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Oxidative stress and glutathione in TGF-β-mediated fibrogenesis

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Abstract

Transforming growth factor beta (TGF-β) is the most potent and ubiquitous profibrogenic cytokine and its expression is increased in almost all the fibrotic diseases and in experimental fibrosis models. TGF-β increases ROS production and decreases the concentration of glutathione (GSH), the most abundant intracellular free thiol and an important antioxidant, in various types of cells, which mediates many of TGF-β's fibrogenic effects. A decreased GSH concentration is also observed in human fibrotic diseases and in experimental fibrosis models. Although the biological significance of GSH depletion in the development of fibrosis remains obscure, GSH and N-acetylcysteine (NAC), a precursor of GSH, have been used in clinics for the treatment of fibrotic diseases. This review summarizes recent findings in the field to address the potential mechanism whereby oxidative stress mediates TGF-β's fibrogenesis and the potential therapeutic values of antioxidant treatment in fibrotic diseases.

Keywords

TGF-β; oxidative stress; fibrosis; glutathione

INTRODUCTION

Fibrosis, characterized by increased deposition of extracellular matrix (ECM) proteins in basement membrane and interstitial tissue, is a common pathological feature and terminal stage of many diseases involved in almost all the organ systems. Currently, there is no FDA-approved antifibrotic drug or efficacious treatment for fibrotic disease due to incomplete understanding of underlying pathogenesis. Many cytokines, chemokines, and growth factors are involved in the development of fibrosis; however, transforming growth factor beta (TGF-β) is considered to be the most potent and ubiquitous profibrogenic cytokine. TGF-β stimulates the production of reactive oxygen species (ROS) in various types of cells, while ROS activate TGF-β and mediate many of TGF-β's fibrogenic effects. Glutathione (GSH), a tripeptide, is the most abundant intracellular free thiol and an important antioxidant. GSH concentration decreases in experimental fibrosis models and in human fibrotic diseases. The mechanism and biological significance of GSH depletion in fibrotic diseases, however, remains unclear. Importantly, TGF-β administration decreases GSH in various types of cells *in vitro* while GSH replenishment suppresses TGF-β's fibrogenic activity, suggesting an important role of GSH depletion in TGF-β's fibrogenesis. Nonetheless, although intensive studies have been

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conducted, the molecular mechanism underlying stimulation of ROS production by TGF-β and whereby ROS mediate TGF-β's fibrogenic effects remains obscure. This review is intended to summarize recent studies to address the role of oxidative stress and GSH depletion in TGF-β's fibrogenesis, the underlying molecular mechanism, and the potential therapeutic value of antioxidant treatment in fibrotic diseases.

INCREASED OXIDATIVE STRESS IS A COMMON PATHOLOGICAL FEATURE OF FIBROSIS

There is a substantial body of evidence supporting the hypothesis that increased oxidative stress plays an important role in the development of fibrotic diseases in humans. The lung is a major target for oxidant damage due to its direct exposure to the atmosphere. Accumulating evidence suggests that oxidants generated endogenously or inhaled from the environment play an essential role in the pathogenesis of various pulmonary fibrotic disorders including idiopathic pulmonary fibrosis (IPF), cystic fibrosis (CF), asthma, acute respiratory distress syndrome (ARDS), and chronic obstructive pulmonary disease (COPD) [1–7]. Oxidized lipid and protein products including 8-isoprostane and carbonylated proteins have been identified in the exhaled air, bronchoalveolar lavage fluid (BALF), or lung tissue from patients with these fibrotic diseases; antioxidants, on the other hand, ameliorate the clinical symptoms [1,3–14]. Silicosis and asbestosis are two important occupational lung fibrotic diseases; ROS and reactive nitrogen species (RNS) have been shown to play a key role in the development of silicosis and asbestosis [15–17]. Workers exposed to silica or asbestos fiber have increased oxidative DNA damage, measured by 8-hydroxy-2′-deoxyguanosine (8-OHdG), and nitrotyrosine adducts [15–17]. Oxidative stress markers have also been detected in silica and/or asbestos-induced lung fibrosis animal models [18–20]. The bleomycin-induced animal model of lung fibrosis has been widely used to study the pathogenesis of fibrosis and the mechanism underlying antifibrotic effects of various compounds. It has been well documented that bleomycin treatment increases ROS production, which mediate bleomycin-induced fibrosis [21–24].

In addition to lung fibrosis, ROS/RNS have also been shown to play critical roles in the development of fibrosis in many other organs. Liver fibrosis/cirrhosis is a final stage of the liver diseases commonly associated with chronic alcohol consumption and hepatitis B or C virus infection. Both alcohol and hepatitis virus core proteins have been shown to increase ROS/RNS production independently and synergistically [25–28]; ROS/RNS play a central role in the development of liver fibrosis/cirrhosis under these pathological conditions [25,27–36]. Oxidative stress markers have been detected in the serum or biopsy samples from liver cirrhosis patients and in experimental liver fibrosis/cirrhosis animals. In liver biopsies, areas of fibrosis were localized to areas with increased 4-hydroxy-2′-nonenal (4-HNE), a marker of lipid peroxidation [31,37]. Renal fibrosis occurs under various pathological conditions including diabetes, hypertension, and urinary tract obstruction. Although the primary causes of renal fibrosis are different, oxidative stress has been found to be a common pathological feature of these diseases and contributes importantly to the development of renal fibrosis [38–43]. The levels of serum 8-isoprostane, an oxidation product of lipids, have been shown to correlate with severity of fibrosis in patients with systemic sclerosis [44]. Accumulating evidence also shows that oxidative stress plays a role in several aspects of fibrotic cardiac repair/remodeling after infarction, including apoptosis, inflammatory and fibrogenic responses, by increasing advance glycated end products or inducing the expression of profibrogenic cytokines such as TGF-β [45–49]. Together, the data indicate that oxidative stress is a common pathological feature of fibrosis and plays an important role in the development of fibrosis under various pathological conditions.

ROS AND TGF-β'S FIBROGENESIS

TGF-β and fibrosis

TGF-β, existing in three isoforms (TGF-β1, TGF-β2, and TGF-β3), is a multiple function protein involved in the regulation of cell proliferation, differentiation, apoptosis, adhesion, and migration, and therefore plays a pivotal role in the pathogenesis of many diseases. Various cytokines, chemokines, and growth factors are involved in the development of fibrosis; however, TGF-β is considered to be the most potent and ubiquitous profibrogenic cytokine. TGF-β mRNA and/or protein expression is increased in fibrotic diseases involved in almost all the organ systems, including pulmonary fibrosis, liver cirrhosis, renal fibrosis, systemic sclerosis, and cardiac fibrosis [50–61]. Increased TGF-β expression is also detected in experimental fibrosis models induced by different stimuli [62–68]. Whole body overexpression of TGF-β in TGF-β transgenic mice leads to multiple organ/tissue fibrosis [69–74] while specifically increasing the expression of constitutively active TGF-β in the lung and peritoneum by adenovirus-mediated gene transfer technique leads to lung and peritoneal fibrosis, respectively [75–78]. On the other hand, therapeutic administration of TGF-β binding proteins, treatment of mice with anti-TGF-β antibody or inhibitor to activin receptor-like kinase 5 (ALK5, a TGF-β type 1 receptor), or overexpression of dominant negative TGF-β type II receptor ameliorates fibrosis in different organ systems induced by different stimuli [77,79–86]. All these lines of evidence indicate that TGF-β is essential for the development of fibrosis.

TGF-β stimulates ROS production

The mechanism underlying the induction of fibrosis by TGF-β has been studied intensively in the past years. Increasing evidence indicates that ROS play a central role in TGF-β's fibrogenesis although the underlying mechanism remains largely undetermined. TGF-β increases ROS production in numerous types of non-phagocytic cells, such as endothelial cells, epithelial cells, smooth muscle cells, and fibroblasts [87–98]. Although it is still controversial, studies have shown that TGF-β can stimulate ROS production from different cellular compartments in different types of cells. Thannickal et al. reported that TGF-β stimulated ROS production through activation of cell-membrane associated oxidase, which led to an increased release of H_2O_2 to extracellular space in human lung fibroblasts and bovine pulmonary artery endothelial cells [88–90]. Yoon et al., on the other hand, reported that TGF-β induced a prolonged mitochondrial ROS production through decreasing complex IV activity in Mv1Lu cells, a mink lung epithelial cell line [92]. Herrera et al. also showed that TGF-β increased ROS production in mitochondria in rat hepatocytes [99,100]. Using different inhibitors, Albright et al. demonstrated that mitochondria and microsomes (cytochrome P450 1A1) were the major source of ROS in TGF-β treated rat hepatocytes [99].

NAPDH oxidase (Nox) enzymes are a family of heme-containing proteins with primary function being transporting electrons from NADPH to oxygen, forming ROS. Seven members have been identified in the Nox family including Nox1, Nox2, Nox3, Nox4, Nox5, Dual oxidase1 (Duox1), and Dual oxidase 2 (Duox2), with different Nox family members being expressed in different cell types and tissues/organs. Emerging evidence suggests that Nox/Duox family members are important ROS producers not only for phagocytic but also for non-phagocytic cells although the biological functions of Nox/Duox in non-phagocyes are still mostly unknown. Importantly, it has been reported that TGF-β activates/induces Nox4 in various types of cells *in vitro* [91,93–98,101–103] and *in vivo* [103], associated with a stimulation of ROS production, although the effects of TGF-β on the expression/activities of other Nox/Duox family members remain largely unclear at the moment. The prototype NADPH oxidase, Nox2, also called $gp91^{phox}$, is mainly expressed in phagocytes and located on the plasma membrane; Nox4, however, is expressed in many types of non-phagocytic cells and associated with other cell compartments such as endoplasmic reticulum (ER) [101,104–106],

perinuclear space [107], and nucleus [101,108,109]. Nonetheless, the biological function of Nox4 in these cell compartments as well as its role in TGF-β's signaling process are unclear at the moment and are currently under intensive investigation.

In addition to directly stimulating ROS production through Nox4, mitochondria, and/or microsomes, TGF-β may increase ROS/RNS levels by suppressing the expression of several antioxidant enzymes including glutaredoxin, catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx) [110–112], and by decreasing the concentration of glutathione, the most abundant intracellular free thiol and an important antioxidant (the effect of TGF-β on GSH metabolism is detailed in the following section). Islam et al. reported that TGF-β inhibited the mRNA expression as well as the activities of catalase and glutathione peroxidase in a hamster pancreatic beta-cell line (HIT), associated with an increase in intracellular ROS level and protein oxidation [111]. Kayanoki et al. showed on the other hand that TGF-β1 suppressed the expression of manganese-superoxide dismutase, copper, zinc-superoxide dismutase, glutathione-S-transferase, and catalase in cultured rat hepatocytes in time- and cell density-dependent manners [110]. Inhibition of the expression of these antioxidant enzymes was followed by an increase in peroxide levels in the cells, suggesting that suppression of antioxidant enzyme expression augments the production of hydrogen peroxide [110].

ROS activate latent TGF-β and induce TGF-β gene expression

TGF-β isoforms are secreted by some cells as a latent complex consisting of TGF-β and its latency association protein (LAP), called the small latent complex. Most cells, however, secrete TGF-β as a large latent complex, in which a latent TGF-β binding protein (LTBP) is associated with the small latent TGF-β complex through a covalent disulfide bond [113,114]. Currently, 4 LTBP isoforms have been identified and differences in isoforms of the LTBP family proteins account for the differential localization and availability of the latent TGF-β complex [115]. Release of TGF-β from its LAP, a process called latent TGF-β activation, is required for the binding of TGF-β to its receptors. Multiple mechanisms have been proposed for the activation of latent TGF-β, including conformation changes induced by thrombospondin-1 [116,117], binding to integrins αvβ6 and αvβ8 [118,119], proteolytic cleavage of LAP by plasmin and matrix metalloproteinase [120–123], and oxidative modification of LAP or activation of MMPs, which then cleave LAP to release active TGF-β [124–128].

ROS have been shown to play important roles in the development of fibrosis under various pathological conditions. One potential mechanism whereby ROS promote fibrosis is activating latent TGF-β [124–126,128–131]. In a cell-free model, recombinant human latent TGF-β was activated after exposure to ionizing radiation or metal iron plus ascorbate, which lead to ROS production [124]. Pociask et al. also showed that recombinant human latent TGF-β was activated in a cell free system in the presence of asbestos and ascorbic acid and that such an activation was reduced significantly by addition of superoxide dismutase, catalase, or deferoxamine, an iron chelator [126]. ROS produced by activated human CD4+CD25− T cells have also been shown to activate TGF-β and such activation was inhibited by antioxidant MnTBAP [128]. Two potential mechanisms have been proposed for ROS activation of latent TGF-β: direct oxidation of LAP and indirectly through activation of MMPs such as MMP-2 and MMP-9, which in turn cleave LAP to release active TGF-β. Vodovotz et al. reported that treatment of recombinant LAP with nitric oxide (NO) reduced its ability to neutralize active TGF-β1 although NO had no direct effect on the activation or the activity of recombinant latent TGF-β1 [125]. The activation of latent TGF-β by asbestos-ascorbate mediated generation of ROS apparently also resulted from oxidative modification in LAP, leading to a loss of its ability to bind to TGF-β1 [126]. On the other hand, ROS generated by ultraviolent light B (UVB) have been shown to activate TGF-β in keratinocytes through stimulating MMP-2 and MMP-9

activities as a MMP-2 and MMP-9 inhibitor diminished the UVB-induced increase in the amount of the active TGF-β but had no effect on the total amount of TGF-β [129].

TGF-β exists as three isoforms and all of them are secreted as a latent form. Interestingly, Jobling et al. showed that redox-mediated activation was restricted to the LAP/TGF-β1 isoform [130]. They also showed that exposure of TGF-β1 to ROS had no effect on its association with LAP while exposing LAP/TGF-β1 to ROS prohibited their association and that reducing agents restored the ability of LAP to neutralize TGF-β [130]. These data suggest that the oxidative modification leading to activation of latent TGF-β occurs in LAP. Using site-specific mutation, they identified a methionine residue at amino acid position 253 unique to LAP-TGFβ1 as a critical target for ROS-mediated activation of latent TGF-β1 [130]. Therefore, they proposed that latent TGF-β1 contained a redox switch centered at methionine 253, which allowed latent TGF-β1 to act uniquely as an extracellular sensor of oxidative stress in tissues [130].

In addition to activating latent TGF-β, ROS can also stimulate the expression and secretion of TGF-β in many types of cells [49,131–135]. In macrophages, the lipid peroxidation product 4-HNE upregulated the expression of TGF-β [132]. In human alveolar epithelial cells, ROS generated by xanthine/xanthine oxidase system induced TGF-β expression through a transcription mechanism while RNS increased TGF-β expression by a post-transcription mechanism [133]. Angiotensin II (AngII) induces TGF-β expression and fibrosis in various animal models. Zhao et al. showed that AngII induced Nox2 and TGF-β expression, accompanied by extensive perivascular and interstitial fibrosis in both ventricles of rat heart; the antioxidants apocynin and tempol, on the other hand, blocked AngII-induced Nox2 and TGF-β expression [49]. The iron chelator deforoxamine and the free radical scavenger T-0970 have also been shown to suppress AngII-induced TGF-β expression in rat heart [134]. Single-walled carbon nanotubes induced TGF-β expression, which was significantly attenuated in Nox2 null mice [135]. These data suggest an important role of Nox2 in the induction of TGF-β. A schematic diagram illustrating the potential relationship between TGF-β induction/activation and ROS production is presented in Fig 1.

Potential mechanisms whereby ROS mediate TGF-β's fibrogenesis

Stimulation of fibroblast proliferation and activation—Myofibroblasts are the major players in the development of fibrosis. Fibroblast proliferation and activation to become myofibroblasts therefore is a key step in the initiation of fibrosis. One of the major mechanisms by which TGF-β induces fibrosis is stimulating fibroblast proliferation and activation. TGF-β has been shown to stimulate the proliferation and activation of fibroblasts originating from different organ systems [136–141] and ROS/RNS play critical role in this process [98,103]. Cucoranu et al. reported that TGF-β treatment stimulated the expression of Nox4 and alpha smooth muscle actin (α-SMA), a myofibroblast marker, in primary human cardiac fibroblasts while knockdown of Nox4 by siRNA reduced TGF-β-stimulated production of ROS and α-SMA mRNA expression, suggesting that ROS mediate TGF-β-induced differentiation of cardiac fibroblasts to myofibroblasts [98]. The same study further showed that ROS mediated TGF-β-induced differentiation of myofibroblasts by activating Smad2/3, although the detailed mechanism underlying Smad2/3 activation by ROS was not explored [98]. A study published in Nature Medicine recently further showed that TGF-β1 specifically induced mRNA and protein expression of the Nox4 isoform in human fetal lung mesenchymal cells (hFLMCs), in myofibroblastic foci/mesenchymal cells from IPF patient lungs, and in lung tissue of bleomycin or hapten FITC treated mice [103]. Most importantly, they showed that inhibition of Nox4 expression by siRNA technique or pharmaceutical agent blocked TGF-β1-stimulated ROS production and myofibroblast activation [103]. These studies demonstrated clearly that ROS/RNS mediate TGF-β's fibrogenesis in part by activating fibroblasts.

In addition to resident fibroblasts, circulating fibrocytes, which derive from bone marrow mesenchymal progenitor cells, also serve as an important source of fibroblasts (myofibroblasts) found in the fibrosis foci [142–145]. TGF-β has been shown to play a critical role in the differentiation of circulating fibrocytes to myofibroblasts [145–147] although it is not clear at the moment whether ROS/RNS are involved in this process.

Induction of EMT—Although a long standing paradigm for the origination of myofibroblasts is that myofibroblasts differentiate from resident fibroblasts upon stimulation, emerging evidence indicates that myofibroblasts may also arise from epithelial cells through epithelial-mesenchymal transition (EMT), a process initially observed in embryogenesis and carcinogenesis and characterized by the loss of epithelial markers and the acquisition of a mesenchymal phenotype [148–152]. EMT has been shown to occur in many fibrotic diseases, including idiopathic pulmonary fibrosis [153–155], and to play an essential role in the development of fibrosis in several animal models induced by different stimuli [78,143,156–159]. In addition to epithelial cells, recent studies have shown that endothelial cells may also undergo such mesenchymal transition, termed endothelial-mesenchymal transition (EndMT) and that EndMT contributes importantly to the development of cardiac fibrosis, kidney fibrosis, and corneal fibrosis [160–162]. Many cytokines, chemokines, and growth factors are involved in EMT/EndMT; however, TGF-β is considered to be a key inducer of EMT/EndMT. TGF-β induces EMT and EndMT in various types of cells *in vitro* [163–167] and in animal models *in vivo* [78,158]. Increased TGF-β expression is also associated with EMT phenotypic changes under various human fibrotic disease conditions [153,154,157,167]. Blocking TGF-β signaling with Smad 7 has been shown to suppress EndMT in several fibrosis models including injury-induced corneal fibrosis model and pressure overload or chronic allograft rejection induced cardiac fibrosis model [161,162]. Together, the evidence suggests that both EMT and EndMT are involved in the development of fibrosis and that one potential mechanism whereby TGF-β stimulates fibrosis is induction of EMT/EndMT.

ROS have been shown to function as signaling molecules mediating the induction of EMT by different stimuli in different cell types [152,168–170]. Several signaling pathways including Smad [164,171–174], Ras/Raf/MAPK [170,174,175] and PI3K-Akt [176–179] pathways are implicated in the induction of EMT by TGF-β. Some of these pathways including the Ras/Raf/MAPK and PI3K-Akt pathways are redox sensitive. Therefore, it is speculated that ROS/RNS play an important role in TGF-β induction of EMT. This speculation is confirmed by a recent study [170]. Rhyu et al. reported that TGF-β increased ROS production and induced epithelial-mesenchymal transition (increased the expression of α-SMA, a mesenchymal marker, and decreased the expression of E-cadherin, an epithelial marker) in rat renal tubular epithelial cells [170]. Suppression of cellular ROS signaling with antioxidants blocked TGF-β-induced EMT, suggesting that ROS mediate the induction of EMT by TGF-β in renal epithelial cells [170].

Induction of apoptosis—Apoptosis and fibrosis are juxtaposed in some fibrotic diseases. Accumulating evidence suggests that apoptosis may be directly linked to the development of fibrosis [73,180–182]. Using an externally regulated triple transgenic system, Lee et al. showed that increasing TGF-β1 expression in airway epithelial cells induced a transient wave of epithelial apoptosis that was followed by mononuclear-rich inflammation and tissue fibrosis [73]. They also showed that a caspase inhibitor blocked TGF-β1-induced apoptosis and ameliorated TGF-β1-induced fibrosis [73]. These results suggest that apoptosis is involved in the development of fibrosis in this animal model. Apoptosis is commonly triggered in hepatocytes through activation of death receptors such as Fas death receptor. Canbay showed that 3-day bile duct ligation induced more TUNEL-positive hepatocytes (apoptosis), higher serum ALT values (liver damage), and profound liver fibrotic changes (increased expression of α-SMA, TGF-β, and collagen mRNAs as well as collagen staining) in wild type mice than in Fas-deficient mice, suggesting that Fas-mediated hepatocyte apoptosis is mechanistically

linked to liver fibrogenesis [180]. Using a pancaspase inhibitor, IDN-6556, they further concluded that hepatocyte apoptosis initiated cascades culminating in liver injury and fibrosis [181]. TGF-β induces apoptosis in variety of cell types, associated with an increase in ROS production, while antioxidants blocked TGF-β-induced apoptosis [95,96,99,100,111,183–186]. These data suggest that ROS mediate TGF-β's fibrogenic effects in part by inducing apoptosis.

Induction of ECM gene expression and suppression of ECM protein degradation—TGF-β upregulates the expression of extracellular matrix (ECM) proteins, including collagens as well as protease inhibitors such as plasmin activator inhibitor 1 (PAI-1) and tissue inhibitor of metalloproteinases (TIMPs), leading to increased ECM production and decreased ECM degradation. Importantly, ROS have been shown to be involved in the induction of ECM proteins and protease inhibitor expression by TGF-β [187–193]. Both Garcia-Trevijano and Cao showed that TGF-β1 increased H_2O_2 production and induced procollagen-α1(I) mRNA expression in hepatic stellate cells while treatment of the cells with catalase reduced H_2O_2 levels and prevented the induction of procollagen by TGF-β, suggesting that ROS mediate TGF-β induction of procollagen expression in these cells [187,188]. ECM degradation is mediated mainly by the matrix metalloproteinases (MMPs) and the plasminogen activators (PAs)/plasmin systems [194–196]. The activities of MMPs and PAs/plasmin, on the other hand, are inhibited by TIMPs and PAI-1. PAI-1, a physiological inhibitor of tissue type and urokinase type plasminogen activators (tPA and uPA), suppresses ECM degradation by inhibiting tPA/uPA activity and thus the activation of plasminogen and MMPs [197]. TGF-β induces the expression of both TIMPs and PAI-1, which contributes importantly to the development of fibrosis under various pathological conditions. Li et al. reported that TGF-β1 increased ROS production and induced TIMP-3 in primary human and bovine chondrocytes while ROS scavenger and antioxidant NAC blocked TGF-β1-induced TIMP-3 mRNA and protein expression [191]. Studies have also shown that the induction of PAI-1 by TGF-β is mediated by ROS in different types of cells [190,192,193]. Our previous studies showed that TGF-β increased the production of ROS as well as the expression of TGF-β1, procollagen α2(I), and PAI-1 mRNAs in murine embryo fibroblast cells (NIH3T3 cells) [192]. Exogenous glutathione selectively inhibited TGF-β-induced PAI-1 expression, accompanied by a restoration of plasmin activity and collagen degradation, with no effect on TGF-β-induced TGF-β1 or procollagen α2(I) mRNA expression [192]. Together, the data suggest that ROS play a central role in TGF-β's fibrogenesis by mediating TGF-β-induced fibroblast proliferation/activation, EMT, epithelial/endothelial apoptosis, and ECM accumulation (Fig 2).

ROS and TGF-β's signaling

Although numerous studies have shown that ROS mediate the induction of many TGF-β responsive genes the underlying mechanism remains equivocal. TGF-β signaling through the Smad pathway has been well described in the past decades. Following the binding of TGF-β to the type II receptor (TβR-II), the type I receptor (TβR-I) is phosphorylated, which further phosphorylates Smad2 or Smad3. Phosphorylated Smad2 and Smad3 then form a heteromeric complex with Smad4, which translocates to the nucleus and binds to the promoters of TGF-β responsive genes to regulate their expression. In addition to the Smad pathway, several other pathways including mitogen activated protein kinase (MAPK) pathways and PI3K-Akt pathway are also important in TGF-β signaling. MAPK pathways, including mainly JNK, p38, and ERK MAPK pathways, are sensitive to the redox environment. Numerous studies including ours have shown that ROS mediate TGF-β's fibrogenic effects by modulating the activities of MAPK pathways although the underlying molecular mechanism remains unclear [97,193, 198–202].

MAPK activation results from the activation of upstream MAPK kinase kinase, which leads to phosphorylation of tyrosine and serine/threonine residues on MAPKs, or inactivation of MAPK phosphatases (MKPs), which dephosphorylate tyrosine, serine/threonine, or both. Although it is still not clear whether ROS can directly oxidize MAPK, numerous studies have shown that protein phosphatases including protein tyrosine phosphatases (PTPs) such as PTP1B, serine/threonine phosphatases such as protein phosphatase 2A (PP2A), and some dual specific MAPK phosphatases (DS-MKPs) such as MKP-1 and MKP-3 can be inactivated by ROS through oxidation of critical cysteine residues in their active sites, associated with a sustained activation of MAPK [203–213]. Interestingly, TGF-β has been shown to inhibit the activity of PP2A [214–219], phosphatase and tensin homolog (PTEN) [220,221], a dual-specificity phosphatase, and protein tyrosine phosphatase [222] in different cell types. Therefore, one potential mechanism whereby ROS mediate TGF-β activation of MAPK pathways and therefore the expression of TGF-β responsive genes could be the inactivation of MKPs (Fig 3).

In addition to MAPK pathways, emerging evidence shows that the activity of the Smad pathway can also be affected the redox status of cells [170,191,223]. Li et al. reported that the induction of TIMP-3 by TGF-β1 was Smad2-dependent and redox sensitive as NAC or reduced GSH blocked TGF-β1-stimulated Smad2 phosphorylation and TIMP-3 expression [191]. Rhyu also showed that, in TGF-β-treated renal tubular epithelial cells, antioxidants effectively blocked not only the activation of p38 and ERK MAPKs but also the phosphorylation of Smad 2, associated with an inhibition of EMT [170]. The molecular mechanism underlying the activation of the Smad pathway by ROS is unclear. MAPKs have been shown to interact with Smad signaling in different ways. In addition to affecting phosphorylation and nuclear translocation of Smad proteins [170,191,224–226], MAPKs may also interfere with the binding of Smad proteins to the promoters through modifying the binding of other transcription factors [193,227–231]. Our previous studies showed that GSH had no effect on TGF-β-induced Smad 2/3 phosphorylation or Smad2/3/4 nuclear translocation in fibroblasts; however, it blocked TGF-β-induced binding of transcription factors to not only AP-1 and SP-1 but also Smad *cis* elements in the PAI-1 promoter [193]. Therefore, it is speculated that AP-1 and SP-1 may be involved in the binding of Smad to its *cis* elements in the PAI-1 promoter and that ROS mediate the induction of PAI-1 by TGF-β through increasing the binding of AP-1 and SP-1, and consequently Smad complex, to the PAI-1 promoter [193].

TGF-β, GSH, and FIBROSIS

Glutathione: functions and homeostasis

Glutathione (GSH), a tripeptide, is the most abundant intracellular free thiol. GSH plays a critical role in regulating a variety of cellular functions including detoxification of xenobiotics, synthesis of DNA and other endogenous compounds, modulation of gene expression, and the regulation of the cell cycle. However, the most important and well-known function of GSH is antioxidant. Glutathione can reduce hydrogen peroxide and lipid peroxide through glutathione peroxidase (GPx) catalyzed reactions, and conjugate and therefore detoxify electrophiles spontaneously or through glutathione S-transferase (GST) catalyzed reactions. Another important mechanism whereby GSH exerts its antioxidant function is to keep protein cysteine residues in their reduced form through reactions catalyzed by glutaredoxin (Grx) and sulfiredoxin (Srx) (Fig 4). Cysteine residues are important for protein structure and function and they are sensitive to oxidation. During oxidant challenge, cysteine residues, especially those in cysteine thiolate ($Cys\text{-}S^{-}$) form, can be oxidized to the sulfenic (RSOH), sulfinic (RSO_2H), and sulfonic (RSO_3H) acid, depending on the intensity of oxidant insults. Although conversion to sulfonic acid is considered to be irreversible, sulfinic acid can be reduced back to sulfenic acid through Srx catalyzed reaction [232–236]. Sulfenic acid can then react with vicinal protein thiols to form disulfides or with GSH to form protein mixed disulfides

(glutathionylation), which can be reduced back to free thiol form by Grx [237–239] or Srx [240,241] catalyzed reactions using GSH as a reductant (Fig 4). Many transcription factors such as NF*k*B, AP-1, and NrF-2 as well as signaling molecules such as protein phosphatases including PTPs, PP2A, and MKP-1, and MKP-3 contain redox sensitive cysteine residues in their active sites and undergo reversible oxidative modifications upon stimulation by growth factors or oxidants [204,208,210,212,217,242–248]. Such reversible oxidative modifications of protein cysteine residues have been increasingly recognized as an important mechanism whereby reactive oxygen/nitrogen species (ROS/RNS) regulate protein functions and cell signaling [249–252]. As the intracellular GSH concentrations (ranging from 1 to 10 mM) are much higher than most other redox active compounds, the ratio of glutathione disulfide (GSSG) and GSH determines cell redox status [253]. Therefore, by detoxifying H_2O_2 and lipid peroxides through glutathione peroxidase catalyzed reactions and by modifying the redox status of protein cysteine residues in these signaling molecules through Grx and Srx catalyzed reactions, GSH plays a central role in regulating cell redox signaling [237,247,253–257] (Fig 4).

As GSH is the most highly concentrated antioxidant in the cell and the determinant factor for cellular redox status, maintenance of intracellular GSH homeostasis is vital for normal cell functions [247]. There are several mechanisms by which cells maintain their intracellular GSH homeostasis, mainly by GSH redox cycling, direct uptake, and *de novo* synthesis. GSH redox cycling, catalyzed by GSSG reductase (GR), prevents the loss of GSH in the form of GSSG that is generated during the reduction of various oxidants with GSH by reducing GSSG back to GSH. A few types of cells can also directly uptake intact GSH from surrounding fluid. Most cells, under both normal and oxidative stress conditions, however, tend to depend on the *de novo* synthesis to maintain their intracellular GSH levels. *De novo* GSH synthesis is a two step process catalyzed by glutamate-cysteine ligase (GCL, EC6.3.2.2), also known as γ-glutamylcysteine synthetase (GCS), and GSH synthetase (EC6.3.2.3) [258]. GCL, which catalyzes the first reaction, is the rate-limiting enzyme in *de novo* GSH synthesis [258]. Accumulating evidence suggests that the regulation of GCL gene expression and activity is a major determinant of intracellular GSH homeostasis under physiological condition and upon oxidant challenge.

Glutathione depletion in fibrotic diseases

GSH concentration has been found to be decreased in human subjects with various fibrotic diseases. In patients with lung fibrotic diseases such as cystic fibrosis [259,260], COPD [261], ARDS [262–266], IPF [267–273], and sarcoidosis [274], GSH concentration is decreased in the lung lining fluid. Such a decrease in GSH concentration is associated with increased oxidative damage of macromolecules. Exposure to asbestos or silica leads to lung fibrosis. It has been shown that workers exposed to asbestos, silica, or related chemicals have a decreased GSH level in the plasma and in the lung lining fluid [275,276]. GSH depletion is also a common feature for chronic liver diseases associated with chronic alcohol consumption or hepatitis B or C infection [277–283]. Loguercio et al. reported that liver cirrhosis patients have decreased levels of glutathione and other antioxidants and increased levels of oxidative stress markers [279]. Using a 2-step infusion protocol, Bianchi et al. found that the primary defect responsible for the decrease in glutathione concentration in liver cirrhosis is reduced production [277]. Alcohol consumption leads to decreases in GSH concentrations not only in liver but also in the lung in humans. It was reported that chronic ethanol ingestion in human subjects causes 80–90% depletion of extracellular glutathione and an increase in oxidative stress in the alveoli, which is accompanied by an increased risk of developing ARDS and acute lung injury [284].

GSH depletion has also been observed in various experimental fibrosis models involved in different organ systems and induced by different insults [24,285–288]. Lung fibrosis is one of the major side effects caused by bleomycin, a chemotherapy drug used for the cancer treatment. It has been reported that bleomycin decreases GSH and induces lung fibrosis in mice and rats [287,289–293]. Paraquat is a broad spectrum contact herbicide that is known to cause progressive pulmonary fibrosis in human and animals [294,295]. It has been reported that paraquat-induced lung fibrosis is associated with a decline in GSH content in the lung [286, 295]. A significant depletion of GSH was also observed in the lung and red blood cells in chrysotile-exposed rats during different developmental stages of asbestosis [296]. Decreased GSH level is also a common pathological feature of liver fibrosis/cirrhosis induced by different stimuli including alcohol [297,298], carbon tetrachloride (CCl4) [299,300], and dimethylnitrosamine [301,302]. Such a decrease in GSH level was associated with an elevated ROS/RNA production, lipid peroxidation, and fibrosis [303]. Taken together, the data indicate that decreased GSH concentration is a common pathological feature of fibrosis. Nonetheless, although it has been well documented that GSH concentration decreases in fibrotic diseases, the mechanism and biological significance of GSH depletion in the development of fibrosis remain unclear and warrant further investigation.

TGF-β decreases intracellular GSH

Although the mechanism underlying GSH depletion in fibrotic diseases is unclear TGF-β has been shown to decrease GSH in various types of cells including endothelial cells, hepatocytes, epithelial cells, and fibroblasts *in vitro* [304–312]. Sanchez et al. reported that TGF-β-induced apoptosis in fetal hepatocytes was preceded by an induction of reactive oxygen species production and a decrease in the intracellular GSH concentration [305]. Cycloheximide, a protein synthesis inhibitor, prevented the depletion of intracellular GSH, increase in ROS production, and apoptosis induced by TGF-β [306]. Our previous studies showed that TGF-β decreased intracellular GSH in murine embryo fibroblasts (NIH3T3 cells), which was accompanied by increased collagen accumulation [312]. Replenishment of intracellular GSH by exogenous GSH, NAC, or GSH ester blocked TGF-β-induced collagen accumulation and PAI-1 expression, and stimulated collagen degradation, suggesting an important role of GSH depletion in TGF-β's fibrogenesis [192,193,312]. Nonetheless, whether increased expression of TGF-β is responsible for the depletion of GSH in fibrotic diseases in humans remains to be determined.

The mechanism whereby TGF-β decreases intracellular GSH has been explored in several studies. GCL is the rate-limiting enzyme in *de novo* GSH synthesis and is composed of two subunits, the catalytic subunit (GCLC) and the modifier subunit (GCLM). It has been reported that TGF-β suppresses the expression of GCLC mRNA and protein in a human lung epithelial cell line (A549), accompanied by a decrease in GCL activity and GSH level [307]. It has also been reported that TGF-β inhibits GCLC gene transcription, measured by nuclear run-on assay, but had no effect on the stability of GCLC mRNA [307]. Jardine et al. reported that TGF-β suppressed the GCLC promoter activity in alveolar epithelial cells and that the increased binding of c-Jun and Fra-1 dimers to AP-1 site within the antioxidant response element (ARE) in the promoter of GCLC gene might be responsible for the down-regulation of GCLC gene by TGF-β in these cells [309]. Bakin et al. further showed that TGF-β suppressed the activity of the human GCLC proximal promoter bearing ARE; ectopic expression of constitutively active Smad3 (Smad3E) alone inhibited the reporter activity whereas dominant negative Smad3 (Smad3A) blocked TGF-β's effects [311]. They also demonstrated that TGF-β increased the protein level of activating transcription factor3 (ATF3), a stress-inducible transcription repressor, and that ectopic expression of ATF3 suppressed GCLC promoter activity and endogenous GCLC expression [311]. Their studies suggest that the Smad3-ATF3 pathway mediates TGF-β suppression of GCLC expression. Importantly, Tiitto et al. showed that GCL

expression was decreased in the fibroblast foci in IPF lung [313]. Together, the data suggest that decreased GSH biosynthesis capacity resulting from inhibition of GCLC gene expression by TGF-β may underlie the decrease in GSH concentrations observed in human fibrotic diseases. A schematic diagram elucidating the potential mechanism whereby TGF-β depletes GSH and GSH antagonizes TGF-β's fibrogenesis is presented in Fig 5.

THERAPEUTIC VALUE OF THIOLS IN FIBROTIC DISEASES

Antifibrotic effects of NAC and GSH in animal models

As GSH concentration decreases in many fibrotic diseases and in experimental fibrosis models, it is reasonable to assume that treatments that increase the concentration of GSH and other antioxidants in the target tissue would prevent the progression of fibrotic diseases. Numerous studies have been conducted to elucidate the antifibrotic potential of thiol reagents in experimental fibrosis models. NAC, which easily passes the cell membrane and is hydrolyzed intracellularly to donate sulfhydryl groups for GSH synthesis [314], is commercially available with few known side effects. Therefore, NAC has been extensively studied as a potential treatment for fibrotic diseases. It has been reported that NAC attenuates liver cirrhosis in different animal models [315–318]. In a Nonalcoholic Steatohepatitis (NASH) model, NAC was found to be able to prevent many aspects of NASH and decrease Picrosirius red staining of collagen, a marker of fibrosis, although it did not block development of steatosis [315]. The bile-duct ligation (BDL) model has been widely used to induce liver cirrhosis to study the pathology of liver cirrhosis and the mechanism of antifibrotic effects of chemicals. Yang et al. showed that NAC administration decreased BDL-induced expression of procollagen III mRNA and protein, associated with a reduction of portal venous pressure and intrahepatic resistance, two liver cirrhosis indicators [316]. NAC has also been shown to reduce CCl4 [318] and dimethylnitrosamine [317] induced liver cirrhosis in animal models.

The antifibrotic effect of NAC has also been well documented in lung fibrosis models. Oral administration of NAC at doses between 2.45 and 3 mmol/kg/day reduced bleomycin-induced lung fibrosis in mice [319] and in rats [22,320,321] associated with an increase in GSH concentration. Aerosol administration of NAC also attenuated bleomycin-induced lung fibrosis in mice [322]. Nonetheless, although many studies have demonstrated the protective effects of NAC/GSH in various fibrosis models the underlying mechanism remains unclear. In almost all of these studies, NAC treatment was initiated before bleomycin treatment and a significant inhibition of the inflammatory response was observed. Therefore, it is not clear whether GSH/NAC attenuated fibrosis by suppressing early-stage inflammatory response or whether they also have direct anti-fibrotic effect.

NAC and GSH clinical trials

Despite severe outcomes (e.g. the patients usually die within 3–5 years after diagnosis of IPF), there is no efficacious treatment for these devastating fibrotic diseases such as IPF and liver cirrhosis due to a poor understanding of the complex pathological process. It was speculated in the past that fibrosis results from an unremitting inflammatory response to an exogenous insult, which leads to fibroblast activation/proliferation and eventually culminates in progressive fibrosis. Therefore, anti-inflammatory agents alone or in combination with cytotoxic drugs have been used in clinics as a standard therapeutic regimen for the treatment of fibrotic diseases. However, there is little evidence indicating that these agents alter the natural history of the disease or improve survival of the patients [323–326]. Although other therapeutic strategies are in development or in early clinical trials, including interferon gamma (INFγ) and anti-TGF-β or anti-connective tissue growth factor (CTGF) antibody, the efficacies of these treatments remain unclear [327–330].

Based on the findings that GSH concentration is decreased in the lung lining fluid of fibrotic disease patients and oxidative stress plays a critical role in the development of fibrosis, GSH and NAC have been used clinically for the treatment of various lung fibrotic diseases including IPF and CF. Studies have shown that administration of GSH or NAC increased GSH concentration in the lung lining fluid in IPF and CF patients. GSH/NAC has also been shown to slow deterioration of the lung functions of CF or IPF patients in some clinic trials. However, the efficacy of GSH and NAC in the treatment of lung fibrotic diseases remains to be further evaluated. Behr et al. reported that there was an increase in the total and reduced glutathione in native BAL fluid and in the epithelial lining fluid in fibrosing alveolitis patients after treatment with 600 mg NAC three times daily for 12 weeks plus immunosuppressive therapy. The increase in GSH concentration was accompanied by a decrease of methionine sulfoxide content, an indicator of oxidative stress at the alveolar surface, and a significant improvement of pulmonary function [270]. In another pilot study [331], 30 patients with IPF were randomly assigned to either NAC or placebo group and received NAC (176 mg, twice a day) or bromhexine hydrochloride (4 mg daily) for 12 months. Efficacy was assessed by the changes in pulmonary function, the 6-min walking test, high-resolution CT, health-related quality of life, and serum KL-6 (Krebs von den Lungen-6) value before and after the treatment. The results showed that there were significant differences between the NAC and control groups in terms of mean changes in lowest Saturation of Oxygen (SaO_2) during the 6-min walking test, serum KL-6, and the ground-glass score on high-resolution CT. However, differences between two groups in pulmonary function, 6-min walking distance, or quality of life were not significant [331]. In a double-blind randomized placebo-controlled multicenter trial, IPF patients were divided into two groups with one group receiving the standard therapy of prednisone plus azathioprine while another group receiving NAC (600 mg 3 times daily) plus standard therapy. The changes between baseline and after 12-month therapy in vital capacity (VC) and single-breath carbon monoxide diffusing capacity (DL_{co}) were monitored. The results showed that NAC treatment slowed the deterioration of VC and DLco (9% and 24% improvement for VC and DLco, respectively). However, the differences in the mortalities between two groups during the study were not statistically significant (9% for NAC plus standard therapy group vs. 11% for standard therapy only group) [332].

Cystic fibrosis (CF) is caused by an autosomal recessive mutation of CF transmembrane conductance regulator (CFTR) protein, which has been shown to function as a GSH transporter [333–335]. It has been well documented that GSH concentration decreases in the lung lining fluid of CF patients and that oxidative stress contributes importantly to the airway pathogenesis of CF. Therefore, GSH and NAC have been on clinical trial for the treatment of CF patients for many years. A report from a double-blind placebo controlled clinical trial showed that the lung function was improved in CF patients who received NAC for six months as compared with placebo controls [336]. In a 14-day glutathione inhalation trial (thrice-daily of 300–400 mg GSH), Griese et al. reported that GSH concentration in lavage of CF patients was increased 3–4 folds 1 hour post inhalation and was still double 12 hours post inhalation. FEV1 was increased compared with pretreatment values although a transient drop was observed at beginning [337]. The same group of investigators further showed that although inhalation of GSH has no effect on oxidative stress markers, it decreased BALF levels of PGE2 and increased CD4+ and CD8+ lymphocytes, which correlated inversely and positively with lung function, respectively [338]. In a randomized, double-blind, placebo-controlled pilot study, Bishop et al. found that after inhalation of buffered reduced glutathione at dose of 66 mg/kg/day for 8 weeks, the peak flow was dramatically increased in GSH group as compared with placebo group [339]. Self-reported average improvement in GSH group was also bigger than placebo group. Of 13 primary and secondary outcomes examined, 11 outcomes favored GSH treatment, suggesting a potential therapeutic value of GSH in the treatment of CF [339].

Overall, while NAC and GSH show a clear inhibitory effect on the development of fibrosis in animal models, their effects in clinics are not as dramatic as in animal studies, although promising. It should be emphasized that, in almost all animal studies, GSH/NAC supplement started before or simultaneously with treatment of bleomycin or other fibrogenic stimuli, which usually induce an early stage inflammatory response. Although inflammation may not be essential in the progression of IPF, animal studies have shown that early inflammatory response is critical for the development of fibrosis. As GSH and NAC are potent anti-inflammation agents, GSH and NAC may exert their antifibrotic effects in animal models by suppressing the early inflammatory response and potentially the later fibrotic response. In IPF, on the other hand, the early inflammatory stage has passed and lung fibrosis has developed when patients seek medical help. Under those circumstances, the efficacy of GSH/NAC may not be as dramatic as that observed in pretreated animal models. One important question that needs to be addressed therefore is whether antioxidant treatment is still effective when given in the later stages of fibrosis in animal models. Otherwise, identification of early stages of IPF patients is critical for efficacious treatment with antioxidants. It should also be emphasized that in addition to its role in antioxidant defense, GSH has many other functions, including detoxification of various toxicants, especially electrophiles, by forming conjugates with these compounds. NAC may provide a similar protective effect after conversion to GSH. Therefore, the therapeutic effects observed with NAC in clinical trials may result from the detoxification of other drugs, such as immunosuppresive drugs administered at the same time [325,332]. An ongoing clinical trial [PANTHER (Prednisone, Azathioprine and N-acetylcysteine: A Study THat Evaluates Responses in IPF) to be conduced by IPF*net*] will compare the therapeutic effects of NAC versus placebo versus NAC plus immunosuppressive therapy (prednisone and azathioprine) in the early stage of IPF patients. The results from this clinical trial should provide direct evidence whether NAC has direct benefit for IPF.

CONCLUSION

ROS/RNS play an important role in the development of fibrosis and in TGF-β-mediated fibrogenesis. The molecular mechanism by which ROS/RNS mediate TGF-β-induced fibrosis, however, remains undetermined. GSH is the most abundant intracellular free thiol and an important antioxidant. GSH concentration decreases in the fibrotic diseases and in experimental fibrosis models. Although TGF-β decreases GSH in various types of cells *in vitro*, whether increased TGF-β expression is responsible for the decrease in GSH concentration in fibrotic diseases in humans is unknown. Furthermore, the biological significance of GSH depletion in the development of fibrosis is also unclear. GSH and NAC have been used in clinics for the treatment of various fibrotic diseases. Although promising, the therapeutic value and mechanism underlying their therapeutic effects remain to be further evaluated. As TGF-β plays a vital role in the development of fibrosis under almost all the pathological conditions and ROS mediate many of TGF-β's fibrogenic effects, to uncover the molecular mechanism underlying redox regulation of TGF-β's fibrogenesis will be critical for the development of efficacious treatment for these devastating diseases.

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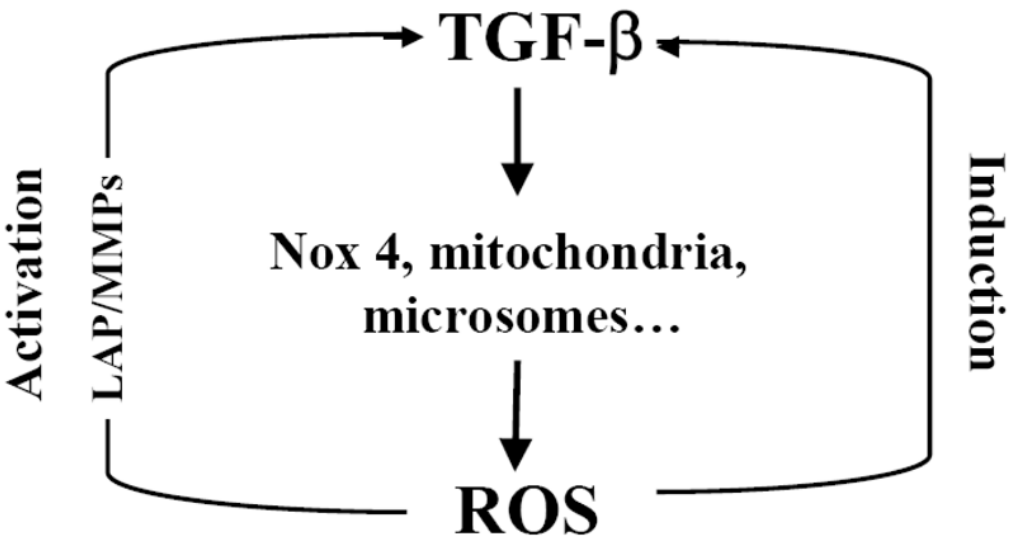

Fig 1. TGF-β increases ROS production and ROS activate/induce TGF-β

TGF-β increases ROS production by Nox4, mitochondria, and microsomes. On the other hand, ROS induce TGF-β gene expression and activate TGF-β through oxidizing latency associated protein (LAP) or activating MMPs, which in turn release LAP.

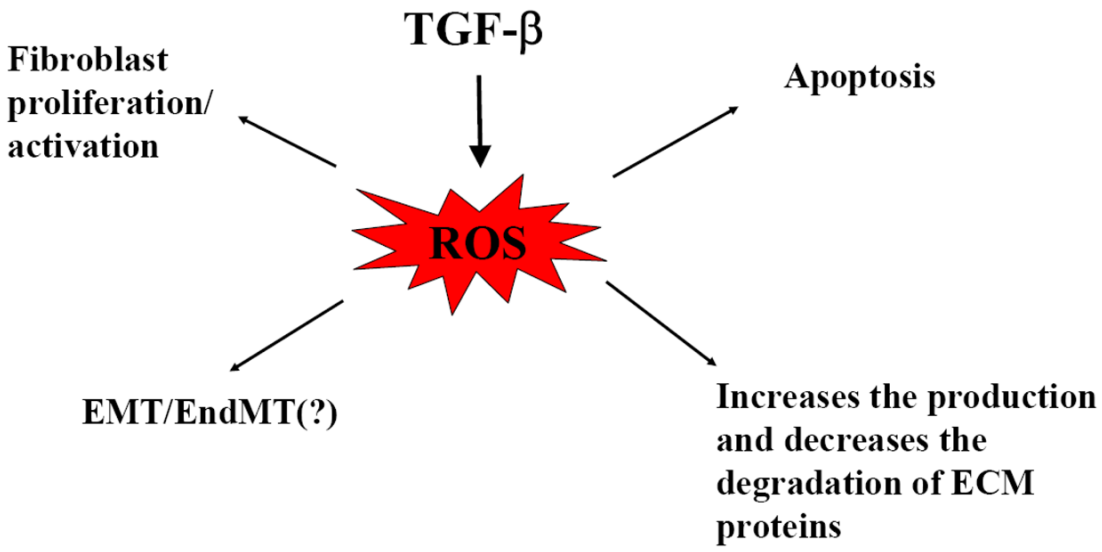

Fig 2.
ROS mediate many of TGF-β's fibrogenic effects.

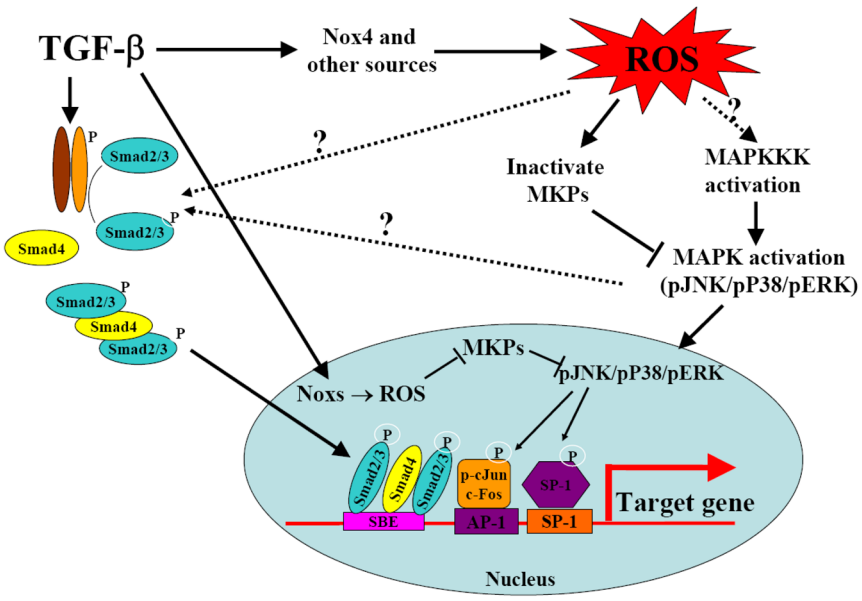

Fig 3. ROS and TGF-β signaling

TGF-β increases ROS production, which inactivates MAPK phosphatases (MKPs). Inactivation of MKPs leads to sustained activation of MAPKs, which induces the expression of TGF-β responsive genes by activating (phosphorylating) transcription factors such as AP-1 and SP-1 or by increasing phosphorylation, nuclear translocation, and/or DNA binding of the Smad proteins.

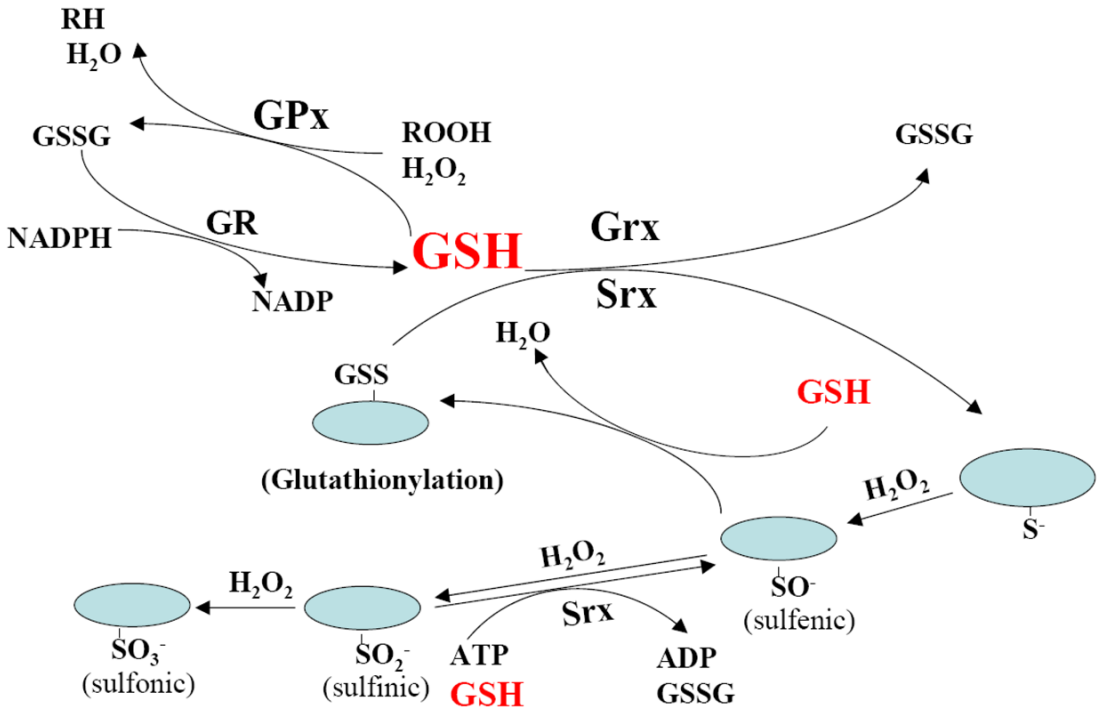

Fig 4. GSH in antioxidant defense and in redox signaling

GSH reduces hydrogen peroxide (H_2O_2) and lipid peroxide (ROOH) through glutathione peroxidase (GPx) catalyzed reactions. Oxidized glutathione (GSSG) formed is then reduced back to GSH by glutathione reductase (GR) catalyzed reaction in consumption of NADPH. GSH also plays a critical role in protein redox signaling through glutaredoxin (Grx) and sulfiredoxin (Srx) catalyzed reactions. During oxidant challenge, protein cysteine residues, especially those in thiolate form ($Cys\text{-}S^{-}$), can be oxidized to the sulfenic (RSOH), sulfinic (RSO_2H) and sulfonic (RSO_3H) acid, depending on the intensity of oxidative insults. Although conversion to sulfonic acid form is considered to be irreversible, sulfinic acid can be reduced back to sulfenic acid through Srx catalyzed reaction. Sulfenic acid then reacts with GSH to form protein mixed disulfides (glutathionylation), which can be reduced back to free-thiol form (deglutathionylation) through Grx or Srx catalyzed reactions using GSH as a reductant.

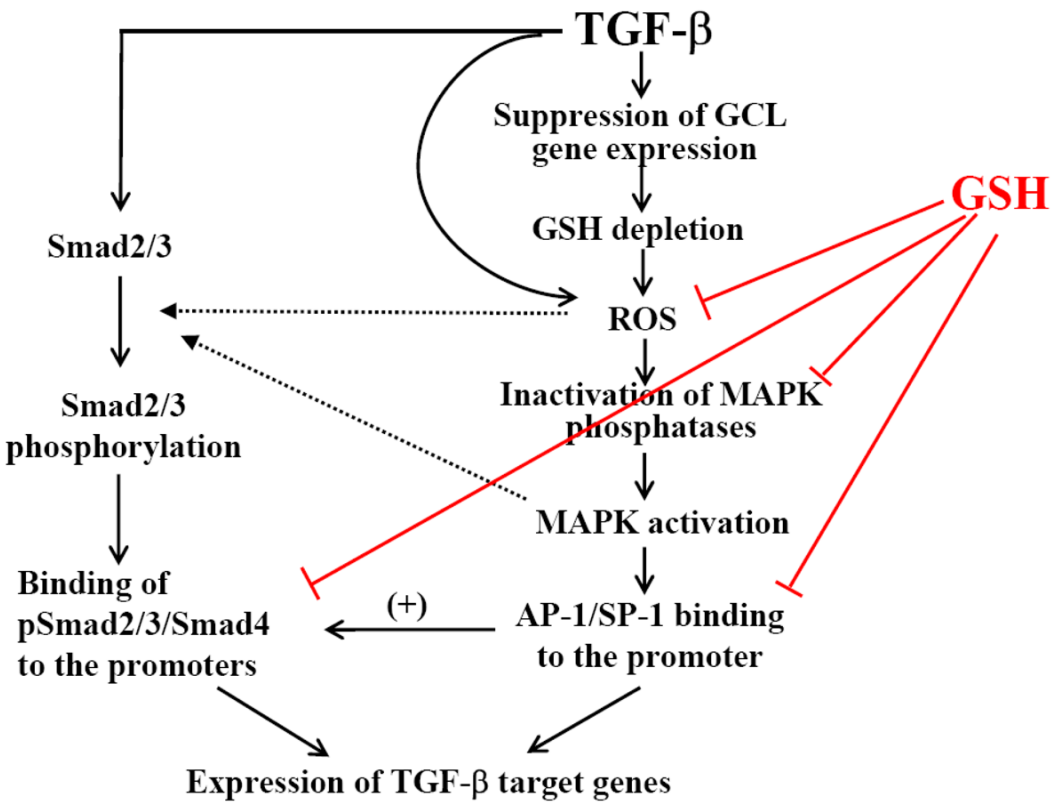

Fig 5. GSH and TGF-β-mediated fibrogenesis

TGF-β decreases intracellular GSH by suppressing the expression of glutamate cysteine ligase (GCL), the rate-limiting enzyme in *de novo* GSH synthesis. On the other hand, GSH suppresses TGF-β-induced fibrogenic effects by reducing intracellular ROS levels and by preventing oxidative modifications/inactivation of MAPK phosphatases and thus MAPK activation. GSH may also block TGF-β-induced Smad pathway activity indirectly by inhibiting MAPK pathways.