The stress-related hormone norepinephrine induced upregulation of Nix, contributing to ECM protein expression

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Abstract Organ fibrosis has been viewed as a major medical problem that leads to progressive dysfunction of the organ and eventually the death of patients. Stress-related hormone norepinephrine (NE) has been reported to exert fibrogenic actions in the injured organ. Nix plays a critical role in pressure overload-induced cardiac remodeling and heart failure through mediating cardiomyocyte apoptosis. However, cardiac remodeling also includes fibrosis. Whether Nix is involved in stress-induced fibrosis remains unclear. The present study was designed to determine the role of Nix in NE-induced NIH/3T3 fibroblasts. The results showed that Nix was upregulated and closely associated with cell proliferation, collagen and fibronectin expression in NIH/3T3 fibroblasts following NE treatment. Overexpression of Nix promoted collagen and fibronectin expression, whereas the suppression of Nix resulted in a strong reduction in collagen and fibronectin expression. Moreover, the increases in collagen and fibronectin expression induced by NE were successively increased when Nix was overexpressed and reduced when Nix was inhibited. Furthermore, we demonstrated that the PKC activation is responsible for the upregulation of Nix induced by NE. Inhibition of Nix expression with α -adrenoceptor antagonist, β -adrenoceptor antagonist or PKC inhibitor attenuated NE-induced collagen and fibronectin expression. Our data revealed that Nix is a novel mediator of NE-induced fibrosis. Thus, it would provide a new insight into the development of effective preventative measures and therapies of tissue fibrosis.

Keywords Norepinephrine · Nix · Fibrosis · NIH/3T3 fibroblasts

Introduction

Numerous studies have established the existence of stress, a concept describing the process in which environmental or psychosocial factors exceed the adaptive capacity of an organism, resulting in psychological and biological changes that may place persons at risk and promote the progression for many diseases (Taylor et al. 2010), but the mechanism remains poorly characterised. One major effect of such stress is remodeling of extracellular matrix (ECM) known to facilitate the biomechanical signals in response to abnormal physiological conditions (Kohli et al. 2013). The extracellular matrix not only provides physical support for tissues by regulating the attachment, proliferation, migration and differentiation of cells through the ECM-cell interaction but also serves as a reservoir for growth factors and cytokines and plays crucial roles during the development of fibrosis. Organ fibrosis is a major component of the pathophysiology of many common diseases, including cardiovascular disease, cancer and chronic diseases of the liver, lung and kidney. Fibrosis is characterised by the excessive production and abnormal accumulation of the ECM, which can ultimately lead to organ structural damage and dysfunction and even failure. This highly conserved response to repetitive injury and inflammation ultimately results in disordered tissue architecture and organ failure, making fibrosis a major cause of mortality worldwide (Greenhalgh et al. 2013). Studies of fibrosis in various organs have flourished over the last decade and have identified a growing number of molecules that regulate the development of fibrosis in experimental models (Oka and Komuro 2012; Liu 2011; Kimura and Iwano 2009); however, therapies based on these mechanisms still cannot effectively prevent organ fibrosis. Stress should be considered as a risk factor for fibrosis. The effect and mechanism of stress-related hormone on fibrosis remains to be illustrated.

Stress can activate the sympathetic-adrenal-medullary (SAM) axis, eliciting the release of catecholamines

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(Goldstein 2003; Okuneva et al. 2009). Catecholamines, which exhibit strong vasoconstrictor functions, regulate a number of physiological and pathological processes (Bassi et al. 2006; Oberbeck 2006). Moreover, various cells express some adrenoceptor subtypes, and stimulation with norepinephrine (NE) induces cell proliferation and collagen gene expression (Yang et al. 2008; Oben et al. 2003; Flint et al. 2013). Norepinephrine, which is involved in the development of fibrosis, plays an important role in fibrosis (Akiyama-Uchida et al. 2002), but the mechanism of NE-induced fibrosis remains poorly characterised. Nix is a nearly ubiquitous protein that is expressed at very low levels in normal hearts, but it has been shown to be transcriptionally upregulated during pathological cardiac hypertrophy in response to hemodynamic overload or neurohormonal excess (Galvez et al. 2006; Yussman et al. 2002; Dorn and Kirshenbaum 2008). Transgenic Nix expression greatly accelerated the progression to left ventricular remodeling and heart failure in adult rats with TAC (Dorn 2005). In subsequent studies, Nix has been shown to play an important role in myocardial cell apoptosis during cardiac remodeling (Diwan et al. 2009). However, organ remodeling also includes tissue fibrosis. The effect of Nix on fibrosis in fibroblasts remains unknown. Nix expression appeared constitutive, with modest transcript levels in almost all tissues. Whether Nix is involved in NE-induced fibrosis remains to be discussed.

In the present study, NIH/3T3 fibroblasts were used for the experiments which have been widely used to study a variety of tissue fibrosis. We found that Nix expression increased in cultured NIH/3T3 fibroblasts undergoing NE-induced proliferation, collagen and fibronectin overexpression. Additionally, we demonstrated that Nix promotes fibrosis by upregulating fibronectin and collagen expression, indicating that Nix is an important mediator during fibrosis. Moreover, we found that Nix was upregulated by the NE-AR-PKC pathway. Together, these results have significant implications for understanding the role of Nix in NE-induced fibroblasts. Fibrosis is characterised as a major cause of mortality for many diseases. The norepinephrine induced upregulation of Nix may accelerate the development of disease through promoting fibrosis, which suggested new therapeutic targets for pathological fibrosis.

Materials and methods

Cell culture and transfection

NIH/3T3 fibroblasts were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % of heat-inactivated foetal bovine serum (FBS), 100 units/ml of penicillin and 100 µg/ml of streptomycin at 37 °C in a CO₂ humidified chamber. The cells

were transfected with control-small interfering RNA (siRNA), Nix-siRNA, empty vector or Nix expression vector using Lipofectamine^{TM} 2000 reagent (Invitrogen) according to the manufacturer's instructions. Thirty-six hours after the transfection, the cells were collected for cell proliferation rate analysis, cell cycle distribution assay and Western blot analysis.

NIH/3T3 cells model

NIH/3T3 fibroblasts were serum-starved for 12 h prior to NE (Sigma) treatment and then were incubated with 1, 10 and 100 µM of NE for 24 h or incubated with 10 µM of NE for 4, 8, 12 and 24 h, respectively. To detect the role of PKC activation, the direct PKC activator phorbol myristate acetate (PMA, 10 nM, Sigma) were used to treat the cells for 24 h. Also, NIH/3T3 fibroblasts were pretreated with or without α -adrenoceptor antagonist phenoxybenzamine (PHE, 50 µM, Sigma), β -adrenoceptor antagonist propranolol (PRO, 50 µM, Sigma) or PKC inhibitor bisindolylmaleimide I (BIM, 25 µM, Calbiochem) for 30 min prior to treatment with 10 µM of NE for 24 h.

Design and application of small interfering RNAs

Three siRNAs against Nix were designed according to guidelines published by Reynolds et al. (Reynolds et al. 2004) (sequence 1: 5'-GGC AGA UCA UGU UUG AUG UTT-3', sequence 2: 5'-CCC AAA GAG UUC CAU UUC ATT-3', sequence 3: 5'-GGC CUA GAC AUA CAU GAA ATT-3'). The siRNAs against Nix and a negative control siRNA (5'-UUC UCC GAA CGU GUC ACG UTT-3') synthesised by GenePharma. siRNAs were transfected into NIH/3T3 fibroblasts using Lipofectamine^{TM} 2000 reagent (Invitrogen) according to the manufacturer's instructions. Thirty-six hours after transfection, expression of Nix was determined by Western blot analysis. The most effective siRNA was used for our experiments.

Western blot analysis

Equal amounts of the protein samples underwent SDS-polyacrylamide gel electrophoresis (PAGE) and were transferred to polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies (anti-Nix, anti-fibronectin, anti-PKC α and anti-P-PKC α : Santa Cruz Biotechnology, anti-collagen I and collagen III: abcam, GAPDH: KangChen Bio-tech) and then probed with horseradish peroxidase-conjugated secondary antibodies. The blots were visualised using the SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL), and the images were captured with an ImageQuant LAS 4000 (GE, USA).

MTT assays

MTT powder (Sigma-Aldrich, St. Louis, MO) was dissolved at 5 mg/ml in distilled H₂O and sterilised through a 0.22- μ m filter before use. The cells were seeded in a 96-well plate (5,000 cells/well, total 100 μ l) and incubated overnight. Then, the cells were treated with the processing mode. After treatment, 10 μ l of the MTT stock solution was added to each well, and the cells were incubated with 5 % of CO₂ at 37 °C for 4 h. Finally, we removed the medium, added 100 μ l of DMSO to each well and incubated the cells at room temperature for 15 min. The absorbance was measured at 490 nm using a microplate reader. All experiments were performed in eight wells of each group and repeated three times.

Cell cycle assays

Briefly, after incubation, the cells were harvested, washed with PBS (pH 7.4) three times, centrifuged and fixed with 70 % anhydrous ethanol overnight at 4 °C. The cells were incubated RNase for 30 min and stained with propidium iodide (PI) for 15 min at 37 °C in the dark. The samples were detected using flow cytometry (FACSCalibur, BD, USA) and analysed with ModFit LT software (FACSCalibur, BD, USA).

Statistical analysis

The data are expressed as the sample mean and their standard errors. Comparisons were performed using Student's *t* test or an ANOVA, as appropriate. A *P* value of <0.05 denoted statistical significance.

Results

Nix expression increased along with cell proliferation and ECM protein overexpression in NIH/3T3 fibroblasts following NE treatment.

Catecholamines participate in the pathogenesis of fibrosis in various organs. Under these conditions, NE secretion increased. NIH/3T3 fibroblasts were incubated with 10⁻⁶–10⁻⁴ M NE for 24 h to assess the effect of NE on fibrosis in vitro. Compared with the parental controls, a significant increase was observed in the cell proliferation rate of the NE-induced NIH/3T3 fibroblasts, as measured using an MTT assay (Fig. 1a). To identify a potential mechanism for the NE-induced cell proliferation, the cell cycle distribution was assessed using flow cytometry, and this analysis showed that the percentage of cells in the DNA synthesis phase (S phase) significantly increased in the NE-induced NIH/3T3 fibroblasts (Fig. 1b). Under these conditions, collagen and fibronectin, the important ECM components, were also increased in the

NE-induced NIH/3T3 fibroblasts (Fig. 1c). Exposure to NE is associated with fibroblast proliferation and the secretion of ECM proteins, which contribute to the pathophysiology of fibrosis. Moreover, Western blot analysis showed that NE had a stimulatory effect on Nix expression in NIH/3T3 fibroblasts (Fig. 1d).

To determine the time effects of NE on fibrosis and Nix expression, NIH/3T3 fibroblasts were then stimulated with 10⁻⁵ M NE for the indicated times. Western blot analysis showed that NE had a time-dependent stimulatory effect on Nix expression (Fig. 2a). The cell proliferation rate, the percentage of cells in the S phase significantly increased in the NE-induced NIH/3T3 cells in a time-dependent manner, as measured using an MTT assay and flow cytometry analysis, respectively (Fig. 2b, c). Compared with the parental controls, a significant increase was also observed in the expression of collagen and fibronectin (Fig. 2d). These results demonstrated that Nix expression is closely related to NE-induced cell proliferation, cell cycle distribution and ECM protein expression, indicating that Nix may be an important mediator in NE-induced fibrosis.

Nix promotes collagen and fibronectin expression

To determine the role of Nix, three siRNAs targeting the Nix gene were synthesised to suppress Nix expression and were tested using Western blot analysis. We used the most effective siRNA for our experiments. The Nix expression vector was provided by the Center for Pharmacogenomics, Washington University. NIH/3T3 fibroblasts were transfected with Nix-siRNA or Nix expression vector. After transfection, the cells were collected, and Nix expression was evaluated using Western blot analysis. The results showed that the Nix-siRNA and Nix expression vector suppressed and increased Nix expression, respectively (Fig. 3a, b). Then, we examined ECM protein expression and cell proliferation by assessing the effect of Nix-siRNA and Nix expression vector in NIH/3T3 fibroblasts. A significant decrease and increase in the expression of fibronectin and collagen was observed after transfection of Nix-siRNA and Nix expression vector, respectively (Fig. 3a, b), but no significant effects on the cell proliferation rate and cell cycle distribution were observed according to MTT assay and flow cytometry, respectively (Fig. 3c, d). These results indicate that Nix regulated ECM protein expression, but had no significant effects on the cell proliferation in NIH/3T3 fibroblasts.

To evaluate the effect of Nix on the NE-induced fibrosis, the transfected NIH/3T3 fibroblasts were treated with 10 μ M of NE. The cells were collected, and Nix expression was analysed by Western blot. The results showed that Nix-siRNA and Nix expression vector suppressed and increased the NE-induced Nix expression, respectively (Fig. 4a, b). No significant effects on the NE-induced cell proliferation rate or cell cycle distribution were observed after the transfection of

Fig. 1 Nix expression increased in NIH/3T3 fibroblasts treated with NE. NIH/3T3 fibroblasts were treated with 0, 1, 10 or 100 μmol/l of NE for 24 h. **a** Proliferation was measured using an MTT assay. **b** NIH/3T3 fibroblasts were stained with PI, and the cell population at each cell cycle stage was detected using FACS; the data were analysed using the Mod-Fit program. **c** Western blot analysis showing the NE-induced fibronectin

expression. The blots were re-probed with an anti-GAPDH antibody to confirm equal loading. **d** Western blot analysis showing the NE-induced Nix expression. The blots were probed with an anti-GAPDH antibody to confirm equal loading. Each experiment repeated three times. Data are expressed as the mean ± SEM, * $P < 0.05$ vs. control

Nix-siRNA or Nix expression vector, respectively (Fig. 4c, d). However, NE had a stimulatory effect on collagen and fibronectin expression, which decreased following transfection with Nix-siRNA (Fig. 4a). In contrast, the overexpression of Nix aggravated the NE-induced collagen and fibronectin expression (Fig. 4b). These results indicated that Nix promotes NE-induced fibrosis by regulating ECM protein expression, not cell proliferation.

NE-AR-PKC pathway is essential for the upregulation of Nix

NE, the major hormone released under stress and pathological conditions, can specifically bind to α - or β -adrenoceptor (AR) to activate PKC. To examine the mechanism of NE-induced Nix expression in NIH/3T3 fibroblasts, we first measured the phosphorylation levels of PKC α (P-PKC α) by Western blot. The results showed that NE had a dose-dependent

Fig. 2 Nix expression is closely related to cell proliferation and fibronectin expression in NE-induced NIH/3T3 fibroblasts. NIH/3T3 cells were treated with 10^{-5} M of NE for 0, 4, 8, 12 or 24 h. **a** Western blot analysis showing the effects of 10^{-5} M of NE treatment for various times on the expression of Nix. The blots were re-probed with an anti-GAPDH antibody to confirm equal loading. **b** Proliferation was measured using an

MTT assay. **c** Flow cytometry analysis showing the cell cycle distribution for the experiment described above. **d** Western blot analysis showing the NE-induced collagen and fibronectin expression. The blots were re-probed with an anti-GAPDH antibody to confirm equal loading. Each experiment repeated three times. Data are expressed as the mean ± SEM, * $P < 0.05$ vs. control

stimulatory effect on P-PKC $_{\alpha}$ expression, indicating that NE induced PKC $_{\alpha}$ activation (Fig. 5a). Also, we found that PKC $_{\alpha}$ activation is closely related to NE-induced Nix expression (Figs. 1d and 5a). NIH/3T3 fibroblasts were pretreated with or without α -adrenoceptor antagonist phenoxybenzamine (PHE), β -adrenoceptor antagonist propranolol (PRO) or PKC inhibitor bisindolylmaleimide I (BIM) for 30 min prior to treatment with 10 μ M of NE for 24 h. Western blot analysis showed that the increases of phosphorylation levels of PKC $_{\alpha}$ induced by NE were reduced when the cells were pretreated with PHE, PRO and BIM. Also, we found that PHE, PRO and BIM were all capable of attenuating the NE-induced Nix expression, as measured using Western blot analysis (Fig. 5b). Moreover, the direct PKC activator PMA had a

significantly stimulatory effect on PKC activation and Nix expression (Fig. 5b). These results indicated that NE induced Nix expression through the AR-PKC pathway. Furthermore, the inhibition of Nix expression by PRO, PHE or BIM attenuated the NE-induced collagen and fibronectin expression (Fig. 5c). These results provide evidence that Nix is upregulated and promotes ECM expression during NE-induced fibrosis through the PKC signaling pathway.

Discussion

Organ fibrosis is a common end-stage feature of many chronic diseases. Most chronic fibrotic disorders can stimulate the

Fig. 3 Nix promotes collagen and fibronectin expression but not cell proliferation. NIH/3T3 fibroblasts were transfected with plasmid DNA (empty vector or Nix expression vector) or siRNA (control-siRNA or Nix-siRNA) for 36 h. **a**, **b** Western blot analysis showing the effects of Nix-siRNA or Nix expression vector on Nix, collagen and fibronectin expression. The blots were re-probed with an anti-GAPDH antibody to

confirm equal loading. **c** Proliferation was measured using an MTT assay. **d** Cell cycle stage was determined using FACS, and the data were analysed using the ModFit program. Each experiment repeated three times. Data are expressed as the mean ± SEM, * $P < 0.05$ vs. control-siRNA or empty vector

deposition of connective tissue elements, which progressively remodel and destroy the normal tissue architecture, seriously threatening human health and life (Edgley et al. 2012; Noble et al. 2012; Ahmad and Ahmad 2012; Igreja et al. 2012; Lewis and Scullion 2012). Stress is defined as the effects of environmental or psychosocial factors on physical or mental well-being. Stress can activate the sympathetic-adrenal-medullary (SAM) axis, eliciting the release of catecholamines (NE and E) resulting in many diseases. A series of studies have indicated that catecholamines respond to different stimuli and mediate the fibrotic response (Rassler et al. 2012; Shao et al. 2013; Bernini et al. 2008; Oben et al. 2004). During fibrosis, fibroblasts are activated to proliferate and synthesise ECM molecules. The mouse NIH/3T3 fibroblasts share features that are present in active fibroblasts, such as remarkable proliferation, substantial ECM production and stable cell growth. In

the present study, we demonstrated that the stress-related hormone norepinephrine induced cell proliferation and fibronectin overexpression, which was accompanied by an increase in Nix expression in NIH/3T3 cells. The overexpression or suppression of Nix promoted or inhibited, respectively, the NE-induced collagen and fibronectin expression in cultured NIH/3T3 cells, which provides the first evidence that Nix plays an important role in NE-induced fibrosis through the upregulation of ECM protein expression. These results suggested that Nix is a novel regulator of stress-induced fibrosis.

Recently, it has been demonstrated that the stress-related hormones NE and epinephrine, especially NE, can directly induce gene expression and are involved in the progression of cancer progression. NE stimulates cell proliferation and collagen secretion, suggesting that it directly induces the fibrogenic effect (Yang et al. 2008; Oben et al. 2003;

Fig. 4 The role of Nix in NE-induced collagen and fibronectin expression. NIH/3T3 fibroblasts were treated with 10^{-5} M of NE after transfection with siRNA (control-siRNA or Nix-siRNA) or plasmid DNA (empty vector or Nix expression vector). **a, b** Western blot analysis showing the effects of Nix-siRNA or Nix expression vector on Nix, collagen and fibronectin expression. The blots were re-probed with an anti-GAPDH

antibody to confirm equal loading. **c** Proliferation was measured using an MTT assay. **d** Cell cycle stage was determined using FACS, and the data were analysed using the ModFit program. Each experiment repeated three times. Data are expressed as the mean ± SEM, * $P < 0.05$ vs. control-siRNA or empty vector, # $P < 0.05$ vs. control-siRNA+NE or empty vector+NE

Akiyama-Uchida et al. 2002), but the important regulator of NE-induced fibrosis is still unknown. Fibroblasts, the predominant secretory cells that produce ECM proteins, are important targets of NE and the key cell type of tissue fibrosis (Flint et al. 2013; Kim and Padanilam 2013). In practice, NIH/3T3 fibroblasts are often used as a desirable cell model for investigations into the mechanisms of fibrosis and anti-fibrotic drugs. Here, we found NE induced cell proliferation and cell cycle progression. Fibronectin and collagen, the important ECM components, are also markedly upregulated in the NE-

induced NIH/3T3 cells. Recently, Nix was shown to be highly transcriptionally upregulated in Gq-mediated and pressure overload cardiac hypertrophy, including human hypertensive heart disease (Galvez et al. 2006; Yussman et al. 2002). In vitro, phenylephrine, an analogue of norepinephrine, has been reported to regulate Nix promoter activity in cardiac myocytes, whereas isoproterenol or angiotensin II cannot (Galvez et al. 2006). In this study, the results showed that Nix expression increased during the procession of NE-induced NIH/3T3 fibroblasts. Importantly, we noticed that

Fig. 5 The NE-AR-PKC pathway is essential for the upregulation of Nix. **a** NIH/3T3 fibroblasts were treated with 0, 10^{-6} , 10^{-5} or 10^{-4} M of NE for 24 h. Western blot analysis showing the NE-induced p-PKC α expression is shown. The blots were re-probed with an antibody to total PKC α as a loading control. **b** NIH/3T3 fibroblasts were pretreated with or without PRO, PHE or BIM for 30 min prior to NE treatment or treated with 10 nM of PMA for 24 h. Western blot analysis showing the effects of NE, PRO, PHE, BIM or PMA on the expression of Nix and p-PKC α is shown. The blots were re-probed with an anti-PKC α antibody or an anti-GAPDH antibody. **c** NIH/3T3 fibroblasts were pretreated with or without PRO, PHE or BIM for 30 min prior to NE treatment. Western blot analysis showing the effects of NE, PRO, PHE and BIM on the NE-induced collagen and fibronectin expression is shown. The blots were re-probed with an anti-GAPDH antibody to confirm equal loading. Each experiment repeated three times. Data are expressed as the mean $\pm$ SEM, * P <0.05 vs. control, # P <0.05 vs. NE

Nix expression was closely related to NE-induced cell proliferation, the percentage of cells in the DNA synthesis phase, collagen and fibronectin expression, indicating that Nix may be an important mediator in NE-induced fibrosis.

In vivo, the overexpression of Nix greatly accelerated the progression to left ventricular remodeling and heart failure in adult rats treated with TAC by mediating cell apoptosis (Dorn 2005; Diwan et al. 2008). Pathological remodeling not only involves the reactivation of cardiomyocyte death but also cardiac fibroblasts proliferation and ECM accumulation (Kehat and Molkentin 2010; Delcayre and Swynghedauw 2002; Polyakova et al. 2011). The direct effect of Nix on fibrosis in fibroblasts is still unclear. We found that Nix expression was closely related to NE-induced cell proliferation and ECM protein expression. Northern blot analysis of Nix transcripts in different tissues appeared constitutive, with modest transcript levels in almost all tissues assayed (Galvez

et al. 2006). Nix may be a regulator of tissue fibrosis. In this study, Western blot analysis showed that Nix overexpression increased the collagen and fibronectin levels, whereas its knockdown inhibited collagen and fibronectin expression. However, Nix expression had no significant effect on cell proliferation or cell cycle distribution. These data suggest that Nix is responsible for ECM production but not cell proliferation. Furthermore, NE had a stimulatory effect on collagen and fibronectin expression, which decreased following the transfection of Nix-siRNA or successively increased when Nix was overexpressed. Collagen accumulation is an essential feature of connective tissue fibrosis during tissue injury. These results indicated that Nix is involved in NE-induced fibrosis by promoting ECM production. Fibronectin is not only a key ECM protein but also an important regulator for the assembly of the collagen matrix in vitro (Sottile and Hocking 2002; Velling et al. 2002). Its continuous presence also supports

matrix integrity both in vitro and in vivo (Sottile and Hocking 2002; Bentmann et al. 2010). Nix-induced upregulation of fibronectin may contribute to collagen protein expression. In addition, many studies have reported that TGF- β -smad signaling is the classical pathway during fibrosis. Ca^{2+} is an important regulator of the TGF- β -smad signal pathway. It has been demonstrated that Nix increases ER-SR calcium stores and mitochondrial calcium uptake, indicating that it affects intracellular Ca^{2+} . So, Ca^{2+} -TGF- β /smad signal pathway may contribute to the effect of Nix on NE-induced fibrosis. Taken together, the stress-related hormone norepinephrine induced upregulation of Nix contributed to the development of fibrosis.

A subsequent study focused on the mechanism of NE-induced Nix expression. Sequencing of the Nix promoter revealed the absence of consensus TATA or CCAAT transcription initiator elements but showed the presence of several GC boxes at the transcription start site (Galvez et al. 2006). As previous reports have linked the responsiveness of GC box-rich promoters in several genes with the regulated binding of the transcription factor Sp1 to GC elements, the mechanism of Sp1-mediated regulation of signaling genes has been reported as induction of this factor by PKC (Kim et al. 2004; Tanaka et al. 2000; Sakamoto and Taniguchi 2001; Chou et al. 2003). The direct PKC activator PMA treatment increased Nix transcriptional activity in cultured neonatal rat cardiac myocytes (NRCM) (Galvez et al. 2006). In our studies, Nix expression increased in PMA-induced NIH/3T3 fibroblasts, which is consistent with the previous studies in NRCM. Moreover, we found that NE-induced Nix expression was inhibited by the PKC antagonist BIM. As is well known, NE binds to a specific adrenoceptor on the cell membrane to induce PKC activation. Both the α - and β -adrenoceptor antagonists attenuated the NE-induced Nix expression. These results demonstrated that Nix expression was induced by NE via the AR-PKC pathway. Moreover, the inhibition of Nix expression by PRO, PHE or BIM attenuated the NE-induced expressions of collagen and fibronectin, which further indicates that Nix has profibrotic activities in NE-induced NIH/3T3 fibroblasts.

Taken together, our findings demonstrated that Nix was upregulated during NE-induced fibrosis through the AR-PKC pathway. The stress-related hormone norepinephrine induced upregulation of Nix plays an important role in fibrosis through promoting fibronectin and collagen expression. This study suggests a potential link between Nix and NE-induced fibrosis, which both improves our understanding of the molecular mechanisms involved in fibrosis and indicates new therapeutic targets for tissue fibrosis. Further exploration of the unknown functions of Nix and the related signaling mechanisms in fibrosis may provide new insights into future therapeutic targets for various fibrogenic diseases.

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References

- Ahmad A, Ahmad R (2012) Understanding the mechanism of hepatic fibrosis and potential therapeutic approaches. *Saudi J Gastroenterol Off J Saudi Gastroenterol Assoc* 18(3):155–167. doi:10.4103/1319-3767.96445
- Akiyama-Uchida Y, Ashizawa N, Ohtsuru A, Seto S, Tsukazaki T, Kikuchi H, Yamashita S, Yano K (2002) Norepinephrine enhances fibrosis mediated by TGF-beta in cardiac fibroblasts. *Hypertension* 40(2):148–154
- Bassi G, Radermacher P, Calzia E (2006) Catecholamines and vasopressin during critical illness. *Endocrinol Metab Clin N Am* 35(4):839–857. doi:10.1016/j.ecl.2006.09.012, x
- Bentmann A, Kawelke N, Moss D, Zentgraf H, Bala Y, Berger I, Gasser JA, Nakchbandi IA (2010) Circulating fibronectin affects bone matrix, whereas osteoblast fibronectin modulates osteoblast function. *J Bone Miner Res Off J Am Soc Bone Miner Res* 25(4):706–715. doi:10.1359/jbmr.091011
- Bernini G, Galetta F, Franzoni F, Bardini M, Taurino C, Moretti A, Bernini M, Berti P, Miccoli P, Salvetti A (2008) Normalization of catecholamine production following resection of pheochromocytoma positively influences carotid vascular remodeling. *Eur J Endocrinol Eur Fed Endocr Soc* 159(2):137–143. doi:10.1530/EJE-08-0126
- Chou SF, Chen HL, Lu SC (2003) Sp1 and Sp3 are involved in up-regulation of human deoxyribonuclease II transcription during differentiation of HL-60 cells. *Eur J Biochem FEBS* 270(8):1855–1862
- Delcayre C, Swynghedauw B (2002) Molecular mechanisms of myocardial remodeling. The role of aldosterone. *J Mol Cell Cardiol* 34(12):1577–1584
- Diwan A, Wansapura J, Syed FM, Matkovich SJ, Lorenz JN, Dorn GW 2nd (2008) Nix-mediated apoptosis links myocardial fibrosis, cardiac remodeling, and hypertrophy decompensation. *Circulation* 117(3):396–404. doi:10.1161/CIRCULATIONAHA.107.727073
- Diwan A, Matkovich SJ, Yuan Q, Zhao W, Yatani A, Brown JH, Molkentin JD, Kranias EG, Dorn GW 2nd (2009) Endoplasmic reticulum-mitochondria crosstalk in NIX-mediated murine cell death. *J Clin Invest* 119(1):203–212. doi:10.1172/JCI36445
- Dorn GW 2nd (2005) Physiologic growth and pathologic genes in cardiac development and cardiomyopathy. *Trends Cardiovasc Med* 15(5):185–189. doi:10.1016/j.tcm.2005.05.009
- Dorn GW 2nd, Kirshenbaum LA (2008) Cardiac reanimation: targeting cardiomyocyte death by BNIP3 and NIX/BNIP3L. *Oncogene* 27(Suppl 1):S158–S167. doi:10.1038/onc.2009.53
- Edgley AJ, Krum H, Kelly DJ (2012) Targeting fibrosis for the treatment of heart failure: a role for transforming growth factor-beta. *Cardiovasc Ther* 30(1):e30–e40. doi:10.1111/j.1755-5922.2010.00228.x
- Flint MS, Baum A, Episcopo B, Knickelbein KZ, Liegey Dougall AJ, Chambers WH, Jenkins FJ (2013) Chronic exposure to stress hormones promotes transformation and tumorigenicity of 3T3 mouse fibroblasts. *Stress* 16(1):114–121. doi:10.3109/10253890.2012.686075
- Galvez AS, Brunskill EW, Marreez Y, Benner BJ, Regula KM, Kirschenbaum LA, Dorn GW 2nd (2006) Distinct pathways regulate proapoptotic Nix and BNIP3 in cardiac stress. *J Biol Chem* 281(3):1442–1448. doi:10.1074/jbc.M509056200
- Goldstein DS (2003) Catecholamines and stress. *Endocr Regul* 37(2):69–80

- Greenhalgh SN, Iredale JP, Henderson NC (2013) Origins of fibrosis: pericytes take centre stage. *F1000 Prime Rep* 5:37. doi:[10.12703/P5-37](https://doi.org/10.12703/P5-37)
- Igreja AC, Mesquita Kde C, Cowper SE, Costa IM (2012) Nephrogenic systemic fibrosis: concepts and perspectives. *An Bras Dermatol* 87(4):597–607
- Kehat I, Molkentin JD (2010) Molecular pathways underlying cardiac remodeling during pathophysiological stimulation. *Circulation* 122(25):2727–2735. doi:[10.1161/CIRCULATIONAHA.110.942268](https://doi.org/10.1161/CIRCULATIONAHA.110.942268)
- Kim J, Padanilam BJ (2013) Renal nerves drive interstitial fibrogenesis in obstructive nephropathy. *J Am Soc Nephrol JASN* 24(2):229–242. doi:[10.1681/ASN.2012070678](https://doi.org/10.1681/ASN.2012070678)
- Kim E, Muga SJ, Fischer SM (2004) Identification and characterization of a phorbol ester-responsive element in the murine 8S-lipoxygenase gene. *J Biol Chem* 279(12):11188–11197. doi:[10.1074/jbc.M313291200](https://doi.org/10.1074/jbc.M313291200)
- Kimura K, Iwano M (2009) Molecular mechanisms of tissue fibrosis. *Nihon Rinsho Men'eki Gakkai Kaishi Jpn J Clin Immunol* 32(3):160–167
- Kohli S, Chhabra A, Jaiswal A, Rustagi Y, Sharma M, Rani V (2013) Curcumin suppresses gelatinase B mediated norepinephrine induced stress in H9c2 cardiomyocytes. *PLoS One* 8(10):e76519. doi:[10.1371/journal.pone.0076519](https://doi.org/10.1371/journal.pone.0076519)
- Lewis D, Scullion J (2012) Palliative and end-of-life care for patients with idiopathic pulmonary fibrosis: challenges and dilemmas. *Int J Palliat Nurs* 18(7):331–337
- Liu Y (2011) Cellular and molecular mechanisms of renal fibrosis. *Nat Rev Nephrol* 7(12):684–696. doi:[10.1038/nrneph.2011.149](https://doi.org/10.1038/nrneph.2011.149)
- Noble PW, Barkauskas CE, Jiang D (2012) Pulmonary fibrosis: patterns and perpetrators. *J Clin Invest* 122(8):2756–2762. doi:[10.1172/JCI60323](https://doi.org/10.1172/JCI60323)
- Oben JA, Yang S, Lin H, Ono M, Diehl AM (2003) Norepinephrine and neuropeptide Y promote proliferation and collagen gene expression of hepatic myofibroblastic stellate cells. *Biochem Biophys Res Commun* 302(4):685–690
- Oben JA, Roskams T, Yang S, Lin H, Sinelli N, Torbenson M, Smedh U, Moran TH, Li Z, Huang J, Thomas SA, Diehl AM (2004) Hepatic fibrogenesis requires sympathetic neurotransmitters. *Gut* 53(3):438–445
- Oberbeck R (2006) Catecholamines: physiological immunomodulators during health and illness. *Curr Med Chem* 13(17):1979–1989
- Oka T, Komuro I (2012) Molecular and cellular mechanisms of organ fibrosis. *Nihon Rinsho Jpn J Clin Med* 70(9):1510–1516
- Okuneva V, Zhvania M, Japaridze N, Gelazonia L, Lordkipanidze T (2009) Stress-system: corticotropin-releasing hormone and catecholamines (review). *Georgian Med News* 172–173:65–69
- Polyakova V, Loeffler I, Hein S, Miyagawa S, Piotrowska I, Dammer S, Risteli J, Schaper J, Kostin S (2011) Fibrosis in endstage human heart failure: severe changes in collagen metabolism and MMP/TIMP profiles. *Int J Cardiol* 151(1):18–33. doi:[10.1016/j.ijcard.2010.04.053](https://doi.org/10.1016/j.ijcard.2010.04.053)
- Rassler B, Marx G, Schierle K, Zimmer HG (2012) Catecholamines can induce pulmonary remodeling in rats. *Cell Physiol Biochem Int J Exp Cell Physiol Biochem Pharmacol* 30(5):1134–1147. doi:[10.1159/000343304](https://doi.org/10.1159/000343304)
- Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A (2004) Rational siRNA design for RNA interference. *Nat Biotechnol* 22(3):326–330. doi:[10.1038/nbt936](https://doi.org/10.1038/nbt936)
- Sakamoto S, Taniguchi T (2001) Identification of a phorbol ester-responsive element in the interferon-gamma receptor 1 chain gene. *J Biol Chem* 276(40):37237–37241. doi:[10.1074/jbc.M105543200](https://doi.org/10.1074/jbc.M105543200)
- Shao Y, Redfors B, Stahlman M, Tang MS, Miljanovic A, Mollmann H, Troidl C, Szardien S, Hamm C, Nef H, Boren J, Omerovic E (2013) A mouse model reveals an important role for catecholamine-induced lipotoxicity in the pathogenesis of stress-induced cardiomyopathy. *Eur J Heart Fail* 15(1):9–22. doi:[10.1093/eurjhf/hfs161](https://doi.org/10.1093/eurjhf/hfs161)
- Sottile J, Hocking DC (2002) Fibronectin polymerization regulates the composition and stability of extracellular matrix fibrils and cell-matrix adhesions. *Mol Biol Cell* 13(10):3546–3559. doi:[10.1091/mbc.E02-01-0048](https://doi.org/10.1091/mbc.E02-01-0048)
- Tanaka T, Kurabayashi M, Aihara Y, Ohyama Y, Nagai R (2000) Inducible expression of manganese superoxide dismutase by phorbol 12-myristate 13-acetate is mediated by Sp1 in endothelial cells. *Arterioscler Thromb Vasc Biol* 20(2):392–401
- Taylor M, Barr M, Stevens G, Bryson-Taylor D, Agho K, Jacobs J, Raphael B (2010) Psychosocial stress and strategies for managing adversity: measuring population resilience in New South Wales, Australia. *Popul Health Metrics* 8:28. doi:[10.1186/1478-7954-8-28](https://doi.org/10.1186/1478-7954-8-28)
- Velling T, Risteli J, Wennerberg K, Mosher DF, Johansson S (2002) Polymerization of type I and III collagens is dependent on fibronectin and enhanced by integrins alpha 11beta 1 and alpha 2beta 1. *J Biol Chem* 277(40):37377–37381. doi:[10.1074/jbc.M206286200](https://doi.org/10.1074/jbc.M206286200)
- Yang Q, Zhang YW, Liu HY, Song YH, Tang WX (2008) Effects of norepinephrine on the proliferation and activation of rat hepatic stellate cells. *Zhonghua Gan Zang Bing Za Zhi Zhonghua Ganzangbing Zazhi Chin J Hepatol* 16(11):844–848
- Yussman MG, Toyokawa T, Odley A, Lynch RA, Wu G, Colbert MC, Aronow BJ, Lorenz JN, Dorn GW 2nd (2002) Mitochondrial death protein Nix is induced in cardiac hypertrophy and triggers apoptotic cardiomyopathy. *Nat Med* 8(7):725–730. doi:[10.1038/nm719](https://doi.org/10.1038/nm719)