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Original Article

Role of reactive oxygen species in the transforming growth factor-β_1-induced collagen production and differentiation of cardiac fibroblasts into myofibroblasts

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Abstract
The aim of the present study was to determine whether transforming growth factor-β_1 (TGF-β_1)-induced collagen production in cardiac fibroblasts is affected by reactive oxygen species (ROS). Cardiac fibroblasts (passage 2) 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 1 h and further incubated with(out) TGF-β_1 at various concentrations and for 1, 2, 4, 24 or 48 h. TGF-β_1 induced a dose-dependent increase in the soluble collagen production in cardiac fibroblasts after 48 h of incubation. No significant effect of TGF-β_1 (600 pmol/l) on collagen production and on α-smooth muscle actin (α-SMA) protein expression was found after 1, 2 and 4 h of incubation. After 24 and 48 h, TGF-β_1 stimulated collagen production and α-SMA protein expression in cardiac fibroblasts. Intracellular ROS were not affected by TGF-β_1 after 0.5 and 1 h of incubation, began to rise after 2 h, and reached a maximal increase after 4 h. The TGF-β_1-stimulated ROS and collagen production is reduced by the ROS inhibitor diphenyleneiodonium chloride (DPI). DPI also decreased the TGF-β_1-stimulated α-SMA protein expression in rat cardiac fibroblasts. Our data indicate that the TGF-β_1-induced increase in ROS preceded the rise in collagen production and α-SMA protein expression and that ROS inhibition diminished the conversion of fibroblasts into myofibroblasts.

INTRODUCTION

A chronic increase in reactive oxygen species (ROS) plays a critical role in the development and progression of cardiac remodeling associated with heart failure [1]. Oxidative stress is indeed increased in heart failure, hypertension, cardiac fibrosis and hypertrophy [2-4]. *In vitro* exposure of cardiac fibroblasts to superoxide anion stimulates their proliferation by increasing the production of transforming growth factor-β_1 (TGF- β_1), a potent fibrogenic cytokine [5].

Ventricular fibroblasts activation and cardiac fibrosis are primary events in ventricular remodeling rather than secondary to cardiomyocyte injury [6]. In normal cardiac tissue collagen synthesis and deposition are exclusively carried out by cardiac fibroblasts with relatively low turnover of extracellular matrix proteins [7]. Contractile myofibroblasts are the relevant phenotypic variants in wound healing or in hypertrophied and failing hearts [7-9]. Signals for fibroblasts to myofibroblasts phenoconversion are TGF-β_1 and mechanical loading. An increase in α-smooth muscle actin (α-SMA) in these cells is a marker of myofibroblasts and is synonymous with enhanced contractile force generation [10]. An increased α-SMA expression is also found in myofibroblasts in fibrotic hearts after volume or pressure overload or in the infarct scar of postmyocardial infarction hearts [7].

Previously we have shown, in adult rat cardiac fibroblasts, that TGF-β_1 induces the differentiation of fibroblasts to myofibroblasts, which have a higher activity for collagen production than fibroblasts [11]. ROS are also released during the phenotypic transformation of fibroblasts to myofibroblasts and are important in the control of collagen production [12, 13]. The angiotensin II-stimulated production of collagen and ROS in cardiac fibroblasts is blocked by the membrane associated NADPH oxidase inhibitor, apocynin and diphenyleneiodonium chloride (DPI) [14, 15]. Exposure of cardiac fibroblasts to the superoxide inhibitor diethyldithiocarbamic acid (DETC) increases superoxide anion and collagen production [15], indicating a role of the generated ROS in the myocardial accumulation of collagen.

The aim of the present study was to determine the role of ROS in the TGF-β_1-induced collagen production and differentiation of cardiac fibroblasts into myofibroblasts.

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 [11]. When cultures reached confluence, the medium was replaced with fresh phenol red free DMEM with 0% FBS for 24 h. The cells, identified as proliferative myofibroblasts [16] were then preincubated with(out) the tested inhibitors for 1 h and then further incubated with(out) TGF-β_1 (600 pmol/l) for 0.5, 1, 2, 4, 24 or 48 h in this medium.

Immunoblot

Western blotting [15] was performed in cell lysates and cytosolic extracts prepared by NE-PER extraction reagents (Pierce Biotechnology, Rockford, IL, USA). Antibodies for α-SMA and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Sigma Chemicals Co. (St. Louis, MO, USA) and the corresponding secondary HRP-conjugated antibodies from Chemicon Inc. (Temecula, CA, USA).

Collagen assay

Collagen production was assayed as [^{3}H]proline incorporation and expressed as desintegrations/min per 10^6 cells [17], and using the Sircol assay (Biocolor, Belfast, Northern Ireland) and expressed as μg/10^6 cells [15]. Soluble collagen has been measured in the conditioned medium and non-soluble collagen in the cell layer.

Assay of reactive oxygen species.

A fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCF-DA) was used for the assessment of intracellular [18, 19] ROS formation (such as hydrogen peroxide, hydroxyl and peroxyl radicals and hydroperoxides) in cardiac fibroblasts treated with(out) TGF-β_1.

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

TGF-β_1-induced collagen production in cardiac fibroblasts

TGF-β_1 at concentrations from 1 to 600 pmol/l induced a dose-dependent increase in the production of soluble and non-soluble collagen after 48 h of incubation (Fig.1A). In the concentration range from 0.12 to 40 pmol/l, TGF-β_1 did not affect the soluble collagen production. A maximal increase in soluble collagen production was obtained at 600 pmol/l or 15 ng/ml. No significant effect of TGF-β_1 (600 pmol/l) on soluble collagen production was found after 1, 2 and 4 h of incubation (Fig.2A). After 24 and 48 h TGF-β_1 significantly ($P < 0.01$) stimulated soluble collagen production (2.5- and 4.7-fold, respectively).

TGF-β_1 upregulation of α-SMA in cardiac fibroblasts

TGF-β_1 dose-dependently induced an increase in the protein expression of α-SMA, a marker of myofibroblasts (Fig.1B).

The induction of α-SMA by TGF-β_1 after 24 and 48 h is 2.5- and 5-fold, respectively (Fig.2B). No induction of α-SMA is found after 1, 2 and 4 h. The TGF-β_1-induced appearance of α-SMA paralleled with the TGF-β_1 stimulated soluble collagen production

TGF-β_1 stimulation of ROS production in cardiac fibroblasts

To determine whether TGF-β_1 stimulates the intracellular ROS production, cardiac fibroblasts treated with TGF-β_1 were incubated with DCF-DA, a

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peroxide-sensitive dye to DCF-DA oxidation as a measure of levels of intracellular ROS.

Intracellular ROS production was not affected by TGF-β_1 after 0.5 and 1 h of incubation, began to rise significantly after 2 h, and reached a maximal increase (2.2 ± 0.5 fold, $P < 0.05$) after 4 h (Fig.3). The increase in ROS production preceded the rise in collagen production and α-SMA production.

The TGF-β_1-stimulated ROS production is reduced ($P < 0.05$) by DPI, an inhibitor of flavin-containing oxidoreductases such as NADPH oxidase; by tempol, a superoxide dismutase mimetic and ROS scavenger and by N-acetylcysteine (NAC), a glutathione precursor and scavenger of H_2O_2 (Fig.4). DPI and NAC, but not tempol, inhibited the ROS production in control fibroblasts (Fig.4).

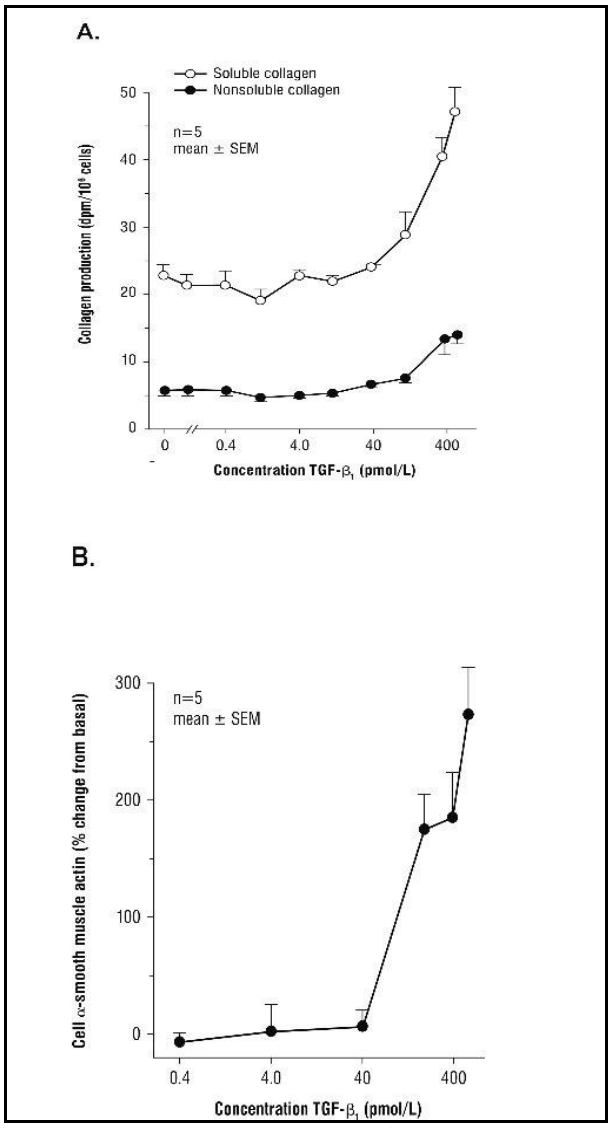

Figure 1. Dose-dependent stimulation of (non)soluble collagen production **(A)** and α-smooth muscle actin protein expression **(B)** by TGF-β_1 in adult rat cardiac fibroblasts.

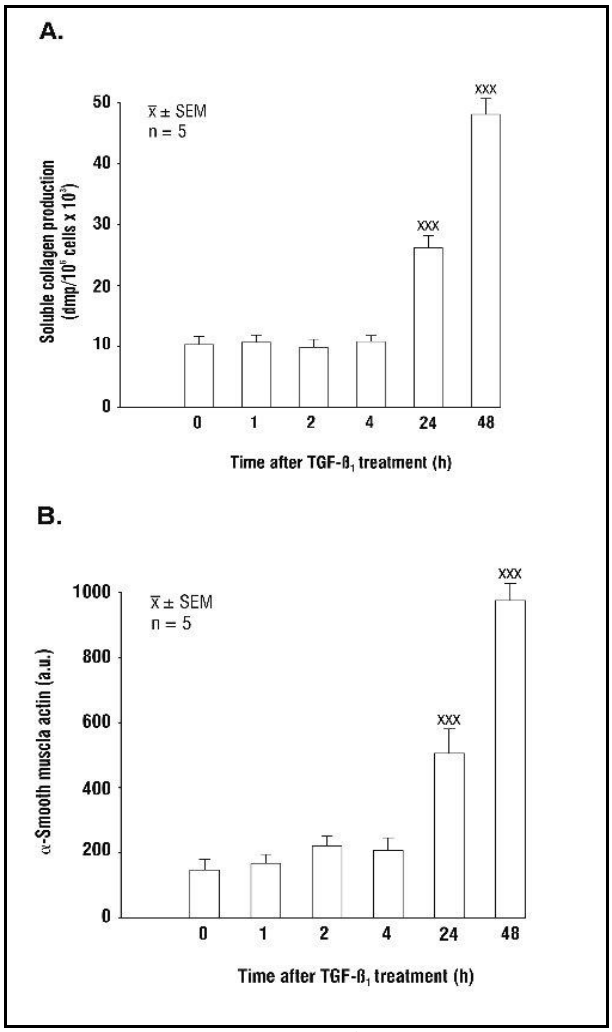

Figure 2. Time-dependent stimulation of soluble collagen production **(A)** and α-smooth muscle actin protein expression **(B)** in cardiac fibroblasts cultured for 0, 1, 2, 4, 2 and 48 h with 600 pmol/l TGF-β_1.

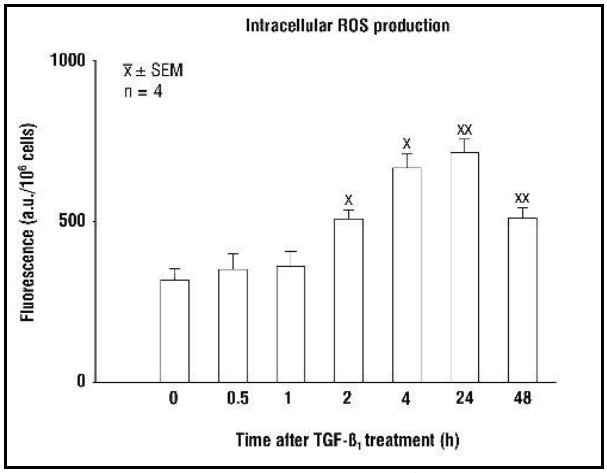

Figure 3. Time-dependent stimulation of intracellular ROS production in cardiac fibroblasts cultured for 0, 1, 2, 4, 2 and 48 h with 600 pmol/l TGF-β_1.

TGF-β_1 upregulation of collagen and α-SMA is ROS-sensitive

Previously we have shown that the soluble collagen production in basal cardiac fibroblasts is reduced by ROS inhibitors such as DPI [14], apocynin [14] tempol [15] and EUK-8 (a superoxide dismutase and catalase mimetic) [15]. The TGF-β_1-stimulated soluble collagen production (30.1 ± 5.9 μg/10^6 cells) compared to control (n = 4) cardiac fibroblasts (12.6 ± 0.4 μg/10^6 cells) is also reduced ($P < .05$) by DPI to 9.1 ± 0.9 μg/10^6 cells.

To determine whether ROS are necessary for the expression of α-SMA in response to TGF-β_1, cardiac fibroblasts were treated with the ROS inhibitor DPI and then the protein expression of α-SMA was examined. As shown in Fig.5, DPI decreased ($P < 0.05$) the TGF-β_1-upregulated α-SMA protein expression by $31.4 \pm 3.2\%$ (n = 4).

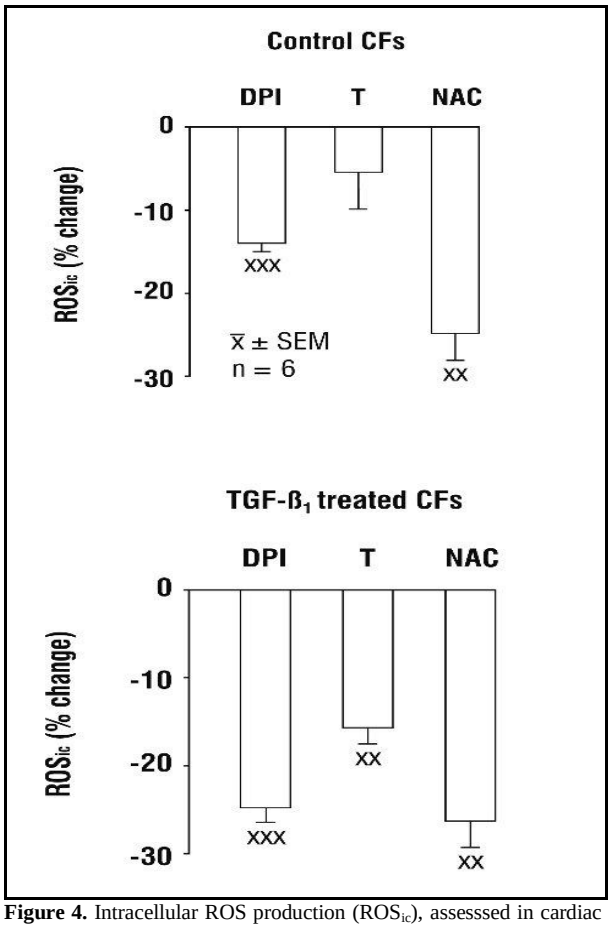

Figure 4. Intracellular ROS production (ROS_{ic}), assesssed in cardiac fibroblasts treated with(out) TGF-β_1 (600 pmol/l) for 24 h after preincubation with DPI (10 μmol/l), tempol (1 mmol/l) and NAC (10 mmol/l) for 1 h and then incubated with 2',7'-dichlorofluorescein diacetate (20 μmol/l) for 30 min. ROS production in control and TGF-β_1- treated cardiac fibroblasts averaged, respectively, 338 ± 29 and 716 ± 76 a.u./10^6 cells; $^{xxx}P < 0.001$, $^{xx}P < 0.01$.

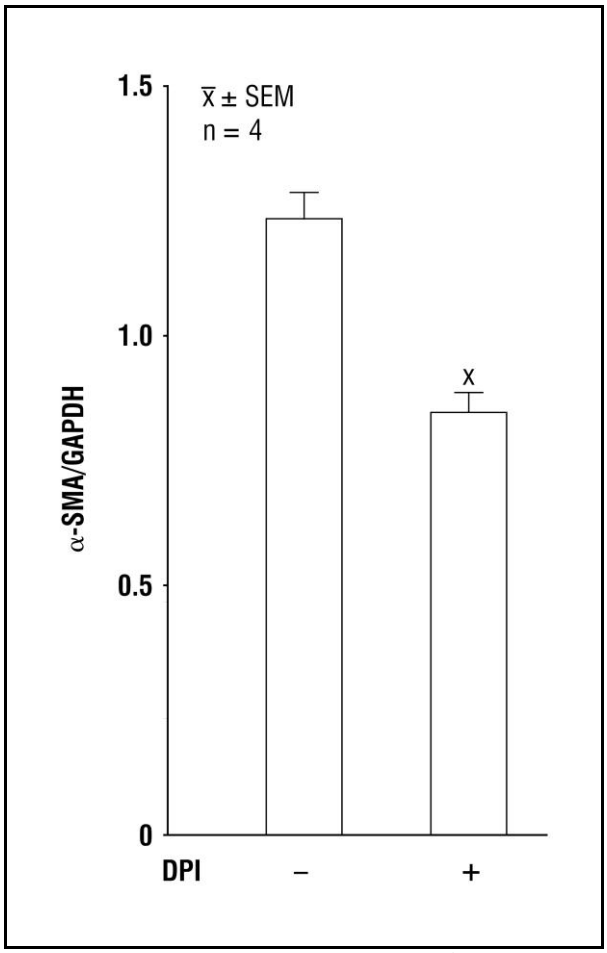

Figure 5. α-SMA/GAPDH protein ratio in cardiac fibroblasts treated with TGF-β_1 (600 pmol/l) for 24 h after preincubation with DPI (10 μmol/l) for 1 h; $^{x}P < 0.05$ compared with samples without DPI.

DISCUSSION

The present *in vitro* study in adult rat cardiac fibroblasts has shown that TGF-β_1 increased the intracellular ROS production and this rise precedes the TGF-β_1-stimulated collagen production and α-SMA expression (Figs.2&3). TGF-β_1 is a multifunctional cytokine that participates in many cellular functions such as control of growth, proliferation, differentiation and apoptosis. In the cardiovascular system TGF-β_1 plays pivotal roles in the pathogenesis of hypertension, restenosis after percutaneous coronary intervention, atherosclerosis, cardiac hypertrophy and heart failure [20]. TGF-β_1 contributes to pathogenic cardiac remodeling via increased ROS formation and cellular redox imbalance.

Accumulating evidence supports a causative role of oxidative stress in various tissues including liver, lung, arteries, nervous system and heart [14, 15, 21, 22]. Indeed, ROS are shown to upregulate the expression of TGF-β_1 and collagen type I in various tissues [23, 24].

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In vitro studies have shown that ROS promote collagen type I gene expression and fibroblast proliferation in cardiac fibroblasts [25].

The stimulatory effect of TGF-β_1 on ROS production has been demonstrated in bovine pulmonary artery endothelial cells, human lung and cardiac fibroblasts [26]. In rat ventricular myocytes TGF-β_1 (1 ng/ml) for 3-4 h increased ROS production by 90% and decreased intracellular glutathione (GSH_{ic}) by 34%; both changes were prevented by DPI, an inhibitor of flavin-containing oxidoreductase such as NADPH oxidase, or by Mn(III)terakis(1-methyl-4-pyridyl)porphyrin-penta-chloride (MnTMPyP), a free radical scavenger [27]. The changes in ROS and GSH_{ic} paralleled a decrease in the rate of myocyte shortening and relaxation, whereas fractional shortening was not affected. The TGF-β_1-induced cardiomyocyte contractile dysfunction is associated with enhanced ROS production and oxidative alterations in Ca^{2+} handling proteins regulated by endogenous GSH.

Antioxidants however reduce cardiac TGF-β_1 expression, induced by angiotensin II infusion, which sequentially attenuated the differentiation of myofibroblasts, type I collagen synthesis and matrix metalloproteinase inhibition, thus attenuating cardiac fibrosis [28].

The superoxide anion mediates the conversion of skin fibroblasts into myofibroblasts via paracrine release of TGF-β_1 in a skin model of wound healing [29]. In primary human cardiac fibroblasts Cucoranu *et al* [13] have shown that TGF-β_1-upregulated α-SMA expression is abrogated by DPI, by NAC (a glutathione precursor and scavenger of H_2O_2 and by ebselen (a nonspecific H_2O_2 scavenger).

The present study also shows that DPI decreased the conversion of adult rat cardiac fibroblasts to myofibroblasts. A recent study also reveals that tryptase, a cardiac mast cell product, induces a cardiac myofibroblastic phenotype and is implicated in the development of cardiac fibrosis [30].

Our *in vitro* data indicate that ROS are involved in the TGF-β_1-induced collagen production and appearance of myofibroblasts in cardiac fibroblasts and suggest a role of ROS in the modulation of TGF-β_1-induced myocardial remodeling.

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The authors declare no conflicts of interest.

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