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

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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-κB, 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 ng·kg⁻¹·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 measured by a sandwich ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Briefly, endogenous peroxidase was blocked with peroxidase-blocking agent and sections were incubated with anti-4-hydroxyynonenal (1:1,000 dilution; Alph Diagnostics, San Antonio, TX), anti-smooth muscle α-actin (1:1,000; DAKO), anti-CD43 (1:1,000; Serotec, Raleigh, NC), and anti-hypoxia-induced factor (HIF)-2α (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 0.79 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.

![Fig. 1](http://ajpgi.physiology.org/)  
**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 10.220.33.4 on June 25, 2017 http://ajpgi.physiology.org/ Downloaded from

*Serum biochemical measurements.* Blood samples were collected from all rats at death. Serum alanine aminotransferase, aspartate aminotransferase, γ-glutamyltranspeptidase, and bilirubin levels were measured by standard enzymatic procedures by the Pathology Department, University of North Carolina. Serum TNF-α 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-QCL; Whittaker Bioproducts, Walkerville, 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 deparaffinized, 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-hydroxyynonenal (1:1,000 dilution; Alph Diagnostics, San Antonio, TX), anti-smooth muscle α-actin (1:1,000; DAKO), anti-CD43 (1:1,000; Serotec, Raleigh, NC), and anti-hypoxia-induced factor (HIF)-2α (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).

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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 MgCl₂, 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 α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), and smooth muscle α-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 chemiluminescence (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 MgCl₂, 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 MgCl₂, 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 32P-labeled probe containing the AP-1 (5'-GTAAAGCATTAGTCAAGACACTC-3') or NF-κB (top: 5'-GACAGAGG-GACTTTCCGAAGA-3'; bottom: 5'-GTTCGGAAAGTCCCC-TCTG-3') consensus sites in buffer containing (in mM) 10 HEPES, 2 MgCl₂, 50 KCl, 1 DTT, 0.1 EDTA and 20% glycerol.
in the presence of single-stranded oligonucleotide (25 µg/ml) and poly(dI/dC) (25 µg/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-1β and TNF-α. Liver tissue samples were homogenized in lysis buffer containing 25 mM HEPES, 0.1% CHAPS, 5 mM MgCl₂, 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-1β and TNF-α (R&D Systems, Minneapolis, MN) was performed by using 1:5 dilutions according to the manufacturer’s instructions.

NF-κB-responsive luciferase assay. Rat HSCs were infected with a recombinant adenoviral vector expressing a luciferase reporter gene driven by NF-κB transcriptional activation (Ad5NF-κBLuc) for 12 h as described (20). Medium was replaced, and cells were stimulated with ANG II (10⁻⁸ M) for 8 h. NF-κB-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 ± 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⁻¹·min⁻¹) infusion were well tolerated. ANG II at 50 ng·kg⁻¹·min⁻¹ increased mean arterial pressure, whereas infusion of 15 ng·kg⁻¹·min⁻¹ 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⁻¹·min⁻¹) compared with control rats (Fig. 1B). A small amount of ascitic fluid was detected in 40% of rats receiving 50 ng·kg⁻¹·min⁻¹ 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 ± 0.03, 1.2 ± 0.2, and

Fig. 4. A: representative photomicrographs of portal tracts from saline- and ANG II (50 ng·kg⁻¹·min⁻¹)-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, ×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, ×400.
1.8 ± 0.5 ng/ml in controls and rats receiving 15 and 50 ng·kg⁻¹·min⁻¹ ANG II, respectively; *P < 0.01 vs. saline). ANG II infusion increased alanine aminotransferase, aspartate aminotransferase, and γ-glutamyltranspeptidase serum levels in a concentration-dependent manner, whereas bilirubin levels remained unchanged (Fig. 2). ANG II-infused rats showed higher serum TNF-α levels compared with control rats (3.3 ± 1.3, 8.4 ± 2.1, and 9.3 ± 3.1 in controls and rats receiving 15 and 50 ng·kg⁻¹·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 ± 0.8, 23.6 ± 3.4, and 37.8 ± 5.5 pg/ml in controls and rats receiving 15 and 50 ng·kg⁻¹·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 ng·kg⁻¹, min⁻¹)-infused livers showed preserved hepatic parenchyma with no apparent hepatocyte damage. Thirty percent of rats receiving ANG II at pressor doses (50 ng·kg⁻¹·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 50% 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 α-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 α-actin-positive stellate cells, mainly observed in the pericentral area (0.1 ± 0.02, 8.2 ± 1.1, and 12.1 ± 2.2 positive cells per field in rats infused with saline and with 15 and 50 ng·kg⁻¹·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 ± 0.1, 11.2 ± 2.6, and 14.1 ± 3.5 positive cells per field in rats infused with saline and with 15 and 50 ng·kg⁻¹·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-2α 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 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 protein adducts. Positive staining was observed in livers of ANG II-treated rats. Original magnifications: A, ×200; B, ×100; C, ×400; D, ×40.
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-κB. ANG II infusion increased phosphorylation of c-Jun (a specific substrate of JNK) and ERK2 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-κB 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 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 α-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-κB, as assessed by luciferase-driven NF-κB assay. All of these effects were prevented by the AT1 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
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-2α 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...
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DISCLOSURES

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