Original Article

Angiotensin II–Induced Regulation of the Expression and Localization of Iron Metabolism–Related Genes in the Rat Kidney

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Due to recent discoveries of novel genes involved in iron metabolism, our understanding of the molecular mechanisms underlying iron metabolism has dramatically increased. We have previously shown that the administration of angiotensin II alters iron homeostasis in the rat kidney, which may in turn aggravate angiotensin II–induced renal damage. Here we have investigated the effect of angiotensin II administration on the localization and expression of transferrin receptor (TfR), divalent metal transporter 1 (DMT1), ferroportin 1 (FPN), and hepcidin mRNA in the rat kidney. Weak expression of TfR, DMT1, FPN, and hepcidin mRNA was observed in the kidneys of control rats. In contrast, after 7 days of angiotensin II infusion by osmotic minipump, the expression of these mRNAs was more widely distributed. Staining of serial sections revealed that some, but not all, of the renal tubular cells positive for these genes contained iron deposits in the kidney of angiotensin II–infused animals. Real-time polymerase chain reaction (PCR) showed that the mRNA expression of TfR, iron-responsive element–negative DMT1, FPN, and hepcidin mRNA increased ~1.9-fold, ~1.7-fold, ~2.3-fold, and ~4.7-fold, respectively, after angiotensin II infusion as compared with that of untreated controls, and that these increases could be suppressed by the concomitant administration of losartan. Our data demonstrate that these genes were unequivocally expressed in the kidney and could be regulated by angiotensin II infusion. The relative contribution, if any, of these genes to renal and/or whole-body iron homeostasis in various disorders in which the renin angiotensin system is activated should be investigated in future studies. (*Hypertens Res* 2007; 30: 195–202)

***Key Words*: angiotensin II, iron metabolism, hypertension, gene regulation**

Introduction

Iron is an essential element that is required for fundamental cell functions in all living organisms. On the other hand, excess body iron is potentially harmful because of its ability to catalyze the conversion of hydrogen peroxide to toxic free radicals. Thus, maintaining an appropriate balance of iron in the body is important. Recently, our understanding of the mechanisms underlying iron metabolism has dramatically increased due to discoveries of novel genes related to iron metabolism. Divalent metal transporter 1 (DMT1), also referred to as natural resistance–associated macrophage protein 2 (Nramp2) or divalent cation transporter (DCT1), is a

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12-segment transmembrane-spanning integral membrane protein (*1*) that is expressed in the duodenum, where it transports divalent metals across the apical membrane of enterocytes. A G185R point mutation of this gene causes microcytic hypochromic anemia in rodents (*2*, *3*), thus indicating the fundamental role played by this gene in iron homeostasis. Ferroportin 1 (FPN), also referred to as metal transporter protein (MTP1) or iron-regulated protein 1 (Ireg1), is a multiple transmembrane-spanning protein that transports iron out of cells. FPN is expressed strongly in the basolateral region of absorptive duodenal enterocytes and in tissue macrophages in the liver, *i.e.*, Kupffer cells (*4*, *5*). Hepcidin (hepc) is a recently discovered cysteine-rich 25-amino acid peptide that has antimicrobial properties and also acts as a negative regulator of intestinal iron absorption and macrophage iron release, and its overproduction may contribute to the anemia associated with inflammation (*6*). Hepc was initially isolated from human urine (*6*); however, it is mainly produced in the liver, from where it is released into the systemic circulation. Binding of hepc to the iron exporter FPN leads to the internalization and degradation of FPN (*7*, *8*); it is presumably by this mechanism that hepc functions as a negative regulator of intestinal iron absorption.

In general, the kidney is not considered to be the major expression site of these newly discovered iron metabolism–related genes. Although expression of the transferrin receptor (TfR) (*9*, *10*), DMT1 (*11*, *12*), FPN (*13*), and hepc (*14*) has been demonstrated in the kidney, information about the physiological importance and the regulation of these genes has been limited to date. We previously reported that the administration of angiotensin II to rats causes prominent iron deposition in the kidney, which occurs primarily in the proximal tubular epithelial cells; this deposition is thought to be associated with increased proteinuria and the upregulation of fibrosis-related genes (*15*, *16*). Here, we have characterized the renal expression patterns of these iron metabolism–related genes and investigated how their expression might be regulated by angiotensin II in the kidney.

Methods

Generation of Animal Models

The experiments were performed in accordance with the guidelines for animal experimentation approved by the Animal Center for Biomedical Research, Faculty of Medicine, University of Tokyo. Angiotensin II was continuously infused into male Sprague-Dawley rats by subcutaneous implantation of an osmotic minipump (Alzet model 2001; Alza Pharmaceutical, Mountain View, USA) as described previously. In brief, Val5-angiotensin II was infused at doses of 0.7 mg/kg/day for 7 days by subcutaneously implanted osmotic minipumps (Alza Pharmaceutical) which exerted hypertensive effects (192±5 mmHg [$n=12$], $p<0.01$ *vs.* control rats, 131±3 mmHg [$n=6$]). In some experiments, the selective angiotensin type 1 (AT_1) receptor antagonist, losartan (25 mg/kg/day) or the nonspecific vasodilator, hydralazine (15 mg/kg/day) (Sigma Chemical, St. Louis, USA) was given in the drinking water, beginning 2 days before pump implantation and throughout angiotensin II infusion (angiotensin II+losartan, 126±5 mmHg [$n=7$]; angiotensin II+hydralazine 126±3 mmHg [$n=7$]). In some experiments, norepinephrine was infused at a dose (2.8 mg/kg/day) which exerted hypertensive effects (192±4 mmHg) comparable to those of angiotensin II. Systolic blood pressure was measured in conscious rats by tail-cuff plethysmography (Ueda Seisakusho, Tokyo, Japan).

In Situ Hybridization, Histological Analyses

Rat cDNAs corresponding to rat sequences of TfR, DMT1, FPN1, and hepc were obtained by subcloning the reverse-transcription (RT)–polymerase chain reaction (PCR) product using rat kidney mRNA. Sense and antisense primers were as follows: 5′-TACCTGTGCAGACGATCTCAAGAG-3′ and 5′-AGGACGACTTTATCCAGATTAAT-3′, respectively, for TfR; 5′-CTACCTGGATCCAGGAAACATT-3′ and 5′-AAGTACTTATTGGCTTCTCGAA-3′, respectively, for DMT1; 5′-AGACCCCTGCTCTGGCT GTA-3′ and 5′-AGA CACATTAGCATAAGCAT-3′, respectively, for FPN; and 5′-GGCAGGACAGAAGGCAAGAT-3′ and 5′-GGTAGG ACAGGAATAAATAAT-3′, respectively, for hepc. The sequence targeted for the amplification of DMT1 was a common region of DMT1 with or without iron-responsive element (IRE), which was designated here as IRE(+)DMT1 and IRE(−)DMT1, respectively. Rat cDNA corresponding to these iron metabolism–related genes were subcloned into a pGEM-T vector, and then *in situ* hybridization was performed as described previously (*17*). After digestion with a restriction enzyme and linearization of the plasmid, antisense and sense cRNA riboprobes were transcribed *in vitro* using the DIG RNA labeling Kit SP6/T7 (Roche Diagnostics, Basel, Switzerland). Hybridization was performed using *In Situ* Hybridization Reagents (Nippon Gene, Tokyo, Japan). *In situ* hybridization was performed on either formalin-fixed specimens or un-fixed frozen specimens. Prussian blue staining was used for iron staining, and Oil red O staining was used to detect the accumulation of lipid in unfixed frozen tissue sections.

RNA Extraction, Northern Blot Analysis, and Real Time RT-PCR

Total RNA was isolated from homogenized aorta by the acid guanidinium thiocyanate–phenol chloroform method as described previously (*18*). To investigate the mRNA expression by quantitative PCR with gene-specific HybriProbes was performed by LightCycler (Roche Diagnostics). The following respective sense and antisense primers were used: 5′-AAGTCCTGCTGAGCGAAGAT-3′ and 5′-TGGTCCCTA

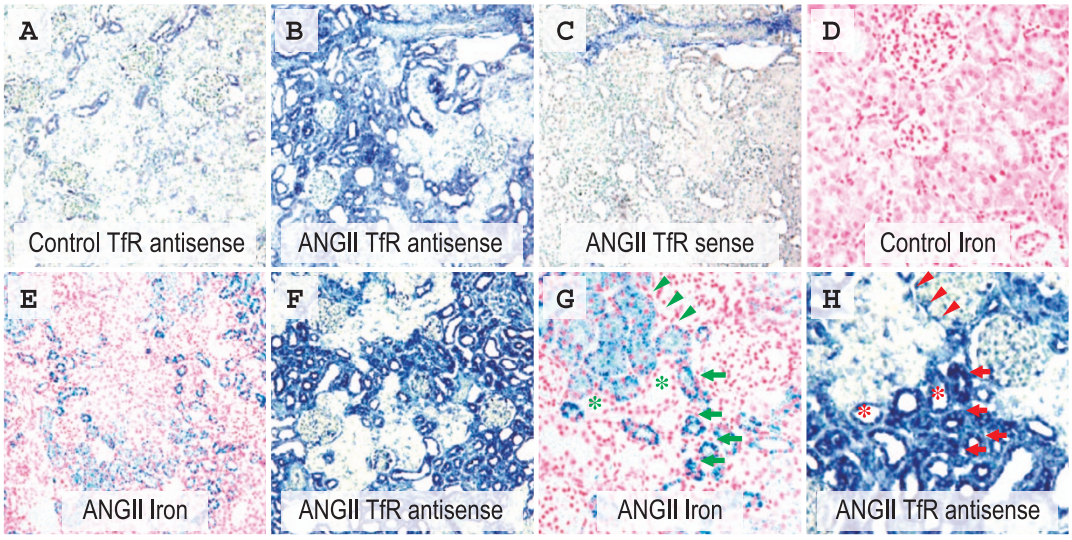

Fig. 1. *Expression and localization of TfR mRNA. Samples were from the kidney of control (A, D) and angiotensin II (ANGII)–infused (B, C, E–H) rats. B-C, E-F, and G-H are serial specimens. A, B, F, H:* In situ *hybridization using the TfR antisense probe. C:* In situ *hybridization using the TfR sense probe (background). D, E: Prussian blue staining for iron. TfR mRNA staining was weak in control rat kidney (A). After angiotensin II infusion, TfR mRNA expression became more widely distributed in the tubular and glomerular cells (B). No staining for iron was observed in the untreated control rat kidney (E). Localization of iron deposits (E) and TfR mRNA (F) showed overlap, although not completely. Higher magnification microscopy showed that some tubular cells that were positive for iron were also positive for TfR mRNA (G, H, arrows). By contrast, some tubular cells that were positive for iron deposition were negative for TfR (G, H, arrowheads), and* vice versa *(G, H, asterisks). Original magnifications, × 100 (A–C, E, F) and × 200 (D, G, H).*

AATGCAGTCTG-3′ for IRE(+)DMT1; 5′-TCTACCTCC TGAACACCGTG-3′ and 5′-CGTTAGCTTTACCCGACT CC-3′ for IRE(–)DMT1; 5′-CCAGATTATGACATTCGGT-3′ and 5′-TTGGCTCAGTATCTTTAGGT-3′ for FPN; and 5′-GGCAACAGACGAGACAGACT-3′ and 5′-ATGCAA CAGAGACCACAGGA-3′ for hepc. The primers used for TfR and GAPDH have been described previously (*19*). The mRNA expression of these genes was normalized to GAPDH mRNA expression and is presented here as the percentage of the values from the aortas of untreated animals.

Statistical Analysis

Data are expressed as the mean±SEM. We used ANOVA followed by a multiple comparison test to compare raw data, before we expressed the results as a percentage of the control value using statistical analysis software, StatView ver. 5.0 (SAS Institute, Cary, USA). A value of $p<0.05$ was considered to be statistically significant.

Results

Localization of the Expression of Iron Metabolism–Related Genes

In situ hybridization revealed that TfR mRNA was weakly expressed, primarily in the tubular and glomerular cells in the kidneys of untreated animals (Fig. 1A). After angiotensin II treatment, TfR mRNA expression was more widely distributed in these regions (Fig. 1B, C). As we reported previously (*15*), angiotensin II infusion led to iron deposition, primarily in the proximal tubular epithelial cells, as detected by Prussian blue staining (Fig. 1D, E). Staining of serial specimens showed the possible overlap of iron and TfR staining (Fig. 1E, F). Higher magnification microscopy showed that levels of TfR mRNA expression were also increased in the glomerular cells and that some TfR-positive cells were positive for iron (Fig. 1G, H, arrows), whereas some iron-positive cells were negative for TfR (Fig. 1G, H, arrowheads), and some TfR-positive cells were negative for iron (Fig. 1G, H, asterisks). DMT1 mRNA expression could be observed in the tubular and glomerular cells in the untreated rat kidney and was markedly increased after angiotensin II infusion (Fig. 2A–C). Similar to TfR mRNA expression, some tubular cells were positive for both iron and DMT1 (Fig. 2D, E, arrows), whereas others were positive for iron but negative for DMT1 (Fig. 2D, E, arrowheads) or *vice versa* (Fig. 2D, E, asterisks). Staining for FPN mRNA was very weak in the control kidney (Fig. 2F), but was substantially increased after angiotensin II infusion (Fig. 2G, H). Some tubular cells were positive for both iron and FPN (Fig. 2I, J, arrows), whereas others were positive for iron but negative for FPN (Fig. 2I, J, arrowheads)

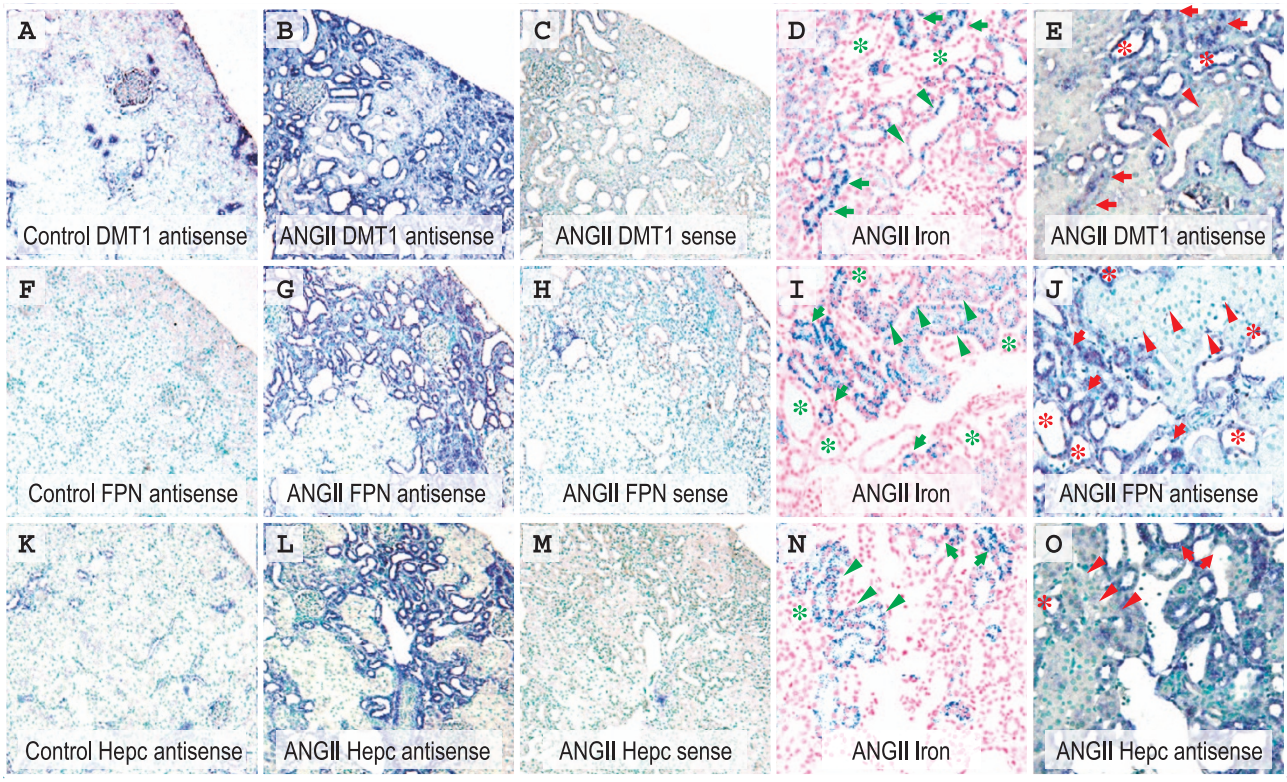

Fig. 2. *Expression and localization of DMT1, FPN, and hepc mRNA and iron deposits. Samples were from the kidney of control (A, F, K) and angiotensin II (ANGII)–infused (B–E, G–J, L–O) rats. B-C, D-E, G-H, I-J, L-M, and N-O are serial specimens. A, B, E:* In situ *hybridization using the DMT1 antisense probe. C:* In situ *hybridization using the DMT1 sense probe (background). F, G, J:* In situ *hybridization using the FPN antisense probe. H:* In situ *hybridization using the FPN sense probe (background). K, L, O:* In situ *hybridization using the hepc antisense probe. M:* In situ *hybridization using the hepc sense probe (background). D, I, N: Prussian blue staining for iron. mRNA expression of DMT1, FPN, and hepc mRNA was more widely distributed after angiotensin II infusion (A, B, F, G, K, L). As in the case of TfR mRNA, some tubular cells that were positive for iron were also positive for DMT1, FPN, or hepc mRNA (D, E, I, J, N, O, arrows). By contrast, some tubular cells that were positive for iron deposition were negative for TfR (D, E, I, J, N, O, arrowheads), and* vice versa *(D, E, I, J, N, O, asterisks). Original magnifications, × 100 (A–C, F–H, K–M) and × 200 (D, E, I, J, N, O).*

or *vice versa* (Fig. 2I, J, asterisks). Hepc mRNA was also expressed weakly in the tubular and glomerular cells in the untreated control rat kidney, and it was also upregulated by angiotensin II (Fig. 2K–O).

Quantification of Iron Metabolism–Related Gene Expression in the Kidney

Real time RT-PCR showed a ~1.9-fold increase in TfR mRNA expression after the infusion with angiotensin II; this increase was suppressed by both hydralazine and losartan (Fig. 3A). The expression of IRE(−)DMT1 mRNA also increased after angiotensin II infusion, which was suppressed by both hydralazine and losartan (Fig. 3B). IRE(+)DMT1 expression was not significantly increased by angiotensin II infusion (Fig. 3C). The expression of FPN mRNA was increased by angiotensin II; this increase was not affected by hydralazine, but was suppressed by losartan. Hepc mRNA expression showed more than a four-fold increase after angiotensin II infusion; this increase was suppressed by both hydralazine and losartan (Fig. 3E). Norepinephrine infusion increased the levels of expression of hepc mRNA, but not those of TfR, IRE(−)DMT1, IRE(+)DMT1, or FPN.

Comparison of the Localization of Lipid Deposits and Iron Metabolism–Related Gene mRNA

We previously found that angiotensin II infusion causes a marked accumulation of lipids in the tubular epithelial cells, and this lipid deposition co-localized with the expression of transforming growth factor-β1 (TGF-β1) mRNA (*17*). We therefore characterized the localization of lipid deposition in relation to the expression of iron metabolism–related genes (Fig. 4). Only a small fraction of TfR, DMT1, and hepc mRNA was found to co-localize with lipid deposition in the angiotensin II–treated rat kidney. By contrast, there was con-

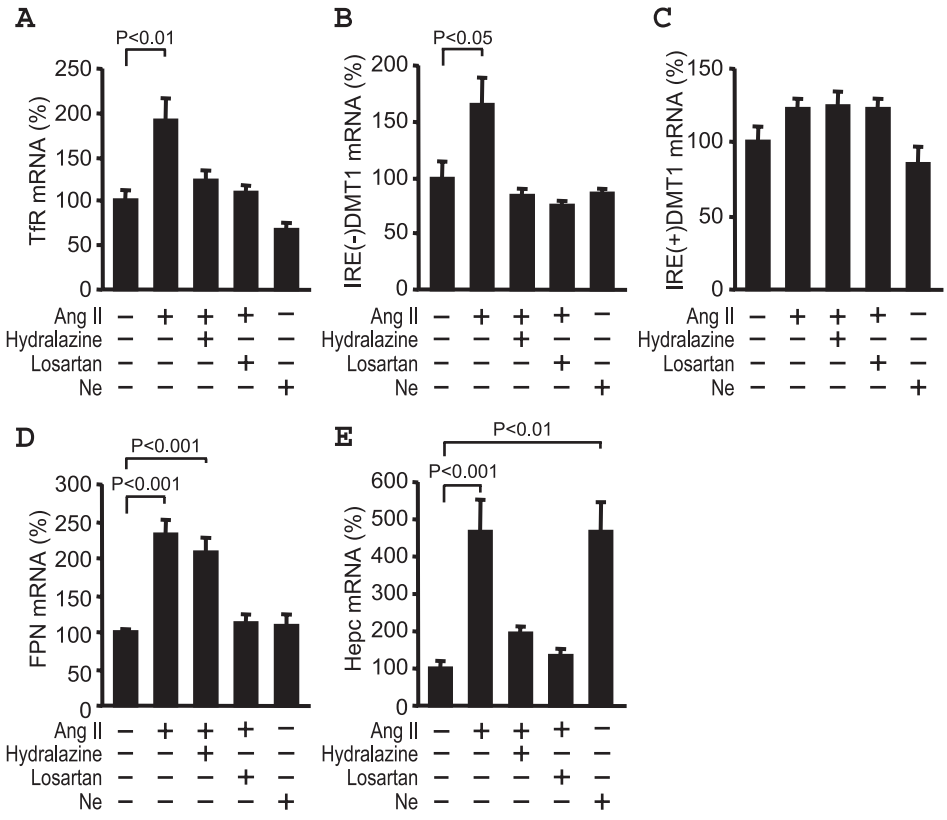

Fig. 3. *Quantification of the expression of iron metabolism–related genes. Expression of TfR (A), IRE(–)DMT1 (B), IRE(+)DMT1 (C), FPN (D), and hepc (E) mRNA was analyzed by quantitative PCR using the HibriProbe system and a LightCycler (Roche). Shown is a summary of the results from quantitative RT-PCR from 6 to 10 samples. Ne, norepinephrine.*

siderable co-localization of FPN mRNA and lipid particles (Fig. 4E, F).

Discussion

Here we have investigated the expression patterns of several iron metabolism–related genes and their regulation by angiotensin II at the mRNA level. We found that the expression of all genes tested (TfR, IRE(–)DMT1, FPN, hepc), with the exception of IRE(+)DMT1, was upregulated at the mRNA level by angiotensin II infusion. Angiotensin II infusion induced slightly different regulatory effects according to the genes tested, in terms of localization and dependency on hypertension *per se*. The causal or resultant relationship between iron deposition and regulation of the expression of these genes remains to be elucidated; however, our results suggest that expression of these iron metabolism–related genes in the kidney may play a role in the modulation of the homeostasis of iron at either the whole-body or the local level. We found that after angiotensin II infusion, some proximal tubular cells exhibiting iron deposition showed increased expression of the iron metabolism–related genes, although some discrepancies were observed (Figs. 1, 2); these results stood in contrast with the exclusive co-localization of ferritin and heme oxygenase-1 (*15*). In addition, only a fraction of the mRNA expression of the genes tested was co-localized with lipid deposition, again in contrast to the exclusive co-localization of TGF-β1 mRNA and lipid deposition in the kidney (*16*, *17*).

TfR, which facilitates the efficient cellular uptake of holotransferrin (a ferric-iron bound transferrin) (*20*) is expressed in the kidney. Recent studies have suggested that, in addition to reabsorbing the iron compounds filtered from the glomerulus, TfR might act as an immunoglobulin (Ig)A1 receptor and might be involved in the pathogenesis of IgA nephropathy (*21*, *22*). It remains of interest whether or not the modulation of TfR expression underlies the renoprotective effects of angiotensin II converting enzyme inhibitor and the AT_1 receptor blocker in IgA nephropathy. DMT1 is expressed at the absorptive epithelium of the duodenum. A mutation of the transmembrane domain of DMT1 in anemic *mk* mice and Belgrade (b) rats (*2*, *3*) causes impaired iron uptake at the intestinal brush border, thus indicating that DMT1 plays a pivotal role as an iron transporter. DMT1 has been shown to be expressed in other organs, including the placenta, brain, and kidney. Alternative splicing of the DMT1 gene produces two different mRNAs, namely, IRE(–)DMT1 and IRE(+)DMT1 (*2*, *3*). In the current study, *in situ* hybridization

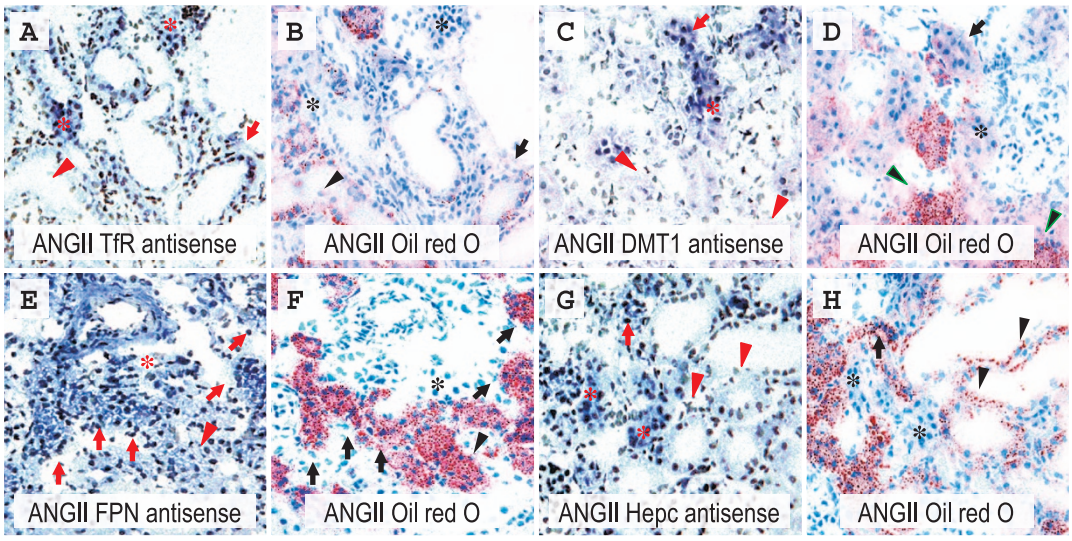

Fig. 4. *Localization of DMT1, FPN, and hepc mRNA and lipid deposition. Unfixed, frozen samples from the kidneys of angiotensin II–infused rats were used. A-B, C-D, E-F, and G-H are serial specimens. A:* In situ *hybridization using the TfR antisense probe. B, D, F, H: Oil red O staining. C:* In situ *hybridization using the DMT1 antisense probe. E:* In situ *hybridization using the FPN antisense probe. G:* In situ *hybridization using the hepc antisense probe. Some tubular cells that were positive for lipid deposition were also positive for the mRNA tested (arrows). By contrast, some tubular cells that were positive for iron deposition were negative for the mRNA tested (arrowheads), and some tubular cells that were negative for iron deposition were positive for the mRNA tested (asterisks). Original magnification, × 200.*

was used to reveal that DMT1 was expressed in the cortex in the kidney of untreated rats, and we demonstrated that both IRE(–) and IRE(+) splicing variants were expressed by RT-PCR; these findings are consistent with those of previous studies (*11*, *12*, *23*, *24*). Because of its localization in the kidney, DMT1 is thought to play a role in the reabsorption of iron in the kidney (*11*). If this is indeed the case, then angiotensin II–induced upregulation of renal DMT1 might enhance the reuptake of filtered iron into the tubular cells, resulting in tubular iron deposition, which would be in accordance with the co-localization (albeit partial co-localization) of DMT1 mRNA and iron deposition in the kidney of angiotensin II–infused rats. This possibility must be carefully validated, however, because DMT1 expression has been found to be reduced in the kidney of an animal model of diabetes (*10*), in which iron accumulation in the tubular cells has also been reported (*25*).

FPN, an iron exporter, is involved in the release of iron from enterocytes of the duodenum and tissue macrophages, and mutation of FPN results in a hemochromatosis-like phenotype (*26*, *27*). It is presumed that in the duodenum, iron is transported into enterocytes across the apical membrane by DMT1, and is then exported out of the cell and into the portal circulation across the basolateral membrane *via* FPN. As we found in the current study, FPN is expressed in the kidney (*4*, *5*); however, little is known about the regulation of renal FPN expression. It has been reported that FPN expression may not be affected by an altered dietary intake of copper (*13*). In the current study, FPN was clearly upregulated in the renal cells after angiotensin II infusion. It is possible that FPN mRNA was upregulated in response to the deposition of iron in some tubular epithelial cells in the kidney of angiotensin II–infused rats. However, a recent study has shown that mutation of the FPN gene causes iron accumulation in hepatic macrophages (*5*, *28*), but not in the enterocytes (*29*), suggesting that FPN haploinsufficiency affects iron export from Kupffer cells, but not from enterocytes. Therefore, the iron export system in parenchymal cells may differ from that in the tissue macrophages.

Hepc, which is expressed most abundantly in the liver, plays a pivotal role in the development of anemia associated with inflammation, innate immunity, and iron metabolism (*30*). Kulaksiz and co-workers have reported that hepc protein is also expressed strongly in the thick ascending limb of the cortex and in the connecting tubules in the rat kidney (*14*). Our findings demonstrated that the expression of both FPN and hepc mRNA was induced by angiotensin II. The role of the angiotensin II–induced upregulation of hepc mRNA awaits further investigation.

In the present study, the expression of all tested genes except for IRE(+)DMT1 was upregulated in the kidney of angiotensin II–infused rats, although the pressor-dependency may differ slightly. For example, the expression of hepc was upregulated in response to both angiotensin II and norepinephrine, suggesting that hypertension *per se* may play a role in the regulation of the hepc expression. To date, little is

known about the regulation of iron homeostasis in hypertensive patients. Piperno *et al*. have reported that increased serum ferritin was more frequent in subjects with essential hypertension than in normotensive subjects (*31*). In their study, increased resistance appeared to be among the possible mechanisms underlying iron overload in an animal model of hypertension (*32*). Urinary transferrin excretion has been reported to be increased when albuminuria is present, not only in diabetic hypertensive cases (*33*), but also in non-diabetic hypertensive cases (*34*); this increase might also in part account for the link between altered iron homeostasis in the kidney and hypertension. It has been reported that treatment of diabetic patients with low-dose candesartan slightly decreased blood pressure, and this in turn reversed the increase in the urinary excretion of transferrin over time (*35*).

In the current study, we targeted the regulation of the expression of several newly discovered iron metabolism–related genes in the kidney. It is well known that the whole-body iron balance is maintained by the regulation of iron absorption by the intestine, as essentially no pathway for iron excretion is present in humans (*20*). Considering that anemia is among the possible side effects of all commercially available AT_1 receptor blockers in Japan, regulation of the expression of iron metabolism–related genes by angiotensin II or by the renin angiotensin system in intestinal cells should also be closely investigated in future studies.

In conclusion, we have characterized the expression patterns of several iron metabolism–related genes, including TfR, DMT1, FPN, and hepc, and their regulation by angiotensin II in the rat kidney. Further studies are necessary for analyzing the relative contribution of these genes to renal iron homeostasis and, presumably, to tubular iron reabsorption in terms of the activity and involvement of the renin angiotensin system.

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