Article

The α1-adrenergic receptor is involved in hepcidin upregulation induced by adrenaline and norepinephrine via the STAT3 pathway†

Running title: hepcidin regulation via α1-adrenergic receptor

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Abstract:

Elevated body iron stores are associated with hypertension progression, while hypertension is associated with elevated plasma catecholamine levels in patients. However, there is a gap in our understanding of the connection between catecholamines and iron regulation. Hepcidin is a key iron-regulatory hormone, which maintains body iron balance. In the present study, we investigated the effects of adrenaline (AD) and norepinephrine (NE) on hepatic hepcidin regulation. Mice were treated with AD, NE, phenylephrine (PE, α1-adrenergic receptor agonist), prazosin (PZ, α1-adrenergic receptor antagonist), and/or propranolol (Pro, β-adrenergic receptor antagonist). The levels of hepcidin, as well as signal transducer and activator of transcription 3 (STAT3), ferroportin 1 (FPN1), and ferritin-light (Ft-L) protein in the liver or spleen, were assessed. Six hours after AD, NE, or PE treatment, hepatic hepcidin mRNA levels increased. Pretreatment with PZ, but not Pro, abolished the effects of AD or NE on STAT3 phosphorylation and hepatic hepcidin expression. When mice were treated with AD or NE continuously for 7 d, an increase in hepatic hepcidin mRNA levels and serum hepcidin concentration was also observed. Meanwhile, the expected downstream effects of elevated hepcidin, namely decreased FPN1 expression and increased Ft-L protein and non-heme iron concentrations in the spleen, were observed after the continuous AD or NE treatments. Taken together, we found that AD or NE increase hepatic hepcidin expression via the α1-adrenergic receptor and STAT3 pathways in mice. The elevated hepatic hepcidin decreased FPN1 levels in the spleen, likely causing the increased iron accumulation in the spleen.

Key terms: adrenaline, α1-adrenergic receptor, hepcidin, norepinephrine, STAT3

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Introduction

Iron is an essential element in the human body. Iron plays essential roles in oxygen transport and storage (hemoglobin and myoglobin) and electron transfer and serves as a cofactor for many enzyme systems. However, iron is a double-edged sword in living systems; both iron deficiency and iron overload are deleterious conditions. Iron deficiency leads to clinical anemia [Clark, 2008], while excess iron can harm tissues by causing oxidative stress through the production of hydroxyl radicals via the Fenton/Haber–Weiss reaction [Kruszewski, 2003]. Iron deposition has been proposed to play a pathogenic role in age-related diseases, including Parkinson's disease [Levenson, 2003] and Alzheimer's disease [Lehmann et al., 2006].

In 1981, Sullivan suggested for the first time that body iron stores may be positively related to the risk of cardiovascular diseases [Sullivan, 1981]. He proposed that the greater incidence of heart disease in men and postmenopausal women, compared with the incidence in premenopausal women, is due to higher levels of stored iron in these two groups. Sullivan also suggested that iron accumulation within the arterial wall may promote atherosclerosis progression [Sullivan, 2007; Sullivan, 2009]. Therefore, the corollary that iron depletion protects against cardiovascular disease has been proposed. In animal studies, dietary iron restriction attenuated the development of hypertension and renal injury in aldosterone/salt-induced hypertensive mice [Sawada et al., 2015]. Depletion of iron levels by phlebotomy, systemic iron chelation or dietary iron restriction reduces atherosclerotic lesion size and increases plaque stability [Sullivan, 2009].

It has been shown that men with hypertension exhibit increased serum ferritin (a marker of iron accumulation) more frequently than healthy controls [Piperno et al., 2002]. However, the exact mechanism of iron overload in hypertension remains unclear. Hepcidin is synthesized by the liver in

the form of an 84 amino acid propeptide and is detected in the plasma as an active peptide of 25 amino acids [Park et al., 2001]. Hepcidin has been proposed to act as the principal iron-regulatory hormone to maintain iron homeostasis. Hepcidin can decrease cellular iron export by activating the internalization and degradation of Ferroportin 1 (FPN1), which is the only known cellular iron exporter [Nemeth et al., 2004]. It has also been proposed that hepcidin promotes plaque destabilization by preventing iron release from macrophages within atherosclerotic lesions, and the absence of this mobilization may result in increased cellular iron load, lipid peroxidation, and progression to foam cells [Sullivan, 2007]. Hepcidin levels have been observed to be inappropriately elevated in idiopathic pulmonary arterial hypertension (IPAH) [Rhodes et al., 2011]. However, the underlying mechanisms of hepcidin regulation in the situation of hypertension are largely unknown.

It is well known that sympathetic neural mechanisms are involved in the development and progression of hypertension [Grassi et al., 2015]. The rise in blood pressure is, at least in part, mediated by over-activity of the sympathetic nervous system. On the other hand, suppression of the activity of the sympathetic nervous system has shown promise in the treatment of hypertension [Currie et al., 2012]. Catecholamines, including adrenaline (AD) and norepinephrine (NE), are produced by the adrenal medulla and postganglionic nerve fibres of the sympathetic nervous system. High blood pressure was indeed associated with elevated plasma catecholamine values in some patients with essential hypertension [Goldstein, 1983]. The aim of our study was to evaluate whether AD and NE are associated with hepcidin regulation in the liver and, if so, the underlying mechanism.

In contrast to the human, rat, dog, and pig genomes, which contains only one copy of the gene, there is a gene duplication in mice, resulting in murine hepcidin 1 and hepcidin 2. However, the two mouse hepcidins are considerably divergent at the level of the corresponding mature 25-amino acid

peptide, and hepcidin 2 does not act on iron metabolism [Lou et al., 2004]. Therefore, our study only examines the regulation and role of hepcidin 1.

Materials and methods

Animals and Treatment

Five to six-month-old male Kunming mice were used in this study. The mice and standard feed were purchased from Hebei Medical University (Shijiazhuang, China). Mice were housed in stainless steel rust-free cages at 22°C–24°C and 45%–55% relative humidity. All mice were provided with free access to food and distilled water. The Animal Care and Use Committee of the Hebei Science and Technical Bureau in the PRC approved the experimental protocol.

Male Kunming mice were randomized into several groups. Each group consisted of 6 animals. In the AD group, mice were injected intraperitoneally (i.p.) with AD (Wuhan Yuanda Pharmaceutical Co., Ltd., Wuhan, China) at a dose of 250 μg/kg. In the NE group, mice were injected (i.p.) with NE (Wuhan Yuanda Pharmaceutical Co., Ltd., Wuhan, China) at a dose of 500 μg/kg. In the NS group, mice were injected (i.p.) with the same dose of normal saline as a control for the AD or NE group. To further determine the role of adrenergic receptor (AR) subtypes, which may be involved in the regulation of hepatic hepcidin expression, mice were treated with phenylephrine (PE, α1-AR agonist; Sigma, St. Louis, MO, USA), prazosin (PZ, α1-AR antagonist; Shanghai Xinyi Pharmaceutical Co., Ltd., Shanghai, China), or propranolol (Pro, β-AR antagonist; Shanxi Yunpeng Pharmaceutical Co., Ltd., Shanxi, China). In the PE group, mice were injected (i.p.) with PE at a dose of 10 mg/kg. In the PZ group, mice were administered intragastrically (i.g.) with PZ twice a day, at 4 mg/kg each time, with a 12 hour interval between the two doses. In the AD+PZ or NE+PZ group, AD or NE was injected (i.p.)

at a dose of 250 μg/kg or 500 μg/kg, respectively, 2 h after the PZ was administered (i.g.) (twice a day, at 4 mg/kg each time, with a 12 hour interval between the two doses). In the Pro group, mice were administered (i.g.) Pro twice a day, at 5 mg/kg each time, with a 12 hour interval between the two doses. In the Pro+AD or Pro+NE group, AD or NE was injected (i.p.) at a dose of 250 μg/kg or 500 μg/kg, respectively, 2 h after the Pro was administered (i.g.) (twice a day, at 5 mg/kg each time, with a 12 hour interval between the two doses). In the PZ+Pro group, mice were administered (i.g.) with the mixture of PZ (4 mg/kg each time) and Pro (5 mg/kg each time) twice a day, with a 12 hour interval between the two doses. In the PZ+Pro+AD or PZ+Pro+NE group, AD or NE was injected (i.p.) at a dose of 250 μg/kg or 500 μg/kg, respectively, 2 h after the mixture of PZ and Pro was administered (i.g.) (twice a day, at 4 mg/kg and 5mg/kg each time, respectively, with a 12 hour interval between the two doses).

In order to study the long-term effects of AD or NE on hepcidin expression, mice were injected (i.p.) with AD or NE for 7 d, twice a day, at 250 μg/kg or 500 μg/kg each time, respectively, with a 12 hour interval between the two doses. In order to detect whether the α1- AR antagonist (PZ) can also ameliorate the 7 day AD/ NE -induced hepcidin upregulation, AD or NE was injected (i.p.) for 7 d at a dose of 250 μg/kg or 500 μg/kg, respectively, at 2 h after the PZ was administered (i.g.) (twice a day, at 4 mg/kg each time, with a 12 hour interval between the two doses). Blood pressure was measured at 2 hours after the final treatment with a Non-Invasive Blood Pressure device (CODA, Kent Scientific Corporation, USA), according to the manufacturer's instructions.

Sampling of Blood and Tissue

At 6 hours after the final treatment, the mice from each group were anesthetized with pentobarbital sodium (40 mg/kg body weight, i.p.). Blood samples were collected by orbital puncture. Serum was obtained by centrifugation of whole blood at 3,000 rpm for 15 min at 4°C. Mice were perfused with ice-cold phosphate-buffered saline (PBS) through the left ventricle. The liver and spleen were excised, frozen in liquid nitrogen, and stored at -70°C for further analysis.

RT-PCR analysis

Total RNA was isolated from liver using TRIzol® Reagent (Invitrogen, San Diego, CA, USA), according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed in a 20 μl reaction system using Reverse Transcription System (Promega, Madison, WI, USA). After denaturation of RNA at 70°C for 10 min in a thermal cycler, the reaction was performed for 1 h at 42°C. The cDNA was then subjected to PCR amplification using primers specific for hepcidin and β-actin. The primer sets were as follows: Hepcidin 1 (forward, 5'- CCTATCTCCATCAACAGATG -3', reverse, 5'- AACAGATACCACACTGGGAA -3') and β-actin (forward, 5'- AGCCATGTACGTAGCCATCC -3', reverse, 5'- TTTGATGTCACGCACGATTT -3') [Guo et al., 2013]. Gene amplification was performed in a 20 μl PCR amplification reaction (TaKaRa Taq DNA Polymerase Kit, Takara Biotechnology Co., Dalian, China) with initial denaturation at 95°C for 4 min, followed by 22 cycles (β-actin) or 24 cycles (hepcidin) at 94°C for 45s, 60°C for 45s, 72°C for 45s, and a single final extension at 72°C for 10 min (Eppendorf Mastercycler gradient PCR System, Hamburg, Germany). The PCR products were analyzed on a 1% agarose gel using Gel-Pro Analyzer® Analysis software (Media Cybernetics, Bethesda, MD, USA). Gene expression values were normalized to β-actin mRNA values.

Serum hepcidin analyses

Mouse serum was prepared from whole blood. Serum hepcidin levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Elabscience Biotechnology Co., Ltd, Wuhan, China) according to the manufacturer's instructions.

Western blot analysis

Western blotting was used to detect the expression of proteins as described previously [Kong et al., 2008]. Briefly, tissue was homogenised supersonically in TBS buffer. After clarification of the lysates by centrifugation, protein concentrations in the supernatant were assayed by the Bradford method. Aliquots of the extract containing 30 μg protein were resolved by reducing SDS-PAGE (10%), and electroblotted onto nitrocellulose (NC) membranes at 4°C. The blots were blocked using 5% nonfat milk in a solution of Tris-buffered saline with Tween-20 (TBS-T) for 2 h at room temperature. The blots were then separately incubated with rabbit anti-STAT3 antibody, rabbit anti-p-STAT3 antibody (cat. 4904, cat. 9145, Cell Signaling Technology Inc., Beverly, MA, USA), rabbit anti-Ft-L antibody (cat. ab109373, Abcam, Cambridge, UK), or rabbit anti- FPN1 antibody (cat. MTP11-S, Alpha Diagnostic International, San Antonio, TX, USA) overnight at 4°C. After being washed with TBS-T, the blots were incubated in goat anti-rabbit secondary antibody-conjugated horseradish peroxide (cat. RPN4301; Amersham, Buckinghamshire, UK) for 1.5 h at room temperature. Peroxidase activity was detected with the SuperSignal WestPico chemiluminescent substrate (Pierce Biotechnology, Waltham, MA, USA) and visualized and digitized with ImageQuant (Fujifilm LAS-4000, Tokyo, Japan). Optical densities of bands were analyzed using Multi Gauge V3.1 (Fuji-

film, Tokyo, Japan). To ensure even loading of the samples, the same membrane was probed with anti-β-actin antibody (cat. A5060, Sigma, St. Louis, MO, USA).

Measurement of tissue non-heme iron levels

The non-heme iron concentrations of tissues were measured as described previously [Kong et al., 2014; Kong et al., 2015]. An acid mixture was prepared with 0.6 M trichloroacetic acid in hydrochloric acid and added to tissue homogenates. After mixing and closing the tube, the content was heated in a water bath at 65°C for 20 h. After centrifugation, a clear acid extract was obtained and then transferred into another set of iron-free tubes. The color was developed by the addition of 1 ml chromogen solution containing 0.2% thioglycolic acid, and the absorbance of the solution was measured using a spectrophotometer at a wavelength of 540 nm against a reagent blank. The chromogen solution was freshly prepared by dissolving 0.5 mM bathophenanthroline in sodium acetate. The absorbance of the reagent blank at 540 nm against iron-free water was less than 0.010.

Statistical analysis

Statistical analyses were performed using the SPSS12.0 statistics package. Data are presented as the mean ± SEM. The significant differences between groups was determined by one-way ANOVA with Tukey's HSD post-hoc test. A probability value of $p < 0.05$ was taken to be statistically significant.

Results

Effect of AD or NE on hepcidin mRNA levels in the mouse liver

To investigate whether AD or NE can regulate hepcidin expression in the liver of mice, AD or NE was injected (i.p.) at a dose of 250 μg/kg or 500 μg/kg, respectively. Normal saline was used as a negative control. The expression of hepatic hepcidin mRNA in mice was tested by RT-PCR analysis. As shown in Figure 1, the hepcidin mRNA levels were significantly increased in AD- or NE-treated mice ($p < 0.01$ or $p < 0.05$ respectively) compared with that in the negative controls at 6 h post injection. In addition, the increase of hepcidin mRNA levels induced by AD was more efficient than that induced by NE.

Effect of α1-AR agonists and antagonists on hepcidin mRNA and p-STAT3 protein levels in the mouse liver

In order to determine the role of α1-AR in the regulation of hepatic hepcidin expression induced by AD or NE, mice were treated with PE (α1-AR agonist) or PZ (α1-AR antagonist). The expression of hepatic hepcidin mRNA in mice was examined by RT-PCR analysis. The levels of p-STAT3 protein was assessed by western blot analysis.

As shown in Figure 2, compared with control mice, treatment of mice with PE increased hepcidin expression in the liver (Figure 2A, $p < 0.01$) and induced a phosphorylation of STAT3 (Figure 2B, $p < 0.01$). On the contrary, the AD- or NE-induced hepcidin expression (Figure 2A, $p < 0.01$) and STAT3 phosphorylation (Figure 2B, $p < 0.01$) was blocked by the selective α1-AR antagonist, PZ ($p < 0.05$).

Effect of β-AR agonists on hepcidin mRNA and p-STAT3 protein levels in the mouse liver

In order to determine the role of β-AR in the regulation of hepatic hepcidin expression induced by AD or NE, mice were treated with Pro (β-AR antagonist). The expression of hepatic hepcidin mRNA in mice was determined by the RT-PCR analysis. The levels of p-STAT3 protein were assessed by western blot analysis.

As shown in Figure 3A, compared with control mice, the expression of hepatic hepcidin significantly increased 6 h after AD or NE treatment ($p < 0.05$). However, no significant difference was observed in the hepatic hepcidin mRNA levels between the AD or NE groups and Pro+AD or Pro+NE groups ($p > 0.05$). In contrast, the elevated hepatic hepcidin expression induced by AD or NE treatment was markedly suppressed by PZ plus Pro pretreatment ($p < 0.05$).

As shown in Figure 3B, in parallel with changes in hepatic hepcidin mRNA expression, the levels of p-STAT3 protein increased at 6 h after AD or NE treatment. There was also no significant alteration in p-STAT3 protein levels in the liver of mice between the AD or NE groups and Pro+AD or Pro+NE groups. However, pretreatment with a mixture of PZ and Pro significantly abolished the STAT3 phosphorylation induced by AD or NE ($p < 0.05$).

Effect of AD or NE on the blood pressure and the expression of hepatic hepcidin in mice after seven consecutive days of treatment

Mice were injected (i.p.) with AD or NE for 7 d at 250 or 500 μg/kg, respectively. PZ was also administered (i.g.) for 7 d at 2 h before AD/NE treatment. As shown in Figure 4, the systolic or diastolic blood pressures were elevated by AD/NE treatment ($p < 0.05$ or $p < 0.01$), and the AD/NE -increased blood pressure was reduced by PZ administration ($p < 0.05$ or $p < 0.01$).

The expression of hepatic hepcidin mRNA in mice was evaluated by RT-PCR analysis. As shown in Figure 5A, the hepcidin mRNA levels were significantly higher in AD- or NE-treated mice than in control mice after continuous treatment for 7 days ($p < 0.001$). This increase was ameliorated by PZ administration ($p < 0.001$). Consistent with the hepatic hepcidin mRNA levels, the serum hepcidin protein concentration was significantly higher in AD/NE-treated mice than in control mice (Figure 5B, $p < 0.05$), and the AD/NE -induced serum hepcidin increase was also abolished by PZ administration (Figure 5B, $p < 0.05$ or $p < 0.01$).

Effect of AD and NE on splenic FPN1, Ft-L protein levels, and splenic non-heme iron concentrations in mice after seven consecutive days of treatment

The levels of FPN1 protein in the spleen markedly decreased after administration of AD or NE for 7 d (Figure 6A, $p < 0.05$ or $p < 0.01$). The expression of Ft-L in the spleen of mice significantly increased after AD or NE treatment, compared with control mice (Figure 6B, $p < 0.05$). Non-heme iron levels in the spleen of mice were also significantly increased after AD or NE treatment for 7 d (Figure 7, $p < 0.05$ or $p < 0.01$). AD/NE treatment induced changes in splenic FPN1 protein levels (Figure 6A, $p < 0.05$ or $p < 0.01$), Ft-L protein levels (Figure 6B, $p < 0.05$), and splenic non-heme iron concentrations (Figure 7, $p < 0.05$ or $p < 0.01$), all of which were sensitive to PZ administration.

Discussion

Hepcidin is the principal iron-regulatory hormone responsible for the maintenance of organismal iron homeostasis. Despite the connections between hypertension and iron metabolism, the mechanisms of hepcidin regulation in hypertension are poorly understood. Our results show that hepcidin

expression was enhanced 6 h after AD or NE injection. In order to study the long-term effects of AD or NE on hepcidin expression, mice were injected (i.p.) daily with AD or NE for 7 days. The increase in hepatic hepcidin mRNA levels was also observed in mice in both the AD and NE groups. Ours is the first report to show that AD or NE can increase hepcidin expression in the liver.

The central and peripheral actions of AD and NE are mediated by adrenergic receptors (ARs). These receptors belong to the family of seven transmembrane domain receptors, coupled to G proteins. The ARs are classified as α1, α2, and β1, β2, β3 based on their pharmacology, structure and signaling mechanisms [Limbird, 2011]. Adult hepatocytes express both α1-AR and three β-AR subtypes (β1, β2, β3), although the abundance of β1-AR and β3-AR mRNA in liver is lower than β2-AR [Han et al., 2008; Jin, 2010]. We investigated which AR subtype was more prominently involved in the regulation of hepatic hepcidin expression induced by AD or NE. Our results show that a specific α1-AR agonist (PE) enhances the hepatic hepcidin expression in mice, while a α1-AR-specific blocker (PZ) was effective at preventing AD- or NE-induced expression of hepcidin in the mouse liver. In addition, we observed an inability of β-AR blockers (Pro) to prevent AD- or NE-induced hepatic hepcidin expression. These findings suggest that α1-AR and not β-AR are important in the regulation of hepcidin expression.

The underlying mechanisms by which α1-ARs regulate hepatic hepcidin expression remain to be further defined. Classically, α1-ARs are coupled to Gq/11protein, phospholipase C activation and increased intracellular calcium [Minneman, 1988]. Additionally, α1-AR has been linked to other signaling pathways, including activation of phospholipase A2 [Xing and Insel, 1996], phospholipase D [Ruan et al., 1998], MAP kinases [Williams et al., 1998], and activation of NADPH oxidase [Xiao et al., 2002]. In 2008, a novel α1-AR-mediated STAT3 activation that involves Gq/11, Src and EGFR in

hepatocytes was demonstrated [Han et al., 2008]. Phosphorylated STAT3 translocates to the nucleus where it binds to tissue-specific transcription factors and cofactors to mediate the transcriptional activation of target genes. The hepcidin gene promoter contains a STAT3-binding site. Verga Falzacappa et al. [Verga Falzacappa et al., 2007] previously identified STAT3 as a critical transcription factor for the basal expression of hepcidin mRNA under resting conditions, as well as for the increased expression in inflammation, via the STAT binding motif located at position –64/–72 of the hepcidin promoter. Many studies have demonstrated that STAT3 is the key transcription factor responsible for hepcidin gene expression in the liver [Pietrangelo et al., 2007; Zhang et al., 2016]. In contrast, a transgenic mouse model with a hepatocyte-specific deletion of STAT3 showed no increase in hepatic hepcidin levels after subcutaneous turpentine oil injection[Sakamori et al., 2010]. We investigated whether STAT3 is involved in the regulation of hepatic hepcidin expression induced by AD or NE. Our findings show that activation of α1-AR by the selective agonist, PE, induced STAT3 phosphorylation, and elevated hepcidin expression in the mouse liver. This effect was completely blocked by the selective α1-AR antagonist, PZ. The AD- or NE-induced STAT3 phosphorylation and the increase in hepcidin expression was not blocked by the β-AR antagonist (Pro). These findings further substantiate our conclusion that α1-AR, and not β-AR, mediates STAT3 activity in hepatic hepcidin gene upregulation induced by AD or NE.

Wrighting et al. showed that the inflammatory cytokine, interleukin-6 (IL-6), directly regulates hepcidin through induction and subsequent promoter binding of STAT3 [Wrighting and Andrews, 2006]. Furthermore, AD administration induced a dose-dependent increase in plasma IL-6 concentrations in rats [DeRijk et al., 1994]. A study by Liao et al. also demonstrated that AD significantly increased IL-6 bioactivity from isolated perfused rat liver effluent [Liao et al., 1995]. NE can directly

increase IL-6 mRNA expression in hepatocytes [Jung et al., 2000] and macrophages [Li et al., 2015]. Therefore, one may conjecture that AD/NE activates STAT3 signaling through IL-6 production. The induction of IL-6 by AD can be blocked by the β-adrenergic receptor antagonist (Pro) [DeRijk et al., 1994; Liao et al., 1995]. Additionally, the stimulatory effect of NE on IL-6 mRNA expression in hepatocytes has been shown to be blocked by the use of α- or β-adrenergic receptor antagonists [Jung et al., 2000], while a β-adrenergic receptor antagonist (Pro) can block NE-induced IL-6 mRNA expression and protein levels in macrophages [Li et al., 2015]. In our study, the AD- or NE-induced STAT3 phosphorylation and the increase in hepcidin expression was not abrogated by the β-adrenergic receptor antagonist (Pro). Therefore, we conclude that the increased hepatic hepcidin expression induced by AD/NE is more likely to be a direct effect of AD/NE than through IL-6 production.

Previous studies have shown that body iron accumulation is associated with hypertension [Piperno et al., 2002; Sullivan, 2007]. Hepcidin exerts its effects by decreasing cellular iron release through the down-regulation of FPN1 [Nemeth et al., 2004]. Consequently, excess intracellular iron is safely sequestered in ferritin (Ft), a cytosolic protein comprising 24 subunits consisting of H- (heavy) and L- (light) chain Ft subunits. Ft is regulated post-transcriptionally by cellular "free" iron levels through the iron-responsive element (IRE)-iron-regulatory protein (IRP) system [Munro, 1993]. Thus, Ft can be used as a proxy for intracellular iron storage.

We next explored whether the increased hepcidin induced by AD/NE affects FPN1 and Ft-L protein levels. In our preliminary experiments, we observed no significant changes in FPN1 protein levels in the mouse spleen 6 h after AD or NE administration (data not shown). We hypothesized that this time interval is too short and therefore insufficient to elicit a detectable change in

FPN1 protein expression. Therefore, we examined the long-term effects of AD or NE on hepcidin and FPN1 expression. Mice treated with AD or NE for 7 d exhibited a decrease in FPN1 expression and dramatic increases in Ft-L protein and non-heme iron levels. Our results are consistent with previous reports. Rivera et al. demonstrated that radiolabeled hepcidin accumulates in FPN1-rich organs, such as liver, spleen, and the proximal duodenum [Rivera et al., 2005], while Nemeth et al. revealed that hepcidin can decrease the activity of FPN1 by directly binding to it and causing its internalization and degradation, leading to a decreased export of cellular iron [Nemeth et al., 2004].

The pathogenesis of hypertension is complex. The sympathetic nervous system, renin-angiotensin-aldosteron system, and the endothelin system all play important roles in the pathophysiology of hypertension. Similarly, the regulation of hepcidin expression is dependent on various factors in addition to the STAT3 pathway [Rishi et al., 2015]. The bone morphogenetic protein (BMP) and small mothers against decapentaplegic (SMAD) pathway has a central role in the regulation of hepcidin in response to body iron levels [Parrow and Fleming, 2014]. Growth differentiation factor 15 (GDF15) [Tanno et al., 2007], twisted gastrulation (TWSG1) [Tanno et al., 2009], and erythroferrone (ERFE) [Kautz et al., 2014] have all been proposed as stress-erythropoeisis specific erythroid regulators of hepcidin. Additionally, there are many potential mediators and pathways that can affect the hypoxic repression of hepcidin, including the transcription factor hypoxia-inducible factor (HIF) [Peyssonnaux et al., 2007], erythopoietin (EPO) [Mastrogiannaki et al., 2012], or platelet-derived growth factor (PDGF) [Sonnweber et al., 2014].

Considering the above, it is not surprising that the mechanisms of hepcidin regulation during hypertension are complex. For example, Tajima et al. showed that mice treated with angiotensin II (ANG II) exhibit decreased levels of BMP6 and C/EBPa, resulting in reduced hepatic hepcidin ex-

pression [Tajima et al., 2015]. Therefore, ANG II may be a negative regulator of hepatic hepcidin through the BMP6/C/EBPa signaling pathway, mediated via the ANG II receptor (AT1). Further study is necessary to fully understand hepcidin regulation in hypertension.

In summary, our results demonstrate a novel AD/α1-AR/STAT3 or NE/α1-AR/STAT3 mediated hepcidin expression activation pathway in the mouse liver. FPN1 expression in the spleen was inhibited by the increased hepcidin expression induced by AD or NE treatment. The decreased levels of FPN1 led to a decrease in iron export and thus increased iron storage in the spleen. Based on our results, we infer that high levels of hepcidin are associated with the elevated plasma catecholamine values in hypertension. Increased hepatic hepcidin can decrease the levels of FPN1 protein, which is expected to increase body iron accumulation. Furthermore, abnormalities in iron homeostasis may predispose one to hypertension [Maatta et al., 2015; Nikkari et al., 2017]. This could explain why elevated body iron stores are associated with hypertension progression. The findings of our current study will be useful in establishing novel therapeutic targets and agents for the treatment of hypertension.

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Figure legends

Fig. 1. Effect of AD or NE on hepcidin mRNA levels in the mouse liver. In the AD or NE group, mice were injected (i.p.) at a dose of 250 μg/kg or 500 μg/kg, respectively. Normal saline (NS) was used as a negative control. Six hours after the final dose, the mice in each group were anesthetized and perfused. The livers were excised. The expression of hepatic hepcidin mRNA was assayed by RT-PCR analysis. Gene expression values were normalized to β-actin mRNA. The results are expressed as the mean ± SEM, n=6. *P < 0.05 versus NS group, **P < 0.01 versus NS group.

Fig. 2. Effect of α1-AR agonists and antagonists on hepcidin mRNA and p-STAT3 protein levels in the mouse liver. In the AD or NE group, mice were injected (i.p.) at a dose of 250 μg/kg or 500 μg/kg, respectively. Normal saline was used as a negative control. In the PE group, the mice were injected (i.p.) with PE at a dose of 10 mg/kg. In the PZ group, mice were administered intragastrically (i.g.) with PZ twice a day, at 4 mg/kg each time, with a 12 hour interval between the two doses. In the AD+PZ or NE+PZ group, AD or NE was injected (i.p.) at a dose of 250 μg/kg or 500 μg/kg, respectively, 2 h after the PZ was administered (i.g.) (twice a day, at 4 mg/kg each time, with a 12 hour interval between the two doses). Six hours after the last dose, the mice in each group were anesthetized and perfused. The livers were excised. (A) The expression of hepatic hepcidin mRNA was investigated by RT- PCR analysis. Gene expression values were normalized to β-actin mRNA. (B) The levels of p-STAT3 protein were assessed by western blot analysis. The p-STAT3 protein concentrations in each specimen were normalized for total STAT3 in that specimen. The results are expressed as the mean ± SEM, n=6. **P < 0.01 versus NS group, $^{\#}$P < 0.05 versus AD group, $^{\&}$P < 0.05 versus NE

group.

Fig. 3. Effect of β-AR agonists on hepcidin mRNA expression and p-STAT3 protein levels in the mouse liver. In the AD or NE group, mice were injected (i.p.) at a dose of 250 μg/kg or 500 μg/kg, respectively. Normal saline was used as a negative control. In the Pro group, the mice were administered (i.g.) Pro twice a day, at 5 mg/kg each time, with a 12 hour interval between the two doses. In the Pro+AD or Pro+NE group, AD or NE was injected (i.p.) at a dose of 250 μg/kg or 500 μg/kg, respectively, 2 h after the Pro was administered (i.g.) (twice a day, at 5 mg/kg each time, with a 12 hour interval between the two doses). In the PZ+Pro group, the mice were administered (i.g.) a mixture of PZ (at 4 mg/kg each time) and Pro (at 5 mg/kg each time) twice a day, with a 12 hour interval between the two doses. In the PZ+Pro+AD or PZ+Pro+NE group, AD or NE was injected (i.p.) at a dose of 250 μg/kg or 500 μg/kg, respectively, 2 h after the mixture of PZ and Pro was administered (i.g.; twice a day, at 4 mg/kg or 5 mg/kg each time, respectively, with a 12 hour interval between the two doses). At 6 hours after the final dose, the mice in each group were anesthetized and perfused. The livers were excised. (A) The expression of hepatic hepcidin mRNA was evaluated by RT- PCR analysis. Gene expression values were normalized to β-actin mRNA. (B) The levels of p-STAT3 protein were estimated by western blot analysis. The p-STAT3 protein concentrations in each specimen were normalized to total STAT3 in that specimen. The results are expressed as the mean ± SEM, n=6. *$P < 0.05$ versus NS group, **$P < 0.01$ versus NS group, $^{\#}P < 0.05$ versus Pro+AD group, $^{\&}P < 0.05$ versus Pro+NE group.

Fig. 4. Effect of 7 consecutive days of injection of AD or NE on blood pressure in mice. In the AD or NE group, mice were injected (i.p.) with AD or NE for 7 d, twice a day, at 250 μg/kg or 500 μg/kg each time, respectively, with a 12 hour interval between the two doses. Normal saline was used as a negative control. In the AD+PZ or NE+PZ group, AD or NE was injected (i.p.) for 7 d at a dose of 250 μg/kg or 500 μg/kg, respectively, 2 h after the PZ was administered (i.g.; twice a day, at 4 mg/kg each time, with a 12 hour interval between the two doses). Blood pressure was measured 2 hours after the final dose with a Non-Invasive Blood Pressure device. The results are expressed as the mean ± SEM, n=6. *$P < 0.05$ versus NS group, **$P < 0.01$ versus NS group, $^{\#}P < 0.05$ versus AD group, $^{\#\#}P < 0.01$ versus AD group, $^{\&\&}P < 0.01$ versus NE group.

Fig. 5. Effect of 7 consecutive days of treatment with AD or NE on hepatic hepcidin mRNA expression and serum hepcidin concentration in mice. In the AD or NE group, mice were injected (i.p.) with AD or NE for 7 d, twice a day, at 250 μg/kg or 500 μg/kg each time, respectively, with a 12 hour interval between the two doses. Normal saline was used as a negative control. In the AD+PZ or NE+PZ group, AD or NE was injected (i.p.) for 7 d at a dose of 250 μg/kg or 500 μg/kg, respectively, 2 h after the PZ was administered (i.g.; twice a day, at 4 mg/kg each time, with a 12 hour interval between the two doses). Six hours after the final dose, the mice in each group were anesthetized. Blood samples were collected by retro-orbital bleeding. Serum was obtained by centrifugation of the whole blood. The livers were excised after perfusion. (A) The expression of hepatic hepcidin mRNA was measured by RT-PCR analysis. Gene expression values were normalized to GAPDH mRNA. (B) The concentration of serum hepcidin was analyzed using ELISA kits. The results are expressed as the mean ± SEM, n=6. *$P < 0.05$ versus NS group, ***$P < 0.001$ versus NS group, $^{\#}P <$

0.05 versus AD group, ###P < 0.001 versus AD group, &&P < 0.01 versus NE group, &&&P < 0.001 versus NE group.

Fig. 6. Effect of 7 consecutive days of treatment with AD or NE on FPN1 and Ft-L protein levels in the mouse spleen. In the AD or NE group, mice were injected (i.p.) with AD or NE for 7 d, twice a day, at 250 μg/kg or 500 μg/kg each time, respectively, with a 12 hour interval between the two doses. Normal saline was used as a negative control. In the AD+PZ or NE+PZ group, AD or NE was injected (i.p.) for 7 d at a dose of 250 μg/kg or 500 μg/kg, respectively, 2 h after the PZ was administered (i.g.; twice a day, at 4 mg/kg each time, with a 12 hour interval between the two doses). Six hours after the final dose, the mice in each group were anesthetized and perfused. The spleens were excised. The levels of splenic FPN1 and Ft-L protein were investigated by western blot analysis. FPN1 and Ft-L protein concentrations in each specimen were normalized for GAPDH in that specimen. The results are expressed as the mean ± SEM, n=6. *P < 0.05 versus NS group, **P < 0.01 versus NS group, &P < 0.05 versus NE group, #P < 0.05 versus AD group , ##P < 0.01 versus AD group.

Fig. 7. Effect of 7 consecutive days of treatment with AD or NE on tissue non-heme iron concentrations in the mouse spleen. In the AD or NE group, mice were injected (i.p.) with AD or NE for 7 d, twice a day, at 250 μg/kg or 500 μg/kg each time, respectively, with a 12 hour interval between the two doses. Normal saline was used as a negative control. In the AD+PZ or NE+PZ group, AD or NE was injected (i.p.) for 7 d at a dose of 250 μg/kg or 500 μg/kg, respectively, 2 h after the PZ was administered (i.g.; twice a day, at 4 mg/kg each time, with a 12 hour interval between the

two doses). Six hours after the last dose, the mice in each group were anesthetized and perfused. The spleens were excised. The non-heme iron concentration in the spleen was determined as described in the Materials and Methods section. The results are expressed as the mean ± SEM, n=6. *P < 0.05 versus NS group, **P < 0.01 versus NS group, $^{\#}$P < 0.05 versus AD group, $^{\&\&}$P < 0.01 versus NE group.

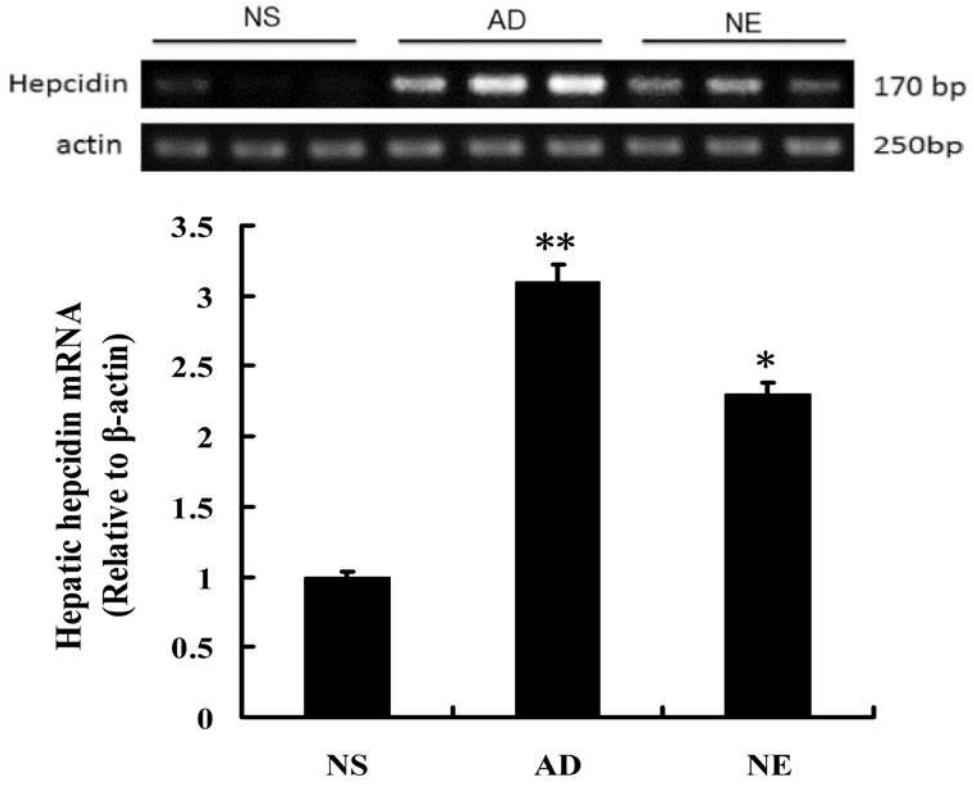

Figure 1

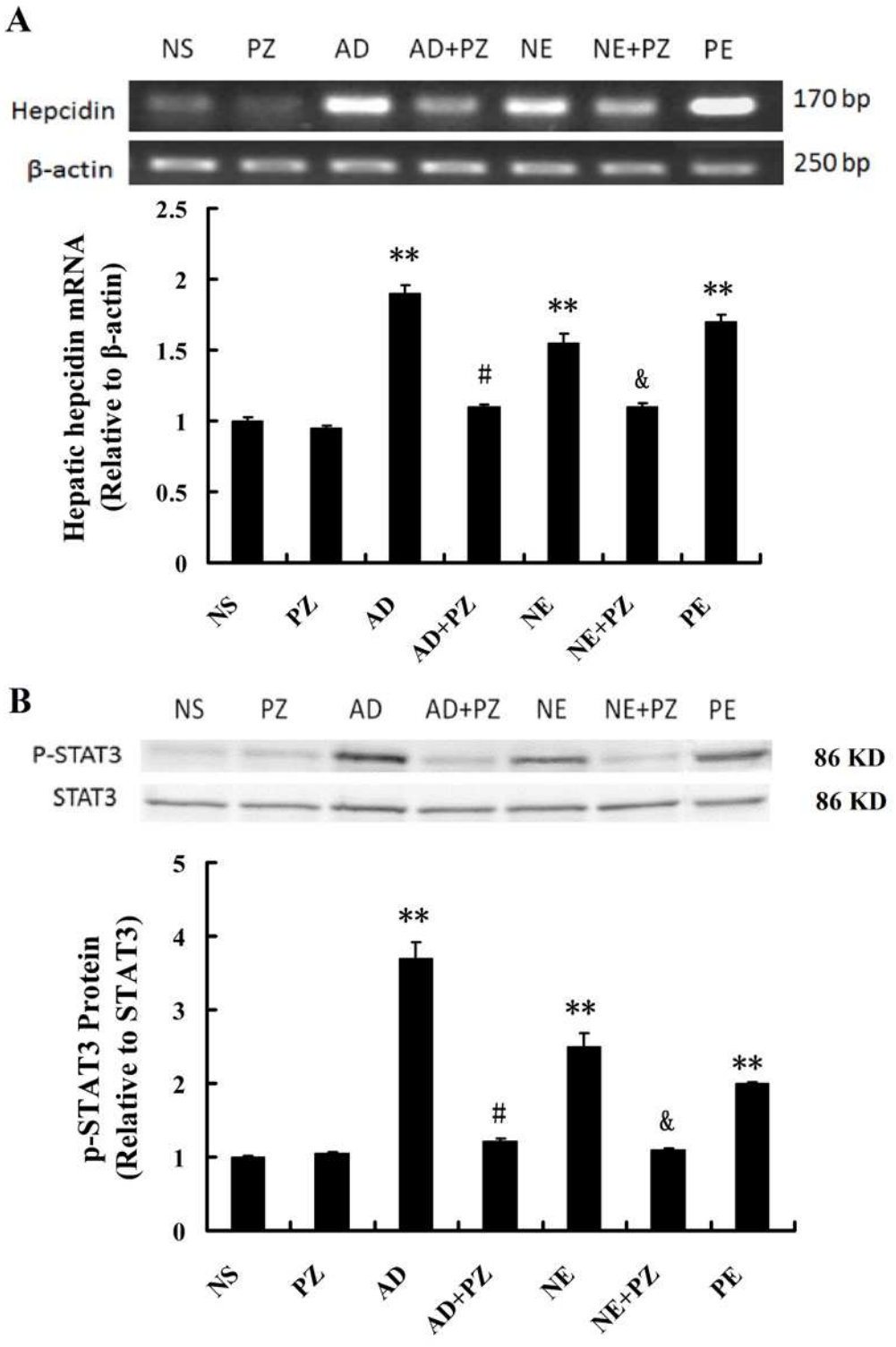

Figure 2

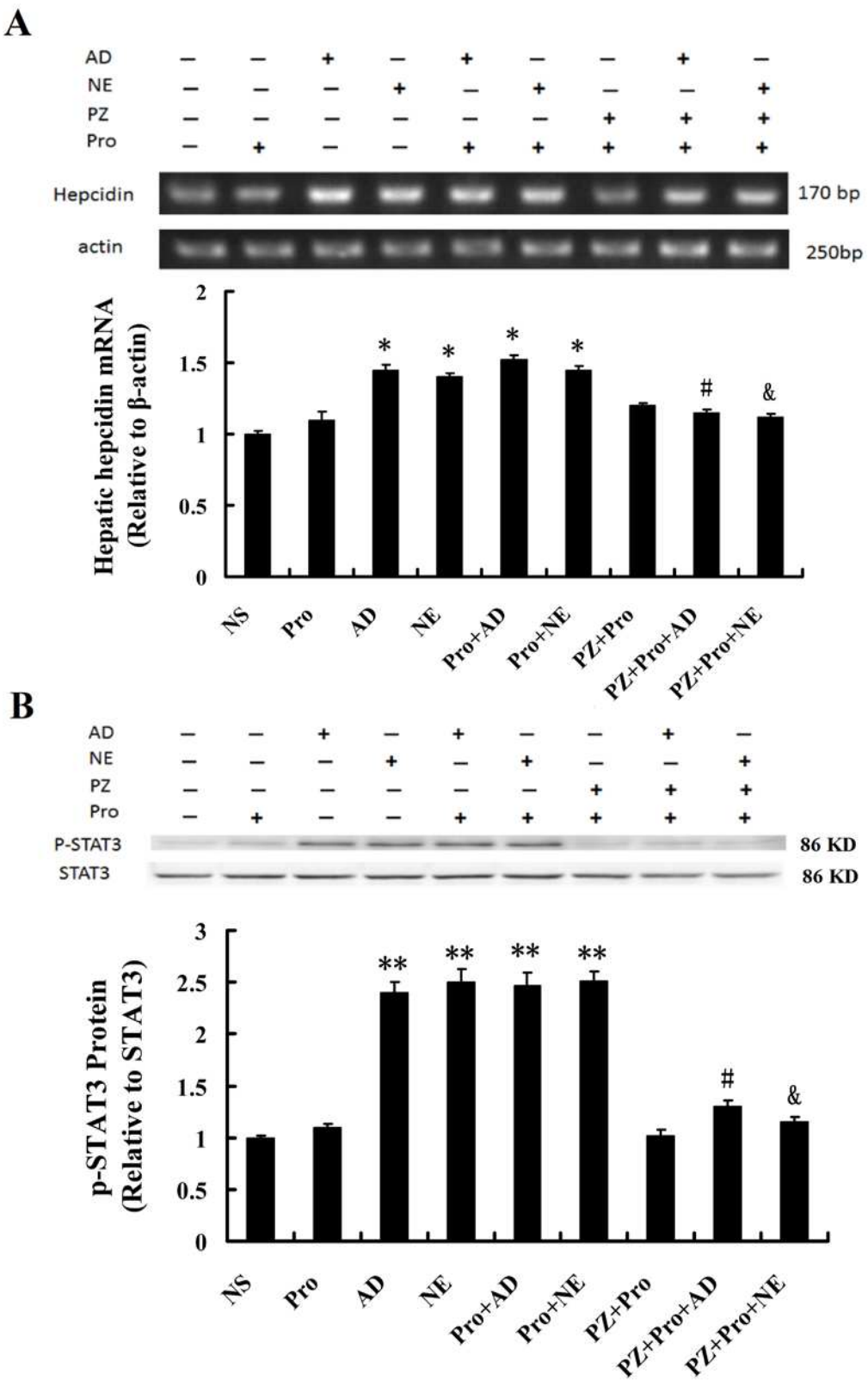

Figure 3

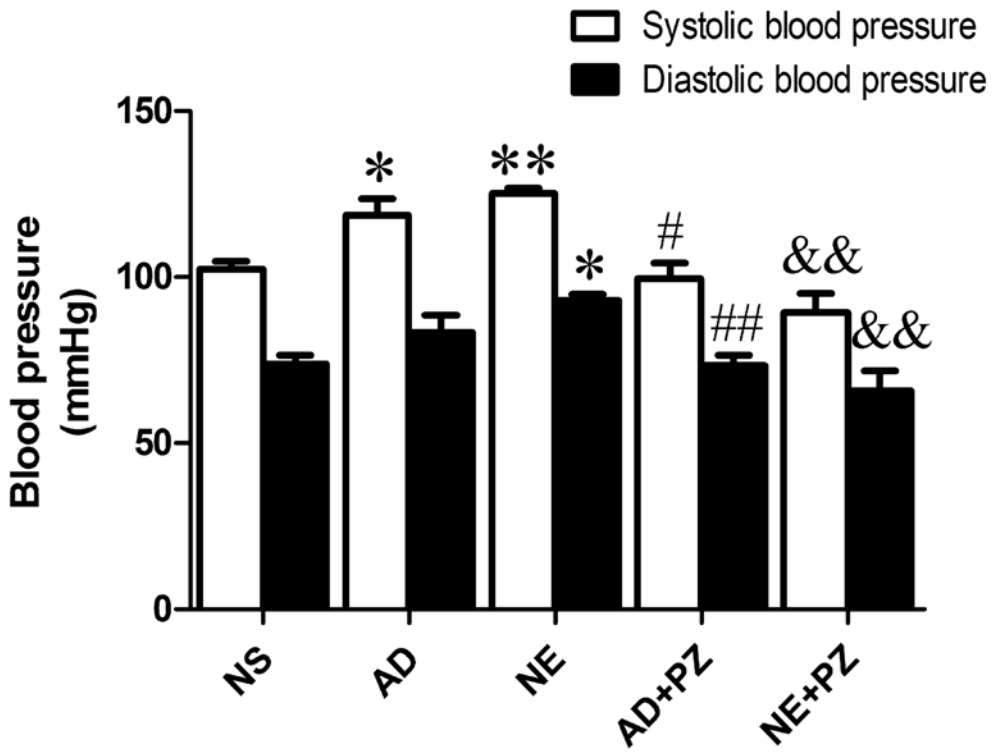

Figure 4

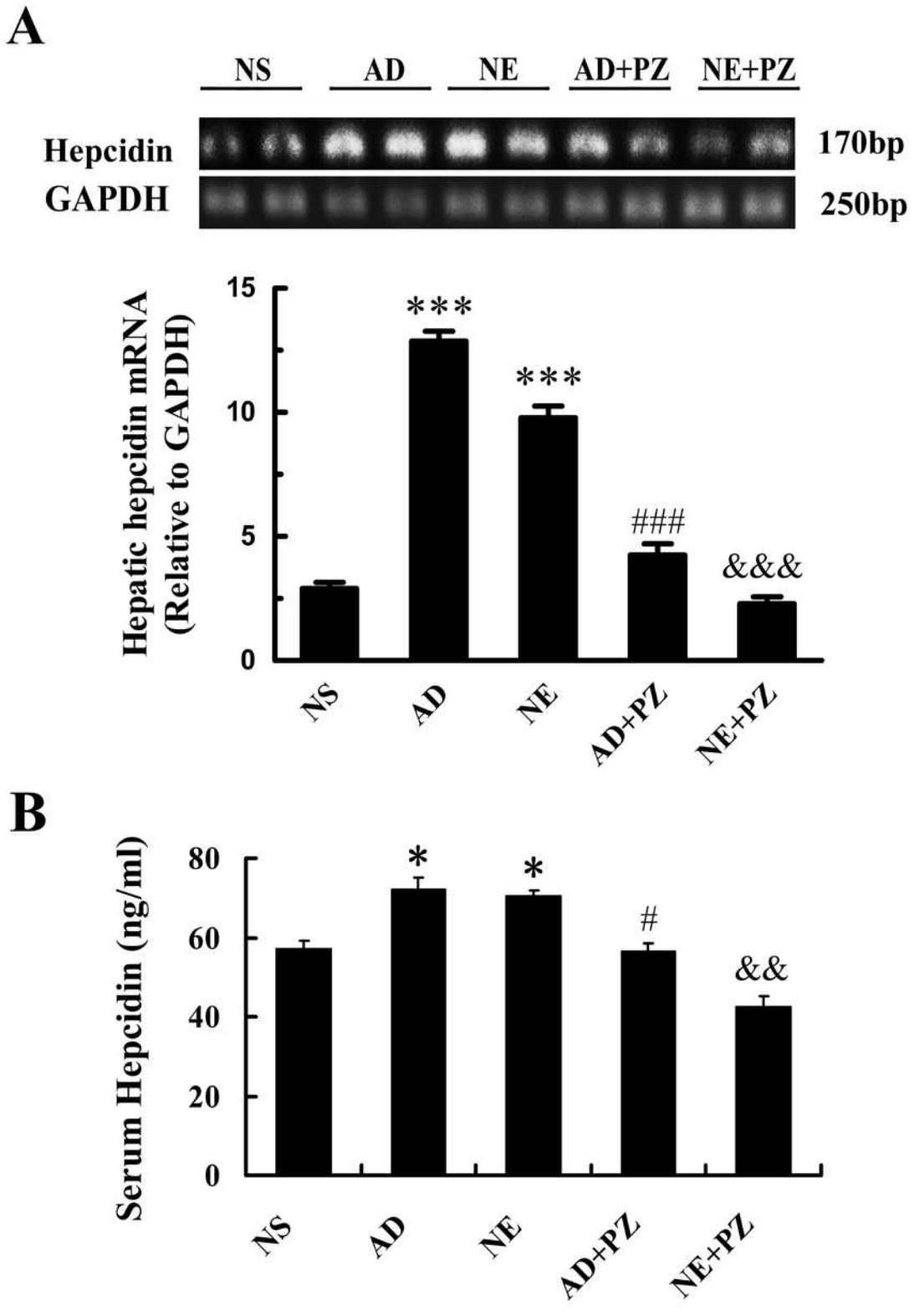

Figure 5

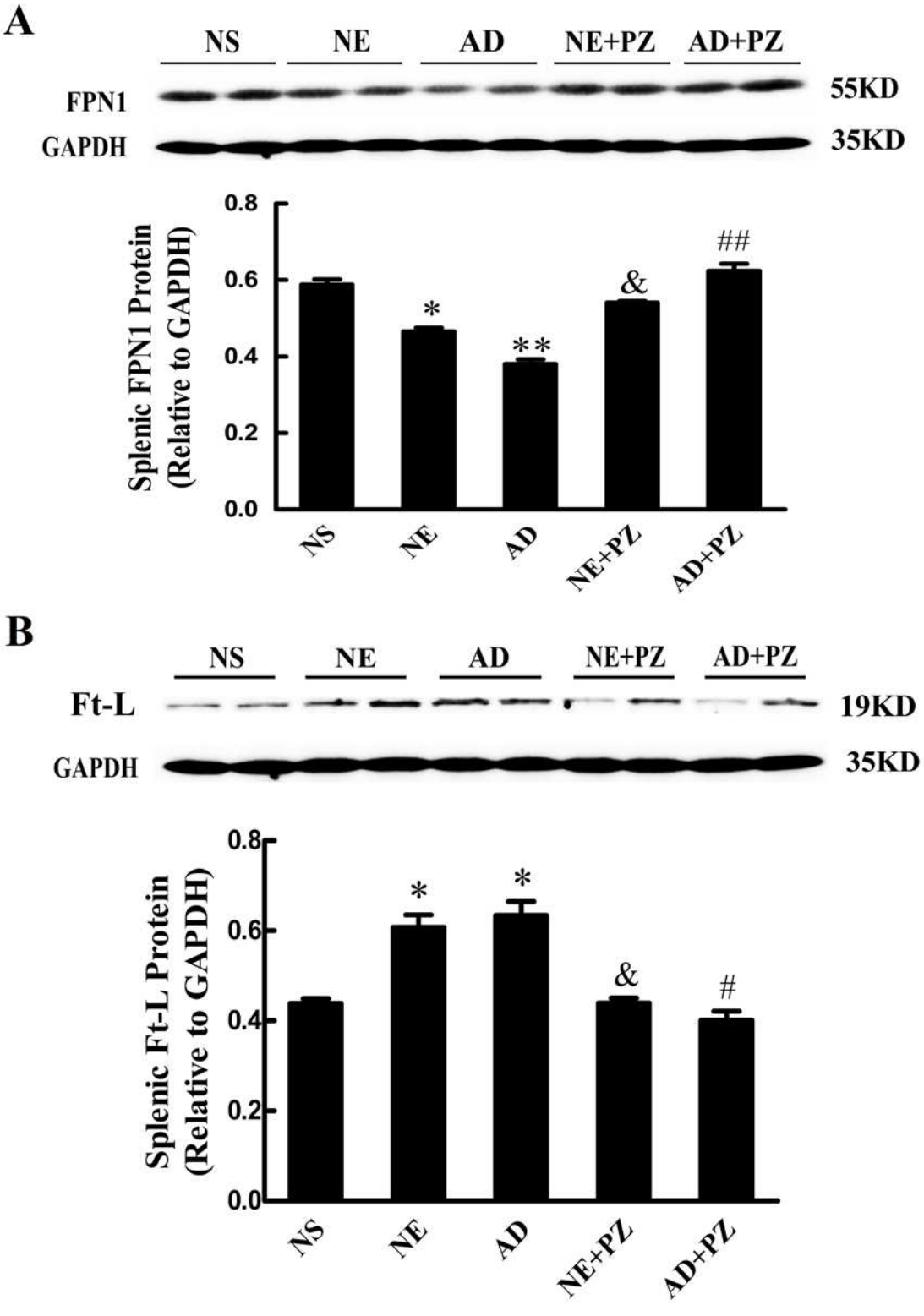

Figure 6

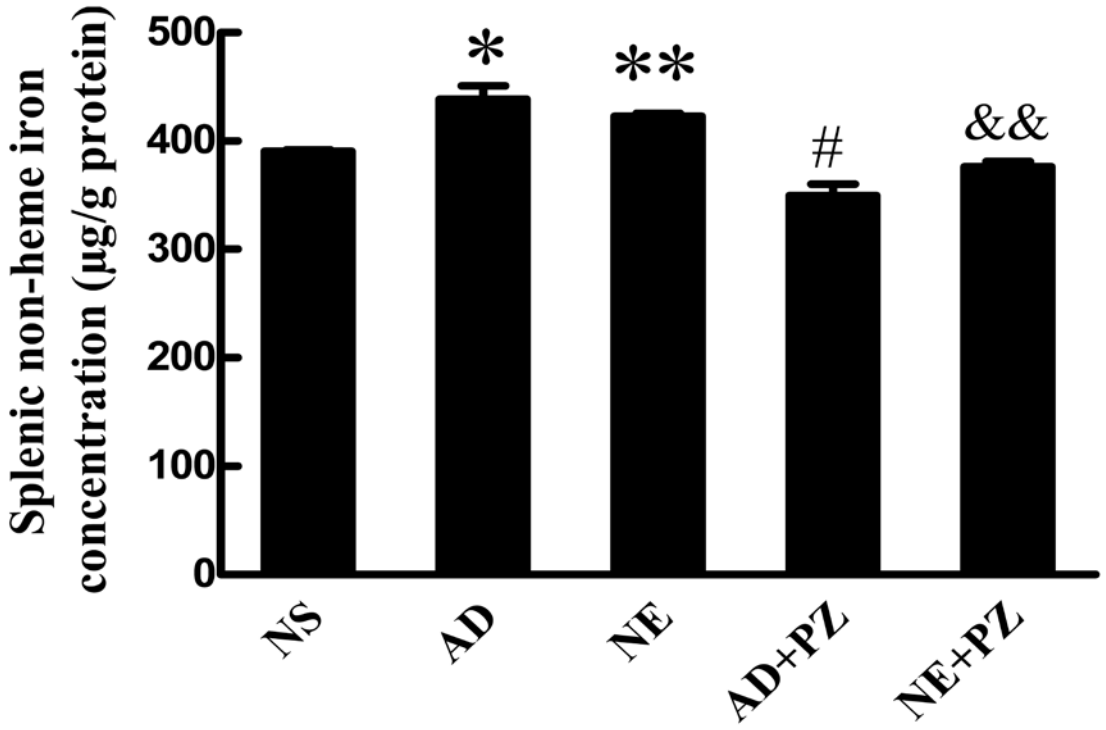

Figure 7