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Original Research

Downmodulation of peroxiredoxin-3 expression by angiotensin II in cardiac fibroblasts through phosphorylation of FOXO3a by Akt

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Key Words
Akt; Angiotensin II; Cardiac fibroblasts; FOXO3a; Peroxiredoxin-3

Abstract
The aim of this study was to determine whether angiotensin II (ANG II) affects the protein and mRNA expression of the mitochondrial antioxidant peroxiredoxin-3 (Prx-3) in cardiac fibroblasts through inducing the phosphorylation of the proteins Akt and FOXO3a. Cardiac fibroblasts from normal male adult rats were cultured to confluency and incubated in serum-free Dulbecco's modified Eagle's medium for 24 h. The cells were then preincubated with(out) the tested inhibitors for 30 min to 1 h and further incubated with(out) ANG II (1 µmol/l) for 24 h. ANG II decreased ($p < 0.01$) the mRNA and protein expression of Prx-3 by 36.9 ± 3.0% and 29.7 ± 2.7% ($n = 4$), respectively. The likely mechanism through which ANG II produces the effect of reducing Prx-3 expression is by reducing the extent of binding of FOXO3a to the Prx-3 promoter. In control fibroblasts inhibition of FOXO3a transcription with small-interfering RNA (siRNA) led to a reduction in Prx-3 gene expression. Our data also showed that when Akt is phosphorylated by ANG II, P-Akt is translocated from the cytoplasm to the nucleus, subsequently nuclear phosphorylation of FOXO3a by P-Akt leads to relocalization of FOXO3a from the nucleus to the cytosol, resulting in a decrease its transcriptional activity, and consequently in Prx-3 expression.

INTRODUCTION

Cardiac fibroblasts are protected from oxidative stress, triggered by inflammation after myocardial injury or induced by angiotensin II (ANG II) or growth factors, by expressing potent antioxidant defenses such as superoxide dismutases, catalases, glutathione peroxidases and peroxiredoxins [1, 2]. Multiple peroxiredoxins or thioredoxin peroxidases, (Prx-1 through -6) are identified in mammalian cells in different intracellular locations and protect cells and tissues from damage caused by reactive oxygen species (ROS) [3-6] . All six peroxiredoxins are present in cardiac fibroblasts. Prx-1, -2 and -6 are localized in the cytosol, Prx-3 in mitochondria, Prx-4 in the extracellular space and Prx-5 is localized intracellularly to cytosol, mitochondria and peroxisomes [7, 8]. Prx-3 is found exclusively in the mitochondria [3] and uses mitochondrial thioredoxin (Trx-2) as the electron donor for its peroxidase activity [9].

Mitochondria are considered the main intracellular source of ROS [10]. ROS production, mitochondrial DNA damage and respiratory chain impairment are linked to one another to create a vicious cycle that leads to progressive decline in mitochondrial bioenergetics and subsequent cardiac dysfunction. Prx-3 functions not only by removing H_2O_2 formed after the superoxide dismutase (SOD)-catalysed dismutation but also by detoxifying peroxynitrite [11].

In adult rat cardiac fibroblasts we have reported that ANG II increased superoxide anion production and intracellular formation of reactive oxygen species [12, 13]. It has also been shown that the serine/threonine protein kinase Akt-regulated Forkhead transcription factor FOXO3a protected cells from oxidative stress by directly increasing Mn-SOD mRNA and protein expression [14]. Upon phosphorylation of Akt (protein kinase B) by ANG II in cardiac fibroblasts, P-Akt is translocated from the cytoplasm to the nucleus and nuclear phosphorylation of FOXO3a by P-Akt leads to relocalisation of FOXO3a from the nucleus to the cytosol, thus resulting in a decrease in its transcriptional activity and in Mn-SOD expression [15].

The aim of the present study was to investigate whether ANG II also affects Prx-3 mRNA and protein expression in rat cardiac fibroblasts through induction of phosphorylation of Akt and FOXO3a and repression of the FOXO3a binding to the Prx-3 promoter and consequently Prx-3 gene expression.

MATERIALS AND METHODS

Cell cultures

All animal procedures were in accordance with the laws, regulations and administrative provisions of the Member States of the European Community (Council Directive 86/609/EEC of November 24, 1986) regarding the protection of animals for experimental and other scientific purposes. This research protocol was also approved by the Ethical Committee for Animal Experiments of the Catholic University of Leuven (KULeuven), Belgium.

Cardiac ventricular fibroblasts obtained from male adult Wistar rats were grown in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% fetal bovine serum (FBS) and used in passage 2 as previously described [16]. When cultures reached confluence, the medium was replaced with fresh phenol red free DMEM with 0.5% FBS for 24 h. The cells, identified as proliferative myofibroblasts [17], were then preincubated with(out) the tested inhibitors for 30 min to 1 h and then further incubated with(out) ANG II (1 μmol/l) for 24 h in this medium.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Total RNA was isolated from control and ANG II-treated cardiac fibroblasts [12] with Trizol (Gibco BRL, Life Technologies Ltd, Paisley, UK), followed by chloroform extraction and precipitation with isopropanolol. RNA pellets were washed with 80% ethanol, air dried and dissolved in distilled water. Single-stranded cDNA was synthetised from 5 μg of total RNA, using a commercial cDNA mix (Life Technologies). The mRNA levels were quantified by the RT-PCR method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal standard. The PCR primers for target cDNAs were as follows:

Prx-3, sense GCTGAGTCTCGAACGACTTTAAGGG and antisense CTTGATCGTAGGGGACTCTGGTGT [18];

Prx-1, sense TGTGGATTCTCACTTCTGTCATCTG and antisense TGCGCTTGGGATCTGATACC [18];

FOXO3a, sense CGGACAAACGGCTCACTT and antisense TCGGCTCTTGGTGTACTTG [19];

GAPDH, sense ACCACAGTCCATGCCATCAC and antisense TCCACCACCCTGTTGCTGTA [15].

Ten microliters of PCR products were separated on 1.2-1.3% agarose gels, stained with ethidium bromide and photographed [17]. Densitometric analysis of the stained images was performed using a Sharp Scanner JX-325 and a Software Image Master (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). The densities of Prx-3 and FOXO3a bands in relation to the bands obtained for GADPH were expressed as Prx-3 or FOXO3a mRNA/GADPH mRNA.

FOXO3a binding site in the rat Prx-3 promoter

FOXO3a binding to Prx-3 promoter in nuclear extracts (30 μg) of control and ANG II-treated fibroblasts was detected by a NoShift Transcription Assay kit (EMD Biosciences Inc., Merck, Darmstadt, Germany) using an anti-FOXO3a antibody (Cell Signaling, Danvers, MA, USA), a biotinylated double stranded oligonucleotide CACACAAGGTTAACAAAACAGTGGGAAATATG GAAACAAATACCTAATGC [20] and an anti-rabbit IgG-HRP(Horseradish peroxidase) (Clontech Labs Inc, Mountain View, CA, USA) as secondary antibody.

Silencing(si)RNA transfection

FOXO3a siRNA SMARTpool, (a mixed pool of 4 target sequences GCACGGAGCUGGAUGACGU, UGGAUGACCUGCUAGAUAA, GAACGUUGUUG GUUUGAAC and CGUCAUGGGUCACGACAAG), synthetised by Dharmacon (Lafayette, CO, USA) according to rat LOC294515(XM_215421)FOXO3a, was delivered to cardiac fibroblasts at 100 nM final concentration for 24-48 hours through a lipid-mediated DharmaFECT transfection reagent.

Prx-3 siRNA (NM_022540) was synthetised by Qiagen (Hilden, Germany) according to rat LOC502175.

Immunoblot

Western blotting [15] has been performed in cell lysates and in nuclear and cytosolic extracts prepared

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by NE-PER extraction reagents (Pierce Biotechnology, Rockford, IL, USA). Antibodies for FOXO3a, phospho-FOXO3a (Ser253), Akt and phospho-Akt (Ser473) were from Cell Signaling, antibodies for Prx-1 from Abcam Inc. (Cambridge, MA, USA), for Prx-3 from LabFrontier (Seoul, Korea) and for GAPDH from Sigma Chem. Co. (St Louis, MS ,USA) and the corresponding secondary HRP-conjugated antibodies from Chemicon Inc. (Temecula, CA, USA).

Assay of reactive oxygen species

A fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCF-DA) was used for the assessment of intracellular [21, 22] ROS formation (such as hydrogen peroxide, hydroxyl and peroxyl radicals and hydroperoxides) in cardiac fibroblasts treated with(out) ANG II .

Statistical analysis

Values are expressed as mean ± SEM. The statistical methods used were repeated measures of variance (Tukey's) and Students 2-tailed test for (un)paired data when appropriate. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effect of ANG II on Prx-3 protein and mRNA expression in cardiac fibroblasts

ANG II reduced dose-dependently the mRNA expression of the mitochondrial antioxidant Prx-3 (Fig.1). Addition of ANG II (1 µmol/l) decreased ($p < 0.01$) the mRNA expression of Prx-3 by 36.9 ± 3.0% (n = 4) while mRNA expression of the cytosolic homologue Prx-1 was unaffected by ANG II (Fig.2).

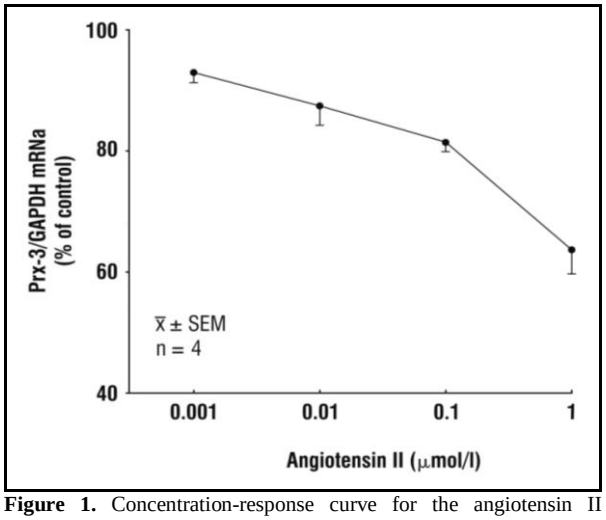

Figure 1. Concentration-response curve for the angiotensin II (ANG II)-induced changes in peroxiredoxin-3 (Prx-3) mRNA expression. Cardiac fibroblasts were treated with(out) ANG II (0.001, 0.01, 0.1 and 1 µmol/l) for 24 h.

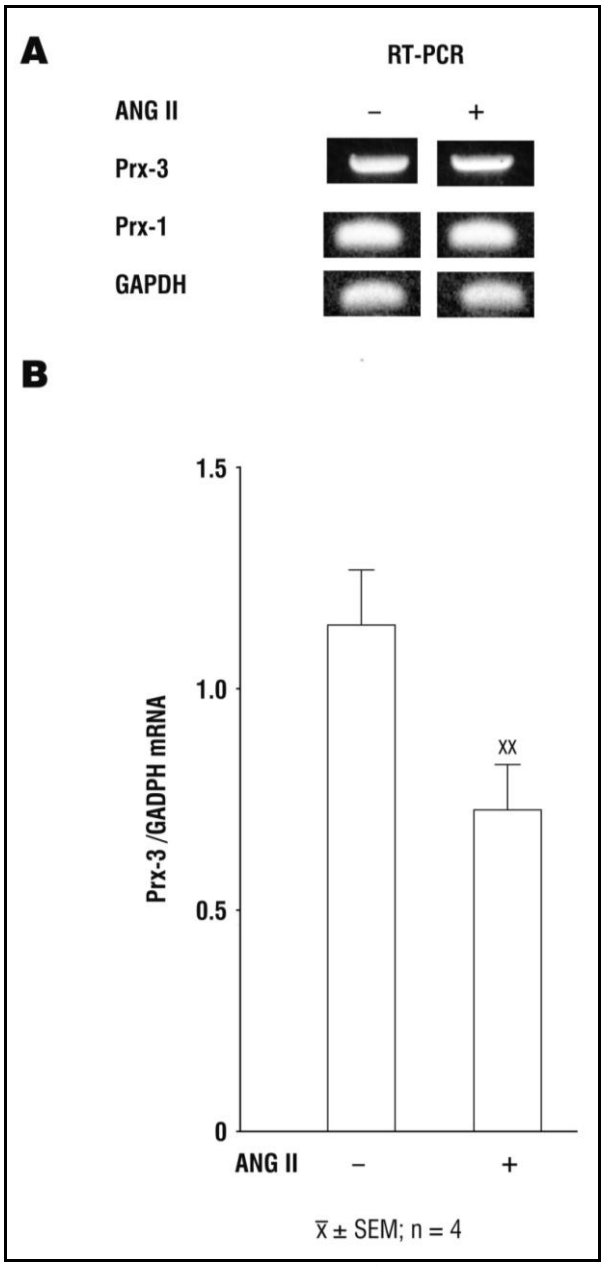

Figure 2. (A) Representative gels of mRNA expression of peroxiredoxin-3 (Prx-3), peroxiredoxin-1 (Prx-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cardiac fibroblasts treated with(out) ANG II (1 µmol/l) for 24 h. **(B)** Prx-3/GAPDH mRNA expression in cardiac fibroblasts treated with(out) ANG II for 24h; **xx** $p < 0.01$ as compared to control.

The Prx-3 protein level was also reduced ($p < 0.01$) by 29.7 ± 2.7% (n = 4) in ANG II-treated fibroblasts compared to controls; the protein expression of Prx-1 was however not affected by ANG II (Fig.3).

ANG II-induced cellular ROS production in cardiac fibroblasts

To determine whether ANG II affects intracellular H_2O_2, cardiac fibroblasts treated with(out) ANG II

were incubated with DCF-DA, a peroxide-sensitive dye that is incorporated into the cell. ANG II caused a 1.8-fold increase ($p < 0.001$) in DCF-DA fluorescence from 389 ± 36 to 695 ± 66 a.u./10^6 cells ($n = 10$), indicating ROS generation. 5-Hydroxydecanoate and glibenclamide, specific and nonspecific inhibitors of mitochondrial ATP-sensitive potassium channels, respectively, suppressed ($p < 0.01$) the ROS generation in control ($42.0 \pm 7.1\%$ and $71.6 \pm 8.7\%$, respectively) and ANG II-treated ($32.5 \pm 5.8\%$ and $45.8 \pm 4.9\%$, respectively) cardiac fibroblasts (Fig.4).

The complex I inhibitor rotenone increased ($p < 0.05$) ROS production in control and ANG II-treated fibroblasts by 27.6 ± 3.5 and $27.3 \pm 5.3\%$, respectively. Antimycin A, which blocks the Q_i site located at the inner membrane and facing the mitochondrial matrix, enhanced ($p < 0.01$) ROS release by complex III in control ($41.9 \pm 8.6\%$) and ANG II-treated ($45.6 \pm 2.9\%$) fibroblasts. The complex II inhibitor thenoyltrifluoroacetone (TTFA) did not affect the ROS production in cardiac fibroblasts (Fig.4).

Intracellular ROS production was also reduced by the phosphoinositide inhibitors wortmannin and LY 294002 in control and ANG II-treated fibroblasts (Fig.5).

Effect of ANG II on FOXO3a binding to Prx-3 promoter

The FOXO3a binding activity to the Prx-3 DNA was lower in nuclear extracts of ANG II-treated fibroblasts as compared to control fibroblasts (Fig.6). ANG II induced a time dependent reduction in FOXO3a binding activity to the Prx-3 promoter. The reduction was 21.8 ± 4.9 % at 30 min, with a further reduction of $83.1 \pm 3.5\%$ at 2 h after ANG II treatment.

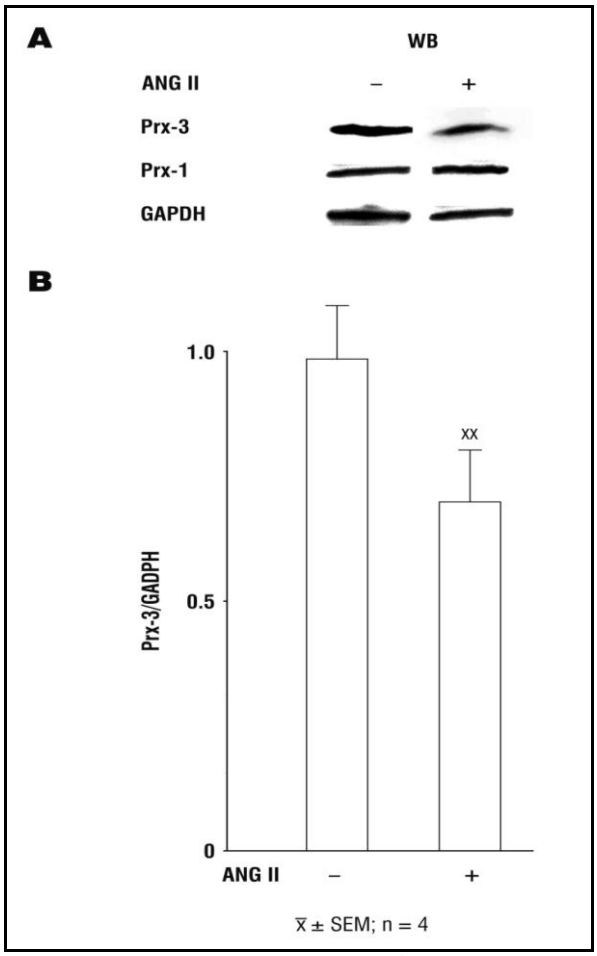

Figure 3. A-Representative Western blots of peroxiredoxin-3 (Prx-3), peroxiredoxin 1 (Prx-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cardiac fibroblasts treated with(out) ANG II (1 µmol/l) for 24 h. **B**-Prx-3/GAPDH protein expression in control and ANG II-treated cardiac fibroblasts; **xx** $p < 0.01$ as compared to control.

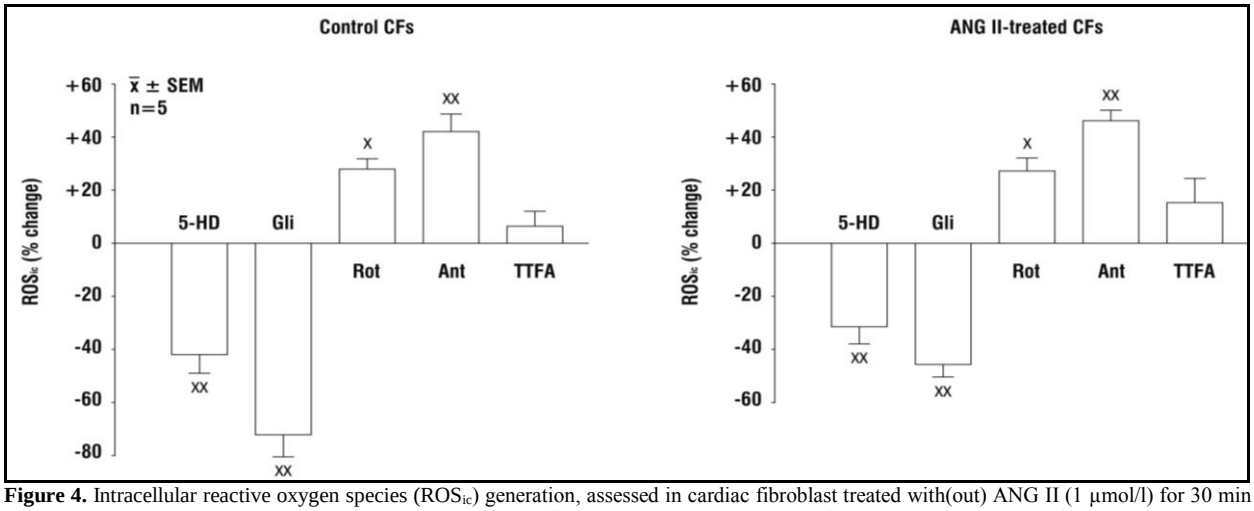

Figure 4. Intracellular reactive oxygen species (ROS_{ic}) generation, assessed in cardiac fibroblast treated with(out) ANG II (1 µmol/l) for 30 min after preincubation with 5-hydroxydecanoate (5-HD, 100 µmol/l), glibenclamide (Gli, 20 µmol/l), rotenone (Rot, 10 µmol/l), Antimycin A (Ant, 10 µmol/l) or thenoyltrifluoroacetone (TTFA, 10 µmol/l) for 1 h. ROS_{ic} in control and ANG II-treated cardiac fibroblasts averaged, respectively, 388 ± 37 and 715 ± 54 a.u./10^6 cells. **xx** $p < 0.01$, **x** $p < 0.05$ compared to samples with(out) ANG II.

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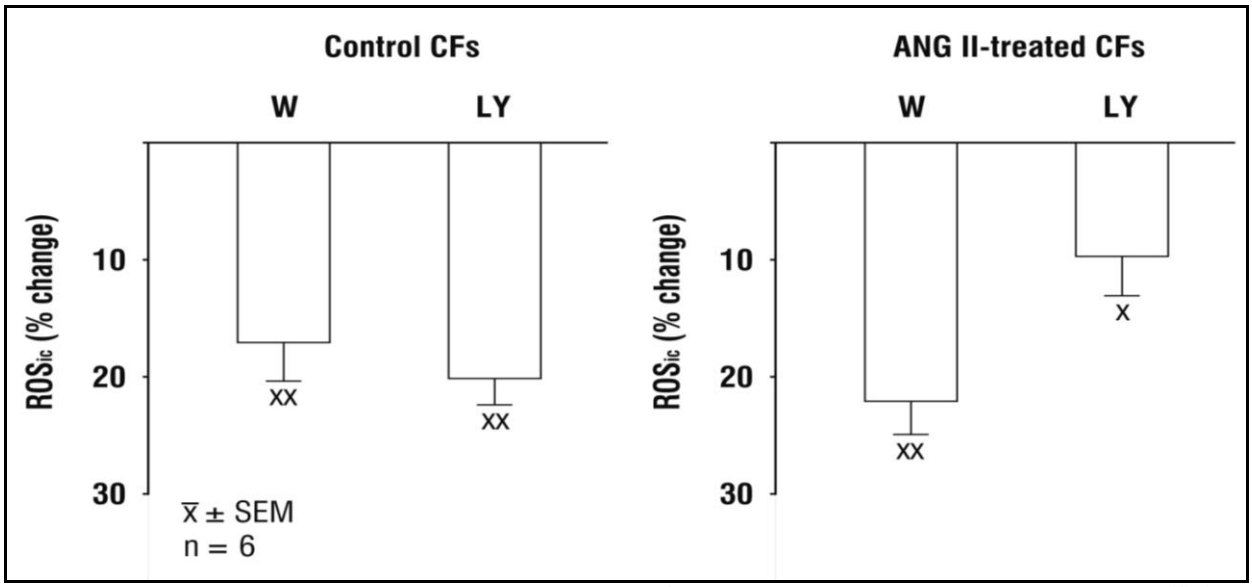

Figure 5. Intracellular reactive oxygen species (ROS_{ic}) generation in cardiac fibroblasts treated with(out) angiotensin II (ANG II, 1 μmol/l) for 30 min after preincubation with(out) LY 294002 or wortmannin (1 μmol/l) for 1 h. ROS_{ic} in control and ANG II-treated cardiac fibroblasts averaged, respectively, 450 ± 23 and 805 ± 50 a.u./10^6 cells. **xx** $p < 0.01$, **x** $p < 0.05$ compared to samples with(out) ANG II.

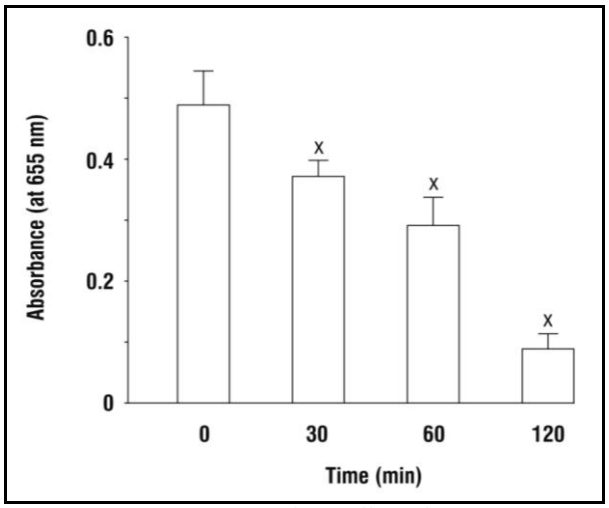

Figure 6. Time dependency of the effect of angiotensin II on FOXO3a binding to peroxiredoxin-3 DNA in nuclear extracts of cardiac fibroblasts. **x** $p < 0.05$ compared vs. controls.

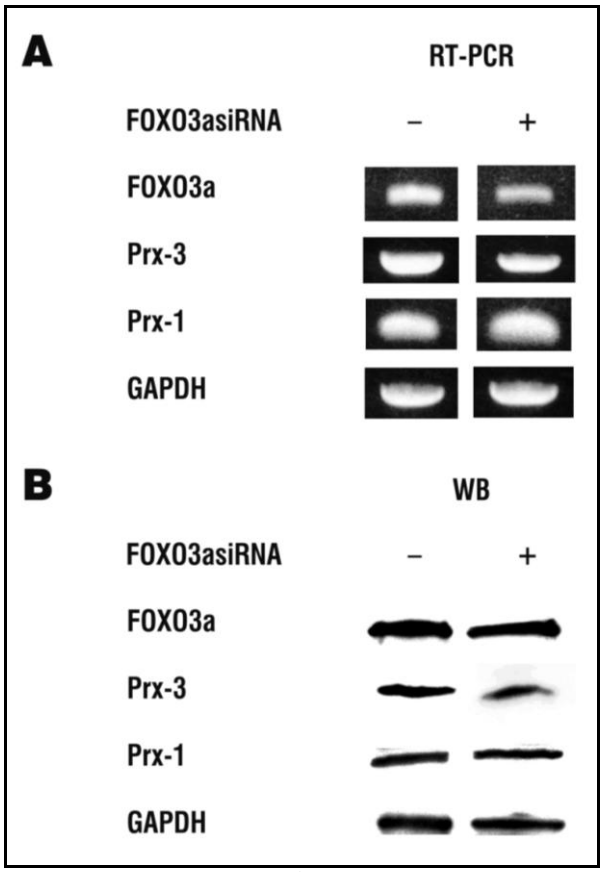

Figure 7. Representative gels of mRNA **(A)** and protein **(B)** expression of FOXO3a, peroxiredoxin-3 (Prx-3), peroxiredoxin-1 (Prx-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cardiac fibroblasts transfected with(out) FOXO3a siRNA. **RT-PCR**, reverse transcription-polymerase chain reaction; **WB**, Western blots show FOXO3a in the nucleus and Prx-3, Prx-1 and GAPDH in the cytosol.

FOXO3a controls Prx-3 transcription

Previously we have shown that ANG II phosphorylated Akt on Ser^{475} and increased FOXO3a ($pSer^{253}$) levels in cardiac fibroblasts [15].

In order to establish whether FOXO3a is causally involved in the transcriptional regulation of the Prx-3 gene, the siRNA technique was used. Cardiac fibroblasts treated with FOXO3a siRNA demonstrated a ~69% reduction of FOXO3a mRNA level, a ~43% reduction of nuclear FOXO3a protein and a decrease in Prx-3 mRNA and protein levels of, respectively, ~38% and ~29%; but no change in Prx-1 mRNA and protein levels (Fig.7). These data indicate that FOXO3a

controls transcription and show consistent changes between Prx-3 mRNA and protein expression levels using the same approach.

In order to elucidate whether the ANG II-mediated regulation of Prx-3 mRNA expression is under control of PI3K-Akt signaling, ANG II-treated fibroblasts were preincubated with the PI3K-inhibitors wortmannin and LY 294002. The Prx-3 mRNA expression was increased ($p < 0.05$) in ANG II-treated cells relative to the values before incubation with the inhibitors (Fig.8).

The ANG II-reduced levels of FOXO3a transcripts in cardiac fibroblasts were abolished in cells pretreated with wortmannin and LY 294002 (Fig.8), indicating a role of PI3K in the control of FOXO3a at the transcriptional level.

PI3K is an upstream mediator of Akt activation in cardiac fibroblasts [15]. Western blot analyses were carried out in nuclear and cytosolic extracts of control fibroblasts to demonstrate differences in the localization of P-FOXO3a and P-Akt. Western blot analysis indicated that the phosphorylation of Akt at Ser^{472} in the nucleus was increased 2.7 fold after exposure to ANG II for 30 min. The same blot, reprobed with an antibody to P-FOXO3a at Ser^{253}, showed minimal changes in P-FOXO3a in the nucleus, but a 4.3 fold rise in the cytosol, indicating that P-FOXO3a was transported to the cytosol after ANG II treatment (Fig.9).

Cellular ROS production in Prx-3- and FOXO3a-depleted fibroblasts

In order to test whether the upregulation of Prx-3 by FOXO3a is required for protection against cellular ROS protection, we depleted fibroblasts from Prx-3 or FOXO3a by performing transfections with siRNA against Prx-3 or FOXO3a and measured ROS production. Depletion of FOXO3a increased ($p < 0.01$) the ROS production in cardiac fibroblasts by $70 \pm 8\%$ ($n = 6$). Depletion of FOXO3a had a more potent effect on ROS production than Prx-3 depletion ($17.4 \pm 1.1\%$) indicating that Prx-3-FOXO3a axis is required to protect against oxidative stress induced by Ang II.

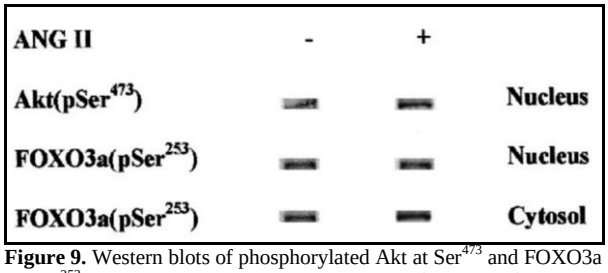

Figure 9. Western blots of phosphorylated Akt at Ser^{473} and FOXO3a at Ser^{253} in nuclear and cytosolic extracts of cardiac fibroblasts treated with(out) ANG II (1 µmol/l) for 30 min.

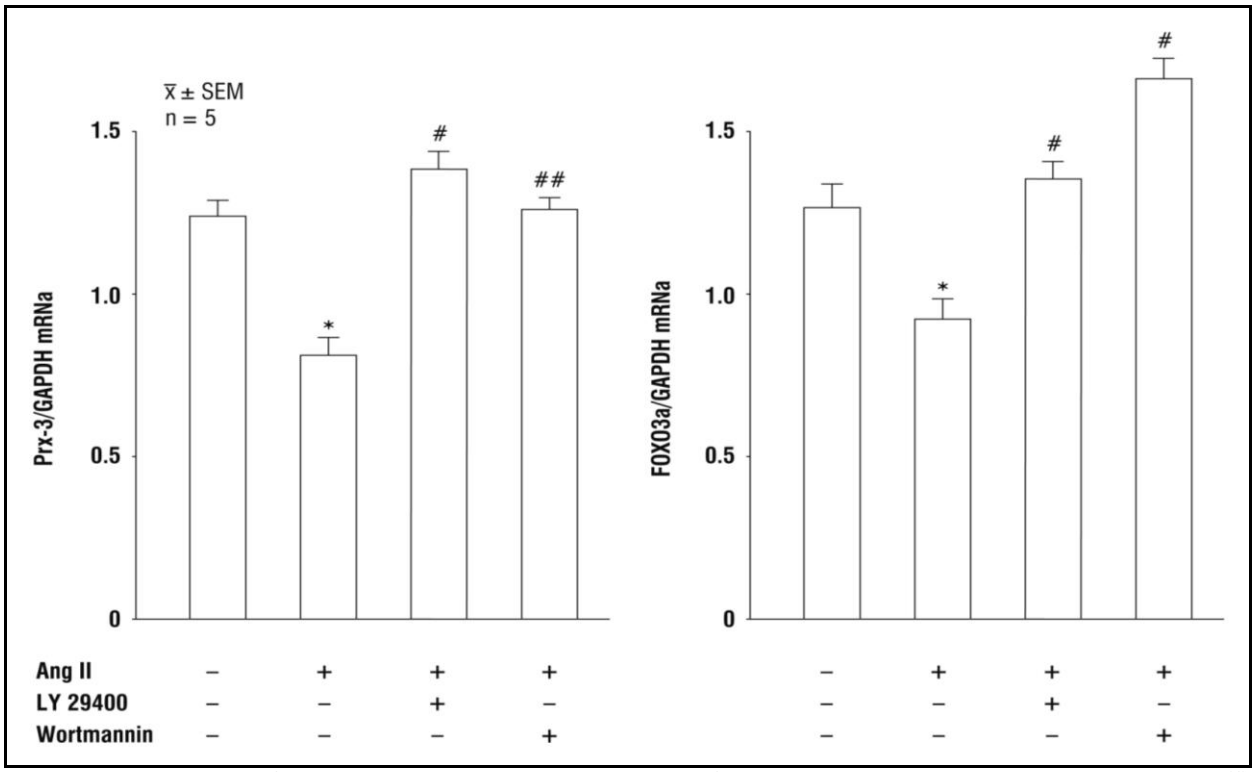

Figure 8. mRNA expression of peroxiredoxin-3 (Prx-3) and FOXO3a in cardiac fibroblasts treated with(out) angiotensin II (ANG II, 1 µmol/l) for 24 h after preincubation with LY 294002 or wortmannin (1 µmol/l) for 1 h. **x** $p < 0.05$ as compared to controls without ANG II, LY and wortmannin; ## $p < 0.01$, # $p < 0.05$ as compared to samples with ANG II and without LY and wortmannin.

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DISCUSSION

The present *in vitro* data have shown, in cultured rat cardiac fibroblasts, that ANG II decreased the mRNA and protein expression of the mitochondrial specific antioxidant Prx-3, while the cytosolic Prx-1 fraction was unaffected (Figs.1-3). This downregulation of Prx-3 is linked to a downregulation of its mRNA levels, indicating that the alterations in Prx-3 expression result from an altered gene expression rather than from alterations in post-transcriptional regulation. Previously we have shown that the protein expression of α-smooth muscle actin, a marker of the differentiation of fibroblasts into myofibroblasts, was not significantly increased by ANG II, indicating that the effects of ANG II on Prx-3 expression do not result from changes in the phenotype of the cultured fibroblasts [15].

A downregulation of Prx-3 has been described in various experimental models that are characterized by an increased cellular oxidative stress [23-25]. In human heart failure Brixius *et al* [26] also reported a selective downregulation of the mitochondrial Prx-3, while the cytosolic Prx-1 and Prx-2 isoforms were unaffected by the enhanced ROS production. Thus reducing Prx-3 sensitizes cells to oxidative stress [19].

ANG II also induces oxidative stress by increasing intracellular ROS production in cardiac fibroblasts (Fig.4). In quiescent cells most of ROS are produced through an univalent reduction of molecular oxygen to superoxide ($\bullet O_2^-$) by electrons that leak from complex I and III of the mitochondrial electron transport chain [27-28]. $\bullet O_2^-$ does not readily cross membranes due to its charged nature. It inhibits mitochondrial function by inactivating the Fe-S centers in complex I and III and the tricarboxylic acid cycle (aconitase) [29]. The burden of $\bullet O_2^-$ is largely countered by the mitochondrial enzyme Mn-SOD. Although Mn-SOD relieves oxidative stress in mitochondria caused by $\bullet O_2^-$, it generates H_2O_2, a mild oxidant which is readily converted to the more powerful oxidant •OH.

Indeed, recent findings have demonstrated that ANG II stimulation induces the opening of mitochondrial K_{ATP} channels and further amplifies ROS formation from mitochondria [30]. ROS produced initially in the mitochondria have been shown to provoke a positive feedback with mitochondria responding to elevated levels of ROS by increasing their own ROS production in a process known as ROS-induced ROS release [31].

Blockade of the mitochondrial K_{ATP} channels by glibenclamide or 5-hydroxydecanoate suppressed the ROS production in control and ANG II-treated cardiac fibroblasts (Fig.4). On the contrary inhibitors of complex I and III, rotenone and antimycin A stimulated ROS production at these sites, while the complex II inhibitor TTFA had no effect on ROS production in control or ANG II-treated cardiac fibroblasts (Fig.4). Antimycin A blocks the electron transfer from the Q_i to Q_o sites of complex III and increases ROS production in the innermembrane space of mitochondria [32, 33]. Rotenone blocks complex I near the binding site for ubiquinol, the electron acceptor of complex I [33]. Blockade of complex I at this distal site in the complex increases the reduction of the NADH dehydrogenase of complex I, increasing the electron leak to ROS. Thus rotenone blockade enhances oxyradical production by complex I. Exposure of cardiac fibroblasts to ANG II can thus lead to increased oxidative stress because of downregulation of the mitochondrial antioxidant Prx-3.

Indeed, Prx-3 knockdown by siRNA increased mitochondrial ROS [25] and Prx-3 knock-out mice are more susceptible to lipopolysaccharide-induced oxidative stress than their wild-type litternates [34]. Higher levels of ROS are also detectable in macrophages derived from these mice and they release increased amounts of TNFα [35].

Overexpression of Prx-3 protects the heart against post-MI remodeling and failure in mice [36]. It reduces LV cavity dilatation and dysfunction as well as myocyte hypertrophy, interstitial fibrosis and apoptosis of the non-infarcted myocardium. These beneficial effects of Prx-3 gene overexpression were associated with the attenuation in oxidative stress, mitochondrial DNA decline and dysfunction [36]. Prx-3 overexpression has also been shown to improve glucose homeostasis, with transgenic mice displaying resistance to diet-induced elevations in blood glucose and increased glucose clearance [37].

Taken all data together suggest that the loss of Prx-3 results in increased susceptibility to oxidative stress. Thus, it may be concluded that an increase in cellular oxidative stress seems to be paralleled by a downregulation of mitochondrial Prx-3.

Chronic increases in mitochondrial ROS production can lead to a catastrophic cycle of further oxidative stress and ultimate cellular injury [38]. This deleterious process may play an important role in the development and progression of myocardial remodeling and failure [39]. Given that mitochondria contain Prx-3 30 times more abundant than glutathione peroxidase [29], Prx-3 is thought to be a primary line of defense against H_2O_2 produced by the mitochondrial respiratory chain, as Mn-SOD does against $\bullet O_2^-$.

Approx. 90% of mitochondrial hydrogen peroxide reacts with Prx-3. Although glutathione peroxidase has a higher rate constant than Prx-3, its lower abundance limits its ability to compete directly with Prx-3 [40]. The specific localization of Prx-3 in the mitochondria suggests that mitochondrial oxidative stress plays an important role in the development and progression of

heart failure and the antioxidant localized specifically within the mitochondria provides a primary line of defense against oxidative stress-mediated myocardial injury [41]. Antioxidant strategies specifically targeting Prx-3 or Mn-SOD can have therapeutic benefit in preventing a wide spectrum of adverse cardiovascular outcomes.

FOXO (Forkhead box class O) transcription factors may be important in the regulation of the antioxidant defense in many species [14]. In human cardiac fibroblasts FOXO3a mediates the expression of peroxiredoxin-3, which functions to protect mitochondria against oxidative stress by scavenging H_2O_2 [20]. In rat cardiac fibroblasts ANG II was shown to reduce the binding of FOXO3a to the Prx-3 promoter (Fig.6). Inhibition of FOXO3a transcription with siRNA led to a reduction in FOXO3a binding to the Prx-3 promoter, and a concomitant reduction in Prx-3 gene expression in control cardiac fibroblasts (Fig.6), thereby suggesting that FOXO3a upregulates Prx-3.

In FOXO3a-depleted fibroblasts the reduced Prx-3 expression was also associated with an increase in ROS production, which was more pronounced than in Prx-3-depleted cells. In human cardiac fibroblasts as well as in HEK293 cells [10] deletion of FOXO3a increased total cellular level of H_2O_2 more pronounced (~45%) than Prx-3 depletion (~15%). Our data suggest, therefore, that FOXO3a may be the transcription factor responsible for the ANG II-induced downregulation of Prx-3 in cardiac fibroblasts.

ANG II-stimulated Akt activity may be responsible for the phosphorylation and inactivation of FOXO3a [15] which, in turn, downregulates Prx-3 transcription in rat cardiac fibroblasts. It has indeed been shown [15] that, when Akt is phosphorylated by ANG II, P-Akt is translocated from the cytoplasm to the nucleus, and nuclear phosphorylation of FOXO3a by P-Akt leads to relocalisation of FOXO3a from the nucleus to the cytosol, thereby resulting in a decrease in its transcriptional activity and consequently in Prx-3 expression (Fig.9).

Inhibition of PI3K with wortmannin and LY 294002 led to a decrease in P-Akt [15], an increase in Prx-3 mRNA expression (Fig.8) and a reduction in ROS production (Fig.5). These data indicate that this translocation-relocalisation mechanism contributed to the downregulation of Prx-3 gene expression.

In conclusion, our data show that ANG II inactivated FOXO3a by activating Akt, leading to a reduction in the expression of the antioxidant Prx-3 and thereby contributing to ROS production. ANG II may cause, through this mechanism, an increase in oxidative stress by inhibiting the expression of the mitochondrial enzyme Prx-3 that is involved in ROS breakdown.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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