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Increased Expression of NAPDH Oxidase 4 (NOX4) in Systemic Sclerosis Dermal Fibroblasts: Regulation by Transforming Growth Factor β

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Abstract

Objective—Systemic Sclerosis (SSc) is characterized by severe and often progressive skin and multiple internal organ fibrosis. The mechanisms responsible for these alterations remain obscure, although excessive ROS-mediated oxidative stress has been implicated. NOX4 is one of seven NADPH oxidase isoforms responsible for ROS generation. Here, we examined NOX4 expression in SSc skin and cultured dermal fibroblasts and its regulation by TGF-β.

Methods—NOX4 was assessed in normal and SSc skin employing immunohistology, and in normal and SSc cultured dermal fibroblasts by quantitative PCR, fluorescence microscopy and Western blots. ROS levels were assessed by fluorescence measurements of H_2O_2 production. The TGF-β signaling involved in NOX4 stimulation was studied employing specific kinase inhibitors. NOX4 inhibition/downregulation was induced with a selective NOX4 small molecule inhibitor and NOX4 siRNA.

Results—In contrast with normal skin SSc skin fibroblasts showed intense NOX4 staining. Cultured SSc fibroblasts displayed increased NOX4 expression. TGF-β1 caused potent NOX4 protein and mRNA stimulation in normal and SSc fibroblasts mediated by PKC-δ and SMAD2/3 pathways. NOX4 knockdown in SSc fibroblasts reduced ROS production and lowered collagen I expression.

Conclusion—NOX4 expression and production are constitutively elevated in SSc skin and cultured SSc dermal fibroblasts. TGF-β1 stimulates NOX4 expression in normal and SSc fibroblasts through PKC-δ and Smad2/3 signaling pathways. A small molecule NOX4 inhibitor decreased fibroblast collagen and fibronectin production and NOX4 siRNA knockdown reduced SSc fibroblast ROS and collagen production. These results demonstrate NOX4 involvement in SSc-associated fibrosis and suggest NOX4 inhibitors as novel therapeutic approaches for SSc.

Keywords

Systemic Sclerosis; NOX4; ROS; fibrosis; TGF-β; SMAD 2/3; PKC-δ; c-Abl Kinase

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INTRODUCTION

The pathogenesis of Systemic Sclerosis (SSc) is complex and despite numerous studies that examined diverse aspects of its intricate picture the exact mechanisms involved are not well understood [1–4]. However, it is apparent that the most severe clinical and pathologic manifestations of the disease are the result of a fibrotic process characterized by the excessive and often progressive deposition of collagen and other connective tissue macromolecules in skin and numerous internal organs [5–8] and by a severe fibroproliferative vasculopathy preferentially affecting the microvasculature [9–11]. The mechanisms involved in the initiation and progression of the remarkable fibrotic and vasculoproliferative processes in SSc remain largely unknown. Extensive studies have shown that Transforming Growth Factor β (TGF-β) plays a crucial role in SSc pathogenesis owing to its potent and pleitropic profibrogenic effects [12–14]. Thus, the most promising novel therapeutic approaches for SSc and other fibrotic disorders include modifiers of TGF-β1 activation and signaling and are currently being intensively investigated [15–17]. However, owing to the important functions of TGF-β in numerous homeostatic processes a therapeutic approach involving global inhibition of its activity may be associated with undesirable adverse effects. Thus, there is a strong interest in the identification of downstream mediators of TGF-β profibrotic effects for the therapy of SSc and other fibrotic disorders with the expectation that their inhibition may lead to potent anti-fibrotic effects without the serious side effects that may result from global TGF-β inhibition.

One novel pathway that has been recently recognized as a potentially important participant in various fibrotic processes including idiopathic pulmonary fibrosis (IPF) and SSc involves reactive oxygen species (ROS). Although ROS are produced by normal fibroblasts and are essential for numerous important intracellular reactions, several studies have implicated excessive oxidative stress with the generation of deleterious ROS in the pathogenesis of SSc and other fibrotic disorders [18–23]. Indeed, numerous studies have provided strong evidence for the participation of ROS in the fibrotic process in IPF [24]. However, the role of ROS-induced oxidative stress in SSc pathogenesis has not been studied as extensively, although, it has been shown that SSc fibroblasts produce elevated ROS levels constitutively and it has been suggested that elevated ROS may be involved in the increased collagen expression in these cells [18, 21]. The presence of severe oxidative stress and high oxidative DNA damage in SSc has been confirmed by the observation of increased urinary 8-oxodG levels [20], and other studies have shown elevated ROS levels in monocytes and plasma from SSc patients [25–27] providing strong evidence for the important contribution of oxidative stress in SSc pathogenesis.

Although there are multiple sources of intracellular ROS, including ROS production by mitochondria, extensive studies have shown that most ROS production derives from the activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system [28–30]. NOX4 is one of seven NADPH isoforms and like other NADPH isoforms its structure consists mainly of a six-transmembrane domain known as the gp91phox domain. Despite their extensive similarity in structure and enzymatic function members of the NOX family differ in their mechanism of activation. NOX4 lacks potential regulatory subunits, therefore, it is generally accepted that NOX4 is constitutively active and the regulation of its

effects occurs mainly at the expression level [31]. Thus, the regulation of NOX4 expression may play a crucial role in the pathogenesis of ROS-mediated disorders. Although the role of NOX4 as an important downstream mediator of TGF-β-induced profibrotic effects has been demonstrated [32] and the participation of NOX4 in the pathogenesis of tissue fibrosis in various fibrotic disorders such as IPF and liver fibrosis has been the subject of several recent studies [33–35], the possible role of NOX4 in the initiation or establishment of the SSc-associated fibrotic process has not been examined extensively. We describe here studies demonstrating increased levels of NOX4 epitopes in fibroblastic cells in the dermis of affected SSc skin compared to normal skin samples and the increased expression of NOX4 transcripts in cultured SSc dermal fibroblasts. Furthermore, we describe studies demonstrating a potent stimulation of NOX4 gene expression by TGF-β and examining the signaling pathway involved in TGF-β regulation of NOX4 expression. We also report that a small molecule selective NOX4 inhibitor causes a dose-related reduction in fibroblast collagen and fibronectin production and that siRNA NOX4 knockdown reduces SSc fibroblast ROS and type I collagen production.

MATERIALS AND METHODS

Materials

Trypsin and bacterial collagenase were from Worthington Biochemical Corporation (Lakewood, NJ). DMEM, L-glutamine, penicillin, streptomycin and vitamins were from Corning Cellgro (Manassas, VA). Fungizone was from Life-Technologies (Carlsbad, CA), and FBS from Atlanta Biologicals (Lawrenceville, GA; now Thermo Scientific Pierce, Rockford, IL). The NOX4 polyclonal antibody was from ABR Affinity BioReagents, (Golden, CO) and the collagen type I polyclonal antibody from SouthernBiotech (Birmingham, AL). The β-actin monoclonal antibody, TGF-β, and ascorbic acid were from Sigma (St. Louis, MO). Rottlerin was from Axxora (San Diego, CA), imatinib from LC Laboratories (Woburn, MA), and SB431542 from Cayman Chemicals (Ann Arbor, MI). Five- (and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; C369) was from Invitrogen, Life Technologies (Thermo Fisher Scientific, Waltham, MA). GKT137831(GKT) is a specific dual NOX4/NOX1 inhibitor synthetized by GenKyoTex S.A. displaying very high NOX4/NOX1 specificity [36–40]. This compound was made available to us through a collaborative study with Dr. Patrick Page from GenKyoTex S.A. (Plan-les-Ouates, Switzerland).

Isolation and culture of fibroblasts

Normal dermal fibroblasts were obtained from the volar surface of forearms of healthy volunteers following their informed consent. SSc fibroblasts were obtained from skin biopsy specimens from the proximal edge of forearm lesions of patients with diffuse SSc of recent onset, as described previously [41, 42]. The cells were cultured and maintained at 37°C in a 5% CO_2 humidified atmosphere in DMEM (Corning Cellgro) containing 10% FBS, 2 mM L-glutamine, 100 U penicillin, 100 μg/ml streptomycin. Fibroblasts were used for experiments between passages 3 and 6.

Immunohistology of normal and SSc skin

Normal skin was obtained from the dorsal forearm of normal human donors with their informed consent following IRB approved protocols. SSc skin was obtained from skin biopsies from patients with diffuse cutaneous SSc of recent onset as described previously [41, 42] performed at the Scleroderma Center of Thomas Jefferson University for diagnostic purposes. The normal and SSc skin tissues were fixed in buffered formalin phosphate and subsequent paraffin embedding and sectioning. Sections were examined by immunohistology with a polyclonal antibody against NOX4 (R&D Systems, Inc. Minneapolis, MN), following the streptavidin-biotin method with AEC as chromogen using the Histostain-Plus kit (Invitrogen) according to the manufacturer's instructions.

Immunofluorescence

Normal and SSc dermal fibroblasts were cultured in plastic chamber slides and cells were fixed using 10% buffered formalin phosphate (Fisher, Pittsburg, PA) for 20 min and permeabilized with TritonX-100 (Sigma). Cells were incubated with NOX4 antibody (1:100 dilution) for 18 h and stained with Cy3 secondary antibody (1:50 dilution) for 1 h at 37°C. Cells were mounted following nuclear counterstaining with 4,6-diamidino-2-phenylindole staining (DAPI) and visualized by fluorescence microscopy. Sections were analyzed using a Zeiss LSM 510 META confocal laser scanning microscope system. The breakthrough of the DAPI signal into the red or the green channel was recorded separately. Zeiss META enhancement software was used in balancing the signal strength, and the image was scanned to separate signal from noise. Panels were assembled using Photoshop software without any RGB modification.

ROS detection

Normal and SSc human dermal fibroblasts were treated either with TGF-β1 (10 ng/mL) alone or with TGF-β1 plus GKT137831 (GKT; 10 μM) for 4–6 h at 37°C. ROS detection was performed using fluorescence of H_2O_2 production employing 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2CDCFDA) oxidation [43] according to the manufacturer's instructions. Briefly, cells were washed twice with HBSS buffer, incubated with 10 μM CM-H_2DCFDA for 15 min at 37°C, then washed with PBS three times and fixed with 2% paraformaldehyde. Samples were then mounted immediately prior to imaging by fluorescence microscopy. Images were obtained following 1 min of exposure to the microscope's light source.

Fluorescence analysis

The intensity of fluorescence of a specific microscopic field was analyzed with ImageJ software (NIH, Bethesda, MD), calculating the sum of the intensity of each pixel and was expressed as corrected total cell fluorescence (CTCF). The CTCF value measures the overall fluorescence intensity in a given microscopic field calculated with the integrated density of fluorescence and corrected for the surface area examined and the background. The average of the CTCF values was used in statistical analysis using a two-tailed unpaired *t*-test. $P < 0.05$ was considered statistically significant.

RNA extraction and semi-quantitative PCR

Normal and SSc fibroblasts were grown in culture under control conditions (untreated) or were treated with TGF-β1 (10ng/mL), or with TGF-β1 plus either the selective PKC-δ inhibitor rottlerin (5 μM), or specific inhibitors of c-Abl (imatinib; 10 μg/mL), and Smad 2/3 (SB431542; 10 μM), or various concentrations of GKT. Cells were harvested following 24 h of treatment and total RNA was extracted using the TRIzol reagent (Invitrogen). RT-PCR amplification of target mRNA was performed with 1 μg of RNA to generate first strand cDNA by using the SuperScript II reverse transcriptase (Invitrogen). PCR was performed using specific primers for NOX4 and β-actin. The primers used for PCR are listed in Supplementary Table 1. Quantitative real time PCR was performed utilizing the QuantiTect SYBR green PCR kit (Qiagen, Valencia, CA) on an ABI PRISM 7700 real time PCR machine. PCR was performed utilizing the cycling conditions of 95°C for 3 min followed by 40 cycles of 95°C for 30 sec/55°C for 30 sec. The method of relative quantitation {ΔΔCt} was employed to determine the level of gene expression in cultured dermal fibroblasts. ΔCt values were computed from the Ct (cycle threshold number) values of the human NOX4 gene and the housekeeping control gene.

Western blots

Human dermal fibroblasts were harvested and lysed in RIPA lysis buffer containing protease inhibitors and 30 μg of the extracts were separated by electrophoresis in SDS polyacrylamide gels under reducing conditions as described previously [41, 42]. Media from cell cultures were collected after 48 h and 15 μL were electrophoresed on 3–12% polyacrylamide gradient gels. Separated proteins were electroblotted onto nitrocellulose membranes (Invitrogen) and the membranes were blocked in PBS/5% dry milk/0.1% normal goat serum for 1 h at room temperature. The transferred proteins were reacted for 18–20 h at 4°C with NOX4 and β-actin antibodies for cell extracts and collagen type I for medium proteins. Appropriate secondary antibodies coupled to peroxidase and the ECL detection system (Thermo Scientific Pierce) were employed for detection. Densitometry quantification was performed using ImageJ software (NIH) and the signals were normalized to those of β-actin or total protein loading assessed from coomasie blue staining of the gels.

PKC-δ and NOX4 siRNA transfections

Normal human dermal fibroblasts were cultured as described above and specific PKC-δ siRNA transfection was performed using HiPerfect reagent (Qiagen) following the manufacturer's instructions. Briefly, 3×10^4 cells were cultured until 70–80% confluence and were transfected with the transfection reaction prepared in serum free media containing 10 nM PKC-δ siRNA and 6 μL of Hiperfect reagent preincubated for 10 min at room temperature. For NOX4 siRNA transfection SSc human dermal fibroblasts were cultured as described above and specific NOX4 siRNA transfection was performed using Lipofectamine (Invitrogen) following the manufacturer's instructions. Briefly, cells were cultured until 50% confluency and transfected with the transfection reaction prepared in Opti-MEM media containing NOX4 siRNA to 33nM final concentration and Lipofectamine preincubated for 20 min at room temperature. Cells were incubated with this transfection reaction for 12 h. All Stars siRNA (Qiagen) was used as control.

Statistical Analysis

The significance of differences between the control and the experimental groups was determined by statistical analysis using ANOVA or Student's t-test assuming equal variances. P-values <0.05 were considered statistically significant. Data are represented as either the mean ± SEM or the mean ±SD.

RESULTS

NOX4 expression in normal and SSc skin and in cultured normal and SSc dermal fibroblasts

To demonstrate and compare the NOX4 expression levels in cultured normal and SSc human dermal fibroblasts immunofluorescence studies were performed. The results showed that NOX4 was constitutively expressed by normal human dermal fibroblasts and that its expression was significantly higher in cultured SSc fibroblasts compared to cultured normal fibroblasts as illustrated for one representative experiment with one normal and one SSc cell line (Figure 1A). The increased NOX4 expression levels were confirmed by Western blot analysis of fibroblast cell extracts from cultured fibroblasts from three healthy donors and from four SSc patients. The results corroborated the increased levels of NOX4 protein in SSc dermal fibroblasts observed by immunofluorescence (Figure 1B, upper panel). ImageJ analysis of the Western blots following correction for the total amount of protein loaded into the gels (from comassie blue staining loading control) showed that on average NOX4 protein levels measured in three different normal fibroblast lines compared to four different SSc fibroblast lines were greater than 3-fold higher in SSc fibroblasts (Figure 1B, lower panel). This difference was highly significant ($p = 0.018$).

Immunohistological detection of NOX4 epitopes was performed in skin biopsy samples from 3 normal controls and from 4 SSc patients with diffuse cutaneous SSc recent onset [41, 42]. The results showed numerous fibroblastic cells displaying intense NOX4 staining in the samples of SSc skin (black arrows in Figure 1C) in contrast with negative staining in normal skin samples.

Stimulation of NOX4 expression by TGF-β1

To study the mechanisms responsible for the increased levels of NOX4 in SSc fibroblasts we examined whether TGF-β, the potent profibrotic growth factor generally accepted to play a crucial role in the pathogenesis of tissue fibrosis in SSc, was capable of stimulating NOX4 expression. To evaluate this possibility we examined the effects of TGF-β on NOX4 expression by cultured normal and SSc dermal fibroblasts. The results showed that TGF-β1 induced a greater than 4-fold stimulation of NOX4 transcripts in normal dermal fibroblasts as shown in Figure 2A (upper panel). Although SSc fibroblasts displayed increased baseline levels of NOX4 transcripts compared to normal fibroblasts TGF-β treatment also induced a greater than two-fold increase in NOX4 mRNA levels in SSc fibroblasts as shown in Figure 2A (lower panel).

Regulation of NOX4 expression by PKC-δ

Because previous studies showed that PKC-δ was a crucial kinase involved in the expression of numerous fibrotic tissue components in SSc [42], we examined the effect of PKC-δ inhibition on TGF-β stimulated NOX4 transcript levels. The results showed that the stimulation of NOX4 gene expression induced by TGF-β was almost completely abrogated by the selective PKC-δ inhibitor rottlerin (Figure 2A). A similar pattern was found at the protein level assessed by Western blots for NOX4 (Figure 2B). However, owing to the controversy related to the specificity of rottlerin as an inhibitor of PKC-δ, an alternative study using small PKC-δ interfering RNA (siRNA) was performed in order to address these concerns. Normal dermal fibroblasts were transfected with either a negative control siRNA or with various combinations of specific siRNA directed against PKC-δ. RNA and cell extracts were isolated and Real Time PCR performed. PCR analysis showed a 50% reduction of PKC-δ mRNA expression using one of the siRNA combinations. The reduction of PKC-δ mRNA was accompanied by a similar reduction of NOX4 mRNA expression (Figure 2C). PKC-δ siRNA also caused a greater than 60% reduction in the potent stimulation of NOX4 induced by TGF-β as shown in Figure 2D.

Regulation of NOX4 expression by other kinases

To examine whether the canonical TGF-β intracellular pathway was involved in the stimulation of NOX4 by TGF-β, normal dermal fibroblasts were treated with TGF-β1 alone or in combination with either the cAbl specific inhibitor imatinib, or the Smad2/3 inhibitor, SB431542. Levels of NOX4 mRNA expression were determined by Real Time-PCR (Figure 3A) and Western blots were performed to assess NOX4 protein levels in total cell protein extracts (Figure 3B). The results showed that imatinib caused only a 20–25% decrease in NOX4 mRNA expression and a 25–30% reduction in NOX4 protein levels whereas the Smad 2/3 inhibitor completely abrogated NOX4 mRNA expression in TGF-β1 treated fibroblasts (Figure 3A). The Smad2/3 inhibitor also reduced by greater than 50% the levels of NOX4 protein assessed by Western blots (Figure 3B).

Effects of stimulation or inhibition of NOX4 expression or activity on ROS production in normal dermal fibroblasts

To study the effects of NOX4 expression on ROS production by dermal fibroblasts the levels of ROS in live cultured cells were examined by analysis of fluorescence using carboxy-H_2DCFDA. This compound permeates live cells and is deacetylated by nonspecific intracellular esterases. In the presence of ROS the reduced fluorescein compound is oxidized and emits bright fluorescence. In order to identify ROS specific production induced by NOX4, normal dermal fibroblasts were treated for 24 h with TGF-β1 to stimulate NOX4 expression as described above, followed by incubation with GKT, the small molecule specific dual inhibitor of NOX4/NOX1. The results shown in Figure 4 demonstrate increased ROS production induced by TGF-β and a marked reduction of TGF-β stimulated ROS production by the specific NOX4/NOX1 inhibitor.

Effects of inhibition of NOX4 activity on type I collagen and fibronectin expression in normal dermal fibroblasts

To assess whether inhibition of NOX4 activity resulted in changes in the expression of type I collagen and fibronectin we incubated normal dermal fibroblast cultures with increasing concentrations of GKT in the presence or absence of TGF-β1 to induce progressively greater inhibition of NOX4 activity and analyzed the effects on type I collagen and fibronectin transcript levels by real time PCR and on the production of type I collagen employing Western blots. The results (Figure 5) showed that GKT caused a dose-related reduction in type I collagen and fibronectin transcript levels both in unstimulated cultures and in cultures stimulated by TGF-β1 (Figure 5A), as well as a reduction in type I collagen protein production assessed by Western blots (Figure 5B).

Effect of NOX4 knockdown on ROS production and collagen type I production in SSc dermal fibroblasts

To directly examine whether NOX4 expression was involved in the increased production of Type I collagen in SSc cells SSc dermal fibroblasts were transfected with either a negative control siRNA or with various combinations of siRNA directed against NOX4. PCR analysis showed a 60% reduction of NOX4 mRNA expression using one of the siRNA combinations (Figure 6A). ROS production was notably reduced by siRNA NOX4 knockdown as assessed by fluorescence measures (Figure 6B). Analysis of type I collagen protein levels secreted into the culture media showed that NOX4 siRNA caused a 20–30% reduction of collagen 1 protein levels depending of the SSc cell line studied (Figure 6C).

DISCUSSION

Several recent studies have suggested that oxidative stress is involved in SSc-associated fibrosis [18–23]. The mechanisms responsible for increased oxidative stress in SSc are a reduction of endogenous antioxidants and a parallel overproduction of ROS. ROS play both a deleterious and a beneficial dual role. Under physiological conditions ROS perform important functions in cellular signaling pathways as well as in host defense, however, in pathological states ROS can induce oxidative stress causing damage to proteins, lipids, and DNA, as well as activating various deleterious redox-sensitive pathways. Although ROS are produced by normal fibroblasts and are essential for numerous homeostatic intracellular functions, including regulation of cellular proliferation, there is evidence that SSc dermal fibroblasts produce increased ROS [18, 21, 44] and it has been suggested that elevated ROS levels may be involved in the increased collagen expression in these cells. The experiments described here showed increased levels of NOX4 epitopes in fibroblastic cells of skin biopsies from patients with diffuse cutaneous SSc of recent onset in comparison to samples of normal skin. *In vitro* studies employing cultures of normal and SSc dermal fibroblasts showed a marked increase in NOX4 expression and ROS production by SSc dermal fibroblasts in culture in comparison with normal dermal fibroblasts. Furthermore, it was shown that most of ROS are produced by NOX4 since inhibition of its activity by a highly selective NOX4 inhibitor or downregulation by specific NOX4 siRNA transfection caused a profound decrease in ROS levels. It should be emphasized, however, that because the small

molecule inhibitor we employed also inhibits NOX1 a role of NOX1 in regulation of ROS production cannot be completely excluded.

TGF-β signaling has been implicated as one of the most crucial mechanisms responsible for the fibrotic process in SSc pathogenesis [1–6,12–14]. In agreement with studies from other investigators [45–47] we found that TGF-β caused a potent stimulation of NOX4 expression in normal dermal fibroblasts as well as in SSc fibroblasts. Owing to the fact that NOX4 lacks regulatory subunits and is generally accepted that the expression of NOX mRNA and the resulting protein levels determine the extent of its activity [31], these observations strongly implicate ROS as a participant in the SSc fibrotic process. We also investigated some of the canonical and non-canonical signaling pathways involved in TGF-β stimulation of NOX4. Among the non-canonical TGF-β pathways substantial interest has been recently focused on the PKC-δ and c-Abl kinase pathways [1,8,16,42]. Several studies have suggested a prominent role of PKC-δ in the pathogenesis of fibrotic diseases including SSc. Indeed, cultured dermal fibroblasts from SSc patients were shown to contain higher PKC-δ levels than normal cells and further studies demonstrated that PKC-δ was capable of inducing a potent stimulation of collagen gene expression [42]. The results described here indicate that PKC-δ mediated-pathways participate in the upregulation of NOX4 transcription induced by TGF-β since PKC-δ inhibition employing rottlerin, a highly selective PKC-δ inhibitor, and PKC-δ knockdown employing siRNA abrogated or decreased TGF-β induction of NOX4 expression. Furthermore, Smad2/3 was also shown to be involved in this regulation since the specific Smad2/3 inhibitor SB431542 caused essentially complete abrogation of TGF-β1 stimulation of NOX4 expression. In contrast, inhibition of c-Abl with Imatinib caused only a minor reduction of 20–25% indicating that the c-Abl contribution to ROS production in SSc fibroblasts is minor and fail to support the existence of the mutually stimulatory interaction between c-Abl and PKC-δ in response to oxidative stress described previously [48]. Therefore, our results demonstrate that both canonical (Smad2/3) and non-canonical pathways (PKC-δ) pathways are implicated in the stimulation of NOX4 expression induced by TGF-β but that c-Abl does not appear to be involved. One important observation in these studies was that inhibition of NOX4 activity with the small molecule inhibition GKT caused a dose-related inhibitory effect in type I collagen and fibronectin transcript levels and a decrease in type I collagen protein levels in normal dermal fibroblasts. Of greater relevance to the pathogenesis of SSc were our observations that siRNA-induced knockdown of NOX4 expression resulted in a substantial reduction ROS production which was accompanied by a reduction in the levels of type I collagen produced by SSc fibroblasts providing strong support for the important pro-fibrogenic role of NOX4 and as a downstream mediator of the TGF-β-induced cascade of pro-fibrotic reactions. Following submission of our paper a recent publication also demonstrated upregulation of NOX4 expression in SSc fibroblasts and showed that treatment of the cells with a non-selective NOX inhibitor or silencing of NOX4 with siRNA decreased type I collagen and α-SMA expression in these cells [49]. Thus, the results from the studies described here together with those described by Spadoni et al. [49] collectively suggest an important role of this constitutively expressed NADPH oxidase in the pathogenesis of tissue fibrosis in SSc and in the regulation of TGF-β-induced pro-fibrotic molecular pathways. These results

further suggest that targeting the downstream TGF-β pathway by inhibiting NOX4 may represent a potentially effective anti-fibrotic therapy for this currently incurable disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Jimenez SA, Derk CT. Following the molecular pathways toward an understanding of the pathogenesis of systemic sclerosis. Ann Intern Med. 2004; 140:37–50. [PubMed: 14706971]
2. Katsumoto TR, Whitfield ML, Connolly MK. The pathogenesis of systemic sclerosis. Annual Rev Pathol. 2011; 6:509–537. [PubMed: 21090968]
3. Balbir-Gurman A, Braun-Moscovici Y. Scleroderma-new aspects in pathogenesis and treatment. Best Pract Res Clin Rheumatol. 2012; 26:13–24. [PubMed: 22424190]
4. Assassi S, Mayes MD. What does global gene expression profiling tell us about the pathogenesis of systemic sclerosis? Curr Opin Rheumatol. 2013; 25:686–691. [PubMed: 24061076]
5. Denton CP, Black CM, Abraham DJ. Mechanisms and consequences of fibrosis in systemic sclerosis. Nat Clin Pract Rheumatol. 2006; 2:134–144. [PubMed: 16932673]
6. Varga J, Abraham D. Systemic sclerosis: a prototypic multisystem fibrotic disorder. J Clin Inves. 2007; 117:557–567.
7. Varga JA, Trojanowska M. Fibrosis in systemic sclerosis. Rheum. Dis. Clin. North America. 2008; 34:115–143.
8. Bhattacharyya S, Wei J, Varga J. Understanding fibrosis in systemic sclerosis: shifting paradigms, emerging opportunities. Nat Rev Rheumatol. 2011; 8:42–54. [PubMed: 22025123]
9. Fleming JN, Schwartz SM. The pathology of scleroderma vascular disease. Rheum Dis Clin North Am. 2008; 34:41–55. [PubMed: 18329531]
10. Matucci-Cerinic M, Kahaleh B, Wigley FM. Review: evidence that systemic sclerosis is a vascular disease. Arthritis Rheum. 2013; 65:1953–1962. [PubMed: 23666787]
11. Altorok N, Wang Y, Kahaleh B. Endothelial dysfunction in systemic sclerosis. Curr Opin Rheumatol. 2014; 26:615–620. [PubMed: 25191994]
12. Varga J, Whitfield ML. Transforming growth factor-beta in systemic sclerosis (scleroderma). Front Biosci (Schol. Ed.). 2009; 1:226–235. [PubMed: 19482698]
13. Jimenez SA, Castro SV, Piera-Velazquez S. Role of Growth factors in the pathogenesis of tissue fibrosis in Systemic Sclerosis. Curr Rheumatol Rev. 2010; 6:283–294. [PubMed: 25693043]
14. Lafyatis R. Transforming growth factor β-at the centre of systemic sclerosis. Nat Rev Rheumatol. 2014 [Epub ahead of print].
15. Varga J, Pasche B. Antitransforming growth factor-beta therapy in fibrosis: recent progress and implications for systemic sclerosis. Curr Opin Rheumatol. 2008; 20:720–728. [PubMed: 18946334]
16. Rosenbloom J, Jimenez SA. Molecular ablation of transforming growth factor beta signaling pathways by tyrosine kinase inhibition: the coming of a promising new era in the treatment of tissue fibrosis. Arthr Rheum. 2008; 58:2219–2224. [PubMed: 18668575]
17. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. Nat Med. 2012; 18:1028–1040. [PubMed: 22772564]

18. Sambo P, Baroni SS, Luchetti M, Paroncini P, Dusi S, Orlandini G, et al. Oxidative stress in scleroderma: maintenance of scleroderma fibroblast phenotype by the constitutive up-regulation of reactive oxygen species generation through the NADPH oxidase complex pathway. Arthr Rheum. 2001; 44:2653–2664. [PubMed: 11710721]
19. Gabrielli A, Svegliati S, Moroncini G, Pomponio G, Santillo M, Avvédimento EV. Oxidative stress and the pathogenesis of scleroderma: the Murrell's hypothesis revisited. Semin Immunopathol. 2008; 30:329–337. [PubMed: 18548250]
20. Avouac J, Borderie D, Ekindjian OG, Kahan A, Allanore Y. High DNA oxidative damage in systemic sclerosis. J Rheumatol. 2010; 37:2540–2547. [PubMed: 20843906]
21. Shi-wen X, Thompson K, Khan K, Liu S, Murphy-Marshman H, Baron M, et al. Focal adhesion kinase and reactive oxygen species contribute to the persistent fibrotic phenotype of lesional scleroderma fibroblasts. Rheumatology (Oxford). 2012; 51:2146–2154. [PubMed: 22977060]
22. Gabrielli A, Svegliati S, Moroncini G, Amico D. New insights into the role of oxidative stress in scleroderma fibrosis. Open Rheumatol J. 2012; 6:87–95. [PubMed: 22802906]
23. Grygiel-Górniak B, Puszczewicz M. Oxidative damage and antioxidative therapy in systemic sclerosis. Mediators Inflamm. 2014; 2014:389582. [PubMed: 25313270]
24. Hecker L, Cheng J, Thannickal VJ. Targeting NOX enzymes in pulmonary fibrosis. Cell Mol Life Sci. 2012; 69:2365–2371. [PubMed: 22618245]
25. Sambo P, Jannino L, Candela M, Salvi A, Donini M, Dusi S, et al. Monocytes of patients with systemic sclerosis (scleroderma) spontaneously release in vitro increased amounts of superoxide anion. J Invest Dermatol. 1999; 112:78–84. [PubMed: 9886268]
26. Allanore Y, Borderie D, Lemarechal H, Ekindjian OG, Kahan A. Acute and sustained effects of dihydropyridine-type calcium channel antagonists on oxidative stress in systemic sclerosis. Am J Med. 2004; 116:595–600. [PubMed: 15093755]
27. Savas E, Aksoy N, Pehlivan Y, Sayiner ZA, Oztürk ZA, Tabur S, et al. Evaluation of oxidant and antioxidant status and relation with prolidase in systemic sclerosis. Wien Klin Wochenschr. 2014; 126:341–346. [PubMed: 24825595]
28. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev. 2007; 87:245–313. [PubMed: 17237347]
29. Leto TL, Morand S, Hurt D, Ueyama T. Targeting and Regulation of Reactive Oxygen Species Generation by Nox Family NADPH Oxidases. Antiox Redox Signal. 2009; 11:2607–2619.
30. Montezano AC, Burger D, Ceravolo GS, Yusuf H, Montero M, Touyz RM. Novel Nox homologues in the vasculature: focusing on Nox4 and Nox5. Clin Sci (Lond). 2011; 120:31–41.
31. Serrander L, Cartier L, Bedard K, Banfi B, Lardy B, Plastre O, et al. NOX4 activity is determined by mRNA levels and reveals a unique pattern of ROS generation. Biochem J. 2007; 406:105–114. [PubMed: 17501721]
32. Jiang F, Liu GS, Dusting GJ, Chan EC. NADPH oxidase-dependent redox signaling in TGF-β-mediated fibrotic responses. Redox Biol. 2014; 2:267–272. [PubMed: 24494202]
33. Amara N, Goven D, Prost F, Muloway R, Crestani B, Boćzkowski J. NOX4/NADPH oxidase expression is increased in pulmonary fibroblasts from patients with idiopathic pulmonary fibrosis and mediates TGFβ1-induced fibroblast differentiation into myofibroblasts. Thorax. 2010; 65:733–738. [PubMed: 20685750]
34. Hecker L, Vittal R, Jones T, Jagirdar R, Luckhardt TR, Horowitz JC, et al. NADPH oxidase-4 mediates myofibroblast activation and fibrogenic responses to lung injury. Nat Med. 2009; 15:1077–1081. [PubMed: 19701206]
35. Sancho P, Mainez J, Crosas-Molist E, Roncero C, Fernández-Rodriguez CM, Pinedo F, et al. NADPH oxidase NOX4 mediates stellate cell activation and hepatocyte cell death during liver fibrosis development. PloS One. 2012; 7:e45285. [PubMed: 23049784]
36. Laleu B, Gaggini F, Orchard M, Fioraso-Cartier L, Cagnon L, Houngninou-Molango S, et al. First in class, potent, and orally bioavailable NADPH oxidase isoform 4 (Nox4) inhibitors for the treatment of idiopathic pulmonary fibrosis. J Med Chem. 2010; 53:7715–7730. [PubMed: 20942471]
37. Gaggini F, Laleu B, Orchard M, Fioraso-Cartier L, Cagnon L, Houngninou-Molango S, et al. Design synthesis and biological activity of original pyrazolo-pyrido-diazepine,-pyrazine and -

oxazine dione derivatives as novel dual Nox4/Nox1 inhibitors. Bioorg Med Chem. 2011; 19:6989–6999. [PubMed: 22041175]

38. Jiang JX, Chen X, Serizawa N, Szyndralewiew C, Page P, Schröder K, et al. Liver fibrosis and hepatocyte apoptosis are attenuated by GKT137831, a novel NOX4/NOX1 inhibitor in vivo. Free Radical Biol Med. 2012; 53:289–296. [PubMed: 22618020]
39. Green DE, Murphy TC, Kang BY, Kleinhenz JM, Szyndralewiew C, Page P, et al. The Nox4 inhibitor GKT137831 attenuates hypoxia-induced pulmonary vascular cell proliferation. Am J Respir Cell Mol Biol. 2012; 47:718–726. [PubMed: 22904198]
40. Aoyama T, Paik YH, Watanabe S, Laleu B, Gaggini F, Fioraso-Cartier L, et al. Nicotinamide adenine dinucleotide phosphate oxidase in experimental liver fibrosis: GKT137831 as a novel potential therapeutic agent. Hepatology. 2012; 56:2316–2327. [PubMed: 22806357]
41. Del Galdo F, Sotgia F, de Almeida C, Jasmin JF, Musick M, Lisanti MP, Jimenez SA. Decreased expression of caveolin-1 in systemic sclerosis: crucial role in the pathogenesis of tissue fibrosis. Arthritis Rheum. 2008; 58:2854–2865. [PubMed: 18759267]
42. Jimenez SA, Gaidarova S, Saitta B, Sandorfi N, Herrich DJ, Rosenbloom JC, et al. Role of protein kinase C-delta in the regulation of collagen gene expression in scleroderma fibroblasts. J Clin Invest. 2001; 108:1395–1403. [PubMed: 11696585]
43. Hempel SL, Buettner GR, O'Malley YQ, Wessels DA, Flaherty DM. Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison with 2',7'-dichlorodihydrofluorescein diacetate, 5(and 6)-carboxy-2'7'-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123. Free Radic Biol Med. 1999; 27:146–159. [PubMed: 10443931]
44. Svegliati S, Cancello R, Sambo P, Luchetti M, Paroncini P, Orlandini G, et al. Platelet-derived growth factor and reactive oxygen species (ROS) regulate Ras protein levels in primary human fibroblasts via ERK1/2. Amplification of ROS and Ras in systemic sclerosis fibroblasts. J Biol Chem. 2005; 280:36474–36482. [PubMed: 16081426]
45. Sturrock A, Cahill B, Norman K, Huecksteadt TP, Hill K, Sanders K, et al. Transforming growth factor-beta1 induces Nox4 NAD(P)H oxidase and reactive oxygen species-dependent proliferation in human pulmonary artery smooth muscle cells. Am J Lung Cell Mol Physiol. 2006; 290:L661–L673.
46. Boudreau HE, Casterline BW, Rada B, Korzeniowska A, Leto TL. Nox4 involvement in TGF-beta and SMAD3-driven induction of the epithelial-to-mesenchymal transition and migration of breast epithelial cells. Free Radic Biol Med. 2012; 53:1489–1499. [PubMed: 22728268]
47. Bai G, Hock TD, Logsdon N, Zhou Y, Thannickal VJ. A far-upstream AP-1/Smad binding box regulates human NOX4 promoter activation by transforming growth factor-β. Gene. 2014; 540:62–67. [PubMed: 24560583]
48. Sun X, Majumder P, Shioya H, Wu F, Kumar S, Weichselbaum R, et al. Activation of the cytoplasmic c-Abl tyrosine kinase by reactive oxygen species. J Biol Chem. 2000; 275:17237–17240. [PubMed: 10770918]
49. Spadoni T, Svegliati Baroni S, Amico D, Albani L, Moroncini G, Avvedimento EV, et al. A reactive oxygen species-mediated loop maintains the increased expression of NOX2 and NOX4 in skin fibroblasts from patients with systemic sclerosis. Arthritis Rheumatol. 2015 [Epub ahead of print].

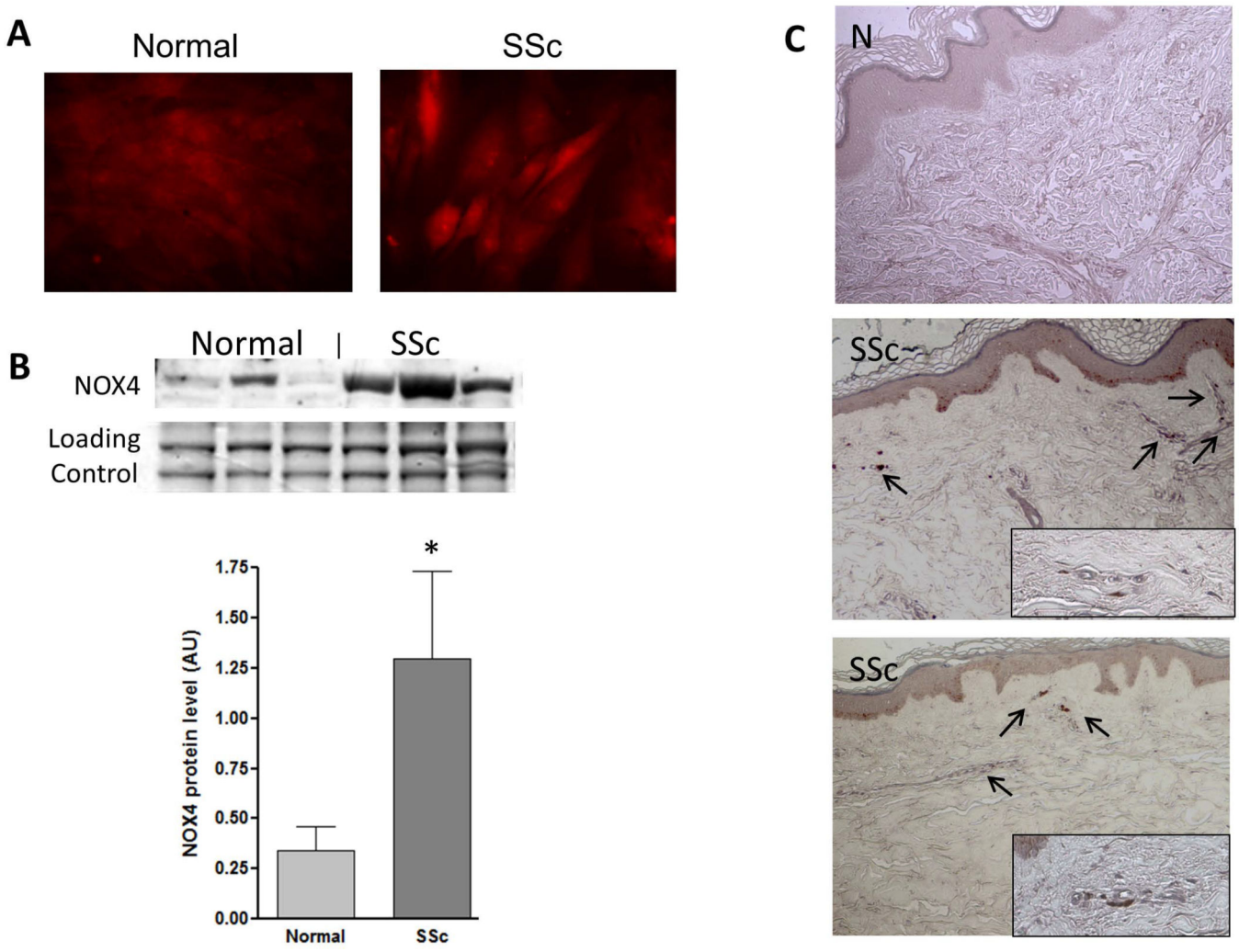

Figure 1.

A. Immunofluorescence analysis of NOX4 protein levels in cultured normal and SSc dermal fibroblasts. Representative NOX4 immunofluorescence in confluent cultures of one normal and one SSc dermal fibroblast cell line.

B. Western blot analysis of equal amounts of cell extracts from three normal and three SSc dermal fibroblasts probed with a specific NOX4 antibody (upper panel). The middle panel shows the same gel stained with comassie blue which was used as loading control for protein concentrations (middle panel). The bar graph (lower panel) represents the average NOX4 protein levels from 3 normal and 4 SSc cultured dermal fibroblast cell lines corrected for protein loading.* p value = 0.018. AU= arbitrary units of fluorescence.

C. Immunohistological staining of normal and SSc skin tissues for NOX4 epitopes. The images shown are representative of images obtained from 3 samples of normal skin and 4 samples of affected SSc skin. Note intensely-stained NOX4 epitopes in numerous fibroblastic cells (black arrows) present only in SSc tissues (Magnification 10×). The insets show greater magnification images (Magnification 40×) of NOX4-positive cells in the SSc skin samples.

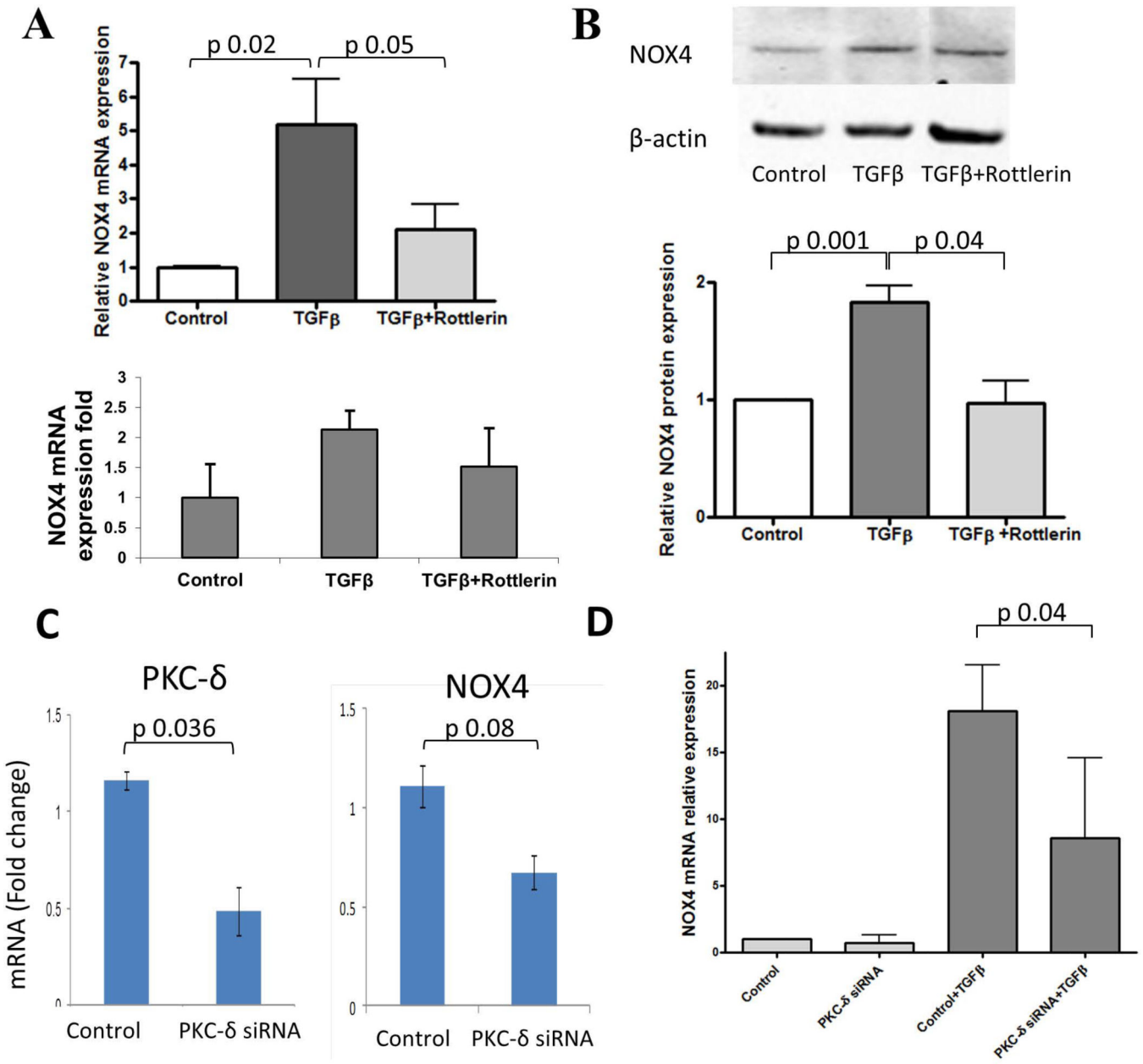

Figure 2.

A. NOX4 mRNA expression levels assessed in triplicate by Real Time PCR analysis in four normal dermal fibroblast cell lines under treatment with TGF-β1 and rottlerin. GAPDH was used as endogenous control. Lower panel shows the effect of TGF-β1 and rottlerin on three SSc cell lines.

B. Representative Western blot analysis of NOX4 and β-actin (as protein control) using cell extracts from four normal dermal fibroblast cell lines treated with TGF-β1 alone or TGF-β1 plus rottlerin. Bar graph represents NOX4 protein levels following correction for the intensity of the β actin band.

C. Bar graphs of PKC-δ and NOX4 mRNA expression by normal dermal fibroblasts following treatment with specific siRNA against PKC-δ. The data shown are the average of the siRNA effect on three normal dermal fibroblast cell lines.

D. Bar graph of PKC-δ expression by normal dermal fibroblasts following treatment with TGF-β1 and a specific siRNA against PKC-δ. The data shown are the average of duplicate experiments examining the PKC-δ siRNA effect on two normal dermal fibroblast cell lines.

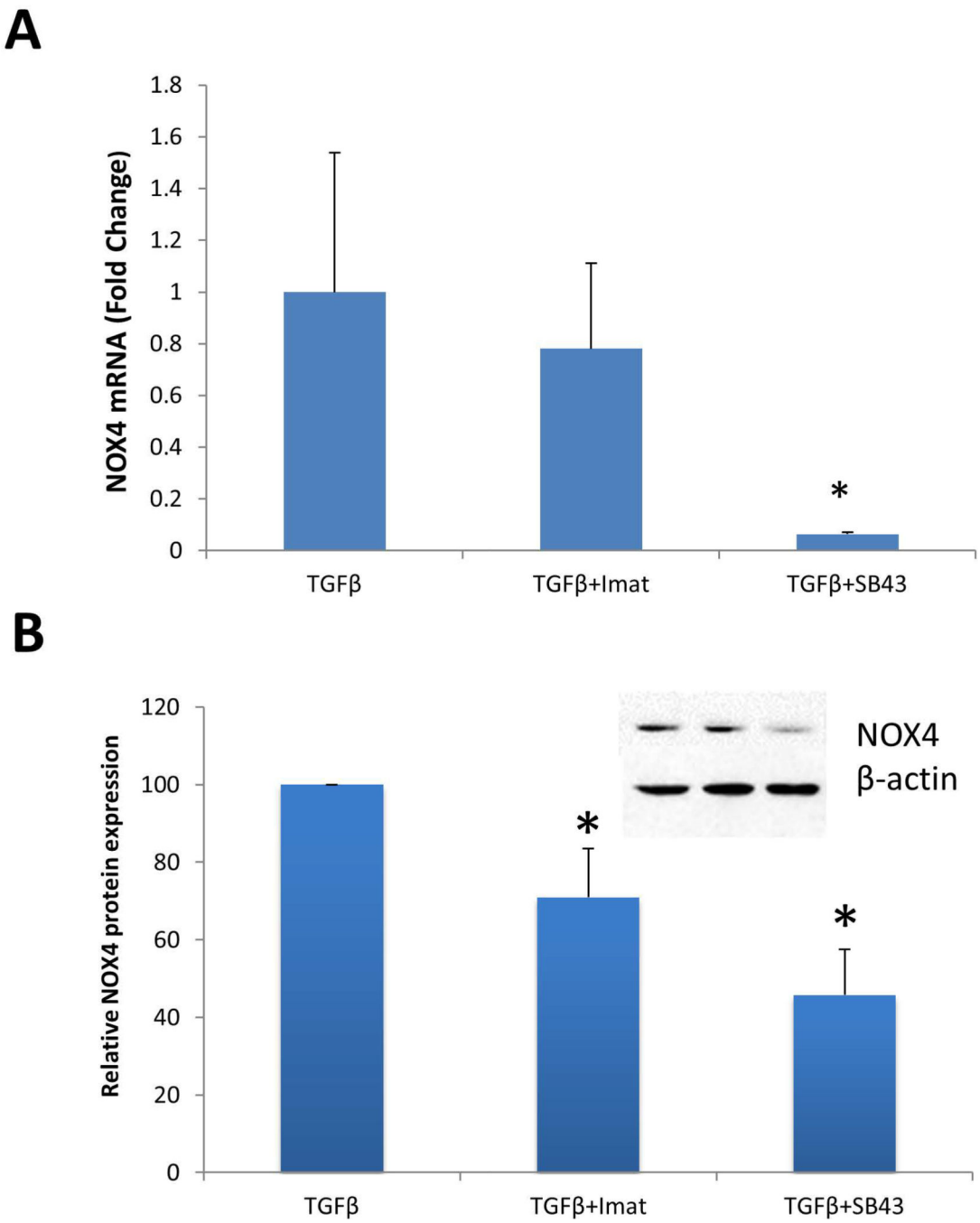

Figure 3.

Real Time PCR analysis of NOX4 expression (A) and protein levels of NOX4 (B) by normal dermal fibroblasts under treatment with either TGF-β1 alone or with TGF-β1 plus either imatinib mesylate (Imat; cAbl inhibitor) or SB4315421 (SB43; Smad2/3 specific inhibitor). The results are the average of three separate experiments with three different cell lines. * p value < 0.05. A representative Western blot is shown in the inset.

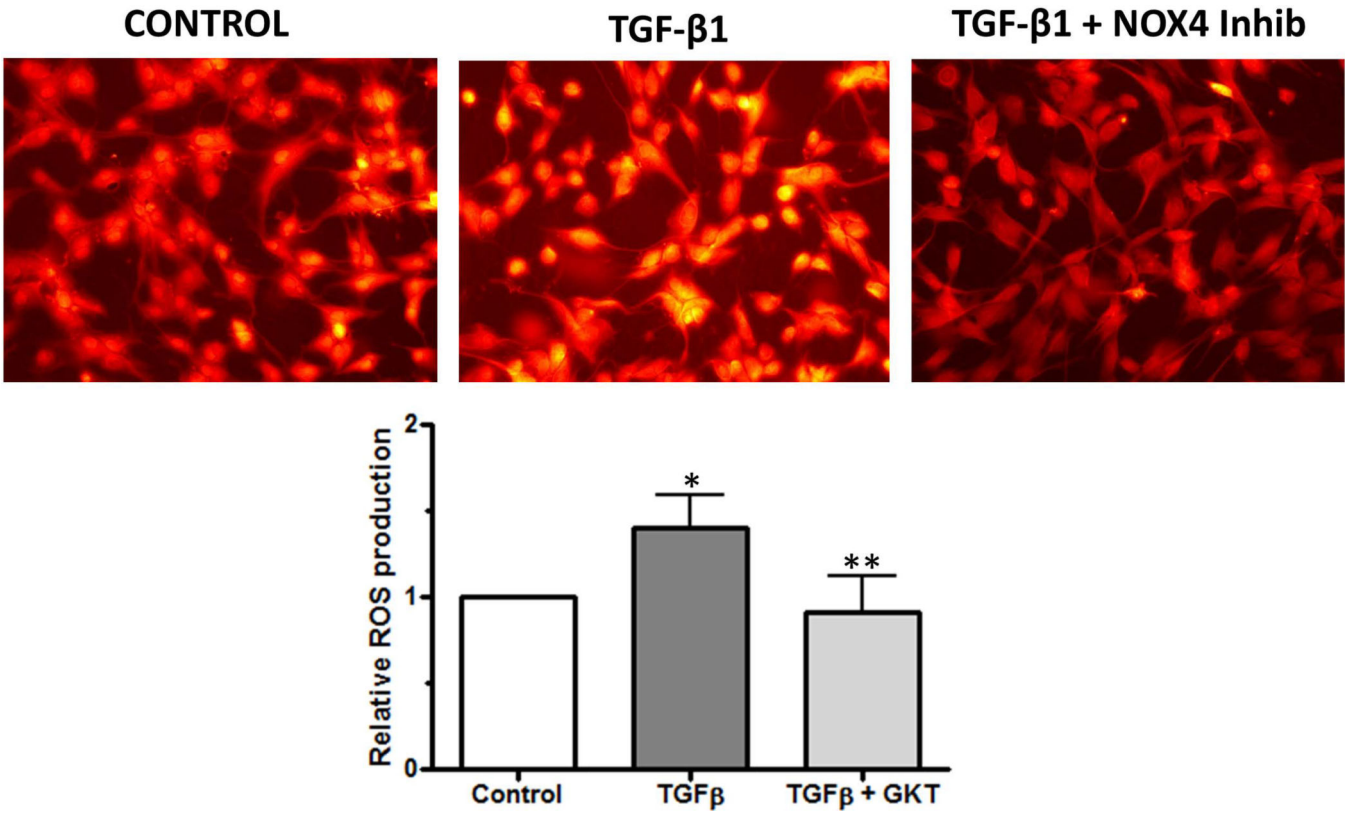

Figure 4.

Fluorescence microscopy images of ROS levels in normal dermal fibroblasts treated with either TGF-β1 alone or TGF-β1 plus the NOX4/NOX1 small molecule inhibitor GKT137831 (GKT). The bar graph (lower panel) represents corrected total cell fluorescence (CTCF) of relative ROS production of TGF-β1 or TGF-β1 plus GKT inhibitor *vs* untreated cells in four separate experiments with two normal dermal fibroblast cell lines.* p value = 0.005, ** p value = 0.02.

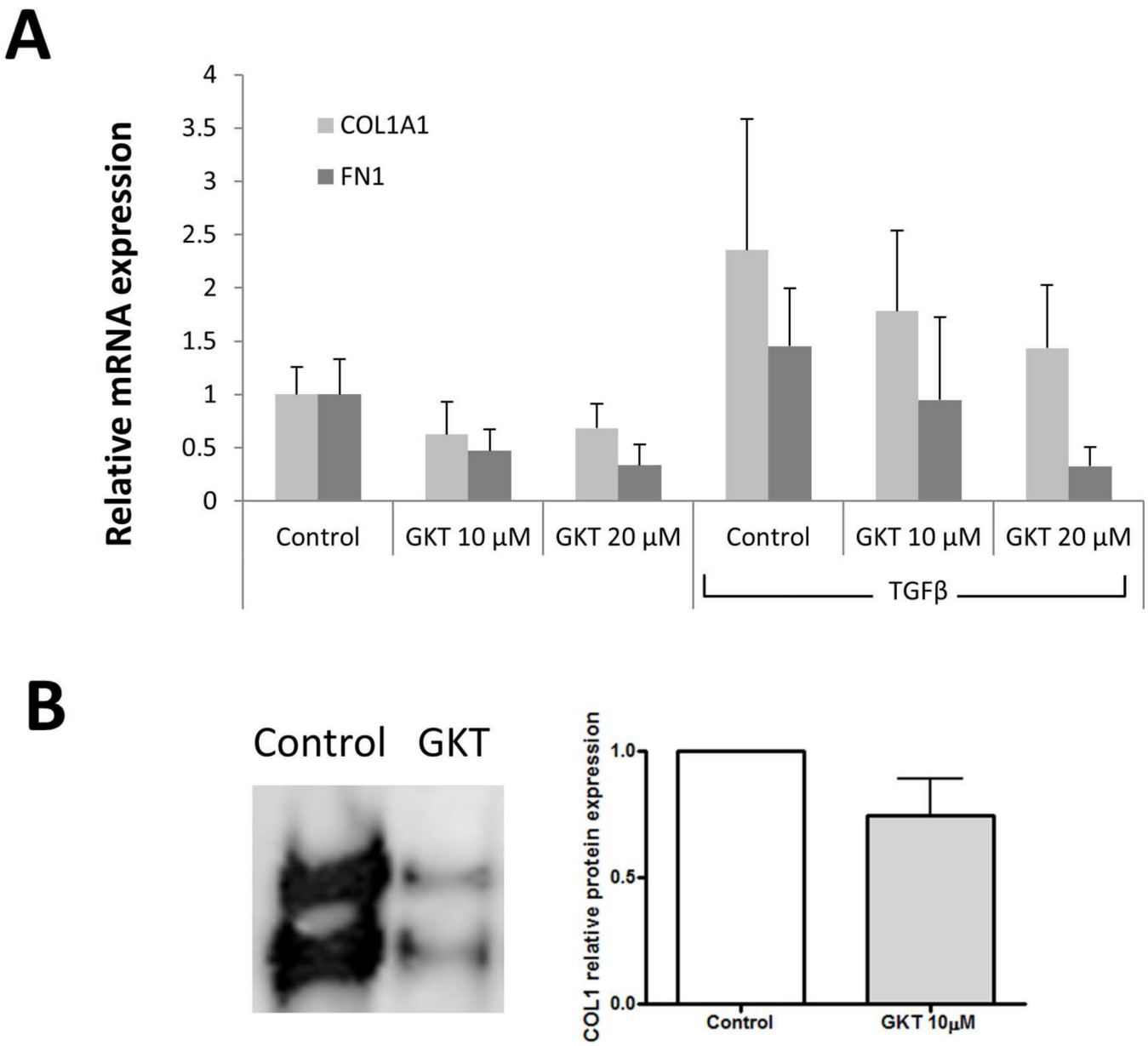

Figure 5.

A. Real Time PCR analysis of COL1A1 and FN1 expression by normal dermal fibroblasts treated with increasing concentration of the NOX4/NOX1 specific inhibitor GKT137831 (GKT) and either culture media alone or culture media containing TGF-β1. The data shown are the average of the results obtained in three separate experiments performed with a normal cell line.

B. Representative image of a Western blot analysis for secreted collagen Type I from normal dermal fibroblasts treated with 10 µM GKT. The bar graph shows the average results obtained with four different cell lines.

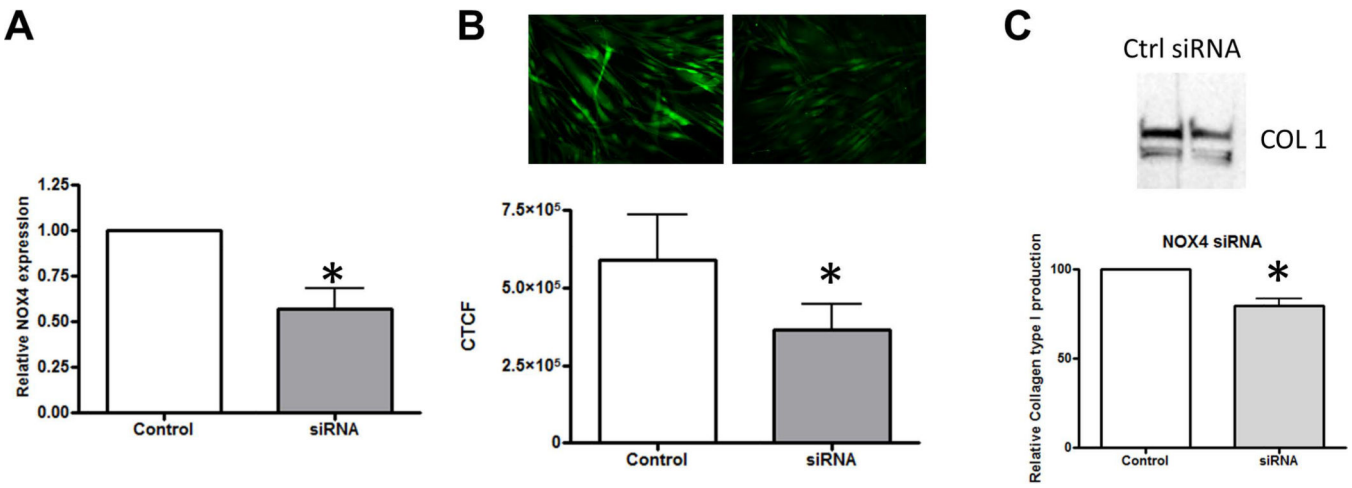

Figure 6.

A. Bar graphs of NOX4 mRNA expression by SSc dermal fibroblasts following treatment with specific siRNA against NOX4. The data shown are the average of the siRNA effect on four SSc dermal fibroblast cell lines * p value < 0.05.

B. Fluorescence microscopy images and quantification of ROS levels in SSc dermal fibroblasts following treatment with specific siRNA against NOX4. The graph represents the average intensity of fluorescence (CTCF) of two SSc dermal fibroblast cell lines examined in duplicate * p value < 0.05.

C. Western blot analysis of secreted collagen Type I from the culture media of SSc dermal fibroblasts following NOX4 siRNA knockdown. Control cells were transfected with a scrambled inactive siRNA. The bar graph shows the average of results obtained with three separate SSc dermal fibroblast cell lines * p value < 0.01.