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TGF- β Signaling in Tissue Fibrosis: Redox Controls, Target Genes and Therapeutic Opportunities

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Abstract

During development of TGF- β 1-initiated fibroproliferative disorders, NADPH oxidases (NOX family members) generate reactive oxygen species (ROS) resulting in downstream transcription of a subset genes encoding matrix structural elements and profibrotic factors. Prominent among the repertoire of disease-implicated genes is the TGF- β 1 target gene encoding the potent profibrotic matricellular protein plasminogen activator inhibitor-1 (PAI-1 or SERPINE1). PAI-1 is the major physiologic inhibitor of the plasmin-based pericellular cascade and a causative factor in the development of vascular thrombotic and fibroproliferative disorders. ROS generation in response to TGF- β 1 stimulation is rapid and precedes PAI-1 induction; engagement of non-SMAD (e.g., EGFR, *Src* kinase, MAP kinases, p53) and SMAD2/3 pathways are both required for PAI-1 expression and are ROS-dependent. Recent findings suggest a novel role for p53 in TGF- β 1-induced PAI-1 transcription that involves ROS generation and p53/SMAD interactions. Targeting ROS and ROS-activated cellular events is likely to have therapeutic implications in the management of fibrotic disorders, particularly in the context of prolonged TGF- β signaling.

Keywords

TGF- β ; NOX; NADPH oxidases; PAI-1; ROS; p53; EGFR; SMADs

ROS and NOX Involvement in Tissue Fibrosis

Fibrosis is a frequent and progressive response to chronic injury, non-resolving inflammation or long-standing metabolic disease (e.g., diabetes, hypersension). Increased accumulation of extracellular matrix (ECM) components, produced largely by persistently activated interstitial (myo)fibroblasts coupled with defects in matrix turnover or clearance, ultimately disrupts normal tissue architecture culminating in eventual organ failure [1,2]. While etiology may vary, cellular stress due to ischemia/reperfusion injury or chronic

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exposure to fibrotic “initiators” (e.g., chemical toxins, elevated glucose levels) increases expression of ROS-generating enzymes (e.g., NADPH oxidases; NOX proteins) with concomitant reductions in ROS scavengers (e.g., glutathione peroxidase [Gpx], catalase, manganese/zinc superoxide dismutases [Mn/Zn SOD]) [3]. Increased oxidative state and associated downstream redox-dependent genomic reprogramming subsequently affects cellular growth and initiates an exuberant repair process [1]. In three organ systems (pulmonary, renal, cardiovascular), NOX isoforms and their constituent subunit complexes regulate stromal myofibroblast differentiation and fate. As such, NOX family members are effectors of normal as well as pathologic tissue repair [4–7] impacting expression controls on critical fibrogenic genes.

NOX proteins are membrane-localized multi-subunit enzymes that catalyze the reduction of oxygen using the electron donor NADPH. NOX-generated ROS impact various signaling pathways contributing to the pathophysiology of chronic wounds [3,8,9]. NOX expression is, in fact, up-regulated in several models of induced fibrosis [10–12]. The mechanism(s) of ROS generation, however, varies depending on the specific NOX repertoire expressed and/or functional in different cell types or organ systems and dictated by the initiating stimulus. The potent profibrogenic factor TGF- β 1, for example, activates NOX4 and mediates myofibroblast recruitment in the kidney and bleomycin-injured lung as well as in idiopathic pulmonary fibrosis [5,8,13–15]. Myofibroblast density, moreover, is suppressed by NOX4 silencing or NADPH inhibition [5], consistent with the reduced fibrotic burden in bleomycin-challenged NOX4-null mice [12,16], implicating a TGF- β 1→NOX4 pathway in development of lung disease. Kidney fibrosis in response to experimental ureteral obstruction (i.e., UUO), a largely TGF- β 1-driven disease and an excellent model of renal inflammation/interstitial fibrosis [17], is accompanied by increases in the NOX subunits p22phox, p47phox and p67phox and ROS induction [18,19]. Obstructive renal injury in a catalase-deficient genetic background, moreover, further enhances p22phox/p47phox levels, augments collagen deposition and accelerates fibrosis [19,20]. The disease relevance of this system is highlighted by the finding that transgenic catalase gene “rescue”, or treatment with apocynin and perindopril, reduced renal fibrosis and the associated hypertension in angiotensinogen-overexpressing mice [21–23].

NOX proteins 1, 2, 4 and 5 are also expressed in the vasculature although myeloperoxidase, xanthine oxidase, lipoxygenase, nitric oxidase synthetase and the mitochondrial respiratory system contribute to vascular ROS production as well [24–27]. NOX4/5 appear important in the etiology of vascular dysfunction, particularly in hypertensive and atherosclerotic disease, and are likely major factors in vessel inflammation/fibrosis [28]. Indeed, NOX1 targeting is atheroprotective [28,29]. TGF- β 1 stimulates vascular ROS generation by inducing NOX expression, increasing NOX activity, mobilizing the mitochondrial respiratory chain or by suppression of antioxidant systems while transgenic overexpression of TGF- β 1 in an Apo-E-deficient background increases ROS production in atherosclerotic arteries [30].

The TGF- β 1-Induced, Redox-Sensitive, PAI-1 Gene in Disease Causation

TGF- β 1 activates a subset of genes implicated in wound healing and fibrotic disorders several of which are ROS-dependent [13,31–35]. Transcriptional reprogramming in the context of increasing renal TGF- β 1 levels, moreover, is superimposed on the time course of tubulointerstitial scarring [36–42]. Among the disease-relevant ROS-dependent genomic targets, TGF- β 1 stimulates expression of the potent profibrotic matricellular PAI-1 (SERPINE1) and CTGF (CCN2) genes as well as those encoding ECM structural elements (fibronectin, collagen I) [13,31,35, 43–46]. PAI-1 is one of the most highly-upregulated members of the TGF- β 1/SMAD3-induced set [33,47–50], a prominent ROS-responsive gene [51–55] and causatively involved in tissue inflammation and fibrosis [37,56–63]. As the

major physiologic inhibitor of plasmin generation, PAI-1 limits ECM degradation facilitating accumulation of matrix structural elements at the injury site [57,62] (Fig. 1). In UUO-induced tubulointerstitial fibrosis, PAI-1 deficiency is, in fact, renal-protective [62] whereas transgenic PAI-1 overexpression promotes an increased fibrotic response with associated recruitment of inflammatory cells, macrophages and myofibroblasts [37]. The latter likely reflecting the newly-recognized chemoattractant properties of PAI-1 that appear unrelated to its function as a clade E SERPIN. As proof-of-concept, unilateral ureter-obstructed PAI-1^{-/-} mice develop a significantly attenuated inflammatory response suggesting that PAI-1 directly promotes infiltration of macrophages and T-cells [62]. Although the actual mechanism is not clear, there is considerable experimental evidence in support of this apparently non-proteolytic inhibitory function of PAI-1. Indeed, PAI-1 stimulates pulmonary macrophage accumulation, a collagen I-producing cell type, upon type II alveolar epithelial injury by recruitment of Ly6C^{high} monocytes [64]. The same Ly6C^{high} monocyte subpopulation homes to the UUO-stressed kidney and differentiates into three distinct macrophage cell types [65]. The dramatic effects of PAI-1 on cellular motility [56] may underlie its potent chemoattractant properties [35,56].

TGF- β 1/ROS/p53 Axis in Fibrosis: SMAD and NON-SMAD Signal Integration

TGF- β 1-induced PAI-1 expression involves ROS signaling to regulate a highly interacting SMAD and non-SMAD axis [31,66,67] that involves the c-Src-EGFR-MEK-ERK cascade [49,68,69] and ALK5 (a type I TGF- β R)-directed SMAD2/3 phosphorylation [49,70] (Fig. 2). TGF- β 1-initiated, ROS-dependent, c-Src^{Y416} activation and subsequent c-Src-directed phosphorylation of the EGFR^{Y845} site appears required engage the MEK-ERK1/2 cascade and downstream PAI-1 transcription [31,49,68,69]. Current evidence also suggests that ROS maintain pSMAD2/3 levels by c-Src-mediated caveolin^{Y14} site phosphorylation activating a c-Src/caveolin-1/RhoA/ROCK pathway that negatively regulates the SMAD2/3 phosphatase PPM1A, providing for retention of pSMAD2/3 levels and continued SMAD-occupancy of TGF- β 1 target gene promoters [31,49]. PPM1A silencing, in fact, enhanced TGF- β 1-stimulated PAI-1 induction, at least in renal fibroblasts and vascular smooth muscle cells [31]. Recent findings also implicate the tumor suppressor p53 as a critical co-factor for several key fibrotic and cell cycle effectors (e.g., TGF- β 1, CTGF, PAI-1, p21, angiotensin) [46,71–76]. Increased oxidative stress associated with the fibrotic process is both a likely initiator [77] and an upstream mediator of p53 signaling in the injured tissue. H₂O₂ promotes p53^{S15} phosphorylation consistent with the upstream role of ROS-mediated mechanisms in p53 activation in mouse embryo fibroblasts [46]. p53 also regulates tubular G2/M cell cycle arrest and elevated TGF- β 1 and CTGF expression associated with the progression of renal fibrosis in several models of acute and chronic kidney injury [71]. Administration of the p53 inhibitor pifithrin- α effectively attenuated renal experimental fibrogenesis consistent with decreased CTGF and TGF- β 1 levels [71] linking p53 to the induction of profibrotic factors [46]. Catalase overexpression and the subsequent reduced oxidative stress, moreover, reduced both p53 expression and renal fibrosis, suggesting a causative role of upstream ROS via p53-mediated mechanisms in at least one experimental model of diabetic nephropathy [78].

p53 participates in a subset of TGF- β 1 responses, in part, due to interactions between phosphorylated p53 and pSMAD2 resulting in the formation of transcriptionally-active multi-protein complexes [75,79,submitted]. Indeed, TGF- β 1 stimulates p53^{S15} phosphorylation [46] and acetylation creating activated pSMAD2/3-p53 complexes. p53 and SMAD2/3 map to defined binding elements in the PAI-1 promoter [80–84]. These sites have functional relevance in the context of TGF- β 1 signaling to target genes since p53 genetic deficiency or silencing of endogenous p53 blocked TGF- β 1-dependent PAI-1 induction while the p53 inhibitor, pifithrin- α , eliminated PAI-1 promoter-driven reporter

construct expression [46]. Interactions between p53 and SMADs are apparently crucial for radiation-associated PAI-1 induction via TGF- β 1 suggesting a role for SMAD and non-SMAD (e.g., p53 and ROS) cross-talk in other forms of tissue injury as well [85].

While the molecular basis for p53 activation downstream of ROS remains to be defined, ATM/ATR, CK1, CK2 and MAP kinases are likely candidates. p53^{S15} phosphorylation directs acetylation (by the histone acetyltransferase, CBP/p300), a necessary event in transcriptional activation [86]. SMAD2/3 also can be acetylated by p300 in response to TGF- β 1 leading to creation of a multi-component complex (e.g., SMADs, p53, transcriptional co-factors) permissive for maximal PAI-1 induction [87–89]. Upstream stimulatory factor 2 (USF2), for example, a helix-loop-helix member of the MYC transcription factor family, is induced in diabetic and obstructive renal injuries and post-translationally modified by growth factors/stress inducers [46,83,84,90–93]. Some of the same potential activators of p53 (e.g., ERK, p38) that are responsive to TGF- β 1 are also implicated in USF phosphorylation [e.g., 84,93]. USF proteins induce PAI-1 expression by occupancy of the PE2-region E box motif (5'-CACGTG-3') adjacent to three SMAD-binding sequences, likely providing a platform for core complex formation with pSMAD2/3 and pp53. Binding proximity, however, may not be a requirement, at least for p53-mediated PAI-1 transcription since PE2 E box recognition by USF2 facilitates DNA bending towards the minor groove [94] potentially promoting interactions between downstream-tethered p53 with PE2 site-bound pSMADs [95].

Therapeutic Opportunities

TGF- β 1 stimulates ROS production by various mechanisms that, in turn, engage downstream signaling pathways (e.g., SMADs, EGFR, *Src* and MAP family kinases) resulting in expression of a subset of profibrotic genes (e.g., PAI-1, CTGF, TGF- β 1, angiotensinogen) [96] (Table 1). Key ROS-generating elements, moreover, likely differ as a function of injury site suggesting complex, perhaps tissue-specific, controls on the fibrotic process. NOX4-produced ROS, for example, is critical for bleomycin-induced lung fibrosis while NOX1/2 dependent events are involved in hepatic disease [5,97]. TGF- β 1 and angiotensin similarly utilize components of the NADPH oxidase system in a tissue- or cell type-specific manner [96]. TGF- β 1 also promotes SMAD-dependent renal induction of NOX4, thereby, orchestrating a second wave of free radical generation necessary for myofibroblast generation [13]. Indeed, administration of ROS inhibitors (e.g., NAC, ebselen, apocynin, DPI) reduces fibrosis in the lung and the kidney consistent with the outcomes of NOX4 knock-out studies and gene silencing approaches in animals. In clinical trials, NAC improves lung function in patients with chronic obstructive pulmonary disease highlighting the potential benefit of ROS-directed therapy [98]. Development of more specific inhibitors of ROS generation may provide improved anti-fibrotic therapies. A major redox sensitive downstream target of TGF- β 1, PAI-1 is a causative factor in the progression of lung and kidney fibrosis as well as cardiovascular disease. The translational importance of this SERPIN is highlighted by the therapeutic efficacy of recently characterized small molecule PAI-1 inhibitors (e.g., TM5275) to attenuate lung fibrosis induced by intra-nasal adenoviral TGF- β 1 delivery [99]. Thus, targeting TGF- β 1-initiated, ROS-dependent, PAI-1 induction (as well as other profibrotic genes) provides an attractive clinical option to suppress progression of organ fibrosis.

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Highlights

The paper submitted by Samarakoon et al presents novel insights as to the fundamental basis for initiation and progression of human fibrotic disorders with an emphasis on signaling events that regulate transcription of disease-causative genes. During development of TGF- β 1-initiated fibroproliferative disorders, NADPH oxidases (NOX family members) generate reactive oxygen species (ROS) resulting in downstream transcription of a subset genes encoding matrix structural elements and profibrotic factors. Prominent among the repertoire of disease-implicated genes is the TGF- β 1 target gene encoding the potent profibrotic matricellular protein plasminogen activator inhibitor-1 (PAI-1 or SERPINE1). PAI-1 is the major physiologic inhibitor of the plasmin-based pericellular cascade and a causative factor in the development of vascular thrombotic and fibroproliferative disorders. ROS generation in response to TGF- β 1 stimulation is rapid and precedes PAI-1 induction; engagement of non-SMAD (e.g., EGFR, *Src* kinase, MAP kinases, p53) and SMAD2/3 pathways are both required for PAI-1 expression and are ROS-dependent. Recent findings suggest a novel role for p53 in TGF- β 1-induced PAI-1 transcription that involves ROS generation and p53/SMAD interactions; these and other signaling pathways involved in PAI-1 gene control are reviewed in this paper. Targeting ROS and ROS-activated cellular events is likely to have therapeutic implications in the management of fibrotic disorders, particularly in the context of prolonged TGF- β signaling.

Thank you.

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Figure 1. The plasmin-dependent/MMP axis in pericellular proteolytic control

uPA, tethered to its receptor (uPAR), converts plasminogen receptor- (PlgR-) bound plasminogen to the broad-spectrum protease plasmin that, in turn, activates several MMP family members. Collectively, plasmin and MMPs regulate, both in time and space, ECM proteolysis and stromal remodeling. TGF- β 1-induced up-regulation of PAI-1 at the injury site can shift this proteolytic balance. In normal tissue repair, PAI-1 “titrates” the extent and locale of collagen matrix remodeling. Chronically-elevated PAI-1 levels in response to persistent profibrotic stimulation (e.g., TGF- β 1, angiotensin II, elevated tissue glucose) commonly accompany the development of such diverse pathologies as, inflammation, hypertrophic scarring, atherosclerosis, thrombosis, myocardial infarction, diabetes, and the obesity-associated metabolic syndrome.

Figure 2. A model for ROS involvement in PAI-1 induction by TGF-β1

TGF-β1 receptor activation consequent to the ligand engagement initiates both Smad 2/3 (by phosphorylation of ALK5/TGF-β1 receptor 1 type) as well as non-Smad (e.g. EGFR, MAPK, Akt, Rho-ROCK) mediated signaling cascades with downstream consequences of gene expression (e.g. PAI-1, ECM molecules) as well as phenotypic modifications (e.g. excessive matrix deposition/fibrosis, epithelial plasticity, myofibroblast induction, proliferation, growth arrest and apoptosis). Rapid ROS generation consequent to TGF-β1 stimulation appears critical for initiation of non-SMAD (e.g. EGFR, Src) and modulation of SMAD (e.g. maintenance of SMAD phosphorylation) mediated signaling events. It is also becoming clear that p53 transcription factor integrates transcriptional contributions from both SMAD and non-SMAD cascades at the level of gene transcription (e.g. PAI-1) and ROS generation by TGF-β1 is crucial for p53 activation (by phosphorylation and acetylation).

Table 1**Targetable Elements in the Design of Anti-Fibrotic Therapies**

A. Signaling Proteins Regulated by ROS		
<u>Target</u>	<u>Approach</u>	<u>Target Genes</u>
p53	Pifithrin- α	PAI-1, CTGF, Angiotensinogen
EGFR	Erlotinib	TGF- β 1, PAI-1, CTGF, ECM
NOX4	siRNA, DPI	ECM Genes, α -SMA
NADPH Oxidases	DPI, Apocynin	ECM genes, PAI-1
SMAD3	SIS3	ECM molecules, NOX4, PAI-1
B. ROS-Dependent Target Genes		
<u>Target</u>	<u>Approach</u>	<u>Outcomes</u>
PAI-1	TM5275	↓PAI-1, TGF- β 1, ECM molecules
CTGF	Neutralizing Antibody	↓ECM molecules
Angiotensinogen	RAAS/ROS inhibitors	↓RAAS target genes (PAI-1, CTGF)
TGF- β 1	Receptor inhibitor, Neutralizing Antibody	↓TGF- β 1 and its genetic targets