Administration of Bilins and High-Dose Biotin May Replicate the Beneficial Impact of Heme Oxygenase-1 Induction on Pathogenic Fibrosis

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Abstract

Clinical fibrotic syndromes are usually driven by excessive transforming growth factor-beta (TGF-beta) activity. Heme oxygenase-1 (HO-1), which is induced by TGF-beta in many tissues, has antifibrotic effects which are at least partially attributable to down-regulation of TGF-beta signal transduction. This down-regulation is mediated by bilirubin – a physiological inhibitor of NADPH oxidase that suppresses the oxidant stress required for optimal TGF-beta activity – and by carbon monoxide, which acts via guanylate cyclase, cGMP, and protein kinase G to inhibit the nuclear translocation of phosphorylated Smads while also impeding TGF-beta activation by blocking thrombospondin-1 transcription. It may be feasible to mimic these protective effects of HO-1 in clinical fibrotic syndromes by administering bilins (biliverdin or phycocyanobilin) and high-dose biotin – the former can function as clinical NADPH oxidase inhibitors, whereas the latter stimulates guanylate cyclase activity. This strategy may prove useful in the prevention and management of clinical fibrotic syndromes in which TGF-beta activity plays a central mediating role – likely including hepatic cirrhosis, glomerulosclerosis, interstitial fibrosis, ventricular hypertrophy, atrial fibrosis with fibrillation, pulmonary hypertension, cystic fibrosis, idiopathic pulmonary fibrosis, asthma, pancreatic fibrosis, scleroderma, and fibrostenosis in Crohn's disease.

Heme Oxygenase-1 is Protective in Fibrotic Syndromes

Boosting the activity of heme oxygenase-1 (HO-1), either by administration of inducing agents, or transfection with the HO-1gene, has shown marked antifibrotic effects in rodent models of hepatic, renal, pulmonary, and cardiac fibrosis.[1-12] Conversely, human HO-1 deficiency is associated with severe renal interstitial fibrosis.[13]

Transforming growth factor-beta (TGF-beta) activity is a central mediator of fibrosis in most fibrotic syndromes; this reflects its ability to promote synthesis of extracellular matrix proteins, while suppressing secretion of proteases and boosting that of protease inhibitors.[14-28] TGF-beta also promotes HO-1 expression at the transcriptional level,[29-32] and there is reason to suspect that this represents a protective feedback mechanism, helping to prevent an overexuberant fibrogenic response;[7] as we shall see, HO-1 acts in several complementary ways to oppose TGF-beta signal transduction.

Modulators of TGF-beta Signal Transduction

The main intracellular effects of TGF-beta are believed to reflect serine phosphorylations of the transcription factors Smad2 and Smad3, catalyzed by activated TGF-beta receptors.[33-35] These phosphorylated Smads, after binding to Smad4, can be translocated to the nucleus, where they act as transcriptional activators for TGF-beta target genes. However, TGF-beta also exerts other effects – including activation of MAP kinases – via routes that are still somewhat obscure.[36-38] In particular, TGF-

beta induces oxidant stress in many tissues by increasing NADPH oxidase activity.[39-48] This often reflects a dramatic up-regulation of Nox4 at the transcriptional level – likely reflecting Smad2/3 activation.[45-47] However, TGF-beta also promotes rapid activation of pre-existing NADPH oxidase in some tissues, an effect which may contingent on concurrent activation of Src family kinases.[42-44] In many tissues, the pro-fibrotic effects of TGF-beta are largely contingent on the increased oxidative stress evoked by NADPH oxidase activation, as demonstrated by the fact that inhibitors of this enzyme complex, as well as certain antioxidants (e.g. N-acetylcysteine, catalase), suppress the ability of TGF-beta to induce increased expression of downstream targets such as collagen type 1 and smooth muscle alpha-actin.[39-42, 45, 47, 49] Antioxidants also abrogate the pro-proliferative impact of TGF-beta on smooth muscle cells.[46] In some cell systems, the oxidative stress induced by TGF-beta up-regulates the ability of this hormone to catalyze activating phosphorylations of Smads 2 and 3.[39-41, 47] Oxidative stress may also influence Smad transcriptional activity by promoting activation of p38 and other MAP kinases.[39, 50] A number of studies indicate that p38 and/or Erk activity plays a co-factor role in Smad-dependent transcriptional activation, for reasons that remain unclear. [51-61]

Oxidant stress also promotes TGF-beta activity by boosting transcription of the TGF-beta1 gene; in this way, TGF-beta can promote its own synthesis.[62-67] This inductive effect appears to be mediated by AP-1 and NF-kappaB transcription factors, which are often activated during oxidant stress.[68-70] Oxidant stress also promotes activation of the latent TGF-beta protein; TGF-beta is synthesized in a latent precursor form that requires activation before TGF-beta can express its hormonal activity. Oxidant stress can achieve TGF-beta activation by a direct effect,[71, 72] and possibly also by stimulating production of thrombospondin-1, a protein which binds to latent TGF-beta in a way that achieves its activation.[73-75] Angiotensin II promotes transcription of the thrombospondin-1 gene via activation of the p38 and JNK MAP kinases,[76, 77] and it is reasonable to expect that oxidants may have a comparable effect. The ability of oxidant stress to promote the synthesis and activation of TGF-beta presumably explains why certain hormones which activate NADPH oxidase in their target tissues – most notably angiotensin II, endothelin, and perhaps marinobufagenin – tend to promote fibrosis in these tissues.

Another way in which oxidant stress can promote TGF-beta activity is by interfering with nitric oxide activity; as is well known, superoxide directly quenches nitric oxide, and the resulting peroxynitrite can inhibit NO synthase activity by oxidizing its cofactor tetrahydrobiopterin.[78, 79] A key role of nitric oxide is to activate guanylate cyclase, leading to increased activity of cGMP-dependent protein kinase G (PKG). PKG activity has been shown to antagonize TGF-beta signaling in several types of cells, apparently because PKG inhibits the nuclear translocation of phosphorylated Smads.[80, 81] This effect, in turn, may reflect increased proteasomal degradation of activated Smads.[81] Not surprisingly, various measures which activate PKG have shown antifibrotic effects in rodent studies.[82-88]

PKG activity can also oppose activation of TGF-beta. Recent studies with rat mesangial cells show that PKG inhibits the transcription of thrombospondin-1 by decreasing the availability of the USF2 transcription factor; this in turn is associated with reduced TGF-beta bioactivity.[89-91]

HO-1 Opposes TGF-beta Activity via Bilirubin and Guanylate Cyclase

What does any of this have to do with HO-1 activity? HO-1 generates both biliverdin and carbon monoxide (CO) by degrading heme.[92, 93] This biliverdin is rapidly reduced to bilirubin by the ubiquitously expressed enzyme biliverdin reductase.[94, 95] Bilirubin, in the nanomolar concentrations that

can be achieved intracellularly, is now known to function physiologically as a highly potent inhibitor of NADPH oxidase[96-98] – a phenomenon that at last rationalizes the potent antioxidant activity of this compound in cell cultures.[99] Oxidative stress – including that induced by TGF-beta – promotes HO-1 induction via interaction of Nrf2 with antioxidant response elements;[100-102] the resulting production of bilirubin often achieves feedback inhibition of this oxidant stress by suppressing NADPH oxidase activity. Thus, HO-1 activity would be expected to interfere with TGF-beta signal transduction by inhibiting TGF-beta's ability to evoke oxidant stress.

One of the key functions of the CO produced by HO-1 activity is to stimulate guanylate cyclase activity, an effect analogous to that of nitric oxide (albeit the maximal response to nitric oxide is considerably greater).[103-106] In the context of oxidant stress, the production and stability of nitric oxide is often compromised – whereas superoxide fails to interact with CO. Thus, when HO-1 is induced by oxidant stress, the CO that is generated may be viewed as "pinch-hitting" for nitric oxide, at least with respect to guanylate cyclase activation. As noted above, guanylate cyclase activity, via PKG, impedes TGF-beta's Smad-mediated signaling activity, while also suppressing TGF-beta activation. Thus, both the bilirubin and CO generated by HO-1 activity have the potential to dampen TGF-beta's bioactivity.

CO also promotes activation of p38 MAP kinase via MKK3 in some cell lines; this effect is independent of guanylate cyclase activation.[106, 107] This activation of p38 MAP kinase may have a countervailing impact on TGF-beta bioactivity, as inhibitors of this kinase negatively influence TGF-beta bioactivity and activation. Nonetheless, the net effect of HO-1 activity appears to be anti-fibrogenic in most models studied. Thus, TGF-beta-mediated HO-1 induction may be viewed as a protective feedback mechanism that prevents an overexuberant fibrotic response.

Orally Administrable Bilins and High-Dose Biotin May Mimic HO-1's Antifibrotic Activity

Some authors have suggested that inducers of HO-1 might be used clinically to treat or prevent fibrotic syndromes. However, an alternative approach may be feasible. Oral administration of biliverdin or of structurally homologous phycobilins found in cyanobacteria (e.g., phycocyanobilin in spirulina) has the potential to achieve systemic suppression of NADPH oxidase activity[99, 108] – mimicking the impact of locally induced HO-1 in this regard. Moreover, in slightly supraphysiological concentrations, the vitamin biotin achieves a moderate (several-fold) activation of guanylate cyclase comparable to that produced by CO.[109, 110] Biotin is well tolerated in high doses (e.g. 100 mg daily or more),[111] and intriguing effects of high-dose biotin in rodents or humans have been interpreted as reflecting guanylate cyclase activation.[112-116] Potentially, biotin's efficacy in this regard could be complemented in certain tissues by phosphodiesterase 5 inhibitors such as sildenafil.[117, 118]

These considerations suggest that, once biliverdin and or phycobilins are commercially available for oral administration, and once the pharmacokinetics of these agents and of high-dose biotin have been clarified in humans, it may be feasible to prevent and treat a range of fibrotic clinical syndromes by supplementing with bilins and high-dose biotin. Furthermore, rodent studies suggest that orally administered phycocyanobilin may be bioavailable when administered in whole spirulina or in phycocyanin (the spirulina protein which carries phycocyanobilin as a covalently-linked chromophore); [99, 108, 119] if this is also true in humans, ample dietary intakes of spirulina might be expected to have clinically useful anti-fibrotic activity.

The range of disorders in which these measures could conceivably be employed for prevention or therapy might include such disparate clinical syndromes as hepatic cirrhosis,[120, 121] glomerulosclerosis,[122, 123] tubulointerstitial fibrosis,[123, 124] cystic fibrosis,[125-127] idiopathic pulmonary fibrosis,[128, 129] asthma,[23] ventricular hypertrophy,[130-133] atrial fibrosis with fibrillation,[134-136] pulmonary hypertension,[137-139] post-radiation fibrosis,[140-142] pancreatic fibrosis,[143, 144] scleroderma,[145, 146] and fibrostenosis in Crohn's disease[28] – in all of which TGF-beta is suspected to play a central pathogenic role.

References

(1) Liu X, Simpson JA, Brunt KR, Ward CA, Hall SR, Kinobe RT, Barrette V, Tse MY, Pang SC, Pachori AS, Dzau VJ, Ogunyankin KO, Melo LG. Preemptive heme oxygenase-1 gene delivery reveals reduced mortality and preservation of left ventricular function 1 yr after acute myocardial infarction. *Am J Physiol Heart Circ Physiol* 2007 July;293(1):H48-H59.

(2) Lee SH, Seo GS, Kim HS, Woo SW, Ko G, Sohn DH. 2',4',6'-Tris(methoxymethoxy) chalcone attenuates hepatic stellate cell proliferation by a heme oxygenase-dependent pathway. *Biochem Pharmacol* 2006 November 15;72(10):1322-33.

(3) Tsui TY, Lau CK, Ma J, Glockzin G, Obed A, Schlitt HJ, Fan ST. Adeno-associated virus-mediated heme oxygenase-1 gene transfer suppresses the progression of micronodular cirrhosis in rats. *World J Gastroenterol* 2006 April 7;12(13):2016-23.

(4) Liu X, Pachori AS, Ward CA, Davis JP, Gnecchi M, Kong D, Zhang L, Murduck J, Yet SF, Perrella MA, Pratt RE, Dzau VJ, Melo LG. Heme oxygenase-1 (HO-1) inhibits postmyocardial infarct remodeling and restores ventricular function. *FASEB J* 2006 February;20(2):207-16.

(5) Pachori AS, Melo LG, Zhang L, Solomon SD, Dzau VJ. Chronic recurrent myocardial ischemic injury is significantly attenuated by pre-emptive adeno-associated virus heme oxygenase-1 gene delivery. *J Am Coll Cardiol* 2006 February 7;47(3):635-43.

(6) Kim JH, Yang JI, Jung MH, Hwa JS, Kang KR, Park DJ, Roh GS, Cho GJ, Choi WS, Chang SH. Heme oxygenase-1 protects rat kidney from ureteral obstruction via an antiapoptotic pathway. *J Am Soc Nephrol* 2006 May;17(5):1373-81.

(7) Mark A, Hock T, Kapturczak MH, Agarwal A, Hill-Kapturczak N. Induction of heme oxygenase-1 modulates the profibrotic effects of transforming growth factor-beta in human renal tubular epithelial cells. *Cell Mol Biol (Noisy -le-grand)* 2005 September 30;51(4):357-62.

(8) Gaedeke J, Noble NA, Border WA. Curcumin blocks fibrosis in anti-Thy 1 glomerulonephritis through up-regulation of heme oxygenase 1. *Kidney Int* 2005 November;68(5):2042-9.

(9) Rezzani R, Rodella L, Buffoli B, Goodman AA, Abraham NG, Lianos EA, Bianchi R. Change in renal heme oxygenase expression in cyclosporine A-induced injury. *J Histochem Cytochem* 2005 January;53(1):105-12.

(10) Liu X, Yang C, He X. Expression of HO-1 in chronic renal insufficiency. Rat kidney and implication. *J Huazhong Univ Sci Technolog Med Sci* 2003;23(3):271-4.

(11) Li L, Grenard P, Nhieu JT, Julien B, Mallat A, Habib A, Lotersztajn S. Heme oxygenase-1 is an antifibrogenic protein in human hepatic myofibroblasts. *Gastroenterology* 2003 August;125(2):460-9.

(12) Tsuburai T, Suzuki M, Nagashima Y, Suzuki S, Inoue S, Hasiba T, Ueda A, Ikehara K, Matsuse T, Ishigatsubo Y. Adenovirus-mediated transfer and overexpression of heme oxygenase 1 cDNA in lung prevents bleomycin-induced pulmonary fibrosis via a Fas-Fas ligand-independent pathway. *Hum Gene Ther* 2002 November 1;13(16):1945-60.

(13) Ohta K, Yachie A, Fujimoto K, Kaneda H, Wada T, Toma T, Seno A, Kasahara Y, Yokoyama H, Seki H, Koizumi S. Tubular injury as a cardinal pathologic feature in human heme oxygenase-1 deficiency. *Am J Kidney Dis* 2000 May;35(5):863-70.

(14) Verrecchia F, Mauviel A. Transforming growth factor-beta and fibrosis. *World J Gastroenterol* 2007 June 14;13(22):3056-62.

(15) Ihn H. Autocrine TGF-beta signaling in the pathogenesis of systemic sclerosis. *J Dermatol Sci* 2007 July 10.

(16) Willis BC, Borok Z. TGF-{beta}-induced EMT: mechanisms and implications for fibrotic lung disease. *Am J Physiol Lung Cell Mol Physiol* 2007 July 13.

(17) Ruiz-Ortega M, Rodriguez-Vita J, Sanchez-Lopez E, Carvajal G, Egido J. TGF-beta signaling in vascular fibrosis. *Cardiovasc Res* 2007 May 1;74(2):196-206.

(18) Gagliardini E, Benigni A. Therapeutic potential of TGF-beta inhibition in chronic renal failure. *Expert Opin Biol Ther* 2007 March;7(3):293-304.

(19) Lim H, Zhu YZ. Role of transforming growth factor-beta in the progression of heart failure. *Cell Mol Life Sci* 2006 November;63(22):2584-96.

(20) Bujak M, Frangogiannis NG. The role of TGF-beta signaling in myocardial infarction and cardiac remodeling. *Cardiovasc Res* 2007 May 1;74(2):184-95.

(21) Leask A. TGFbeta, cardiac fibroblasts, and the fibrotic response. *Cardiovasc Res* 2007 May 1;74(2):207-12.

(22) Sheppard D. Transforming growth factor beta: a central modulator of pulmonary and airway inflammation and fibrosis. *Proc Am Thorac Soc* 2006 July;3(5):413-7.

(23) Howell JE, McAnulty RJ. TGF-beta: its role in asthma and therapeutic potential. *Curr Drug Targets* 2006 May;7(5):547-65.

(24) Liu X, Hu H, Yin JQ. Therapeutic strategies against TGF-beta signaling pathway in hepatic fibrosis. *Liver Int* 2006 February;26(1):8-22.

(25) Breitkopf K, Haas S, Wiercinska E, Singer MV, Dooley S. Anti-TGF-beta strategies for the treatment of chronic liver disease. *Alcohol Clin Exp Res* 2005 November;29(11 Suppl):121S-31S.

(26) Mauviel A. Transforming growth factor-beta: a key mediator of fibrosis. *Methods Mol Med* 2005;117:69-80.

(27) Ellenrieder V, Schneiderhan W, Bachem M, Adler G. Fibrogenesis in the pancreas. *Rocz Akad Med Bialymst* 2004;49:40-6.

(28) Burke JP, Mulsow JJ, O'Keane C, Docherty NG, Watson RW, O'Connell PR. Fibrogenesis in Crohn's disease. *Am J Gastroenterol* 2007 February;102(2):439-48.

(29) Kutty RK, Nagineni CN, Kutty G, Hooks JJ, Chader GJ, Wiggert B. Increased expression of heme oxygenase-1 in human retinal pigment epithelial cells by transforming growth factor-beta. *J Cell Physiol* 1994 May;159(2):371-8.

(30) Ning W, Song R, Li C, Park E, Mohsenin A, Choi AM, Choi ME. TGF-beta1 stimulates HO-1 via the p38 mitogen-activated protein kinase in A549 pulmonary epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2002 November;283(5):L1094-L1102.

(31) Lin CC, Chiang LL, Lin CH, Shih CH, Liao YT, Hsu MJ, Chen BC. Transforming growth factor-beta1 stimulates heme oxygenase-1 expression via the PI3K/Akt and NF-kappaB pathways in human lung epithelial cells. *Eur J Pharmacol* 2007 April 10;560(2-3):101-9.

(32) Traylor A, Hock T, Hill-Kapturczak N. Specificity Protein 1 and Smad-Dependent Regulation of Human Heme Oxygenase-1 Gene by Transforming Growth Factor-{beta}1 in Renal Epithelial Cells. *Am J Physiol Renal Physiol* 2007 June 13.

(33) Moustakas A, Souchelnytskyi S, Heldin CH. Smad regulation in TGF-beta signal transduction. *J Cell Sci* 2001 December;114(Pt 24):4359-69.

(34) ten DP, Hill CS. New insights into TGF-beta-Smad signalling. *Trends Biochem Sci* 2004 May;29(5):265-73.

(35) Liu F. Receptor-regulated Smads in TGF-beta signaling. *Front Biosci* 2003 September 1;8:s1280-s1303.

(36) Kaminska B, Wesolowska A, Danilkiewicz M. TGF beta signalling and its role in tumour pathogenesis. *Acta Biochim Pol* 2005;52(2):329-37.

(37) Utsugi M, Dobashi K, Ishizuka T, Masubuchi K, Shimizu Y, Nakazawa T, Mori M. C-Jun-NH2-terminal kinase mediates expression of connective tissue growth factor induced by transforming growth factor-beta1 in human lung fibroblasts. *Am J Respir Cell Mol Biol* 2003 June;28(6):754-61.

(38) Chen G, Khalil N. TGF-beta1 increases proliferation of airway smooth muscle cells by phosphorylation of map kinases. *Respir Res* 2006;7:2.

(39) Rhyu DY, Yang Y, Ha H, Lee GT, Song JS, Uh ST, Lee HB. Role of reactive oxygen species in TGF-beta1-induced mitogen-activated protein kinase activation and epithelial-mesenchymal transition in renal tubular epithelial cells. *J Am Soc Nephrol* 2005 March;16(3):667-75.

(40) Li WQ, Qureshi HY, Liacini A, Dehnade F, Zafarullah M. Transforming growth factor Beta1 induction of tissue inhibitor of metalloproteinases 3 in articular chondrocytes is mediated by reactive oxygen species. *Free Radic Biol Med* 2004 July 15;37(2):196-207.

(41) Meurer SK, Lahme B, Tihaa L, Weiskirchen R, Gressner AM. N-acetyl-L-cysteine suppresses TGF-beta signaling at distinct molecular steps: the biochemical and biological efficacy of a multifunctional, antifibrotic drug. *Biochem Pharmacol* 2005 October 1;70(7):1026-34.

(42) Sato M, Kawai-Kowase K, Sato H, Oyama Y, Kanai H, Ohyama Y, Suga T, Maeno T, Aoki Y, Tamura J, Sakamoto H, Nagai R, Kurabayashi M. c-Src and hydrogen peroxide mediate transforming growth factor-beta1-induced smooth muscle cell-gene expression in 10T1/2 cells. *Arterioscler Thromb Vasc Biol* 2005 February;25(2):341-7.

(43) Sharma K, Cook A, Smith M, Valancius C, Inscho EW. TGF-beta impairs renal autoregulation via generation of ROS. *Am J Physiol Renal Physiol* 2005 May;288(5):F1069-F1077.

(44) Joo CK, Kim HS, Park JY, Seomun Y, Son MJ, Kim JT. Ligand release-independent transactivation of epidermal growth factor receptor by transforming growth factor-beta involves multiple signaling pathways. *Oncogene* 2007 July 16.

(45) Rocic P, Lucchesi PA. NAD(P)H oxidases and TGF-beta-induced cardiac fibroblast differentiation: Nox-4 gets Smad. *Circ Res* 2005 October 28;97(9):850-2.

(46) Sturrock A, Cahill B, Norman K, Huecksteadt TP, Hill K, Sanders K, Karwande SV, Stringham JC, Bull DA, Gleich M, Kennedy TP, Hoidal JR. Transforming growth factor-beta1 induces Nox4 NAD(P)H oxidase and reactive oxygen species-dependent proliferation in human pulmonary artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2006 April;290(4):L661-L673.

(47) Cucoranu I, Clempus R, Dikalova A, Phelan PJ, Ariyan S, Dikalov S, Sorescu D. NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts. *Circ Res* 2005 October 28;97(9):900-7.

(48) Jallali N, Ridha H, Thrasivoulou C, Butler P, Cowen T. Modulation of Intracellular Reactive Oxygen Species Level in Chondrocytes by IGF-1, FGF, and TGF-beta1. *Connect Tissue Res* 2007;48(3):149-58.

(49) Cao Q, Mak KM, Lieber CS. DLPC decreases TGF-beta1-induced collagen mRNA by inhibiting p38 MAPK in hepatic stellate cells. *Am J Physiol Gastrointest Liver Physiol* 2002 November;283(5):G1051-G1061.

(50) Chiu C, Maddock DA, Zhang Q, Souza KP, Townsend AR, Wan Y. TGF-beta-induced p38 activation is mediated by Rac1-regulated generation of reactive oxygen species in cultured human keratinocytes. *Int J Mol Med* 2001 September;8(3):251-5.

(51) Cao Q, Mak KM, Lieber CS. DLPC decreases TGF-beta1-induced collagen mRNA by inhibiting p38 MAPK in hepatic stellate cells. *Am J Physiol Gastrointest Liver Physiol* 2002 November;283(5):G1051-G1061.

(52) Furukawa F, Matsuzaki K, Mori S, Tahashi Y, Yoshida K, Sugano Y, Yamagata H, Matsushita M, Seki T, Inagaki Y, Nishizawa M, Fujisawa J, Inoue K. p38 MAPK mediates fibrogenic

signal through Smad3 phosphorylation in rat myofibroblasts. *Hepatology* 2003 October;38(4):879-89.

(53) Kimoto K, Nakatsuka K, Matsuo N, Yoshioka H. p38 MAPK mediates the expression of type I collagen induced by TGF-beta 2 in human retinal pigment epithelial cells ARPE-19. *Invest Ophthalmol Vis Sci* 2004 July;45(7):2431-7.

(54) Suzuki H, Uchida K, Nitta K, Nihei H. Role of mitogen-activated protein kinase in the regulation of transforming growth factor-beta-induced fibronectin accumulation in cultured renal interstitial fibroblasts. *Clin Exp Nephrol* 2004 September;8(3):188-95.

(55) Tsukada S, Westwick JK, Ikejima K, Sato N, Rippe RA. SMAD and p38 MAPK signaling pathways independently regulate alpha1(I) collagen gene expression in unstimulated and transforming growth factor-beta-stimulated hepatic stellate cells. *J Biol Chem* 2005 March 18;280(11):10055-64.

(56) Rhyu DY, Yang Y, Ha H, Lee GT, Song JS, Uh ST, Lee HB. Role of reactive oxygen species in TGF-beta1-induced mitogen-activated protein kinase activation and epithelial-mesenchymal transition in renal tubular epithelial cells. *J Am Soc Nephrol* 2005 March;16(3):667-75.

(57) Nakagawa T, Lan HY, Glushakova O, Zhu HJ, Kang DH, Schreiner GF, Bottinger EP, Johnson RJ, Sautin YY. Role of ERK1/2 and p38 mitogen-activated protein kinases in the regulation of thrombospondin-1 by TGF-beta1 in rat proximal tubular cells and mouse fibroblasts. *J Am Soc Nephrol* 2005 April;16(4):899-904.

(58) Davies M, Robinson M, Smith E, Huntley S, Prime S, Paterson I. Induction of an epithelial to mesenchymal transition in human immortal and malignant keratinocytes by TGF-beta1 involves MAPK, Smad and AP-1 signalling pathways. *J Cell Biochem* 2005 August 1;95(5):918-31.

(59) Sugaru E, Sakai M, Horigome K, Tokunaga T, Kitoh M, Hume WE, Nagata R, Nakagawa T, Taiji M. SMP-534 inhibits TGF-beta-induced ECM production in fibroblast cells and reduces mesangial matrix accumulation in experimental glomerulonephritis. *Am J Physiol Renal Physiol* 2005 November;289(5):F998-1004.

(60) Deaton RA, Su C, Valencia TG, Grant SR. Transforming growth factor-beta1-induced expression of smooth muscle marker genes involves activation of PKN and p38 MAPK. *J Biol Chem* 2005 September 2;280(35):31172-81.

(61) Chen G, Khalil N. TGF-beta1 increases proliferation of airway smooth muscle cells by phosphorylation of map kinases. *Respir Res* 2006;7:2.

(62) Iglesias-de la Cruz MC, Ruiz-Torres P, Alcami J, ez-Marques L, Ortega-Velazquez R, Chen S, Rodriguez-Puyol M, Ziyadeh FN, Rodriguez-Puyol D. Hydrogen peroxide increases extracellular matrix mRNA through TGF-beta in human mesangial cells. *Kidney Int* 2001 January;59(1):87-95.

(63) Wilmer WA, Dixon CL, Hebert C, Lu L, Rovin BH. PPAR-alpha ligands inhibit H2O2-mediated activation of transforming growth factor-beta1 in human mesangial cells. *Antioxid Redox Signal* 2002 December;4(6):877-84.

(64) Ha H, Lee HB. Reactive oxygen species as glucose signaling molecules in mesangial cells cultured under high glucose. *Kidney Int Suppl* 2000 September;77:S19-S25.

(65) Lee HB, Yu MR, Yang Y, Jiang Z, Ha H. Reactive oxygen species-regulated signaling pathways in diabetic nephropathy. *J Am Soc Nephrol* 2003 August;14(8 Suppl 3):S241-S245.

(66) Hamada Y, Miyata S, Nii-Kono T, Kitazawa R, Kitazawa S, Higo S, Fukunaga M, Ueyama S, Nakamura H, Yodoi J, Fukagawa M, Kasuga M. Overexpression of thioredoxin1 in transgenic mice suppresses development of diabetic nephropathy. *Nephrol Dial Transplant* 2007 June;22(6):1547-57.

(67) Chao J, Li HJ, Yao YY, Shen B, Gao L, Bledsoe G, Chao L. Kinin infusion prevents renal inflammation, apoptosis, and fibrosis via inhibition of oxidative stress and mitogen-activated protein kinase activity. *Hypertension* 2007 March;49(3):490-7.

(68) Lee KY, Ito K, Hayashi R, Jazrawi EP, Barnes PJ, Adcock IM. NF-kappaB and activator protein 1 response elements and the role of histone modifications in IL-1beta-induced TGF-beta1 gene transcription. *J Immunol* 2006 January 1;176(1):603-15.

(69) Cohen MP, Shea E, Chen S, Shearman CW. Glycated albumin increases oxidative stress, activates NF-kappa B and extracellular signal-regulated kinase (ERK), and stimulates ERK-dependent transforming growth factor-beta 1 production in macrophage RAW cells. *J Lab Clin Med* 2003 April;141(4):242-9.

(70) Weigert C, Brodbeck K, Bierhaus A, Haring HU, Schleicher ED. c-Fos-driven transcriptional activation of transforming growth factor beta-1: inhibition of high glucose-induced promoter activity by thiazolidinediones. *Biochem Biophys Res Commun* 2003 May 2;304(2):301-7.

(71) Barcellos-Hoff MH, Dix TA. Redox-mediated activation of latent transforming growth factor-beta 1. *Mol Endocrinol* 1996 September;10(9):1077-83.

(72) Jobling MF, Mott JD, Finnegan MT, Jurukovski V, Erickson AC, Walian PJ, Taylor SE, Ledbetter S, Lawrence CM, Rifkin DB, Barcellos-Hoff MH. Isoform-specific activation of latent transforming growth factor beta (LTGF-beta) by reactive oxygen species. *Radiat Res* 2006 December;166(6):839-48.

(73) Schultz-Cherry S, Lawler J, Murphy-Ullrich JE. The type 1 repeats of thrombospondin 1 activate latent transforming growth factor-beta. *J Biol Chem* 1994 October 28;269(43):26783-8.

(74) Yevdokimova N, Wahab NA, Mason RM. Thrombospondin-1 is the key activator of TGF-beta1 in human mesangial cells exposed to high glucose. *J Am Soc Nephrol* 2001 April;12(4):703-12.

(75) Murphy-Ullrich JE, Poczatek M. Activation of latent TGF-beta by thrombospondin-1: mechanisms and physiology. *Cytokine Growth Factor Rev* 2000 March;11(1-2):59-69.

(76) Naito T, Masaki T, Nikolic-Paterson DJ, Tanji C, Yorioka N, Kohno N. Angiotensin II induces thrombospondin-1 production in human mesangial cells via p38 MAPK and JNK: a mechanism for activation of latent TGF-beta1. *Am J Physiol Renal Physiol* 2004 February;286(2):F278-F287.

(77) Zhou Y, Poczatek MH, Berecek KH, Murphy-Ullrich JE. Thrombospondin 1 mediates angiotensin II induction of TGF-beta activation by cardiac and renal cells under both high and low glucose conditions. *Biochem Biophys Res Commun* 2006 January 13;339(2):633-41.

(78) Milstien S, Katusic Z. Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. *Biochem Biophys Res Commun* 1999 October 5;263(3):681-4.

(79) Kohnen SL, Mouithys-Mickalad AA, by-Dupont GP, Deby CM, Lamy ML, Noels AF. Oxidation of tetrahydrobiopterin by peroxynitrite or oxoferryl species occurs by a radical pathway. *Free Radic Res* 2001 December;35(6):709-21.

(80) Li P, Oparil S, Novak L, Cao X, Shi W, Lucas J, Chen YF. ANP signaling inhibits TGF-beta-induced Smad2 and Smad3 nuclear translocation and extracellular matrix expression in rat pulmonary arterial smooth muscle cells. *J Appl Physiol* 2007 January;102(1):390-8.

(81) Saura M, Zaragoza C, Herranz B, Griera M, ez-Marques L, Rodriguez-Puyol D, Rodriguez-Puyol M. Nitric oxide regulates transforming growth factor-beta signaling in endothelial cells. *Circ Res* 2005 November 25;97(11):1115-23.

(82) Keil A, Blom IE, Goldschmeding R, Rupprecht HD. Nitric oxide down-regulates connective tissue growth factor in rat mesangial cells. *Kidney Int* 2002 August;62(2):401-11.

(83) Ozaki M, Kawashima S, Yamashita T, Hirase T, Ohashi Y, Inoue N, Hirata K, Yokoyama M. Overexpression of endothelial nitric oxide synthase attenuates cardiac hypertrophy induced by chronic isoproterenol infusion. *Circ J* 2002 September;66(9):851-6.

(84) Valente EG, Vernet D, Ferrini MG, Qian A, Rajfer J, Gonzalez-Cadavid NF. L-arginine and phosphodiesterase (PDE) inhibitors counteract fibrosis in the Peyronie's fibrotic plaque and related fibroblast cultures. *Nitric Oxide* 2003 December;9(4):229-44.

(85) Zhou Z, Song R, Fattman CL, Greenhill S, Alber S, Oury TD, Choi AM, Morse D. Carbon monoxide suppresses bleomycin-induced lung fibrosis. *Am J Pathol* 2005 January;166(1):27-37.

(86) Wang Y, Kramer S, Loof T, Martini S, Kron S, Kawachi H, Shimizu F, Neumayer HH, Peters H. Stimulation of soluble guanylate cyclase slows progression in anti-thy1-induced chronic glomerulosclerosis. *Kidney Int* 2005 July;68(1):47-61.

(87) Wang Y, Kramer S, Loof T, Martini S, Kron S, Kawachi H, Shimizu F, Neumayer HH, Peters H. Enhancing cGMP in experimental progressive renal fibrosis: soluble guanylate cyclase stimulation vs. phosphodiesterase inhibition. *Am J Physiol Renal Physiol* 2006 January;290(1):F167-F176.

(88) Masuyama H, Tsuruda T, Kato J, Imamura T, Asada Y, Stasch JP, Kitamura K, Eto T. Soluble guanylate cyclase stimulation on cardiovascular remodeling in angiotensin II-induced hypertensive rats. *Hypertension* 2006 November;48(5):972-8.

(89) Wang S, Shiva S, Poczatek MH, rley-Usmar V, Murphy-Ullrich JE. Nitric oxide and cGMP-dependent protein kinase regulation of glucose-mediated thrombospondin 1-dependent transforming growth factor-beta activation in mesangial cells. *J Biol Chem* 2002 March 22;277(12):9880-8.

(90) Wang S, Wu X, Lincoln TM, Murphy-Ullrich JE. Expression of constitutively active cGMP-dependent protein kinase prevents glucose stimulation of thrombospondin 1 expression and TGF-beta activity. *Diabetes* 2003 August;52(8):2144-50.

(91) Wang S, Skorczewski J, Feng X, Mei L, Murphy-Ullrich JE. Glucose up-regulates thrombospondin 1 gene transcription and transforming growth factor-beta activity through antagonism of cGMP-dependent protein kinase repression via upstream stimulatory factor 2. *J Biol Chem* 2004 August 13;279(33):34311-22.

(92) Bach FH. Heme oxygenase-1 as a protective gene. *Wien Klin Wochenschr* 2002;114 Suppl 4:1-3.

(93) Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* 2003 August;24(8):449-55.

(94) Baranano DE, Rao M, Ferris CD, Snyder SH. Biliverdin reductase: a major physiologic cytoprotectant. *Proc Natl Acad Sci U S A* 2002 December 10;99(25):16093-8.

(95) Sedlak TW, Snyder SH. Bilirubin benefits: cellular protection by a biliverdin reductase antioxidant cycle. *Pediatrics* 2004 June;113(6):1776-82.

(96) Lanone S, Bloc S, Foresti R, Almolki A, Taille C, Callebert J, Conti M, Goven D, Aubier M, Dureuil B, El-Benna J, Motterlini R, Boczkowski J. Bilirubin decreases nos2 expression via inhibition of NAD(P)H oxidase: implications for protection against endotoxic shock in rats. *FASEB J* 2005 November;19(13):1890-2.

(97) Matsumoto H, Ishikawa K, Itabe H, Maruyama Y. Carbon monoxide and bilirubin from heme oxygenase-1 suppresses reactive oxygen species generation and plasminogen activator inhibitor-1 induction. *Mol Cell Biochem* 2006 October;291(1-2):21-8.

(98) Jiang F, Roberts SJ, Datla S, Dusting GJ. NO modulates NADPH oxidase function via heme oxygenase-1 in human endothelial cells. *Hypertension* 2006 November;48(5):950-7.

(99) McCarty MF. "Iatrogenic Gilbert sydrome" - a strategy for reducing vascular and cancer risk by increasing plasma unconjugated bilirubin. *Medical Hypotheses* 2007;accepted for publication.

(100) Gong P, Stewart D, Hu B, Li N, Cook J, Nel A, Alam J. Activation of the mouse heme oxygenase-1 gene by 15-deoxy-Delta(12,14)-prostaglandin J(2) is mediated by the stress response elements and transcription factor Nrf2. *Antioxid Redox Signal* 2002 April;4(2):249-57.

(101) Li N, Alam J, Venkatesan MI, Eiguren-Fernandez A, Schmitz D, Di SE, Slaughter N, Killeen E, Wang X, Huang A, Wang M, Miguel AH, Cho A, Sioutas C, Nel AE. Nrf2 is a key transcription factor that regulates antioxidant defense in macrophages and epithelial cells: protecting against the proinflammatory and oxidizing effects of diesel exhaust chemicals. *J Immunol* 2004 September 1;173(5):3467-81.

(102) Zhang J, Ohta T, Maruyama A, Hosoya T, Nishikawa K, Maher JM, Shibahara S, Itoh K, Yamamoto M. BRG1 interacts with Nrf2 to selectively mediate HO-1 induction in response to oxidative stress. *Mol Cell Biol* 2006 November;26(21):7942-52.

(103) Ingi T, Cheng J, Ronnett GV. Carbon monoxide: an endogenous modulator of the nitric oxide-cyclic GMP signaling system. *Neuron* 1996 April;16(4):835-42.

(104) Stone JR, Marletta MA. Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochemistry* 1994 May 10;33(18):5636-40.

(105) Verma A, Hirsch DJ, Glatt CE, Ronnett GV, Snyder SH. Carbon monoxide: a putative neural messenger. *Science* 1993 January 15;259(5093):381-4.

(106) Kim HP, Ryter SW, Choi AM. CO as a cellular signaling molecule. *Annu Rev Pharmacol Toxicol* 2006;46:411-49.

(107) Otterbein LE, Otterbein SL, Ifedigbo E, Liu F, Morse DE, Fearns C, Ulevitch RJ, Knickelbein R, Flavell RA, Choi AM. MKK3 mitogen-activated protein kinase pathway mediates carbon monoxide-induced protection against oxidant-induced lung injury. *Am J Pathol* 2003 December;163(6):2555-63.

(108) McCarty MF. Clinical potential of spirulina as a source of phycocyanobilin. *J Medicinal Food* 2007;in press.

(109) Vesely DL. Biotin enhances guanylate cyclase activity. *Science* 1982 June 18;216(4552):1329-30.

(110) Spence JT, Koudelka AP. Effects of biotin upon the intracellular level of cGMP and the activity of glucokinase in cultured rat hepatocytes. *J Biol Chem* 1984 May 25;259(10):6393-6.

(111) Mock DM. Biotin. In: Ziegler EE, Filer LJJr, eds. *Present Knowledge in Nutrition*. 7trh ed. Washington,D.C.: ILSI Press; 1996. p. 220-35.

(112) Chauhan J, Dakshinamurti K. Transcriptional regulation of the glucokinase gene by biotin in starved rats. *J Biol Chem* 1991 June 5;266(16):10035-8.

(113) Dakshinamurti K, Li W. Transcriptional regulation of liver phosphoenolpyruvate carboxykinase by biotin in diabetic rats. *Mol Cell Biochem* 1994 March 30;132(2):127-32.

(114) Koutsikos D, Agroyannis B, Tzanatos-Exarchou H. Biotin for diabetic peripheral neuropathy. *Biomed Pharmacother* 1990;44(10):511-4.

(115) McCarty MF. High-dose biotin, an inducer of glucokinase expression, may synergize with chromium picolinate to enable a definitive nutritional therapy for type II diabetes. *Med Hypotheses* 1999 May;52(5):401-6.

(116) Revilla-Monsalve C, Zendejas-Ruiz I, Islas-Andrade S, Baez-Saldana A, Palomino-Garibay MA, Hernandez-Quiroz PM, Fernandez-Mejia C. Biotin supplementation reduces plasma triacylglycerol and VLDL in type 2 diabetic patients and in nondiabetic subjects with hypertriglyceridemia. *Biomed Pharmacother* 2006 May;60(4):182-5.

(117) Corbin J. PDE5 inhibition and fibrosis. *Int J Impot Res* 2005 November;17(6):546.

(118) Ferrini MG, Kovanecz I, Sanchez S, Vernet D, Davila HH, Rajfer J, Gonzalez-Cadavid NF. Long-term continuous treatment with sildenafil ameliorates aging-related erectile dysfunction and the underlying corporal fibrosis in the rat. *Biol Reprod* 2007 May;76(5):915-23.

(119) Romay C, Gonzalez R, Ledon N, Remirez D, Rimbau V. C-phycocyanin: a biliprotein with antioxidant, anti-inflammatory and neuroprotective effects. *Curr Protein Pept Sci* 2003 June;4(3):207-16.

(120) Liu X, Hu H, Yin JQ. Therapeutic strategies against TGF-beta signaling pathway in hepatic fibrosis. *Liver Int* 2006 February;26(1):8-22.

(121) Gressner AM, Weiskirchen R. Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-beta as major players and therapeutic targets. *J Cell Mol Med* 2006 January;10(1):76-99.

(122) Chen S, Jim B, Ziyadeh FN. Diabetic nephropathy and transforming growth factor-beta: transforming our view of glomerulosclerosis and fibrosis build-up. *Semin Nephrol* 2003 November;23(6):532-43.

(123) Gagliardini E, Benigni A. Therapeutic potential of TGF-beta inhibition in chronic renal failure. *Expert Opin Biol Ther* 2007 March;7(3):293-304.

(124) Ina K, Kitamura H, Tatsukawa S, Takayama T, Fujikura Y, Shimada T. Transformation of interstitial fibroblasts and tubulointerstitial fibrosis in diabetic nephropathy. *Med Electron Microsc* 2002 June;35(2):87-95.

(125) Arkwright PD, Laurie S, Super M, Pravica V, Schwarz MJ, Webb AK, Hutchinson IV. TGF-beta(1) genotype and accelerated decline in lung function of patients with cystic fibrosis. *Thorax* 2000 June;55(6):459-62.

(126) Lewindon PJ, Pereira TN, Hoskins AC, Bridle KR, Williamson RM, Shepherd RW, Ramm GA. The role of hepatic stellate cells and transforming growth factor-beta(1) in cystic fibrosis liver disease. *Am J Pathol* 2002 May;160(5):1705-15.

(127) Drumm ML, Konstan MW, Schluchter MD, Handler A, Pace R, Zou F, Zariwala M, Fargo D, Xu A, Dunn JM, Darrah RJ, Dorfman R, Sandford AJ, Corey M, Zielenski J, Durie P, Goddard K, Yankaskas JR, Wright FA, Knowles MR. Genetic modifiers of lung disease in cystic fibrosis. *N Engl J Med* 2005 October 6;353(14):1443-53.

(128) Sheppard D. Transforming growth factor beta: a central modulator of pulmonary and airway inflammation and fibrosis. *Proc Am Thorac Soc* 2006 July;3(5):413-7.

(129) Willis BC, Borok Z. TGF-{beta}-induced EMT: mechanisms and implications for fibrotic lung disease. *Am J Physiol Lung Cell Mol Physiol* 2007 July 13.

(130) Rosenkranz S. TGF-beta1 and angiotensin networking in cardiac remodeling. *Cardiovasc Res* 2004 August 15;63(3):423-32.

(131) Huang J, Qin GH, Hu CX, Gong LY, Cheng FZ, Ma YX, Lu ZY. Effects of transforming growth factor-beta1 and signal protein Smad3 on rat cardiomyocyte hypertrophy. *Chin Med J (Engl)* 2004 October;117(10):1471-5.

(132) Ikeuchi M, Tsutsui H, Shiomi T, Matsusaka H, Matsushima S, Wen J, Kubota T, Takeshita A. Inhibition of TGF-beta signaling exacerbates early cardiac dysfunction but prevents late remodeling after infarction. *Cardiovasc Res* 2004 December 1;64(3):526-35.

(133) Khan R. Examining Potential Therapies Targeting Myocardial Fibrosis through the Inhibition of Transforming Growth Factor-Beta 1. *Cardiology* 2007 February 15;108(4):368-80.

(134) Verheule S, Sato T, Everett T, Engle SK, Otten D, Rubart-von der LM, Nakajima HO, Nakajima H, Field LJ, Olgin JE. Increased vulnerability to atrial fibrillation in transgenic mice with selective atrial fibrosis caused by overexpression of TGF-beta1. *Circ Res* 2004 June 11;94(11):1458-65.

(135) Khan R, Sheppard R. Fibrosis in heart disease: understanding the role of transforming growth factor-beta in cardiomyopathy, valvular disease and arrhythmia. *Immunology* 2006 May;118(1):10-24.

(136) Everett TH, Olgin JE. Atrial fibrosis and the mechanisms of atrial fibrillation. *Heart Rhythm* 2007 March;4(3 Suppl):S24-S27.

(137) Sturrock A, Cahill B, Norman K, Huecksteadt TP, Hill K, Sanders K, Karwande SV, Stringham JC, Bull DA, Gleich M, Kennedy TP, Hoidal JR. Transforming growth factor-beta1 induces Nox4 NAD(P)H oxidase and reactive oxygen species-dependent proliferation in human pulmonary artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2006 April;290(4):L661-L673.

(138) Chen YF, Feng JA, Li P, Xing D, Zhang Y, Serra R, Ambalavanan N, Majid-Hassan E, Oparil S. Dominant negative mutation of the TGF-beta receptor blocks hypoxia-induced pulmonary vascular remodeling. *J Appl Physiol* 2006 February;100(2):564-71.

(139) Arciniegas E, Frid MG, Douglas IS, Stenmark KR. Perspectives on endothelial-to-mesenchymal transition: potential contribution to vascular remodeling in chronic pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2007 July;293(1):L1-L8.

(140) Xavier S, Piek E, Fujii M, Javelaud D, Mauviel A, Flanders KC, Samuni AM, Felici A, Reiss M, Yarkoni S, Sowers A, Mitchell JB, Roberts AB, Russo A. Amelioration of radiation-induced fibrosis: inhibition of transforming growth factor-beta signaling by halofuginone. *J Biol Chem* 2004 April 9;279(15):15167-76.

(141) Barcellos-Hoff MH. Radiation-induced transforming growth factor beta and subsequent extracellular matrix reorganization in murine mammary gland. *Cancer Res* 1993 September 1;53(17):3880-6.

(142) Canney PA, Dean S. Transforming growth factor beta: a promotor of late connective tissue injury following radiotherapy? *Br J Radiol* 1990 August;63(752):620-3.

(143) Menke A, Adler G. TGFbeta-induced fibrogenesis of the pancreas. *Int J Gastrointest Cancer* 2002;31(1-3):41-6.

(144) Patel M, Fine DR. Fibrogenesis in the pancreas after acinar cell injury. *Scand J Surg* 2005;94(2):108-11.

(145) Verrecchia F, Mauviel A, Farge D. Transforming growth factor-beta signaling through the Smad proteins: role in systemic sclerosis. *Autoimmun Rev* 2006 October;5(8):563-9.

(146) Ihn H. Autocrine TGF-beta signaling in the pathogenesis of systemic sclerosis. *J Dermatol Sci* 2007 July 10.