ARTHRITIS & RHEUMATISM
Vol. 64, No. 6, June 2012, pp 1978–1989
DOI 10.1002/art.34336
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Effect of Oxidative Stress on Protein Tyrosine Phosphatase 1B in Scleroderma Dermal Fibroblasts

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Objective. **Platelet-derived growth factor (PDGF) and its receptor, PDGFR, promote fibrosis in systemic sclerosis (SSc; scleroderma) dermal fibroblasts, and such cells in scleroderma skin lesions produce excessive reactive oxygen species (ROS). PDGFR is phosphorylated upon PDGF stimulation, and is dephosphorylated by protein tyrosine phosphatases (PTPs), including PTP1B. This study was undertaken to determine whether the thiol-sensitive PTP1B is affected by ROS in SSc dermal fibroblasts, thereby enhancing the phosphorylation of PDGFR and synthesis of type I collagen. This study also sought to investigate the effect of a thiol antioxidant, *N*-acetylcysteine (NAC), in SSc.**

Methods. **Fibroblasts were isolated from the skin of patients with diffuse SSc and normal healthy donors for cell culture experiments and immunofluorescence analyses. A phosphate release assay was used to determine the activity of PTP1B.**

Results. **Levels of ROS and type I collagen were significantly higher and amounts of free thiol were significantly lower in SSc fibroblasts compared to normal fibroblasts. After stimulation with PDGF, not only were PDGFR and ERK-1/2 phosphorylated to a greater extent, but also the ability to produce PTP1B was hampered in SSc fibroblasts. The activity of PTP1B was significantly inactivated in SSc fibroblasts as a result of cysteine oxidation by the raised levels of ROS, which was confirmed by the oxidation of multiple PTPs, including PTP1B, in SSc fibroblasts. Decreased expression of PTP1B in normal fibroblasts led to increased expression of type I collagen. Treatment of the cells with NAC restored the activity of PTP1B, improved the profile of PDGFR phosphorylation, decreased the numbers of tyrosine-phosphorylated proteins and levels of type I collagen, and scavenged ROS in SSc fibroblasts.**

Conclusion. **This study describes a new mechanism by which ROS may promote a profibrotic phenotype in SSc fibroblasts through the oxidative inactivation of PTP1B, leading to pronounced activation of PDGFR. The study also presents a novel molecular mechanism by which NAC may act on ROS and PTP1B to provide therapeutic benefit in SSc.**

Systemic sclerosis (SSc; scleroderma) is characterized by Raynaud's phenomenon, proliferative vascular lesions, and fibrosis of the skin and internal organs. The exact mechanism of tissue scarring is still unknown. However, the abnormal phenotype has been suggested to be due to excessive production of extracellular matrix components, such as type I collagen, by fibroblasts (1). These cells proliferate rapidly and stain positive for α-smooth muscle actin, and therefore possess a myofibroblast phenotype (2).

Supported by the NIH (grant AR-48267 to Dr. Koch and Center for Translational Science Activities grant UL1-RR-024986), the Office of Research and Development, Medical Research Service, Department of Veterans Affairs, the Frederick G. L. Huetwell and William D. Robinson, MD, Professorship in Rheumatology (to Dr. Koch, University of Michigan), the Scleroderma Research Foundation, the University of Michigan Clinical Research Unit, the American College of Rheumatology Research and Education Foundation (Academic Reentry Award to Dr. Phillips), the Linda Dolce Scleroderma Research Fund, and the Marvin and Betty Danto and the Jonathan and Lisa Rye Endowments for Scleroderma Research at the University of Michigan.

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Submitted for publication March 25, 2011; accepted in revised form December 6, 2011.

Previous results have suggested that platelet-derived growth factor (PDGF) and its receptor, PDGFR, are key elements in fibrosis. Increased PDGF activity has been observed in plasma-derived serum from patients with SSc (3). Immunohistologic studies of skin biopsy samples have revealed that the expression of PDGF and PDGFR is increased in the skin of patients with SSc (4,5). Cytokines, such as transforming growth factor β (TGFβ) and interleukin-1α, stimulate the expression of PDGFR and the production of PDGF in SSc fibroblasts, and appear to be critical for promoting fibrosis in SSc fibroblasts (6,7). PDGF stimulates the Ras–ERK-1/2–reactive oxygen species (ROS) signaling pathway, which results in stimulation of genes encoding human collagen (COL) (8). All of these studies provide evidence of the importance of PDGFR signaling in the development of skin lesions in SSc.

ROS, such as superoxide ($O_2^{\cdot -}$), act as messengers to maintain cellular functions through redox signaling (9). However, when the production of ROS exceeds the cellular antioxidant capacity and disturbs the balance of the normal redox state, this can lead to oxidative damage of proteins, lipids, and DNA. Increased oxidative damage by ROS has been suggested to be a pathogenetic mechanism of SSc (8,10–14). The imbalance of the redox state can cause endothelial dysfunction and injury (15), fibroblast proliferation (16), increased type I collagen synthesis (11), and skewing of autoantibody production toward a Th2 profile (17); all of these features are major characteristics of SSc. Free radical generators, such as bleomycin, can result in a skin fibrosis similar to that in SSc (18), and, in fact, induction of fibrosis through the injection of bleomycin into mouse skin serves as an animal model of SSc (19). Recently, an animal model of SSc was established by injecting different forms of ROS subcutaneously (20). *N*-acetylcysteine (NAC), a thiol antioxidant, decreases the production of $O_2^{\cdot -}$ in vitro (11), and shows promising results in alleviating the symptoms of SSc (14,21–23). NAC is also used as a treatment for idiopathic pulmonary fibrosis (24). These studies imply that there may be a thiol-oxidation mechanism involved in the development of oxidative stress in SSc, since NAC not only acts as a free radical scavenger (25), but also acts specifically on protein thiols to support glutathione synthesis and generate free sulfhydryl groups (27).

The protein tyrosine phosphatase (PTP) family comprises a group of PTPs that act as critical regulators of a variety of cellular signaling pathways, including the PDGFR pathway. These PTPs act by dephosphorylating the activation signals elicited by protein tyrosine kinases. PTPs are characterized by their signature motif, $HC(X)_5R$, which contains a cysteine (Cys) residue that is essential for their catalytic activity. The low pKa of this Cys residue allows it to function as a nucleophile, but also makes it susceptible to oxidation. Several studies have shown that the Cys residue in various PTPs is oxidized in the presence of ROS (28,29). These observations imply that the increased levels of ROS in scleroderma skin lesions can oxidize PTPs that are involved in the PDGFR signaling pathway.

In this study, we examined the effect of excessive oxidative stress on PTP1B, a phosphatase that has been shown to dephosphorylate the PDGFR (30,31). The effect of the thiol antioxidant NAC on cellular ROS production, PDGFR phosphorylation, tyrosine-phosphorylated proteins, and PTP1B activity was also investigated.

MATERIALS AND METHODS

Cell culture. Both normal and SSc dermal fibroblasts (32) were kindly provided by Dr. Sergio Jimenez (Thomas Jefferson University, Philadelphia, PA). SSc cells were obtained from the forearm of patients with diffuse SSc (ages 32–57 years). All fibroblasts were maintained in RPMI medium containing 10% fetal bovine serum (FBS) with penicillin and streptomycin. The cells were switched to RPMI medium containing 0.1% FBS and incubated for 24 hours, before being stimulated with PDGF-BB (30 ng/ml; R&D Systems). Cells at passages 6–12 were used.

Superoxide detection. Two days before immunostaining analyses were performed, normal and SSc dermal fibroblasts were switched to RPMI medium containing 0.1% FBS, to avoid the effect of FBS on $O_2^{\cdot -}$ production. In some experiments, 20 m*M* NAC (Sigma-Aldrich) was added to the cell culture medium. Cellular $O_2^{\cdot -}$ was measured in the cells using dihydroethidium, while the nuclei were stained with DAPI (both from Invitrogen). Fluorescence was detected using an Olympus FV-500 confocal microscope, and photographs were taken at a magnification of $\times$400.

Extraction of messenger RNA (mRNA) and quantitative reverse transcription–polymerase chain reaction (qRT-PCR). Total RNA was isolated using RNeasy Mini isolation kits (Qiagen). Complementary DNA (cDNA) was prepared using Verso cDNA synthesis kits (Thermo Scientific). Quantitative PCR was performed using Platinum qPCR SYBR Green Supermix-UDG (Invitrogen), with specific primers for human PTP1B, COL1, and β-actin. All samples were run in duplicate using a Mastercycler ep realplex thermal cycler (Eppendorf), with results analyzed using Eppendorf software.

Detection of PDGFR phosphorylation. Both normal and SSc dermal fibroblasts were incubated with or without 20 m*M* NAC overnight, and then stimulated with PDGF. Cell lysates were obtained, and an equal amount of lysate proteins was incubated overnight at 4°C with immobilized mouse anti-human monoclonal antibodies to phosphorylated tyrosine (Cell Signaling Technology). Rabbit anti-human antibodies to

PDGFRβ (Cell Signaling Technology) were used to probe for phosphorylated PDGFR after the cells had been subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The immunoprecipitated tyrosine-phosphorylated proteins were detected using the mouse anti-human phosphorylated tyrosine antibody.

Immunofluorescence. For immunofluorescence analyses, the cells were grown in 8-well chambers and then fixed and blocked, before being probed with mouse anti-human type I collagen monoclonal antibodies (Abcam). The cells were subsequently incubated with Alexa Fluor–conjugated donkey anti-mouse antibodies (Invitrogen). The nuclei were stained using DAPI.

Western blotting and immunoprecipitation. Equal amounts of cell lysate were loaded onto polyacrylamide gels and separated by SDS-PAGE. The proteins were then transferred onto nitrocellulose membranes via Western blotting. The blots were probed with either PTP1B (Abcam), phosphorylated or total ERK-1/2 (both from Cell Signaling Technologies), oxidized PTPs (R&D Systems), or β-actin (Sigma-Aldrich). The blots were scanned and densitometric analysis was carried out using Un-Scan-It software (Silk Scientific). Immunoprecipitation was performed by incubating equal amounts of protein from normal and SSc dermal fibroblasts with a human anti-rabbit PTP1B antibody (20 μg/mg protein; Novus Biologicals). Rabbit IgG (Thermo Scientific) was incorporated as a negative control. The proteins captured by the Protein A/G beads were eluted and subjected to SDS-PAGE and Western blotting. The blot was then probed with a mouse anti-human oxidized PTP antibody and rabbit anti-human PTP1B antibody.

PTP1B activity assay. The PTP1B activity assay was carried out using a PTP1B DuoSet IC kit (R&D Systems). Mouse anti-human PTP1B antibodies that capture both active and inactive PTP1B were immobilized. After washing away unbound proteins, a synthetic phosphopeptide substrate that was dephosphorylated by active phosphatases to generate free phosphate and unphosphorylated peptide was added. The free phosphate was detected by a sensitive dye-binding assay, using malachite green and molybdic acid. By calculating the rate of phosphate release, the activity of the phosphatase was determined.

PTP1B-knockdown studies. Normal dermal fibroblasts were plated in 6-well plates and allowed to grow to ~60% confluence. The cells were then transfected with either 50 n*M* control small interfering RNA (siRNA) (Invitrogen) or 50 n*M* PTP1B siRNA (Santa Cruz Biotechnology) for 72 hours in RPMI medium containing 10% FBS. Both PTP1B and COL1 levels were then quantified by qPCR.

Analysis of free thiol content. Free sulfhydryl groups on proteins were determined using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Briefly, the cell lysates were mixed with Tris-EDTA buffer (0.25*M* Tris base and 20 m*M* EDTA; pH 8.2). The absorbance of the mixture was measured at 412 nm. DTNB in methanol was then added, followed by incubation for 15 minutes, and the absorbance was again measured at 412 nm. After subtracting the absorbance obtained before adding DTNB, the net absorbance was used to calculate the thiol content in the samples using a molar extinction coefficient of 13,600*M*/cm at 412 nm, which was normalized to the level of total cellular protein, with results expressed as μM/mg protein.

Statistical analysis. Results are expressed as the mean ± SEM. The significance of differences between groups was determined by Student's *t*-test. *P* values less than 0.05, in 2-tailed analyses, were considered significant.

RESULTS

Superoxide production in dermal fibroblasts. As shown in Figure 1, our results indicated that $O_2^{\cdot -}$ production was significantly higher in SSc dermal fibroblasts compared to normal cells. In the presence of NAC, a thiol antioxidant, the level of $O_2^{\cdot -}$ was significantly reduced in SSc dermal fibroblasts.

Thiol content in normal and SSc dermal fibroblasts. In normal dermal fibroblasts, the mean ± SEM total free thiol content was 464 ± 18 μM/mg protein, while in SSc dermal fibroblasts, it was significantly lower (344 ± 15 μM/mg protein) ($P < 0.01$; n = 3 per group). These results suggest that a portion of the protein thiols in SSc dermal fibroblasts was modified into oxidation products that do not react with DTNB. These findings also confirm the observed pattern of $O_2^{\cdot -}$ staining in the cells (Figure 1) and suggest that there is increased oxidative stress in SSc dermal fibroblasts compared to normal cells.

Phosphorylation of PDGFR after PDGF stimulation. In normal dermal fibroblasts, PDGF-stimulated phosphorylation of PDGFR reached maximum levels after 10 minutes of stimulation ($P < 0.05$ versus unstimulated cells) and returned to basal levels at 1 hour, as shown in Figure 2A. In contrast, in SSc dermal fibroblasts, PDGFR was maximally phosphorylated after 10 minutes of PDGF stimulation but remained phosphorylated after 1 hour of stimulation ($P < 0.05$ versus unstimulated cells at both time points). Within 30 minutes of stimulation, the extent of phosphorylation of PDGFR in SSc dermal fibroblasts was significantly higher than that in normal cells ($P < 0.05$). These results suggest that phosphatases that dephosphorylate the PDGFR, such as PTP1B, might be deficient or inactivated in SSc dermal fibroblasts.

To determine whether oxidative stress plays a role in the prolonged PDGFR phosphorylation seen in SSc dermal fibroblasts, NAC was added to the cell culture medium and tyrosine-phosphorylated proteins were immunoprecipitated. As shown in Figure 2B, in the presence of NAC, phosphorylation of the PDGFR occurred after 10 minutes of PDGF stimulation and continued up to 30 minutes in normal dermal fibroblasts. A similar pattern was observed in SSc dermal fibroblasts, although lower levels of phosphorylation were observed

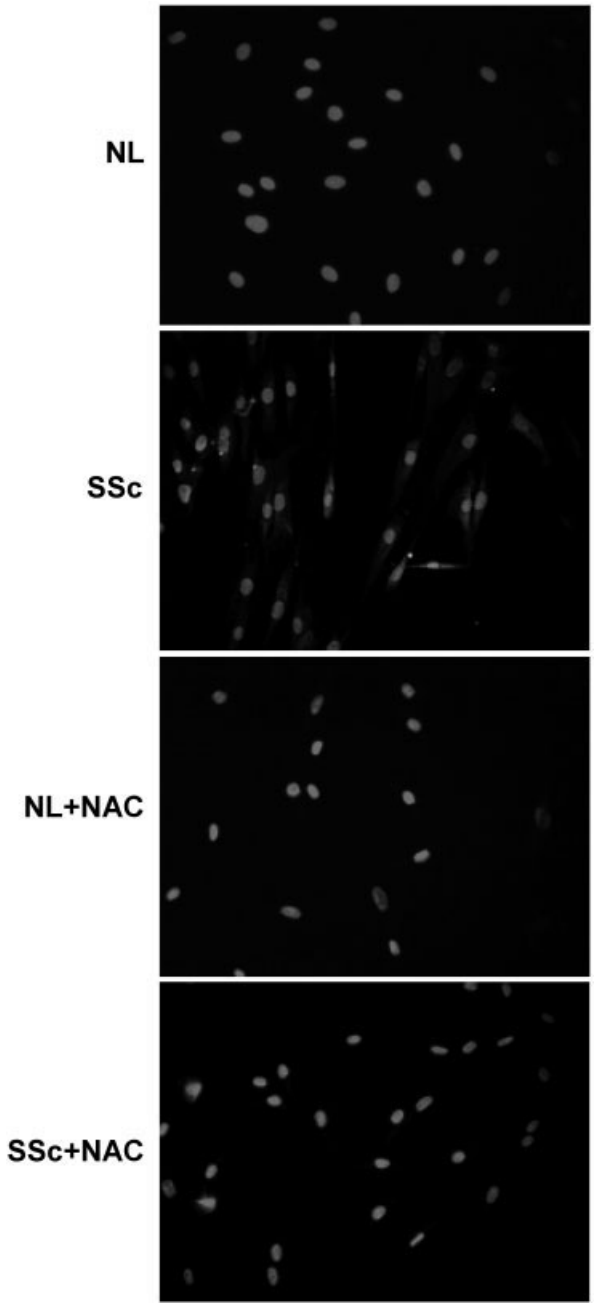

Figure 1. Production of superoxide ($O_2^{\cdot -}$) in normal (NL) and systemic sclerosis (SSc) dermal fibroblasts, as detected by fluorescence microscopy. Normal dermal fibroblasts and SSc dermal fibroblasts were treated with dihydroethidium, an $O_2^{\cdot -}$ trap that releases red fluorescence when it encounters $O_2^{\cdot -}$. Nuclei were counterstained with DAPI. Staining was detected using a fluorescence microscope and images were obtained at an original magnification of ×400. Before treatment with *N*-acetylcysteine (NAC), a significant amount of $O_2^{\cdot -}$ was detected in SSc fibroblasts compared to normal fibroblasts. In the presence of NAC, $O_2^{\cdot -}$ production was decreased in SSc fibroblasts, whereas treatment with NAC had no effect on normal fibroblasts. Representative images of cells from 1 of 3 normal subjects and 1 of 3 SSc patients are shown.

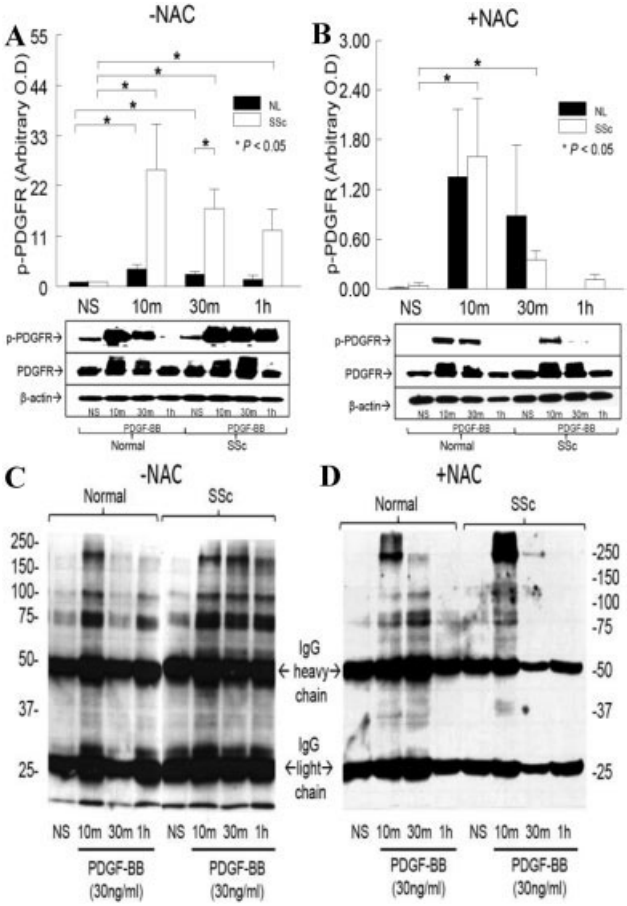

Figure 2. Platelet-derived growth factor (PDGF) stimulation of phosphorylation of the PDGF receptor (PDGFR) and other proteins in normal (NL) and systemic sclerosis (SSc) dermal fibroblasts. **A** and **B,** Stimulation of PDGFR phosphorylation in the absence or presence of *N*-acetylcysteine (NAC). In the absence of NAC (**A**), PDGF significantly stimulated PDGFR phosphorylation, when compared to basal (not-stimulated [NS]) levels, in normal cells after 10 minutes and 30 minutes, while in SSc fibroblasts, PDGFR phosphorylation increased significantly from basal levels up to 1 hour. At 30 minutes, the level of PDGFR phosphorylation in SSc dermal fibroblasts was significantly higher than that in normal cells. In the presence of NAC (**B**), PDGFR phosphorylation was observed after 10 minutes and 30 minutes of stimulation in both normal and SSc dermal fibroblasts. PDGF stimulated significant levels of PDGFR phosphorylation, when compared to basal levels, in SSc dermal fibroblasts at 10 and 30 minutes. The intensity of PDGFR phosphorylation was significantly lower in the presence of NAC in both normal and SSc dermal fibroblasts. Results in **A** and **B** are the mean ± SEM arbitrary units of optical density (OD) in 3 samples per group, with detection by Western blotting using β-actin as a positive control. **C** and **D,** Phosphorylation of multiple proteins in the absence or presence of NAC, as detected by Western blotting. In the absence of NAC (**C**), multiple proteins were phosphorylated in both normal and SSc dermal fibroblasts after stimulation with PDGF for up to 1 hour, and more tyrosine-phosphorylated proteins were induced in SSc cells compared to normal cells. In the presence of NAC (**D**), PDGF stimulated protein tyrosine phosphorylation in both normal and SSc dermal fibroblasts, but with less intensity compared to that in the absence of NAC.

in SSc fibroblasts compared to normal cells after 30 minutes of stimulation.

We found that the addition of NAC significantly decreased the intensity of PDGFR phosphorylation in both normal and SSc dermal fibroblasts. When we compared the time course of PDGFR phosphorylation in the presence and absence of NAC (Figures 2A and B), we found that activation of the PDGFR occurred through dimerization after PDGF stimulation of the cells for a period of 10 minutes up to 30 minutes.

Protein tyrosine phosphorylation after PDGF stimulation. To examine the effect of NAC on other tyrosine-phosphorylated proteins, the immunoprecipitated proteins (as shown in Figures 2A and B) were subjected to Western blotting and probed with mouse anti-human protein tyrosine phosphorylation antibodies. In these blots, the 25-kd and 50-kd bands represent the light and heavy chains of IgG, respectively.

The expression patterns of the tyrosine-phosphorylated proteins in the absence of NAC (Figure 2C) showed that PDGF induced phosphorylation of multiple proteins in both normal and SSc dermal fibroblasts. Most of these phosphorylated proteins were still visible at 1 hour after stimulation with PDGF. Moreover, more proteins were phosphorylated in SSc dermal fibroblasts compared to normal cells. In the presence of NAC (Figure 2D), fewer proteins were phosphorylated by PDGF in both normal and SSc dermal fibroblasts, consistent with the results observed for phosphorylation of the PDGFR.

These results suggest that in addition to the PDGFR pathway, many more proteins are phosphorylated in SSc dermal fibroblasts, and NAC affects not only the PDGFR, but also many other proteins. Since NAC decreases the production of cellular ROS, these findings indicate that the prolonged phosphorylation of PDGFR in SSc dermal fibroblasts might be attributed to oxidative inactivation of multiple thiol-sensitive phosphatases that dephosphorylate the PDGFR. Since PTPs tend to have multiple substrates, we hypothesized that the increased number of phosphorylated proteins observed might be attributed to the inactivation of a certain PTP.

Phosphorylation of ERK-1/2 after PDGF stimulation. When normal dermal fibroblasts were stimulated with PDGF, ERK-1/2 phosphorylation reached maximum levels at 45 minutes of stimulation, and returned to baseline levels at 4 hours (Figure 3A). In contrast, in SSc dermal fibroblasts, significant phosphorylation of ERK-1/2 was observed after 10 minutes of PDGF stimulation, and persisted up to 4 hours.

The extent of ERK-1/2 phosphorylation was significantly different between normal and SSc dermal fibroblasts ($P < 0.05$) after 4 hours of stimulation of the cells with PDGF. These results are consistent with the observed patterns of PDGFR phosphorylation (Figure 2A), in that the enhanced and prolonged phosphorylation of PDGFR led to enhanced and prolonged activation of its downstream signaling pathway, ERK-1/2, in SSc dermal fibroblasts, while in normal cells, the profile of PDGFR phosphorylation paralleled that of ERK-1/2 phosphorylation, with both showing a shorter time course and less pronounced phosphorylation compared with that in SSc cells.

Although the basal level of phosphorylated ERK-1/2 was increased in both normal and SSc dermal fibroblasts ($P > 0.05$) (Figure 3A), incubation of the cells in the presence of NAC did not result in further significant ERK-1/2 phosphorylation in either normal or SSc fibroblasts ($P > 0.05$) (Figure 3B). The slight increase in the basal level of phosphorylated ERK-1/2 in the presence of NAC might be due to the effects of NAC on negative regulators of the MAPK pathway. These results also suggest that the beneficial effect of NAC on type I collagen production might be due, in part, to some other factors, such as an effect on the expression of matrix metalloproteinases. Taken together, these results show that the PDGFR and its downstream signaling pathway were activated to a greater extent in SSc dermal fibroblasts, and NAC, a thiol antioxidant, could alter the extent of PDGF stimulation in both normal and SSc dermal fibroblasts.

Expression of type I collagen in normal and SSc dermal fibroblasts. At baseline and after 10 minutes of PDGF stimulation, COL1 mRNA levels were significantly higher in SSc dermal fibroblasts compared to normal dermal fibroblasts ($P < 0.05$) (Figure 3C). After 4 hours of PDGF stimulation, COL1 mRNA levels were significantly decreased in SSc dermal fibroblasts when compared to the levels in unstimulated SSc cells. Treatment with NAC significantly decreased the COL1 mRNA levels in SSc dermal fibroblasts at several time points after incubation of the cells with PDGF, whereas NAC had no significant effect on normal cells. At the protein level, there was more staining for type I collagen in SSc dermal fibroblasts compared to normal cells (Figure 3D). In the presence of NAC, the expression of type I collagen in SSc dermal fibroblasts decreased to levels similar to those observed in normal cells.

These results characterize the final phase of the response following PDGFR activation in SSc dermal fibroblasts. In summary, enhanced PDGFR activation in

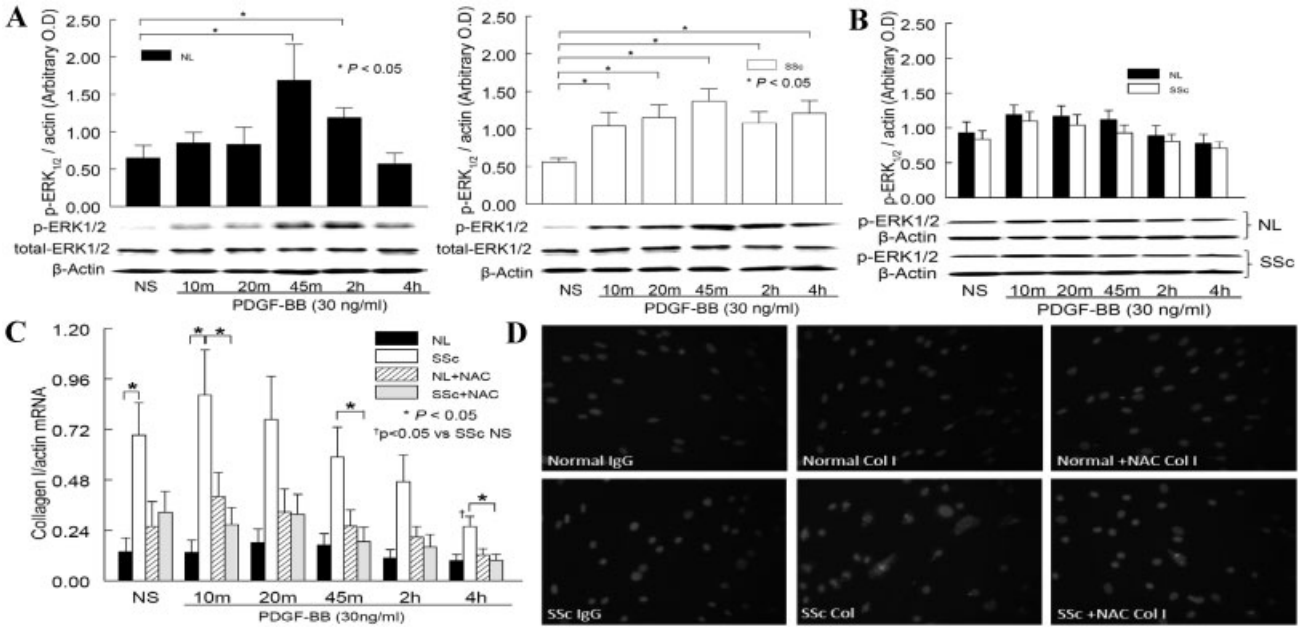

Figure 3. ERK-1/2 phosphorylation patterns and type I collagen production in normal (NL) and systemic sclerosis (SSc) dermal fibroblasts. **A** and **B,** Platelet-derived growth factor (PDGF) stimulation of ERK-1/2 phosphorylation in the absence or presence of *N*-acetylcysteine (NAC). In the absence of NAC **(A)**, PDGF stimulated significant ERK-1/2 phosphorylation, when compared to basal (not-stimulated [NS]) levels, in normal cells after 45 minutes and 2 hours. In SSc fibroblasts, ERK-1/2 phosphorylation increased significantly from 10 minutes up to 4 hours. The level of ERK-1/2 phosphorylation in SSc dermal fibroblasts was significantly higher than that in normal cells after 4 hours of stimulation. In the presence of NAC **(B)**, although the basal level of ERK-1/2 phosphorylation was increased slightly in both normal and SSc cells, PDGF stimulation did not result in further significant ERK-1/2 phosphorylation. Results in **A** and **B** are the mean ± SEM arbitrary units of optical density (OD) in 3 samples per group, with detection by Western blotting using β-actin as a positive control. **C** and **D,** Expression of mRNA and protein for type I collagen. In the absence of NAC, mRNA levels of the gene encoding type I collagen **(C)** were significantly higher in SSc fibroblasts compared to normal fibroblasts. Treatment with NAC significantly decreased the mRNA levels in SSc cells, but had no significant effect on normal dermal fibroblasts. Similarly, in the absence of NAC, a significant amount of type I collagen (Col I) protein **(D)** was detected in SSc fibroblasts compared to normal fibroblasts, and treatment with NAC led to decreased protein expression in SSc fibroblasts. Bars in **C** show the mean ± SEM mRNA expression, relative to β-actin, in 3 samples per group. In **D,** representative images of cells from 1 of 3 normal subjects and 1 of 3 SSc patients are shown (original magnification × 400); rabbit IgG was used as a negative control.

SSc dermal fibroblasts leads to increased ERK-1/2 phosphorylation, which thereby induces more synthesis of type I collagen. Treatment with NAC, which acts by scavenging ROS and deactivating the PDGFR pathway, results in a decrease in the synthesis of type I collagen in SSc dermal fibroblasts.

Expression of PTP1B in normal and SSc dermal fibroblasts. In the presence of PDGF, the levels of PTP1B mRNA in normal dermal fibroblasts were slightly increased after 2 hours and 4 hours of stimulation, while the expression of PTP1B mRNA in SSc dermal fibroblasts remained the same, as shown in Figure 4A. When normal dermal fibroblasts were stimulated with PDGF, the expression of PTP1B protein increased gradually and was maximal after 45 minutes of stimulation, and remained elevated after 2 hours of stimulation ($P < 0.05$ versus unstimulated cells at both time points), whereas after 4 hours of stimulation, there was no difference in PTP1B protein expression between stimulated and unstimulated normal dermal fibroblasts (Figure 4B). In contrast, similar to the results with regard to mRNA expression (Figure 4A), when SSc dermal fibroblasts were stimulated with PDGF for up to 4 hours, the expression of PTP1B protein remained the same (Figure 4B).

In the basal state, there was no difference in PTP1B protein expression between normal and SSc dermal fibroblasts (mean ± SEM optical density 0.46 ± 0.10 in unstimulated normal dermal fibroblasts versus 0.49 ± 0.14 in unstimulated SSc dermal fibroblasts) (Figure 4B). After 45 minutes of stimulation, the level of PTP1B protein in SSc dermal fibroblasts was significantly lower than that in normal cells ($P < 0.05$). Therefore, based on these results, it appears that in an

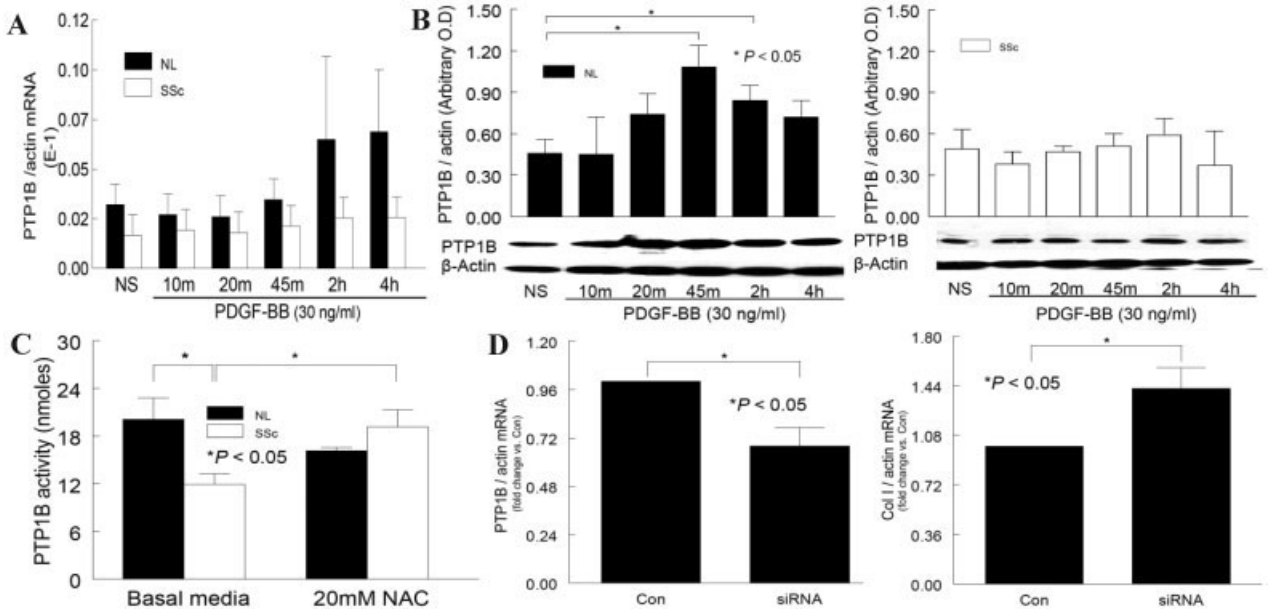

Figure 4. Expression and enzymatic activity of protein tyrosine phosphatase 1B (PTP1B) in normal (NL) and systemic sclerosis (SSc) dermal fibroblasts. **A** and **B,** Platelet-derived growth factor (PDGF) stimulation of PTP1B mRNA and protein expression. The expression of PTP1B mRNA **(A)** was not significantly different in normal and SSc dermal fibroblasts at basal (not-stimulated [NS]) levels or up to 4 hours after stimulation with PDGF. At the protein level **(B)**, PDGF-stimulated PTP1B production was significantly higher, when compared to basal levels, after 45 minutes and 2 hours of stimulation in normal cells. However, in SSc cells, incubation of the cells with PDGF did not alter the expression of PTP1B protein. At 45 minutes of stimulation with PDGF, there was a significant difference in PTP1B protein expression between normal and SSc cells ($P < 0.05$). Results in **A** are the mean ± SEM mRNA expression relative to β-actin and results in **B** are the mean ± SEM arbitrary units of optical density (OD) in 4 samples per group, along with Western blots using β-actin as a positive control. **C,** PTP1B activity in the absence (basal media) or presence of *N*-acetylcysteine (NAC). In medium without NAC, the PTP1B activity was significantly lower in SSc dermal fibroblasts compared to normal dermal fibroblasts. In the presence of NAC, the PTP1B activity was restored in SSc cells. **D,** Effects of small interfering RNA (siRNA) knockdown of PTP1B in normal dermal fibroblasts. When PTP1B mRNA expression was significantly knocked down by the siRNA (left), this was paralleled by a significant increase in the expression of type I collagen (Col I) mRNA (right) in normal dermal fibroblasts, mimicking the findings in SSc dermal fibroblasts. Bars in **C** and **D** show the mean ± SEM of 4 samples per group. Con = control.

unstimulated state, the same amount of PTP1B is present in normal dermal fibroblasts and SSc dermal fibroblasts. However, when the cells are stimulated with PDGF, the level of PTP1B increases in normal cells, but not in SSc dermal fibroblasts.

Enzyme activity of PTP1B in normal and SSc dermal fibroblasts. PTP1B activity was significantly lower in SSc dermal fibroblasts compared to normal cells (mean ± SEM 11.9 ± 1.3 nmoles versus 20.1 ± 2.7 nmoles; $P < 0.05$) (Figure 4C). In contrast, in the presence of the antioxidant NAC, PTP1B activity was restored in SSc dermal fibroblasts (mean ± SEM 19.2 ± 2.1 nmoles), compared to a level of 16.2 ± 0.4 nmoles in normal dermal fibroblasts in the presence of NAC. Therefore, these findings indicate that when excess $O_2^{\cdot -}$ is present in SSc dermal fibroblasts, the activity of PTP1B is inactivated, whereas when $O_2^{\cdot -}$ is eliminated by NAC, the activity of PTP1B is restored. These results imply that the increased oxidative stress in SSc dermal fibroblasts inactivates PTP1B, resulting in increased PDGFR phosphorylation, ERK-1/2 activation, and increased type I collagen synthesis. The presence of NAC not only eliminates $O_2^{\cdot -}$ in these cells, but also restores PTP1B activity, thus decreasing the extent of PDGFR phosphorylation and its downstream events.

PTP1B expression and type I collagen production. To elucidate the direct involvement of PTP1B in type I collagen production, we knocked down PTP1B expression in normal dermal fibroblasts and assessed the expression of COL1 mRNA. As shown in Figure 4D, PTP1B mRNA expression was significantly knocked down by siRNA treatment ($P < 0.05$ versus control). Under this condition, COL1 mRNA expression in normal dermal fibroblasts increased by ~40% ($P < 0.05$ versus control). These results suggest that PTP1B, possibly through the PDGFR pathway, controls type I

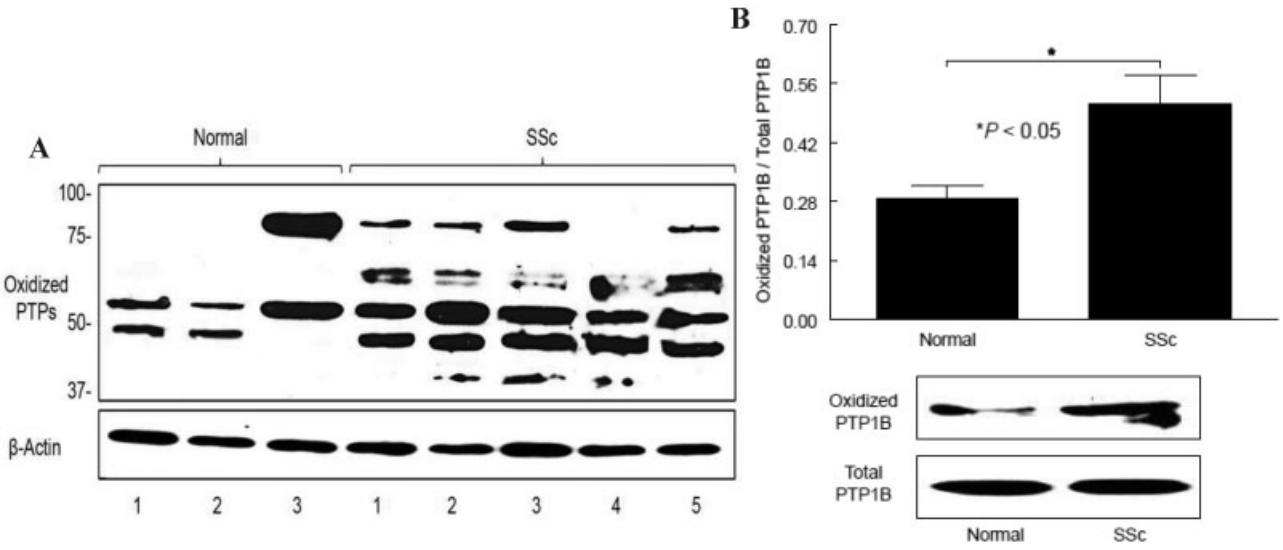

Figure 5. Oxidation of protein tyrosine phosphatases (PTPs) in normal and systemic sclerosis (SSc) dermal fibroblasts. Oxidized PTPs in normal and SSc dermal fibroblasts were visualized on Western blots using mouse anti–oxidized human PTP antibodies. Equal amounts of protein were subjected to immunoprecipitation using rabbit anti-human PTP1B antibodies. The blot was then probed with mouse anti–oxidized human PTP antibodies. **A,** Overall, more PTPs were oxidized in SSc dermal fibroblasts (n = 5) compared to normal dermal fibroblasts (n = 3). In normal cells, 2 bands were observed in all subjects, although at different molecular weights. In SSc dermal fibroblasts, at least 3 PTPs were oxidized. The 50-kd protein, which is postulated to be PTP1B, was oxidized in all SSc dermal fibroblasts. β-actin was used as a positive control. **B,** Results of immunoprecipitation confirmed that the 50-kd protein was PTP1B. There was significantly more oxidized PTP1B in SSc dermal fibroblasts compared to normal dermal fibroblasts. Bars show the mean ± SEM of 3 samples per group.

collagen production in dermal fibroblasts. This further supports the notion that PTP1B plays a significant role in skin fibrosis.

Oxidation of PTPs in normal and SSc dermal fibroblasts. To examine whether PTPs are oxidized in dermal fibroblasts, oxidized PTPs were probed using antibodies that recognize the oxidized Cys residue at their active site. The oxidized PTP profiles in 3 normal healthy subjects and 5 patients with SSc are shown in Figure 5A. In the dermal fibroblasts from the 3 normal subjects, 2 protein bands were observed, although in the fibroblasts from subject 3, a stronger signal intensity was observed and one of the bands was located at a higher molecular weight than that in fibroblasts from the other 2 subjects. In SSc dermal fibroblasts, at least 3 PTPs were oxidized, all of which had stronger signal intensities compared to those in normal dermal cells.

The profile of PTP expression appeared slightly different in each SSc patient, although oxidation was consistent for the PTP at the 50-kd band. This latter band could represent PTP1B. We then probed PTP1B in the cell lysates with the same antibody as used in Western blotting. The results showed that ~2-fold more oxidized PTP1B was present in SSc dermal fibroblasts compared to normal dermal fibroblasts ($P < 0.05$; n = 3) (Figure 5B). These results suggest that the inactivation of PTP1B can be attributed to oxidation of its active site.

DISCUSSION

In this study, we provide evidence that PTP1B is inactivated by ROS in SSc dermal fibroblasts, leading to prolonged phosphorylation of the PDGFR. As summarized in Figure 6, we have shown that the levels of ROS are elevated in SSc dermal fibroblasts compared to normal cells. The increased oxidative stress leads to a significant decrease in the total protein thiol groups in SSc dermal fibroblasts, as well as oxidation at the Cys-active site of PTP1B. Oxidation of PTPs leads to inactivation of their phosphatase activity, resulting in a profile of prolonged phosphorylation of PDGFR and ERK-1/2 as well as increased type I collagen expression in SSc dermal fibroblasts. In addition, we have demonstrated a direct relationship between PTP1B expression and COL1 synthesis, using PTP1B-knockdown experiments in normal dermal fibroblasts. Our findings with regard to the inclusion of NAC, a thiol antioxidant, in the cell cultures reconfirm the impact of oxidative stress on PTP1B, as lower PTP1B activity was observed to be present in conjunction with high levels of $O_2^{\cdot -}$ in SSc

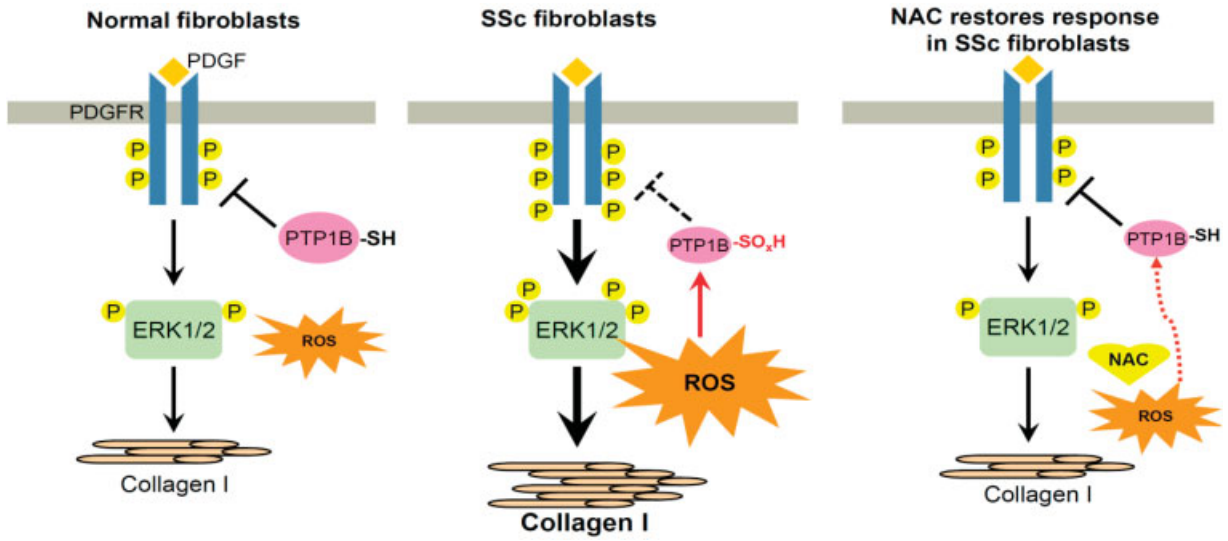

Figure 6. Summary of the mechanisms of protein tyrosine phosphatase 1B (PTP1B) inactivation by oxidative stress in systemic sclerosis (SSc) dermal fibroblasts. In normal dermal fibroblasts, adequate amounts of reactive oxygen species (ROS) perform their role in redox signaling and are not harmful in oxidizing cellular proteins. When platelet-derived growth factor (PDGF) binds to its receptor, PDGFR, the receptor is phosphorylated and downstream signaling pathways, such as ERK-1/2, are activated, leading to a response, exemplified by type I collagen production. PTP1B is active and controls the extent of phosphorylation on the PDGFR. In SSc dermal fibroblasts, the increased production of ROS inactivates PTP1B through oxidation of the cysteine residue in its active site. When PDGF binds to its receptor, the prolonged phosphorylation of the PDGFR due to the inactivation of PTP1B results in an increased downstream response. In SSc fibroblasts in the presence of the thiol antioxidant *N*-acetylcysteine (NAC), levels of ROS decrease, the oxidized cysteine residue on PTP1B is reduced, the PTP1B activity is restored, and the response pattern returns to that observed at baseline. The **red solid arrow** indicates oxidation, the **broken arrow** indicates reduced oxidation, the **solid crossbar** indicates dephosphorylation, and the **broken crossbar** indicates inability to dephosphorylate. P = tyrosine phosphorylation; SH = free thiols; SO_xH = oxidized thiols.

dermal fibroblasts, and when the $O_2^{\cdot -}$ was eliminated by NAC, not only was the PTP1B activity restored, but also the levels of PDGFR and its downstream events, including type I collagen expression, were similar to those found in normal cells. Our results suggest a new mechanism by which oxidative stress promotes a profibrotic phenotype in SSc dermal fibroblasts through oxidative inactivation of phosphatases, leading to pronounced PDGFR activation.

Our findings of increased ROS production in SSc dermal fibroblasts are consistent with those reported previously (8,11,12,14,33). The source of $O_2^{\cdot -}$ is believed to be NADPH oxidase. When SSc dermal fibroblasts were treated with diphenylene iodonium, which acts as an NADPH oxidase inhibitor, $O_2^{\cdot -}$ levels were diminished (11). In addition, increased expression of the NADPH oxidase subunits was observed in monocytes and dermal fibroblasts isolated from SSc patients (11,12). Because protein thiols are very sensitive to ROS and can be oxidized, we measured free thiols in the cells, as another marker of oxidative stress. We showed that the free thiol content in SSc dermal fibroblasts was significantly lower than that in normal cells, again suggesting the existence of oxidative stress in SSc. Significantly reduced levels of total plasma thiols in SSc patients compared with normal subjects have been demonstrated previously (34,35).

In this study we hypothesized that the prolonged phosphorylation of the PDGFR in SSc fibroblasts is a result of decreased expression of, or ROS inactivation of, PTPs. We cannot rule out the role of protein kinases in driving PDGFR phosphorylation, as studies have emphasized the importance of these enzymes in the pathogenesis of SSc (36). However, it appears that protein tyrosine kinases are not direct targets of ROS. In addition, protein tyrosine phosphorylation is controlled through the coordinated actions of tyrosine kinases and PTPs. These proteins not only have housekeeping functions, but also are highly regulated and play crucial roles in many cell functions and in different diseases. A recent study showed that the expression of protein phosphatase 2A was significantly lower in SSc fibroblasts (37). The

authors suggested that this led to constitutive activation of the TGFβ pathway and enhanced ERK-1/2 phosphorylation, which was also seen in the present study.

We examined the expression and function of PTP1B, which has been reported to be a negative regulator of receptor tyrosine kinases such as PDGFR (30,31). The association between PTP1B and PDGFR is further supported by a study using PTP1B-knockout mice (38). In addition to the PDGFR pathway, PTP1B appears to be a negative regulator in both insulin and leptin signaling pathways (39,40). Epidermal growth factor receptor (41) and tyrosine-phosphorylated proteins such as STAT-5 (42) have also been shown to be targets of PTP1B. Although the expression of PTP1B was similar in normal dermal fibroblasts and SSc dermal fibroblasts (Figure 4B), the activity of PTP1B was significantly lower in SSc fibroblasts, suggesting that only a portion of the expressed PTP1B is active in SSc fibroblasts.

We further showed that the inactivation of PTP1B is due to oxidation of its active site. Oxidation of PTPs is not rare. In fact, reversible and transient oxidation of PTPs is regarded as a redox regulatory mechanism for receptor tyrosine kinases to control their phosphorylation state (43). However, when a constant and large amount of ROS is present, prolonged inactivation of PTP activity may occur. Indeed, it has been shown that in human cancer cells, increased production of ROS can lead to production of sulfenic, sulfinic, and sulfonic acids at the active site of PTP1B, resulting in its inactivation (44). Similar to these findings, we showed that increased levels of ROS in SSc dermal fibroblasts not only inactivated PTP1B, but also oxidized at least 3 PTPs. The decreased antioxidant capacity in SSc (45) may also exacerbate these mechanisms of inactivation.

The reason for the increased expression of PTP1B in normal cells after PDGF stimulation requires further investigation. Since protein synthesis requires a prolonged time course, we postulate that the increase in PTP1B might be due to release of PTP1B from the pool of phosphorylated or sumoylated proteins, since the antibody that was used recognizes only free PTP1B. This might be a defense mechanism in normal cells to counteract the activation of the PDGFR. However, this mechanism appears to be lost in SSc dermal fibroblasts, as we found that the expression of PTP1B did not change after PDGF stimulation of SSc cells.

Since oxidative stress in SSc dermal fibroblasts was accompanied by a significant reduction in free thiol content, we hypothesize that NAC, a thiol antioxidant (25–27) that has shown beneficial effects in SSc (11,14,20–23), will benefit the cells by decreasing cellular ROS levels and replenishing free cellular thiols. Indeed, we showed that treatment of the cells with NAC not only decreased the cellular levels of $O_2^{\cdot -}$, but also restored PTP1B activity, which led to improvement in PDGFR phosphorylation and in the type I collagen expression profile. Our results are consistent with those in a previous study in which NAC prevented serum-induced PDGFR phosphorylation and its downstream signaling pathways by scavenging $O_2^{\cdot -}$ produced by NADPH oxidase (46). Another study showed that in cultured vascular smooth muscle cells, NAC reduced PDGFR phosphorylation and increased PTP activity (47).

PTP1B is not the only phosphatase that regulates the phosphorylation state of PDGFR. A number of thiol-sensitive phosphatases, such as SH2 domain–containing phosphatase 2 (SHP-2) (30) and density-enhanced phosphatase 1 (DEP-1) (48), have been shown to dephosphorylate the PDGFR. Preliminary results show that the expression of SHP-2 seems to differ in SSc fibroblasts compared to normal cells, when the cells are treated with PDGF. In addition, in cultures of unstimulated cells, the expression of DEP-1 appears to be higher in SSc fibroblasts compared with normal fibroblasts (results not shown). Whether the phosphatase activities of DEP-1 and SHP-2 are affected by ROS needs further investigation. If indeed DEP-1 and SHP-2 are inactivated, the antioxidative effect of NAC on PDGFR phosphorylation and protein tyrosine phosphorylation may therefore be attributed to restoration of the activities of a combination of phosphatases. Nonetheless, we showed that at least one phosphatase, PTP1B, is inactive when excessive $O_2^{\cdot -}$ is present in SSc dermal fibroblasts, and its activity is restored when NAC is present, with a simultaneous change in the PDGFR phosphorylation pattern.

In conclusion, our findings provide evidence that the increased oxidative stress in SSc dermal fibroblasts oxidizes PTP1B and renders it inactive, and thereby amplifies the PDGFR signaling pathway. We not only introduce a new mechanism for the role of oxidative stress in the pathogenesis of fibrosis in SSc, but also provide a novel molecular pathway by which NAC therapy may act on ROS and PTP1B to benefit SSc patients.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Koch had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Tsou, Piera-Velazquez, Jimenez, Seibold, Koch.
Acquisition of data. Tsou, Talia, Pinney, Kendzicky, Seibold.
Analysis and interpretation of data. Tsou, Pinney, Kendzicky, Phillips.

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