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# Hyperglycemia and angiotensin II cooperate to enhance collagen I deposition by cardiac fibroblasts through a ROS-STAT3-dependent mechanism

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## ABSTRACT

Cardiac fibroblasts significantly contribute to diabetes-induced structural and functional changes in the myocardium. The objective of the present study was to determine the effects of high glucose (alone or supplemented with angiotensin II) in the activation of the JAK2/STAT3 pathway and its involvement in collagen I production by cardiac fibroblasts. We observed that the diabetic environment 1) enhanced tyrosine phosphorylation of JAK2 and STAT3; 2) induced nuclear localization of tyrosine phosphorylated STAT3 through a reactive oxygen species-mediated mechanism, with angiotensin II stimulation further enhancing STAT3 nuclear accumulation; and 3) stimulated collagen I production. The effects were inhibited by depletion of reactive oxygen species or silencing of STAT3 in high glucose alone or supplemented with exogenous angiotensin II. Combined, our data demonstrate that increased collagen I deposition in the setting of high glucose occurred through a reactive oxygen species- and STAT3-dependent mechanism. Our results reveal a novel role for STAT3 as a key signaling molecule of collagen I production in cardiac fibroblasts exposed to a diabetic environment.

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## 1. Introduction

Extracellular matrix is an important component of myocardial architecture and regulator of function of the left ventricle (LV), and is dynamically regulated by cardiac fibroblasts [1]. Fibroblast proliferation and a dysregulated balance between enhanced extracellular matrix synthesis and degradation can evolve into a state of fibrosis with important effects on function by increasing LV stiffness. A prominent component of diabetic cardiomyopathy is cardiac fibrosis, a pathological condition characterized by alterations in the structure and function of the heart that includes increases in deposition of extracellular matrix proteins (e.g., collagen I) in the absence of coronary artery disease [2–4].

The activation of fibrogenic pathways can be evoked by stimuli independent of cardiomyocyte loss. High glucose (25 mM) was found to promote fibrosis in vitro by increasing cardiac fibroblast protein and

collagen synthesis, decreasing matrix metalloproteinase (MMP) activity, and up-regulating mRNA expression of angiotensin II (Ang II) AT1 receptor [5]. Ang II plays a critical role in LV remodeling by promoting cardiomyocyte hypertrophy and cardiac fibroblast proliferation, synthesis, and secretion of adhesion molecules and extracellular matrix proteins [6].

High glucose also importantly affects Ang II receptor signaling. In non-failing cardiomyocytes incubated in a diabetic environment, Ang II induces reactive oxygen species (ROS)-dependent JAK2 tyrosine phosphorylation [7] followed by signal transducer and activator of transcription 3 (STAT3) tyrosine phosphorylation and B cell lymphoma (Bcl)-xL overexpression. The transcriptional activity of STAT3 is coordinated by ERK1/2 activation [8]. STAT3 belongs to the family of STAT proteins, activated in response to extracellular signaling proteins [9,10]. The phosphorylation of STAT3 at tyrosine 705 permits dimerization followed by transport of the dimer into the nucleus [11], where the dimer binds to a consensus DNA sequence in the promoters of its target genes to regulate transcription [12]. STAT3 participates in the transcription of target genes in ischemia/reperfusion injury [13,14], pressure-overload hypertrophy [15], cardio-protection [16], and angiogenesis [17]. Recently, the protective role of STAT3 was challenged by evidence showing that IL-6 mediated STAT3 activation may also induce interstitial fibrosis [18]. In addition, the STAT3 cardio-protective response was

*Abbreviations:* HG, high glucose; NG, normal glucose; LV, left ventricle; MMP, matrix metalloproteinase

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blunted in failing myocytes [8]. Although STAT3 plays contrasting actions in the physiology of the heart (protective and pro-fibrotic), the molecular mechanism responsible for fibrosis involving STAT3 has not been fully elucidated in cardiac fibroblasts.

Based on this background, the current manuscript evaluated the effect of exposing cardiac fibroblasts to a high glucose alone or supplemented with Ang II. Our findings revealed novel roles for STAT3 to enhance collagen I synthesis in the setting of high glucose.

## 2. Materials and methods

### 2.1. Materials

Unless specified, all reagents were obtained from Sigma. Anti-actin (sc-1615), anti-JAK2 (sc-278), anti-p-JAK2-Tyr 1007 (sc-101717), anti-MMP-2 (sc-10736) antibodies, STAT3 siRNA (sc-270027) and scramble siRNA were from Santa Cruz Biotechnology. Antibody anti p-STAT3-Tyr 705 (catalog number 91315) was from Cell Signaling. Antibodies anti-collagen I (ab34710) was from Abcam. TransIT-TKO Transfection Reagent was from Mirus (Catalog number MIR2154). 488Alexa Fluor-conjugated secondary antibodies and 2',7'-dichlorofluorescein-diacetate (DCF-DA) were from Molecular Probe (catalog number D-399). Amicon Ultra centrifugal filters (10 K) were from Millipore. Bradford solution for protein assay detection was from Biorad (catalog number 500-0006).

### 2.2. Cardiac fibroblast isolation, culture, and stimulation with Ang II

Cardiac fibroblasts were isolated from the LV of 5-day-old Sprague–Dawley rats. In brief, LVs were harvested, cut into small pieces (~1–2 mm), placed in a Petri dish and covered with a glass slide. A total of 10 mL of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin (100 U/mL) was added to Petri dishes. Petri dishes are maintained at 37 °C under 5% CO<sub>2</sub> in a humidified incubator to permit outgrowth of the fibroblasts from the tissue. Only cells that adhered to the plate grew and were passaged. The purity of the fibroblast population was about 99%, as assayed by vimentin, cytokeratin, and  $\alpha$ -smooth muscle actin expression (Suppl. Fig. 1). Cells from passages 2 or 3 were used for all experiments. Cells were maintained in DMEM containing 5.5 mM (normal glucose, NG) or 25 mM glucose (high glucose, HG) corresponding to plasma levels of 100 and 450 mg/dL, respectively. Differences in osmolarity between normal glucose and high glucose media were corrected by adding 19.5 mM of mannitol to the normal glucose medium.

Fibroblasts were treated with serum-free normal glucose medium overnight. For high glucose samples stimulated with Ang II, high glucose medium was added to fibroblasts 2 h before Ang II addition. In all experiments, high glucose is maintained for the entire time and never removed. For Ang II stimulation, cells were serum deprived overnight before stimulation with Ang II (100 nM final). The generic ROS scavenger N-acetyl-cysteine (NAC) (20 mM final) was added to the medium 15 min before Ang II stimulation and maintained throughout the experiment.

### 2.3. STAT3 silencing

Specific silencing of STAT3 was performed by fibroblast transfection with small-interfering-RNA (siRNA) using TransIT Transfection Reagent according to manufacturer's instruction. Briefly, 48 h after silencing, fibroblasts were serum-deprived for 24 h in normal glucose medium and treated with high glucose or normal glucose for additional 24 h (both culture media with or without Ang II, 100 nM final). Not-targeting siRNA (scramble) was used as a negative control. Viability of STAT3-silenced cells was evaluated by MTT assay.

### 2.4. Immunoblot analysis

Cardiac fibroblasts were lysed for 20 min on ice in 500  $\mu$ L of complete radio-immunoprecipitation assay (RIPA) buffer. Lysates were clarified by centrifugation, and total protein contents were obtained using a Bradford assay. A total of 20  $\mu$ g of total protein for each sample were separated by SDS-PAGE and transferred onto PVDF membranes. PVDF membranes were incubated in 2% milk, probed with primary antibodies, and incubated with secondary antibodies conjugated with Horseradish Peroxidase. Band intensity was measured by ImageJ. Actin and total STAT3 or JAK2 was used for normalization.

### 2.5. ROS evaluation

ROS generation in cardiac fibroblasts was measured using 2',7'-dichlorofluorescein-diacetate (DCF-DA), a fluorogenic dye that binds to ROS. Within the cell, DCFDA is deacetylated by cellular esterase to a non-fluorescent compound, which is later oxidized by cellular ROS into the highly fluorescent compound 2',7'-dichlorofluorescein (DCF). A total of 5  $\mu$ M DCF-DA was added to sub-confluent cells for 3 min. Cells were lysed in 1 mL RIPA buffer containing 1% Triton X-100 and fluorescence was immediately analyzed using a fluorescence spectrophotometer (excitation wavelength: 488 nm, emission wavelength: 510 nm). The fluorescence values were normalized for total protein content.

### 2.6. Nuclear localization of phosphorylated Tyr 705 STAT3 by confocal analysis

Cardiac fibroblasts were seeded onto coverslips, stimulated with Ang II (100 nM) for 1 h, washed with phosphate buffered saline, and fixed in 3% paraformaldehyde for 20 min at 4 °C. Fixed cells were permeabilized with three washes with 0.1% Triton X-100 (in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl) (TBST) and blocked with 5.5% horse serum in TBST for 1 h at room temperature. Cells were incubated with p-Tyr 705 STAT3 primary antibodies overnight at 4 °C. Cells were incubated with 488 Alexa Fluor-conjugated secondary antibodies (diluted 1:100) for 1 h at room temperature in TBST containing 3% bovine serum albumin. Nuclei were identified by labeling the cells with propidium iodide (50 mg/L) for 10 min at room temperature. After extensive washes in TBST, cells were mounted with glycerol plastine and observed under a laser scanning confocal microscope (Leica TCS SP5).

### 2.7. Zymography

Zymography was performed on collected culture media. A total of  $3 \times 10^5$  fibroblasts were cultured in 1.5 mL of medium. Conditioned media (20  $\mu$ L) was subjected to electrophoresis on 8% SDS-PAGE copolymerized with 0.1% (w/v) type A gelatin. Equal loading was ensured by measuring total protein content for each sample. Gels were washed twice in 2.5% v/v Triton X-100 for 30 min and incubated in 50 mM Tris-HCl, pH 7.4, 200 mM NaCl and 5 mM CaCl<sub>2</sub> at 37 °C for 24 h. After incubation, the gels were stained with 0.1% Coomassie brilliant blue in acetic acid, methanol, and distilled water (1:2:3) for 1 h at room temperature. After destaining, the gels were immersed in distilled water. Bands of gelatinase activity appeared as transparent area against a blue background. The intensity of the bands was measured using ImageJ. Conditioned medium of the fibrosarcoma cell line HT1080 is used as positive control of MMP-2.

### 2.8. Analysis of collagen I deposition

Fibroblasts ( $3 \times 10^5$  cells) were cultured in 1.5 mL of culture medium. Conditioned medium had the same total protein content in each sample as revealed by protein assay (using Bradford method) and by anti-actin immunoblot. Conditioned medium (1 mL) was concentrated to 100  $\mu$ L.

using Amicon Ultra centrifugal filters (with 10 K cut-off). A total of 20  $\mu$ L each of the concentrated medium was subjected to immunoblot analysis for collagen I. The intensities of the bands were analyzed by ImageJ.

### 2.9. Statistical analysis

Data are presented as mean  $\pm$  S.D. from at least three independent experiments. Analysis of densitometry was performed using ImageJ. Statistical analysis of the data was performed by Student's *t*-test or by one-way ANOVA using Graph Pad Prism 4.0. *p*-Values <0.05 were considered statistically significant.

## 3. Results

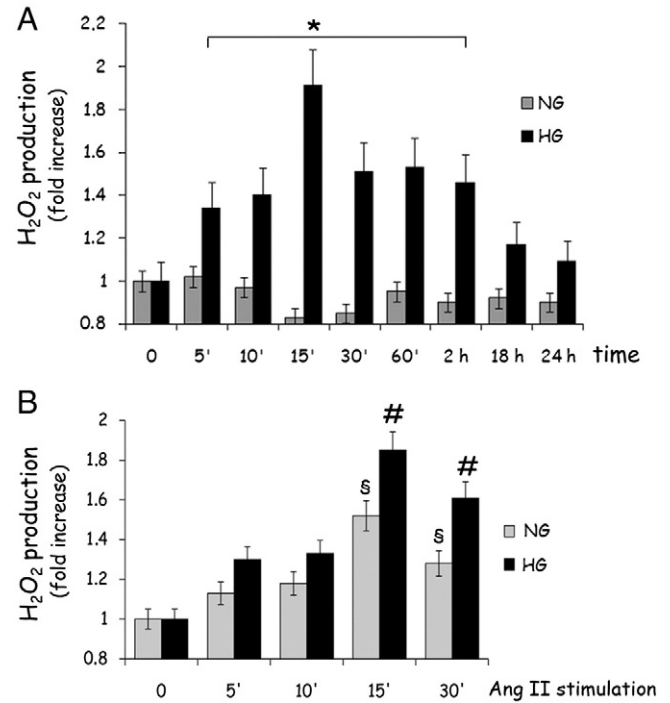
### 3.1. Effects of glucose on JAK2 and STAT3 tyrosine phosphorylation in cardiac fibroblasts

Phosphorylation on Tyr-1007 of JAK2 (Fig. 1A) and Tyr-705 of STAT3 (Fig. 1B) was 2-fold and 5-fold enhanced under high glucose, compared to normal glucose. This response in high glucose was independent of reduction in either salt content or osmolarity.

### 3.2. Effects of glucose and Ang II stimulation in ROS generation

To verify the influence of hyperglycemic environment on ROS production, we performed a time-course experiment of ROS generation under high glucose conditions. High glucose provoked a transient burst of ROS, peaking 15 min after high glucose addition (about 2 fold enhancement vs time zero). Conversely, fibroblasts in normal glucose produced ROS levels that did not change for any of the times examined (Fig. 2A).

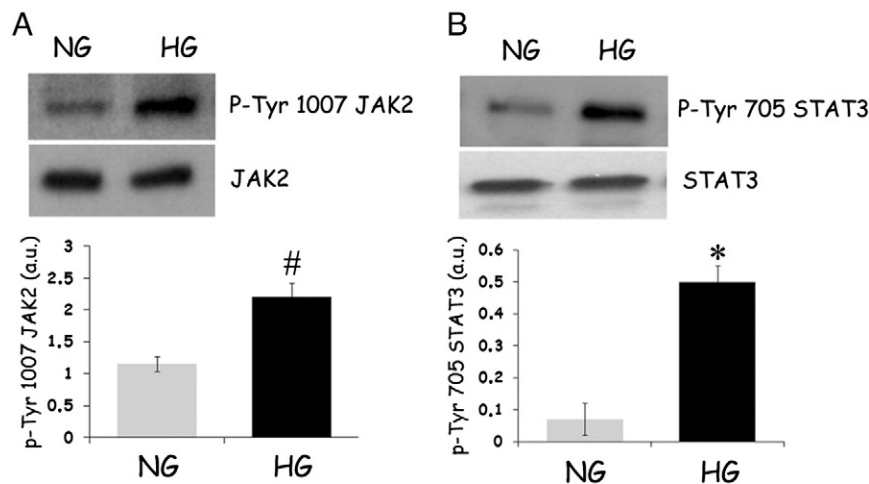
Further, we compared ROS production in fibroblasts stimulated with Ang II under normal and high glucose conditions. Ang II stimulation in normal glucose induced a transient burst of ROS, with peak of production 15 min after stimulation (Fig. 2B). Ang II stimulation in high glucose induced a similar timing of effect, with ROS production also peaking 15 min after stimulation, but at higher levels than observed in normal glucose (Fig. 2B).



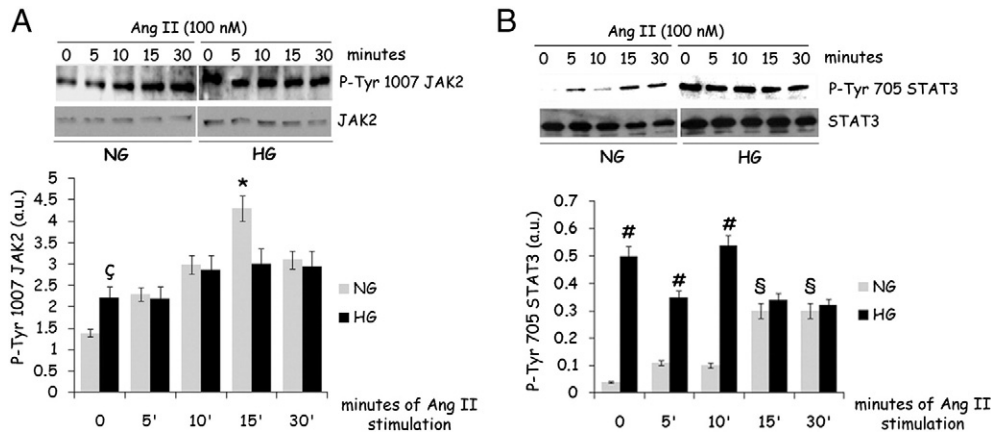
**Fig. 2.** Effect of glucose and Ang II stimulation on ROS production in cardiac fibroblasts. A) Time-course of ROS production in cardiac fibroblasts under normal and high glucose levels. B) ROS generation following Ang II stimulation in normal glucose and after two-hour incubation in high glucose. ROS values were normalized on total protein content and reported in the bar graph as fold increase vs time 0, with time 0 equal to 1. \**p* < 0.01 and #*p* < 0.01 vs corresponding time in normal glucose. §*p* < 0.01 vs time 0 in normal glucose. Data are representative of three independent experiments.

### 3.3. Effect of Ang II stimulation in normal or high glucose medium on tyrosine phosphorylation of JAK2 and STAT3

Ang II stimulation of cardiac fibroblasts in normal glucose induced 1.3-fold increased JAK2 phosphorylation (Fig. 3A) and >10-fold increased STAT3 tyrosine phosphorylation (Fig. 3B), with peak activation occurring 15–30 min after stimulation. Ang II stimulation of cardiac



**Fig. 1.** Analysis of JAK2 and STAT3 tyrosine phosphorylation in normal and high glucose conditions. Cardiac fibroblasts were cultured in media containing normal glucose (5 mM) for 24 h. Cells were treated with high glucose for two h. A) Basal phosphorylation level of Tyr 1007 JAK2 phosphorylation in normal and high glucose conditions, by immunoblotting. B) Basal phosphorylation level of Tyr 705 STAT3 in normal and high glucose conditions, by immunoblotting. Bar graph in A) and B) shows the phosphorylation level of JAK2 and STAT3 obtained from the ratio between the phosphorylated and total amount of protein and reported as arbitrary units (a.u.). #*p* < 0.01 vs normal glucose; \**p* < 0.001 vs normal glucose. The immunoblots are representative of three independent analyses with similar results.

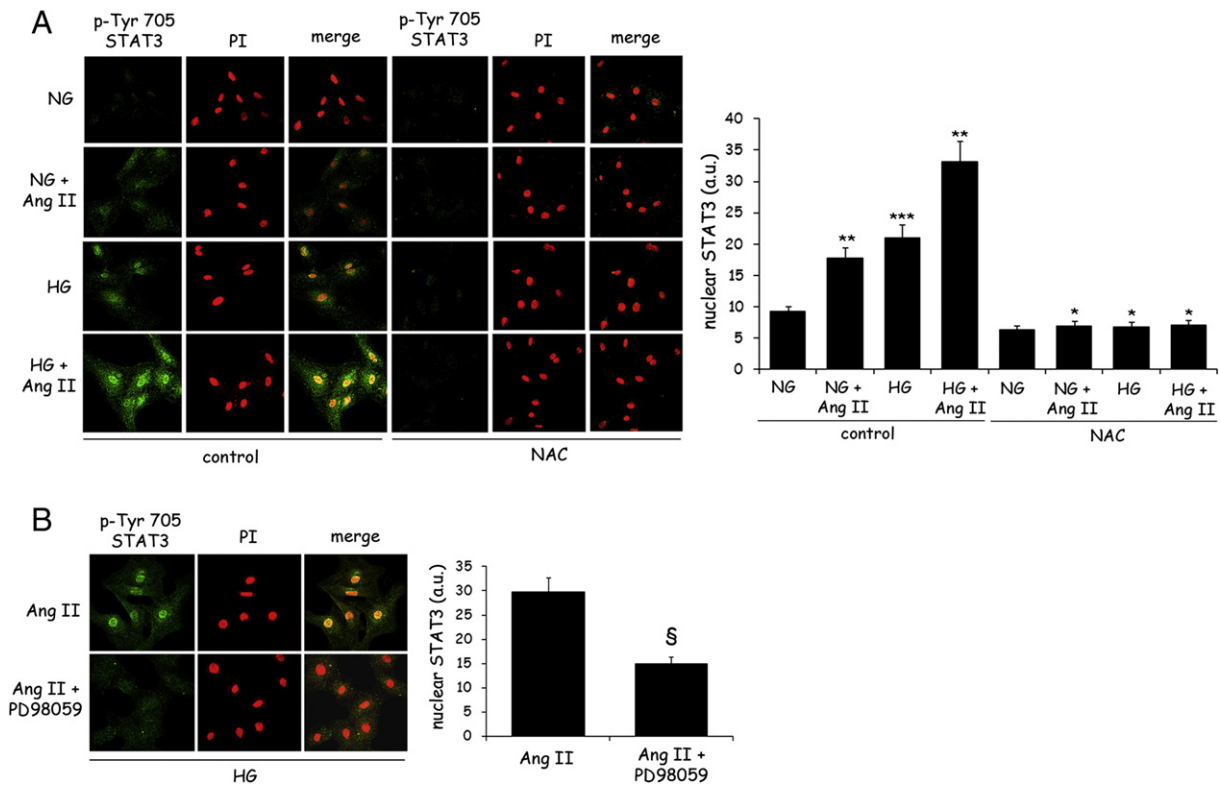


**Fig. 3.** JAK2 and STAT3 tyrosine phosphorylation by Ang II stimulation in normal and high glucose environments. Fibroblasts were serum-deprived for 24 h in normal glucose. High glucose treatment was performed for 2 h. Cells were then stimulated with Ang II (100 nM) for the indicated period. A) Representative immunoblot shows the phosphorylation of Tyr 1007 of JAK2 using phospho-specific antibodies. B) Representative immunoblot performed using anti-phospho-Tyr 705 STAT3 antibodies. Bar graph indicates the ratio between the phosphorylated and the total amount of protein reported as arbitrary unit (a.u.).  $\zeta$   $p < 0.01$  vs time 0 in normal glucose. \*  $p < 0.01$  and #  $p < 0.001$  vs corresponding time in normal glucose. §  $p < 0.01$  vs time 0 in normal glucose. The immunoblots are representative of three different and independent experiments.

fibroblasts in high glucose did not induce JAK2 or STAT3 phosphorylation, even after 2 h of stimulation (Fig. 3B). The addition of hydrogen peroxide to fibroblasts in normal glucose stimulated tyrosine phosphorylation of both JAK2 and STAT3, indicating the involvement of ROS in JAK2 and STAT3 activation (Suppl. Fig. 2).

### 3.4. Nuclear accumulation of pTyr-705 STAT3

Nuclear translocation initiated by Tyr705 phosphorylation is central to STAT3 transcription factor function [11]. Ang II stimulation of cardiac fibroblasts under normal glucose condition significantly



**Fig. 4.** Nuclear localization of phosphorylated-Tyr 705 of STAT3 in normal and high glucose conditions. A) Cells were seeded on coverslips, serum-deprived for 24 h in normal glucose and then treated with high glucose for 2 h. Ang II (100 nM) stimulation was carried out for 1 h. B) The analysis of ERK1/2 involvement in the nuclear localization of phospho-Tyr 705 STAT3 was performed by treating the fibroblasts as described in A). Before Ang II stimulation the MEK1 inhibitor PD98059 (10  $\mu$ M final) was added to fibroblasts. Nuclear localization of phosphorylated-Tyr 705 STAT3 was carried out by labeling the cells with anti-phospho-Tyr705 STAT3 antibodies and then with Alexa Fluor 488-conjugated secondary antibodies (green staining). Nuclei were detected by labeling the cells with propidium iodide (red staining). For the experiment with NAC, cells were pre-treated with NAC (20 mM) for 1 h before adding high glucose medium. Fluorescence intensity of the nuclei was analyzed by ImageJ choosing at least six random fields in each experiment. The bar graph shows the mean of fluorescence intensity calculated on the number of cells examined (100 cells/experiment) and reported as arbitrary unit (a.u.). One-way ANOVA followed by Tukey's post-test was used to find statistical differences between groups. \*  $p < 0.001$  vs corresponding sample without NAC; \*\*  $p < 0.01$  vs corresponding sample without Ang II; \*\*\*  $p < 0.01$  vs normal glucose. §  $p < 0.01$  vs corresponding sample without PD98059. The images are representative of four independent experiments.



enhanced Tyr-705 phosphorylation of STAT3 (Fig. 4A). A significant increase in the nuclear accumulation of STAT3 was also observed in fibroblasts after 2 h incubation in high glucose conditions (Fig. 4A). Furthermore, Ang II stimulation in a high glucose environment caused a marked increase in the nuclear accumulation of phosphorylated STAT3 (about a 40% increase compared to high glucose). Pre-incubation with N-acetyl-cysteine (NAC), before shifting cells to high glucose, abolished nuclear accumulation of phosphorylated STAT3 (Fig. 4A), demonstrating that nuclear localization of Tyr-705 phosphorylated STAT3 occurred through a ROS-dependent mechanism.

To study the involvement of the ERK1/2 signaling pathway in the enhancement of nuclear STAT3 localization observed following Ang II stimulation in high glucose, fibroblasts were treated with the MEK inhibitor PD98059 that specifically inhibits MEK-1-mediated activation of the ERK1/2 pathway. Pre-treatment of fibroblasts with PD98059 provoked a significant decrease in nuclear STAT3 accumulation (Fig. 4B), indicating the participation of ERK1/2 in the enhancement of nuclear STAT3 localization due to Ang II stimulation under high glucose conditions.

### 3.5. Collagen I and MMP-2 secretion in conditioned media of cardiac fibroblasts after 24 h of incubation in normal or high glucose medium

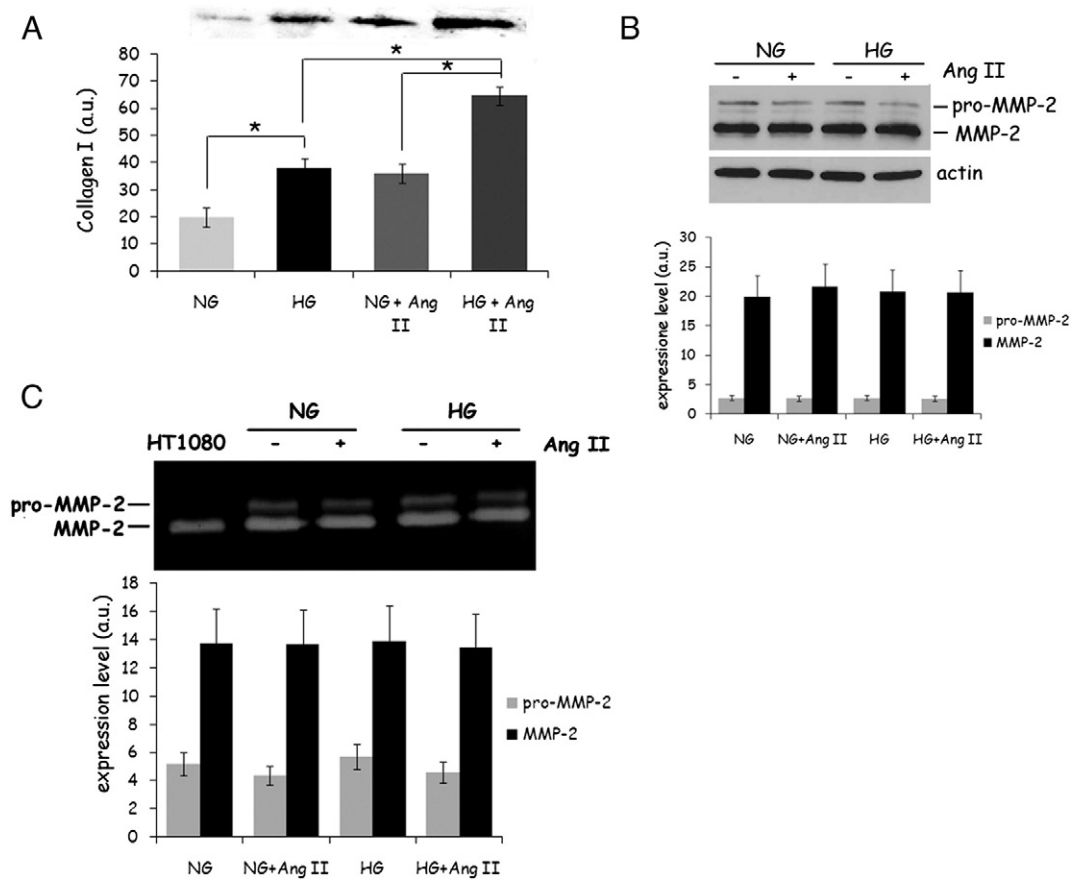
Collagen I is a major component of the LV extracellular matrix, and dysregulation of collagen I synthesis leads to cardiac fibrosis. A diabetic environment with high glucose increased collagen I deposition about

two-fold in comparison to normal glucose conditions (Fig. 5A). In addition, treatment of fibroblasts with Ang II synergized with high glucose to further enhance collagen I deposition (1.5-fold vs Ang II in high glucose; Fig. 5A).

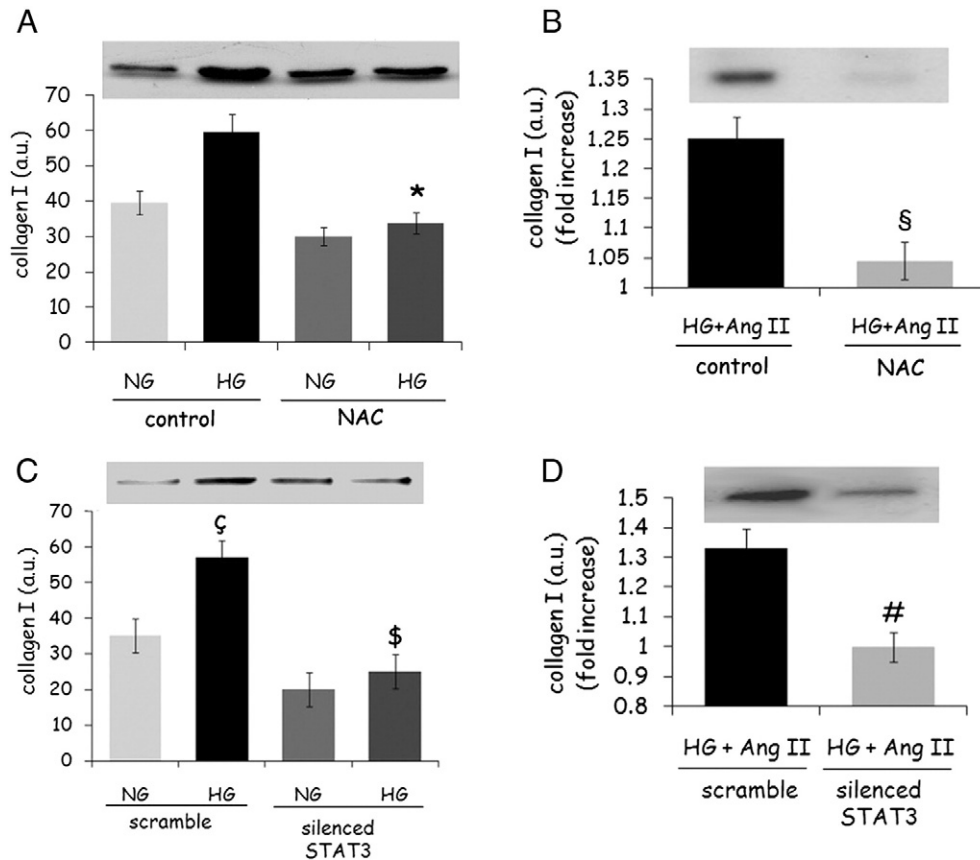
Extracellular matrix deposition is strictly regulated by the balance between synthesis and degradation processes. Degradation is regulated by the zinc-dependent matrix metalloproteinases (MMPs), particularly MMP-2 [19,20]. Cardiac fibroblast MMP-2 expression levels (both intracellular and extracellular) were not difference under high either glucose or Ang II stimulation (Fig. 5B and C).

### 3.6. Increased collagen I deposition in high glucose occurs through a ROS- and STAT3-dependent mechanism

The depletion of ROS by NAC inhibited the increase in collagen I synthesis under high glucose (Fig. 6A). Likewise, ROS participated by additionally increasing collagen I deposition observed in high glucose and Ang II treatment, since the addition of NAC decreased collagen I formation (Fig. 6B). To assess the involvement of STAT3 in collagen I formation, we silenced STAT3 in cardiac fibroblasts. Viability was unaffected by STAT3 silencing (Suppl. Fig. 3). Conversely, STAT3 silencing provoked a decrease of collagen I deposition both under normal and high glucose conditions (Fig. 6C and Suppl. Fig. 4A). In addition, STAT3 silencing greatly decreased the enhancement of collagen I formation due to Ang II stimulation (Fig. 6D and Suppl. Fig. 4B), thus demonstrating STAT3



**Fig. 5.** Analysis of collagen I and MMP-2 in normal and high glucose conditioned media. Cardiac fibroblasts were serum-deprived in normal glucose for 24 h and then high glucose medium was added for an additional 24 h. Where indicated, cells were stimulated for Ang II (100 nM) for 24 h. A) Analysis of collagen I deposition. Collagen I deposition was assayed by immunoblot analysis using specific antibodies. The intensity of the bands was analyzed by ImageJ, and the mean value is reported in the bar graph as arbitrary units (a.u.). One-way ANOVA followed by Tukey's post-test was used to evaluate statistical differences between groups. \* $p < 0.01$ . B) Representative immunoblot of MMP-2 expression in cardiac fibroblasts. Immunoblot shows the pro-MMP-2 (MW: 72 KDa) and the active MMP-2 (MW: 63 KDa). Bar graph represents the mean value reported as arbitrary unit (a.u.). C) MMP-2 secretion was assayed in cardiac fibroblast conditioned media by zymography. Conditioned medium of the fibrosarcoma cell line HT1080 is used as positive control of MMP-2. Data are representative of three independent experiments with similar results.



**Fig. 6.** Increase of collagen I deposition is mediated by a ROS- and STAT3-dependent mechanism. A) Collagen I formation in the setting of high glucose is ROS-dependent. Cardiac fibroblasts were serum-deprived in normal glucose for 24 h and then in high glucose for an additional 24 h. Where indicated, NAC (20 mM) was added to the media and left throughout the experiment. Collagen I deposition was assayed as reported in *Materials and methods* section. B) Involvement of ROS in collagen I deposition after Ang II stimulation for 24 h. Collagen I is reported as fold increase vs high glucose or vs high glucose + NAC. C) and D) Collagen I deposition occurs through a STAT3-dependent mechanism. 48 h after silencing STAT3 in fibroblasts, the cells were serum-deprived for 24 h in normal glucose and then treated with normal or high glucose for an additional 24 h. Where indicated, Ang II (100 nM) was added to the culture media. Silencing of STAT3 was detected by immunoblot using an anti-STAT3 antibody (Suppl. Fig. 4) while collagen I release was detected as described in *Material and methods* section. \* $p < 0.01$  vs high glucose; § $p < 0.001$  vs high glucose + Ang II; # $p < 0.05$  vs high glucose + scrambled Ang II; ζ $p < 0.01$  vs normal glucose; \$ $p < 0.001$  vs high glucose. Bar graph reports the mean of three independent analysis, while the immunoblots are representative images of three independent experiments.

as a key molecule for the enhancement of collagen I formation under hyperglycemic conditions.

#### 4. Discussion

This paper examined the role of high glucose (alone or in combination with Ang II) on the activation of the JAK2/STAT3 signaling pathway in cardiac fibroblasts. Further, we analyzed STAT3 involvement in collagen I formation in cardiac fibroblasts in hyperglycemic conditions. The most significant findings were: i) 2 h of pre-incubation of cardiac fibroblasts in high glucose induced JAK2 and STAT3 tyrosine phosphorylation; ii) high glucose increased STAT3 nuclear localization that was further enhanced by Ang II stimulation; iii) high glucose enhanced collagen I production that was further increased by Ang II treatment; and iv) increased collagen I, observed in high glucose and high glucose supplemented with Ang II, occurred through ROS- and STAT3-dependent mechanisms.

Hyperglycemia participates in the onset and worsening of cardiovascular disease. In particular, hyperglycemia may sustain the progression of heart failure through excessive interstitial myocardial collagen accumulation, thus leading to impaired diastolic and systolic function [21]. Cardiac fibroblasts, one of the major cell types contributing to structural and functional properties of the heart [1], are greatly affected by glucose levels. Indeed, a direct association between heart failure and dysregulation of cardiac fibroblast proliferation and extracellular matrix deposition has been reported [22–24]. Here we show that hyperglycemia alone induces the tyrosine phosphorylation of the JAK2/STAT3 signaling

pathway. The link between JAK2 and STAT3 in CFs has been previously reported [25]. Although an increased JAK2 and STAT3 tyrosine phosphorylation due to high glucose has been previously shown in mesangial cells [26], this is the first evidence in CFs. We observed a remarkable increase of STAT3 tyrosine phosphorylation in comparison to JAK2 in hyperglycemic condition, indicating that high glucose leads to STAT3 tyrosine phosphorylation by a JAK2-independent fashion. The tyrosine kinase Src may be a good candidate for JAK2-independent STAT3 tyrosine phosphorylation. Indeed, Src undergoes to redox activation following different kind of stimuli leading to ROS production. In turn, oxidized-activated Src promotes the phosphorylation/activation of downstream targets, culminating in specific cellular responses [27]. STAT3 is one of the several downstream substrates of Src and the involvement of the Src-STAT3 signaling pathway in the cardio protection has been recently reported [28]. Furthermore, another JAK2-independent STAT3 activation mechanism, involving the small GTPase Rac1, has been reported in atrial myocytes and fibroblasts [29]. Hyperglycemia induces the increase of ROS production provoking apoptosis in cardiomyocytes [30]. As consequence, oxidative stress leads to the reduction of myocardial contractility and the increased of myocyte fibrosis [31]. In agreement with the previous findings [32], we show an increased ROS production in high glucose. Addition of Ang II, after 2 h of pre-incubation of cells in high glucose, further enhanced ROS production. Ang II participates in inducing cardiac myocyte hypertrophy, fibroblast proliferation and collagen deposition [33–36]. Both cardiac myocytes and fibroblasts produce Ang II leading to an autocrine/paracrine mechanism [37–39]. Particularly in the diabetic

environment, fibroblasts are a considerable source of Ang II secretion that leads to the up-regulation of transforming growth factor  $\beta$  and to increased collagen I production [6,40].

The enhanced Tyr705 phosphorylation of STAT3, observed in the high glucose condition, increased STAT3 nuclear localization, in agreement with the previous findings showing that STAT3 tyrosine phosphorylation is a key event for the STAT3 nuclear migration [9]. The raise of ROS production observed with high glucose and Ang II stimulation was important for nuclear STAT3 accumulation since ROS depletion greatly decreased STAT3 migration. It was intriguing to observe that Ang II addition, after 2 h of pre-incubation of CFs in high glucose, did not enhance JAK2 and STAT3 tyrosine phosphorylation. This result was completely different from the previous observations in cardiomyocytes. In healthy human ventricular myocytes Ang II activates JAK2 phosphorylation only in the presence of high glucose leading to enhancement of ROS generation. Conversely, in failing myocytes, high glucose induces JAK2 phosphorylation via an angiotensin converting enzyme-dependent Ang II autocrine production [7]. In addition, Ang II-induced JAK2 activation is followed by STAT3 phosphorylation in healthy cardiac myocytes [8]. In the latter condition, Ang II contemporary activates different intracellular pathways. Ang II induces the overexpression of the associated B cell lymphoma (Bcl)-xL through a JAK2- and extracellular signal-regulated kinase (ERK) 1/2-dependent mechanism, while p38MAPK cascade leads to the activation of the Fas ligand (Fas-L) [8]. Although JAK2 and STAT3 phosphorylation is unaffected by Ang II stimulation in high glucose, a significant increase of nuclear STAT3 has been detected in response to Ang II in high glucose medium. Recent results show that hyperglycemic condition induces CF proliferation by activating STAT1/STAT3 and ERK1/2 signaling pathways [41]. In particular, ERK1/2 pathway is involved in collagen types I and III production in fibroblasts exposed to high glucose concentrations [42]. ERK1/2 activation due to Ang II stimulation in fibroblasts has been reported [43,44]. Our findings suggest that the enhanced nuclear STAT3 localization involves ERK1/2 activation. Activated ERK1/2 is involved in the rapid STAT3 serine 727 phosphorylation which modulates STAT3 transcriptional activity [45, 46]. Although further studies are needed to know deeply the mechanism underlying the increased nuclear STAT3 due to Ang II in the setting of high glucose, the participation of the Ang II-ERK1/2-STAT3 pathway can be hypothesized.

Our findings show that glucose and Ang II act in synergy (at least in the early phase of hyperglycemic condition) leading to the increased amount of phosphorylated STAT3 in the nucleus. Transcriptional activity of STAT3 is enhanced by p300 co-factor [47] and inhibited by protein inhibitors of STAT3 [48]. Transcriptional regulation of COL1A2 gene, responsible for collagen I biosynthesis during development and in fibrotic conditions, occurs through *cis*-acting elements and *trans*-acting factors implicated in constitutive, cytokine-modulated and tissue-specific expression of COL1A2 [49]. Currently, there are no data regarding the presence of STAT3 elements in the collagen I promoter, thus suggesting that STAT3 may act through an indirect mechanism. Although it is clear that hyperglycemia leads to enhanced fibrosis, the underlying molecular mechanism is not fully elucidated. The increased collagen production due to fibroblasts exposed to high glucose is not associated with enhanced extracellular matrix degradation by MMPs [5]. As consequence, there is an imbalance between synthesis and degradation of extracellular matrix which is shifted toward matrix accumulation [50]. Here, we observe that MMP-2 secretion is unaffected by both glucose and Ang II, thus contributing to the enhancement of extracellular matrix deposition.

The previous findings suggest that ROS are involved in Ang II-dependent collagen production [51] and focused on STAT3 as a responsible molecule for collagen deposition in the onset of fibrosis [52]. In conclusion, we report for the first time that the increased collagen I deposition in high glucose occurred through a ROS- and STAT3-dependent mechanism. Indeed, both ROS depletion and the STAT3 silencing inhibit collagen I production due to high glucose in CFs. Ang II stimulation in

the early phase of hyperglycemia provokes an increased burst of ROS leading to enhancement of STAT3 nuclear localization through a mechanism involving ERK1/2.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbamcr.2014.07.009>.

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