Preproendothelin-1 Expression Is Negatively Regulated by IFN-gamma During Hepatic Stellate Cell Activation

Tianxia Li*#, Zengdun Shi*#, and Don C. Rockey*

*UT Southwestern Medical Center, Department of Internal Medicine¹, Dallas, TX 753901

#These authors made an equal contribution to the research work.

Author Names:

1. *#Tianxia Li: tli@rx.umaryland.edu
2. *#Zengdun Shi: Zengdun.shi@utsouthwestern.edu
3. *Don C. Rockey (corresponding author): don.rockey@utsouthwestern.edu

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Contact Information:

Don C. Rockey, M.D., Division of Digestive and Liver Diseases
University of Texas Southwestern Medical Center
5323 Harry Hines Blvd. Dallas, TX 75390-8887.
Phone: 214-645-6344; Fax: 214-648-8446. Email: don.rockey@utsouthwestern.edu

List of Abbreviations: AP-1, activator protein 1; ET-1, endothelin-1; IFN\(\gamma\), interferon-gamma; TGF\(\beta\), transforming growth factor beta; p-ERK, phospho-extracellular signal-regulated kinase; p-JNK, phospho-c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; p-c-Jun, phospho-c-Jun; NE, nuclear extracts; HSC, Hepatic stellate cell; SM, smooth muscle; SRF, serum response factor.

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Abstract

Endothelin-1 (ET-1), a powerful vasoconstrictor peptide, is produced by activated hepatic stellate cells (HSC) and promotes cell proliferation, fibrogenesis, and contraction, the latter of which has been thought to be mechanistically linked to portal hypertension in cirrhosis. Interferon-gamma (IFNγ), a Th1 cytokine produced by T cells, inhibits stellate cell proliferation, fibrogenesis, and muscle specific gene expression. Whether IFNγ-induced inhibitory effects are linked to regulation of ET-1 expression in activated stellate cells remains unknown. Here we examined IFNγ’s effects on preproET-1 mRNA expression and the signal pathways underlying this process. We demonstrated that preproET-1 mRNA expression in HSCs was prominently increased during cell culture-induced activation; IFNγ significantly inhibited both preproET-1 mRNA expression and ET-1 peptide production. Similar results were found in an in vivo model of liver injury and intraperitoneal administration of IFNγ. PreproET-1 promoter analysis revealed that IFNγ-induced inhibition of preproET-1 mRNA expression was closely linked to AP-1 and Smad3 pathways. Further, IFNγ reduced JNK phosphorylation, which tightly associated with decreased phosphorylation of downstream factors c-Jun and Smad3 and the decreased binding activity of c-Jun and Smad3 in the preprpET-1 promoter. Importantly, IFNγ reduced both c-Jun mRNA and protein levels. Given the important role of ET-1 in wound healing, our results suggest a novel negative signaling network by which IFNγ inhibits preproET-1 expression, highlighting one potential molecular mechanism for IFNγ-induced host immunomodulation of liver fibrogenesis.
Key words: Liver, stellate cells, cytokine, gene expression, signal pathway
**Introduction**

Endothelin-1 (ET-1), a 21 amino-acid bioactive peptide, exerts important biological effects on multiple physiological and pathological processes in liver (see review (7, 24)). Under normal conditions, the sinusoidal endothelium is the major source of ET-1, which appears to be important in intrahepatic circulatory homeostasis. However, during liver injury, ET-1 synthesis shifts to activated HSCs (21, 27), themselves an apparent target of ET-1 by virtue of their expression of ET-1 A and B receptors (14, 21). These transmembrane guanine nucleotide-binding protein-coupled receptors (GPCRs) stimulate a variety of downstream signaling pathways including MAPKs, PI3-K/Akt, Jun, and others (5), which modulate multiple biological processes, including cell proliferation, survival and contraction (24). Thus, ET-1 has been thought to be a potent agonist in liver fibrosis. However, liver fibrogenesis is prominently modulated by the host immune response, for example, Interferon-gamma (IFNγ) knockout deficient mice developed more liver fibrosis, while Th1 cytokine dominant mice or animals with administration of IFNγ exhibited reduced liver fibrosis (26, 31). Those findings suggest that ET-1 expression may be regulated through IFNγ. Whether IFNγ-mediated inhibitory effects on HSC links to regulation of ET-1 expression remains elusive.

ET-1 biosynthesis has been extensively studied (7). Regulation of ET-1 expression appears to be complicated (32). It has been shown that PreproET-1 expression is regulated by both transcriptional and posttranscriptional mechanisms (29, 20). The preproET-1 gene promoter contains several putative cis-acting elements including AP-1
and Smad. AP-1 is required for constitutive ET-1 promoter activity. In contrast, Smad binding appears to be required for TGFβ-induced ET-1 expression via activation of the Smad signaling pathway (29). The 3’ UTR region of preproET-1 contains three AUUUA motifs, which destabilize ET-1 transcripts through an AUF1-proteasome pathway (20). The preproET-1 mRNA is labile with a half-life of less than 1 hour (15). Such rapid turnover of ET-1 mRNA allows for stringent control over its expression in response to extracellular environmental stimuli.

We have recently shown that IFNγ inhibits muscle-specific gene expression in HSCs through targeting SRF (30). However, ET-1 appears not to be a SRF target gene since no functional SRF binding site in the preproET-1 promoter has been identified. In the present study, we have demonstrated that the preproET-1 gene is a novel target of IFNγ in activated HSCs, and the mechanism by which IFNγ inhibits preproET-1 expression appears to be via suppression of JNK phosphorylation, which leads to decreased phospho-c-Jun and phospho-Smad3, resulting in decreased preproET-1 transcription. Further, IFNγ may inhibit preproET-1 transcription via targeting c-Jun expression. Our results highlight a novel mechanism whereby IFNγ modulates myofibroblast-mediated liver fibrosis through targeting preproET-1 expression.
Materials and Methods

Animals and Cell Culture

HSCs were isolated from retired breeder Sprague Dawley rats (Charles River Laboratory) as described previously (30). Isolated HSCs were subjected to activation by incubation in 199OR medium containing 20% serum (10% horse serum and 10% calf serum; Invitrogen, Carlsbad, CA) for 4-5 days. Activated HSCs were then subjected to starvation in 0.2% serum 199OR medium overnight before exposure to IFNγ (500 IU/mL for luciferase assays and 1,000 IU/mL for other experiments) (PBL Biomedical) as described before (10, 12), or pharmacological inhibitors against MEK/ERK (U0126), p38-MAPK (SB203580), JNK (SP600125) and TGFβ type I receptor (SB431542) (Calbiochem). For in vivo experiment, Alzet osmotic pumps (model 1002) with murine IFNγ (140,000 IU per pump, which releases 10,000 IU/day for 14 days, PeproTech) or carrier control (1 x PBS containing 1% BSA) were implanted into peritoneal cavity of C57/black 6 male mice (22-25 g). Then, the animals were gavaged with carbon tetrachloride (CCL4) once a week for two weeks (26, 31, 16). Animals were cared for and experiments were performed in accordance with National Institutes of Health (NIH) guidelines. All experimental procedures were approved by the UT Southwestern IACUC.

Plasmids and luciferase assay

The preproET-1 gene promoter (ppET-1pro) was cloned from a mouse HSC genomic DNA. The specific PCR primers were designed according to the sequence from GenBank
database (NT_039580. Mus strain C56BL/6J Chromosome 13). Sense primer: 5’-CAGAGGTCCCTCAGCTGAAGG-3’ and antisense primer: 5’-AGATCTCAGCGCGTCCTCAG-3’, which span 2,252bp of the preproET-1 5’ flank region between +131 and -2121bp. The cloned ppET-1 pro fragment was ligated into pGL3 basic luciferase reporter vector (Promega) at HindIII and XhoI sites. The luciferase constructs harboring truncated ppET-1 gene promoter fragments were generated by PCR method and mutant AP-1 (from TGACTA to TATCTA) and/or Smad3 (from CAGAC to TACAT) sites were created by site-directed mutagenesis (Stratagene). All constructs were confirmed by sequencing (UT Southwestern Sequencing Core). JNK1/2-KM (dominant active form) and JNK1/2-DN (dominant negative form) expression plasmids were obtained from Dr. Lin (The University of Chicago).

Activated HSCs were transiently transfected by using Lipofectamine 2000 (Invitrogen). The cells were exposed to IFNγ (500 IU/mL) for 2 days and luciferase activity was assayed by using dual luciferase assay system (Promega). Transfections were performed in triplicates, and the experiments were repeated three times. The promoter activity was presented as a fold change relative to the activity of promoter-less luciferase basic reporter (mean ± SD, n=3, p<0.05 for comparison of IFNγ to control). For cotransfection experiments, 1μg of each plasmid was used (total 2μg of plasmid DNA per transfection).

**RNase Protection Assay (RPA) and RT-PCR**

RPA was performed as described (31). Briefly, a cRNA probe used to detect preproET-1 (ppET-1) mRNA was generated by TA cloning (Invitrogen) of a 213 base pair fragment,
encoding the first 71 amino acids of ppET-1. The primer sequences were as follows: sense 5’-ATGGATTATTTTCCCCTGGATC-3’; antisense 5’-GATGTCAGGGTGCAAGAAGTA-3’). 32P-labeled probes were generated with an in vitro transcription system (Promega) and purified by NucTrap column (Stratagene). A control probe (GAPDH) was obtained from Ambion; and tRNA was used as negative control. The specific signals were quantitated by image analysis (Syngene, Frederick, MD). The average raw volume from the first control samples (IFNγ -) in each experiment was arbitrarily set to 1 or 100% and experimental data were presented as relative abundance (30).

RT-PCR (real-time PCR) was performed as in (30). The primer sequences for c-jun were as follows: sense 5’-ACGACCTTCTACGACGATGCC-3’; antisense 5’-TGCCCATTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3’. For ppET-1, sense 5’-GACATCATCTGGTGCTGGACT GGATG-3'.

Immunoblot

HSCs were lysed in RIPA buffer containing protease inhibitors (Roche) as described (30). Samples were subjected to SDS-PAGE and immunoblotting with specific antibody against ERK, phospho-ERK, p38, phospho-p38, JNK, phospho-JNK, c-Jun, phospho-c-Jun, Smad3, phosphor-Smad3 (Cell Signaling), smooth muscle α-actin (Sigma) and
collagen type 1 (Rockland, PA). Following incubation with secondary antibody, specific
signals were visualized using an enhanced chemiluminescence detection kit (Pierce).
Specific bands were quantitated by image analysis (Bio Image system, Syngene). The
raw value for controls (IFNγ -) in the first lane of each experiment was arbitrarily set to
100 and the data were presented as percent change (30).

**ET-1 peptide measurement**

Conditioned cell culture medium was harvested and cleared with centrifugation at 500g
for 7 minutes. The supernatants were subjected to sandwich enzyme-linked
immunosorbent assay (ELISA) kits according to manufacturer’s instruction (Assay
Designs) and as previously described (36).

**Electrophoresis mobility shift assays (EMSA)**

EMSA was performed as described (30). The sequence of the sense strand
oligonucleotide for preproET-1 promoter harboring Smad3 site was
5’CTGGATTGTACGAGGCGGCGTGC3’. The sequence of the sense strand
oligonucleotide for preproET-1 promoter harboring AP-1 site was
5’GGTGCTGTGGGTACTACACACAAAC3’ and the mutant preproET-1 AP-1
was 5’GGTGCTGTGGGGCAACTACACACAAAC3’. To verify specific DNA-
protein interaction, a cold probe or a specific antibody (Cell Signaling) were added in
some of the reactions and preincubated for 30 min before incubation with radioactive
labeled probe.
Statistical analysis

All data are presented as the average of three individual experiments plus standard deviation (mean ± SD). Student’s $t$ tests were used for statistical analysis; values of $P < 0.05$ were considered significant.
Results

**IFN\(\gamma\) inhibits activation-mediated ET-1 synthesis through down-regulation of preproET-1 transcription.** In a (primary) cell culture model system of stellate cell activation that closely recapitulates *in vivo* stellate cell activation, we examined the correlation between smooth muscle \(\alpha\)-actin (SM \(\alpha\)-actin) and preproET-1 mRNA expression over time. As shown in Figure 1A, both SM \(\alpha\)-actin (top panel) and preproET-1 mRNA (middle panel) were expressed in increasing proportions over time. This expression pattern strongly suggested that preproET-1 expression was closely associated with HSC activation (Figure 1A, lower panel). We next examined the effect of IFN\(\gamma\) on hepatic stellate cell activation. As shown in Figure 1B (lower left panel), activated HSCs were spread and had an activated appearance on the fifth day of culture. This activation process was prominently blocked by exposure to IFN\(\gamma\) (Figure 1B, upper panel and lower right panel), and HSCs were rounded and appeared refractile (but were still viable as evidenced by exclusion of propidium iodide). Further, we examined the effect of IFN\(\gamma\) on preproET-1 expression in activated HSCs. As shown in Figure 1C, IFN\(\gamma\) exposure for 12 hours led to a reduction in preproET-1 mRNA expression. The inhibitory effect remained prominent at later time points also (Figure 1C). As expected, ET-1 peptide synthesis was significantly reduced in HSCs after IFN\(\gamma\) exposure (Figure 1D). Of note, ET-2 mRNA, and ET-3 mRNA (measured by RT-PCR) were detected at levels of less than 10% of the abundance of ET-1 mRNA. Further, expression of ECE-1 mRNA and protein was not affected by IFN\(\gamma\) exposure. These results suggest that ET-1 is a novel target of IFN\(\gamma\) in activated HSCs, and that the mechanism of IFN\(\gamma\)'s affect is
likely to be at the level of preproET-1 regulation.

Since mRNA stability plays a critical role in determining gene expression (11), we further evaluated preproET-1 mRNA stability in our system. Following actinomycin D exposure for 15 minutes, preproET-1 mRNA was almost completely degraded after 2 hours (Figure 1E, left panel). Next, we examined whether IFNγ had an effect on preproET-1 mRNA decay. As shown in Figure 1E (right panel), IFNγ did not enhance preproET-1 mRNA degradation. The results indicated that IFNγ-mediated down-regulation of preproET-1 mRNA expression occurs mainly through transcriptional regulation.

IFNγ exerts a prominent negative effect on preproET-1 promoter activity. To explore the mechanisms by which IFNγ inhibits preproET-1 expression, we first examined IFNγ’s effect on preproET-1 transcription. As shown in Figure 2A, IFNγ reduced preproET-1 promoter activity up to 2-3 fold; in contrast, ET-1 robustly increased its own promoter activity. However, the ET-1-mediated increase in preproET-1 promoter activity was almost completely abolished by IFNγ exposure. Next, we generated a series of deletion mutants in the preproET-1 promoter to explore preproET-1 transcriptional responses to IFNγ (Figure 2B). IFNγ inhibited luciferase activity in all truncated preproET-1 promoter constructs – suggesting that IFNγ-induced inhibitory effects on preproET-1 promoter activity may be mediated through multiple pathways. Since previous studies were shown that AP-1 and Smad3 are critical for preproET-1 promoter activity (18, 29), we hypothesized that the IFNγ-induced inhibitory effect on
preproET-1 promoter activity might be associated with one or both of them. Mutation of the AP-1 site led to a remarkable reduction in promoter activity (Figure 2C), suggesting that AP-1 is critical in regulating preproET-1 promoter activity in stellate cells. Of note, compared to the 2.0-fold reduction of the promoter activity caused by IFNγ with the wild type ppET-1 promoter construct, IFNγ induced only a 0.7-fold decrease in the promoter activity with the mutated AP-1 binding site promoter construct. A similar phenomenon was found with the Smad3 mutant, although it was not as prominent as that in the AP-1 mutation construct. Further, mutation of both AP-1 and Smad3 binding sites in the preproET-1 promoter essentially abolished all promoter activity. These findings suggested that both AP-1 and Smad3 signaling pathways are required to induce preproET-1 transcription and that IFNγ-induced inhibitory effects on preproET-1 promoter activity appear to closely associate with AP-1 and Smad3 pathways.

JNK-c-Jun pathway plays a key role in IFNγ-induced inhibition of preproET-1 transcription. Since the preproET-1 promoter has an AP-1 binding site (29) and MAP kinase mediates a variety of cell activities (5), we hypothesized that IFNγ-induced inhibition of ET-1 expression might proceed through the MAK kinase pathway. Inhibition of ERK, P38 or JNK phosphorylation with specific inhibitors led to prominent reduction in preproET-1 promoter activity, which was further reduced by IFNγ (Figure 3A). This finding implied that all three MAP kinases could be important in the regulation of ET-1 expression and mediation of IFNγ's inhibitory effect. Next, we examined MAP kinase family member phosphorylation following IFNγ exposure. Importantly, p-JNK was prominently reduced early after IFNγ exposure, while the phosphorylation of ERK
and P38 did not appear to be significantly affected (Figure 3B). Therefore, we explored JNK further. Since c-Jun is downstream of JNK, we hypothesized that given interferon’s effect on p-JNK, there would likely also be an effect on p-c-Jun. Thus, we examined IFNγ’s effect on c-Jun phosphorylation over several time points (Figure 3C); p-c-Jun was reduced shortly after exposure to IFNγ and prominently decreased through 60 minutes of exposure (Figure 3C). To confirm that the decrease in c-Jun phosphorylation was a result of reduced JNK phosphorylation, we used a specific JNK inhibitor to block JNK phosphorylation (Figure 3D, upper panel); as expected, it also blocked c-Jun phosphorylation (Figure 3D, middle panel). Finally, we examined the correlation between JNK phosphorylation and preproET-1 expression by using the JNK inhibitor. As shown in (Figure 3E), preproET-1 mRNA expression was significantly reduced following JNK inhibition, which was similar to the effect with IFNγ treatment (Figure 1C). Taken together, these data suggested that JNK-c-Jun pathway might play an important role in IFNγ-induced inhibition of preproET-1 mRNA expression.

**JNK plays an important role in IFNγ’s inhibitory effect on preproET-1 promoter activity.** To further explore the functional importance of JNK in IFNγ-induced inhibition of preproET-1 promoter activity, we over-expressed a dominant active or negative form of JNK in HSCs. As expected, preproET-1 promoter activity was significantly increased by overexpression of the dominant active JNK1/2 (JNK1/2-KM) (Figure 4A). However, overexpression of the dominant negative JNK1/2 (JNK1/2-DN) did not obviously affect preproET-1 promoter activity. Notably, overexpression of the dominant active JNK1/2 significantly blocked IFNγ-induced inhibition of preproET-1 promoter activity (0.9-fold
reduction) compared to the control (2.0-fold reduction), but overexpression of the dominant negative JNK1/2 prominently potentiated IFNγ-induced inhibition of preproET-1 promoter activity (3.2-fold reduction) (Figure 4A). Since AP-1 was essential to preproET-1 promoter activity (Figure 2C), we explored whether JNK-mediated effect on preproET-1 promoter activity was AP-1 dependent. As shown in Figure 4B, mutation of the AP-1 site in the preproET-1 promoter led to dramatically decreased promoter activity (similar to that shown in Figure 2C) and overexpression of the dominant active JNK1/2 failed to significantly increase preproET-1 promoter activity. Moreover, IFNγ-induced inhibitory effect on the preproET-1 promoter activity was obviously compromised compared to that in Figure 4A. Taken together, these data suggested that JNK was a key factor to mediate IFNγ-induced inhibition of preproET-1 promoter activity via AP-1, but in addition to AP-1, other factors might also mediate IFNγ’s effect via JNK pathway.

**IFNγ-induced inhibition of ET-1 expression through a TGFβ-Smad pathway.**

Previous data has suggested that IFNγ antagonizes TGFβ’s effect on collagen type 1 gene expression via a TGFβ-Smad pathway (13). Additionally, the TGFβ-Smad pathway appears to play a role in regulation of preproET-1 expression in endothelial cells (29). These previous data led us to postulate that IFNγ might inhibit TGFβ-Smad-mediated preproET-1 transcriptional activation in the current system. To explore this possibility, we first examined the effect of IFNγ on Smad3 signaling. Total Smad3 levels were not affected following IFNγ exposure, but Smad3 phosphorylation was reduced at all time points compared to controls (Figure 5A, left panel), which led to a relative decrease in...
phosphorylation (i.e. the ratio between p-Smad and total Smad in IFNγ-treated stellate cells was reduced (Figure 5A, right panel)). Since phosphorylation appears to be an important prerequisite for Smad3 binding activity (19), we further examined Smad3 binding activity in the preproET-1 promoter. As shown in Figure 5B, a weaker shifted band (lane 3) was detected in the sample with IFNγ exposure for 16 hours compared to the control (lane 2). To confirm the specificity of binding, an antibody against Smad3 (lane 6, 7) and the cold probe (lane 4, 5) were also examined. Notably, the DNA-protein complexes in lane 2 and 3 were essentially depleted by Smad3 antibody or cold probe. We further postulated that reduced Smad3 phosphorylation was likely a result of the effect of IFNγ on JNK phosphorylation (Figure 3). We found that inhibition of JNK phosphorylation with a JNK specific inhibitor (SP600125) substantially abrogated Smad3 phosphorylation (Figure 5C, left panel). However, blocking Erk phosphorylation did not reduce Smad3 phosphorylation (Figure 5D, right panel) – suggesting that JNK, but not Erk, activates Smad3 signaling in our system. Taken together, these data indicate that targeting endogenous TGF-β signaling through a JNK-Smad pathway is an important route for IFNγ-mediated inhibition of preproET-1 mRNA expression in stellate cells.

**IFNγ directly inhibits c-Jun expression.** c-Jun is an important component of the AP-1 transcription complex, which is integrally related to cell proliferation and differentiation (34). We hypothesized that IFNγ might directly target c-Jun, which in turn might mediate IFNγ’s inhibitory effect on preproET-1 mRNA expression. As shown in Figure 6A, c-Jun mRNA was decreased about 30% compared to the control following IFNγ exposure for 12 hours. We also examined c-Jun protein expression following IFNγ...
Interestingly, c-Jun protein levels were prominently reduced at 12 hours and IFNγ maintained the inhibitory effect through 24 hours. Further, we examined c-Jun binding activity in the preproET-1 promoter (Figure 6C). As expected, IFNγ exposure led to reduced binding of c-Jun (lane 3) compared with no IFNγ exposure (lane 2). The specific binding was confirmed by using cold probe (lane 4 and 5), specific antibody against c-Jun (lane 6 and 7) and AP-1 site mutant probe (lane 8 and 9). Notably, the cold probe or AP-1 site mutant probe completely abrogated the DNA-protein complex formation as that occurred in lane 2 and 3, which was prominently reduced by anti-c-Jun antibody (indicated by arrow). Taken together, these data demonstrated that IFNγ signals to c-Jun to negatively regulate preproET-1 transcription.

**IFNγ** down regulates preproET-1 mRNA expression *in vivo*. Previous studies demonstrated that IFNγ reduced liver fibrogenesis in animal (rat or mouse) models (26, 16). To explore the *in vivo* effect of IFNγ, we examined whether IFNγ inhibits preproET-1 mRNA expression in stellate cells following CCl4-induced liver injury. Using osmotic pumps (26), IFNγ was consistently released for two weeks during CCl4 exposure. As shown in Figure 7A, preproET-1 mRNA levels in isolated stellate cells from mice receiving IFNγ was significantly decreased compared to control. Smooth muscle α-actin, a molecular marker for stellate cell activation, as well as col1α1 (collagen type 1) was also reduced in stellate cells from mice exposed to IFNγ compared to control (Figure 7B). In aggregate, our data link IFNγ's negative regulatory effect to preproET-1 transcription during stellate cell activation (Figure 7C).
Discussion

The importance of ET-1 in wound healing has been well established (2, 35). However, in liver wound healing the molecular mechanisms underlying preproET-1 regulation remain unclear (24, 32). In this study, we have demonstrated a novel signaling pathway in which ET-1 synthesis is down regulated by IFNγ in HSCs. We have shown that IFNγ inhibits JNK phosphorylation and consequently leads to decreased c-Jun and Smad3 phosphorylation as well as reduced total c-Jun expression and diminished Smad3 and c-Jun binding activity in the preproET-1 promoter, which are required for ET-1 transcriptional activation. Our results link IFNγ signaling to a preproET-1 transcriptional regulation through JNK, Smad3 and c-Jun (Figure 6D).

ET-1 together with ET-2 and ET-3 comprise the endothelin family of 21 amino acid peptides produced in various cells and tissues, especially endothelial and epithelial lineages. These endothelins are derived from three individual genes (7). Compared to ET-1, ET-2 and ET-3 mRNA levels were almost undetectable in our system, suggesting that ET-1 is the major species of endothelins produced by stellate cells, which further serve as an autocrine target of ET-1 (Figure 1A). ET-1 not only induces stellate cell proliferation, but also increases extracellular matrix synthesis (such as collagen type I expression) (24). Simultaneously, activated HSCs also respond to IFNγ - including reduced fibrogenesis, reduced proliferation, and inhibition of the smooth muscle gene program typical of myofibroblasts (3, 28). Our data extend these effects to include an inhibitory effect on preproET-1 mRNA expression (Figure 1C, D), which likely contributes to IFNγ’s
antiproliferative and anti-fibrogenic effects (Figure 1B).

We emphasize that our data suggest one potential mechanism for IFNγ’s anti-fibrogenic effect in stellate cells. Indeed, it is widely accepted that IFNγ inhibits ECM/collagen expression in stellate cells by virtue of a number of effects on stellate cells (3, 15, 28). In fact, IFNγ appears to have diverse signaling effects in stellate cells, including beyond those on the ET-1 system.

Transcriptional regulation of preproET-1 expression involves multiple transcription factors (32). However, AP-1 and Smad3 appear to be critical in our system since simultaneous mutation of their binding sites in the preproET-1 promoter essentially abolished all promoter activity, which is very similar to a previous study in bovine aortic endothelial cells (18). Compared to Smad3, AP-1 appears to be more important in mediating the IFNγ-induced inhibitory effect on preproET-1 transcriptional activation since mutation of AP-1 binding site in the promoter alone prominently compromised IFNγ’s effect (Figure 2B and C). Although all members of the MAPK signaling family (i.e. ERK and P38) appeared to associate with preproET-1 transcriptional activation (Figure 3A), we found that p-JNK was prominently reduced by IFNγ (Figure 3B). Not unexpectedly, we also found IFNγ potently inhibited p-c-Jun (Figure 3), consistent with previous data emphasizes that c-Jun is downstream of JNK (22). Interestingly, overexpression of a dominant-negative JNK greatly facilitated IFNγ-induced inhibition of preproET-1 promoter activity (Figure 4). Furthermore, inhibition of JNK phosphorylation by a pharmaceutical inhibitor led to a reduction of preproET-1 mRNA
expression (Figure 3D and E) similar to that induced by IFNγ. Our results likely provide a novel insight into the mechanism of decreased liver fibrosis in JNK1 deficient mice or pan-JNK inhibition in vivo models (17). Notably, ET-1 can robustly increase its own promoter activity (Figure 2A), which coincides with cotransfection of a dominant-active JNK (Figure 4A). It has been demonstrated that ET-1 signals to JNK (5). Such signal transduction feedback loop from ET-1 to JNK and JNK to preproET-1 transcription appears to play a critical role in HSC activation. Our highly reproducible data link IFNγ's inhibitory effect on ET-1 expression with JNK-c-Jun pathway.

TGFβ-Smad3 pathway played an important role in regulation of preproET-1 expression (Figure 2C) (29). Inhibition of endogenous TGFβ signaling by IFNγ appears to be an important mechanism in IFNγ-induced inhibition of preproET-1 transcriptional activation. It was demonstrated that phosphorylation of Smad3 is essential step for Smad3’s DNA binding activity and TGFβ signaling (19). Indeed, IFNγ inhibited Smad3 phosphorylation, which further led to a reduced Smad3 binding in the preproET-1 promoter (Figure 5C). Previous studies indicated that JNK could physically interact with Smad3 and led to Smad3 phosphorylation (33). Thus, IFNγ-induced inhibition of Smad3 phosphorylation likely came from the effect of IFNγ on JNK since we blocked JNK activity and resulted in essential abrogation of Smad3 phosphorylation (Figure 5D).

c-fos and c-Jun are both key components of the AP-1 transcription factor complex, which is critical for regulation of many genes, including collagen type 1 and ET-1 (29, 9). IFNγ has been shown to negatively regulate c-fos expression (23). We here demonstrated
that c-Jun was also negatively regulated by IFNγ in our system (Figure 6). Thus, IFNγ-
induced total c-Jun reduction and decreased c-Jun binding activity further support the
position that AP-1 is a major molecular target of IFNγ-JNK-c-Jun pathway in preproET-1
transcriptional regulation.

Our data have therapeutic implications. For example, it has been previously
demonstrated that abrogation of each the IFNγ and ET-1 signaling systems are capable of
reducing fibrosis in animal models. For example it was shown that inhibition of ET-A
receptors (6) or both ET-A and -B receptors (25) inhibited fibrosis. It has also been well
established that IFNγ inhibits fibrosis in animal models (26, 3, 31, 16); thus whether a
blocking IFNγ and ET-1 in a combined fashion is more effective than either alone is an
intriguing and attractive possibility.

It has been well demonstrated that ET-1 is important in regulation of cell proliferation,
muscle-specific gene expression and cellular contraction, which are all closely associated
with the pathological wound healing responses, vasculopathies, and even cancer
progression (1, 4, 24). Our data in a model of liver wound healing (both \textit{in vitro} and \textit{in vivo}), which demonstrate not only a potent effect of IFNγ on ET-1, but also on wound
healing suggest a novel mechanism for the effect of IFNγ (i.e. through ET-1). The data
also highlight an important signaling network linking IFNγ to preproET-1 transcription
through JNK-Smad3 and c-Jun pathways (Figure 7C), which may provide a potentially
important molecular mechanism for IFNγ’s effect in wound healing.
Acknowledgements

We thank Dr. Lin (The University of Chicago) for JNK1/2-KM and JNK1/2-DN expression plasmids.
References


**Figure 1.** *IFN*γ* inhibits ET-1 expression in hepatic myofibrolasts.* In (A), HSCs were cultured as in Methods and total RNA was isolated at the indicated times. SM α-actin and PreproET-1 mRNA (ppET-1) levels were detected by RPA and signals from specific bands were normalized to control GAPDH mRNA. In the upper panel is shown a representative assay, and quantitative data are presented graphically below (n = 3 for each time point). In (B), freshly isolated HSCs were cultured in 20% serum medium for 1 day and then incubated in 0.2% serum medium with or without IFNγ (1,000 IU/ml) as in upper panel and phase images (lower panel) were taken. An arrow indicates an inactivated HSC following IFNγ exposure. In (C) and (D), activated HSCs were incubated in 0.2% serum medium overnight and then exposed to IFNγ (1,000 IU/mL) for 0, 12, or 24 hours. Total RNA was isolated and preproET-1 mRNA was detected as in Methods (C) (n = 3; *p < 0.05 for IFNγ vs. controls); the conditioned supernatants were collected at the indicated times; ET-1 peptide was measured by ELISA, and normalized to the total protein content from the monolayer cells (D) (n = 3; *p < 0.05 for IFNγ vs. controls). In (E), stellate cells were incubated in 0.2% serum medium with actinomycin D (10μg/mL) for the indicated time points. Total RNA was isolated, preproET-1 mRNA was detected by RPA (left panel). In (right panel), stellate cells were cultured as in (left panel), with the exception that cells were exposed to IFNγ (1,000 IU/mL) for the indicated times. A representative example of 2 different experiments is shown.
Figure 2. Transcriptional analysis of IFNγ-induced effect on preproET-1 promoter. In (A), HSCs were transduced with a preproET-1 promoter (ppET-1pro) luciferase reporter plasmid and then exposed to IFNγ (500 IU/ml) or ET-1 (20nM) for 48 hours. Cell lysates were assayed for luciferase activity (n = 3; *p < 0.05 vs. control and #p < 0.05 vs. control with ET-1). In (B), a series of the truncated preproET-1 promoter luciferase reporter plasmids were generated (left panel) and stellate cells were transfected and exposed to IFNγ as in (A) (n = 3; *p < 0.05 vs. control). In (C), preproET-1 promoter luciferase reporter plasmid harboring a mutant AP-1 binding site or Smad3 binding site or both was prepared (upper panel) and stellate cells were transfected and exposed to IFNγ as in (A) (n = 3; *p < 0.05 for IFNγ vs. control and #p < 0.05 for wild type preproET-1 luciferase reporter plasmid vs. site mutants).

Figure 3. The JNK/c-Jun pathway mediates IFNγ-induced inhibition of preproET-1 transcription. In (A), HSCs were transduced with a preproET-1 promoter/luciferase reporter construct (ppET-1-2121+131) and pre-exposed to U0126 (U, 10uM, MEK/ERK inhibitor), SB203580 (SB, 10uM, p38-MAPK inhibitor) or SP600125 (SP, 10uM, JNK inhibitor) for 30 minutes followed by the addition of IFNγ (500 IU/mL). Luciferase activity was measured (n = 3; *p < 0.05 vs. corresponding control with no IFNγ; #p < 0.05 vs. no IFNγ). In (B), HSCs were incubated in 0.2% serum medium with or without IFNγ (1,000 IU/mL) for the indicated times, and whole cell extracts were subjected to immunoblotting with antibodies to detect phospho-ERK1/2 (p-ERK1/2), ERK1/2, phospho-p38 (p-p38), p38, phospho-JNK (p-JNK) and JNK. Representative images (of 3 others) are shown. In (C), stellate cells were incubated in 0.2% serum medium with or
without IFNγ (1,000 IU/mL) as in (B) and whole cell lysates were subjected to immunoblot with anti-phospho-c-Jun (p-c-Jun) or c-Jun antibodies. Quantitative data are shown in the lower panel (n = 3). In (D), stellate cells were exposed to SP600125 (SP, 10μM) for 30min, and cell lysates were immunoblotted with p-JNK (top panel) or p-c-Jun (middle panel). In (E), HSCs were incubated in 0.2% serum medium with or without SP600125 (SP, 10μM) for the indicated times. PreproET-1 mRNA was detected as in Methods and signals corresponding to specific bands were normalized to control GAPDH mRNA signals. The quantitative data are presented graphically (deviation = 3; *p < 0.05 vs. control).

Figure 4. JNK mediates IFNγ-induced inhibition of preproET-1 promoter activity. In (A), HSCs were co-transfected with preproET-1 promoter luciferase reporter plasmid (ppET1-2121+131) with expression plasmid containing dominant-negative JNK1/2 (JNK1/2-DN) or dominant-active JNK1/2 (JNK1/2-KM) or control expression vector for overnight, exposed to IFNγ (500 IU/mL) for 2 days. The luciferase activity was assayed (n = 3; *p < 0.05 vs. no IFNγ; #p < 0.05 vs. control). In (B), cotransfection was performed as the same as in (A) except ppET1-2121+131 construct was replaced with the ppET1-2121+131 containing AP-1 site mutation (n = 3; *p < 0.05 vs. no IFNγ).

Figure 5. IFNγ inhibits Smad3 phosphorylation and binding in the preproET-1 promoter. In (A), HSCs were cultured in 0.2% serum medium and exposed to IFNγ (1,000 IU/mL) for the indicated time periods. Phosphor-Smad3 (p-Smad3) and total Smad3 were detected by immunoblotting and the signals were quantitated as in Methods. The relative
phosphorylation ratio was determined by p-Smad vs. total Smad and was presented graphically (right panel). In (B), HSCs were cultured in 0.2% serum medium and exposed to IFNγ (1,000 IU/mL) for 16 hours. Nuclear extracts were used for EMSA (specific shifted band was indicated by an arrow). Lane 1 is a control (no nuclear extract); cold probe: lane 4 and 5; anti-Smad3 antibody: lane 6 and 7. In (D), p-Smad3 or Smad3 were detected after exposure to SP600125 (SP, 10μM) (left panel) or SB203580 (SB, 10μM) (right panel) for the indicated time periods by immunoblotting.

Figure 6. *IFNγ directly targets c-Jun.* In (A), following incubation in 0.2% serum medium overnight, HSCs were exposed to IFNγ (1,000 IU/mL) for 12 hours and total RNA was subjected to RT-PCR for c-Jun expression analysis (a representative image (left) and quantitative data (right); n = 3; *p* < 0.05 vs. control)). In (B), HSCs were exposed to IFNγ (1,000 IU/mL) as indicated time points and whole cell lysates were subjected to immunoblotting (n = 3; *p* < 0.05 vs. control). In (C), HSCs were exposed to IFNγ (1,000 IU/mL) for 16 hours and nuclear extracts were subjected to EMSA (the specific shifted band is highlighted with by an arrow). Lane 1 is a control (no nuclear extract); cold probe: lane 4 and 5; anti-c-Jun antibody: lane 6 and 7; mutant probe lane 8 and 9. The experiments in (B) and (C) were repeated twice.

Figure 7. *IFNγ reduces preproET-1 expression during liver injury.* In (A), osmotic pumps containing IFNγ (140,000 IU/100 μL) or carrier (BSA-PBS/100 μL) were implanted into the peritoneal cavity of the mice and then gavaged with CCl₄ once a week for 2 weeks, beginning 2 days after surgery. In (A), stellate cells were isolated and
harvested as in methods and a representative image including specific bands is shown.

On the right, preproET-1 mRNA was measured by RT-PCR (n = 5, *p < 0.05 vs. control).

In (B), on the left, cell lysates were immunoblotted to detect smooth muscle α-actin, collagen type 1 (coll1α1), and β-actin. On the right, specific bands were scanned, normalized to β-actin, and presented graphically (n = 5, *p < 0.05 vs. control). In (C), a diagram highlights putative IFNγ-endothelin-1 signaling pathways.
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Figure 2
Figure 3
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Figure 5

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Relative phosphorylation ratio (p-Smad/total Smad)

- IFNγ

- + IFNγ
Figure 6

(A) IFNγ, c-Jun, and GAPDH expression levels measured by RT-PCR. IFNγ mRNA levels are shown as a histogram with bar graphs for different time points (12 hours) under both negative (−) and positive (+) conditions.

(B) Western blot analysis showing c-Jun and β-actin abundance over time (12 and 24 hours) under different IFNγ conditions. Relative abundance is plotted on a y-axis with time points (12 and 24 hours).

(C) EMSA gel showing binding of AP-1 and mu to cold probe with IFNγ and c-jun Ab under different conditions. Relative abundance is quantified and compared across different time points (12 and 24 hours).
Figure 7