxidase inhibitors indicates that activated NAD(P)H oxidase is the source of intracellular ROS. A similar pharmacological strategy to demonstrate that NAD(P)H oxidase is responsible for ROS generation in myotubes and T-tubules have been previously described [32,33]. Moreover, the inhibition of NAD(P)H oxidase by apocynin suggests that a phagocytic-like oxidase (NAD(P)H oxidase type 2 is involved in the Ang-II-induced ROS generation in skeletal muscle cells.

Inhibition of PKC activity significantly diminished ROS production, fibronectin and CTGF levels induced by Ang-II. These effects were probably produced by inhibition of NAD(P)H oxidase since calcium-dependent PKC-mediated serine phosphorylation is the main mechanism for activating NAD(P)H oxidase [34,35]. The PI3-K and PKB/Akt pathways have also been reported to activate NAD(P)H oxidase in the presence or absence of PKC activity [36]. However, further experiments are needed to determine whether these pathways are involved.

Our results indicate that Ang-II induced a significant increase in intracellular calcium levels which was not inhibited by NAD(P)H oxidase blockers, suggesting that Ang-II-induced ROS are not involved in the increase in calcium. Thus, Ang-II-induced ROS signals act differently to previously observed in myotubes and isolated triads (containing T-tubules), in which NAD(P)H oxidase-generated ROS induced an increase in calcium levels [32,33]. Further experiments must be performed to determine the mechanism by which

Ang-II increases calcium levels. In our model, it is possible to hypothesize that the Ang-II-induced calcium increment was required to activate PKC and thus to promote NAD(P)H oxidase activation, ROS generation, and the subsequent Ang-II-induced fibrotic effect. However, the direct participation of PKC and which PKC isoform could be involved in the NAD(P)H oxidase-induced ROS and Ang-II-induced fibrosis has not yet been demonstrated.

To summarize, in this report we show that Ang-II induces fibrosis in skeletal muscle through its AT-1 receptor, inducing ROS production via a PKC-dependent NAD(P)H oxidase mechanism. These results strongly show the importance of Ang-II in the fibrotic phenotype in skeletal muscle and support the use of ARB or ACE inhibitors as possible therapeutic drugs to decrease skeletal muscle fibrosis associated with dystrophies such as DMD.

Conflict of interest

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 doi:10.1016/j.bbrc.2011.06.051.

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