Nifedipine Increases Iron Content in WKPT-0293 Cl.2 Cells via Up-Regulating Iron Influx Proteins

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Nifedipine was reported to enhance urinary iron excretion in iron overloaded mice. However, it remains unknown how nifedipine stimulates urinary iron excretion in the kidney. We speculated that nifedipine might inhibit the TfR1/DMT1 (transferrin receptor 1/divalent metal transporter1)-mediated iron uptake by proximal tubule cells in addition to blocking L-type Ca2+ channels, leading to an increase in iron in lumen-fluid and then urinary iron excretion. To test this hypothesis, we investigated the effects of nifedipine on iron content and expression of TfR1, DMT1 and ferroportin1 (Fpn1) in WKPT-0293 Cl.2 cells of the S1 segment of the proximal tubule in rats, using a graphite furnace atomic absorption spectrophotometer and Western blot analysis, respectively. We demonstrated for the first time that nifedipine significantly enhanced iron content as well as TfR1 and DMT1 expression and had no effect on Fpn1 levels in the cells. We also found that ferric ammonium citrate decreased TfR1 levels, increased Fpn1 expression and had no effect on DMT1 content, while co-treatment with nifedipine and FAC increase TfR1 and DMT1 expression and also had no effect on Fpn1 levels. These findings suggest that the nifedipine-induced increase in cell iron may mainly be due to the corresponding increase in TfR1 and DMT1 expression and also imply that the effects of nifedipine on iron transport in proximal tubule cells can not explain the increase in urinary iron excretion.

Keywords: nifedipine, iron transport proteins, WKPT-0293 Cl.2 cells, urinary iron, cell iron

INTRODUCTION
Plasma iron is tightly bound to Tf, a 78-kDa circulating protein that is assumed to not cross the glomerulus filter, because of its low protein permeability (Aisen, 1994; Linder, 2013). The kidney has therefore been regarded as more or less irrelevant in terms of iron handling, and for a long time, iron metabolism in the kidney also received relatively little attention (Smith and Thévenod, 2009; Thévenod and Wolff, 2016). However, a number of recent studies have provided evidence for the presence of many, if not all of the iron transporters and receptors in renal tubular cells, including TfR1 (Zhang et al., 2007; Moulouel et al., 2013), cubilin (a novel Tf receptor) (Kozyraki et al., 2001), divalent metal transporter 1 (DMT1) (Canonne-Hergaux and Gros, 2002) at the apical membrane, and ferroportin 1 (Fpn1)

Abbreviations: BSA, bovine serum albumin; DMT1, divalent metal transporter1; DMT1 + IRE, divalent metal transporter1 with iron response element; DMT1 − IRE, divalent metal transporter1 without iron response element; FAC, ferric ammonium citrate; Fpn1, ferroportin1; Tf, transferrin; TR1, transferrin receptor 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
Unless otherwise stated, all chemicals, including MTT and mouse monoclonal anti-β-actin, were obtained from the Sigma Chemical Company, St. Louis, MO, USA. The mouse anti-rat TIR1 antibody was purchased from Invitrogen, Carlsbad, CA, USA, and the rabbit polyclonal anti-mouse Fpn1 antibody from Chemicon International, Temecula, CA, USA. The rabbit anti-rat DMT1 + IRE and DMT1 – IRE polyclonal antibodies were bought from the Alpha Diagnostic International Company, San Antonio, TX, USA, and goat anti-rabbit or anti-mouse IRDye 800 CW secondary antibodies from LI-COR Biosciences, Lincoln, NE, USA. Dulbecco’s Modified Eagle’s Medium (DMEM)/Ham’s F-12 was obtained from GIBCO, Grand Island, NY, USA and BSA from the Calbiochem-Novabiochem Corporation, San Diego, CA, USA. The scintillation cocktail and tubes were obtained from the Beckman Coulter Company, Fullerton, CA, USA. All experimental protocols were performed according to the Animal Management Rules of the Ministry of Health of China, and approved by the Animal Ethics Committees of Fudan University and The Chinese University of Hong Kong.

**Cell Culture**

Immortalized cells (WKPT-0293 Cl.2) from the S1 segment of the proximal tubule of normotensive Wistar-Kyoto rats (RPTC) were cultured as previously described (Woost et al., 1996). Briefly, cells were maintained in renal tubular epithelium medium composed of DMEM and F-12 [nutrient mixture F-12 (Ham)] at a ratio of 1:1, supplemented with 15 mM HEPES, 1.2 mg/ml NaHCO3, 5 µg/ml Tf, 10 ng/ml epidermal growth factor, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 10% fetal calf serum. Cells were plated at a density of 5 × 104/ml on collagen-coated flasks, passed at 80% confluency, and split 1:10, twice a week.

**Assessment of Cell Viability**

Cell viability was assessed using an MTT assay as previously described (He et al., 2008; Du et al., 2009). Briefly, a total of 25 µl MTT (1 g/L in PBS) was added to each well before incubation was conducted at 37°C for 4 h. The assay was stopped by the addition of a 100 µl lysis buffer (20% SDS in 50% N’Ndimethylformamide, pH 4.7). Optical density (OD) was measured at the 570 nm wavelength by the use of an ELX-800 microplate assay reader (Bio-tek, Winoski, VT, USA) and the results were expressed as a percentage of the absorbance measured in the control cells.

**Graphite Furnace Atomic Absorption Spectrophotometer**

Total iron in the WKPT-0293 Cl.2 cells was determined using a graphite furnace atomic absorption spectrophotometer (GFAAS, Perkin Elmer, Analyst 100) as previously described (Chang et al., 2005; Ke et al., 2005). The cells were diluted 1:20 (wt/v) with HEPES buffer and homogenized with a sonicator (MSE Soniprep 150 Ultrasonic Disintegrator, MSE Scientific Instruments, England). A 50-µl portion of the homogenate was added to an equal volume of ultra-pure nitric acid in a 0.5 ml polystyrene microfuge tube, digested for 48 h at 50°C, and diluted 1:40 with 3.12 mmol/L nitric acid for iron analysis. Standard curves (0–40 ppb) were prepared by diluting iron standard with blanks prepared from homogenization reagents.
FIGURE 1 | Effect of nifedipine and FAC treatment on the viability of WKPT-0293 Cl.2 cells. WKPT-0293 Cl.2 cells were treated with FAC (100 µM) or nifedipine (1, 10, 100 µM) alone for 16 h (A), or FAC (100 µM) with nifedipine (1, 10, 100 µM) for 16 h (B). Cell viability was then evaluated using the MTT assay as described in the Section “Materials and Methods.” Data were presented as means ± SEM (n = 8). *p < 0.05 versus the control.

Western Blot Analysis
WKPT-0293 Cl.2 cells that received different treatments were washed with ice-cold PBS, homogenized with lysis buffer and then subjected to sonication using a Soniprep 150 (MSE Scientific Instruments, London, UK). After centrifugation at 10,000 g for 15 min at 4°C, the supernatant was collected and protein content was determined using the Bradford assay kit (Bio-Rad). Aliquots of the cell extract containing 50 µg of protein were diluted in 2 µl of sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromphenol blue, and 5% β-mercaptoethanol) and heated for 5 min at 95°C before SDS-PAGE on 10% gel and transfer to a pure nitrocellulose membrane. After the transfer, the membrane was blocked with 5% blocking reagent in Tris-buffered saline containing 0.1% Tween-20, overnight at 4°C. The membrane was then rinsed in three changes of Tris-buffered saline/Tween-20, incubated in fresh washing buffer once for 15 min and twice for 5 min, and then incubated overnight at 4°C with primary antibodies: rabbit anti-rat DMT1 + IRE, DMT1-IRE polyclonal antibodies, rabbit anti-mouse Fpn1 polyclonal antibody (1:5000), and mouse anti-rat TfR1 monoclonal antibody (1:1000). After three washes, the blots were incubated with goat anti-rabbit or anti-mouse IRDye 800 CW secondary antibody (1:5000, Li-Cor) for 1 h at room temperature. The intensity of the specific bands was detected and analyzed by the Odyssey infrared imaging system (Li-Cor) (Du et al., 2014; Qian et al., 2014). To ensure
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FIGURE 3 | Nifedipine treatment increased expression of Tfr1, Dmt1 + IRE and Dmt1 − IRE in WKPT-0293 Cl.2 cells. WKPT-0293 Cl.2 cells were treated with nifedipine (1, 10, 100 µM) for 16 h, and the expression of Tfr1 (A), Dmt1 + IRE (B), Dmt1 + IRE (C), and Fpn1 (D) was then measured by Western blot analysis. The data were presented as Mean ± SEM (n = 6, % Control). *p < 0.05 versus the control.

RESULTS

Effects of Nifedipine and Ferric Ammonium Citrate (FAC) Treatment on the Viability of WKPT-0293 Cl.2 Cells

We first investigated the effects of FAC and nifedipine on the viability of WKPT-0293 Cl.2 by treating the cells with FAC (100 µM) (Chen et al., 2005) or various concentrations (1, 10, 100 µM) of nifedipine alone for 16 h, and then evaluated cell viability using an MTT assay. The results showed that there were no significant differences in viability between the control cells and the cells treated with 100 µM of FAC or 1 and 10 µM of nifedipine. However, viability in the cells treated with 100 µM of nifedipine was found to be significantly lower than that of the control cells (Figure 1A).

To find out the effects of co-treatment with FAC and nifedipine on the viability of WKPT-0293 Cl.2 cells, the cells were treated with FAC (100 µM) plus 1, 10 or 100 µM of nifedipine for 16 h. The MTT assay demonstrated that viability in the cells treated with 1 µM of nifedipine plus FAC or FAC alone were not significantly different from that of the control cells, but treatment with 10 or 100 µM of nifedipine plus FAC did induce a significant reduction in cell viability (Figure 1B). The findings here demonstrated that nifedipine could promote the toxic effects of FAC on the viability of WKPT-0293 Cl.2 cells.
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FIGURE 4 | Ferric ammonium citrate treatment decreased TfR1 and increased Fpn1 expression in WKPT-0293 Cl.2 cells. WKPT-0293 Cl.2 cells were treated with FAC (100 µM) for 16 h, and the expression of TfR1 (A), DMT1 + IRE (B), DMT1 − IRE (C), and Fpn1 (D) was then measured by Western blot analysis. The data were presented as Mean ± SEM (n = 6, % Control). *p < 0.05 versus the control.

Nifedipine Treatment Increased Iron Level in WKPT-0293 Cl.2 Cells
We then examined the effects of nifedipine on iron uptake in WKPT-0293 Cl.2 cells. The cells were treated with FAC (100 µM) with different concentrations (0, 1, 10, 100 µM) of nifedipine for 16 h, and iron content was then measured using a GFAAS. It was found that treatment with 100 µM of FAC alone induced a significant increase in iron content, 38.629 fold of that of the control cells (Figure 2A). The addition of nifedipine led to a further increase in cell iron levels. Iron contents in the cells treated with 1, 10, or 100 µM of nifedipine plus 100 µM of FAC were significantly higher than those in the cells treated with 100 µM of FAC alone. There were no significant differences between the cells treated with different concentrations of nifedipine plus FAC (Figure 2B). These results showed that nifedipine has a role in increasing the iron uptake of WKPT-0293 Cl.2 cells.

Nifedipine Treatment Increased Expression of TfR1, DMT1 + IRE and DMT1 − IRE in WKPT-0293 Cl.2 Cells
To find out the potential mechanisms involved in the positive role of nifedipine on iron uptake by WKPT-0293 Cl.2 cells, we investigated the effects of nifedipine on the expression of major iron uptake proteins TfR1, DMT1 + IRE and DMT1 − IRE, as well as iron release protein Fpn1 in the cells. We demonstrated that nifedipine induced a progressive increase in TfR1 (Figure 3A) and DMT1 + IRE (Figure 3B) expression but not in DMT1 − IRE and Fpn1 with the concentrations added. Although the expression of DMT1 − IRE in cells treated with 1, 10, or 100 µM of nifedipine was significantly higher than that of the control cells, there were no significant differences between cells treated with different concentrations of nifedipine (Figure 3C). Treatment with 1 or 10 µM of nifedipine did not induce any significant changes in Fpn1 expression, with no
Co-treatment with nifedipine and FAC increased TfR1, DMT1 + IRE and DMT1 − IRE expression in WKPT-0293 Cl.2 cells. WKPT-0293 Cl.2 cells were treated with 100 µM of nifedipine plus 100 µM of FAC for 16 h, and the expression of TfR1 (A), DMT1 + IRE (B), DMT1 − IRE (C), and Fpn1 (D) was then measured by Western blot analysis. The data were presented as Mean ± SEM (n = 4, % Control). *p < 0.05 versus the control.

Significant differences being found in Fpn1 content between the cells treated with 1 or 10 µM of nifedipine and the controls (Figure 3D). However, treatment with 100 µM of nifedipine was found to induce a significant reduction in Fpn1 expression in the WKPT-0293 Cl.2 cells. The data imply that nifedipine likely increases iron uptake in WKPT-0293 Cl.2 cells via its role in up-regulating expression of iron uptake proteins.

FAC Treatment Decreased TfR1 and Increased Fpn1 Expression in WKPT-0293 Cl.2 Cells

We also investigated the effects of iron-overload on the expression of TfR1, DMT1 + IRE, DMT1 − IRE and Fpn1 by treating WKPT-0293 Cl.2 cells with 100 µM of FAC for 16 h. Western blot analysis showed that FAC treatment induced a significant reduction in TfR1 (Figure 4A) and an increase in Fpn1 (Figure 4D) expression, and had no effect on DMT1 + IRE (Figure 4B) and DMT1 − IRE (Figure 4C) expression in WKPT-0293 Cl.2 cells.

Co-treatment With Nifedipine and FAC Increased TfR1, DMT1 + IRE and DMT1 − IRE Expression in WKPT-0293 Cl.2 Cells

Finally, we investigated the effects of co-treatment with Nifedipine and FAC on the expression of TfR1, DMT1 + IRE, DMT1 − IRE and Fpn1 by treating WKPT-0293 Cl.2 cells with 100 µM of nifedipine plus 100 µM of FAC for 16 h. Western blot
and had no effects on DMT1. FAC alone decreased TfR1 expression, increased Fpn1 expression respectively in WKPT-0293 Cl.2 cells. It was found that nifedipine and/or FAC on the expression of iron transport proteins TfR1, DMT1 + IRE and DMT1 − IRE expression, and had no effect on Fpn1, while co-treatment with nifedipine and FAC led to a significant increase in TfR1, DMT1 + IRE and DMT1 − IRE expression and also had no effect on Fpn1 expression in WKPT-0293 Cl.2 cells. These results implied that the nifedipine-induced increase in iron content in the cells may mainly be due to the increased expression of TfR1 and DMT1 (Figure 6). FAC may be either donor of iron to transferrin in the medium or as an NTBI source. Most of the iron taken up by the cells was probably in the form of Tf-Fe via a TfR1 (cell membrane)/DMT1 (endosomal membrane) pathway because we did not deplete the intracellular store of Tf by suspending the cells in DMEM–HEPES medium for a given period (Chang et al., 2007; Qian et al., 2011) in this study. It is also conceivable, but remains to be tested, that small amount of the iron may be taken up by the cells in the form of NTBI via a DMT1-mediated pathway. In addition, further investigations on how nifedipine enhances TfR1 and DMT1 expression in this type of cells are needed. The relevant mechanisms, especially the effects of nifedipine on the expression of iron regulatory proteins (IRPs) and membrane potential polarization (Du et al., 2016) need to be clarified as well.

Studies in Belgarde rats have shown that DMT1, also known as DCT1 (Gunshin et al., 1997) or NRAMP2 (Fleming et al., 1997), plays a fundamental role in body iron homeostasis (Fleming et al., 1998, 1999; Garrick et al., 1999; Gruenheid et al., 1999). DMT1 is highly expressed in the kidney (Canonne-Hergaux and Gros, 2002) and is also suggested to play a role in urinary iron handling (Martines et al., 2013; Veuthey et al., 2014). Therefore, modulation of renal DMT1 expression may influence renal iron excretion rate. A recent in vivo study showed that altered dietary iron intake is a strong modulator of renal DMT1 expression in male Wistar rats (Wareing et al., 2003). Increasing dietary iron (an iron-enriched diet, 5 g/kg) was found to decrease DMT1 expression and increase urinary iron excretion rate, while decreasing dietary iron (an iron-restricted diet, 50 mg/kg) caused an increase in DMT1 expression and a decrease in urinary iron excretion (Wareing et al., 2003). This finding is different from what was found in the present study, where we found FAC to have no effect on DMT1. The cause of this conflict in results is unknown, although it may be partly due to the differences between experimental conditions in vivo and in vitro.

DMT1 is found not only in proximal tubule cells, but also in late thick ascending limbs, early distal tubules, and intercalated cells of the cortical collecting duct (Ferguson et al., 2001; Abouhamed et al., 2006). The types of epithelial cells found at different segments of nephron are different, which is why different segments have different functions in re-absorption and secretion for practical ions such as Na+ and K+ (Silverthorn, 2001). Currently it is unknown whether iron handling differs between different segments of the nephron. It is also unknown whether the responses of DMT1 to iron differ between different parts of nephron, although it is certainly possible. In addition, it has yet to be determined whether iron within the peritubular capillaries can be moved into the interstitial space, then to tubular

FIGURE 6 | Hypothetical scheme for the possible effects of nifedipine on TfR1, DMT1 and Fpn1 expression and iron re-absorption in proximal tubule cells of kidney.

analysis demonstrated that co-treatment with nifedipine and FAC led to a significant increase in TfR1 (Figure 5A), DMT1 + IRE (Figure 5B) and DMT1-IRE (Figure 5C) expression and had no effect on Fpn1 expression (Figure 5D) in WKPT-0293 Cl.2 cells.

DISCUSSION

The major objective of this study was to find out whether treatment with the L-type Ca2+ channel blocker nifedipine could reduce iron uptake by the proximal tubule cells of the nephron, leading to an increase in the iron content within luminal fluid and, subsequently to also an increase in urinary iron excretion in the kidney. We therefore first investigated the effect of nifedipine on iron content in proximal tubule cells treated with FAC. We demonstrated for the first time that nifedipine treatment has a significant role in enhancing, rather than reducing iron content in WKPT-0293 Cl.2 cells of the S1 segment of the proximal tubule of normotensive Wistar-Kyoto rats (RPTC). This unexpected finding, that is, that nifedipine induces an increase in cell iron content, should predictably lead to a decrease rather than an increase in urinary iron excretion. It implies that the effects of nifedipine on iron transport in proximal tubule cells are not associated with its induced increase in urinary iron excretion in the kidney.

To find out the mechanisms involved in the nifedipine-induced increase in cell iron content, we further examined the effects of nifedipine and/or FAC on the expression of iron transport proteins TfR1, DMT1 + IRE, DMT1 − IRE, and Fpn1 respectively in WKPT-0293 Cl.2 cells. It was found that FAC alone decreased TfR1 expression, increased Fpn1 expression and had no effects on DMT1 + IRE and DMT1 − IRE, nifedipine alone increased TfR1, DMT1 + IRE and DMT1 − IRE expression, and had no effect on Fpn1, while co-treatment with nifedipine and FAC led to a significant increase in TfR1, DMT1 + IRE and DMT1 − IRE expression and also had no effect on Fpn1 expression in WKPT-0293 Cl.2 cells. These results implied that the nifedipine-induced increase in iron content in the cells may mainly be due to the increased expression of TfR1 and DMT1 (Figure 6). FAC may be either donor of iron to transferrin in the medium or as an NTBI source. Most of the iron taken up by the cells was probably in the form of Tf-Fe via a TfR1 (cell membrane)/DMT1 (endosomal membrane) pathway because we did not deplete the intracellular store of Tf by suspending the cells in DMEM–HEPES medium for a given period (Chang et al., 2007; Qian et al., 2011) in this study. It is also conceivable, but remains to be tested, that small amount of the iron may be taken up by the cells in the form of NTBI via a DMT1-mediated pathway. In addition, further investigations on how nifedipine enhances TfR1 and DMT1 expression in this type of cells are needed. The relevant mechanisms, especially the effects of nifedipine on the expression of iron regulatory proteins (IRPs) and membrane potential polarization (Du et al., 2016) need to be clarified as well.

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cells and finally, be deposited into tubular fluid, especially when there is an increase in serum iron. Furthermore, the role of L-type Ca2+ channels in iron transport in different segments of the nephron still need to be clarified. Only after we have the answers to the above questions, can we fully account for how nifedipine stimulates urinary iron excretion in the kidney.

AUTHOR CONTRIBUTIONS

YK and Z-MQ conceived, organized and supervised the study; S-SY, YL, Y-FZ, JL and L-RJ performed the experiments; YK and Z-MQ contributed to the analysis of data, and prepared and wrote the manuscript.

REFERENCES

Abouhamed, M., Gburek, J., Liu, W., Torchalski, B., Wilhelm, A., Wolff, N. A., et al. (2006). Divalent metal transporter 1 in the kidney proximal tubule is expressed in late endosomes/lysosomal membranes: implications for renal handling of protein-metal complexes. *Am. J. Physiol. Renal Physiol*. 290, F1525–F1533. doi: 10.1152/ajprenal.00359.2005

Aisen, P. (1994). “Iron transport proteins,” in *Iron Metabolism in Health and Disease*, Chap. 1, eds J. H. Brock, J. W. Halliday, M. J. Pippard, and L. W. Powell (London: WR Saunders), 1–30.

Alfrey, A. C., and Hammond, W. S. (1990). Renal iron handling in the nephrotic syndrome. *Kidney Int.* 37, 1409–1413. doi: 10.1038/ki.1990.130

Canonne-Hergaux, F., and Gros, P. (2002). Expression of the iron transporter DMT1 in kidney from normal and anemic mk mice. *Kidney Int.* 62, 147–156. doi: 10.1046/j.1523-1755.2002.00405.x

Chang, Y. Z., Qian, Z. M., Du, J. R., Zhu, L., Xu, Y., Li, L. Z., et al. (2007). Ceruloplasmin expression and its role in iron transport in C6 cells. *Neurochem. Int.* 50, 726–733. doi: 10.1016/j.neuint.2007.01.004

Chang, Y. Z., Qian, Z. M., Wang, K., Zhu, L., Yang, X. D., Du, J. R., et al. (2005). Effects of development and iron status on ceruloplasmin expression in rat brain. *J. Cell Physiol.* 204, 623–631. doi: 10.1002/jcp.20321

Chen, Y., Qian, Z. M., Du, J., Duan, X., Chang, Y., Wang, Q., et al. (2005). Iron loading inhibits ferroportin1 expression in PC12 cells. *Neurochem. Int.* 47, 507–513. doi: 10.1016/j.neuint.2005.06.004

Du, F., Qian, Z. M., Luo, Q., Yung, W. H., and Ke, Y. (2014). Hepcidin suppresses brain iron accumulation by downregulating iron transport proteins in iron-overloaded rats. *Mol. Neurobiol.* 52, 101–114. doi: 10.1007/s12035-014-8847-x

Du, F., Qian, Z. M., Zhu, L., Xu, W. M., Yung, W. H., Tsim, T. Y., et al. (2009). L-DOPA neurotoxicity is mediated by up-regulation of DMT1-IRE expression. *PLoS ONE* 4:e4593. doi: 10.1371/journal.pone.0004593

Du, X., Xu, H., Shi, L., Jiang, Z., Song, N., Jiang, H., et al. (2016). Activation of ATP-sensitive potassium channels enhances DMT1-mediated iron uptake in SK-N-SH cells in vitro. *Sci. Rep.* 6:36374. doi: 10.1038/srep36374

Ferguson, C. J., Wareing, M., Ward, D. T., Green, R., Smith, C. P., and Riccardi, D. (2001). Cellular localization of divalent metal transporter DMT-1 in rat kidney. *Am. J. Physiol. Renal Physiol.* 280, F803–F814

Fleming, M. D., Romano, M. A., Su, M. A., Garrick, M. D., and Andrews, N. C. (1998). Nramp2 is mutated in the anemic Belgrade (b) rat: evidence of a role for Nramp2 in endosomal iron transport. *Proc. Natl. Acad. Sci. U.S.A.* 95, 1148–1153. doi: 10.1073/pnas.95.3.1148

Fleming, M. D., Trenor, C. C. III, Su, M. A., Foerenzler, D., Beier, D. R., Dietrich, W. F., et al. (1997). Microcytic anemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nat. Genet.* 16, 383–386.

Fleming, R. E., Migas, M. C., Zhou, X., Jiang, J., Britton, R. S., Brunt, E. M., et al. (1999). Mechanism of increased iron absorption in murine model of hereditary hemochromatosis: increased duodenal expression of the iron transporter DMT1. *Proc. Natl. Acad. Sci. U.S.A.* 96, 3143–3148. doi: 10.1073/pnas.96.6.3143

FUNDING

The studies done in our laboratories were supported by the Competitive Earmarked Grants of The Hong Kong Research Grants Council (GRF 466713, GRF 14106914, GRF 14111815), and the Grants of the National Natural Science Foundation of China (NSFC) (31271132-2012, 31371092-2013, 31571195-2014, 31330035-2013).

ACKNOWLEDGMENT

We would like to thank Christopher Qian for assisting with the preparation and English revision of the manuscript.
Silverthorn, D. U. (ed.). (2001). “The Kidney Chapter 19,” in Human Physiology, 3rd Edn. London: Pearson, 598–624.
Smith, C. P., and Thévenod, F. (2009). Iron transport and the kidney. Biochim. Biophys. Acta 1790, 724–730. doi: 10.1016/j.bbagen.2008.10.010
Thévenod, F., and Wolff, N. A. (2016). Iron transport in the kidney: implications for physiology and cadmium nephrotoxicity. Metallomics 8, 17–42. doi: 10.1039/c5mi00215j
Tsushima, R. G., Wickenden, A. D., Bouchard, R. A., Oudit, G. Y., Liu, P. P., and Backx, P. H. (1999). Modulation of iron uptake in heart by L-type Ca2+ channel modifiers: possible implications in iron overload. Circ. Res. 84, 1302–1309. doi: 10.1161/01.RES.84.11.1302
Veuthey, T., Hoffmann, D., Vaidya, V. S., and Wessling-Resnick, M. (2014). Impaired renal function and development in Belgrade rats. Am. J. Physiol. Renal Physiol. 306, F333–F343. doi: 10.1152/ajprenal.00285.2013
Wareing, M., Ferguson, C. J., Delannoy, M., Cox, A. G., McMahon, R. F., Green, R., et al. (2003). Altered dietary iron intake is a strong modulator of renal DMT1 expression. Am. J. Physiol. Renal Physiol. 285, F1050–F1059. doi: 10.1152/ajprenal.00664.2003
Wareing, M., Ferguson, C. J., Green, R., Riccardi, D., and Smith, C. P. (2000). In vivo characterization of renal iron transport in the anaesthetized rat. J. Physiol. 524(Pt 2), 581–586. doi: 10.1111/j.1469-7793.2000.00581.x
Wolff, N. A., Liu, W., Fenton, R. A., Lee, W. K., Thévenod, F., and Smith, C. P. (2011). Ferroportin 1 is expressed basolaterally in rat kidney proximal tubule cells and iron excess increases its membrane trafficking. J. Cell Mol. Med. 15, 209–219. doi: 10.1111/j.1582-4934.2009.00985.x
Woost, P. G., Crous, D. E., Jin, W., Frisa, P. S., Jacobberger, J. W., Douglas, J. G., et al. (1996). Immortalization and characterization of proximal tubule cells derived from kidneys of spontaneously hypertensive and normotensive rats. Kidney Int. 50, 125–134. doi: 10.1038/ki.1996.295
Zhang, D., Meyron-Holtz, E., and Rouault, T. A. (2007). Renal iron metabolism: transferrin iron delivery and the role of iron regulatory proteins. J. Am. Soc. Nephrol. 18, 401–406. doi: 10.1681/ASN.2006080908
Zhao, N., Sun, Z., Mao, Y., Hang, P., Jiang, X., Sun, L., et al. (2010). Myocardial iron metabolism in the regulation of cardiovascular diseases in rats. Cell Physiol. Biochem. 25, 587–594. doi: 10.1159/000315077

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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