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Angiotensin II alters the expression of duodenal iron transporters, hepatic hepcidin, and body iron distribution in mice

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Abstract

Purpose: Angiotensin II (ANG II) has been shown to affect iron metabolism through alteration of iron transporters, leading to increased cellular and tissue iron contents. Serum ferritin, a marker of body iron storage, is elevated in various cardiovascular diseases, including hypertension. However, the associated changes in iron absorption and the mechanism underlying increased iron content in a hypertensive state remain unclear.

Methods: C57BL6/J mice were treated with ANG II to generate a model of hypertension. Mice were divided into 3 groups: (1) control, (2) ANG II-treated, and (3) ANG II-treated and ANG II receptor blocker (ARB)-administered (ANG II-ARB) groups.

Results: Mice treated with ANG II showed increased serum ferritin levels compared to vehicle-treated control mice. In ANG II-treated mice, duodenal divalent metal transporter-1 (DMT1) and ferroportin (FPN) expression levels were increased and hepatic *hepcidin* mRNA expression and serum hepcidin concentration were reduced. The mRNA expression of bone morphogenetic protein 6 (*BMP6*) and CCAAT/enhancer binding protein alpha (*C/EBPα*), which are regulators of hepcidin, was also down-regulated in the livers of ANG II-treated mice. In terms of tissue iron content, macrophage iron content and renal iron content were increased by ANG II treatment, and these increases were associated

with reduced expression of transferrin receptor 1 and FPN and increased expression of ferritin. These changes induced by ANG II treatment were ameliorated by administration of an ARB.

Conclusions: ANG II altered the expression of duodenal iron transporters and reduced hepcidin levels, contributing to the alteration of body iron distribution.

Keywords: iron metabolism, angiotensin II, hepcidin, ferritin

Introduction

Iron is an essential trace metal for almost all living organisms. However, excess iron can harm tissues by causing oxidative stress through the production of hydroxyl radicals via the Fenton/Haber-Weiss reaction [1]. Therefore, intracellular iron levels are tightly regulated by iron transporters and iron-binding proteins, and in normal physiological states, iron is stored in complexes with various proteins, such as metalloproteins, heme complexes, and oxygen carrier proteins [2].

Iron metabolism is affected by various factors, including hyperglycemia [3] and tumor necrosis factor-α (TNF-α) [4], through alteration of the expression of iron transporters such as transferrin receptor (TfR), divalent metal transporter-1 (DMT1), and ferroportin (FPN). In fact, stress-induced changes in iron transporter expression increase intracellular iron content, leading to oxidative stress. Angiotensin II (ANG II) is a critical hormone that acts as a vasoconstrictor and blood pressure regulator, which affects the function of the heart, vasculature, kidneys, and brain [5]. ANG II has also been shown to be relevant to insulin resistance [6], which was shown to be prevented by treatment with an angiotensin-converting enzyme inhibitor or an ANG II receptor blocker [7,8]. In terms of the relationship between ANG II and iron, ANG II was shown to increase tissue iron content in the liver [9], kidneys [10], and cardiovascular organs [11,12], as well as change the expression of iron transporters in the kidneys [13] and glomerular endothelial

cells [14]. Patients with hypertension [15] and insulin resistance [16] showed increased body iron content, as estimated by serum ferritin levels. We hypothesized that ANG II increases iron absorption by modulating iron transporter expression in the duodenum and contributes to elevated body iron storage. However, the role of ANG II in duodenal iron absorption and its related regulators remain unclear.

In the present study, we demonstrated that duodenal DMT1 and FPN expression were increased, and hepatic hepcidin and bone morphogenetic protein 6 (BMP6) were decreased in ANG II-induced hypertensive mice, which contributed to increased iron content in macrophages and elevated serum ferritin levels. This is the first report of the effects of ANG II on duodenal iron transporters, hepatic hepcidin, and body iron storage.

Methods

Chemicals and reagents

The following commercially available antibodies were used for this study: anti-ferritin heavy chain (FTH) and anti-ferritin light chain (FTL) antibodies, anti-NRAMP2 (DMT1) antibody (Santa Cruz Biotechnology; Santa Cruz, CA, USA); anti-hypoxia inducible factor-2α (HIF-2α) antibody (Abcam Japan; Tokyo, Japan); anti-transferrin receptor 1 (TfR1) antibody (Invitrogen; Carlsbad, CA, USA);

anti-ferroportin (FPN) antibody (Alpha Diagnostics; San Antonio, TX, USA), and an anti-β-actin antibody as a loading control (Cell Signaling Technology; Beverly, MA, USA). Olmesartan was kindly provided by Daiichi Sankyo Co. Ltd. (Tokyo, Japan).

Experimental animals and treatment

All animal experimental procedures were performed in accordance with the guidelines of the Animal Research Committee of the University of Tokushima Graduate School, and all protocols were approved by the Tokushima University Institutional Review Board for animal protection. We used 6-week-old male C57BL6/J mice, which had free access to food (Type NMF; 10 mg Fe/100 g food; Oriental Yeast; Tokyo, Japan) and water during the study. The mice were subcutaneously infused with ANG II (WAKO; Tokyo, Japan) at a rate of 2.0 $mg \cdot kg^{-1} \cdot day^{-1}$ for 4 weeks by an osmotic mini-pump (Alzet model 1004; Alza Corp.; Mountain View, CA, USA) or were sham-infused. At 8 weeks of age, the ANG II-infused mice were divided into 2 groups: 1 group was orally administered olmesartan, an ANG II type 1 (AT1) receptor blocker (ARB) at a rate of 3 $mg \cdot kg^{-1} \cdot day^{-1}$ in 0.5% carboxymethylcellulose, and the other group was administered vehicle only. Mice were maintained on their respective treatments for 2 weeks, and then sacrificed by an injection of overdose pentobarbital and used for analysis.

Macrophage extraction

Peritoneal macrophages were harvested from the experimental mice for use in this study. In brief, the mice were anesthetized and intraperitoneally injected with 5 mL of ice-cold, sterilized phosphate-buffered saline (PBS) buffer. The distended abdomen was lightly massaged for 3 minutes, and then the PBS was recovered from the abdominal cavity. The recovered PBS buffer was centrifuged to pellet peritoneal macrophages.

Measurement of serum ferritin levels

Serum ferritin levels were measured using a Mouse Ferritin enzyme-linked immunosorbent assay (ELISA) kit (Immunology Consultants Laboratory, Newberg, OR, USA) according to the manufacturer's instructions. Serum was diluted by 20% and used for assay.

Measurement of serum iron levels and tissue iron concentrations in the liver, spleen, and kidney, and in peritoneal macrophages

Serum iron levels were measured using an iron assay kit according to the manufacturer's instructions (Metallo Assay; Metallogenics Co., Ltd.; Chiba, Japan). Tissue iron concentrations were measured using the Metallo Assay kit as previously described [17,18]. In brief, the extracted tissues were weighed or protein levels were measured and then the tissues were mechanically homogenized in cell lysis buffer.

The crude lysates were further homogenized with an ultrasonic sonicator and then the crude lysate was mixed with HCl (0.01 M final concentration) and incubated at room temperature for 30 min. The lysate was then centrifuged at 4°C for 15 min, and the supernatant was used in the iron measurement assay. Tissue iron concentration was normalized to tissue wet weight or protein concentration and is expressed as $ng \cdot g^{-1}$ tissue or $ng \cdot \mu g^{-1}$ protein.

Measurement of serum hepcidin-1 levels

Serum mouse hepcidin concentrations were measured by surface-enhanced laser desorption/ionization-time-of-flight mass-spectrometry as previously described [17]. The assay was performed by Medical Care Proteomics Biotechnology Co., Ltd. (Kanazawa, Japan).

Quantitative measurement of mRNA expression levels

The methods of total RNA extraction, cDNA synthesis, and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) were described in detail in our previous manuscript [19]. The primer sets used were as follows: 5′-GAAGGTTGGCTGGAATTTGA-3′ and 5′-ACCTCGCTCACCTTGAAGAA-3′ for mouse *BMP6*, 5′-AATCTTCCTGCAGCCTTTGA-3′ and 5′-ATCTCCACGTGACTCCCAAG-3′ for mouse *Hfe2*, 5′-GTGGACAAGAACAGCAACGA-3′ and

5′-TCACTGGTCAACTCCAGCAC-3′ for mouse *C/EBPα*, 5′-CTGCCTGTCTCCTGCTTCTC-3′ and 5′-AGATGCAGATGGGGAAGTTG-3′ for mouse *hepcidin-1*, 5′-ATTTCTGAGAGCCCAGACGA-3′ and 5′-CCATTTGCTTCCTCCAACAT-3′ for mouse *CD11c*, 5′-CTGTAACCGGATGGCAAACT-3′ and 5′-CTGTACCCACATGGCTGATG-3′ for mouse *F4/80*, 5′-AAGCTGTAGTTTTTGTCACC-3′ and 5′-GGGCAGATGCAGTTTTAA-3′ for mouse monocyte chemoattractant protein-1 *(MCP-1)*, 5′-CTAAGTGGCCTGTGGGTGAT-3′ and 5′-CGAAGTCCTTTGCAGACCTC-3′ for mouse hypoxia-inducible factor-2α *(HIF2-α)*, 5′-GAAGGCCCAGCGTATTTGTA-3′ and 5′-ACGGTTCTCATGGGAGTGAC-3′ for mouse duodenal cytochrome B *(Dcytb)*, 5′-TGATGAAGCTGCAGAACCAG-3′ and 5′-GTGCACACTCCATTGCATTC-3′ for mouse ferritin heavy chain *(FTH)*, 5′-AATGGGGTAAAACCCAGGAG-3′ and 5′-AGATCCAAGAGGGCCTGATT-3′ for mouse ferritin light chain *(FTL)*, and 5′-GCTCCAAGCAGATGCAGCA-3′ and 5′-CCGGATGTGAGGCAGCAG-3′ for *36B4* (an internal control).

Western blotting

Protein expression levels were evaluated by western blot analysis. In brief, protein extracts from tissue samples were homogenized in T-PER reagent (Thermo Fisher Scientific Inc.; Rockford, IL, USA) with a protease inhibitor cocktail and a phosphatase inhibitor (Roche Applied Science; Indianapolis, IN, USA)

as previously described [19]. Semi-quantitative analysis of the immunoblot bands by densitometry was performed using Image J 1.38x software.

Histological analysis

The spleen of each mouse was extracted, fixed in paraformaldehyde, and embedded in paraffin. Samples were cut into 3-μm sections and stained with Perl's Prussian blue to evaluate splenic iron accumulation.

Electrophoretic mobility-shift assay (EMSA)

The interaction between iron regulatory protein (IRP) and iron responsive element (IRE) was measured using an EMSA kit according to the manufacturer's instructions (LightShift Chemiluminescent RNA EMSA Kit; Thermo Fisher Scientific Inc.; Rockford, IL USA). Semi-quantitative analysis of the immunoblot bands by densitometry was performed using Image J 1.38x software.

Statistical analysis

Data are expressed as the means ± standard error of the mean (SEM). For comparisons among 3 groups, statistical significance was confirmed by using two-way analysis of variance and the significance of each difference was determined by post-hoc testing using the Tukey-Kramer method. P-values less than 0.05 were considered statistically significant.

Results

Characteristics of experimental mice

We first evaluated the effect of ANG II on body weight, hematological characteristics, blood pressure, and iron status. As shown in Table 1, there were no significant differences in final body weight among the control, ANG II, and ANG II-ARB groups. No significant differences were seen in hematological characteristics, although hemoglobin and hematocrit levels tended to be higher in the ANGII and ANGII-ARB groups. Systolic and diastolic blood pressures were elevated by ANG II treatment, and ANG II-increased blood pressure was reduced to control levels by olmesartan. There was no difference in heart rate among the 3 groups. The mean serum iron concentration in mice treated with ANG II was significantly lower than that in control mice, and olmesartan reversed the ANG II-induced serum iron reduction. Serum ferritin levels were significantly higher in ANG II-treated mice than in control mice, and olmesartan reduced these increased serum ferritin levels.

Changes in duodenal iron transporter expression induced by ANG II treatment

The expression of FPN and DMT1 protein increased in the duodenum of ANG II-treated mice, and these changes were reversed by olmesartan administration (Fig. 1). HIF-2α plays a crucial role in duodenal iron absorption because it transcriptonally regulates DMT1 and FPN but is itself translationally regulated by IRPs [20]. Mice treated with ANG II showed increased duodenal HIF-2α mRNA and protein expression levels, which were reduced by olmesartan administration. *Dcytb*, a target gene of HIF-2α, tended to be increased by ANG II stimulation, and the change was partly suppressed by olmesartan.

Effect of ANG II on the BMP6-hepcidin axis

Hepatic hepcidin plays a crucial role in interstitial iron absorption through FPN [21], and the BMP6 signaling pathway is upstream of hepcidin expression [22,23]. We examined the effect of ANG II on BMP6-hepcidin signaling in the liver. As shown Fig. 2, *BMP6* and *hepcidin* mRNA expression was lower in the livers of ANG II-infused mice than in control mice, and this decrease was ameliorated and further elevated by olmesartan administration. In contrast, *Hfe2* mRNA expression was higher in the livers of ANGII-treated mice, which was restored to baseline levels by olmesartan. Expression of CCAAT/enhancer binding protein (C/EBP), a transcription factor for hepcidin [24], was reduced by ANG II treatment, and this was also reversed by olmesartan administration (Fig. 2d). Consistent with the hepatic expression findings, the serum hepcidin concentration was significantly lower in ANG II-treated

mice than in control mice. Consistent with the mRNA levels, the ANG II-induced serum hepcidin reduction was also reversed and further increased by olmesartan administration (control mice, 91.0 ± 7.1 ng/mL; ANG II mice, 60.7 ± 5.2 ng/mL; ANG II+ARB mice, 153 ± 11.3 ng/mL).

Iron contents in the liver, spleen, and kidney

Next, we examined the effect of ANG II on tissue iron content. Unexpectedly, no differences in iron content among the 3 groups were seen in the liver or spleen (Fig. 2f). Conversely, renal iron content was higher in ANG II-treated mice, and this was suppressed by olmesartan treatment (Table 2).

ANG II increased iron content and altered iron transporter expression in peritoneal macrophages

Serum ferritin levels were elevated in ANG II-treated mice, and serum ferritin may be derived from macrophages [25]. Therefore, we measured the expression of TfR1, FPN, FTH, and FTL in peritoneal macrophages extracted from the experimental mice. Iron content was elevated in the macrophages from ANG II-treated mice, and this was lowered by olmesartan administration (Table. 2). ANG II decreased TfR and FPN protein expression, and increased FTH and FTL protein expression (Fig. 3a–d). These changes in iron transporter and ferritin expression were almost completely reversed by olmesartan administration. The mRNA expression levels of M1 macrophage markers, including *CD11c*, *F4/80*, and

MCP-1, were also increased by ANG II stimulation, and these levels were reduced by olmesartan treatment (Fig. 3e).

ANG II effects on IRP-IRE RNA-binding activity in peritoneal macrophages and the duodenum

The effect of ANGII on the interaction between IRP and IRE RNA binding was analyzed by EMSA (Fig. 3f). ANG II treatment augmented IRP-IRE binding activity and this change was inhibited by olmesartan in peritoneal macrophages. In contrast, IRP-IRE binding activity was reduced by ANG II administration, which was partly reversed by olmesartan treatment in the duodenum. Duodenal mRNA levels of *FTH* and *FTL*, regulated post-transcriptionally by IRP, were elevated, which was consistent with the reduced IRP activity observed in the duodenum of ANG II-treated mice (Fig. 1b).

Discussion

In the present study, mice treated with excess ANG II presented with increased expression of DMT1 and FPN in the duodenum, reduced expression of BMP6, C/EBPα, and hepcidin in the liver, and reduced serum hepcidin. Although iron content in the liver and spleen was not changed by ANG II treatment, ANG II did augment iron content in the kidney and macrophages, which was associated with reduced expression of TfR1 and FPN and increased expression of FTH and FTL, contributing to

increased serum ferritin levels. Therefore, we propose that ANG II regulates duodenal iron transporter, hepcidin levels through a BMP6/C/EBPα-dependent mechanism, and the alternation of the iron distribution in the body.

ANG II is a crucial hormone that regulates blood pressure through its various actions, including vasoconstriction, tubular sodium reabsorption, and aldosterone secretion, which are mediated by the AT1 receptor [26]. Plasma ANG II concentration is increased in hypertensive patients [27], and inhibition of ANG II by an angiotensin-converting enzyme inhibitor or ARB are effective treatments for patients with hypertension [26]. In experimental studies, ANG II treatment is useful for exploring the mechanism of hypertension-related pathophysiology both *in vivo* [11,12] and *in vitro* [14]. In male patients with essential hypertension, serum ferritin is elevated compared to normal healthy subjects [15]. Serum ferritin is a widely used indicator of body iron storage [28]. Consistent with this, we found that mice treated with ANG II showed increased serum ferritin levels, as well as increased iron content in macrophages compared to control mice, and these changes were ameliorated by treatment with olmesartan, an ARB. Therefore, body iron content might be augmented in a hypertensive state with elevated ANG II levels.

With respect to the relationship between ANG II and iron, several studies have demonstrated the effects of ANG II on iron metabolism. Ishizaka and colleagues showed that ANG II stimulation promoted

tissue iron deposition in the heart, aorta, kidneys, and liver [11,12,10,9], and augmented the expression of TfR1, DMT1, FPN, and hepcidin in the rat kidney, which was ameliorated by administration of the ARB losartan [13]. We also demonstrated that ANG II altered the expression of iron transporters, and increased intracellular free iron levels with 30% saturated transferrin in endothelial cells [14]. In the present study, ANG II treatment induced the expression of DMT1 and FPN in the duodenum of mice, and this was reversed by olmesartan administration. DMT1, an iron importer, is an important transporter for duodenal iron absorption [29]; FPN, an iron exporter, plays a critical role in intestinal iron absorption [30]. Therefore, ANG II is thought to affect duodenal iron absorption by altering duodenal iron transporter expression.

Hepcidin, a secreted protein derived from hepatocytes, was first identified as an antimicrobial peptide, and plays an important role in the regulation of iron metabolism [31]. Hepcidin regulates iron absorption through both internalization and degradation of FPN in the duodenum [21] and ubiquitin-dependent proteasome degradation of DMT-1 [32]. In the present study, ANG II treatment diminished hepatic *hepcidin* mRNA expression, as well as serum hepcidin levels, contributing to increased iron absorption via FPN and DMT-1 up-regulation in the duodenum. Based on these findings, hepatic hepcidin expression is inversely correlated with the expression of iron transporters and iron

absorption [33]. In addition, we showed that ANG II augmented duodenal expression of HIF-2α, which was previously shown to play a critical role in duodenal iron absorption in an investigation of duodenum-specific HIF-2α-deficient mice [20]. Therefore, the findings suggest that the ANG II-induced increases in FPN and DMT-1 expression are dependent on both hepcidin and HIF-2α, and contribute to the promotion of iron absorption in the duodenum.

The regulation of hepcidin expression is dependent on not only iron [34] but also various other factors. BMP6 is an important factor in iron metabolism that regulates hepcidin expression [22,23]. C/EBPα promotes hepcidin expression through a transcriptional-dependent mechanism [24]. In this study, mice treated with ANG II showed decreased levels of *BMP6* and *C/EBPα*□□□□, resulting in reduced hepatic hepcidin expression. Serum levels of hepcidin were lower in ANG II-treated mice than in control or ANG II-treated mice administered olmesartan. ANG II-induced suppression of BMP6/C/EBPα-hepcidin was reversed by olmesartan. Therefore, ANG II might be a negative regulator of hepatic hepcidin through the BMP6/C/EBPα signaling pathway mediated via the AT1 receptor, resulting in increased iron absorption. □□□□□□□□ *hepcidin* mRNA and serum hepcidin levels were much higher in ANG II-ARB mice than in control mice. Further studies are necessary in order to clarify the effect of the AT1 receptor and ARB on hepcidin. □□□□□□□□□□we found that in ANG II-treated

mice, *Hfe2* mRNA expression was increased and *BMP6* mRNA was reduced in the liver. *Hfe2* encodes the hemojuvelin gene and its mutation is responsible for juvenile hemochromatosis [35]. It has also been shown that the *Hfe2* gene regulates hepcidin expression as the function of a BMP co-receptor [36]. Liver-specific *Hfe2*-deficient mice showed reduced *hepcidin* expression and augmented *BMP6* expression in the liver [37,38]. In the present study, the increased *Hfe2* mRNA expression might reflect a compensatory change for the decreased *BMP6* mRNA expression in the liver of ANG II-treated mice.

ANG II promoted iron deposition in tissues [10-12,9]. Although we confirmed that serum ferritin levels were increased in mice treated with ANG II, unexpectedly, no changes in tissue iron content were observed in the liver or spleen, while renal and macrophage iron content was elevated by ANG II stimulation. Although the reason for the lack of ANGII-induced hepatic and splenic iron deposition in our model is not clear, ANGII might be involved in the alteration of iron distribution in the whole body. Recently, Cohen and colleagues reported that serum ferritin is mainly secreted from macrophages [25]. Consistently, ANG II-induced iron accumulation in macrophages contributes to increased serum ferritin levels. In contrast, the expression of FPN and TfR1 proteins was diminished by ANG II treatment, which was restored by olmesartan administration. In contrast with the increased FPN levels in the duodenum, it is difficult to understand the discrepancy between the decreased FPN expression in macrophages and the

reduction of hepcidin in the liver and serum in mice treated with ANG II. Several studies have demonstrated that the phenotype of macrophages plays a crucial role in iron management. Macrophages activated by inflammatory cytokines exhibit M1 characteristics, retain intracellular iron, and exhibit low expression of TfR and FPN and high expression of FTH [39,40]. ANG II promotes macrophage polarization toward the M1 phenotype, which is mediated by the AT1 receptor [41,42]. In the present study, ANG II increased M1 macrophage markers, including *CD11c*, *F4/80* and *MCP-1*, which were suppressed by olmesartan treatment. Therefore, ANG II might directly modulate TfR1 and FPN expression through polarization toward M1 macrophages, leading to increased iron content mediated by the AT1 receptor, independent of hepcidin regulation. Subsequently, the increased iron content would promote ferritin synthesis and secretion, contributing to elevated serum ferritin concentration in ANG II-treated mice. Further examinations are needed to clarify the coordinated regulation of tissue FPN and hepatic hepcidin levels.

IRP is well known to be important for mammalian iron homeostasis through post-transcriptional regulation of TfR, DMT1, FPN, and ferritins [43]. In the present study, ANG II promoted IRP activity in peritoneal macrophages despite the increased iron content of the macrophages. In contrast, duodenal IRP activity was decreased in ANG II-treated mice. The results showing increased IRP activity in peritoneal

macrophages are not consistent with the reduced TfR1 and FPN expression and increased ferritin expression observed in mice treated with ANG II. Moreover, the decreased duodenal IRP activity is also inconsistent with the increased DMT1 and FPN expression. These results suggest that ANG II might have the potential to regulate the expression of iron transporters or ferritins independently of IRP activity. Further study is needed for clarifying the effects of ANG II on the interaction between iron homeostasis and IRP activity.

Ferritin has been shown to play a key regulatory role in mucosal iron absorption and transport in the intestine. Intestinal IRP-deficient mice have impaired iron absorption and mucosal iron retention via ferritin upregulation [44]. Conversely, intestinal ferritin H-knockout mice have been shown to exhibit increased iron absorption [45]. In the present study, IRP activity was reduced and ferritin expression was elevated in the duodenum by ANG II treatment. These findings suggest that the impairment of mucosal iron transport and iron retention in duodenum were induced in mice with ANG II treatment, consequently leading to low serum iron, low hepcidin levels, and no increase in iron deposition in the liver or spleen. However, renal or peritoneal macrophage iron was elevated in mice treated with ANG II, and the positive regulators of hepcidin (i.e., BMP6, C/EBPα, and HFE2) were also upregulated by ANG II, suggesting the

involvement of ANG II on hepcidin regulation independently of iron levels. Thus, further study is necessary for investigating the exact mechanism of ANG II on iron metabolism.

In conclusion, ANG II influences duodenal iron transporter, hepatic hepcidin, and the promotion of iron retention in macrophages, which leads to the alteration of body iron storage. These ANG II-induced changes were ameliorated by ARB administration. To our knowledge, this is the first report showing the effect of ANG II on systemic iron metabolism.

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Figure legends

Fig. 1. (a) Effects of ANG II on the expression of duodenal iron absorption-related proteins. Upper panel: Representative immunoblots for DMT1, FPN, HIF-2α, and β-actin (internal control). Lower panel: Semi-quantitative densitometry of DMT1, FPN, and HIF-2α protein levels normalized to β-actin. Values are expressed as means ± SEM. *$P < 0.05$, **$P < 0.01$. $n = 4$ in each group. (b) ANGII action on *HIF-2α*, *Dcytb*, *FTH*, and *FTL* mRNA expression in the duodenum. Values are expressed as means ± SEM. *$P < 0.05$, **$P < 0.01$. $n = 4$-5 in each group (HIF-2α). $n = 11$-12 in each group (*Dcytb*, *FTH* and *FTL*). CTR: Vehicle-infused mice, ANGII: ANGII-infused mice, ANGII+Olm: ANGII-infused mice with olmesartan administration

Fig. 2. Effects of ANG II on expression of hepcidin, BMP6, and C/EBPα in the liver and on serum hepcidin-25 concentration. Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *hepcidin* (a), *BMP-6* (c), *C/EBPα* (d), *Hfe2* (e) mRNA expression in the liver.

Values are expressed as means ± SEM. *P < 0.05, **P < 0.01. n = 5–7 in each group. (b) Serum hepcidin-1 concentration as measured by SELDI-TOF mass-spectrometry. Values are expressed as means ± SEM. *P < 0.05, **P < 0.01. n = 8–12 in each group. (f) Representative histological findings of Berlin blue staining in the spleen. CTR: Vehicle-infused mice, ANGII: ANGII-infused mice, ANGII+Olm: ANGII-infused mice with olmesartan administration

Fig. 3. Alterations in iron metabolism-related proteins induced by ANG II in mouse peritoneal macrophages. Upper panel: Representative immunoblots of transferrin receptor (TfR) (a), ferroportin (FPN) (b), ferritin heavy chain (FTH) (c), ferritin light chain (FTL) (d) and β-actin (internal control). Lower panel: Semi-quantitative densitometry analysis of TfR (a), FPN (b), FTH (c), and FTL (d) normalized to β-actin. Values are expressed as means ± SEM. *P < 0.05, **P < 0.01. n = 4 in each group. (e) Effects of ANGII on mRNA expression of M1 macrophage markers. Values are expressed as means ± SEM. *P < 0.05, **P < 0.01. n = 6-7 in each group. The action of ANGII on iron regulatory protein (IRP)-iron responsive element (IRE) -binding activity in peritoneal macrophages and the duodenum. (f) Upper panel: Representative IRP-IRE-binding bands. Lower panel: Semi-quantitative densitometry analysis of IRP activity in peritoneal macrophages. Values are expressed as means ± SEM. *P < 0.05,

**$P < 0.01$. n = 4-8 in each group. (g) Upper panel: Representative IRP-IRE-binding bands. Lower panel: Semi-quantitative densitometry analysis of IRP activity in the duodenum. Values are expressed as means ± SEM. *$P < 0.05$, **$P < 0.01$. n = 6 in each group. CTR: Vehicle-infused mice, ANGII: ANGII-infused mice, ANGII+Olm: ANGII-infused mice with olmesartan administration.

2nd revised Fig. 1

Fig. 1

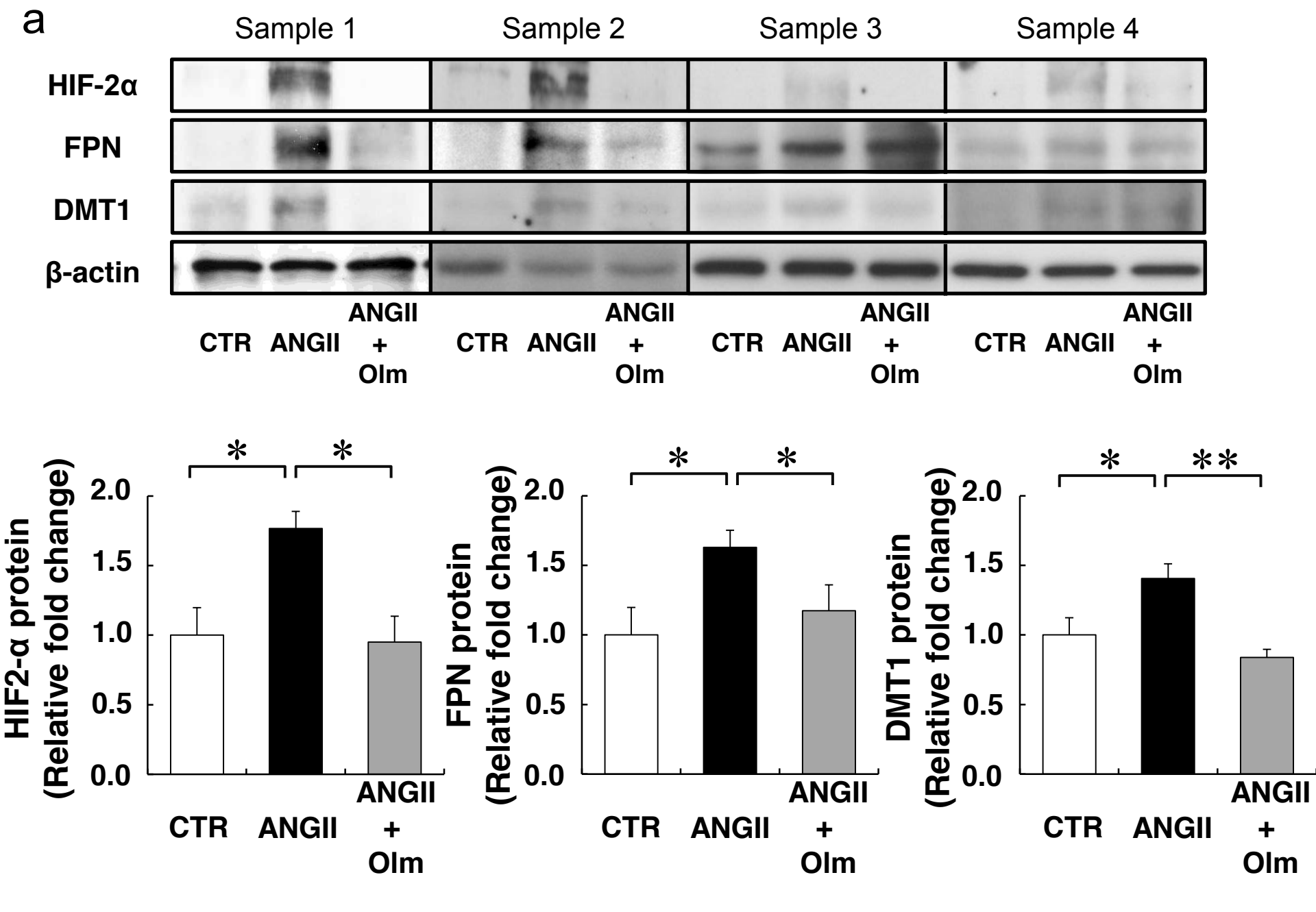
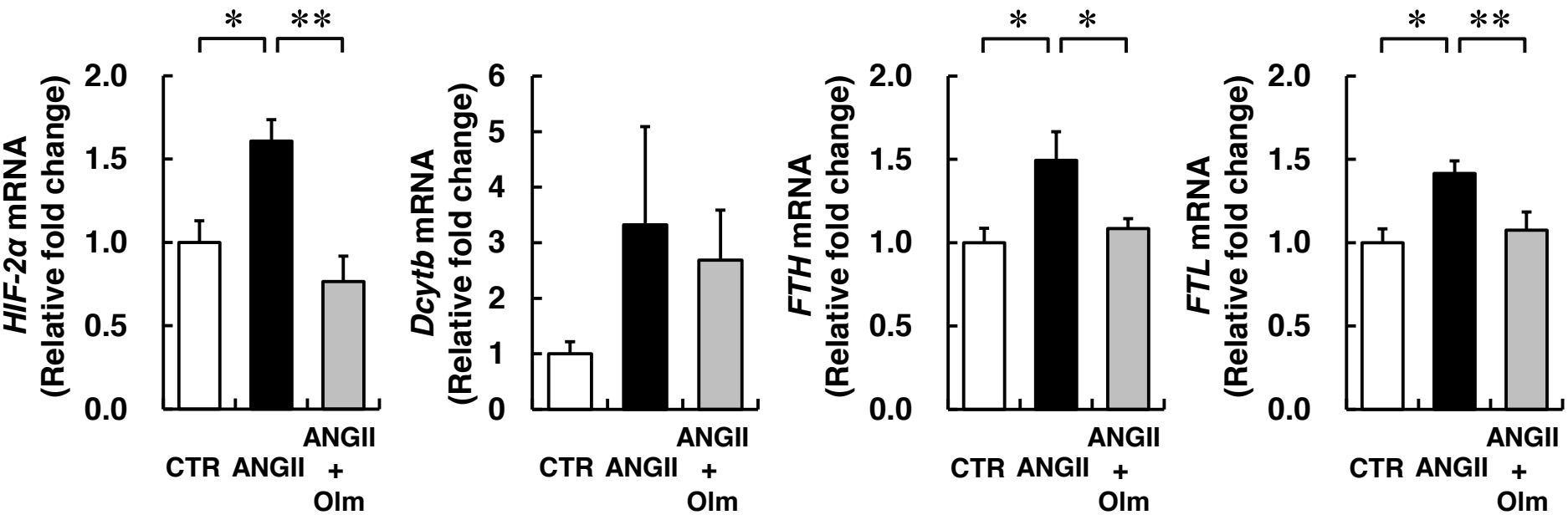


Fig. 1 continued

b



2nd revised Fig. 2 and Fig. 3

Fig. 2

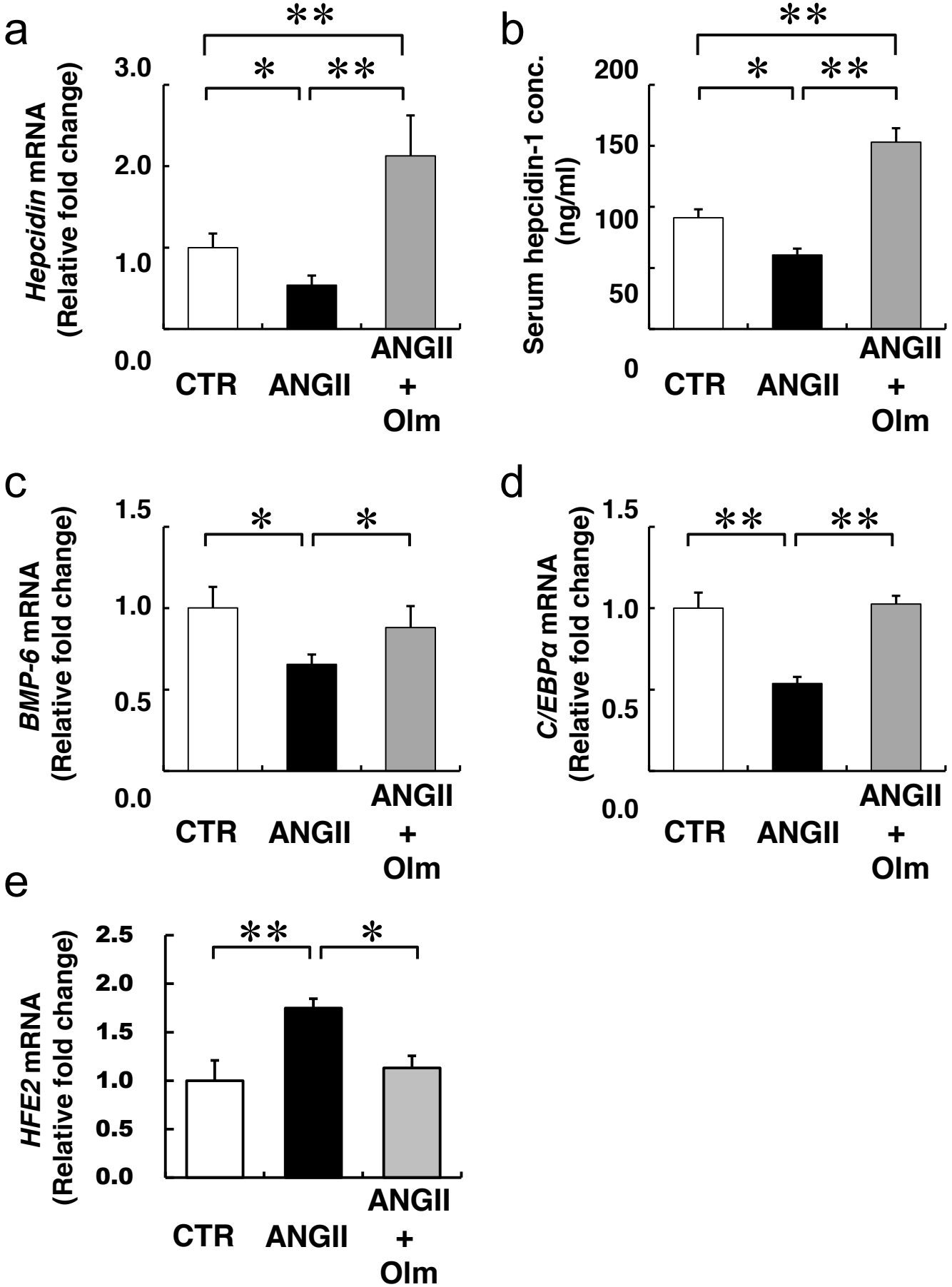


Fig. 2 continued.

f

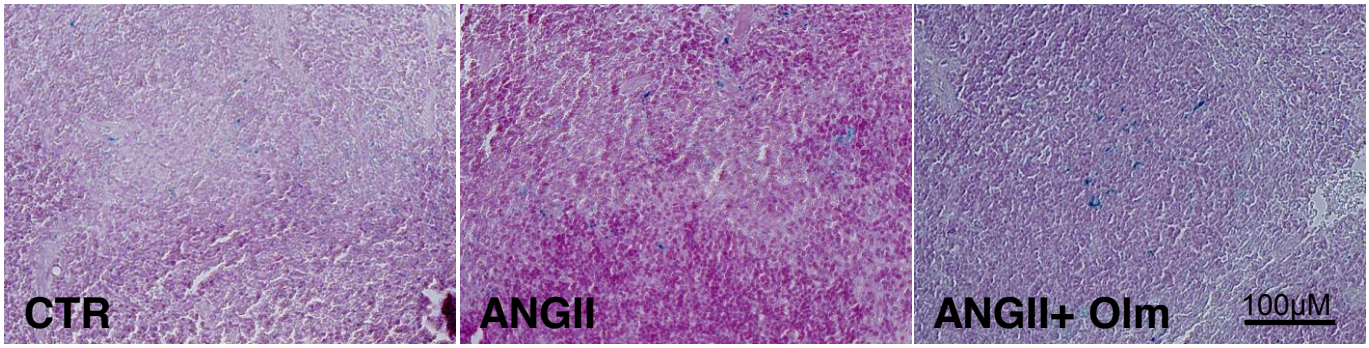


Fig. 3

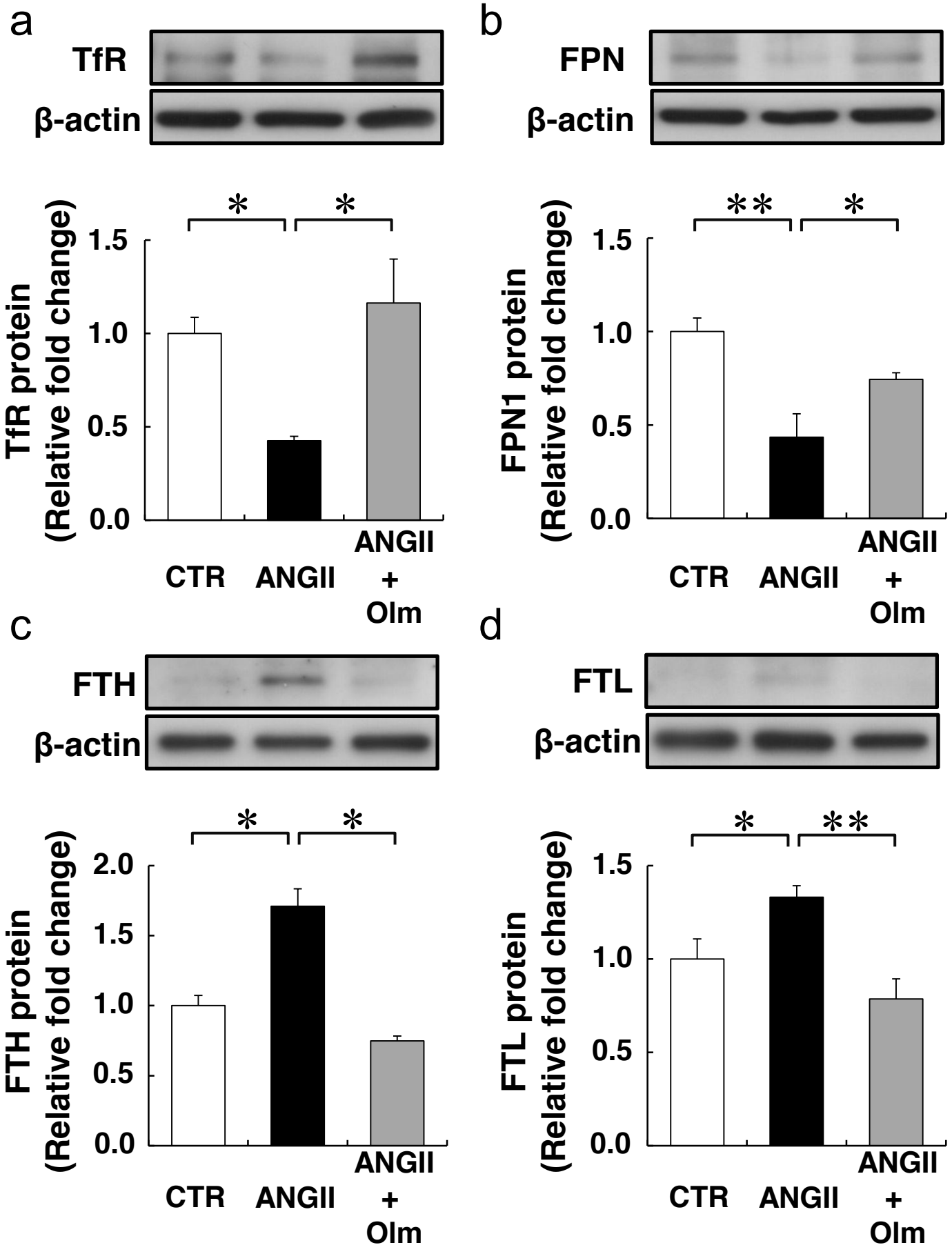

a
TfR
β-actin
TfR protein
(Relative fold change)
1.5
1.0
0.5
0.0
*
*
CTR
ANGII
ANGII + Olm
b
FPN
β-actin
FPN1 protein
(Relative fold change)
**
*
c
FTH
β-actin
FTH protein
(Relative fold change)
2.0
d
FTL
β-actin
FTL protein
(Relative fold change)

Figure 3

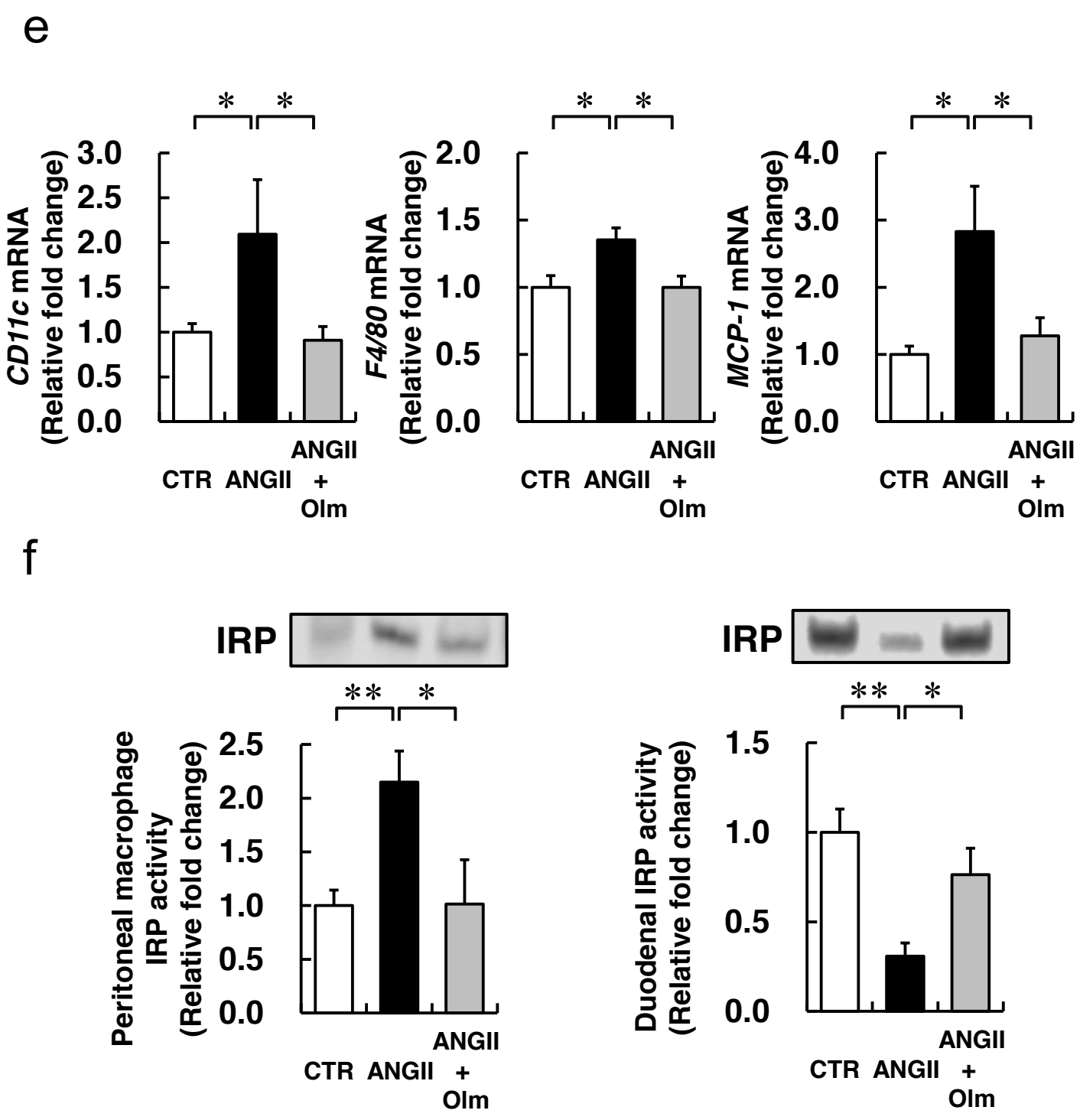

e
CD11c mRNA (Relative fold change)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
* *
CTR
ANGII
ANGII + Olm
F4/80 mRNA (Relative fold change)
2.0
1.5
1.0
0.5
0.0
* *
CTR
ANGII
ANGII + Olm
MCP-1 mRNA (Relative fold change)
4.0
3.0
2.0
1.0
0.0
* *
CTR
ANGII
ANGII + Olm
f
IRP
** *
Peritoneal macrophage IRP activity (Relative fold change)
2.5
2.0
1.5
1.0
0.5
0.0
CTR
ANGII
ANGII + Olm
IRP
** *
Duodenal IRP activity (Relative fold change)
1.5
1.0
0.5
0.0
CTR
ANGII
ANGII + Olm

Table. 1 The characteristics of mice

	CTR	ANG II	ANG II + Olm
Body weight, g	26.1 ± 0.5	24.4 ± 0.8	25.0 ± 0.7
Systolic blood pressure, mmHg	104.8 ± 2.1	132.4 ± 4.2**	100.9 ± 2.6††
Diastolic blood pressure, mmHg	69.1 ± 3.3	85.8 ± 4.2**	71.4 ± 2.5††
Heart rate, beats/min	637 ± 17	629 ± 25	653 ± 16
Hemoglobin, g/dl	12.7 ± 0.6	13.7 ± 0.8	13.7 ± 0.4
Hematocrit, %	38.7 ± 2.2	43.4 ± 0.8	42.4 ± 1.8
Serum iron concentration, μg/dl	98.2 ± 7.3	69.6 ± 6.9**	112.3 ± 9.8††
Serum ferritin concentration, ng/ml	551.3 ± 52.4	827.0 ± 113.0**	613.4 ± 51.9†

Values are expressed as means ± SEM. **P $<$ 0.01 vs. CTR. † P $<$ 0.05; †† P $<$ 0.01 vs. ANGII n = 8 – 12 in each group.

CTR: Vehicle-infused mice, ANGII: ANGII-infused mice, ANGII+Olm: ANGII-infused mice with olmesartan administration

Table. 2 Tissue iron contents

	CTR	ANG II	ANG II + Olm
Liver, μg/g・wet tissue weight	16.8 ± 1.9	16.7 ± 1.2	16.3 ± 2.0
Spleen, μg/g・wet tissue weight	7.6 ± 1.3	7.5 ± 1.1	6.5 ± 1.6
Peritoneal macrophage, μg/g・protein	222.7 ± 59.9	515.4 ± 106.2*	186.8 ± 21.3†
Kidney, μg/g・wet tissue weight	2.0 ± 0.2	2.7 ± 0.3*	2.1 ± 0.1†

Values are expressed as means ± SEM. *P ＜ 0.05 vs. CTR, † P ＜ 0.05 vs. ANGII $n = 8 – 12$ in each group.

CTR: Vehicle-infused mice, ANGII: ANGII-infused mice, ANGII+Olm: ANGII-infused mice with olmesartan administration