

Original Article

Nox2 contributes to cardiac fibrosis in diabetic cardiomyopathy in a transforming growth factor- β dependent manner

Yuqin Liu^{1*}, Jinhua Zhang^{2*}

¹Department of Health Care, The Forth People's Hospital of Jinan City, Jinan 250031, China; ²Department of Endocrinology, Binzhou City Central Hospital, Binzhou 251700, China. *Co-first authors.

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Abstract: Purpose: This study aimed to investigate the effect of Nox2 on cardiac fibrosis and to elucidate the regulatory mechanism of Nox2 in the development of DCM. Methods: We established normal and insulin-resistant cellular model using neonatal rat cardiac fibroblasts. Then Nox2-specific siRNA were transfected into cardiac fibroblasts with Lipofectamine ® 2000 and scrambled siRNA sequence was considered as control. Meanwhile, a part of cells were randomly selected to be treated with or without transforming growth factor- β (TGF- β). Moreover, quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot were respectively performed to determine the expression level of related molecules, such as Nox2, collagen type I and III (COL I and III) and PI3K/AKT and PKC/Rho signaling pathway-related proteins. Results: TGF- β stimulation significantly increased the expression level of Nox2 both in mRNA and protein levels. Suppression of the Nox2 markedly decreased the expression of COL I and COL III in normal and insulin-resistant cellular model with TGF- β stimulation. Moreover, suppression of the Nox2 significantly decreased the expression of PI3K/AKT and PKC/Rho signaling pathway-related proteins in insulin-resistant cellular model with TGF- β stimulation. However, suppression of Nox2 had no effects on these proteins without TGF- β stimulation. Conclusions: Our finding reveals that Nox2 may promote synthesis of COL I and III via involved in PI3K/AKT and PKC/Rho signaling pathway in a TGF- β dependent manner and consequently promote cardiac fibrosis in the development of DCM.

Keywords: Diabetic cardiomyopathy, Nox 2, cardiac fibrosis, transforming growth factor- β

Introduction

Diabetic cardiomyopathy (DCM) is a common complication of longstanding type 2 diabetes mellitus (T2DM) and is a serious cardiac dysfunction caused by structural and functional alterations of the myocardium [1, 2]. It contributes to more than 50% diabetic death and has attracted people's attention due to the increased morbidity and mortality in diabetes population [3]. Despite the broad availability of antidiabetic therapy, it still remains a great challenge to the treatment of this disease. Moreover, to date, the molecular mechanism underlying DCM remains poorly understood.

Emerging evidences have revealed that myocardial fibrosis is considered to be an underlying contributor to early DCM [4-6]. Fibrosis is characterized by cardiac fibroblasts

accumulation and excess extracellular matrix (ECM) deposition. Cardiac fibroblasts is reported not only to produce ECM molecules, in particular collagens types I (COL I) and collagens types III (COL III), but also to secrete growth factors and cytokines which are key players for the maintenance of myocardial function [7, 8]. Cardiac fibrosis is thought to be a major feature of DCM [9]. Moreover, insulin resistance is considered to play an important role in the development of DCM [10]. In an insulin-resistant state, the mitogen-activated protein kinase (MAPK) pathway can be fully activated, thereby inducing the overproduction of transforming growth factor- β (TGF- β) [11]. TGF- β is thought to be a potent stimulator of collagen-producing cardiac fibroblasts [7]. However, antagonizing TGF- β is shown to do not prevent fibrosis completely, suggesting that fibrogenesis is a complex progress caused by multiple molecule alterations in DCM.

The effect of Nox2 on cardiac fibrosis in DCM

Recently, NADPH oxidase-dependent ROS production is shown to play a pivotal role in cardiac pathophysiology, such as myocyte hypertrophy, contractile dysfunction and fibrosis [12, 13]. Nox2 NADPH oxidase has been identified as a key form involved in angiotensin II-induced cardiac hypertrophy and interstitial fibrosis [14, 15]. Moreover, Nox2 upregulation in the myocardium from streptozotocin-induced diabetic rats is paralleled with increased TGF- β expression [16], and angiotensin II-induced TGF- β expression in cultured cardiac fibroblasts is required for TGF- β II-mediated fibrosis [17], suggesting Nox2 may be implicated in cardiac fibrosis. Besides, PI3 kinase (PI3K)/Akt pathway is shown to be activated by NADPH oxidase-derived ROS [18]. Despite these, the role and regulatory mechanism of Nox2 in the development of myocardial fibrosis in DCM has not been investigated.

In the current study, we established normal and insulin-resistant cellular model and utilized Nox2-specific siRNA (siR-Nox2) to suppress its expression. Our study aimed to determine the effect of Nox2 on collagen expressions in normal and insulin-resistant cellular model and to elucidate the regulatory mechanism of Nox2 in the development of DCM. Our findings may provide a new insight to the development therapeutic targets for this disease.

Materials and methods

Cell culture and treatment

Primary cultures of neonatal rat cardiac fibroblasts were prepared by a modification of a protocol reported previously [19]. In brief, the ventricles were firstly isolated from neonatal Wistar rats (Japan Laboratory Animals, Tokyo, Japan). After washed three times by ice-cold PBS, the ventricles were minced carefully and dissociated with 3 ml preheated 0.25% collagenase (Sigma Chemical Co.) for 15 min. Dispersed cells were then incubated in culture dishes for 30 min at 37°C in 5% CO₂ incubator, and non-myocytes were subsequently attached to the dish floor. Then minimum essential medium (MEM) containing 10% fetal calf serum were added into incubate them for 90 min. Afterwards, confluent non-myocytes were trypsinized and subcultured. Subconfluent cardiac fibroblasts from the second to fourth passage were used for subsequently experiments.

Cardiac fibroblasts continued to culture in Dulbecco's Modified Eagle Medium (DEME) containing 1% fetal calf serum for 12 h at 37°C. Then normal model (5.5 mM glucose) and insulin-resistant cellular model (15 mM glucose +10⁴ nU/ml insulin) were established. Meanwhile, cells were randomly selected to be treated with or without TGF- β (0.1 ng/ml).

siRNA transfection

To explore the function of Nox2 in neonatal rat cardiac fibroblasts, we used siRNA to silence its expression. The target sequence for Nox2-specific siRNA (siR-Nox2) was CCATTCGGAGG-TCTTACTT, and scrambled siRNA sequence was considered as control. These sequences were synthesized by GenePharma Co (Shanghai, China). Then siR-Nox2 and scrambled siRNA were transfected into cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Following the manufacturer's instructions, total RNA was extracted using the Trizol reagent (Invitrogen, Burlington, ON, Canada) and the quality of the total RNA was determined by a spectrophotometer (NanoDrop 2000, Thermo Scientific, USA). For reverse transcription, 500 ng of total RNA was reversely transcribed into cDNA with Prime Script reverse transcriptase (Takara, Kyoto, Japan). qRT-PCR was then carried out using SYBR Green real-time PCR MasterMix under ABI 7500 RT-PCR amplification equipment (Applied Biosystems, CA, USA). All values were normalized to the expression of β -actin. Three biological replicates and three technical replicates were performed for each sample. The relative expression level of each gene was calculated using the comparative threshold (Ct) cycle ($2^{-\Delta\Delta Ct}$). Forward and reverse primers used in this study were as follows: Nox-2: forward: 5'-TGCTAGAGAAAAAATCAAGAACCC-3', reverse: 5'-CGCCAAAACCGAACCAACCTC-3'; COL I: forward: 5'-TTCACCTACAGCAGCCTTGT-3', reverse: 5'-TTGGGATGGAGGGAGTTTAC-3'; COL III: forward: 5'-GGTCACTTTCACTGGTTGACGA-3', reverse: 5'-TTGAATATCAAACACGCAAGGC-3'; β -actin: forward: 5'-AGACCTTCAACACCCAG-3', reverse: 5'-CACGATTTCCCTCTC AGC-3'.

Western blot analysis

The cells were washed twice by PBS and then lysed with radio immunoprecipitation assay

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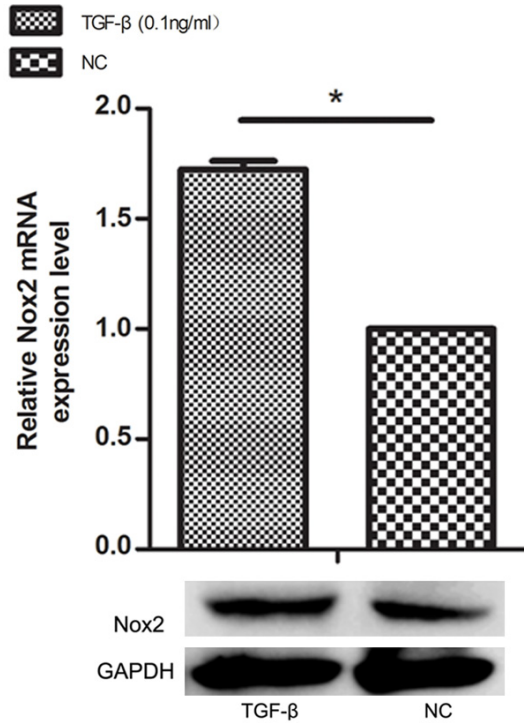


Figure 1. Expression of Nox2 in cardiac fibroblasts treated with and without TGF-β stimulation. The results showed that TGF-β stimulation significantly increased the expression level of Nox2 both in mRNA and protein levels.

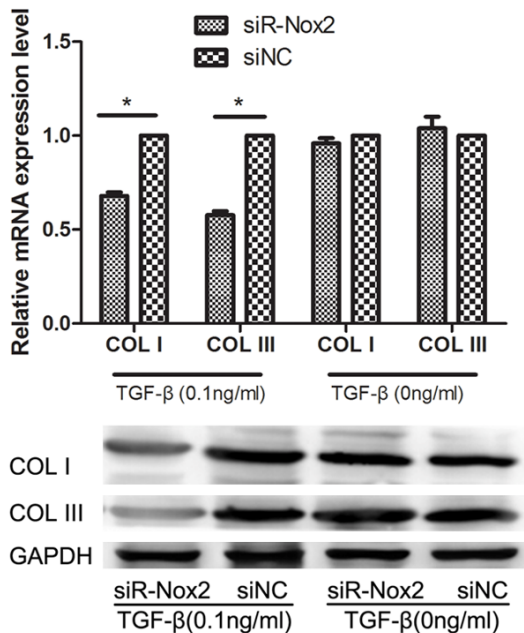


Figure 2. Expression of COL I and COL III in normal model. Suppression of Nox2 expression significantly decrease the expression of COL I and COL III compared to normal control, which was observed only in normal model with TGF-β stimulation.

(RIPA) lysis buffer (BestBio, Shanghai, China) for 48 h, after which the proteins were harvested. Total protein concentration was determined with BCA assay (Beyotime, Haimen, China). An equal amount of protein was loaded onto a 12% sodium dodecyl sulfate (SDS) denaturing-poly-acrylamide gel, separated by electrophoresis and transferred to a nitrocellulose membrane. After blocked, the membranes were incubated with the specific primary antibody at 4°C overnight. The following primary antibodies were used: p-PI3K, p-AKT, p-PKC and p-MYPT-1 (p-Rho) were purchased from Cell Signaling Technology (Beverly, MA). After washed, the membranes were subsequently incubated with the appropriate secondary antibody conjugated to horse radish peroxidase (HRP). The protein bands were visualized using enhanced chemiluminescence kit (Millipore) and quantified based on the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistics analysis

All the data are presented as the mean \pm SD. The significance of differences between or among groups was analyzed by a Student's test or one-way ANOVA using SPSS 20.0. Differences were considered statistically significant at $P < 0.05$.

Results

Analysis of the expression level of Nox2

The effect of TGF-β on the expression level of Nox2 was shown in **Figure 1**. The results showed that TGF-β stimulation significantly increased the expression level of Nox2 both in mRNA and protein levels.

Effect of Nox2 on the expression of COL I and COL III in normal model

To explore the effect of Nox2 on the fibrosis of cardiac fibroblasts, the expression of COL I and COL III was determined in normal model. The results showed that suppression of Nox2 expression in normal model with TGF-β stimulation could significantly decrease the expression of COL I and COL III compared to normal control. However, there were no differences between them without TGF-β stimulation (**Figure 2**).

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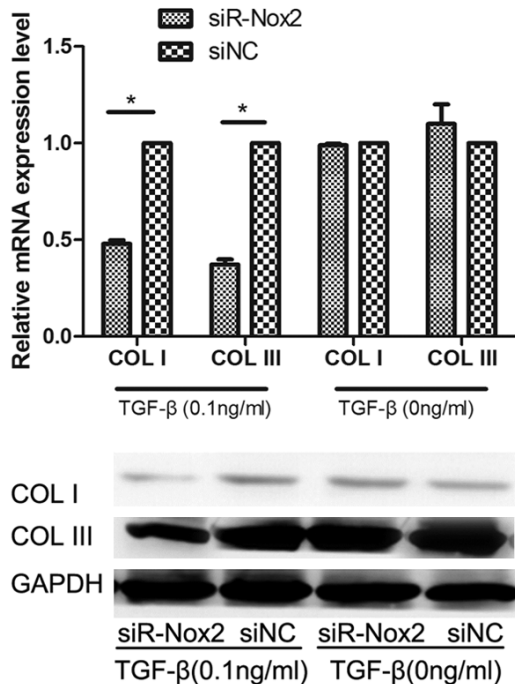


Figure 3. Expression of COL I and COL III in insulin-resistant cellular model. Suppression of Nox2 expression could significantly decrease the expression of COL I and COL III compared to normal control, which was observed only in models with TGF-β stimulation.

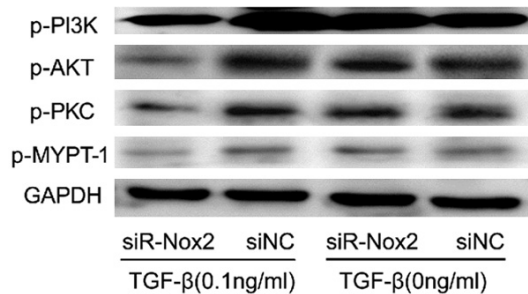


Figure 4. Expression of p-PI3K, p-AKT, p-PKC and p-MYPT-1 determined by Western blot. Suppression of Nox2 expression markedly decreased the expression of these proteins in insulin-resistant state, which was observed only in normal model with TGF-β stimulation.

Effect of Nox2 on the expression of COL I and COL III in insulin-resistant cellular model

To further explore the effect of Nox2 on the fibrosis of cardiac fibroblasts, the expression of COL I and COL III was also determined in insulin-resistant cellular model. In insulin-resistant state, similar results were obtained that suppression of Nox2 expression in insulin-resistant cellular model with TGF-β stimulation

could significantly decrease the expression of COL I and COL III compared to normal control (Figure 3).

Effect of Nox2 on PI3K/AKT and PKC/Rho signaling pathway under insulin-resistant state

To further explore the regulatory mechanism of Nox2 in the fibrosis of cardiac fibroblasts, we investigated the effect of Nox2 on the expression of PI3K/AKT and PKC/Rho signaling pathway-related proteins, such as p-PI3K, p-AKT, p-PKC and p-MYPT-1. As shown in Figure 4, under TGF-β stimulation, suppression of Nox2 expression markedly decreased the expression of these proteins in insulin-resistant state. Notably, in insulin-resistant state, suppression of Nox2 expression had no effects on the expression of these proteins without TGF-β stimulation.

Discussion

In the present study, we investigated the effect of Nox2 on the fibrosis of cardiac fibroblasts under both normal and insulin-resistant state. The results showed that suppression of the Nox2 markedly decreased the expression of COL I and COL III in normal and insulin-resistant cellular model with TGF-β stimulation. Moreover, suppression of the Nox2 significantly decreased the expression of PI3K/AKT and PKC/Rho signaling pathway-related proteins in insulin-resistant cellular model with TGF-β stimulation. However, suppression of Nox2 had no effects on these proteins without TGF-β stimulation. All these findings reveal that Nox2 may facilitate the fibrosis of cardiac fibroblasts via PI3K/AKT and PKC/Rho signaling pathway dependently of TGF-β.

For the first time, we found that the expression of COL I and COL III was inhibited after knock-down of Nox2, indicating that Nox2 could play a significant role in promoting collagen synthesis in cardiac fibroblasts. Our observation is consistent with previous finding that Nox2 can promote collagen synthesis in H₂O₂-stimulated cardiac fibroblasts [20]. Goldsmith et al. demonstrated that enhanced fibrillar collagen synthesis could turnover result in myocardial collagen accumulation, thereby inducing fibrosis [21]. Moreover, increased COL I synthesis and deposition in patients with hypertensive heart disease is reported to be responsible for

the enhancement of myocardial fibrosis of cardiac function [22]. COL III has been shown to play an important role not only in liver fibrosis but also in other fibrosis-related diseases [23]. Besides, COL I and COL III is a key component of ECM for maintaining the balance between ECM synthesis and degradation which has been considered as a good marker to predict the development of diabetes-induced cardiac fibrosis [24]. Taken together, considering the relationship of Nox2 and these collagen proteins, we speculate that Nox2 may promote the cardiac fibrosis in the development of DCM via inducing higher levels of COL I and COL III.

Furthermore, previous study has shown that insulin resistance is characterized by increased collagen [25]. Our results demonstrated that COL I and III expression was markedly decreased concomitantly with significantly decreased Nox2 expression in insulin-resistant cellular model. Recent data have revealed that the expression levels of Nox2 are elevated in insulin-resistant state [26]. Moreover, PI3K/Akt pathway is shown to be involved insulin-resistant in diabetic patients [27]. Prostaglandin F₂ α facilitates collagen synthesis in cardiac fibroblasts via an F-prostanoid receptor/protein kinase C/Rho kinase (PKC/Rho) pathway [28]. Bujak et al. have demonstrated that PKC- β is associated with diabetes-associated cardiac dysfunction, such as collagen deposition, myocyte hypertrophy, and preserved cardiac contractility [29]. Anthocyanin is shown to have protective potential for targeting cardiac hypertrophy and fibrosis in DCM via suppression of PKC-ERK pathway [30]. Thus, to further explore the regulatory mechanism of Nox2 in the cardiac fibrosis, we investigated the relationship between Nox2 and PI3K/AKT and PKC/Rho signaling pathway. Our results observed that suppression of Nox2 expression markedly decreased the expression of PI3K/AKT and PKC/Rho signaling pathway-related proteins in insulin-resistant state. It can therefore be speculated that Nox2 may drive synthesis of COL I and III in the development of DCM via involved in PI3K/AKT and PKC/Rho signaling pathway.

Notably, the effects of Nox2 on the expression of COL I, COL III and PI3K/AKT and PKC/Rho signaling pathway-related proteins were observed only under TGF- β stimulation, whereas there were no effects without TGF- β stimulation, suggesting the role of Nox2 in cardiac

fibrosis via PI3K/AKT and PKC/Rho signaling pathway was played dependently with TGF- β . TGF- β is reported to not only play an important role in promoting ECM deposition in the infarct by upregulating collagen and fibronectin synthesis but also function as a key mediator to induce interstitial fibrosis [29]. Moreover, Martin et al. revealed that reduced TGF- β activity by tranilast may be key mechanism for attenuating cardiac matrix deposition in experimental diabetes rats [31]. Besides, PKC activity was decreased in curcumin treated rats and consequently prevented cardiac fibrosis by downregulation of TGF- β under diabetic conditions [32]. Therefore, we speculate that Nox2 may play an important role in cardiac fibrosis in a TGF- β dependent manner.

In conclusion, our finding reveal that Nox2 may promote synthesis of COL I and III via involved in PI3K/AKT and PKC/Rho signaling pathway in a TGF- β dependent manner and consequently promote cardiac fibrosis in the development of DCM. However, further investigations are warranted to verify our findings.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jinhua Zhang, Department of Health Care, The Forth People's Hospital of Jinan City, 50 Shifan Road, Tianqiao District, Jinan 250031, China. Tel: +86-5328-9751558; Fax: +86-53289751558; E-mail: zhangjinhua253@126.com

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