

## Prolonged infusion of angiotensin II into normal rats induces stellate cell activation and proinflammatory events in liver

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**Bataller, Ramón, Erwin Gäbele, Robert Schoonhoven, Terry Morris, Mark Lehnert, Liu Yang, David A. Brenner, and Richard A. Rippe.** Prolonged infusion of angiotensin II into normal rats induces stellate cell activation and proinflammatory events in liver. *Am J Physiol Gastrointest Liver Physiol* 285: G642–G651, 2003. First published May 28, 2003; 10.1152/ajpgi.00037.2003.—Recent evidence indicates that angiotensin II (ANG II) plays an important role in liver fibrogenesis. However, the underlying mechanisms are largely unknown. In advanced chronic liver diseases, circulating levels of ANG II are frequently elevated. We investigated the hepatic effects of prolonged systemic infusion of ANG II in normal rats. Saline or ANG II at subpressor and pressor doses (15 and 50 ng·kg<sup>-1</sup>·min<sup>-1</sup>, respectively) were infused to normal rats for 4 wk through a subcutaneous osmotic pump. Infusion of ANG II resulted in liver injury, as assessed by elevated serum liver enzymes. Livers from ANG II-perfused rats showed activation of JNK and ERK as well as increased NF-κB and activating protein-1 DNA-binding activity. Moreover, ANG II perfusion induced oxidative stress, increased concentration of proinflammatory cytokines, and upregulated the inflammatory proteins inducible nitric oxide synthase and cyclooxygenase-2. Histological examination of the livers from ANG II-infused rats showed mild portal inflammation as well as thickening and thrombosis of small hepatic vessels. ANG II-treated livers showed accumulation of CD43-positive inflammatory cells and activated hepatic stellate cells (HSCs) at the pericentral areas. A slight increase in collagen synthesis was observed, as assessed by Sirius red staining and hepatic hydroxyproline. All of these effects were observed when ANG II was perfused at subpressor and pressor doses. ANG II also accelerated the activation of primary cultured rat HSCs. In conclusion, increased systemic ANG II can induce liver injury by promoting proinflammatory events and vascular damage. ANG II-induced hepatic effects are not dependent on increase in arterial pressure.

hepatic inflammation; renin-angiotensin system; cytokines

ANGIOTENSIN II (ANG II), the main effector of the renin-angiotensin system, is a multifunctional cytokine that plays an important role in conditions characterized by chronic inflammation and continuous tissue remodeling (28). Both systemic and tissue renin-angiotensin systems exert prooxidant, proinflammatory, and profi-

brogenic actions in the heart, vessels, and kidney (30, 36). As a result, the blockade of ANG II synthesis and/or binding to its AT<sub>1</sub> receptor attenuate tissue damage in a number of experimental models of renal, vascular, and cardiac chronic injury (31). In humans, drugs interfering with ANG II biological actions are widely used to prevent the progression of chronic cardiac and renal failure (9, 14). The cellular and molecular mechanisms underlying the pathogenic effects of ANG II have been extensively studied. ANG II promotes the proliferation of local myofibroblasts and stimulates the synthesis of extracellular matrix proteins (18). Moreover, ANG II exerts proinflammatory actions by stimulating the secretion of proinflammatory chemokines and the expression of cell adhesion molecules, which recruit inflammatory cells at the sites of tissue repair (7, 30). The intracellular mechanisms involve oxidative stress and the subsequent activation of redox-sensitive signaling pathways, including the transcription factors NF-κB and activating protein-1 (AP-1) and the signaling molecules called MAPKs (29).

Recent clinical and experimental evidence suggests that ANG II is involved in the progression of chronic liver diseases. Systemic ANG II is frequently elevated in patients with cirrhosis, and the hepatic renin-angiotensin system is upregulated in both human and rat livers undergoing active fibrogenesis (3, 21). Inhibition of ANG II synthesis and/or the blockade of AT<sub>1</sub> receptors markedly attenuate inflammation and extracellular matrix accumulation after chronic liver injury in rats (10, 22, 26, 42, 44). Moreover, patients with chronic hepatitis C and a genetic polymorphism that results in increased synthesis of ANG II develop more severe fibrosis (25). Most importantly, a recent report suggests that the blockade of AT<sub>1</sub> receptors has antifibrotic effects in patients with chronic hepatitis C (39).

The mechanisms underlying the pathophysiological effects of ANG II in the damaged liver are largely unknown. ANG II induces proinflammatory and profibrogenic effects in hepatic stellate cells (HSCs), a key cell involved in the hepatic wound-healing response to injury (2, 4). However, it is unknown whether increased systemic ANG II induces liver damage and

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which molecular mechanisms are involved. In this study, we investigated the effects of prolonged systemic infusion of ANG II on the normal rat liver. We demonstrate that infusion of ANG II induces oxidative stress, hepatic inflammation, and vascular damage, resulting in liver injury. The intracellular pathways activated by ANG II include NF- $\kappa$ B, AP-1, and MAPK activation.

## EXPERIMENTAL PROCEDURES

**Experimental design.** Male Sprague-Dawley rats (350–400 g) were infused with either saline or ANG II (at doses of 15 and 50  $\text{ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ , dissolved in saline) through an osmotic minipump (Alza, Palo Alto, CA) for 4 wk. Minipumps were placed subcutaneously and replaced after 2 wk. Ten rats were included in each group. Animals were killed at the end of the infusion period, and tissue samples were immediately removed and further processed for histological studies and mRNA/protein extraction. Arterial blood pressure was measured immediately before tissue harvesting. Animals were anesthetized with pentobarbital (50 mg/kg ip), and the right carotid artery was cannulated (PE-90; Transonics Systems, Ithaca, NY). The rat was turned to a prone position, and after a stabilization period of 5 min, mean arterial blood pressure was recorded by using a pressure analyzer (LPA-200; Digi-Med, Louisville, KY) for 10 min. Body temperature was kept at 37°C throughout the whole experiment. The blood pressure value for each group was calculated as the average of four separate animals.

**Isolation of rat HSCs.** HSCs were isolated from adult male Sprague-Dawley rats (>400 g) by in situ perfusion of the livers with collagenase and pronase, followed by arabinogalactan gradient ultracentrifugation, as previously described (32). HSC purity (as estimated by the autofluorescence of the cells by ultraviolet-excited fluorescence microscopy) was >95%. Cells were seeded on uncoated plastic tissue culture dishes and cultured in DMEM (GIBCO-BRL, Grand Island, NY) supplemented with 10% FCS and 2 mM L-glutamine. All animal procedures were performed in accordance with Institutional Animal Care and Use Committee of the University of North Carolina.

**Serum biochemical measurements.** Blood samples were collected from all rats at death. Serum alanine aminotransferase, aspartate aminotransferase,  $\gamma$ -glutamyltranspeptidase, and bilirubin levels were measured by standard enzymatic procedures by the Pathology Department, University of North Carolina. Serum TNF- $\alpha$  and ANG II levels were measured by a sandwich ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

**Measurement of serum endotoxin levels.** Blood samples were collected in endotoxin-free vials and centrifuged at 400 g for 15 min at 4°C. Samples were then diluted 1:10 in pyrogen-free water and heated to 75°C for 30 min to remove inhibitors of endotoxin from plasma. The limulus amoebocyte lysate test (Kinetic-QLC; Whittaker Bioproducts, Walkersville, MD) was used for measurements of endotoxin. Samples were incubated at 37°C for 10 min with limulus amoebocyte lysate. The substrate solution was added, and the incubation continued for 20 min. The reaction was stopped with 25% acetic acid. Samples were read spectrophotometrically at 410 nm.

**Immunohistochemical studies.** Livers were fixed in 10% phosphate-buffered formalin for 24 h at room temperature, washed twice with water, stored in 70% ethanol at 4°C, and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin, Masson trichrome, and Sirius red. For immunohistochemical analysis, sections were depar-

affinized, rehydrated, and stained by using the DAKO Envision system (DAKO, Carpinteria, CA) according to the manufacturer's instructions. Briefly, endogenous peroxidase was blocked with peroxidase-blocking agent and sections were incubated with anti-4-hydroxynonenal (1:1,000 dilution; Alpha Diagnostic, San Antonio, TX), anti-smooth muscle  $\alpha$ -actin (1:1,000; DAKO), anti-CD43 (1:1,000; Serotec, Raleigh, NC), and anti-hypoxia-induced factor (HIF)-2 $\alpha$  (Novus Biologicals, Littleton, CO) for 30 min at room temperature in PBS containing 1% Tween 20 and 1% bovine serum albumin. Slides were incubated with peroxidase-linked secondary antibodies for 15 min, washed, and further incubated with labeled polymer for 10 min at room temperature. Sections were washed twice with PBS, incubated with 3,3-diaminobenzidine substrate chromogen for 8 min, washed with water, incubated with diaminobenzidine enhancer (Innovex Biosciences, Richmond, CA) for 5 min, and washed with water before being counterstained with hematoxylin. As negative controls, all specimens were incubated with an isotype-matched control antibody under identical conditions. The area of positive staining was measured by using a Macintosh-based morphometric analysis system (Apple Computer, Brea, CA) with NIH Image software (version 1.62).

**Hydroxyproline assay.** Hydroxyproline content was quantified colorimetrically in duplicate from ~0.2-g liver samples. Tissue was homogenized in 3 ml of 6 N HCl and hydrolyzed at 110°C for 16 h. The hydrolysate was filtered, aliquots were evaporated under vacuum, and the sediment was redissolved in 1.2 ml of 50% isopropanol. The solution was then incubated with 0.2 ml of 0.84% chloramine T in 42 mM sodium acetate, 2.6 mM citric acid, and 39.5% (vol/vol) isopropanol (pH 6.0) for 10 min at room temperature. Next, 0.248 g *p*-dimethylaminobenzaldehyde, dissolved in 0.27 ml of 60% perchloric acid, and 0.73 ml isopropanol were added and incubated at 50°C for 90 min. Hydroxyproline content was quantified photometrically at 558 nm from a standard curve with the amino acid alone and against a reagent blank. The

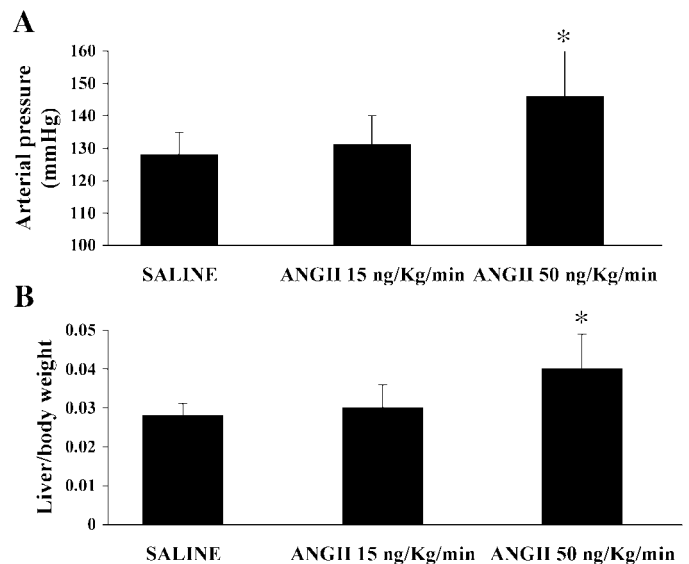


Fig. 1. A: effects of angiotensin II (ANG II) systemic infusion on arterial pressure. Arterial pressure was measured in the carotid artery as described in EXPERIMENTAL PROCEDURES. Only rats receiving 50  $\text{ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  of ANG II showed significant increase in arterial pressure. \* $P < 0.05$  compared with saline. B: effect of ANG II infusion on body-to-liver weight ratio. \* $P < 0.05$  compared with saline.

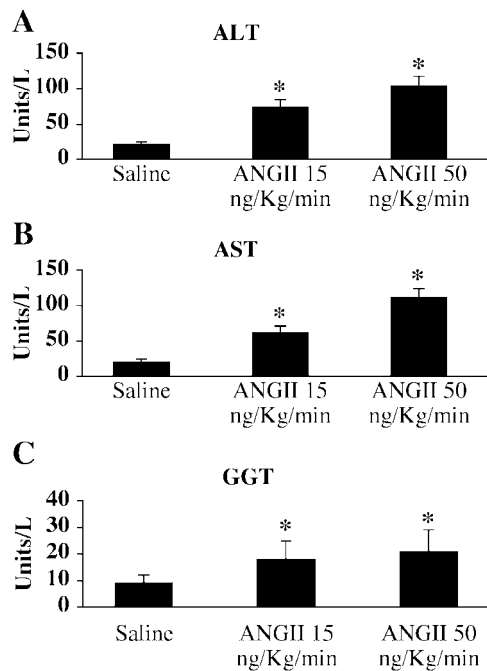


Fig. 2. Effect of ANG II infusion on serum liver enzymes. Both doses of ANG II increase serum alanine aminotransferase (ALT; A), aspartate aminotransferase (AST; B), and  $\gamma$ -glutamyltranspeptidase (GGT; C) levels. Bilirubin serum levels remained unchanged (not shown). Five rats were studied per group. \* $P < 0.01$  vs. control.

results were expressed as grams of hydroxyproline per gram liver.

**Western blot analysis.** Liver samples were homogenized in lysis buffer (10 mM HEPES, 420 mM NaCl, 1.5 mM  $MgCl_2$ ,

0.5 mM DTT, 0.5% NP-40, and 25% glycerol) containing protease and phosphatase inhibitors for 30 min at 4°C. After centrifugation, cleared tissue lysates were collected and stored at -80°C until analysis. Cell lysates were obtained with Triton lysis buffer as described (32). Twenty-five micrograms were loaded onto 10% SDS-acrylamide gels and blotted onto nitrocellulose membranes. Membranes were incubated in blocking buffer containing antibodies against phospho-ERK (Cell Signaling, Beverly, MA), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), phospho-c-Jun and  $\alpha$ -tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), and smooth muscle  $\alpha$ -actin. After extensive washing, the membranes were incubated with blocking buffer containing horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology) at a dilution of 1:1,000. Proteins were detected by enhanced chemoluminescence (Amersham, Arlington Heights, IL). For detection of collagen I secretion, cell media were precipitated with sodium sulfite and resuspended in acetic acid as described (37). After being blotted, membranes were probed with anti-human collagen type I antibody (1:1,000; Biodesign, Saco, ME).

**Electrophoretic mobility shift assay.** Fresh liver tissue samples were homogenized in *buffer A* (in mM: 10 HEPES, pH 7.9, 10 KCl, 1.5  $MgCl_2$ , and 0.5 DTT) containing protease and phosphatase inhibitors, incubated for 30 min at 4°C, and then lysed in 10% NP-40. After centrifugation, nuclei were lysed in *buffer C* (10 mM HEPES, 25% glycerol, 420 mM NaCl, 1.5 mM  $MgCl_2$ , 0.5 mM DTT, and 0.5% NP-40) containing protease and phosphatase inhibitors. Eight micrograms of nuclear proteins were incubated with 100 pg of a  $^{32}P$ -labeled probe containing the AP-1 (5'-GTAAAGCAT-GAGTCAGACACCTC-3') or NF- $\kappa$ B (top: 5'-GCAGAGGG-GACTTTCGAGA-3'; bottom: 5'-GTCTCGGAAAGTCCCC-TCTG-3') consensus sites in buffer containing (in mM) 10 HEPES, 2  $MgCl_2$ , 50 KCl, 1 DTT, 0.1 EDTA and 20% glycerol

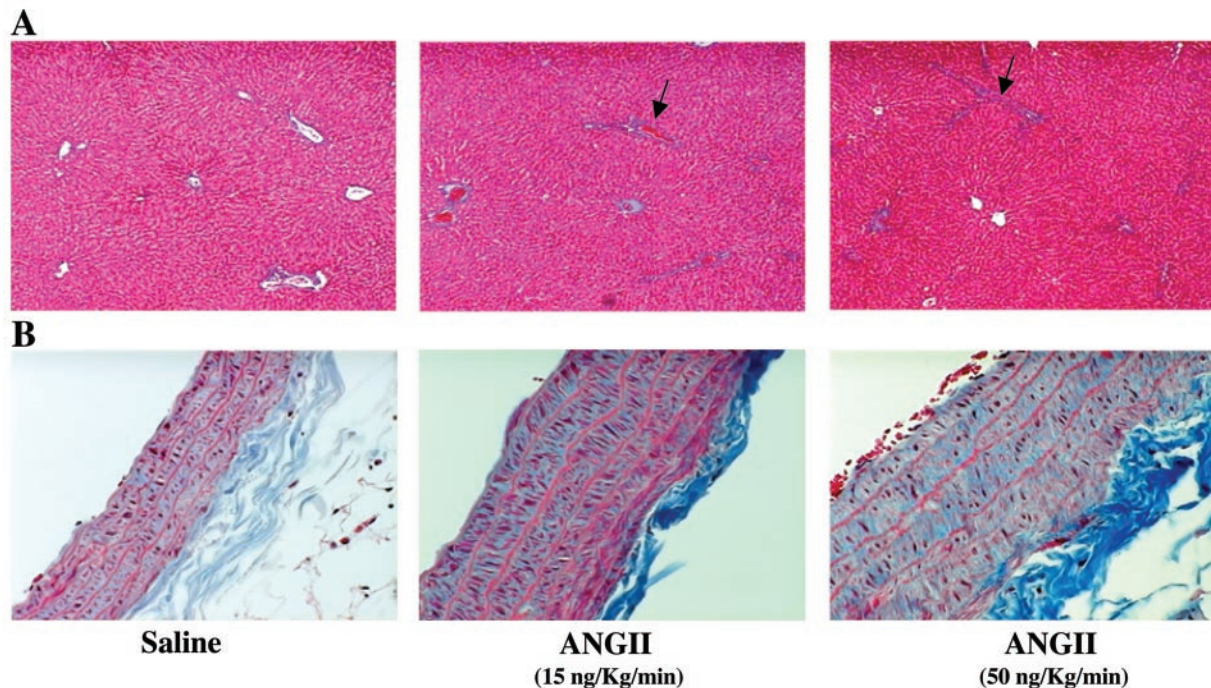


Fig. 3. Representative photomicrographs of liver and abdominal aorta sections. Paraffin-embedded sections were processed for Masson's trichrome staining. A: thickening and thrombosis in the portal tracts (arrow) of ANG II-treated livers are observed. B: section of abdominal aorta from saline and ANG II-infused rats. ANG II infusion induced infiltration of mononuclear cells and a wall thickening in a dose-dependent manner. Original magnification: A,  $\times 40$  and B,  $\times 100$ .

in the presence of single-stranded oligonucleotide (25  $\mu\text{g/ml}$ ) and poly(dI/dC) (25  $\mu\text{g/ml}$ ) for 20 min at room temperature (13). For competition assay, one sample was incubated with 10 ng unlabeled probe. Complexes were separated by electrophoresis on nondenaturing 4% acrylamide gels and assayed by autoradiography and PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

**ELISA for IL-1 $\beta$  and TNF- $\alpha$ .** Liver tissue samples were homogenized in lysis buffer containing 25 mM HEPES, 0.1% CHAPS, 5 mM MgCl<sub>2</sub>, 1.3 mM EDTA, 1 mM EGTA, and phosphatase and protease inhibitors for 30 min at 4°C. After centrifugation, cleared tissue lysates were collected and stored at -80°C. A sandwich ELISA for rat IL-1 $\beta$  and TNF- $\alpha$  (R&D Systems, Minneapolis, MN) was performed by using 1:5 dilutions according to the manufacturer's instructions.

**NF- $\kappa$ B-responsive luciferase assay.** Rat HSCs were infected with a recombinant adenoviral vector expressing a luciferase reporter gene driven by NF- $\kappa$ B transcriptional activation (Ad5NF- $\kappa$ BLuc) for 12 h as described (20). Medium was replaced, and cells were stimulated with ANG II (10<sup>-8</sup> M) for 8 h. NF- $\kappa$ B-mediated transcriptional induction was assessed by the luciferase assay system (BD Pharmingen,

San Diego, CA). Luciferase activity (relative light units) was normalized to the protein concentration.

**Statistical analysis.** Results are expressed as means  $\pm$  SE. Significance was established by using the Mann-Whitney *U*-test. Differences were considered significant if  $P < 0.05$ .

## RESULTS

**Effects of prolonged ANG II infusion on normal rats.** Both saline and ANG II (15 and 50 ng·kg<sup>-1</sup>·min<sup>-1</sup>) infusion were well tolerated. ANG II at 50 ng·kg<sup>-1</sup>·min<sup>-1</sup> increased mean arterial pressure, whereas infusion of 15 ng·kg<sup>-1</sup>·min<sup>-1</sup> of ANG II did not significantly affect arterial pressure (Fig. 1A). The liver-to-body weight ratio increased in rats receiving ANG II (50 ng·kg<sup>-1</sup>·min<sup>-1</sup>) compared with control rats (Fig. 1B). A small amount of ascitic fluid was detected in 40% of rats receiving 50 ng·kg<sup>-1</sup>·min<sup>-1</sup> ANG II.

**Effect of ANG II infusion on serum liver enzymes and endotoxin levels.** ANG II infusion resulted in increased ANG II serum concentration (0.3  $\pm$  0.03, 1.2  $\pm$  0.2, and

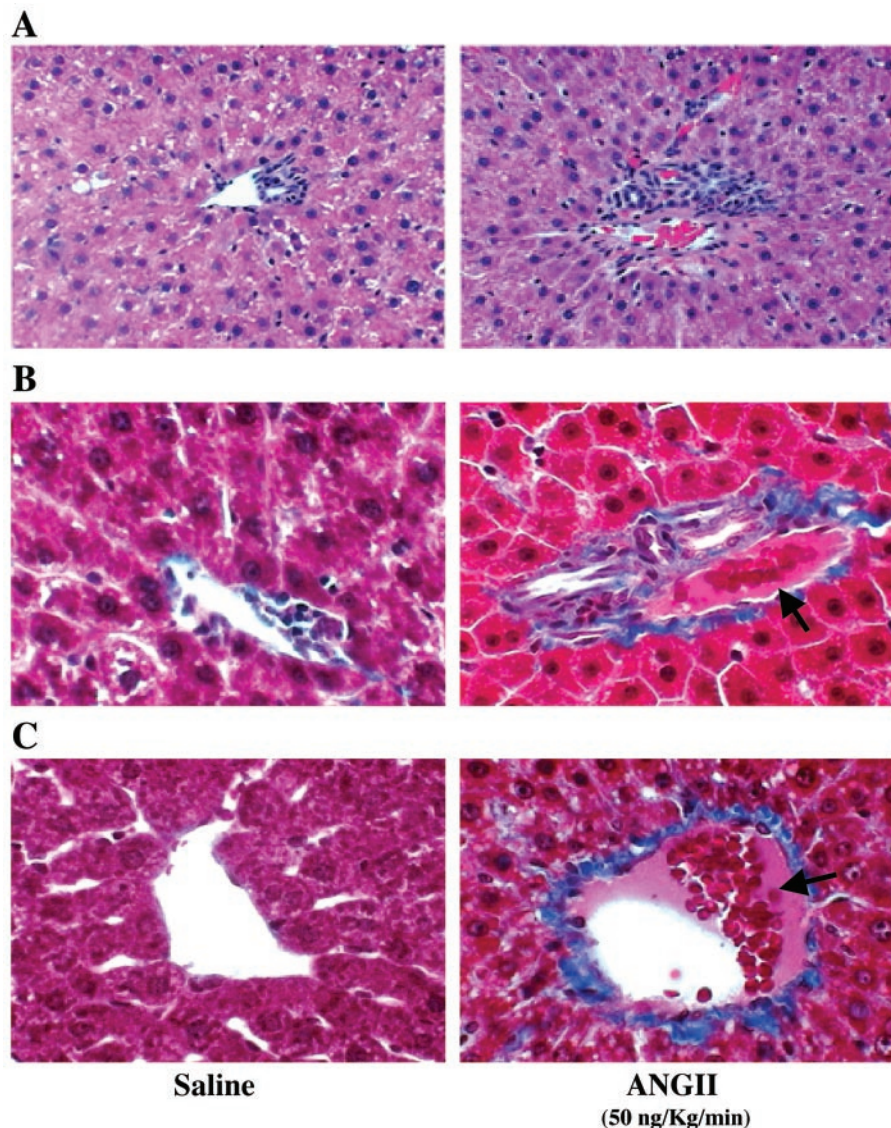


Fig. 4. A: representative photomicrographs of portal tracts from saline- and ANG II (50 ng·kg<sup>-1</sup>·min<sup>-1</sup>)-treated rat livers. Paraffin-embedded sections were processed for hematoxylin and eosin. An inflammatory infiltrate of mononuclear cells and thickening of the limiting membrane was observed in portal tracts from ANG II-treated rats. Original magnification,  $\times 200$ . B and C: Masson trichrome staining showing patent vascular thrombosis in a portal vein (B) and a central vein (C) in ANG II-infused livers (arrows). Original magnification,  $\times 400$ .

$1.8 \pm 0.5$  ng/ml in controls and rats receiving 15 and 50  $\text{ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  ANG II, respectively;  $P < 0.01$  vs. saline). ANG II infusion increased alanine aminotransferase, aspartate aminotransferase, and  $\gamma$ -glutamyl-transpeptidase serum levels in a concentration-dependent manner, whereas bilirubin levels remained unchanged (Fig. 2). ANG II-infused rats showed higher serum TNF- $\alpha$  levels compared with control rats ( $3.3 \pm 1.3$ ,  $8.4 \pm 2.1$ , and  $9.3 \pm 3.1$  in controls and rats receiving 15 and 50  $\text{ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  ANG II, respectively;  $P < 0.05$  vs saline). Finally, ANG II-infused rats showed higher serum endotoxin levels compared with control rats ( $8.7 \pm 0.8$ ,  $23.6 \pm 3.4$ , and  $37.8 \pm 5.5$  pg/ml in controls and rats receiving 15 and 50  $\text{ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  ANG II, respectively;  $P < 0.01$  vs saline).

**Histological changes in livers from ANG II-infused rats.** Histological examination of ANG II ( $15 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ )-infused livers showed preserved hepatic parenchyma with no apparent hepatocyte damage. Thirty percent of rats receiving ANG II at pressor doses (50  $\text{ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) showed signs of mild ischemic hepatitis with necrosis in pericentral areas (not shown). Infiltration of mononuclear cells and thickening of the limiting membrane were observed in portal tracts from ANG II-treated rats (Figs. 3A and 4). Proliferation of bile ducts was observed in 30% of portal tracts in ANG II-treated rats (not shown). Hepatic small vessels showed signs of vasculitis and wall thickening with frequent vascular thrombosis (Fig. 4). As expected, kidneys from ANG II-treated rats show profound inflammation and collagen deposition (not shown). Similarly, the abdominal aorta showed infiltration of mononuclear cells and marked wall thickening (Fig. 3B). These latter changes were more pronounced in rat infused with the pressor doses of ANG II.

**Immunostaining studies.** Because ANG II induces proliferation of culture-activated HSCs (2), we first examined whether ANG II infusion induces the accumulation of smooth muscle  $\alpha$ -actin-positive cells, a marker of activated HSCs, in the liver. Infusion of ANG II induced a marked increase in the number of smooth muscle  $\alpha$ -actin-positive stellate cells, mainly observed in the pericentral area ( $0.1 \pm 0.02$ ,  $8.2 \pm 1.1$ , and  $12.1 \pm 2.2$  positive cells per field in rats infused with saline and with 15 and 50  $\text{ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  ANG II, respectively;  $P < 0.01$  vs saline; Fig. 5A). To investigate the infiltration of inflammatory cells in the liver parenchyma, CD43-positive cells were counted. CD43 is typically expressed by infiltrating mononuclear cells and lymphocytes (29). ANG II perfusion induced the infiltration of CD43-positive cells in the hepatic parenchyma, which were mainly observed at the pericentral areas ( $2.2 \pm 0.1$ ,  $11.2 \pm 2.6$ , and  $14.1 \pm 3.5$  positive cells per field in rats infused with saline and with 15 and 50  $\text{ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  ANG II, respectively;  $P < 0.05$  vs. saline; Fig. 5B). ANG II infusion also induced tissue hypoxia, as demonstrated by increased expression of HIF-2 $\alpha$  in the hepatocytes located in the pericentral areas (Fig. 5C). Finally, because oxidative stress mediates the effects of ANG II in other tissues, we assessed whether ANG II infusion induces oxidative

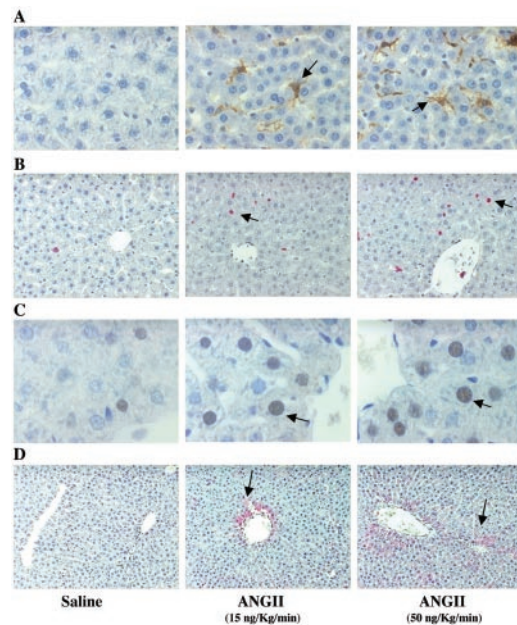


Fig. 5. Representative photomicrographs of the immunohistochemistry studies. A: ANG II infusion induced the accumulation of smooth muscle  $\alpha$ -actin-positive stellate cells (arrows) in the hepatic parenchyma. B: detection of CD43-positive inflammatory infiltrating cells (arrows) in the hepatic parenchyma in ANG II-infused rats. C: ANG II infusion induced the expression of hypoxia-inducible factor-2 $\alpha$  in the nuclei of hepatocytes located in the pericentral areas. D: ANG II infusion induced oxidative stress in the liver, as assessed by detection of 4-hydroxynonenal protein adducts. Positive staining was observed in livers of ANG II-treated rats. Original magnifications: A,  $\times 200$ ; B,  $\times 100$ ; C,  $\times 400$ ; D,  $\times 40$ .

stress in the rat liver. ANG II-infused livers showed a marked increase in lipid peroxidation products, as demonstrated by intense staining for 4-hydroxynonenal-modified proteins (Fig. 5D). These results indicate that systemic infusion of ANG II induces activation of HSCs, infiltration of inflammatory cells, and oxidative stress in the rat liver.

**Effect of ANG II perfusion on hepatic proinflammatory proteins.** To confirm that ANG II exerts inflammatory actions on the rat liver, we measured the expression of cytokines and other proinflammatory proteins in the rat liver. ANG II infusion increased the hepatic concentration of the inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  compared with livers infused with saline (Fig. 6, A and B). Similarly, ANG II infusion induced the expression of typical proinflammatory proteins such as COX-2 and iNOS, as assessed by Western blotting (Fig. 6C). These results further demonstrate that ANG II infusion induces inflammatory events in the rat liver.

**Effect of ANG II perfusion on collagen deposition.** Because blockade of ANG II's biological actions ameliorates collagen deposition in experimental liver fibrosis (26, 39, 42, 44), we explored whether increased systemic ANG II induces collagen accumulation in rat liver. ANG II infusion did not significantly increase collagen content in the hepatic parenchyma, as assessed by Sirius red staining. However, increased Sirius red staining was observed in the small portal

vein and arterial vessel walls, limiting membrane of portal tracts, and central veins (Fig. 7, A and B). Hepatic collagen was also assessed by measuring hepatic hydroxyproline content. Only pressor doses of ANG II induced a slight increase in hydroxyproline content, whereas infusion of ANG II at subpressor doses had no effect (Fig. 7C). These results suggest that infusion of ANG II to normal rats induces thickening and fibrosis of small hepatic vessels but no parenchymal fibrosis.

**Intracellular pathways stimulated by ANG II in the rat liver.** We also explored whether ANG II infusion stimulates redox-sensitive intracellular pathways, including MAPK and the transcription factors AP-1 and NF- $\kappa$ B. ANG II infusion increased phosphorylation of c-Jun (a specific substrate of JNK) and ERK-2 by six- and fivefold, respectively, in the rat liver compared with saline-infused rats, as assessed by Western blot analysis (Fig. 8A). Moreover, ANG II increased DNA-binding activity of the transcription factors AP-1 and NF- $\kappa$ B by four- and threefold, respectively, compared with control rats (Fig. 8B).

**ANG II accelerates the activation of primary rat cultured HSCs.** To test whether ANG II induces activation of HSCs in vitro, early-cultured rat HSCs were

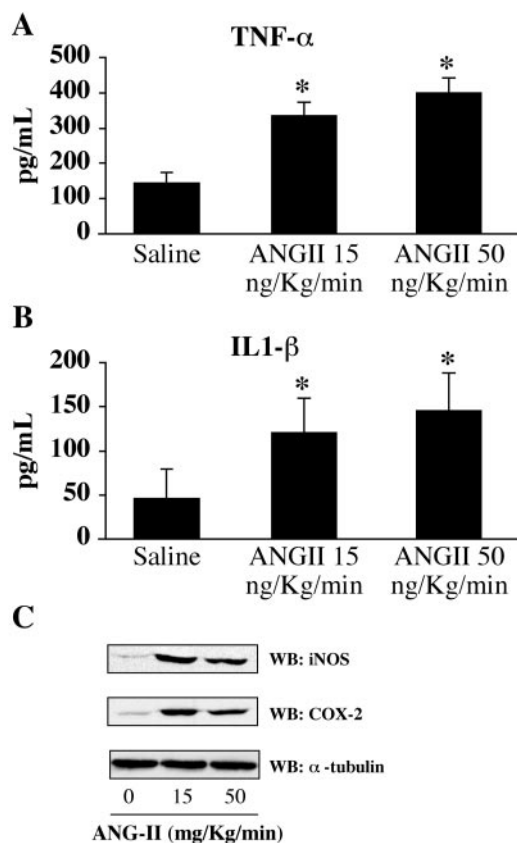


Fig. 6. ANG II infusion increases the hepatic concentration of TNF- $\alpha$  (A) and IL-1 $\beta$  (B). \* $P$  < 0.05 vs. saline. C: ANG II infusion increases cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression in the liver, as assessed by Western blot analysis (see EXPERIMENTAL PROCEDURES). Figure is representative of 3 independent experiments.

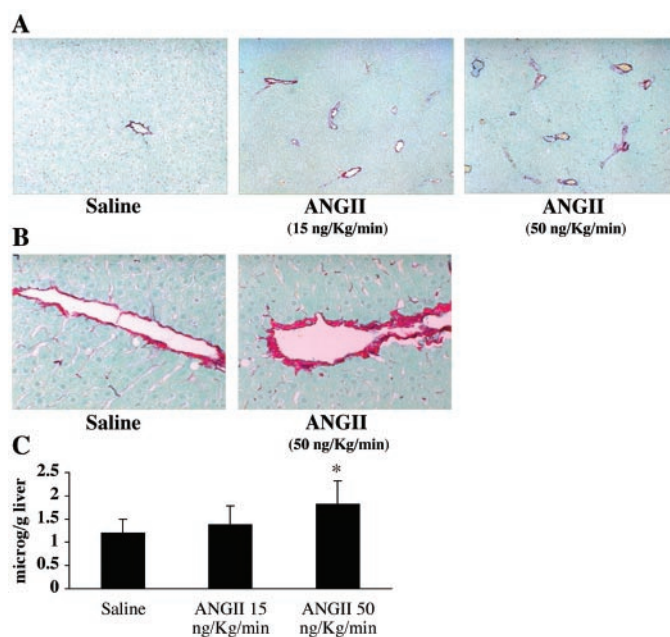


Fig. 7. Representative photomicrographs of livers from saline and ANG II-treated rat livers. Paraffin-embedded sections were processed for Sirius red to assess collagen deposition. A: increased staining was observed in portal tracts and hepatic vessels of ANG II-infused rats. No increased staining in the hepatic parenchyma was observed. Original magnification,  $\times 40$ . B: ANG II infusion (50 ng $\cdot$ kg $^{-1}$  $\cdot$ min $^{-1}$ ) increased Sirius red staining in the small vessel walls. Original magnification,  $\times 100$ . C: effects of ANG II infusion on hepatic hydroxyproline content. Livers from rats receiving pressor doses of ANG II (50 ng $\cdot$ kg $^{-1}$  $\cdot$ min $^{-1}$ ) show a mild increase in hydroxyproline content. \* $P$  < 0.04 vs. control.

incubated for 2 days in the presence or absence of ANG II ( $10^{-8}$  M). As shown in Fig. 9, ANG II increased the expression of smooth muscle  $\alpha$ -actin and increased the secretion of collagen I to the culture medium. Moreover, ANG II ( $10^{-8}$  M) induced the activation of the transcription factor NF- $\kappa$ B, as assessed by luciferase-driven NF- $\kappa$ B assay. All of these effects were prevented by the AT $_1$  receptor antagonist losartan ( $10^{-7}$  M).

## DISCUSSION

The current study demonstrates that prolonged systemic infusion of ANG II into normal rats induces liver inflammation, oxidative stress, and vascular damage, resulting in liver injury. We provide evidence that systemic ANG II induces activation of HSCs in vivo and in culture, confirming that these cells are targets for ANG II. However, systemic ANG II alone is not sufficient to induce parenchymal fibrosis. Our results are in accordance with previous studies showing that increased systemic ANG II induces oxidative stress and profound inflammation in the vasculature and the kidneys (1, 11, 29, 35). On the basis of these data, we propose that increased systemic ANG II may participate in the progression of chronic liver diseases.

Our experimental model consisting of infusing ANG II through a subcutaneous pump into rats has been used by many investigators (1, 11, 15, 29, 35). In the current study, we used both subpressor and pressor

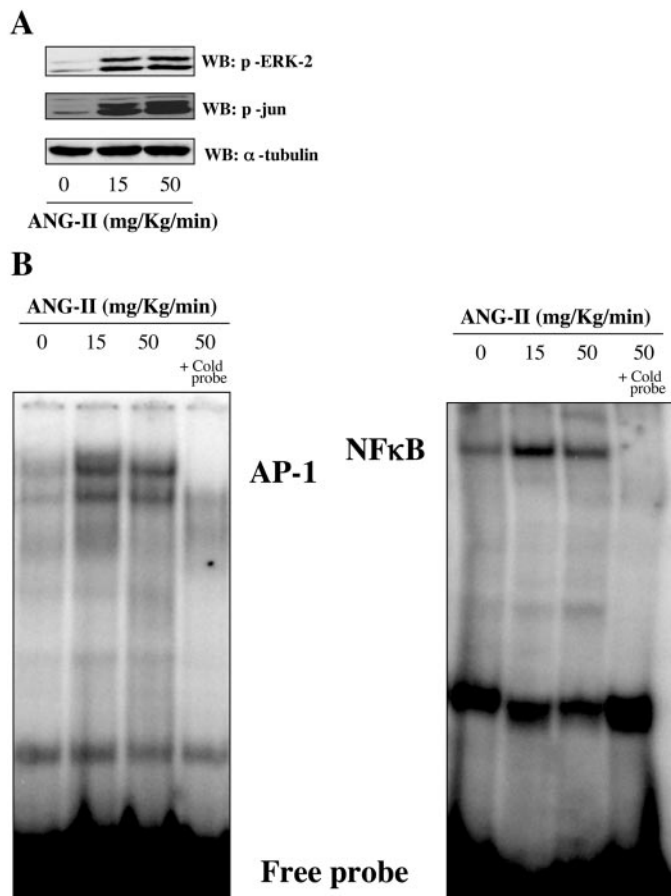


Fig. 8. *A*: effect of ANG II infusion on ERK and c-Jun phosphorylation, as assessed by Western blot analysis. Twenty micrograms of total liver extracts were blotted with anti-phospho-ERK and anti-phospho-c-Jun as described in EXPERIMENTAL PROCEDURES. Figure is representative of 3 independent experiments. Tubulin staining was performed to ensure equal protein loading. *B*: ANG II infusion increases activating protein-1 (AP-1) and NF- $\kappa$ B DNA-binding activities, as assessed by electrophoretic binding shift assays. Eight micrograms of hepatic nuclear proteins were incubated with  $^{32}$ P-labeled AP-1 and NF- $\kappa$ B probes as described in EXPERIMENTAL PROCEDURES. ANG II markedly increased AP-1 and NF- $\kappa$ B DNA binding compared with controls. Figures are representative of 3 independent experiments.

doses of ANG II. Liver injury was more severe when pressor doses of ANG II were used. Although renal function was not measured in the current study, the histological changes found in the kidneys of ANG II-infused rats strongly suggest that renal function is reduced in these animals. However, because of the abnormal liver histology that we observed, it is unlikely that an ANG II-induced rise in serum liver enzymes is solely due to impaired renal function. Importantly, the pathogenic effects were also observed when ANG II was infused at subpressor doses, indicating that these effects are not dependent on an increase in arterial pressure. ANG II infusion caused inflammation and sclerosis of small vessels and thrombosis. These effects may be influenced by hepatic hypoxia, since ANG II infusion induced HIF-2 $\alpha$  in the liver. Moreover, a marked oxidative stress was found, as

assessed by increased lipid peroxidation of protein adducts. Similar *in vivo* effects have been previously described in the heart, the vasculature, and the kidney (1, 29, 35, 43). In these organs, ANG II induces infiltration by mononuclear cells and progressive fibrosis. However, the severity of histological damage observed in the kidneys was more pronounced than that found in the liver, suggesting that the kidneys are more sensitive to increased systemic ANG II levels. The hepatic effects induced by prolonged infusion of ANG II can be due either to local or remote effects. The finding that the aorta and kidneys show profound inflammation strongly indicates that ANG II infusion induces pathological effects in other organs than the liver. A potential mechanism is that ANG II infusion increases gut permeability, leading to gram-negative bacterial translocation. Increased bacterial products in serum would cause hepatic inflammation in the context of a generalized inflammatory response. The finding that ANG II infusion markedly increases endotoxin serum levels supports this hypothesis. Alternatively, ANG II could induce proinflammatory effects in the liver by causing direct stimulation of hepatic cells. ANG II induces proinflammatory effects on HSCs (4). Whether ANG II induces similar effects in other cell types involved in liver inflammation (i.e., Kupffer cells) is unknown.

An important finding of our study is that ANG II induces activation of HSCs *in vivo*, a key event in liver fibrogenesis. Following activation, HSCs secrete abundant extracellular matrix and a number of proinflam-

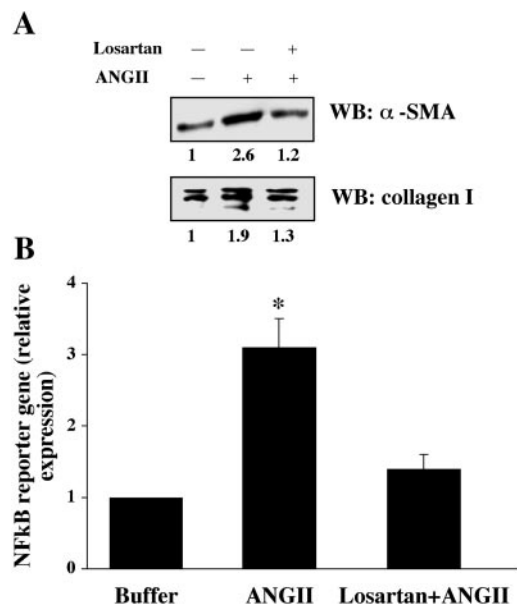


Fig. 9. *A*: ANG II accelerates the activation of primary rat cultured hepatic stellate cells (HSCs). Rat HSCs were incubated at day 2 for 48 h with buffer or ANG II ( $10^{-8}$  M). ANG II increased the expression of smooth muscle  $\alpha$ -actin ( $\alpha$ -SMA) and increased the secretion of collagen I to the culture medium, as assessed by Western blot analysis. Figure is representative of 3 independent experiments. *B*: ANG II ( $10^{-8}$  M) induced the activation of the transcription factor NF- $\kappa$ B, as assessed by luciferase-driven NF- $\kappa$ B reporter gene assay.  $P < 0.05$  vs. cells incubated with buffer. The AT $_1$  receptor antagonist losartan ( $10^{-7}$  M) prevented the effects induced by ANG II.

matory cytokines (27). We also show that ANG II accelerates the activation process of HSCs in culture. Collectively, these results indicate that ANG II is a powerful agonist for these cells. However, although hepatic inflammation was commonly observed in ANG II-treated rats, no extensive parenchymal fibrosis was found. Several reasons can explain this finding. First, it is conceivable that systemic ANG II only increases collagen synthesis when the liver is being damaged by another agent (i.e., alcohol, viral infection). Second, a local renin-angiotensin system has been recently described in both rat and human liver (21). This system is upregulated in damaged livers, suggesting that locally synthesized ANG II could participate in hepatic fibrogenesis. Whether the local, rather than systemic, renin-angiotensin system induces collagen synthesis in the damaged liver is unknown and deserves further investigation.

We provide *in vivo* evidence that ANG II activates the transcription factors NF- $\kappa$ B and AP-1 and stimulates the intracellular signaling molecules ERK and c-Jun in the rat liver. Comparable results have been described in the rat kidney, suggesting that ANG II infusion stimulates similar intracellular pathways in different organs. NF- $\kappa$ B activation mediates the inflammatory actions of ANG II in many tissues and is activated in a number of human and experimental liver diseases (4–6, 24, 29). Importantly, inhibition of NF- $\kappa$ B activation prevents experimental liver injury (41). In the current study, NF- $\kappa$ B-dependent proteins such as COX-2 and iNOS, which are typically involved in inflammatory processes, were upregulated in the ANG II-infused livers. Whether NF- $\kappa$ B inhibition attenuates the effects of ANG II in the liver is unknown. We also found increased AP-1 binding activity in the ANG II-treated livers. AP-1 mediates proinflammatory and profibrogenic actions in HSCs (16). However, its expression during liver fibrogenesis *in vivo* is unknown. Finally, ANG II stimulates the MAPKs JNK and ERK in the rat liver. JNK modulates fibrogenic and inflammatory actions in HSCs and is activated after liver injury (32, 34). Importantly, JNK inhibitors have protective effects in ischemia-reperfusion and liver transplantation in rats (40). ERK is induced after acute liver injury and regulates proliferation and biological actions in many liver cell types (17).

Because of the *in vivo* nature of our study, it is difficult to ascertain which specific cell type mediates the hepatic effects induced by ANG II infusion. Several cell types are potential candidates. Although ANG II induces activation and stimulates NF- $\kappa$ B in rat primary HSCs, other cell types such as hepatocytes and Kupffer cells are potential targets for ANG II. Whether ANG II exerts proinflammatory effects in these cells has not been addressed in the current study and deserves further investigation. Moreover, the relative importance of the different intracellular signaling pathways induced by ANG II infusion (ERK, NF- $\kappa$ B, c-Jun/AP-1) in the pathogenesis of liver inflammation is uncertain.

The results of this study may have important pathophysiological implications. In particular, several of the effects observed after ANG II infusion could contribute to the progression of chronic liver diseases. First, oxidative stress plays an important role in the hepatic fibrogenic response to different etiologic agents. Increased reactive oxygen species and resulting lipid peroxidation products are commonly detected in livers from patients with alcohol abuse, hepatitis C virus infection, iron overload, or chronic cholestasis, as well as in most types of experimental liver fibrogenesis (23). Moreover, antioxidant agents attenuate the development of hepatic fibrosis in rodents and exert beneficial effects in patients with chronic liver diseases (12). Second, ANG II infusion induces vascular thrombosis in the liver. The procoagulant effect of ANG II has been previously reported in other organs (38). Thrombosis of small vessels is frequently found in chronic liver diseases, including alcoholic hepatitis, hepatic rejection, and liver cirrhosis (8, 19). Vascular thrombosis can impair liver perfusion and has been implicated in the pathogenesis of portal hypertension and fibrosis progression (19). Potential mechanisms include impaired liver oxygenation and inflammatory effects of coagulation proteins. Therefore, the procoagulant effect of ANG II may contribute to liver damage. Finally, systemic ANG II could contribute to the increased intrahepatic vascular resistance found in chronic liver diseases. Circulating ANG II levels are markedly increased in patients with cirrhosis and portal hypertension, and ANG II inhibitors decrease portal pressure (10, 33). We previously reported that ANG II increases intracellular  $Ca^{2+}$  concentration and cell contraction in activated, but not quiescent, HSCs (2). The finding that ANG II induces activation of resident HSCs reinforces the hypothesis that ANG II may play a role in the pathogenesis of portal hypertension.

In summary, we provide evidence that prolonged systemic infusion of ANG II to normal rats induces liver damage consisting of areas of necrosis, vasculitis with thrombogenic events, oxidative stress, and activation of HSCs. Moreover, we demonstrate that ANG II induces NF- $\kappa$ B and AP-1 activity in the liver, as well as MAPK activation. These results shed light on the mechanisms involved in the protective effect of ANG II inhibition in chronic liver diseases.

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## DISCLOSURES

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