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

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Short title: ANGIOTENSIN II, IRON, AND TGF-β IN THE LIVER

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ABSTRACT

We previously found that angiotensin II infusion into rat causes iron deposition in the kidney and heart, which may have a role in the regulation of profibrotic genes expression as well as tissue fibrosis. In the present study, we have investigated whether angiotensin 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 angiotensin 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 angiotensin II infusion caused about 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 suppressed by treating angiotensin 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 angiotensin II for 7 days or with non-pressor dose angiotensin II for 30 days, latter of which also caused loss of hepatocytes and intrahetatic hemorrhage in the liver. Taken together, our data suggest that angiotensin II infusion induces aberrant iron homeostasis in the liver, which may have a role in the angiotensin II-induced upregulation of profibrotic gene expression in the liver.

KEY WORDS: angiotensin II; iron metabolism; oxidative stress; profibrotic gene; iron chelator
INTRODUCTION

Recent studies have suggested that activation of the angiotensin II-AT$_1$ receptor axis promotes fibrogenesis and upregulates profibrotic genes, such as 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 angiotensin II increases the expression of TGF-β1 (3, 19). Thus RAS may play a pivotal role in the fibrosclerosis also in the liver.

We recently demonstrated that angiotensin 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 angiotensin II stimulates NAD(P)H 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 angiotensin II infusion causes hepatic iron deposition, and whether angiotensin 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. Angiotensin II was continuously infused into male Sprague-Dawley rats by subcutaneous implantation of an osmotic minipump (Alzet model 2001; Alza Pharmaceutical, CA) as described previously (15). Briefly, Val5-angiotensin II (Sigma, MO) was infused at a dose of 0.7 mg/kg/day for 7 days. In some animals, angiotensin II at non pressor-dose, 0.25 mg/kg/day, was administered for 30 days using the same system. In some experiments, losartan (25 mg/kg/day; a kind gift from Merck) or hydralazine (15 mg/kg/day, Sigma) was given in the drinking water, beginning 2 days before pump implantation and continuing throughout angiotensin II infusion. In some experiments, rats were given daily subcutaneous injections of the iron chelator, deferoxamine (a kind gift from Novartis) at a dose of 200 mg/kg/day. Systolic blood pressure was measured in conscious rats by tail-cuff plethysmography (Ueda Seisakusyo, 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, Japan), and mRNA was subsequently isolated by using oligotex-dT30 (Roche Diagnostics, IN). The rat TGF-β1 cDNA probe was a kind gift from Dr. Shiow-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). 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 \([\alpha-^{32}P]\) dCTP (Amersham Life Sciences, NJ) using commercial kits (Nippon Gene).
Hybridized bands were visualized, and quantified using a bio-imaging analyzer (BAS 2000; Fuji Photo Film, Japan). 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, 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, Germany). Hybridization was performed by using In Situ Hybridization Reagents (Nippongene, Japan) 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, Canada), ferritin (Panapharm, Japan), monocytes/macrophages (ED-1, Chemicon, CA), human TGF-β1 (Chemicon), 4-hydroxynonenal (HNE)-modified proteins (JaICA, 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 co-localization 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 polyvinylidene difluoride membranes (Millipore, MA). Polyclonal antibodies against HO-1 and ferritin were used at a dilution of 1/2,000. The ECL Western blotting system (Amersham Life Sciences, IL) was used for detection. Bands were visualized by a lumino-analyzer (LAS-1000, Fuji Photo Film). Band intensity was calculated by the image analysis software, NIH Image (NIH, Research Service Branch).

Statistical Analysis

Data are expressed as the mean ± SEM. 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 on initial data before expression as a percentage of the control. A value of p<0.05 has been considered to be 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 angiotensin II (p<0.01 versus control). Both losartan and hydralazine completely abolished the hypertensive effects of angiotensin II (blood pressure, 126 ± 5 and 133 ± 8 mmHg, respectively, n=10, ns versus control). Deferoxamine did not significantly affect the blood pressure of angiotensin II-treated (196±7 mmHg, n=10; ns versus angiotensin II-treated rats) or untreated (129 ± 3 mmHg, n=4; ns versus control) rats. Serum levels of both iron and total transferrin in rats treated with angiotensin 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 Angiotensin II-Treated and Untreated Rats

Prussian blue staining detected no iron deposition in the livers of control rats (Figure 1A). In contrast, iron was frequently detected in nonparenchymal cells, but not hepatocytes, after angiotensin II infusion (Figures 1B, 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 angiotensin II infusion (Figures 2A-D). ED-1-positive monocytes/macrophages were also frequently observed in the liver of angiotensin II-treated rats, but only rarely in the liver of untreated rats (Figures 2E, 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 non-specific vasodilator hydrazine, completely suppressed the angiotensin II-induced increase in ED-1 immunoreactivity (Figures 2G-H). Analysis of serial sections showed that cells that were positive for HO-1, ferritin, and ED-1 were positive for iron (Figures 2J-O).

Effects of Antihypertensive Drugs and an Iron Chelator on Angiotensin II-Induced Upregulation of Ferritin and HO-1

Western blot showed that angiotensin II infusion caused about a fourfold increase in
ferritin and HO-1 protein expression in the liver as compared to untreated rats. Losartan, but not hydralazine, suppressed this angiotensin II-induced increase in expression of these proteins. In addition, treatment of the angiotensin II-infused rats with deferoxamine, which does not affect the hypertensive effects of angiotensin II, inhibited the angiotensin II-induced induction of ferritin and HO-1 protein in the liver (Figure 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 (Figure 4A). After angiotensin II infusion, a high expression of TGF-β1 mRNA was observed in the interstitial cells (Figures 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 (Figures 4C-J). Co-localization of TGF-β and iron deposition was also demonstrated by immunohistochemistry using an antibody against TGF-β1 protein (Figures 4K, L).

_Effects of Antihypertensive Drugs and an Iron Chelator on Angiotensin II-Induced Upregulation of TGF-β1 and Collagen Type 1 mRNA_

Northern blotting showed that angiotensin II infusion caused about a twofold increase in both TGF-β1 and collagen type 1 mRNA expression in the liver as compared to untreated rats. Losartan completely suppressed the angiotensin II-induced upregulation of TGF-β1 and collagen type 1 mRNA. By contrast, hydralazine only partially, although statistically significantly, suppressed the angiotensin II-induced upregulation of these genes. In addition, treatment of the angiotensin II-infused rats with deferoxamine inhibited the angiotensin II-induced upregulation of both TGF-β1 and collagen type 1 mRNA (Figure 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 angiotensin II stimulation, increased amounts of HNE-modified protein adducts were detected in the nonparenchymal cells that were positive for iron deposition (Figure 6).

**Effects of Administration of Angiotensin II for 30 days**

Masson’s trichrome staining showed that administration of angiotensin II for 7 days caused, although modestly, an increase in interstitial fibrosis (Figures 7A, B). To elucidate whether interstitial fibrosis was generated specifically by angiotensin II or by hypertension per se, we generated rats that were given non-pressor dose angiotensin II for 30 days. The systolic blood pressure of rats receiving a non-pressor dose of angiotensin II for 30 days was 130 ± 10mmHg (n=5, ns versus untreated control). In the liver of these rats, a loss of hepatic cells accompanied by intrahepatic hemorrhage could occasionally be observed (Figure 7C). Masson's trichrome staining demonstrated that interstitial fibrosis occurred in these regions (Figure 7D). In addition, some interstitial cells showed increased immunoreactivity to α-smooth muscle actin after angiotensin II treatment (Figures 7E, F). These data further suggested that angiotensin II could promote interstitial fibrosis by a pressor-independent mechanism. Northern blot analysis showed that administration of a non-pressor dose angiotensin II for 30 days increased TGF-β1 mRNA expression in liver (Figure 8).

**DISCUSSION**

In the present study, we have demonstrated that administration of angiotensin II for 7 days caused iron deposition in the hepatic ED-1-positive macrophages, where TGF-β1 mRNA expression was also increased. Iron chelation, which abolished the angiotensin
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 angiotensin II-induced upregulation of TGF-β1 mRNA. These data collectively suggest that aberrant iron homeostasis in the liver induced by angiotensin II stimulation may play a role in the angiotensin II-induced regulation of profibrotic gene expression in vivo.

We found that the angiotensin II-induced upregulation of TGF-β1 mRNA was inhibited completely by losartan, but only partially by hydralazine, which indicates that angiotensin II-induced TGF-β1 mRNA upregulation is a pressor-independent event, a suggestion that is supported by our finding that a non-pressor dose of angiotensin II infusion for a more prolonged period upregulated TGF-β1 mRNA expression. These observations are consistent with the finding by Bataller et al. that angiotensin II, even at subpressor doses, increases collagen synthesis in the liver (3). Bataller et al. have also reported that angiotensin 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, angiotensin II apparently increased the number of ED-1-positive inflammatory 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 thus 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 angiotensin 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. have recently reported that the link between hypertension and a higher prevalence of increased iron stores (26), although they did not analyzed the activity of RAS. Although recent studies have shown that angiotensin II type 1 (AT1) receptor blockade ameliorates hepatic fibrosis in animal models (30), little is known whether modulation of iron homeostasis underlies as a mechanism of the anti-fibrotic effects of AT1 receptor antagonist. Interestingly, however, Yokohama et al. have very recently reported that AT1 receptor antagonism not only suppressed the hepatic fibrosis, but also reduced the serum ferritin content (34). In addition, we have demonstrated that angiotensin II administration to rat 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 angiotensin 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 occurring to produce reactive aldehydes in these cells. Similarly, Khan et al. 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 (17). 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 angiotensin 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. have shown that angiotensin II infusion increases HNE-modified protein
adducts in the hepatocytes (3). Here, however, we found that HNE-modified protein adducts did not apparently increase in hepatocytes after angiotensin 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 F$_{2\alpha}$ (8-epi-PGF$_{2\alpha}$), a reliable marker of $in$ vivo oxidative stress, are increased in response to angiotensin II infusion (2); furthermore, this angiotensin II-induced increase in plasma 8-epi-PGF$_{2\alpha}$ is inhibited by the treatment with deferoxamine (27), which suggests that iron metabolism is involved in the enhancement of angiotensin II-induced oxidative stress. In these experiments, however, hydralazine, which did not suppress the angiotensin II-induced upregulation of hepatic TGF-β1 mRNA upregulation in the current study, also suppressed the angiotensin II-induced increase in plasma 8-epi-PGF$_{2\alpha}$ 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 angiotensin 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 angiotensin 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, angiotensin 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 nonparenchymal cells had increased levels of the oxidative stress markers,
HO-1 protein and HNE-modified protein adducts. Treatment of angiotensin II-infused rat with AT$_1$ receptor blocker or an iron chelator suppressed the upregulation of ferritin, HO-1, and TGF-β1 and collagen type 1 mRNA induced by angiotensin II. Taken together, our data suggest that angiotensin II infusion increases profibrotic gene expression in part by modulating iron homeostasis in the liver in vivo that is mediated by AT$_1$ receptor.

ACKNOWLEDGEMENTS

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GRANTS

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REFERENCES


FIGURE LEGENDS

Figure 1. Iron in the liver of control and angiotensin II-infused rats.
A: Section from a control rat; B, C: sections from angiotensin 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 angiotensin II-infused rats (B, C). Original magnifications, × 100 (A, B), × 400 (C).

Figure 2. Expression of heme oxygenase-1 (HO-1) and ferritin in the liver of angiotensin II-infused rats.
A, C, E: Sections from control rats; B, D, F-O: sections from angiotensin 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 angiotensin II infusion, immunoreactivity of HO-1 (B) and ferritin (D) and ED-1 (F), which detects monocytes/macrophages, was increased. Losartan (H), but not hydrazine (I), completely suppresses angiotensin 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).

Figure 3. Western blot analysis of ferritin and heme oxygenase-1 (HO-1) protein expression.
Effects of losartan (LOS), hydralazine (HYD), and deferoxamine (DFO) on ferritin and HO-1 protein expression were analyzed. A: Representative Western blots. B: Summary of data from 5-7 experiments in each group. Treatment of angiotensin II-infused rats with losartan or deferoxamine, but not hydralazine, suppresses the angiotensin II-induced increase in ferritin and HO-1 protein expression. *p<0.01 versus untreated control.
Figure 4. Analysis of TGF-β1 mRNA localization by *in situ* hybridization. A: A section from a control rat; B-L: sections from angiotensin II-infused rats. C and D, E and F, G and H, I and J, and K and L are serial sections. A, B, C, E, G, I, L: Hybridization with a TGF-β1 antisense probe. D, L: Prussian blue (PB) staining. F, H, J, K: Immunohistochemistry using antibodies against heme oxygenase-1 (HO-1) (F), ferritin (H), ED-1 (J), and TGF-β (K). Some interstitial cells with high TGF-β1 mRNA expression are also positive for iron (arrows in C and D), HO-1 (arrows in E and F), ferritin (arrows in G and H), and ED-1 (arrows in I and J). In addition, some interstitial cells with increased TGF-β protein expression are positive for iron (arrows in K and L). Original magnifications, ×250 (A-C, E, G, I, L), and ×200 (D, F, H, J, K).

Figure 5. Northern blot analysis of TGF-β1 mRNA expression. A: Representative Northern blots. B: Summary of data from 5-7 experiments in each group. Losartan (LOS) and deferoxamine (DFO) completely, whereas hydralazine (HYD) only partially, suppresses the angiotensin II-induced upregulation of mRNA expression of TGF-β1 and collagen type 1. *p<0.01 and †p<0.05 versus untreated control.

Figure 6. Localization of 4-hydroxynonenal (HNE)-modified protein adducts in the liver. A: Section from control rats; B-D: sections from angiotensin II-infused rats. A, B: Staining with antibody against HNE-modified protein adducts. D. Prussian blue (PB) staining. C: Co-staining of Prussian blue and HNE-modified protein adducts. B and C are serial sections. Nonparenchymal cells that are positive for HNE-modified protein adducts were positive for iron. Original magnification, ×200.

Figure 7. Fibrosis in the liver of rats given angiotensin II for 7 and 30 days. A, E: Sections from a control rat; B: sections from rats given a pressor dose of angiotensin
II for 7 days; C, D, F: sections from rats given a non-pressor dose of angiotensin II for 30 days. A, B, D. Masson trichrome (MT) staining. C. Hematoxylin-eosin (HE) staining. E, F. Immunohistochemistry using antibodies against rat α-smooth muscle actin (αSMA). In the liver of untreated rats, no apparent fibrosis could be observed (A). In contrast, increased fibrosis was occasionally observed in the liver of rats treated with pressor-dose angiotensin II for 7 days (B). Interstitial was accompanied by loss of hepatic cells and intrahepatic hemorrhage, in rats treated with non-pressor dose angiotensin for 30 days. Some interstitial cells showed increased immunoreactivity to αSMA after angiotensin II treatment compared to those in the liver of untreated rats (E, F). Original magnification, ×200.

Figure 8. Northern blot analysis of TGF-β1 mRNA expression in the liver of rats given angiotensin II for 30 days.
A: Representative Northern blots. B: Summary of data from 4-6 experiments in each group. *p<0.01 versus untreated control.
Figure 1
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Figure 3

A

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B

![Graph showing Ferritin and HO-1 expression](image)

**Ferritin Expression (% Control)**

- ANG II
- LOS
- HYD
- DFO

**HO-1 Expression (% Control)**

- ANG II
- LOS
- HYD
- DFO

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Figure 4
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**Figure 5**
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**Figure 8**

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B

![Bar chart showing TGF-β1/GAPDH (% control)]

- CONTROL
- ANG II (Pressor: 7d)
- ANG II (Non-pressor: 30d)

* indicates statistical significance.

ns indicates non-significance.