Smad7-dependent regulation of heme oxygenase-1 by transforming growth factor-β in human renal epithelial cells

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Running title:
Regulation of heme oxygenase-1 expression by TGF-β

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Summary

Heme oxygenase-1 (HO-1), a 32kD microsomal enzyme, is induced as a beneficial and adaptive response in cells/tissues exposed to oxidative stress. Transforming growth factor-β1 (TGF-β1) is a regulatory cytokine which has been implicated in a variety of renal diseases where it promotes extracellular matrix deposition and pro-inflammatory events. We hypothesize that the release of TGF-β1 via autocrine and/or paracrine pathways may induce HO-1 and serve as a protective response in renal injury. To understand the molecular mechanism of HO-1 induction by TGF-β1, we exposed confluent human renal proximal tubule cells to TGF-β1 and observed a significant induction of HO-1 mRNA at 4h with a maximal induction at 8h. This induction was accompanied by increased expression of HO-1 protein. TGF-β1 treatment in conjunction with actinomycin D and/or cycloheximide demonstrated that induction of HO-1 mRNA requires de novo transcription and in part protein synthesis. Exposure to TGF-β1 resulted in marked induction of Smad7 mRNA with no effect on Smad6 expression. Overexpression of Smad7, but not Smad6, inhibited TGF-β1-mediated induction of endogenous HO-1 gene expression. We speculate that the induction of HO-1 in the kidney is an adaptive response to the inflammatory effects of TGF-β1 and manipulations of the Smad pathway to alter HO-1 expression may serve as a potential therapeutic target.
Introduction

Kidney diseases such as IgA nephropathy, focal and segmental glomerulosclerosis, crescentic glomerulonephritis, lupus nephritis, diabetic nephropathy and chronic rejection are characterized by the deposition of extracellular matrix and have increased expression of transforming growth factor-β1 (TGF-β1) in glomeruli and tubulointerstitium as compared to normal kidneys (1, 2). TGF-β1 is a member of the TGF-β superfamily, which includes the TGF-β’s, the activins/inhibins, and the bone morphogenetic proteins (BMP). TGF-β1 is implicated in a wide range of cellular events such as cell proliferation and migration, wound healing, inflammatory responses, and stimulation of the extracellular matrix components (3, 4). In response to an injury, TGF-β1 and other growth factors are released via autocrine and/or paracrine mechanisms to maintain cellular homeostasis. While chronic elevation of TGF-β1 plays an important role in the progression of renal diseases, TGF-β1 can also stabilize and attenuate tissue injury through the activation of cytoprotective proteins. We hypothesize that the paradoxical effects of TGF-β1 in response to cellular injury may be in part mediated by the induction of an antioxidant enzyme, heme oxygenase-1 (HO-1).

Heme oxygenase is a microsomal enzyme that catalyzes the conversion of heme to biliverdin, releasing equimolar amounts of carbon monoxide (CO) and iron (5). Subsequently, biliverdin is converted to bilirubin by biliverdin reductase (5). Three isoforms of heme oxygenase (HO-1, HO-2 and HO-3) have been described (5, 6). HO-1 is inducible while HO-2 is refractory to most stimuli and, thus, the constitutive form. The third isoform, HO-3, with properties similar to HO-2 has only recently been described (6). HO-1 is induced by heme products and a wide variety of non-heme stimuli which include hydrogen peroxide, ultraviolet A radiation, heavy metals, endotoxin, cytokines and oxidant stress (7-9). The induction of HO-1 is thought to be an
adaptive response that offers cytoprotection to cells/tissue against oxidant stress (9). A previous study reported an increase of HO-1 mRNA and protein expression by TGF-β1 in human retinal pigment epithelial cells and suggested that induction of HO-1 attenuates the adverse effects of elevated TGF-β1 (10). The initial purpose of this study was to evaluate the molecular mechanism of TGF-β1-mediated induction of the human HO-1 gene in renal epithelial cells as well as to elucidate the signaling pathways involved in HO-1 gene induction.

TGF-β initiates signaling through type I (TβR-I) and type II (TβR-II) receptors, binding directly with TβR-II which then interacts transiently with TβR-I forming a heteromeric complex (11). Ligand binding is followed by TβR-II transphosphorylation of TβR-I, mainly in the conserved glycine- and serine-rich domain (GS), resulting in activation of the TβR-I kinase that initiates downstream signaling events. The molecular mechanism of TGF-β signal transduction from the cell surface to the nucleus has been recently identified to occur through a novel group of structurally related proteins called Smads (12, 13). Smad proteins, originally named as the human analogue of the Mad protein (Mothers against decapentaplegic), serve as signaling molecules that mediate the downstream effects of TGF-β. Vertebrate Smads are subdivided based on structural and functional considerations into receptor-regulated Smads, common mediator Smads and antagonistic- or anti-Smads. Receptor-regulated Smads are pathway specific. Smad2 and Smad3 are involved in TGF-β/activin signaling while Smad1 and presumably its close homologues Smad5 and Smad8 are mediators in BMP signaling (12-16). Receptor-regulated Smads are directly phosphorylated by activated TGF-β family receptors. Following phosphorylation, receptor-regulated Smad proteins oligomerize with the common mediator Smad, which includes Smad4 in vertebrates (17). The receptor-activated Smads bind Smad4 in the cytoplasm and carry it into the nucleus (18) where the heteromeric complex can interact directly with DNA and/or
bind to transcription factors such as FAST-1, CEBP/P300 and others to activate targeted genes (19-21). Antagonistic- or anti-Smads (Smad6, Smad7) are inhibitors of TGF-β1 mediated signaling events. At lower concentrations Smad6 is a specific inhibitor of BMP signaling (22) whereas Smad7 inhibits BMP and TGF-β1 signaling (23-25).

The molecular mechanism of TGF-β1 signal transduction through Smads has been studied extensively in cancer models. The anti-Smad pathway has been shown to inhibit activation of other genes such as human plasminogen activator inhibitor-I (PAI-I) and collagen (25-29). However, most of these studies have demonstrated effects of Smad6/7 expression on TGF-β1-responsiveness using only transiently transfected promoter-reporter systems (eg. p3TP-Lux) (25-29). In this regard, our studies have not only demonstrated that TGF-β1 induces HO-1 and Smad7 in human renal epithelial cells but also represent the first demonstration that overexpression of Smad7 inhibits the induction of the endogenous HO-1 gene.
Experimental Procedures

Reagents:

Recombinant human TGF-β1 and anti-TGF-β antibody were obtained from R&D systems, Minneapolis, MN. Another preparation of recombinant human TGF-β1 was obtained from Genzyme (Cambridge, MA). Actinomycin D, anti-FLAG® M2 monoclonal antibody, anti-mouse IgG-agarose, cycloheximide, and hemin were purchased from Sigma Chemical Co., St. Louis, MO. Anti-HA High Affinity rat monoclonal antibody, peroxidase-conjugated goat anti-mouse IgG antibody and streptavidin-POD were purchased from Boehringer Mannheim, Mannheim (Germany). Calf serum, fetal bovine serum, G418 (Geneticin), and transfection reagents Lipofectamine™, Lipofectamine™ Plus and OptiMEM were obtained from Gibco BRL Life Technologies, Gaithersberg, MD. Fugene™ transfection reagent was purchased from Roche Molecular Biochemicals, Indianapolis, IN. Kinase inhibitors PD98059 and SB203580 were obtained from Calbiochem, San Diego, CA.

Cell culture:

Human proximal tubule cells (HPTC; Clonetics, Walkersville, MD) were grown in renal epithelial basal medium (Clonetics) supplemented with fetal bovine serum (5%), gentamicin (50 µg/ml), amphotericin B (50 µg/ml), insulin (5 µg/ml), transferrin (10 µg/ml), triiodothyronine (6.5 ng/ml), hydrocortisone (0.5 µg/ml), epinephrine (0.5 µg/ml), and human epidermal growth factor (10 ng/ml), at 37°C in 95% air and 5% carbon dioxide. HPTC were used over a range of 4 to 5 passages. Human embryonic kidney cells (HEK 293 cells; ATCC, Rockville, MD) were grown in Dulbecco’s minimum essential medium supplemented with calf serum (10%) and...
HEPES buffer (25 mM), at 37°C in 90% air and 10% carbon dioxide.

Northern analysis:

Total RNA was extracted from cultured cells grown in 60 mm plates using the method described by Chomczynski and Sacchi (30) and purified using RNeasy mini kits (Qiagen). The RNA (10-15 µg) was electrophoresed on a 1% agarose gel containing formaldehyde, electrotransferred to a nylon membrane, and hybridized with a 32P-labeled 1.0kb human HO-1 cDNA probe. For Smad6/7 expression, poly A+ RNA was obtained by purifying total RNA using Oligotex mRNA mini kits (Qiagen) and probed with cDNAs for human Smad6/7. The nylon membranes were stripped and rehybridized with a human GAPDH cDNA probe to control for handling and loading. The blots were also reprobed with a TβR-I (TD) cDNA probe to confirm overexpression. Following autoradiography, quantitation was performed by densitometry using NIH Image.

Plasmid constructs and transfections:

Expression constructs for FLAG-tagged-pcDNA3-Smad6 and Smad7 as well as HA-tagged-pcDNA3-constitutively active TβR-I (TD) were generously provided by Takeshi Imamura and Kohei Miyazono and have been described previously (23). Stable transfectants using either vector, Smad6 or Smad7 expression plasmids were made in HEK 293 cells by antibiotic selection (G418, 400µg/ml) and clones confirmed by northern and immunoblot analyses. Transfections were performed using Lipofectamine™ Plus reagent. The expression plasmid for TβR-I (TD), a constitutively active mutant of TβR-I, was transfected into the above stable clones of HEK 293 cells at 50% confluency using Fugene™ 6. Replacement of Thr204 with aspartic...
acid in the GS domain results in constitutive activation of TβR-I such that TGF-β specific responses (e.g. growth inhibition and extracellular matrix deposition) occur in the absence of ligand or TβR-II (31). Transfection efficiency was 40% based on co-transfections using a β-gal expression plasmid (pcDNA3.1/Lac Z).

*Immunoblot analysis and Immunoprecipitation:*

For HO-1 immunoblots, cells were treated with stimulus for the concentrations and times indicated. Cells were then washed twice with ice-cold PBS and lysed in a buffer containing a broad spectrum cocktail of protease inhibitors consisting of 10 µg/ml aprotinin, 5 mM EDTA, 1 µg/ml leupeptin, 0.7 µg/ml pepstatin A, 1 mM PMSF (phenylmethanesulfonyl fluoride) and triton X100. Protein concentration of lysates was assessed by the bicinchoninic acid assay (Pierce, Rockford, IL). Samples were separated in a 10% SDS-polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane. The membranes were incubated for 1.5 h with the anti-HO-1 antibody (1:500 dilution) followed by incubation with peroxidase-conjugated goat anti-rabbit IgG antibody (1:10,000 dilution) for 1 h. Labeled protein bands were examined by using a chemiluminescence method according to the manufacturer’s recommendation.

Flag tagged Smad6, Smad7 and HA-tagged TβR-I (TD) were immunoprecipitated prior to immunoblot analysis. For immunoprecipitation, cells were washed twice with ice-cold PBS and lysed in a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet 40, 0.5% sodium deoxycholate along with a broad spectrum cocktail of protease inhibitors (as described above). The supernatant of cell lysates from vector alone, Smad6 or Smad7 stable transfectants were immunoprecipitated with anti-mouse IgG-agarose (Sigma) bound to anti-FLAG® M2 (Sigma) according to the manufacturers directions. To confirm the presence of TβR-I (TD) protein, the supernatants from cells transiently transfected with TβR-I (TD) were immunoprecipitated with
protein G agarose (Boehringer Mannheim) followed by an anti-HA High Affinity rat monoclonal antibody (Boehringer Mannheim) as indicated by the manufacturer. Protein concentration of lysates was assessed by the bicinchoninic acid assay (Pierce, Rockford, IL).

Immunoprecipitated samples were then subjected to immunoblot analysis by separation on a 7.5% SDS-polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane. For FLAG-tagged Smad6 and Smad7, membranes were incubated with a murine anti-FLAG® M2 monoclonal antibody (10 µg/ml) in 5% non-fat dry milk/TBS-Tween (0.05%) for 1 hr at room temperature. Membranes were washed with TBS-Tween (0.05%) and then incubated with anti-mouse IgG peroxidase conjugate (secondary antibody) at the manufacturers recommended concentration in TBS-Tween (0.05%) for 1 h. For HA-tagged TβR-I (TD), membranes were incubated following manufacturer recommendations with anti-HA High Affinity rat monoclonal antibody (200 ng/ml) in 5% non-fat dry milk/TBS for 1 h, washed with TBS-Tween (0.05%), and incubated with peroxidase-conjugated goat anti-mouse IgG antibody (20 ng/ml; Boehringer Mannheim) for 30 min. The HA-immunoblots were washed, and incubated with streptavidin-POD (15 mU/ml; Boehringer Mannheim) for 30 min. All labeled protein bands (Flag-or HA-tagged) were examined using the chemiluminescence method (Pierce).
Results

*TGF-β1 induces HO-1 mRNA and protein expression in human renal proximal tubule cells.* Increased levels of TGF-β1 play an important role in the pathogenesis of renal injury (1, 2). A previous study by Kutty et al. reported an increase of HO-1 mRNA and protein by TGF-β1 in retinal pigment epithelial cells (10). We hypothesized that the induction of cytoprotective enzymes such as HO-1 may explain the paradoxical effects of TGF-β1 in response to renal injury. Steady-state levels of HO-1 mRNA in HPTC were examined by northern blot analyses after exposure to TGF-β1. HPTC treated with TGF-β1 at concentrations of 0.05, 0.5, and 2.0 ng/ml exhibited a significant, dose-dependent induction in HO-1 mRNA of 6, 12 and 13 fold, respectively, over control (PBS) at 4 h (Fig. 1A). Hemin (5µM) was used as a positive control. Immunoblot analysis demonstrated that induction of HO-1 mRNA by TGF-β1, obtained from two different sources, was accompanied by a 3.5 fold increase in HO-1 protein (Fig. 1B). Furthermore, HO-1 mRNA levels were induced by TGF-β1 (2.0 ng/ml) in a time-dependent manner with a maximum induction observed at 8 h (Fig. 1C).

Neutralization of TGF-β1 activity with anti-TGF-β antibody (1000 ng/ml) completely blocked HO-1 mRNA induction (Fig. 2A) demonstrating the specificity of TGF-β1. To further evaluate the mechanism(s) of TGF-β1 mediated HO-1 induction, confluent HPTC were co-treated with TGF-β1 (1.0 ng/ml) and actinomycin D (4 µM), a transcriptional inhibitor, or cycloheximide (20 µM), an inhibitor of protein synthesis. Actinomycin D completely blocked TGF-β1 mediated HO-1 mRNA induction (Fig. 2B). Cycloheximide attenuated the up-regulation of HO-1 mRNA steady state levels in response to TGF-β1 by 50% (Fig. 2B). These data suggest that HO-1 induction by TGF-β1 is dependent on *de novo* mRNA expression and in part on new
protein synthesis.

**TGF-β1 induces Smad7 mRNA.** Recent studies have reported that TGF-β1 upregulates its inhibitory proteins, Smad6 and Smad7, in mink lung epithelial cells and other TGF-β responsive cell lines (24, 32, 33). While Smad6 is involved mainly in BMP signalling, Smad7 inhibits both BMP and TGF-β1 signalling (22-25). Smad7 translocates from the nucleus to the cytoplasm on ligand binding and inhibits TGF-β1-mediated downstream signalling events through either an association with receptor regulated Smads and TβR-I (23-25) or competition with Smad4 (22). Previous studies have also reported that the anti-Smads inhibit activation of TGF-β1 responsive promoter-reporter systems such as p3TP-lux (25, 27, 29). To extend these observations to activation of an endogenous gene, we evaluated the role of the anti-Smads in TGF-β1-mediated activation of HO-1. To examine the presence/absence of the inhibitory Smad pathway in HPTC, the induction of Smad6 and Smad7 following TGF-β1 stimulation was first examined. Steady-state levels of Smad7 mRNA were examined by northern blot analyses after exposure to TGF-β1. HPTC treated with TGF-β1 (1.0 ng/ml) for 0, 0.5, 1.0, and 1.5 h exhibited a significant induction (12 fold) in Smad 7 mRNA as early as 1 hour (Fig. 3). No induction of Smad6 mRNA was observed (data not shown).

**Smad7, but not Smad6, inhibits TGF-β1 mediated HO-1 induction.** In order to further investigate the effect of anti-Smads on TGF-β1-mediated HO-1 mRNA induction, mammalian expression vectors harboring the cDNAs for Smad6 and Smad7 were stably transfected into HEK 293 cells. Since HEK 293 cells are only minimally responsive to TGF-β1, the stable Smad6 and Smad7 clones were transfected with TβR-I (TD) to mimic the actions of TGF-β. Transfection of the
TβR-I (TD) expression plasmid results in constitutive activation of TβR-I such that TGF-β specific responses occur in the absence of ligand or TβR-II (31). Expression of Smad6 and Smad7 was confirmed by immunoprecipitation using a FLAG antibody (Fig. 4A). Expression of TβR-I (TD) was confirmed by immunoprecipitation using a HA antibody (and/or northern analysis) (Fig. 4B and Fig. 5). 24 and 48 h following transfection of TβR-I (TD) into the stable Smad6 and Smad7 clones, steady-state levels of HO-1 mRNA were examined by northern blot analyses. TβR-I (TD) increased the steady state levels of HO-1 mRNA in vector alone and Smad6 transfected cells (Fig. 5). However, the expression of Smad7 inhibited TβR-I (TD)-mediated induction of HO-1 mRNA (Fig. 5). These data indicate that overexpression of Smad7 can inhibit TGF-β1-mediated induction of the endogenous HO-1 gene.
The effects of TGF-β1 and other growth factors such as platelet derived growth factor (PDGF), epidermal growth factor, fibroblast growth factor (FGF), TGF-β2, TGF-α and insulin like growth factor-1 (IGF) on HO-1 expression is cell-type specific. For example, in retinal pigment epithelial cells, TGF-β1 induces HO-1 while PDGF, FGF, IGF, TGF-α do not (10). PDGF has been reported to induce HO-1 in vascular smooth muscle cells (34). TGF-β1 induces HO-1 expression in bovine choroid fibroblasts but not in Hela, human lung fibroblasts or bovine corneal fibroblasts (10). Furthermore, TGF-β1 downregulates endotoxin-mediated induction of HO-1 in rat vascular smooth muscle cells (35). It is possible that the induction of HO-1 in cells is dependent on the presence/absence of signaling components of the Smad family or a balance between the activator/inhibitor Smads.

While the cellular processes underlying HO-1 induction are complex and tightly regulated, one denominator common to most of the stimuli that upregulate HO-1 is a significant shift in cellular redox. The induction of HO-1 in response to heme, heavy metals (cobalt chloride, cadmium), nitric oxide, oxidized LDL and by cytokines has been demonstrated to be a consequence of de novo transcription (36-41). Consensus binding sites for NF-κB, AP-1, AP-2 and interleukin-6 responsive elements as well as other transcription factors have been reported in the promoter region of the HO-1 gene (36, 42) suggesting a potential role for these trans-acting factors in modulating HO-1 gene expression. AP-1 and NF-κB have been implicated in the induction of other TGF-β1 responsive genes (e.g. PAI-1) (43-45). The elements that control TGF-β1 mediated HO-1 induction have yet to be identified.

The ability of several genes to respond to members of the TGF-β family requires the presence of one or more Smad binding elements (SBE) (46-48). Putative SBEs have been
described in the human PAI-1 promoter which is responsive to TGF-β1 (49, 50). A palindromic sequence, GTCTAGAC, has been described as the Smad3-Smad4 binding element. However, optimal Smad binding is reportedly achieved with a 5bp sequence CAGAC (51, 52). The original palindromic sequence, which may have resulted from dimerization of recombinant Smads used in oligonucleotide selection experiments (52, 53), is not present in the human HO-1 gene. We have evaluated a ~4.5 kb human HO-1 gene promoter fragment, which responds partially to known inducers of the gene (e.g. heme and cadmium), but lacks the cis-acting elements necessary for TGF-β1-dependent gene induction (data not shown). The absence of a response with the 4.5 kb human HO-1 gene promoter fragment to TGF-β1 is further corroborated by the failure of this fragment to respond to other stimuli that directly increase de novo HO-1 gene transcription i.e. linoleyl hydroperoxide, hydrogen peroxide, hyperoxia and iron/hyperoxia (38, 39).

Previous reports indicate the involvement of the mitogen-activated protein kinase (MAPK) pathways in TGF-β1 signaling (26, 54, 55). For example, TGF-β1 activates MAPK in human mesangial cells and plays a role in TGF-β1 stimulated collagen I expression (54). However, it has also been reported that TGF-β1 modulation of cellular proliferation is independent of the MAPK cascade (56). In our study, HO-1 induction by TGF-β1 does not appear to be mediated through the MAPK pathway. Inhibitors of the MAPK pathway, PD98059 or SB203580, did not affect TGF-β1 induction of HO-1 (data not shown). Other inhibitors such as N-acetylcysteine (NAC, antioxidant) and quinacrine (phospholipase A2 inhibitor) have been reported to block induction of HO-1 by cytokines (TNF-α, IL-1α) in endothelial cells (57). In addition, we have previously reported that deferoxamine (DFO, iron chelator) inhibited HO-1 induction by oxidized LDL and hyperoxia in endothelial cells (39, 58). However, TGF-β1
mediated induction of HO-1 was not affected by co-treatment with DFO, NAC or quinacrine (data not shown) in HPTCs suggesting that different pathways are involved.

Recent studies have reported the involvement of the Smad pathway in regulating the expression of TGF-β responsive genes. The anti-Smad pathway has been shown to inhibit activation of other genes such as human PAI-I and collagen (25-29). However, most of these studies have demonstrated effects of Smad6/7 expression on TGF-β responsiveness using transiently transfected promoter-reporter systems (eg. p3TP-Lux). In this regard, our studies represent the first demonstration that expression of Smad7 inhibits the induction of an endogenous gene, in our case, HO-1. The mechanism of inhibition by anti-Smads is complex and not completely understood. It has been demonstrated that anti-Smads inhibit TGF-β signaling by blocking the association of receptor-regulated Smads with TβR-I (23-25). Another mechanism of antagonism recently proposed involves anti-Smads competing with Smad4 for binding to receptor mediated Smads. The anti-Smad and receptor-mediated Smad complex (for example Smad6-Smad1) is inactive and prevents formation of receptor-mediated Smads and Smad4 (active) complexes (22). Similarly, Smad1 and Smad2 compete for binding to the limited pool of Smad4, resulting in transduction of either BMP or activin/ TGF-β signals, but not both (59). Whether the inhibitory Smads have other direct effects on gene transcription is not known.

The dual role of TGF-β1 in renal injury is well documented. While TGF-β1 initiates and modulates tissue repair, excessive TGF-β1 results in increased deposition of extracellular matrix, the main pathological feature in fibrotic disease. TGF-β1 is upregulated in all experimental models of fibrotic disease as well as in several human diseases (1, 2). The physiological significance of HO-1 induction by TGF-β1 may be relevant to the tubulointerstitial disease seen in diabetic nephropathy. High glucose environments in the presence of PDGF are known to
stimulate the release of TGF-β1 by the proximal tubule and mesangial cells, in addition to elaboration of extracellular matrix proteins such as fibronectin and collagen (60, 61). The induction of HO-1 by TGF-β1 may serve as a protective response to block the injurious effects of TGF-β1. In this regard, interventions aimed at manipulating the Smad7 pathway would aid in regulating expression of HO-1, thereby exploiting its cytoprotective effects.

We speculate that the beneficial effects of TGF-β1 may be mediated via the induction of HO-1 and the harmful effects of TGF-β1 are counteracted by HO-1 induction. There are several potential mechanisms of the beneficial effects of HO-1 induction in response to TGF-β1. Injury results in increased cellular heme, derived from ubiquitously disposed heme proteins, such as cytochromes, peroxidases, respiratory burst oxidases, pyrrolases, catalase, nitric oxide synthases, hemoglobin and myoglobin. The heme moiety is pro-oxidant and potentially toxic (62-64) resulting in damage to lipid bilayers, mitochondria, cytoskeleton and components of the nucleus (64). Increased HO-1 activity, therefore, removes excess heme generated during injury. Furthermore, HO-1 is responsible for converting heme, a pro-oxidant, into biliverdin. Biliverdin is subsequently converted to an anti-oxidant, bilirubin, which is capable of scavenging peroxyl radicals and inhibiting lipid peroxidation (65, 66). Ferritin, an intracellular repository for iron, is co-induced with HO-1 allowing safe sequestering of the unbound iron liberated during the degradation of heme (67). Finally, CO has received considerable attention as a signalling molecule similar to nitric oxide. CO has vasodilatory effects mediated via cGMP and also possesses anti-apoptotic and anti-inflammatory functions (68). Therefore, induction of HO-1 may be responsible for some of the wound healing properties associated with TGF-β1 as well as a cytoprotective response to an over abundance of TGF-β1.
In summary, we have demonstrated that TGF-β1 induces HO-1 gene expression in a time and dose dependent manner in renal proximal tubule cells. A neutralizing antibody against TGF-β1 blocked the upregulation of HO-1 thereby confirming specificity and eliminating possible effects of impurities that may be present in TGF-β1. HO-1 induction by TGF-β1 is mediated via transcriptional mechanisms and requires *de novo* protein synthesis. In addition, our studies show that TGF-β1-mediated HO-1 induction occurs via the Smad pathway. Smad7 mRNA is induced by TGF-β1 as early as 1h of incubation. Furthermore, transfection of a constitutively active TβR-I (TD) mutant receptor upregulates HO-1 in cells stably transfected with either vector alone or Smad6, the BMP signaling inhibitory Smad, while cells stably transfected with Smad7, the anti-Smad for TGF-β signaling, blocks upregulation of TGF-β1-mediated HO-1 gene expression. Our data demonstrates that the Smad pathway is involved in TGF-β1-mediated HO-1 induction and provides evidence that Smad7 inhibits expression of an endogenous gene, HO-1.
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Footnotes

1. Abbreviations used in this paper:

BMP, bone morphogenetic protein; CO, carbon monoxide; DFO, deferoxamine; FGF, fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GS, glycine-serine; HO-1, heme oxygenase-1; HPTC, human renal proximal tubular cell; IGF-1, insulin like growth factor; Mad, Mothers against decapentaplegic; MAPK, mitogen activated protein kinase; NAC, N-acetylcysteine; PAI-I, plasminogen activator inhibitor type I; PDGF, platelet derived growth factor; PMSF, phenylmethanesulfonyl fluoride; SBE, smad binding element; TβR-I and II, Type I and II receptor for transforming growth factor-beta; TβR-I (TD), constitutively active mutant Type I receptor for transforming growth factor-beta; TGF-β1, transforming growth factor-beta
Acknowledgments

This work was supported by grants from the National Institutes of Health, K08 DK02446 and R03 DK56279 (to A. Agarwal), and HL39593 (to H.S. Nick). We are grateful to Drs. TakeshiImamura and Kohei Miyazono, Department of Biochemistry, The Cancer Institute of the Japanese Foundation for Cancer Research (JFCR), Toshima-ku, Tokyo, Japan, for providing us with the expression plasmids for human Smad6, Smad7 and TβR-I (TD).
Figure Legends

Fig. 1. Induction of HO-1 mRNA and protein with TGF-β1. (A) Confluent monolayers of HPTCs were incubated with serum free media containing PBS (control) or TGF-β1 as indicated. (A) Total RNA was isolated, electrophoresed, transferred to a nylon membrane and hybridized with a 32P-labeled human HO-1 cDNA probe. Hemin (5 µM) was used as a positive control. The membrane was stripped and reprobed with a human GAPDH cDNA probe to control for loading and transfer. The results are representative of several experiments performed in duplicate. (B) Western blot analysis using a polyclonal anti-HO-1 antibody. The concentration of TGF-β1 used was 1.0 ng/ml. (C) Time course of HO-1 mRNA induction in HPTCs with TGF-β1. Confluent monolayers of HPTCs were incubated with serum free media containing PBS (control) or TGF-β1 (2.0 ng/ml) for the times indicated and Northern analysis was performed. Maximum induction was observed at 8 h. The membrane was stripped and reprobed with a human GAPDH cDNA probe to control for loading and transfer. Results are representative of 3 independent experiments.

Fig. 2. (A) TGF-β1 induction of HO-1 is neutralized by an anti-TGF-β1 antibody. Confluent monolayers of HPTCs were incubated with serum free media containing PBS (control) or TGF-β1 (1.0 ng/ml) in the presence or absence of anti-TGF-β1 antibody (1000 ng/ml) for 8 h. Total RNA was isolated, electrophoresed, transferred to a nylon membrane and hybridized with a 32P-labeled human HO-1 cDNA probe. Anti-TGF-β1 antibody (1000 ng/ml) alone had no effect on HO-1 mRNA. The membrane was stripped and reprobed with a human GAPDH cDNA probe to control for loading and transfer. (B) Effect of actinomycin D and cycloheximide on HO-1 mRNA induction by TGF-β1. Confluent
monolayers of HPTCs were incubated for 8 h with serum free media containing PBS (control) or TGF-β1 alone, actinomycin D (4 µM) alone, actinomycin D + TGF-β1, cycloheximide (20 µM) alone or cycloheximide + TGF-β1. The concentration of TGF-β1 used was 1.0 ng/ml. Total RNA was isolated, electrophoresed, transferred to a nylon membrane and hybridized with a 32P-labeled human HO-1 cDNA probe. The membrane was stripped and reprobed with a human GAPDH cDNA probe to control for loading and transfer. Results are representative of 2 independent experiments.

Fig 3. Effect of TGF-β1 on Smad7 mRNA expression. Confluent monolayers of HPTCs were incubated with serum free media and TGF-β1 (1.0 ng/ml) for the times indicated. Poly A+ RNA was isolated, electrophoresed, transferred to a nylon membrane and hybridized with a 32P-labeled human Smad7 cDNA probe. The membrane was stripped and reprobed with a human GAPDH cDNA probe to control for loading and transfer. Results are representative of 2 independent experiments.

Fig 4. Overexpression of Smad6, Smad7 and TβR-I (TD) in HEK 293 cells. (A) Immunoprecipitation and immunoblot analysis of stable transfectants of vector, Smad6 or Smad7 expression plasmids in HEK 293 cells. Transfections were performed using with Lipofectamine™ Plus reagent and stable transfectants selected using G418. Immunoprecipitation and immunoblot analysis was performed using anti-flag antibody. (B) Immunoprecipitation and immunoblot analysis of stable transfectants (vector, Smad6 and Smad7) transiently transfected with TβR-I (TD) using anti-HA antibody, as described in methods.
Fig 5. Effect of TβR-I (TD) on HO-1 mRNA in HEK 293 cells. Vector, Smad6 and Smad7 stable transfectants were transiently transfected with TβR-I (TD), respective stable transfectants alone were used as controls. Total RNA was isolated 24 and 48 h after transient transfection of TβR-I (TD), electrophoresed, transferred to a nylon membrane and hybridized with a $^{32}$P-labeled human HO-1 cDNA probe. The membrane was stripped and reprobed with a TβR-I (TD) cDNA probe to control for transfection efficiency. The membrane was again stripped and reprobed with a human GAPDH cDNA probe to control for loading and transfer. Results are representative of 3 independent experiments.
Fig. 1

A

Control
TGF-β 0.05 ng/ml
TGF-β 0.5 ng/ml
TGF-β 2.0 ng/ml
Hemin 5 µM

HO-1 mRNA

GAPDH

B

Control
TGF-β (Genzyme)
TGF-β (R&D)

HO-1 protein

32kD

Fig. 1

C

Control
TGF-β 2.0 ng/ml

2 4 8 12 24 48h

HO-1 mRNA

GAPDH
Fig. 2

A

Control  TGF-β  Anti-TGF-β ab  TGF-β + Anti-TGF-β ab

HO-1 mRNA

GAPDH

Fig. 2

B

Control  TGF-β  Actino  TGF-β + Actino  Cyclo  TGF-β + Cyclo

HO-1 mRNA

GAPDH
Fig. 3

TGF-β (1 ng/ml)

0  30  60  90 min

- Smad7 mRNA
- GAPDH mRNA

Fig. 4

A

Vector  Smad6  Flag-Smad6

Vector  Smad7  Flag-Smad7

B

Vector  Smad6  Smad7  HA-TβRI (TD)

-  +  +  -  +  TβRI (TD)
Fig. 5

| Vector TβRI (TD)  | Smad 6 TβRI (TD) | Smad 7 TβRI (TD) |
|-------------------|------------------|------------------|
| C 24 48 h         | C 24 48 h        | C 24 48 h        |
| HO-1              | TβRI (TD)        | TβRI (TD)        |
| GAPDH             |                  |                  |
Smad7-dependent regulation of heme oxygenase-1 by transforming growth factor-β in human renal epithelial cells

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J. Biol. Chem. published online October 3, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M006621200

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