

# Influence of prazosin on systemic iron levels and the associated iron metabolic alterations in spontaneously hypertensive rats

Hengrui Chang<sup>1,2</sup> | Dong Zhang<sup>2</sup> | Zhen Xin<sup>2</sup> | Pengfei Zhang<sup>2</sup> | Wenyuan Ding<sup>1</sup> | Yan-Zhong Chang<sup>2</sup> 

<sup>1</sup>Department of Spinal Surgery, The Third Hospital of Hebei Medical University, Shijiazhuang, Hebei, People's Republic of China

<sup>2</sup>College of Life Science, Hebei Normal University, Shijiazhuang, Hebei, People's Republic of China

## Correspondence

Yan-Zhong Chang, College of Life Science, Hebei Normal University, Shijiazhuang, Hebei Province, People's Republic of China.

Email: chang7676@163.com or yzchang@hebtu.edu.cn

Wenyuan Ding, Department of Spinal Surgery, The Third Hospital of Hebei Medical University, Shijiazhuang, People's Republic of China.

Email: drdingwy@163.com

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## Abstract

The relationship between cardiovascular diseases and iron disorders has gained increasing attention; however, the effects of hypotensive drugs on iron metabolic alterations in hypertension are not well understood. The purpose of this study was to investigate iron metabolic changes after prazosin treatment of spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) rats. Our second objective was to examine the effects of hypertension and anti-hypertensive drugs on bone formation and resorption. SHRs and WKY rats were randomized into either prazosin-treated groups (WKY + PZ and SHR + PZ) or untreated groups (WKY and SHR). After 7 days of intragastric prazosin administration, the rats were sacrificed for analysis; blood samples and organs (the duodenum, liver, kidneys, spleen, and femur) were collected. Both WKY + PZ and SHR groups exhibited iron deficiency in the serum and liver. Prazosin increased the iron levels in the bone tissue of SHRs. Prazosin stimulated the expression of hepcidin mRNA in the liver of SHRs and inhibited the expression of this iron-regulatory hormone in WKY rats. FPN1 expression in the duodenum was increased significantly in SHRs, however markedly decreased after prazosin treatment. The expression of TLR4 and Ctsk was enhanced in the bone tissue of SHRs, whereas CLC-7 expression was inhibited. Both hypotension and hypertension can lead to iron deficiency. Treatment with prazosin restored iron homeostasis in SHRs. The inverse impacts of prazosin on hepatic hepcidin expression in SHRs versus WKY rats indicates differing iron regulatory mechanisms between hypertensive and normal animals. The osteoclast activity was found to be enhanced in SHRs. Further study is needed to address whether the changes in osteoblast and osteoclast activity in SHRs correlates with the effects on iron metabolism.

## KEYWORDS

bone tissue, hepatic, hypertension, iron metabolism, Prazosin

Hengrui Chang and Dong Zhang authors have contributed equally to this work and share first authorship.

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## 1 | INTRODUCTION

Hypertension, responsible for a large proportion (13.5%) of deaths worldwide, is one of the most complicated and prevalent diseases.<sup>1</sup> It is regarded as a major contributor to cardiovascular diseases (CVD), retinopathy, and nephropathy. A combination of drug therapy and lifestyle intervention is effective for blood pressure (BP) control and can greatly reduce cardiovascular risk.<sup>2</sup> However, long-term use of hypotensive drugs can cause side effects. It has been shown that antihypertensive therapy may disturb electrolyte and mineral homeostasis by affecting reabsorption in the intestine and kidney.<sup>3,4</sup> Thus, it is important to explore the metabolic alterations, and associated biological mechanisms, affected by antihypertension drugs in hypertensive models.

Iron is essential to life, participating in DNA synthesis, oxygen utilization, and the activity of the central nervous system (CNS) and immune system.<sup>5</sup> Iron homeostasis is dependent both on cellular and systemic regulation mainly directed by the activity of iron regulatory proteins (IRP) and the hepcidin/ferroportin (FPN) axis, respectively.<sup>6</sup> Since the 1980s, the hypothesis that iron is cardiotoxic, as well as the correlation between iron disorders and cardiovascular diseases, has attracted much attention.<sup>7</sup> Essential hypertension, characterized by sympathetic over activity, accounts for 95% of hypertensive patients.<sup>8</sup> Recent clinical evidence suggests that, among patients with essential hypertension, a greater sympathetic activation was displayed in patients who also exhibited iron overload.<sup>9</sup> Circulating ferritin levels, a marker of iron accumulation, has been linked to a hyperadrenergic state in this group of patients.<sup>9</sup> This finding is consistent with previous evidence that increased iron storage is apparent in men with essential hypertension, indicating that disruption of global iron homeostasis correlates with abnormal blood pressure in hypertensive patients.<sup>10</sup> Furthermore, elevated iron stores with concomitant hyperferritinemia has also been linked to high arterial stiffness in individuals with essential hypertension.<sup>11</sup> However, most studies on this topic focus on clinical investigation, and the examination of iron metabolic alterations in specific organs has not been fully investigated. Moreover, the hypotensive drugs used to treat hypertension often influence the concentration of trace elements in several tissues. It is therefore possible that antihypertensive drugs affect iron metabolism.

High blood pressure has also been correlated with low bone mineral density (BMD).<sup>12,13</sup> However, we have a limited understanding of abnormal bone metabolism as a consequence of hypertension since clinical studies have demonstrated the effect to be multifactorial, depending on smoking, aging, and lack of exercise, among others.<sup>14</sup> In vivo investigations have demonstrated that the spontaneously hypertensive rat (SHR) model displays a loss of trabecular bone thickness and impaired bone healing.<sup>15</sup> At the cellular level, an imbalance of bone remodeling was found, with increased expression of osteoclastogenic markers and decreased alkaline phosphatase (ALP) activity.<sup>14</sup> At present, the specific mechanism in which high-blood pressure affects bone metabolism remains unclear.

Iron overload can disturb bone homeostasis.<sup>16</sup> Yamasaki et al. found that iron overload inversely correlates with osteoblast activity, as

indicated by decreased expression of type I collagen and ALP.<sup>17</sup> Further studies suggested that iron-induced inhibition of osteoblast activity was attributed to ferritin and its ferroxidase activity.<sup>18</sup> Moreover, excessive iron has been shown to generate reactive oxygen species (ROS) through the Fenton reaction, which may enhance the differentiation of osteoclasts (OCs), resulting in reduced bone density.<sup>19</sup> Hepcidin, the main regulator of systemic iron homeostasis, acts on bone metabolism. An in vitro study showed that hepcidin treatment can increase intracellular calcium levels in cultured osteoblasts.<sup>20</sup> Subsequent experiments confirmed that intracellular calcium promotes the differentiation of osteoblasts.<sup>21</sup> Based on the aforementioned evidence, we hypothesized that the abnormal bone metabolism caused by blood pressure variation may be attributed to an iron-mediated mechanism.

Prazosin is  $\alpha$ 1-adrenergic receptor antagonist and is capable of dilating blood vessels and reducing peripheral resistance, thus lowering blood pressure.<sup>22</sup> In the present study, we investigated the effects of treating hypertension with prazosin on iron metabolism in the SHR model. We compared the results with those in Wistar-Kyoto (WKY) rats, with the aim of investigating the role of iron hypertensive and hypotensive processes, so as to uncover novel possibilities for the prevention and treatment of hypertension. Our second objective was to elucidate markers related to bone formation and resorption, and then use those markers to verify if alterations in iron levels may be involved in the abnormal bone metabolism caused by blood pressure variation.

## 2 | MATERIALS AND METHODS

### 2.1 | Animal studies

Twelve-week-old male SHRs and WKY rats (Charles River Laboratories, China) were used to examine the effects of prazosin on iron metabolism. All animals were fed a standard rodent chow and tap water, ad libitum, and housed in a facility with a temperature of  $21 \pm 2^\circ\text{C}$  and humidity of  $55 \pm 10\%$  and under a 12h dark/light cycle. Following a period of 1 week to allow the rats to accommodate to laboratory conditions, the SHRs and WKY rats were randomized into either prazosin groups (WKY + PZ and SHR + PZ,  $n = 6$ ) or untreated groups (WKY and SHR,  $n = 6$ ). In the diet of the treatment group, prazosin was added at a dose of  $0.5 \text{ mg}\cdot\text{kg}^{-1}$  through gavage twice daily for 7 days.

### 2.2 | Organ and serum collection

All rats were fasted for 12h prior to the end of the experiment. Body weight was recorded before tissue collection. At the end of the experiment, the rats were anesthetized with an intraperitoneal injection of 0.8% pentobarbital sodium (0.5 ml/100g). The abdomens were sterilized using 75% alcohol. The heart and its surrounding structures were fully exposed through median sternotomy. Next, the right auricle was dissected, and a blood sample was collected

immediately using 1.5 ml Eppendorf tubes. Each blood sample was left undisturbed at room temperature for 60 min, and then centrifuged for 20 min at 2000×g at 4°C. The supernatant was removed and stored at -80°C for subsequent assays. Finally, organs were harvested once the circulating blood was fully replaced with ice-cold saline through left ventricular perfusion. The duodenum, liver, kidney, and spleen were dissected and stored frozen (-80°C) for further analysis. The thigh muscles of rats were fully detached to obtain bone tissue. The metaphysis was removed and the bone marrow was flushed into sterile tubes and stored in liquid nitrogen.

### 2.3 | Determination of iron and calcium in tissues and serum

Samples of liver, bone, and kidney were digested in 65% spectra pure HNO<sub>3</sub> (Merck, USA) in a Microwave Digestion System (MARS 5, CEM Corp, USA), according to a previously described procedure.<sup>23</sup> The completely digested samples were diluted to 2 ml with ultra-pure water. The concentration of iron and calcium in the solutions was determined using an AA240FS apparatus (Varian, USA), which combines atomic absorption spectrometry with a graphite-furnace atomizer. Standard curves were plotted using serial concentrations of diluted standard solutions, as described previously.<sup>24</sup> Serum iron concentrations were assessed using kits that employ a colorimetric method (NanJing, JianCheng Bioengineering, China) according to the manufacturer's instructions.

### 2.4 | qRT-PCR analysis

Total RNA from bone, bone marrow and liver were isolated using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA (1 µg) of from each sample was used for reverse transcription using an RT kit (Takara, Dalian, China). After denaturation of RNA at 70°C for 10 min, the reaction was performed for 1 h at 42°C. Each PCR reaction (final volume: 20 µl) consisted of 4 µl (20 ng) cDNA, 10 µl SYBR Green PCR master mix (Applied Biosystems Gen Star), and 0.8 µl each PCR primer. PCR amplification was performed with the BIO-RAD CFX Connect™ Optics Module with the following cycling parameters: 95°C for 10 s, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. 18s cDNA was amplified simultaneously as an internal control. Expression of the target gene was normalized to the respective 18s levels. The  $\Delta\Delta C_t$  method was used to compare fold changes in mRNA expression between prazosin groups (WKY+PZ and SHR+PZ) or untreated groups (WKY and SHR). Primer sequences are listed in Table 1.

### 2.5 | Western blot analysis

The levels of proteins involved in iron metabolism were estimated by western blot analysis as described previously.<sup>25</sup> Briefly, tissues

**TABLE 1** Primer sequences used for reverse transcription semi-quantitative PCR

Gene	Primers
18s	Forward5'-GTAACCCGTTGAACCCATT-3' Reverse5'-CCATCCAATCGGTAGTAGCG-3'
Hepcidin	Forward5'-GCAGACCACCTATCTCCAT-3' Reverse5'-AATAATGGGGTTGAGGGGC-3'
Acp5 (Trap)	Forward5'-AGTCAAATCCGCCCTTCTTT-3' Reverse5'-TCGGTCTCCCGGTCTTTTAG-3'
MMP9	Forward5'-CTACAGCTTTGCTGCCCTC-3' Reverse5'-TGGAGGTTTTAGGTCTCGG-3'
Ctsk	Forward5'-CCTTAGTCTACCATTACAGTAGCCA-3' Reverse5'-CTGCTGTTGAGAATCTGTTGTCT-3'
CLC7	Forward5'-TCTAAGAAAGTGCTTGGTCCGGC-3' Reverse5'-GTCTCCTCGTCAGGTTGCC-3'
OCN	Forward5'-CCCGTGTGGTTAATGCCAC-3' Reverse5'-GTCCGCTAGCTCGTCACAAT-3'
ALP	Forward5'-GGGAAGATGTGGCGGTCTTT-3' Reverse5'-TAGTTCTGCTCATGGACGCC-3'
Col1	Forward5'-CCCAGCGGTGGTTATGACTT-3' Reverse5'-AACGCCACCATCTTGAGAC-3'
TLR4	Forward5'-GGCATCATCTTCATTGCTCTTG-3' Reverse5'-AGCATTGTCTCCCACTCG-3'

were homogenized supersonically in Tris-buffered saline(TBS). After clarification of the lysates by centrifugation, protein concentrations in the supernatant were assayed by Bradford method. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously.<sup>26</sup> Protein samples were subjected to SDS-PAGE and transferred to nitrocellulose (NC) membranes. The blots were blocked in 5% blocking reagent (Amersham Biosciences) in a solution of TBS-T(0.05% Tween-20) for 2 h at room temperature, and then incubated separately with rabbit anti rat FPN1 antiserum (1:5000), DcytB antiserum (1:1000), DMT1 (+IRE) antiserum (1:5000), TfR1 antiserum (1:1000), ferritin antiserum (1:1000), or  $\beta$ -actin antiserum (1:1000) (Abcam, UK) at 4°C overnight. After being washed with TBS-T, the blots were incubated with a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:5000) for 1.5 h at room temperature. Blots were developed with ECL reagents (Pierce Biotechnology). Bands were quantified with densitometry software. Protein contents were normalized to  $\beta$ -actin levels.

### 2.6 | Statistical analysis

The results are presented as the mean  $\pm$  standard deviation (SD). Statistical analysis was carried out using the SPSS statistics package (SPSS, Chicago, IL, USA). Multiple comparisons among the groups were performed using a one-way ANOVA test followed by Fisher's

LSD post hoc test. A probability value of  $p < .05$  was taken to be statistically significant.

### 3 | RESULTS

#### 3.1 | The effect of prazosin on tissular iron levels

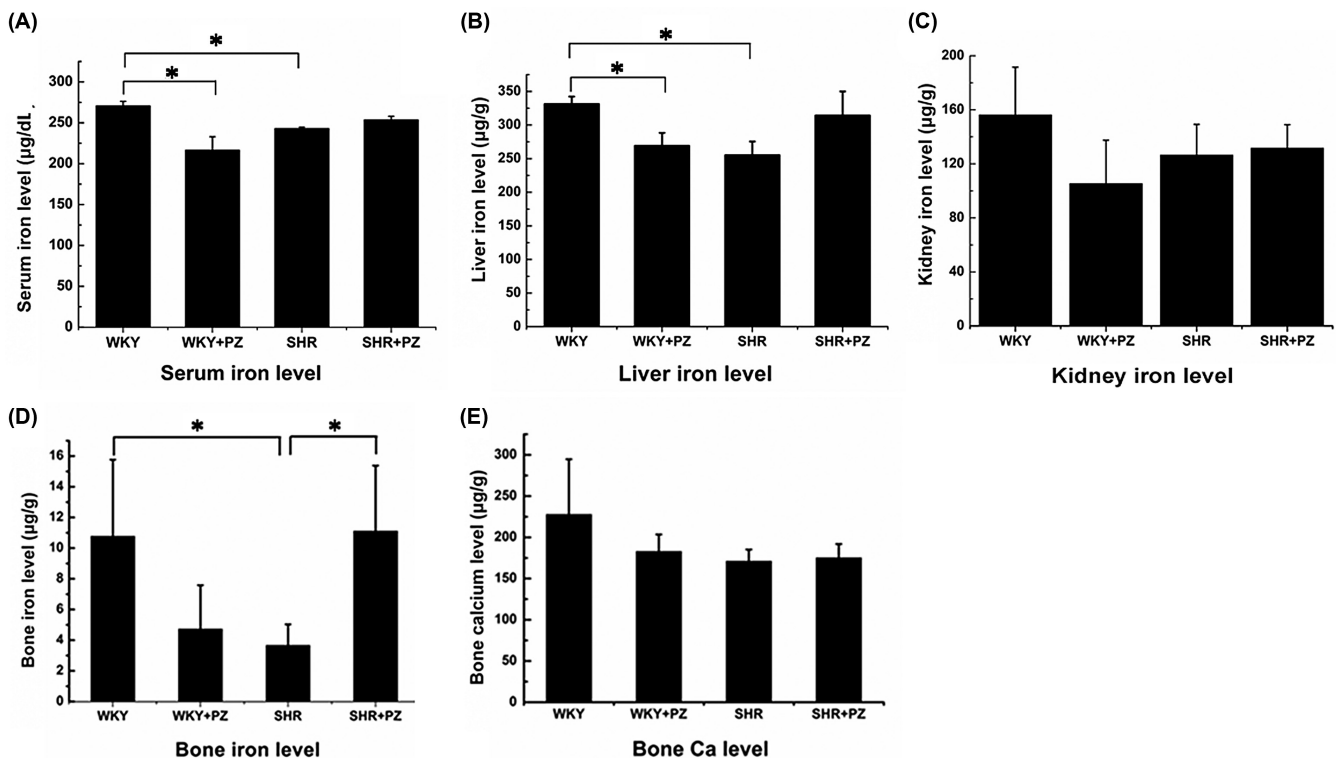
Our results suggest that prazosin interferes with iron homeostasis in WKY rats and SHRs. Compared with the WKY group, a significant decrease in serum and liver iron levels were observed in the WKY+PZ group (Figure 1A and B). Conversely, serum and liver iron levels were slightly increased in the SHR+PZ group compared with the SHR group, though the difference was not significant (Figure 1A and B). With regards to the control groups, markedly lower concentrations of serum and liver iron were seen in the SHR group compared with the WKY group (Figure 1A and B). Although the trend in iron changes was similar in the kidney, no significant differences were found between the four groups (Figure 1C). Importantly, the concentration of iron in the bone tissue was significantly lower in the SHR group than the WKY group (Figure 1D). Treatment with prazosin resulted in a significant increase in bone iron in SHR+PZ rats (Figure 1D); however, there was no significant change in bone calcium levels between the four groups (Figure 1E).

#### 3.2 | Effect of prazosin on hepcidin, ERFE, and EPO mRNA expression

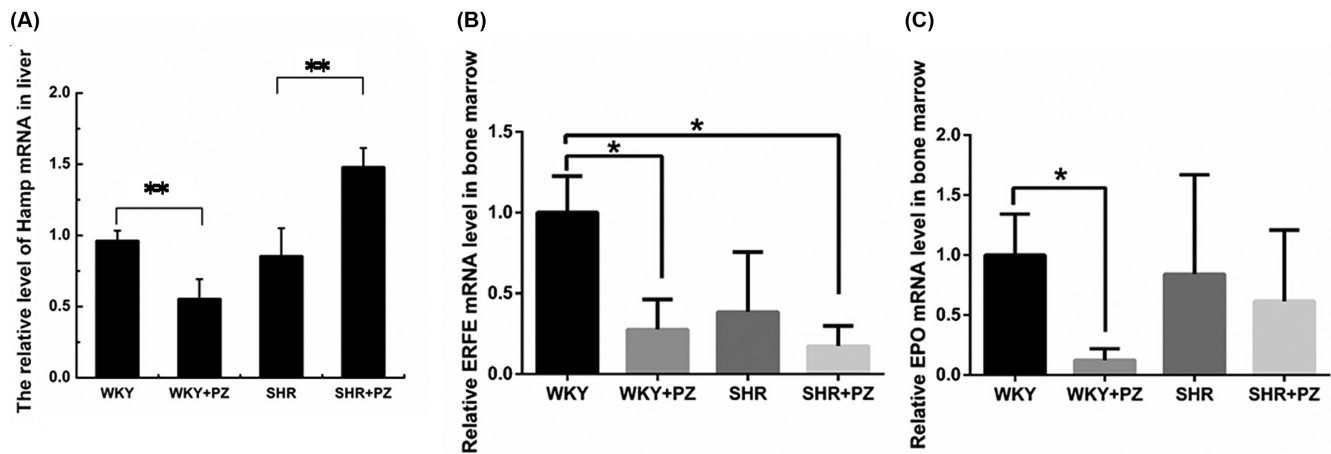
To understand the mechanism of the changes in tissue iron concentrations under prazosin treatment, we examined the level of hepcidin mRNA in the liver by qRT-PCR. Compared with the control groups, a significantly reduced expression of liver hepcidin mRNA was apparent in WKY+PZ rats, while the expression of the mRNA was markedly upregulated in SHR+PZ rats (Figure 2A). However, no difference was found in hepcidin mRNA expression between the WKY and SHR groups (Figure 2A). The hormone, erythroferrone (ERFE), is produced by erythroblasts in response to erythropoietin (EPO) and acts on hepatocytes to suppress hepcidin synthesis.<sup>27</sup> Therefore, we investigated the levels of ERFE and EPO mRNA in the bone marrow; prazosin treatment significantly suppressed the expression of both ERFE and EPO mRNA in WKY rats (Figure 2B–C), while there was no significant effect in the SHRs.

#### 3.3 | Effect of prazosin on the expression of proteins of iron metabolism

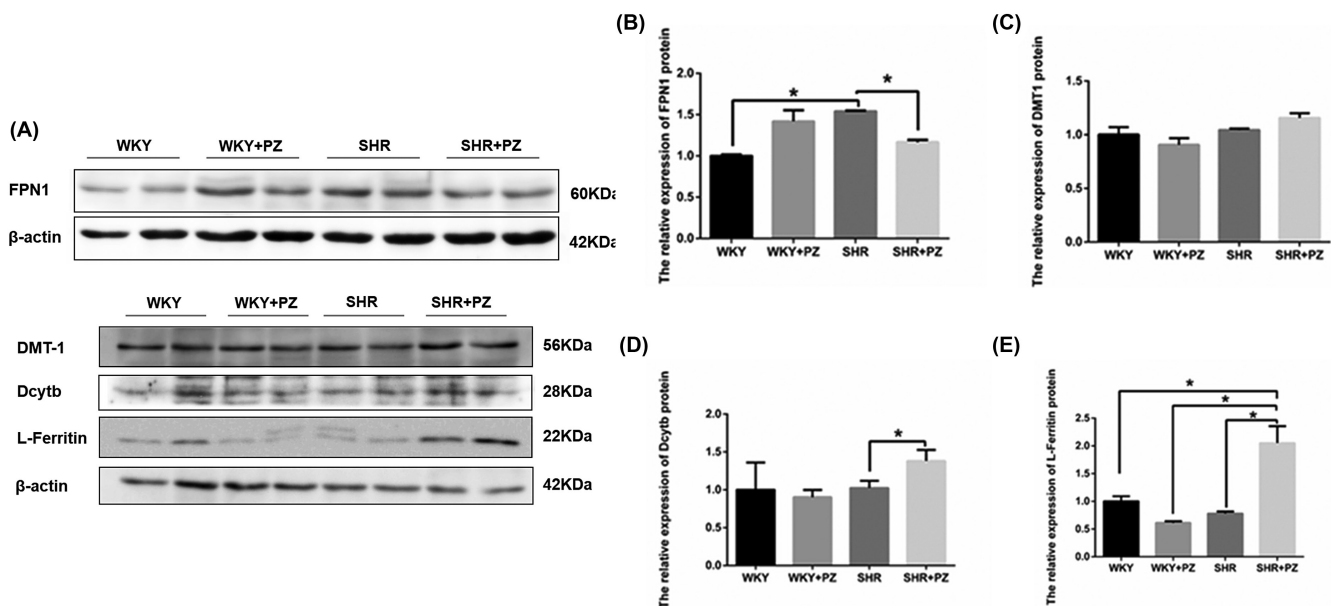
To understand the effects of changes in hepcidin expression in animals treated with prazosin, we assessed the expression of iron metabolism-related proteins in the duodenum, liver, spleen, and



**FIGURE 1** Effects of prazosin on iron levels in serum (A), liver (B), kidney (C), bone (D), and calcium levels in bone tissues (E) of WKY rats and SHRs. WKY: Wistar-Kyoto, PZ: prazosin, SHR: spontaneously hypertensive rat. The results are expressed as the mean  $\pm$  SD,  $n = 6$ . \* $p < .05$ .



**FIGURE 2** Effect of prazosin on hepcidin, ERFE, and EPO mRNA expression was measured by quantitative real-time PCR. (A) hepcidin expression in the liver ( $n = 6$ ). ERFE (B) and EPO (C) mRNA expression in bone marrow ( $n = 4$ ). WKY: Wistar-Kyoto, PZ: prazosin, SHR: spontaneously hypertensive rat. The results are expressed as the mean  $\pm$  SD, \* $p < .05$ , \*\* $p < .01$ .



**FIGURE 3** Effect of prazosin on the expression of FPN1, DMT1+IRE, DcytB, and L-ferritin protein in the duodenal epithelium. The synthesis of FPN1, DMT1+IRE, DcytB and L-ferritin protein was evaluated by western blot analysis. FPN1 (A, B), DMT1+IRE (A, C), DcytB (A, D), and L-ferritin (A, E) protein concentrations in each specimen were normalized to  $\beta$ -actin levels in that specimen. WKY: Wistar-Kyoto, PZ: prazosin, SHR: spontaneously hypertensive rat. The results are expressed as the mean  $\pm$  SD,  $n = 4$ . \* $p < .05$ .

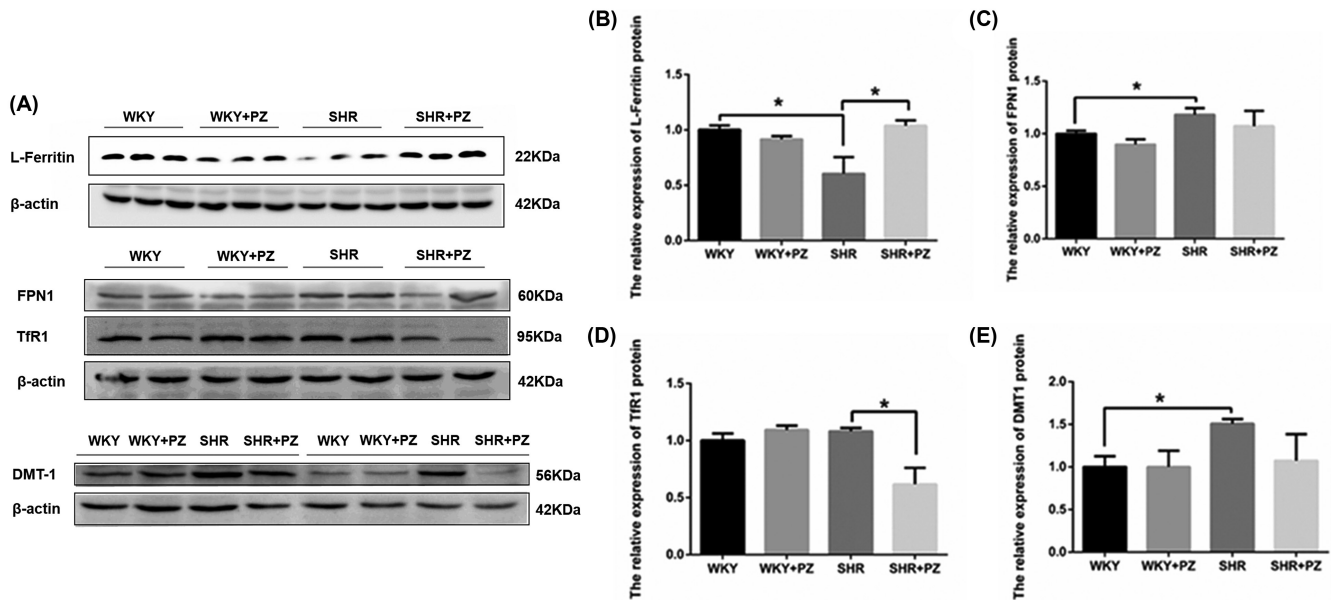
kidney by western blot analysis. Compared with WKY rats, we observed markedly enhanced expression of FPN1 in the duodenum and liver in SHRs (Figures 3A,B and 4A,C), while prazosin treatment led to a significant decrease in the levels of FPN1 in the duodenum and spleen in the SHRs (Figures 3A,B and 5A,C). We also found that SHRs exhibited significantly increased expression of DMT1+IRE in the liver (Figure 4A,E). Treatment of the SHRs with prazosin resulted in lower levels of DMT1+IRE in the spleen (Figure 5A,D). The ubiquitous iron-storage protein, ferritin, plays a crucial role in iron metabolism. Compared with the SHR group, we found markedly higher protein levels of L-ferritin in the duodenum and liver in the SHR+PZ group (Figures 3A,E and 4A,B). However, the expression of

L-ferritin was significantly diminished in the spleen of SHR+PZ rats (Figure 5A,B). Moreover, prazosin up-regulated DcytB protein levels in the duodenum, whereas the drug markedly suppressed the expression of Tfr1 in the liver in SHRs (Figures 3A,D and 4A,D). There were no significant differences in Tfr1 and FPN1 expression in the kidney between the four groups (Figure 6A-C).

### 3.4 | Effect of prazosin on osteoclast activity

To examine whether prazosin affects bone resorption, we examined the effects of prazosin on the expression of osteoclast-associated





**FIGURE 4** Effect of prazosin on the expression of L-ferritin, FPN1, Tfr1, and DMT1+IRE protein in the liver. The synthesis of L-ferritin, FPN1, Tfr1, and DMT1+IRE protein was evaluated by western blot analysis. L-ferritin (A, B), FPN1 (A, C), Tfr1 (A, D), and DMT1+IRE (A, E) protein concentrations in each specimen were normalized to  $\beta$ -actin levels in that specimen. WKY: Wistar-Kyoto, PZ: prazosin, SHR: spontaneously hypertensive rat. The results are expressed as the mean  $\pm$  SD,  $n = 4$ . \* $p < .05$ .

genes by qRT-PCR. Compared with the WKY group, we observed markedly enhanced expression of TLR4 in the SHR group (Figure 7E,  $p < .05$ ). It has been suggested that CIC-7 supports bone resorption by acidifying the resorption lacuna of OCs.<sup>28</sup> The mRNA levels of CIC-7 in the SHR group were significantly lower than those in the WKY group (Figure 7D). Importantly, our results demonstrate a suppression of the mRNA levels of Ctsk, CLC-7, and tartrate-resistant acid phosphatase (Trap) in WKY rats treated with prazosin (Figure 7A,C-D). In addition, prazosin-treated SHRs exhibited decreased expression of Ctsk and TLR4 mRNA, although the inhibition was not statistically significant (Figure 7A and E).

### 3.5 | Effect of prazosin on osteoblast activity

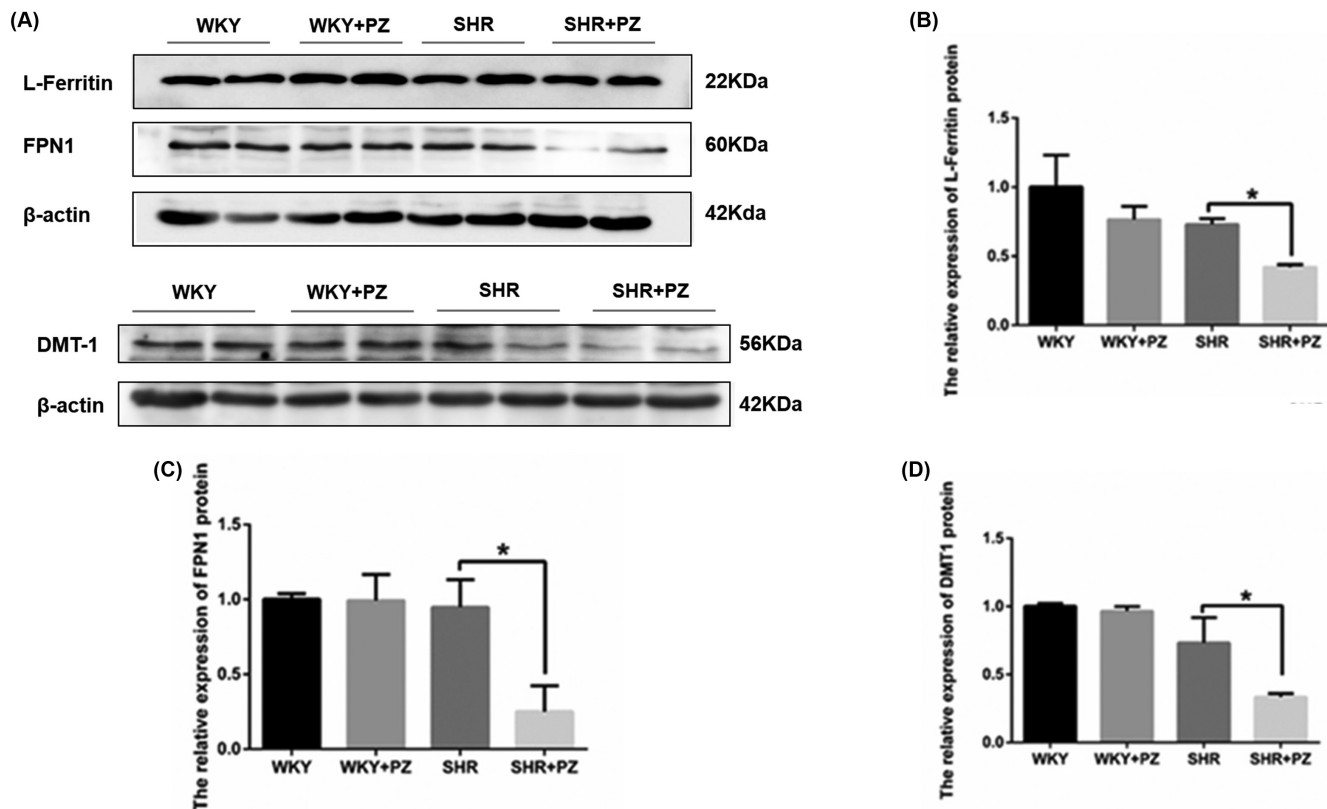
We evaluated the expression levels of the osteoblast differentiation markers, ALP, osteocalcin (OCN), and collagen I (Col I), by qRT-PCR. The mRNA levels of ALP in the SHR group were lower than those in the WKY group, however without statistical significance (Figure 8A). The mRNA levels of ALP and OCN were elevated in SHRs after prazosin treatment, although the enhancement was also not statistically significant (Figure 8A-B).

## 4 | DISCUSSION

Essential hypertension is a multifactorial disease that accounts for 95% of hypertensive patients.<sup>8</sup> The effects of the hypertensive state and anti-hypertensive treatments on iron metabolism have not been rigorously explored.<sup>29</sup> In the present study, we found significantly

lower levels of serum and liver iron in SHRs compared with WKY rats, indicating that hypertension is associated with systemic low-iron status. These results are consistent with a previous study showing that serum iron levels were decreased in SHRs compared with normotensive animals.<sup>30</sup> The hypotensive drug, prazosin, caused slight increases in iron concentration in the serum and liver of SHRs, but marked decreases in WKY rats. Suliburska et al. also found that antihypertensive drugs, perindopril, induced a higher concentration of liver iron in SHRs.<sup>4</sup> Overall, these findings suggest that both hypotension and hypertension lead to iron deficiency in the serum and liver of rats. Hypotensive treatment was able to restore the iron homeostasis in the body under hypertension. The exact underlying mechanism by which the hypotensive process induces iron changes remains unclear.

Systemic iron homeostasis is maintained through the regulation of iron absorption and recycling.<sup>31</sup> The intestinal epithelium acts as the key modulator of iron absorption in the body. Ferric iron in the intestinal lumen can be reduced by DcytB to its ferrous form, and then entered the enterocyte cytosol through DMT-1.<sup>32</sup> Cytoplasmic ferrous iron can then either be stored in ferritin or exported through the basolateral transporter, FPN1, the only known cellular iron exporter.<sup>33</sup> Ferrous iron is then converted to ferric iron by hephaestin, making it available to bind transferrin in the circulation.<sup>32</sup> We therefore examined the expression of DcytB, DMT1, FPN1, and ferritin in the duodenum. SHRs exhibited increased expression of FPN1 protein, and decreased expression of L-ferritin protein, compared with WKY rats, indicating increased system iron absorption phenotype in duodenal enterocytes. A similar mechanism could also explain the changes in FPN1 and L-ferritin expression as a result of prazosin treatment in WKY rats. Following treatment with prazosin,



**FIGURE 5** Effect of prazosin on the expression of L-ferritin, FPN1 and DMT1+IRE protein in the spleen. The synthesis of L-ferritin, FPN1, and DMT1+IRE protein was evaluated by western blot analysis. L-ferritin (A, B), FPN1 (A, C), and DMT1+IRE (A, D) protein concentrations in each specimen were normalized to  $\beta$ -actin levels in that specimen. WKY: Wistar-Kyoto, PZ: prazosin, SHR: spontaneously hypertensive rat. The results are expressed as the mean  $\pm$  SD,  $n = 4$ . \* $p < .05$ .

the expression of FPN1 in the duodenum of SHRs decreased, while the expression of DcytB and L-ferritin increased, suggesting that iron absorption capacity was decreased in the duodenum. This may be a downstream effect of the increased levels of serum and liver iron elicited by prazosin.

A major role for the spleen is turnover of iron from senescent red blood cells via macrophages of the reticuloendothelial system.<sup>34</sup> We found that the splenic expression of FPN1, DMT1, and L-ferritin in SHRs exhibited no significant changes compared with WKY rats. However, hypotensive treatment with prazosin markedly inhibited the expression FPN1, indicating that the release of iron from the spleen was suppressed in SHRs. It is notable that prazosin did not lead to significant changes in splenic iron-metabolism in WKY rats.

The above analysis indicates changes in FPN1, which acts as a key factor in iron regulation. We therefore explored the mechanisms of FPN1 expression changes that accompany the variations in blood pressure. Heparin is the key iron-regulatory hormone. This peptide is synthesized in the liver and is feedback regulated by extracellular iron levels.<sup>25</sup> Heparin activity causes the internalization and degradation of FPN1 from the cell surface of enterocytes and macrophages, thus inhibiting cellular iron efflux to ultimately decrease serum iron concentrations.<sup>35</sup> The present results show that essential hypertension does not markedly alter the expression of hepcidin mRNA. Interestingly, prazosin led to noticeable but opposite effects

on hepcidin expression in SHRs and WKY rats, suggesting that the mechanisms involved in hepcidin regulation in these models may differ. We previously demonstrated that prazosin treatment decreases the expression of hepatic hepcidin in hypertensive mice via adrenaline and noradrenaline, both of which exert their actions through  $\alpha$ 1-adrenergic receptors.<sup>25</sup> These results are in contrast to the findings in the present study. We conjecture that prazosin acts differently on hepcidin expression in hypertension models of different etiologies.

In response to EPO, erythroblasts produce ERFE, which down-regulates hepatic hepcidin expression.<sup>27</sup> We investigated the expression of EPO and ERFE mRNA in bone marrow and found no significant difference between the WKY and SHR groups. Although prazosin treatment suppressed the expression of EPO and EREE in SHRs, the inhibitory effects were not remarkable. However, the expression of both EPO and EREE mRNA was significantly inhibited following treatment of WKY rats with prazosin. Based on the mechanism of ERFE inhibition of hepcidin expression, not possible for us to conclude that ERFE was involved in the regulation of hepcidin in SHRs or prazosin-treated SHRs. Therefore, the specific mechanism of the altered regulation of iron metabolism in the SHR+prazosin group remains to be further explored.

It has been reported that the long bones of SHRs displays marked reductions in trabecular BMD, cortical thickness, and total cross-sectional area, all of which may be due to excessive bone

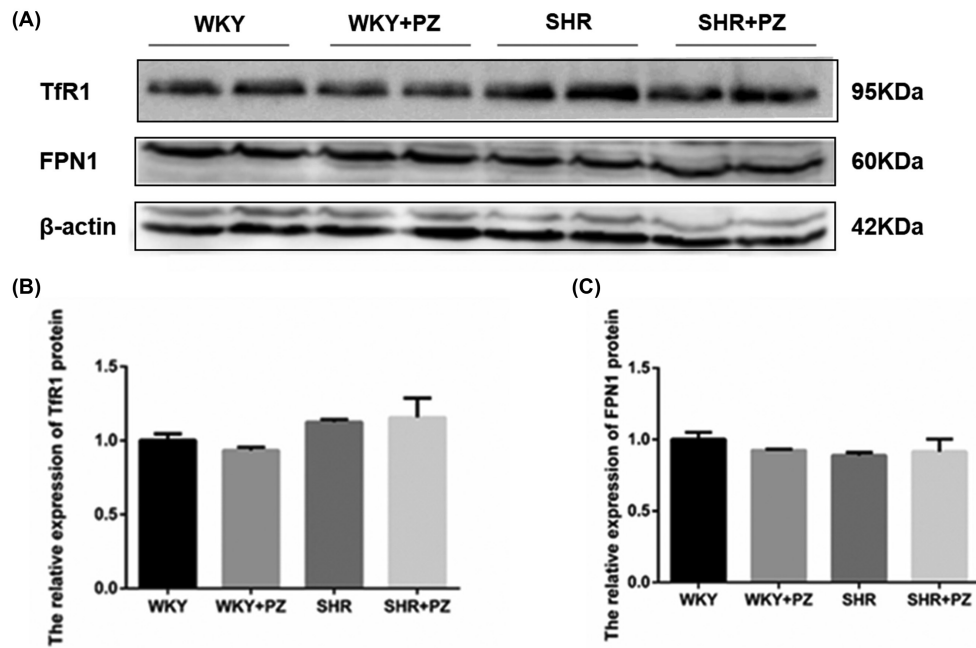


FIGURE 6 Effect of prazosin on the expression of TfR1 and FPN1 protein in the kidney. The synthesis of TfR1 and FPN1 protein was investigated by western blot analysis. TfR1 (A, B) and FPN1 (A, C) protein concentrations in each specimen were normalized to  $\beta$ -actin levels in that specimen. WKY: Wistar-Kyoto, PZ: prazosin, SHR: spontaneously hypertensive rat. The results are expressed as the mean  $\pm$  SD,  $n = 4$ .

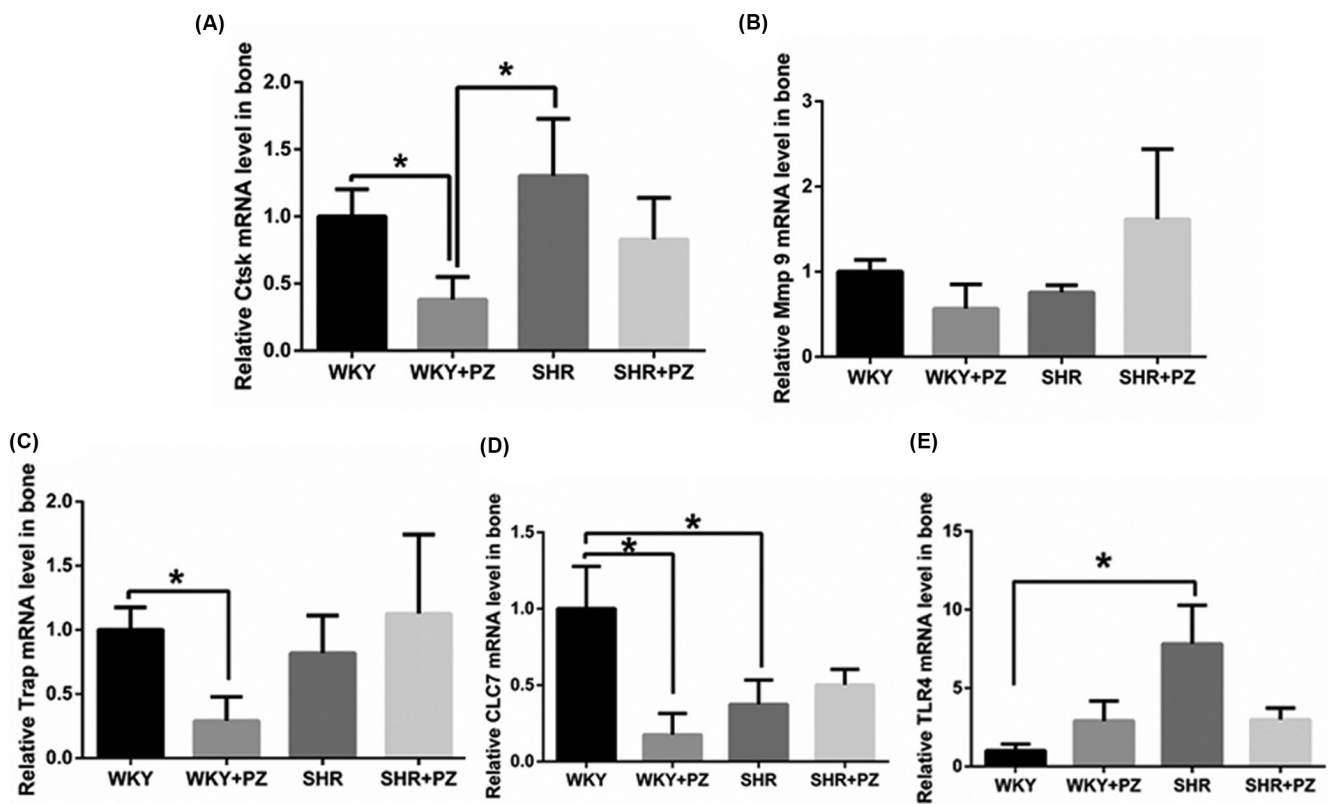
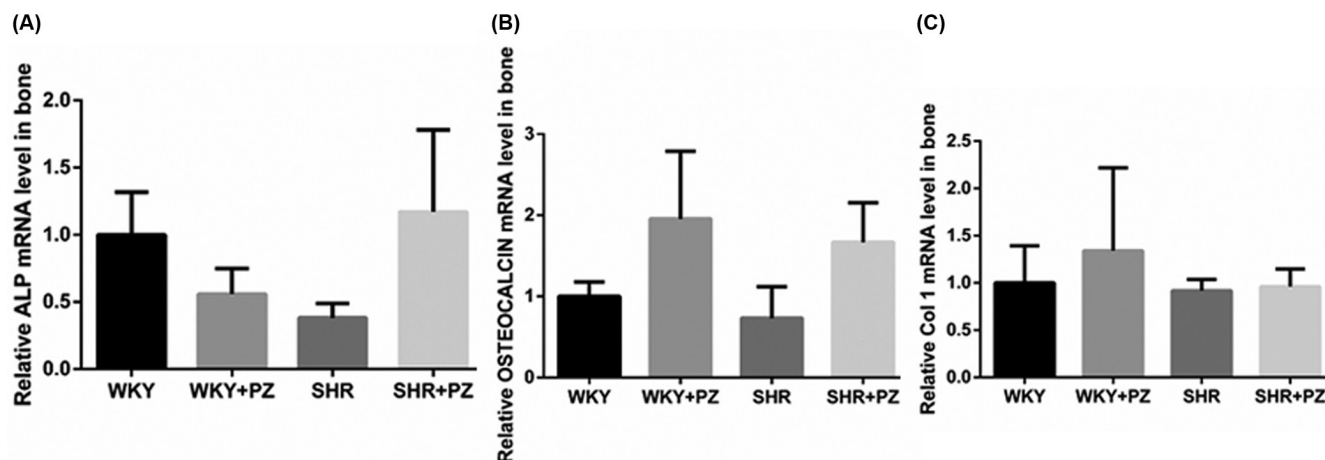


FIGURE 7 Effect of prazosin on mRNA levels of genes relates to osteoclast activity in bone tissue. Gene expression of Ctsk (A), Mmp9 (B), Trap (C), CLC7 (D), and TLR4 (E) in the bone tissue was assessed using quantitative real-time PCR. WKY: Wistar-Kyoto, PZ: prazosin, SHR: spontaneously hypertensive rat. The results are expressed as the mean  $\pm$  SD,  $n = 4$ . \* $p < .05$ .





**FIGURE 8** Effect of prazosin on mRNA levels relating to osteoblast activity in bone tissue. Gene expression of ALP (A), Osteocalcin (B), and Col1 (C) in the bone tissue was measured using quantitative real-time PCR. WKY: Wistar-Kyoto, PZ: prazosin, SHR: spontaneously hypertensive rat. The results are expressed as the mean  $\pm$  SD,  $n = 4$ .

resorption at the cellular level.<sup>14</sup> In this study, we used atomic absorption spectrometry to determine the iron and calcium content in bone tissues. Compared with WKY rats, a lower level of bone calcium was observed in 12-week-old SHRs, although this finding was not statistically significant. In addition, the bone iron concentration was significantly decreased in the SHR group, compared with the WKY rats. Prazosin treatment did not change the bone calcium level of SHRs, but markedly enhanced the iron levels. However, when prazosin was used to treat WKY rats, the bone iron was found to be significantly decreased, and the total calcium content also showed a downward trend. The exact underlying mechanisms by which antihypertensive drugs induce variations in bone iron and bone calcium remain unclear.

Maintenance of the bone mass depends on a balance of osteogenesis and bone resorption by osteoclasts. By examining the presence of osteoclastic genes, we found that the expression of TLR4 was significantly increased and Ctsk presented an increasing tendency in the bone tissues of SHRs, suggesting that the essential hypertension stimulated osteoclast activity. Chloride channel-7 (CLC-7) is involved in the acidification of the resorption lacuna.<sup>28</sup> The expression of CLC-7 in SHRs was significantly decreased, which may be responsible, at least in part, for the disruption of normal bone structure in hypertension. When treated with prazosin, although the expression of Ctsk and TLR4 decreased, no statistical difference was found compared with the non-treatment group, which might be due to the small sample size. Following antihypertensive treatment of WKY rats, the expression of Ctsk and Trap significantly decreased suggesting that the activity of osteoclasts was suppressed in the WKY+PZ group. As regards the osteogenic genes, the expression of ALP was decreased, but without significance in SHRs. Prazosin treatment enhanced the expression of ALP and OCN genes suggesting that the abnormal bone metabolism was alleviated to some extent. Our results also show that prazosin had no significant effect on osteogenic genes in rats with normal blood pressure.

Our study has some limitations. Although our data clarified the effects of prazosin on the iron metabolism in various tissues of SHRs and WKY rats, whether antihypertensive drugs, with different mechanisms of action, may cause similar effects on systemic iron status is unknown. In subsequent studies, we will continue to assess the influence of other antihypertensive drugs and combination treatment on iron homeostasis in rats. Secondly, our study found that essential hypertension disturbed iron homeostasis and caused changes in the activity of osteoblasts and osteoclasts. Further investigation is warranted to address whether the changes of osteoblast and osteoclast activity in SHRs correlates with the variations in iron metabolism, and subsequently elucidate the underlying mechanisms.

#### AUTHOR CONTRIBUTIONS

Hengrui Chang contributed to literature review, the data analysis, wrote the paper and editing. Dong Zhang analyzed and performed the experiments. Zhen Xin performed the experiments. Pengfei Zhang performed the experiments. Yan-Zhong Chang helped with study design and drafted the work; Wenyan Ding performed analysis, substantively revised the paper. All authors approved the final manuscript.

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#### CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

#### HUMAN AND ANIMAL RIGHTS AND INFORMED CONSENT

The experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of

Laboratory Animals, and were approved by the Animal Care and Use Committee of Hebei Science and Technical Bureau in China.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## ORCID

Yan-Zhong Chang  <https://orcid.org/0000-0002-6386-9649>

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