

Angiotensin II Activates I κ B Kinase Phosphorylation of RelA at Ser⁵³⁶ to Promote Myofibroblast Survival and Liver Fibrosis

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See editorial on page 2061.

BACKGROUND & AIMS: The transcription factor nuclear factor- κ B (NF)- κ B promotes survival of hepatic myofibroblasts and fibrogenesis through poorly defined mechanisms. We investigated the activities of angiotensin II and I κ B kinase (IKK) in regulation of NF- κ B activity and the role of these proteins in liver fibrosis in rodents and humans. **METHODS:** Phosphorylation of the NF- κ B subunit RelA at serine 536 (P-Ser⁵³⁶-RelA) was detected by immunoblot and immunohistochemical analyses. P-Ser⁵³⁶-RelA function was assessed using vectors that expressed mutant forms of RelA, cell-permeable blocking peptides, and assays for RelA nuclear transport and apoptosis. Levels of P-Ser⁵³⁶-RelA were compared with degree of fibrosis in liver sections from chronically injured rats and patients with hepatitis C virus-mediated fibrosis who had been treated with the AT1 antagonist losartan. **RESULTS:** Constitutive P-Ser⁵³⁶-RelA is a feature of human hepatic myofibroblasts, both in vitro and in situ in diseased livers. Autocrine angiotensin II stimulated IKK-mediated phosphorylation of RelA at Ser⁵³⁶, which was required for nuclear transport and transcriptional activity of NF- κ B. Inhibition of angiotensin II, the angiotensin II receptor type 1 (AT1), or IKK blocked Ser⁵³⁶ phosphorylation and stimulated myofibroblast apoptosis. Treatment of fibrotic rodent liver with the angiotensin converting enzyme (ACE) inhibitor captopril or the IKK inhibitor sulphasalazine resulted in loss of P-Ser⁵³⁶-RelA-positive myofibroblasts and fibrosis regression. In human liver samples, increased numbers of P-Ser⁵³⁶-RelA-positive cells were associated with fibrosis that regressed following exposure to losartan. **CONCLUSIONS: An auto-crine pathway that includes angiotensin II, IKK, and P-Ser⁵³⁶-RelA regulates myofibroblast survival and can be targeted to stimulate therapeutic regression of liver fibrosis.**

Liver fibrosis is a dynamic process that can either progress to end-stage cirrhosis or regress and enable regeneration of normal functional hepatic tissue.^{1,2} Clinical studies show that effective treatment for the underlying cause of liver injury can provoke regression of fibrosis, although reversion of cirrhosis remains unlikely.^{1,2} Unfortunately, long-term effective control of the causes of chronic liver disease remains a significant challenge and, for the majority of patients, is currently not possible. Development of therapeutics that stimulate regression of preestablished fibrosis would have substantial clinical impact.

Transient accumulation of activated smooth muscle α -actin (α -SMA)-positive myofibroblasts is a pivotal event in tissue injury that enables formation of a temporary scar composed of fibril-forming collagens secreted by the myofibroblast.³ In addition, the contractile properties of the myofibroblast promote wound closure, which protects the injured tissue from infection and further damage.⁴ Resolution of wound healing and remodelling of scar tissue is associated with diminution of the myofibroblast population.^{5,6} By contrast, progressive fibrosis is associated with a steadily increasing myofibroblast population because of proliferation and prolonged survival.^{3,7} Experimental rodent-based models of liver disease have revealed an association between myofibroblast apoptosis and regression of fibrosis.^{5–7} Agents such as gliotoxin, sulphasalazine, and thalidomide promote apoptosis of liver myofibroblasts and augment the rate at which fibrosis spontaneously regresses.^{8–10} Selective targeting of gliotoxin to myofibroblasts provokes regression of fibrosis even under conditions of sustained liver injury.¹¹ Myofibroblast apoptosis is therefore not simply a bystander event in fibrosis regression; rather, it is a naturally occurring phys-

Abbreviations used in this paper: ACE, angiotensin converting enzyme; α -SMA, smooth muscle α -actin; AT1, angiotensin type 1 receptor; I κ B, inhibitor of NF- κ B; IKK, I κ B kinase; NF- κ B, nuclear factor- κ B; P-Ser⁵³⁶-RelA, RelA phosphorylated on serine 536.

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ologic process that enables dynamic remodelling of extracellular matrix and can be stimulated to promote therapeutic regression of fibrosis. The next step toward the translation of these discoveries to the clinic is the identification of fibrogenic-signaling pathways that can be effectively and safely targeted in humans.

Human and rodent liver myofibroblasts express constitutive active nuclear factor- κ B (NF)- κ B (RelA:p50), which promotes survival by stimulating the expression of antiapoptotic proteins Gadd45 β and BCL-2.^{12,13} Specific inhibition of NF- κ B is sufficient to provoke apoptosis of fully mature human myofibroblasts that are relatively resistant to apoptosis.^{13,14} These discoveries raise the important questions of how constitutive NF- κ B is regulated in myofibroblasts and whether the regulatory checkpoints that control NF- κ B activity can provide clinically safe therapeutic targets. A physiologically relevant model for the generation of liver myofibroblasts is the culture-induced transdifferentiation of quiescent hepatic stellate cells (HSC) into activated myofibroblasts.⁶ This model recapitulates the molecular and cellular events involved in sinusoidal fibrosis that are common to alcoholic and fatty liver diseases. Using this model, we have shown that HSC transdifferentiation is accompanied by a sustained transcriptional repression I κ B α , the natural occurring inhibitor of NF- κ B.^{15,16} I κ B α is abundantly expressed in the majority of mammalian cells and forms a direct interaction with NF- κ B that masks its nuclear localization and DNA-binding motifs, resulting in an inactive cytoplasmic location for the transcription factor. Upon stimulation of cells by a variety of agents, including lipopolysaccharides and tumor necrosis factor- α , 2 serine residues (Ser32 and Ser36) on I κ B α are phosphorylated by the I κ B kinase (IKK) β component of the IKK complex.¹⁷ This results in polyubiquitination and proteasome-mediated degradation of I κ B α which releases NF- κ B for nuclear transport and interaction with target genes. Depletion of I κ B α in hepatic myofibroblasts effectively bypasses this regulatory checkpoint. However, the IKK inhibitor sulphasalazine inhibits myofibroblast NF- κ B activity and stimulates apoptosis.⁹ This suggests existence of additional regulatory IKK-dependent checkpoints that control myofibroblast NF- κ B.

In this study, we discover that the NF- κ B RelA subunit is constitutively phosphorylated at residue serine 536 (P-Ser⁵³⁶-RelA) both in cultured HSC and myofibroblasts of diseased human liver. We determine that myofibroblasts express constitutive IKK β activity under the autocrine control of the renin-angiotensin system which is required for maintenance of P-Ser⁵³⁶-RelA, nuclear transport of NF- κ B, and myofibroblast survival. The clinical relevance of this autocrine angiotensin II/IKK β /NF- κ B pathway is demonstrated with animal models of liver fibrosis and human liver biopsy samples that collectively provide strong support that it can be targeted to stim-

ulate fibrosis regression with drugs that are safe for use in humans.

Materials and Methods

Reagents

Jun N-terminal kinase (JNK) inhibitor SP600125 was purchased from Calbiochem (MERK, Nottingham, UK). Angiotensin II and Enalapril were purchased from Sigma (Poole, UK) and the angiotensin II receptor antagonist ZD7155 from Tocris Biochemicals (Bristol, UK). Cell-permeable peptides were synthesized, high-performance liquid chromatography purified, and verified with mass spectroscopy at W. M. Keck Biotechnology Resource Center (New Haven, CA). Peptides sequences were synthesized as fusions of the membrane-translocating peptide sequence derived from antennapedia (PTD)—DRQIK, IWFQN, NRRMK, WKK—with peptide sequences spanning amino acids 271–282 (Ser276) within the nuclear localization sequence of RelA (RelA-P1 = QLRRPSDRELSE) or to a sequence that spans amino acids 525–536 (Ser529/536) in the transactivation domain of RelA (RelA-P6 NGLLSGDEDFSS) or control peptides where serine 529 and/or serine 536 are substituted with alanine.

Cell Culture

Rat hepatic myofibroblasts were isolated from livers of Sprague-Dawley rats and cultured as previously described.⁵ Human hepatic myofibroblasts were isolated from livers of adult male patients after surgical resection as approved by the Newcastle and North Tyneside Local Research Ethics Committee, subject to patient consent. RelA^{-/-} mouse embryonic fibroblasts (MEFS) were a gift of Dr N. Perkins, University of Bristol, and were cultured on plastic in Dulbecco's modified Eagle medium, supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, 2 mmol/L L-glutamine, and 10% fetal calf serum (FCS) and maintained at 37°C in an atmosphere of 5% CO₂.

In Vivo Models of Rodent Liver Fibrosis and Losartan Clinical Trial

Protocols for carbon tetrachloride (CCl₄) and bile duct ligation (BDL) injury models and treatments with sulphasalazine and captopril, respectively, are described in detail elsewhere.^{9,18} Briefly, sulphasalazine was administered 24 hours following the final CCl₄ injection of a 5-week injury model, and control mice were administered vehicle alone.⁹ Captopril treatment was commenced 2 weeks after start of BDL injury and continued for a further 2 weeks; controls were untreated for the entire 4-weeks.¹⁸

Acute CCl₄

Cell-permeable peptides P6 or control (10 mg/kg body weight) was given intraperitoneally (IP) 30 minutes prior to and then at 6 and 20 hours after a single dose of

CCL₄ (CCL₄:olive oil, given at 1 μ L/g body weight). ZD7155 (10 mg/kg body weight) was given IP at 20 or 22 hours after a single injection of CCL₄. In both studies, mice were culled 24 hours after the CCL₄ injury. Extensive methodology of the losartan clinical trial has been published.¹⁹ Fourteen patients with chronic hepatitis C virus (HCV)-induced fibrosis were included in an open-label study investigating antifibrogenic effects of losartan. Inclusion criteria were as follows: (1) age 35–65 years; (2) elevation of serum aminotransferases for >6 months, and positive RNA-HCV; (3) significant histologic fibrosis in liver biopsy (\geq F2 in METAVIR score); and (4) no previous response and/or contraindications to antiviral therapy. Exclusion criteria were as follows: (1) existence of other known liver disease; (2) past history of hepatic decompensations or hepatocellular carcinoma; (3) alcohol consumption (>20 g/day); (4) arterial hypertension; (5) serum creatinine >1.5 mg/dL; (6) treatment with AT1 receptor blockers, angiotensin converting enzyme (ACE) inhibitors, or interferon in the preceding 12 months; or (7) contraindications to oral losartan. Patients were treated for 18 months with oral losartan 50 mg/day (Cozaar; MSD, Wilmington, DE). In all patients, 2 liver biopsies were performed: the first biopsy 24 hours prior to initiation of treatment and the second biopsy on the following day of the last dose of losartan. Fibrosis was blindly evaluated by the same pathologist using the METAVIR scoring system. The protocol was approved by the Ethics

Committee of the Hospital Clínic of Barcelona and the Agencia Española del Medicamento (ARAHEPC 02-0491) and registered into the protocol registration system (NCT00298714). All patients gave written informed consent. Paired biopsy samples from 11 out of the original 14 patients were available for the current study.

Western Blot

Whole-cell extracts were resolved by SDS PAGE and transferred to nitrocellulose. Primary antibodies were employed for detection of IKK α / β (Santa Cruz Biotechnology, Santa Cruz, CA), P-Ser⁵³⁶-RelA and P-IKK α / β (Cell Signaling Technology, Danvers, MA), RelA (Calbiochem), and β -actin (Sigma). Horseradish peroxidase-conjugated secondary antibodies, goat anti-rabbit (Santa Cruz Biotechnology), and goat anti-mouse (Sigma) were used at a dilution of 1:2000.

Plasmid DNA, Cell Transfections, and Reporter Assays

IKB α -Luc (NF- κ B-dependent) and TIMP1 (NF- κ B-independent) reporters have been previously described.^{9,16} Wild-type RelA-EGFP expression construct was generated by insertion of human RelA into pEGFP-N1 (Clontech-Takara, Saint-Germain-en-Laye, France). RelA_{S536A}-EGFP was generated using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Myofibroblasts were

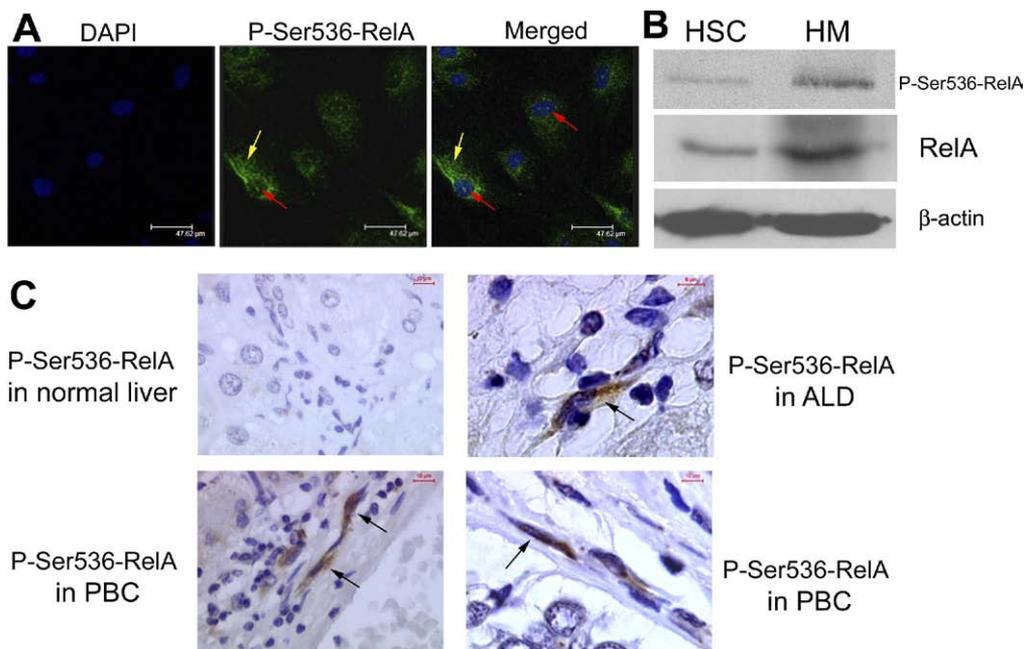


Figure 1. P-Ser⁵³⁶-RelA is a marker of activated hepatic myofibroblasts. (A) Nuclear localization of P-Ser⁵³⁶-RelA in human myofibroblasts (red arrows) and cytoplasmic localization (yellow arrows) was determined by indirect immunofluorescence. Fluorescent images using FITC (P-Ser⁵³⁶-RelA) and DAPI (nuclei) filters were taken using laser scanning confocal microscopy (63 \times 1.9 zoom magnification). Photomicrographs represent 2 independent cell isolations. (B) Whole cell extracts were isolated from quiescent (day 0) and activated (day 10) rat hepatic stellate cells, and levels of RelA and P-Ser⁵³⁶-RelA were assessed by Western blotting (n = 3). (C) Photomicrographs (original magnification, 40 \times) show liver sections from near normal human liver or patients with primary biliary cirrhosis (PBC) and alcoholic liver disease (ALD) immunohistochemically stained for RelA-phospho-Ser⁵³⁶. Arrows indicate high levels of P-Ser⁵³⁶-RelA in myofibroblastic cells, and cell counts show an average of 52.6% \pm 4.6% and 50.4% \pm 2.9% P-Ser⁵³⁶-RelA positive cells in fibrotic bands in PBC and ALD, respectively.

transfected using the Effectene protocol (Qiagen, Crawley, UK) with 0.5 μg of reporter plasmid DNA and 10 ng of control Renilla plasmid pRLTK. Transfection of mouse embryonic fibroblasts with 1 μg of p65-enhanced green fluo-

rescent protein (EGFP) or p65_{S536A}-EGFP, 10 ng pRLTK was achieved using polyethylenimine (Polysciences Inc, Eppelheim, Germany). A transfection efficiency of ≥60% was observed in all experiments.

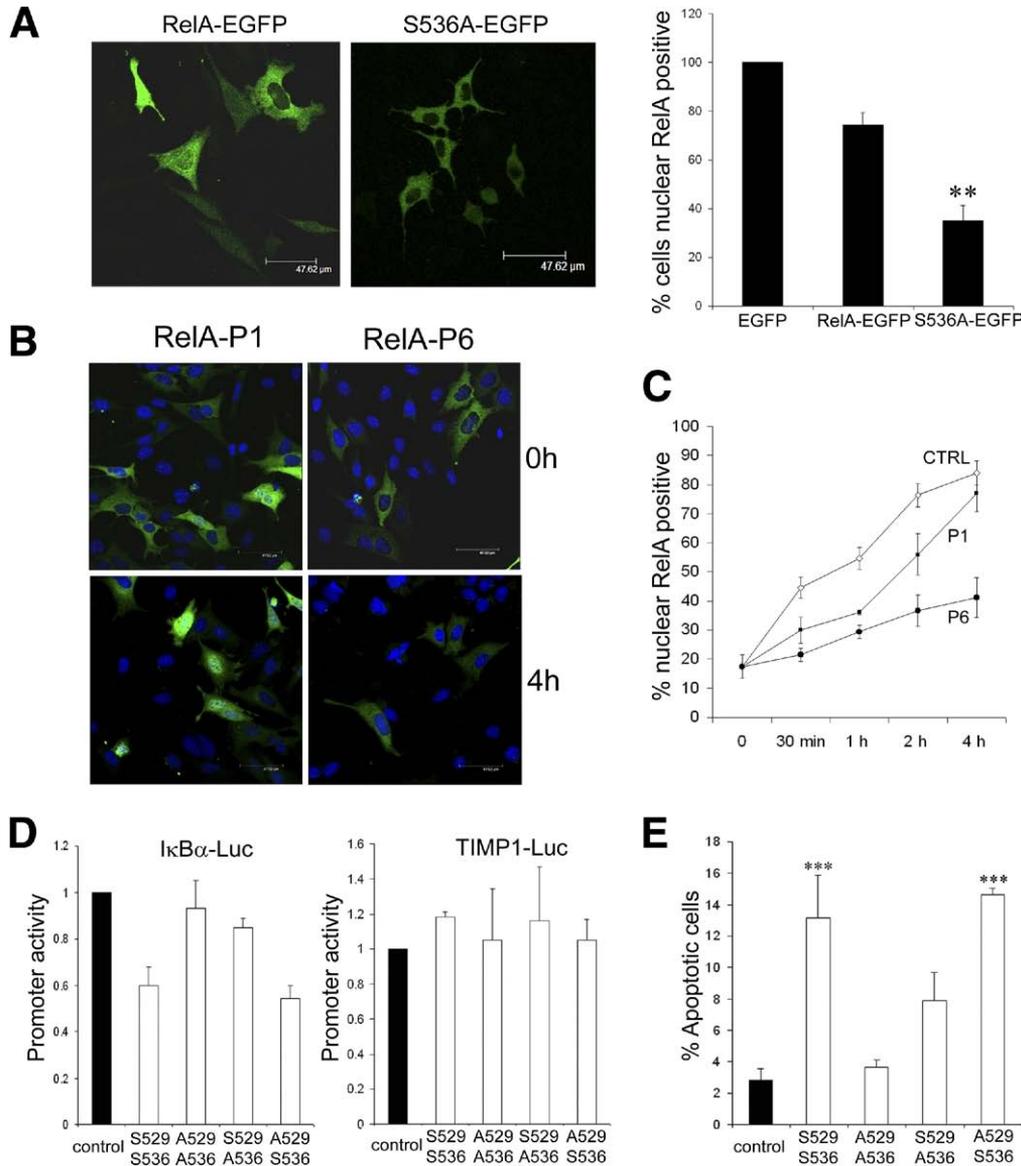


Figure 2. P-Ser⁵³⁶-RelA regulates nuclear translocation and myfibroblast survival. (A) Representative fluorescent confocal scanned images (original magnification, 63×) of RelA^{-/-} mouse embryonic fibroblasts (MEFs) transfected with RelA-EGFP or RelA_{S536A}-EGFP (S536A-EGFP) plasmids. Nuclear positive cells were counted in RelA^{-/-} MEFs transfected with expression vectors: EGFP, RelA-EGFP, and RelA_{S536A}-EGFP. Ser⁵³⁶ to Ala mutation statistically significantly retarded RelA nuclear localization, ***P* < .01. An average of 150 cells/plasmid/experiment were counted, and data are expressed as mean ± SEM, n = 4. (B) RelA^{-/-} MEFs were transfected with RelA-EGFP overnight, serum starved for 24 hours, and then treated with cell-permeable peptides RelA-P6 (Ser529/536) or RelA-P1 (Ser276) for 30 minutes prior to serum stimulation. Representative fluorescent confocal scanned images (original magnification, 63×) of RelA-EGFP (green) and nuclei (blue) at 0 hours or 4 hours serum stimulation with competing peptides. (C) RelA nuclear positive cells were counted at time 0 (24 hours serum free); 30 minutes; and 1, 2, and 4 hours postserum stimulation ± 30-minute pretreatment with 50 μmol/L competing peptides. Results were expressed as average percentage RelA nuclear positive cells/10× field ± SEM, n = 6, vehicle (◇), RelA-P1 (■), and RelA-P6 (●). (D) Rat hepatic myfibroblasts were transiently transfected with IkBα-Luc or TIMP1-Luc promoter reporter plasmid for 16 hours, then treated with cell-permeable RelA-P6 (Ser529/536) or controls for a further 6 hours. (E) Apoptosis of rat myfibroblasts incubated with 50 μmol/L cell-permeable RelA phosphorylation competing peptides for 24 hours was determined using acridine orange staining. Respective *P* values for ser529/536, ala529/536, ser529/ala536, and ala529/ser536 were ****P* < .001, ns, ns, and ****P* < .001. Results were expressed as percentage apoptotic cells in 4 independent experiments ± SEM, and *P* values were determined using 1-way ANOVA.

Total RNA Isolation and Reverse-Transcriptase Polymerase Chain Reaction

Total RNA was isolated from $\sim 5 \times 10^6$ cells or ~ 10 mg liver tissue using the "Total RNA purification kit" (Qiagen) and used to generate complementary DNA. Polymerase chain reaction (PCR) amplification of target genes was conducted using primers recognizing rat and human β -actin, BCL-2, and Gadd45 β described elsewhere.¹³ Primers recognizing human angiotensinogen were forward, CCGTGGTGGCGTGTTC; reverse, TTGCTGGAAAGTGAGACC. Twenty-five cycles were used for β -actin, and 35 cycles for target genes. PCR products were detected and verified as previously described.¹³ Methods for Taqman quantitative reverse-transcription PCR (qRT-PCR) to detect mouse 18S, TIMP-1, α -SMA, and procollagen I have been previously described.²⁰

Indirect Immunofluorescence and Confocal Microscopy

P-Ser⁵³⁶-RelA was visualized by indirect immunofluorescence. Cells were fixed in 4% paraformaldehyde for

20 minutes, permeabilized for 5 minutes with 0.1% Triton X-100, and blocked with phosphate-buffered saline (PBS)/0.1% Triton X-100/5% FCS at 37°C for 2 hours. Slides were incubated overnight at 4°C with mouse monoclonal anti-P-Ser⁵³⁶-RelA antibody (Cell Signaling Technology), 1:250 dilution. Next, slides were PBS washed and incubated with rabbit anti-mouse FITC-conjugated antibody (Dako, Ely, UK). Slides were mounted using VectorShield containing 4,6-diamidino-2-phenylindole (DAPI). FITC and DAPI fluorescent images were taken using a Leica-TCS-SP2UV scanning confocal microscope (Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry

Slides were dewaxed in clearene and dehydrated in alcohol, citric saline antigen retrieval was performed, and endogenous peroxidase activity was blocked using hydrogen peroxide. Inhibition of nonspecific binding was achieved using avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) followed by incubation with complete culture medium for 20 minutes. Slides were

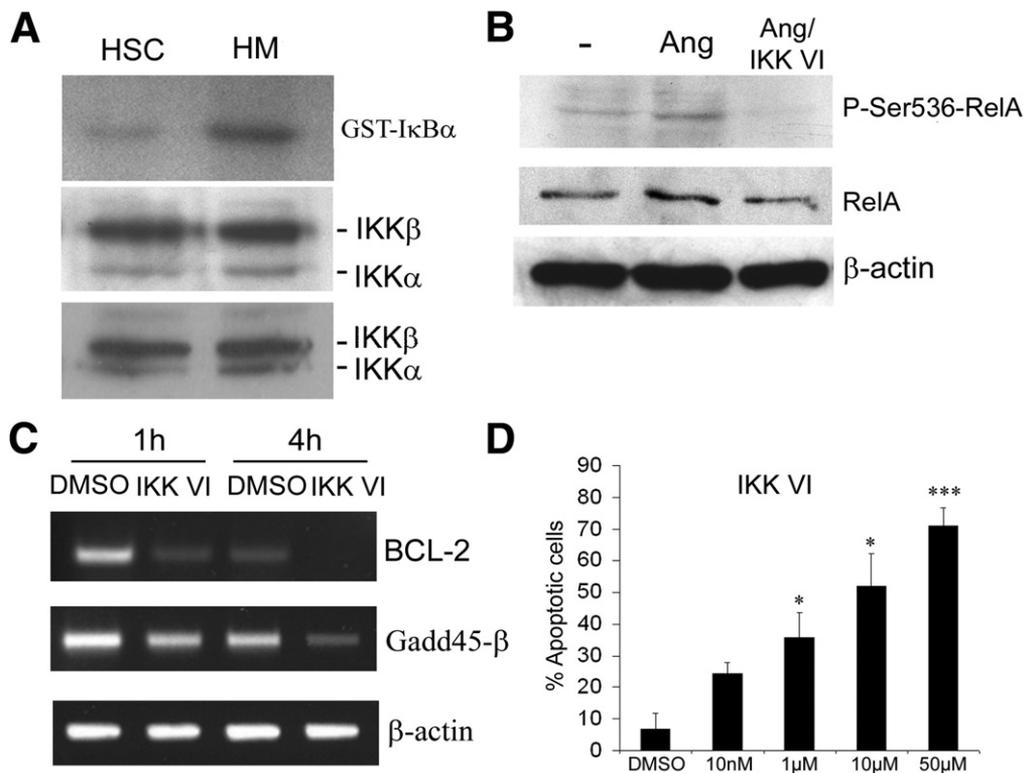


Figure 3. Constitutive IKK activity regulates P-Ser⁵³⁶-RelA and myofibroblast survival. (A) IKK activity assay. Total IKK complex was immunoprecipitated from freshly isolated rat hepatic stellate cells and culture activated rat myofibroblasts using anti-IKK γ and incubated with kinase buffer, γ^{32} P-ATP, and recombinant GST-I κ B α , then quenched with Laemmli buffer. Reactions were resolved by SDS-PAGE, and GST-P-I κ B α was visualized upon exposure to x-ray film. Total levels of IKK α/β were assessed in whole-cell extracts (middle) or input control (bottom) isolated from quiescent and activated rat myofibroblasts by Western blotting. (B) Whole-cell extracts isolated from human myofibroblasts treated $\pm 10^{-8}$ mol/L angiotensin II with or without 50 μ mol/L Calbiochem IKK inhibitor VI, relative levels of total RelA, P-Ser⁵³⁶-RelA, and β -actin were assessed by Western blotting. (C) Relative mRNA levels of BCL-2, Gadd45 β , or β -actin were assessed by RT-PCR in rat myofibroblasts treated ± 50 μ mol/L Calbiochem IKK inhibitor VI for up to 4 hours. (D) Rat myofibroblast apoptosis was induced after 16-hour incubation with 10 nmol/L–50 μ mol/L IKK inhibitor VI and quantified by acridine orange staining with respective *P* values of **P* < .05, ***P* < .01, ****P* < .001 for 1–50 μ mol/L. Results were expressed as percentage apoptotic cells for at least 4 independent experiments \pm SEM, and *P* values were determined using a 1-way ANOVA. Gels are representative of at least 2 or 3 independent experiments.

incubated overnight at 4°C with mouse anti-P-Ser⁵³⁶-RelA antibody (Cell Signaling Technology) diluted 1:500. Slides were washed in PBS, and amplification of antigen was achieved using the Vector Elite mouse IgG kit (Vector Laboratories). P-Ser⁵³⁶-RelA-positive cells were visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB), counted in 20 high-power fields, and expressed as average number positive cells/high-power field \pm SEM. For studies with human biopsy slides, Leica Qwin software (Leica Microsystems) was used for densitometric analysis to quantify percentage area of the slide positively stained; results were expressed as average percentage area positively stained \pm SEM.

IKK Assay

Cells were harvested and resuspended in Lysis buffer: 20 mmol/L Tris, 0.5% Triton X-100, 20 mmol/L sodium chloride, 30 mmol/L EGTA, 30 mmol/L EDTA plus protease and phosphatase inhibitors (Sigma). Immunoprecipitation: 300–500 μ g of protein was incubated with 1.2 μ g anti-IKK γ (Santa Cruz Biotechnology) overnight at 4°C. Twenty microliters protein-G beads was added to the immunoprecipitation mix and incubated at 4°C for 2 hours. Protein/bead complexes were centrifuged at 5000 rpm for 5 minutes, and supernatant was removed. Beads were washed once with lysis buffer and a further 2 times in kinase buffer (20 mmol/L HEPES, 20 mmol/L β -glycerophosphate, 1 mmol/L Dithiothreitol, 20 mmol/L magnesium chloride, plus protease and phosphatase inhibitors), and the supernatant was removed. Kinase reaction mix (10 μ mol/L AP, 5 μ Ci ³²P- γ ATP, and 1 μ g GST-I κ B α) was incubated with the protein/bead complex for 30 minutes at 30°C. The reaction was terminated by addition of 25 μ L Laemelli buffer and boiled.

Quantification of Apoptosis

Apoptotic cells were identified and counted by acridine orange staining as previously described.^{5,9}

Statistical Analysis

All *P* values were calculated using a 2-tailed paired Student *t* test or a 1-way ANOVA, and *P* < .05 was considered statistically significant.

Results

Constitutive P-Ser⁵³⁶-RelA Is Critical for the Survival of Hepatic Myofibroblasts

We examined phosphorylation of the Ser⁵³⁶ residue of RelA, a posttranslational modification that has been associated with transcriptional active NF- κ B in a cell context and signaling-dependent manner.^{21–23} Confocal imaging of cultured human hepatic myofibroblasts immunostained with anti-P-Ser⁵³⁶-RelA revealed substantial levels of the modified protein in both the cytoplasm

and nucleus (Figure 1A). Culture-induced transdifferentiation of HSC was associated with a modest increase in expression of RelA and the appearance of P-Ser⁵³⁶-RelA (Figure 1B). Myofibroblasts expressing active P-Ser⁵³⁶-RelA were observed in situ within fibrotic tissue from livers of patients suffering primary biliary cirrhosis and alcoholic liver disease. This modification was weakly detected in bile ducts of near normal human liver (Figure 1C).

Mutation of Ser⁵³⁶ generated a mutant RelA defective for nuclear transport (Figure 2A). The functional significance of Ser⁵³⁶ phosphorylation was further examined using a cell-permeable peptide (RelA-P6) that spans amino acids 525–536 of RelA and that via competition with Ser⁵²⁹ and Ser⁵³⁶ residues in endogenous RelA inhibits NF- κ B activation.²⁴ RelA-P6 suppressed serum-induced nuclear accumulation of transfected RelA-EGFP (Figure 2B and C). By contrast, peptide RelA-P1 (amino acids 271–282) that spans Ser²⁷⁶ failed to prevent serum-induced nuclear translocation of RelA-EGFP. Peptides competing with Ser⁵³⁶ but not Ser⁵²⁹ inhibited NF- κ B-dependent (I κ B α -Luc) but not NF- κ B-independent (TIMP1-Luc) transcriptional activity in myofibroblasts (Figure 2D). Treatment of myofibroblasts with RelA-P6 stimulated apoptosis; this response was completely abro-

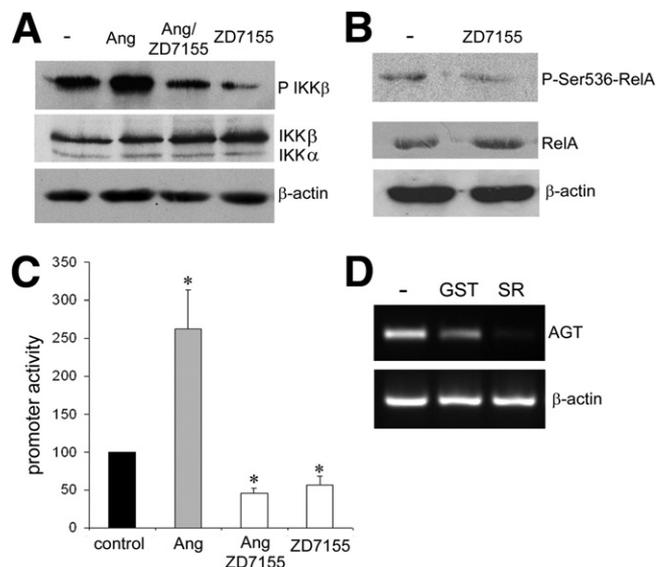


Figure 4. IKK and P-Ser⁵³⁶-RelA are under the autocrine control of angiotensin II. Whole-cell extracts isolated from human myofibroblasts treated with (A) 10⁻⁸ mol/L angiotensin II \pm 10 μ mol/L ZD7155 and (B) relative levels of total and P-IKK α/β or total RelA or P-Ser⁵³⁶-RelA and β -actin were assessed by Western blotting. (C) Human myofibroblasts were transiently transfected with I κ B α -Luc promoter reporter plasmid for 16 hours, serum starved for 24 hours, and treated \pm angiotensin II (10⁻⁸ mol/L) or ZD7155 (10 μ mol/L) for a further 6 hours. Reporter gene activity was expressed as a percentage relative to unstimulated control \pm SEM (n = 4); *P* values, **P* < .05. (D) Relative levels of angiotensinogen (AGT) or β -actin were determined via RT-PCR in human myofibroblasts treated with either 30 μ g/mL TAT-I κ B α -SR (SR) or control protein TAT-GST. Gels are representative of 2 independent cell preparations.

gated when the Ser⁵²⁹ and Ser⁵³⁶ residues were mutated in combination (Figure 2E). However, mutation at Ser⁵²⁹ alone was without significant effect on apoptosis, whereas a single mutation at Ser⁵³⁶ substantially suppressed the apoptosis-promoting properties of RelA-P6. These data functionally implicate P-Ser⁵³⁶-RelA as a critical regulator of myofibroblast survival but additionally implicate phosphorylation at Ser⁵²⁹, which appears to operate synergistically with phospho-Ser⁵³⁶.

A Positive Feedback Loop Regulates Constitutive P-Ser⁵³⁶-RelA, NF- κ B Activity, and Myofibroblast Apoptosis via Angiotensin II Activated IKK β

Ser⁵³⁶ can be a target for IKK-mediated phosphorylation.^{21,25} We show that quiescent HSC lack IKK activity, which is constitutively present in myofibroblasts despite no change in total levels of IKK α/β during transdifferentiation (Figure 3A). A highly selective IKK inhibitor suppressed P-Ser⁵³⁶-RelA (Figure 3B), inhibited expression of antiapoptosis factors BCL-2 and Gadd45 β (Figure 3C), and stimulated apoptosis (Figure 3D). Angiotensin II plays a major role in the fibrogenic response

of the liver.^{26,27} Hepatic myofibroblasts express an auto-crine renin-angiotensin system, secrete angiotensin II, and respond to the hormone via surface expressed AT1 receptors.²⁸ Because AT1 receptors are implicated as activators of IKK,²⁹ we determined whether angiotensin II is responsible for constitutive IKK activity and P-Ser⁵³⁶-RelA in hepatic myofibroblasts. Western blot for Phospho-IKK revealed constitutive phosphorylation of IKK β (a marker of IKK activity) in human myofibroblasts, which was enhanced by exogenous angiotensin II and suppressed by the AT1 antagonist ZD7155 (Figure 4A). Treatment of human myofibroblasts with ZD7155 suppressed Ser⁵³⁶ phosphorylation (Figure 4B) and inhibited basal and angiotensin II-stimulated NF- κ B activity (Figure 4C). These data suggest that angiotensin II signals via AT1 receptors to stimulate IKK β and, in turn, phosphorylation of Ser⁵³⁶ and activation of NF- κ B. Furthermore, inhibition of NF- κ B with a cell-permeable I κ B- α super-repressor protein¹³ blocked expression of the angiotensin II precursor angiotensinogen (Figure 4D) identifying a positive feedback mechanism that promotes constitutive NF- κ B.

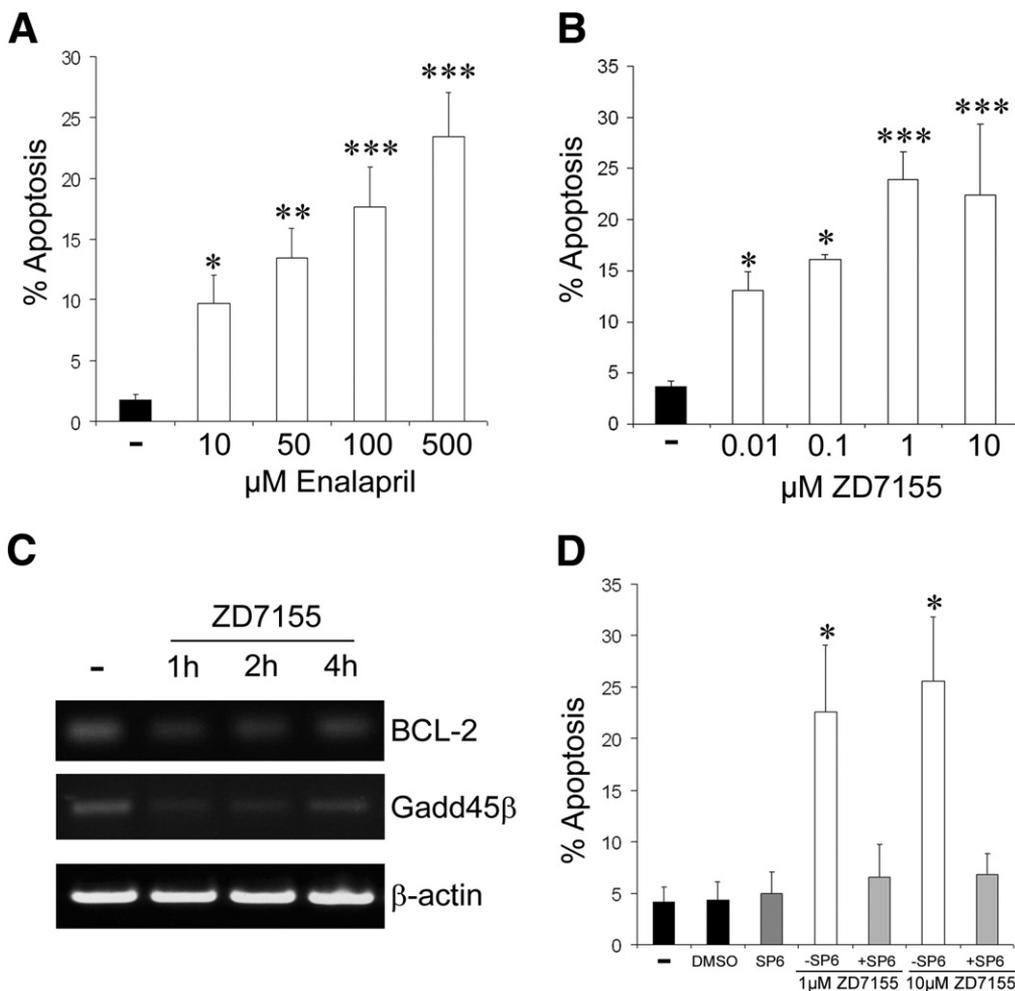


Figure 5. Angiotensin II prevents JNK-dependent myofibroblast apoptosis. (A) Human myofibroblast apoptosis was determined via acridine orange staining after 16-hour exposure to enalapril (10–500 μ mol/L); respective *P* values, **P* < .05, ***P* < .01, and ****P* < .001. (B) Percentage apoptosis was determined in human myofibroblasts treated with ZD7155 (0.01–10 μ mol/L); respective *P* values, **P* < .05, ****P* < .001. (C) Relative mRNA levels of BCL-2, Gadd45 β , and β -actin were determined by RT-PCR after 0–4-hour treatment with 10 μ mol/L ZD7155. (D) Quantification of myofibroblast apoptosis after treatment with 1 and 10 μ mol/L ZD7155 \pm 50 μ mol/L JNK inhibitor SP600125 or vehicle, DMSO; respective *P* values, **P* < .05. Statistical significance was calculated using a 1-way ANOVA.

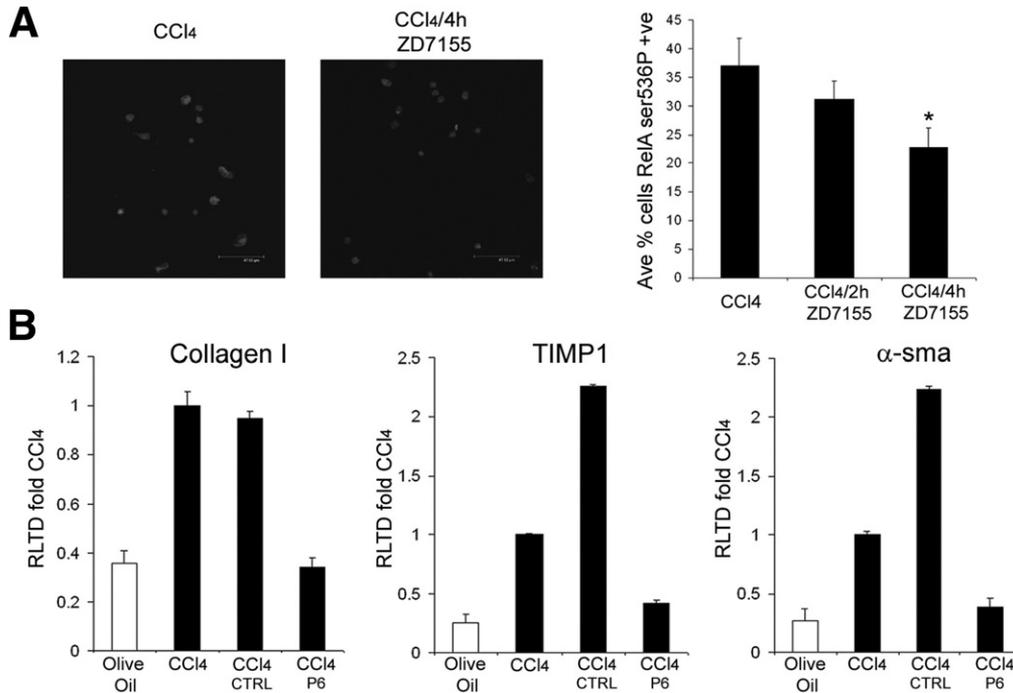


Figure 6. Myofibroblast P-Ser⁵³⁶-RelA is angiotensin dependent and profibrogenic in acute liver injury. (A) Mice were injured once with CCl₄, then treated with ZD7155 (10 mg/kg) at 20 or 22 hours postinjury. Hepatic myofibroblasts were isolated 24 hours after CCl₄ injury and stained for P-Ser⁵³⁶-RelA using indirect immunofluorescence. Fluorescent images using FITC (P-Ser⁵³⁶-RelA) and DAPI (nuclei) filters were taken using laser scanning confocal microscopy (original magnification, 63×). Positively stained cells were expressed as a percentage of total cells counted in 15 fields ± SEM. Statistical significance was **P* < .05 calculated using a 1-way ANOVA. (B) Mice given a single dose of CCl₄, then treated with cell-permeable P-Ser⁵³⁶-RelA competing peptides (P6) or control 30 minutes prior to and 6 and 20 hours postinjury. Livers were harvested; RNA was isolated 24 hours after CCl₄ administration; and mRNA levels of procollagen I, TIMP1, and α-SMA were assessed using Taqman qRT-PCR. Relative level of transcriptional difference between olive oil and treatment groups was calculated and expressed as an average ± SEM, from 4 or 5 independent experiments.

Treatment of human myofibroblasts with ACE inhibitor (enalapril) or ZD7155 provoked apoptosis (Figure 5A and B). ZD7155 also suppressed expression of the NF-κB-regulated antiapoptotic factors BCL-2 and Gadd45β (Figure 5C). Apoptosis induced by ZD7155 was prevented by pretreatment with JNK inhibitor SP600125 (Figure 5D), which we previously reported to block apoptosis induced by NF-κB inhibitors.^{9,13} Angiotensin II therefore promotes survival of myofibroblasts at least in part by stimulating NF-κB-dependent expression of the naturally occurring JNK inhibitor Gadd45β.

In Vivo Phosphorylation at Ser⁵³⁶-RelA in Myofibroblasts Is Angiotensin Dependent and Profibrogenic

Myofibroblasts isolated from livers at 24 hours post-CCl₄ were positive for P-Ser⁵³⁶-RelA (Figure 6A, left photomicrograph). In vivo induction of P-Ser⁵³⁶-RelA in myofibroblasts in response to acute CCl₄-induced liver injury was attenuated by 4-hour treatment with ZD7155 (Figure 6A, right photomicrograph). A criticism of this experiment is that the postisolation culture of myofibroblasts may activate angiotensin II-independent pathways that influence P-Ser⁵³⁶-RelA. However, despite this possibility, our data clearly demonstrate that short-term in

vivo treatment with an angiotensin II inhibitor reduces the number of P-Ser⁵³⁶-RelA-positive myofibroblasts isolated from the injured liver and as such makes a strong case for angiotensin II as a major contributor to myofibroblast NF-κB activity and survival in the injured liver. Administration of cell-permeable peptides competing with P-Ser⁵³⁶-RelA (P6) blocked the induction of the fibrogenic genes procollagen I, TIMP1, and α-SMA in response to acute liver injury. Direct targeting of P-Ser⁵³⁶-RelA is therefore a potential antifibrotic strategy. Of note, the control peptide lacking Ser⁵³⁶ promoted an increase in expression of TIMP1 and α-SMA (Figure 6B), an effect currently under investigation.

Constitutive P-Ser⁵³⁶-RelA Is a Biomarker for Liver Fibrosis That Is Susceptible to Regression in Response to Blockade of Angiotensin II and IKK Signaling

We next determined whether Ser⁵³⁶ phosphorylation is suppressed upon in vivo treatment with ACE and IKK inhibitors. The ACE inhibitor captopril promotes regression of BDL-induced fibrosis despite progressive injury,¹⁸ and the IKK/NF-κB inhibitor sulphasalazine augments spontaneous regression of CCl₄-induced fibrosis.⁹ Both treatments are associated with loss of P-Ser⁵³⁶-

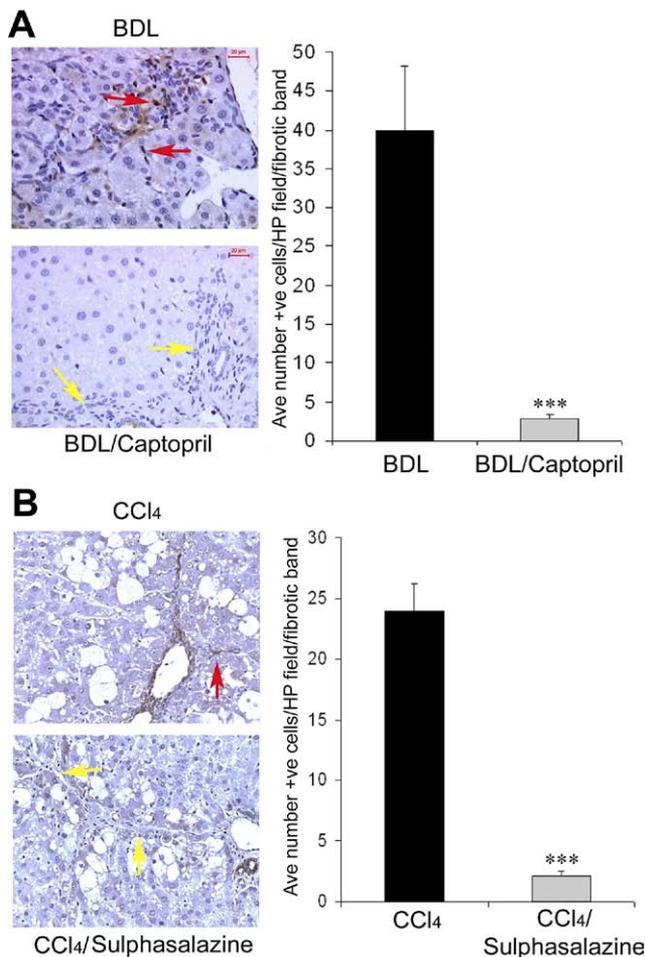


Figure 7. Blockade of IKK activity or angiotensin II signaling promotes regression of established fibrosis in rodents. (A) Rats were treated with captopril (100 mg/kg/day) commencing 2 weeks after BDL and livers harvested after a further 2 weeks injury. Photomicrographs (original magnification, 40×) show rat liver sections without (top) or with captopril treatment (bottom), immunostained with anti-P-Ser⁵³⁶-RelA antibodies. Positive myofibroblastic cells in fibrotic tissue (red arrows) were reduced upon captopril treatment (yellow arrows). Cell counts show the average number of P-Ser⁵³⁶-RelA-positive cells in fibrotic bands, expressed as mean ± SEM, n = 8, and ***P < .001. (B) Photomicrographs (original magnification, 20×) of liver sections from 4 weeks CCl₄ injured rats ± 1 IP administered dose (100 mg/kg) of sulphasalazine, immunostained for P-Ser⁵³⁶-RelA. Graphical representation of cell counts showing the average number of P-Ser⁵³⁶-RelA-positive cells in fibrotic bands from CCl₄ and CCl₄/sulphasalazine-treated rats. Cell counts are the mean of up to n = 5 ± SEM. A statistically significant reduction in P-Ser⁵³⁶-RelA-positive cells was observed in the treatment group, ***P < .001. All P values were determined using an unpaired 2-tailed Student t test.

RelA in cells associated with fibrotic tissue (Figure 7). Of note, both treatments were originally reported to result in roughly 50% reduced numbers of α-SMA⁺ cells relative to controls^{9,18}; however, this would not account for the 90% loss of P-Ser⁵³⁶-RelA observed here and may indicate that diminution of P-Ser⁵³⁶-RelA precedes myofibroblast apoptosis. To translate these discoveries to human disease, we examined P-Ser⁵³⁶-RelA in biopsy slides from the

fibrotic livers of patients with chronic hepatitis C prior to and after 18 months of treatment with the AT1 antagonist losartan. As described elsewhere,¹⁹ this treatment promoted regression of fibrosis in half of the patients (Supplementary Table 1). Staining biopsy slides for P-Ser⁵³⁶-RelA indicated that patients who underwent regression in response to losartan began (prior to treatment) with more P-Ser⁵³⁶-RelA positive cells than found in nonresponders (Figure 8). Following treatment, numbers of P-Ser⁵³⁶-RelA-positive cells diminished in the losartan-responder group to levels around those measured

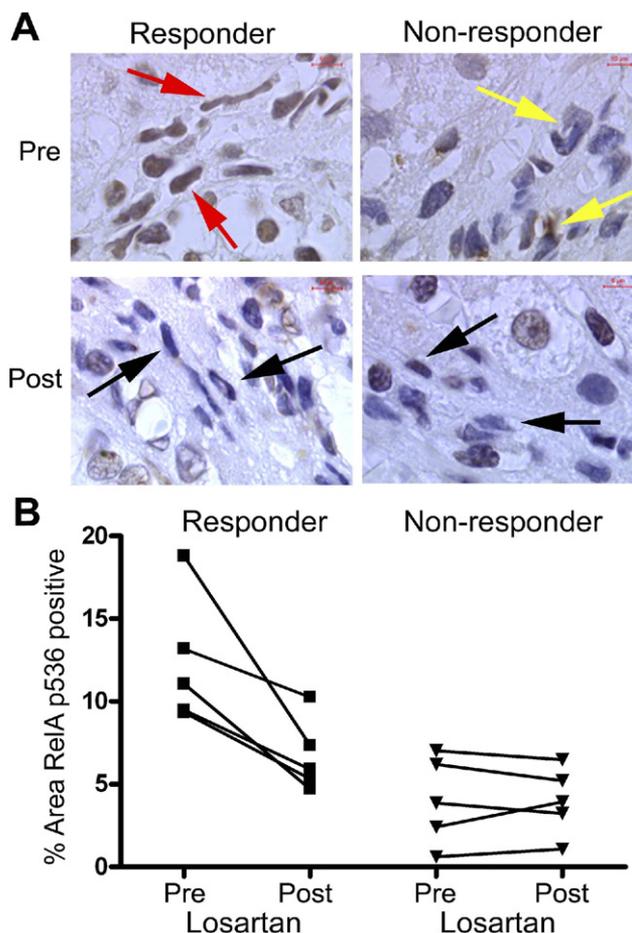


Figure 8. Regression of human fibrosis in response to losartan is associated with loss of P-Ser⁵³⁶-RelA-positive cells. (A) Representative photomicrographs (original magnification, 100×) of human liver biopsy samples taken from hepatitis C virus-infected fibrotic patients pre- and post-18-month treatment with losartan. Sections were immunostained with antibodies recognizing P-Ser⁵³⁶-RelA. Positively stained cells pre-losartan treatment in nonresponders and responders are denoted by yellow and red arrows, respectively, whereas black arrows show cells postlosartan treatment. Images are subdivided into 2 groups: patients who responded to losartan treatment (right) and nonresponders (left). (B) Graphical representations of average percentage area of tissue stained positively for P-Ser⁵³⁶-RelA in fibrotic bands of human liver biopsy specimens. Densitometric analysis was used to calculate mean percentage area of up to 5 patients/group ± SEM. A statistically significant reduction in immunoreactivity was observed in the responder group, P = .025 (2-tailed paired Student t test).

in nonresponders, which were unchanged between pre- and posttreatment biopsies. Loss of P-Ser⁵³⁶-RelA-positive cells is therefore associated with regression of human liver fibrosis in response to treatment with losartan. Moreover, the level of constitutive P-Ser⁵³⁶-RelA may be predictive for patients who are likely to benefit from losartan treatment.

Discussion

We have described a constitutive positive feedback fibrogenic signaling pathway (angiotensin II/IKK β /P-Ser⁵³⁶-RelA/active nuclear NF- κ B/angiotensinogen gene transcription) that promotes survival of human hepatic myofibroblasts. This pathway explains our previous finding that the IKK inhibitor sulphasalazine can repress myofibroblast NF- κ B activity despite low-level expression of I κ B α in these cells.⁹ Although a significant proportion of RelA:p50 dimers in hepatic myofibroblasts are not subject to regulation by I κ B α ,^{12,15,16} they are clearly still subject to additional regulatory checkpoints such as subunit phosphorylation. P-Ser⁵³⁶-RelA is required for efficient nuclear transport of RelA-containing NF- κ B,²² which in hepatic myofibroblasts is regulated by constitutive IKK β (a target for sulphasalazine), and can be suppressed by ACE inhibitors and AT1 antagonists.

Angiotensin II has emerged as an important stimulator of fibrogenesis. Patients with chronic HCV infection carrying a genetic polymorphism associated with increased angiotensin II synthesis develop more severe fibrosis.³⁰ Inhibition of angiotensin II synthesis, blockade of AT1 receptor, or genetic ablation of AT1 receptor attenuates fibrosis in animal models of chronic liver disease.^{26,27} Direct fibrogenic effects of angiotensin II have been described, including stimulation of proliferation and the expression of fibrogenic genes encoding extracellular matrix proteins, cytokines, growth factors, and fibrogenic receptors.²⁶ One mechanism by which angiotensin II mediates these effects is via the phosphorylation of the p47phox component of NADPH oxidase; induction of reactive oxygen species; and subsequent activation of AKT, MAPKs, and AP-1 DNA binding.³¹ Here, we describe a second angiotensin II-activated fibrogenic pathway involving activation of IKK, phosphorylation of RelA, and induction of NF- κ B-dependent transcription of cell survival genes. Taken together, these observations lend support for angiotensin II functioning as a vital paracrine and autocrine regulator of the survival and fibrogenic actions of hepatic myofibroblasts.

The therapeutic potential for targeting this pathway was demonstrated by a correlation between the loss of scar-associated P-Ser⁵³⁶-RelA-positive cells and regression of fibrosis in diseased rodent livers treated with ACE and IKK inhibitors. Moreover, our observation that fibrotic HCV patients who undergo regression of fibrosis in response to treatment with losartan initially express elevated numbers of P-Ser⁵³⁶-RelA (relative to nonre-

sponders) that diminish following treatment suggests that P-Ser⁵³⁶-RelA may be a useful biomarker for fibrosis that is susceptible to treatment with pharmacologic attenuators of the renin-angiotensin system. Hepatic myofibroblasts are a phenotypically diverse and highly plastic population of cells.² There are several distinct mechanisms by which myofibroblasts can avoid apoptosis.⁷ It can be speculated that myofibroblasts may modulate their phenotype to predominantly employ a particular survival mechanism as dictated by apoptotic signals present in the microenvironment. P-Ser⁵³⁶-RelA may be a surrogate for a predominantly angiotensin II-driven survival mechanism and fibrosis that will regress in response to drugs that are well tolerated by humans.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.02.081.

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Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Table 1. Fibrosis Grade Quantified by the Ishak Scoring Method on Liver Biopsy Samples From 11 Hepatitis C Virus Patients Pre- and Post-18 Months, Losartan Treatment

Patient	Histology grade		Average % area of phospho-Ser ⁵³⁶ -RelA-positive staining		Responded
	Prelosartan treatment	Postlosartan treatment	Prelosartan treatment	Postlosartan treatment	
Patient 3	F3	F2	13.176	10.250	Yes
Patient 5	F2	F1	18.800	7.550	Yes
Patient 6	F4	F3	11.055	4.71	Yes
Patient 7	F4	F3	9.344	5.310	Yes
Patient 8	F3	F2	9.467	5.891	Yes
Patient 1	F2	F2	7.018	6.471	No
Patient 2	F2	F2	6.196	5.216	No
Patient 4	F3	F3	7.256	7.61	No
Patient 9	F3	F3	2.410	3.930	No
Patient 10	F2	F4	3.860	3.230	No
Patient 11	F2	F4	0.612	1.070	No

NOTE. Losartan treatment was 50 mg/day (Cozaar; MSD, Wilmington, DE). Average percent area of positive P-Ser⁵³⁶-RelA immunostaining calculated within fibrotic bands of patients with HCV pre- and postlosartan treatment.