Administration of ANG II induces iron deposition and upregulation of TGF-β1 mRNA in the rat liver

Nobukazu Ishizaka,1 Kan Saito,1 Eisei Noiri,2 Masataka Sata,1 Hitoshi Ikeda,3 Akihiko Ohno,4 Jiro Ando,1 Ichiro Mori,5 Minoru Ohno,1 and Ryozo Nagai1

Departments of 1Cardiovascular Medicine, 2Nephrology, and 3Gastroenterology, University of Tokyo Graduate School of Medicine, Tokyo, Japan; 4Department of Gastroenterology and Hepatology, Saitama Medical School, Saitama, Japan; and 5Department of Pathology, Wakayama Medical College, Wakayama, Japan

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We previously found that ANG II infusion into rats causes iron deposition in the kidney and heart, which may have a role in the regulation of profibrotic gene expression and tissue fibrosis. In the present study, we have investigated whether ANG II can also induce iron accumulation in the liver. Prussian blue staining detected frequent iron deposition in the interstitium of the liver of rats treated with pressor dose ANG II for 7 days, whereas iron deposition was absent in the livers of control rats. Immunohistochemical and histological analyses showed that some iron-positive nonparenchymal cells were positive for ferritin and heme oxygenase-1 (HO-1) protein and TGF-β1 mRNA and were judged to be monocytes/macrophages. It was shown that ANG II infusion caused a fourfold increase in ferritin and HO-1 protein expression by Western blot analysis and about a twofold increase in TGF-β1 mRNA expression by Northern blot analysis, which were both suppressed by treating ANG II-infused rats with losartan and deferoxamine. In addition, mild interstitial fibrosis was observed in the liver of rats that had been treated with pressor dose ANG II for 7 days or with nonpressor dose ANG II for 30 days, the latter of which also caused loss of hepatocytes and intrahepatic hemorrhage in the liver. Taken together, our data suggest that ANG II infusion induces aberrant iron homeostasis in the liver, which may have a role in the ANG II-induced upregulation of profibrotic gene expression in the liver.

iron metabolism; oxidative stress; profibrotic gene; iron chelator

Recent studies have suggested that activation of the ANG II type 1 (ANG II-AT1) receptor axis promotes fibrogenesis and upregulates profibrotic genes, such as transforming growth factor-β1 (TGF-β1) in various tissues, including kidney (5), heart (22), and pancreas (33). It has also been shown that inhibition of the renin-angiotensin system (RAS) reduces the expression of TGF-β1 and the extent of liver fibrosis both in animal models (24, 35) and in humans with liver fibrosis (28), and that, conversely, stimulation of hepatic cells with ANG II increases the expression of TGF-β1 (3, 19). Thus the RAS may also play a pivotal role in the fibroerosion in the liver.

We recently demonstrated that ANG II infusion into rats causes iron deposition and the induction of ferritin expression in the kidney (14) and heart (16), which may augment the profibrotic effects of this peptide. Iron catalyzes Fenton and Haber-Weiss reactions to generate toxic hydroxyl radicals and the consequent products of lipid peroxidation. As ANG II stimulates reduced NADP (NADPH) oxidase to generate superoxides in the liver (4), as well as in the kidney and cardiovascular system (12), iron loading in the liver, if any, may further enhance the oxidant-induced tissue injury, resulting in an hepatic fibrosis (18, 25). Intriguingly, recent studies have raised the possibility that aberrant iron homeostasis may play a role in hepatic fibrosis not only in genetic hemochromatosis but also in other chronic liver diseases such as chronic infections of hepatitis C virus (31) and alcoholic liver disease (29).

In the present study, we have investigated whether ANG II infusion causes hepatic iron deposition and whether ANG II-induced aberrant iron homeostasis has any effect on the expression of profibrotic genes in the liver.

Materials and Methods

The protocols in the current study followed the guidelines by the Animal Research Committee of the University of Tokyo. ANG II was continuously infused into male Sprague-Dawley rats by subcutaneous implantation of an osmotic minipump (Alzet model 2001; Alza Pharmaceutical, Mountain View, CA), as described previously (15). Briefly, Val3-ANG II (Sigma, St. Louis, MO) was infused at a dose of 0.7 mg·kg−1·day−1 for 7 days. In some animals, ANG II was administered at the nonpressor dose of 0.25 mg·kg−1·day−1 for 30 days using the same system. In some experiments, losartan (25 mg·kg−1·day−1; a kind gift from Merck, Warehouse Station, NJ) or hydralazine (15 mg·kg−1·day−1; Sigma) was given in the drinking water, beginning 2 days before pump implantation and continuing throughout ANG II infusion. In some experiments, rats were given daily subcutaneous injections of the iron chelator, deferoxamine (a kind gift from Novartis, Basel, Switzerland) at a dose of 200 mg·kg−1·day−1. Systolic blood pressure was measured in conscious rats by tail-cuff plethysmography (Ueda Seisakuyo, Tokyo, Japan). Serum levels of transferrin were measured by nitroso-PSAP (2-nitroso-5-[N-propyl-N-(3-sulfopropyl)amino]phenol) method.

Preparation of mRNA and Northern blot analysis. Total RNA was obtained by using Isogen (WAKO, Tokyo, Japan), and mRNA was subsequently isolated by using oligotex-dT30 (Roche Diagnostics, Pleasanton, CA). The rat TGF-β1 cDNA probe was a kind gift from Dr. Shio-Shih Tang (Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts) and Dr. John S. D. Chan (Centre Hospitalier de l’Université de Montréal-Hôtel-Dieu, Montreal, Quebec, Canada). The probe was labeled with [γ-32P]ATP (1000 Ci/mmol; Amersham, Chicago, IL) by using T4 polynucleotide kinase (500 U; Roche Diagnostics) for 1 h at 37°C. After labeling, the probe was precipitated in ethanol and resuspended in 100 μl of sterile distilled water.

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treal, Quebec, Canada). Rat collagen type I cDNA was obtained by subcloning the RT-PCR product using rat kidney mRNA and validated by DNA sequencing using the dideoxyribonucleotide sequencing method, as described previously (27). These cDNA probes were labeled with $^{32}$P-dCTP (Amersham Life Sciences, Piscataway, NJ) using commercial kits (Nippon Gene, Toyama, Japan). Hybridized bands were visualized and quantified using a bioimaging analyzer (BAS 2000; Fuji Photo Film). Band densities were normalized to the intensities of corresponding GAPDH mRNA bands present in the same samples on the gel.

In situ hybridization. Rat TGF-β1 cDNA was subcloned into pGEM-T vector (Promega, Madison, WI) in sense and antisense orientations by standard methodology. After digestion with a restriction enzyme and linearization of the plasmid, antisense and sense cRNA riboprobes were transcribed in vitro by using a DIG RNA labeling kit SP6/T7 (Roche Diagnostics). Hybridization was per-

![Fig. 1. Iron in the liver of control and ANG II-infused rats. A: section from a control rat; B, C: sections from ANG II-infused rats. Ferric iron was detected by Prussian blue staining. Iron deposition is not apparent in the liver of control rats (A), whereas iron stains positively in the interstitial cells in the liver of ANG II-infused rats (B, C). Original magnifications, ×100 (A), ×400 (C).](http://ajpregu.physiology.org/)

![Fig. 2. Expression of heme oxygenase-1 (HO-1) and ferritin in the liver of ANG II-infused rats. A, C, E: Sections from control rats; B, D, F–O: sections from ANG II-infused rats. A, B, K: HO-1 staining. C, D, M: ferritin staining. E–I, O: ED-1 staining. J, L, N: Prussian blue staining. J and K, L and M, and N and O are serial sections. After ANG II infusion, immunoreactivity of HO-1 (B), ferritin (D), and ED-1 (F), which detects monocytes/macrophages, was increased. Losartan (H), but not hydrazine (I), completely suppresses ANG II-induced increase in ED-1 immunoreactivity. Staining of serial sections shows that some cells that are positive for HO-1 (K), ferritin (M), and ED-1 (O) were positive for iron. Original magnifications, ×100 (A–F), ×200 (G–O).](http://ajpregu.physiology.org/)
formed by using in situ hybridization reagents (Nippon Gene) according to the manufacturer’s instructions.

**Histological and immunohistochemical analyses.** Hepatic iron accumulation was chemically detected by Prussian blue staining. Presence of hepatic fibrosis was analyzed by Masson’s trichrome staining. Immunohistochemistry was performed as described previously (1). Antibodies against rat HO-1 (SPA895, StressGen Biotechnologies, Victoria, British Columbia, Canada), ferritin (Panapharm, Uto, Japan), monocytes/macrophages (ED-1, Chemicon, CA), human TGF-β1 (Chemicon, Temecula, CA), 4-hydroxynonenal (HNE)-modified proteins (Japan Institute for the Control of Aging, Fukuroi City, Japan), and rat α-smooth muscle actin (Sigma) were used at dilutions of 1/200, 1/200, 1/100, 1/200, 1/75, and 1/400, respectively. In some experiments, the same sections stained with these antibodies were subsequently stained with Prussian blue to investigate the colocalization of iron deposition and these markers.

**Protein purification and Western blot analysis.** Protein was isolated by homogenizing samples in the lysis buffer [50 mmol/l HEPES, 5 mmol/l EDTA, and 50 mmol/l NaCl; pH 7.5] containing protease inhibitors [10 μg/ml aprotinin, 1 mmol/l PMSF, and 10 μg/ml leupeptin]. Equal amounts of protein were loaded onto 15% SDS polyacrylamide gels and subsequently blotted onto Immobilon-P polyvinylidine difluoride membranes (Millipore, Billerica, MA). Polyclonal antibodies against HO-1 and ferritin were used at dilutions of 1/2,000. The ECL Western blotting system (Amersham Life Sciences) was used for detection. Bands were visualized by a luminoanalyzer (LAS-1000, Fuji Photo Film). Band intensity was calculated by the image analysis software, NIH Image (National Institutes of Health, Research Service Branch, Bethesda, MD).

**Statistical analysis.** Data are expressed as means ± SE. The results from protein and mRNA expression are presented as a percentage of the control value. ANOVA followed by a multiple comparison test for comparisons was performed on initial data before expression as a percentage of the control. A value of *P* < 0.05 was considered statistically significant.

**RESULTS**

**Systolic blood pressure.** The systolic blood pressure was 131 ± 3 mmHg of control rats, and it was increased to 192 ± 4 mmHg after a 7-day infusion of ANG II (*P* < 0.01 vs. control). Both losartan and hydralazine completely abolished the hypertensive effects of ANG II (blood pressure, 126 ± 5 and 133 ± 8 mmHg, respectively, *n* = 10, not significant vs. control). Deferoxamine did not significantly affect the blood pressure of ANG II-treated (196 ± 7 mmHg, *n* = 10; not significant vs. ANG II-treated rats) or untreated (129 ± 3 mmHg, *n* = 4; not significant vs. control) rats. Serum levels of both iron and total transferrin in rats treated with ANG II for 7 days were found to be slightly, but significantly higher (iron, 204 ± 46 mg/dl, *n* = 5; transferrin 505 ± 13 mg/dl, *n* = 10) than those in the control rats (iron, 139 ± 5 mg/dl, *n* = 10; transferrin 469 ± 8 mg/dl, *n* = 10) (*P* < 0.05).

**Iron deposition in the liver of ANG II-treated and untreated rats.** Prussian blue staining detected no iron deposition in the livers of control rats (Fig. 1A). In contrast, iron was frequently detected in nonparenchymal cells, but not hepatocytes, after ANG II infusion (Fig. 1B and C).

**Immunohistochemistry of ferritin and HO-1.** Immunohistochemical analysis showed that interstitial cells expressing ferritin and heme oxygenase-1 (HO-1), an inducible form of the rate-limiting enzyme in heme degradation, were more frequent after ANG II infusion (Fig. 2, A–D). ED-1-positive monocytes/macrophages were also frequently observed in the liver of ANG II-treated rats, but only rarely in the liver of untreated rats (Fig. 2, E and F). Analysis of serial sections showed that the cells in the interstitium that were expressing HO-1 and ferritin were ED-1-positive monocytes/macrophages and occasionally unidentified cells. The latter cells are not likely to be neutrophils, because neutrophils were only rarely observed in these samples by hematoxylin-eosin staining. Expression of HO-1 and ferritin was also observed occasionally in hepatocytes, especially in the pericentral area (data not shown). The selective AT1 receptor blocker losartan, but not the nonspecific vasodilator hydralazine, completely suppressed the ANG II-induced increase in ED-1 immunoreactivity (Fig. 2, G–H). Analysis of serial sections showed that cells that were positive for HO-1, ferritin, and ED-1 were positive for iron (Fig. 2, J–O).

**Effects of antihypertensive drugs and an iron chelator on ANG II-induced upregulation of ferritin and HO-1.** Western blot showed that ANG II infusion caused about a fourfold increase in ferritin and HO-1 protein expression in the liver compared with untreated rats. Losartan, but not hydralazine, suppressed this ANG II-induced increase in expression of these proteins. In addition, treatment of the ANG II-infused rats with deferoxamine, which does not affect the hypertensive effects of
ANG II, inhibited the ANG II-induced induction of ferritin and HO-1 protein in the liver (Fig. 3).

Localization of TGF-β1 by in situ hybridization. By in situ hybridization, TGF-β1 mRNA was barely detected in the liver of control rats (Fig. 4A). After ANG II infusion, a high expression of TGF-β1 mRNA was observed in the interstitial cells (Fig. 4B). Staining of serial sections showed that many but not all cells that were positive for TGF-β1 were positive for iron, HO-1, ferritin, and ED-1 (Fig. 4, C–J). Colocalization of TGF-β1 and iron deposition was also demonstrated by immunohistochemistry using an antibody against TGF-β1 protein (Fig. 4, K and L).

Effects of antihypertensive drugs and an iron chelator on ANG II-induced upregulation of TGF-β1 and collagen type 1 mRNA. Northern blotting showed that ANG II infusion caused about a twofold increase in both TGF-β1 and collagen type 1 mRNA expression in the liver compared with untreated rats. Losartan completely suppressed the ANG II-induced upregulation of TGF-β1 and collagen type 1 mRNA. By contrast, hydralazine only partially, although statistically significantly, suppressed the ANG II-induced upregulation of these genes. In addition, treatment of the ANG II-infused rats with deferoxamine inhibited the ANG II-induced upregulation of both TGF-β1 and collagen type 1 mRNA (Fig. 5).

Localization of HNE-modified protein adducts. We investigated whether cells that stained positively for iron showed an increase in levels of lipid peroxidation products by an immunohistochemical assessment of HNE-modified protein adducts. As expected, after ANG II stimulation, increased amounts of HNE-modified protein adducts were detected in the nonparenchymal cells that were positive for iron deposition (Fig. 6).

Effects of administration of ANG II for 30 days. Masson’s trichrome staining showed that administration of ANG II for 7 days caused a modest increase in interstitial fibrosis (Fig. 7, A and B). To elucidate whether interstitial fibrosis was generated specifically by ANG II or by hypertension per se, we administered to rats a nonpressor dose of ANG II for 30 days. The systolic blood pressure of rats receiving a nonpressor dose of ANG II for 30 days was 130 ± 10 mmHg (n = 5, not significant vs. untreated control). In the liver of these rats, a loss of hepatic cells accompanied by intrahepatic hemorrhage could occasionally be observed (Fig. 7C). Masson’s trichrome staining demonstrated that interstitial fibrosis occurred in these regions (Fig. 7D). In addition, some interstitial cells showed...
increased immunoreactivity to α-smooth muscle actin after ANG II treatment (Figs. 7, E and F). These data further suggested that ANG II could promote interstitial fibrosis by a pressor-independent mechanism. Northern blot analysis showed that administration of a nonpressor dose ANG II for 30 days increased TGF-β1 mRNA expression in liver (Fig. 8).

DISCUSSION

In the present study, we have demonstrated that administration of ANG II for 7 days caused iron deposition in the hepatic ED-1-positive macrophages, in which TGF-β1 mRNA expression was also increased. Iron chelation, which abolished the ANG II-induced deposition of iron and induction of ferritin and HO-1 proteins, whose gene expression is thought to be linked to oxidative stress, suppressed the ANG II-induced upregulation of TGF-β1 mRNA. These data collectively suggest that aberrant iron homeostasis in the liver induced by ANG II stimulation may play a role in the ANG II-induced regulation of profibrotic gene expression in vivo.

We found that the ANG II-induced upregulation of TGF-β1 mRNA was inhibited completely by losartan, but only partially by hydralazine, which indicates that upregulation of ANG II-induced TGF-β1 mRNA is a pressor-independent event, confirming our finding that a nonpressor ANG II infusion administered over a prolonged period expressed an upregulation of TGF-β1 mRNA expression. These observations are consistent with the finding by Bataller et al. (3) that ANG II, even at subpressor doses, increases collagen synthesis in the...
liver. Bataller et al. have also reported that ANG II infusion induces the recruitment of inflammatory cells that are positive for CD43, which is expressed by infiltrating mononuclear cells and lymphocytes. In the present study, ANG II apparently increased the number of ED-1-positive cells, which were also found to be positive for iron deposition. These ED-1-positive cells are considered to be monocytes and free and fixed macrophages (10), and are more likely to be recruited from the circulation than Kupffer cells (9).

A link between iron, hepatic fibrosis, and cirrhosis has been reported in several conditions of iron loading, such as genetic hemochromatosis, alcoholic liver disease (8), and chronic hepatitis C infection (21), and iron is considered as a comorbid factor in these diseases (11). The formation of highly toxic hydroxyl radicals via Fenton and Haber-Weiss reactions, followed by the subsequent formation of lipid peroxidation products, is postulated to be an underlying mechanism of iron-mediated liver injury (7). We demonstrated here that ANG II infusion caused deposition of iron and induction of ferritin in the liver; however, not much is known about the possible relationship between RAS and iron stores in humans thus far. Piperno et al. (26) have recently reported that the link between hypertension and a higher prevalence of increased iron stores, although they did not analyze the activity of RAS. Although recent studies have shown that AT1 receptor blockade ameliorates hepatic fibrosis in animal models (30), little is known about whether modulation of iron homeostasis underlies the mechanism of the antifibrotic effects of AT1 receptor antagonist. Interestingly, however, Yokohama et al. (34) have very recently reported that AT1 receptor antagonism not only suppressed the hepatic fibrosis, but also reduced the serum ferritin content. In addition, we have demonstrated that ANG II administration to rats caused deposition of iron and induction of ferritin in the heart and that AT1 receptor blockade decreased the extent of fibrosis and expression of ferritin in the heart (16). Together with the current results, it is suggested that ANG II may act to promote organ fibrosis, in part, via modulating iron homeostasis, and this can be suppressed by the AT1 receptor antagonism.

Our study showed that the amount of HNE-modified protein adducts was increased in the iron-laden macrophages, which suggested that the iron-catalyzed peroxidation of lipids was producing reactive aldehydes in these cells. Similarly, Khan et al. (17) have reported that iron loading causes iron deposition and an increase in HNE-modified protein adducts, although they found that these events occurred not in macrophages, but...
in preportal hepatocytes. However, it has been pointed out that hepatic macrophages may also participate in the early events of liver injury involving lipid peroxidation (20). Furthermore, the treatment of macrophage lineage cells with HNE results in an upregulation of TGF-β1 mRNA expression (18). Together with these findings, our data suggest that activation of the ANG II-AT1 receptor axis upregulates hepatic expression of profibrotic genes, which is, in part, mediated by an increase in the production of the lipid peroxidation end products, facilitated by iron accumulation in the liver. Bataller et al. (3) have shown that ANG II infusion increases HNE-modified protein adducts in the hepatocytes. Here, however, we found that HNE-modified protein adducts did not apparently increase in hepatocytes after ANG II infusion. The mechanisms of induction of lipid peroxidation that underlie these differential results should be studied in future studies.

Iron overload has been shown to cause an increase in the levels of circulatory oxidative stress markers (32). We previously showed that plasma levels of 8-epi-prostaglandin F2α (8-epi-PGF2α), a reliable marker of in vivo oxidative stress, are increased in response to ANG II infusion (2); furthermore, this ANG II-induced increase in plasma 8-epi-PGF2α is inhibited by the treatment with deferoxamine (27), which suggests that iron metabolism is involved in the enhancement of ANG II-induced oxidative stress. In these experiments, however, hydralazine, which did not completely suppress the ANG II-induced oxidative stress, also suppressed the ANG II-induced increase in plasma 8-epi-PGF2α levels (2). These data suggest that plasma markers of oxidative stress may not reflect the extent of hepatic iron loading in some animal models, such as rat models of ANG II infusion.

In the present study, the origin of the iron deposited in the ED-1-positive cells was not investigated. We and others have previously found that ANG II induces degradation of skeletal (6) and cardiac (13) muscles. In addition, treatment of animals with hemoglobin, but not with iron dextran, increases tissue expression of HO-1, although both agents increase ferritin content (23). As iron-positive cells had also showed increased levels of HO-1 in the present study, the deposited iron is most likely to have originated from heme proteins, to which a release of myoglobin from muscles may partially contribute.

In conclusion, ANG II infusion increased the expression of TGF-β1 mRNA in the ED-1-positive macrophages in the liver, which also showed positive iron deposition. Iron-positive non-parenchymal cells had increased levels of the oxidative stress markers HO-1 protein and HNE-modified protein adducts. Treatment of ANG II-infused rat with AT1 receptor blocker or an iron chelator suppressed the upregulation of ferritin, HO-1, TGF-β1, and collagen type 1 mRNA induced by ANG II. Taken together, our data suggest that ANG II infusion increases profibrotic gene expression in part by modulating iron homeostasis in the liver in vivo that is mediated by AT1 receptor.

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References


