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Hemodialysis restored iron distribution that was sequestered in the spleen by bilateral nephrectomy

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Kida A, Kuragano T, Furuta M, Otaki Y, Hasuike Y, Matsuda S, Akaike N, Kokuba Y, Nakanishi T. Hemodialysis restored iron distribution that was sequestered in the spleen by bilateral nephrectomy. Am J Physiol Renal Physiol 306: F1393–F1399, 2014. First published April 16, 2014; doi:10.1152/ajprenal.00685.2012.—Acute kidney injury (AKI) is associated with dysregulated iron metabolism, which may play a significant role in cellular injury. The effect of hemodialysis (HD) on iron metabolism in AKI therapy has not been well defined. The effects of HD on iron parameters were tested in control rats and bilateral nephrectomy (BNx) rats. The BNx rats were divided into the following three groups: 1) the sham-operated group (BNx-Sham), 2) the BNx group, and 3) the HD group (BNx-HD), which received HD therapy 40–45 h after BNx. Sections of the liver or spleen were stained with Berlin blue to examine the accumulation of iron. The mRNA levels of hepcidin and ferroportin 1 in the spleen and liver were also quantified using RT-PCR. In the BNx group, the plasma iron and hematocrit levels were decreased, and hepcidin levels were increased. The iron staining in the spleen in the BNx group was significantly more intense than that in the BNx-Sham group; however, after an HD session, splenic iron staining diminished to the level of the sham group along with an increase in plasma iron and a decrease in hepcidin. BNx moved iron from hemoglobin and the plasma to the spleen, which is associated with an increase in plasma hepcidin. A single HD session accelerated the release of iron from the spleen, and the increased plasma iron was linked to the removal of hepcidin. Our data suggested that hepcidin might dynamically modulate the iron metabolism in BNx as well as in HD.

iron metabolism; hepcidin; ferroportin 1; acute kidney injury; hemodialysis

ACUTE KIDNEY INJURY (AKI) is a major clinical problem that has recently been suggested to influence other remote organs (2, 5, 6). The mechanisms of AKI-induced organ failure include the dysregulation of the inflammatory reaction, the innate immune response, oxidative stress, apoptosis, and soluble mediator metabolism. In addition, we suspect that iron may accelerate these processes because iron metabolism can be remarkably altered during an acute loss of renal function. For instance, in a model of bilateral nephrectomy (BNx), severe anemia was a consistent finding, indicated by suppressed erythropoiesis as well as by increased hemolysis (14). In this model, ferrokinetic studies have shown low iron turnover in the bone marrow or low iron incorporation into red blood cells, as well as increased iron storage in the reticuloendothelial cells (15, 17). This significant amount of iron that is not utilized for erythropoiesis could play a significant role in accelerating oxidative stress mediated by macrophages, even in several other tissues. Free iron can catalyze the formation of free radicals and hydroxyl radicals via the Fenton reaction, and these radicals can damage the macromolecular components of the cell, including lipids, proteins and DNA (4, 22). These mechanisms may play a significant role in the disease process as a common theme of cellular injury in the acute loss of renal function. The recently identified hepatic peptide hepcidin has been proposed to be central in iron regulation, acting as the principal iron-regulatory hormone to maintain iron homeostasis (3). We reasonably suspected that hepcidin plays an important role in the iron metabolism of impaired renal function. In addition, patients with AKI sometimes require renal replacement therapy (RRT), but the effect of RRT on iron metabolism has not been well examined.

To clarify whether the acute cessation of renal function affects iron metabolism, the present study examined iron parameters, including hepcidin, in BNx rats. The effects of hemodialysis (HD) therapy on these parameters were also investigated using acetate-free dialysate.

METHODS

Animals

The experiments were performed in male Sprague-Dawley rats (weighing 300–470 g and aged 9 wk). The rats had free access to food and water until the day of the experiment. HD therapy was performed in control rats and in BNx rats. The control rats were divided into two groups: sham-operated rats (C-Sham, n = 4) and sham-operated rats with HD therapy (C-HD, n = 8). The BNx rats were divided into the following three groups: 1) sham-operated rats (BNx-Sham group, n = 8); 2) bilateral kidney resection (BNx group, n = 8); and 3) HD therapy after BNx (BNx-HD group, n = 6). All rats were anesthetized with pentobarbital sodium. For BNx, the renal pedicles were tied off with sutures and then cut distally. The sham surgery consisted of the same procedure without the application of the clamps.

Ethical Approval

This research protocol was approved by the animal ethics committee of the Pharmaceutical Research Laboratories of Ajinomoto, (Ajinomoto Pharma, 20071030).

HD Conditions

For HD, 40–45 h after the BNx or sham operation, the rats in the HD groups (C-HD and BNx-HD) were anesthetized intraperitoneally
with pentobarbital sodium (10–15 mg/kg). Catheters for vascular access were inserted into the right carotid artery and the left femoral vein, and the rats underwent HD through the extracorporeal circulation of blood.

Polysulfone membranes [for each membrane, a 145-cm² membrane surface area and a 50-kDa pore size (MidiKros, Hollow Fiber Modules, Spectrum Laboratories)] and acetate-free dialysate (CARBOSTAR, Ajinomoto Pharmaceuticals, Tokyo, Japan) were used for the HD sessions. Acetate-free dialysate does not contain acetate as a buffering agent but rather contains a small amount of citric acid. The HD treatment time was 4 h; the blood flow rate was 4.25 ml/min, and the dialysate flow rate was 14 ml/min. Heparin sodium at 100 units/body was injected intravenously and subcutaneously as an anticoagulant. The body weight of the animals was maintained by controlling the volume of dialysis effluent with a three-way cock during the HD session.

Measurement of Blood Parameters

Blood was sampled at 2 days after the operation for both the sham and the BNx groups. In the HD group, blood samples were taken before (pre-HD) and after (post-HD) the HD sessions. The plasma levels of urea nitrogen (UN), albumin (Alb), and iron (Fe), as well as the total iron binding capacity [TIBC, as iron level plus unsaturated iron binding capacity (UIBC)] were measured using a HITACHI Automatic Analyzer (7080, Hitachi High-Technologies, Tokyo, Japan). Plasma levels of hepcidin were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS), as previously described (7, 13). Hematocrit (Ht) was measured by a handheld blood analyzer i-STAT (F300, Fuso Pharmaceutical Industries, Osaka, Japan). Transferrin saturation (TSAT) was obtained as the ratio of plasma UN and Fe levels in the BNx group were significantly higher than in the Sham group, while plasma iron, TSAT, and Ht levels were lower (Fig. 1). A single HD session significantly decreased plasma UN and Ht in the BNx-HD (Fig. 2B) and C-HD rats (Fig. 2A). In contrast, plasma iron and TSAT were significantly higher post-HD than pre-HD rats. Plasma hepcidin levels in the BNx group were significantly (P < 0.05) higher than those in the BNx-Sham group (Fig. 1) but decreased significantly after the HD session (Fig. 2B) (P < 0.05).

Fig. 1. Plasma levels of urea nitrogen (UN), iron (Fe), transferrin saturation (TSAT), hepcidin, albumin (Alb), and hematocrit (Ht) 2 days after sham operation (Sham) or bilateral nephrectomy (BNx; n = 8). Values are means ± SD. Differences were analyzed by Mann-Whitney test. *Significantly different (P < 0.05) from the Sham group.

Determination of Iron Content in the Liver and Spleen

Iron content in the liver and spleen was measured using atomic absorption spectroscopy. Pieces of liver and spleen were freeze-dried and mixed with nitric acid. Then, the mixed samples were heated in a closed tube to 105°C for 3 h, cooled, and centrifuged at 500 g for 5 min. Iron content of the lysates was determined using a Z-8200 polarized Zeeman atomic absorption spectrophotometer (Hitachi).

Relative Quantification of Transporter mRNAs by Real-Time PCR

Total RNA was isolated from the livers or spleens of the rats using an RNA extraction reagent (Isogen, Nippon Gene, Tokyo, Japan). To determine the amount of hepcidin and ferroportin-1 (FPN1) in the livers and spleens of the rats, relative quantification was performed using a PCR with TaqMan polymerase and an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA). To correct the variations in total RNA and unequal reverse transcription efficiency, the mRNA quantities of hepcidin and FPN1 were normalized to the amount of mRNA encoding GAPDH.

Statistical Analysis

Normally distributed variables were expressed as the means ± SD, and non-normally distributed variables were expressed as medians (interquartile ranges). The differences among two or three groups were analyzed using Mann-Whitney, Wilcoxon, or Kruskal-Wallis tests. The statistical analyses were performed using the Statistical Package for Social Science (SPSS), version 18.0 (SPSS, Chicago, IL). A P value <0.05 was the threshold for statistical significance.

RESULTS

Laboratory Data

Plasma UN levels in the BNx group were significantly higher than in the Sham group, while plasma iron, TSAT, and Ht levels were lower (Fig. 1). A single HD session significantly decreased plasma UN and Ht in the BNx-HD (Fig. 2B) and C-HD rats (Fig. 2A). In contrast, plasma iron and TSAT were significantly higher post-HD than pre-HD rats. Plasma hepcidin levels in the BNx group were significantly (P < 0.05) higher than those in the BNx-Sham group (Fig. 1) but decreased significantly after the HD session (Fig. 2B) (P < 0.05).
On the other hand, HD did not affect the plasma hepcidin levels in the control rats.

**Iron Accumulation in the Liver and Spleen**

In the histological analysis of the livers stained with Berlin blue (×100), no groups showed iron staining (Fig. 3). However, the level of iron staining in the spleens of the BNx group was significantly (*P* < 0.01) more intense than that in the Sham group. After HD, the level of iron staining in the BNx group had been essentially restored to the level of the Sham group (Fig. 3B). The principal sites of iron deposition were the red pulp.

At 40–45 h after BNx, the amount of iron in the liver (0.47 ± 0.08 vs. 0.42 ± 0.07 µg/mg dry wt tissue) and spleen (1.28 ± 0.28 vs. 1.19 ± 0.26 µg/mg dry wt tissue) did not differ from the Sham group. In addition, they did not change significantly after HD (liver: 0.44 ± 0.05, spleen: 1.13 ± 0.38 µg/mg dry wt tissue).

**Hepcidin and FPN1 Expression in the Liver and Spleen**

There were no significant differences in the mRNA levels of hepcidin in the liver among the BNx rats (BNx-Sham, BNx, and BNx-HD groups) as well as (Fig. 4B) control rats (Fig. 4A). In the spleen, hepcidin mRNA was not detected (data not shown). In the
liver, there was no significant difference in the mRNA levels of FPN1 among the control and BNx rats (Fig. 4A). On the other hand, FPN1 mRNA levels in the spleen were significantly decreased in the BNx and BNx-HD groups compared with those of the BNx-Sham group ($P < 0.05$). The HD session did not affect these levels in the BNx and BNx-HD groups (Fig. 4B).

**DISCUSSION**

In the present study, we first demonstrated the dynamic changes in iron metabolism in the rat model of an acute loss of renal function; the changes include the following: 1) the iron that is not utilized for erythropoiesis is sequestered in the...
spleen in BNx rats; and 2) the iron is released from the spleen to the plasma after a HD session.

We confirmed that BNx changed the distribution of iron from the red blood cells and plasma to the spleen. Simultaneously, plasma iron and Ht levels decreased. Two days after BNx, histological examination showed intense iron deposition in the spleen but not in the liver (Fig. 3). In a dog model of BNx, the rapid development of anemia has been reported to be principally associated with a hemolytic process (12), but we should also consider that the red blood cells in the resected kidneys were lost after BNx. In these animals, erythrophagocytosis in the reticuloendothelial system (RES) could be associated with hemosiderin or pigment deposition within macrophages in the medullary sinuses or in the spleen. Iron deposition in the liver was trivial compared with that in the spleen (11, 12).

The iron deposition in the spleen after BNx might be explained by the increase in plasma hepcidin, which binds to FPN1, the only known cellular iron exporter, resulting in the internalization and degradation of FPN1 and blocked cellular iron export from the cells of the RES (Fig. 3). We suspected that plasma hepcidin levels increase in response to iron loading and inflammation.

The iron content in the liver and spleen from the BNx group did not differ from those of the Sham group and did not change after HD. In addition, based on our preliminary experiment, we did not observe any effect of BNx on FPN1 protein expression in the spleen (data not shown). Although these results are not consistent with the data on serum hepcidin levels and iron staining in the spleen, these discrepancies might be explained by the fact that the iron content and the FPN1 protein levels were determined in the whole spleen. As shown in Fig. 3, iron mainly accumulated in the red pulp of the spleen, where FPN1 expression has been consistently reported (1). Thus the limited localization of iron as well as FPN1 may hamper the exact determination of these changes when they are measured in the whole splenic samples. Similar observations were reported in a rat model of turpentine oil-induced inflammation (23). In these models, the iron content of the liver or spleen between the control and inflammation model animals was variable. The decrease in FPN1 expression was detected in the red pulp macrophages isolated from the spleen, but the expression levels were very low for up to 3 days. The effect of hepcidin on iron metabolism in the whole animal has been only observed using serum iron levels (20), while the direct effects of hepcidin on FPN1 protein levels have mainly been demonstrated in the cultured cell models or by immunohistochemistry (16, 24). Therefore, further investigations are needed to clarify the direct contribution of hepcidin to the changes in FPN1 proteins in
BNx rats, which include iron egress from splenic macrophages and iron absorption in duodenal cells. In addition, we demonstrated the decrease in the FPN1 mRNA levels independently of the increased hepcidin levels in the spleens of the BNx group. Therefore, another possible mechanism should also be considered in these changes. These data could be linked to the increase in several cytokines caused by the nephrectomy; recent animal studies have shown a significant increase in serum TNF-α, IL-1β, IL-6, and IL-12 after nephrectomy or ischemia (5, 18–27). It has been consistently shown that TNF-α and LPS decrease the expression of FPN1, which could block iron egress, in several cells including monocyctic cells (8, 10). However, we should be aware that iron sequestration occurred even in TNF-α or IL-6 knockout mouse (9). Elucidating the exact mechanism for the iron sequestration in BNx, which is the possible contribution of cytokines, requires additional investigation.

In striking contrast to the spleen, the iron deposition in the liver was not remarkable. These observations could be linked to the role of hepcidin in the liver. Several recent observations have shown that hepcidin alters the pattern of cellular iron accumulation, leading to increased iron in the tissue macrophages but decreased iron in the hepatocytes (21, 26). The effects of HD therapy on iron metabolism in BNx rats were unexpectedly remarkable. A single session of HD caused the release of iron from the spleen and an increase in the plasma iron concentration in BNx rats. HD therapy restored the plasma iron concentration, consistent with the disappearance of iron deposits from the spleen. The release of iron from the spleen or macrophages could have been caused by the decrease in plasma hepcidin levels in the HD group, which was the opposite direction of iron sequestration in the BNx group. Therefore, we assumed that the restoration of iron from the spleen to the plasma could be caused by the removal of hepcidin by HD. These data are compatible with our previous clinical study, which showed that serum hepcidin levels decreased after a single HD session and were restored to the pre-HD level within 1 h (7). Hepcidin had been presumed to be cleared efficiently in BNx rats by HD, as it has a very small molecular weight (25).

In the control rats, HD did not affect plasma hepcidin levels and iron content in the liver and spleen, but it did significantly increase the plasma iron levels. Further investigation should be necessary for clarifying the mechanism of the increase in plasma iron.

In this study, the mRNA levels of hepcidin in the liver were unchanged, although the plasma hepcidin levels increased after BNx and decreased after HD. This discrepancy might be explained by the maturation mechanism of hepcidin-25, in which active hepcidin is generated by the removal of its proregion by the prohormone convertase furin, which could be regulated by iron metabolism (19).

There are major limitations to this study. First, we failed to show the effect of HD on hepcidin and iron transport proteins in the Sham-operated rats, although the direct effect of HD on the rats with normal renal function could be more adequate control. Second, we could not determine the time-course of the mRNA levels of hepcidin and FPN1 in this study after BNx and HD. Therefore, we did not know the exact relationship between BNx or HD and the hepcidin and FPN1 transcriptional changes.

Conclusions

After BNx, decreased erythropoiesis due to the depletion of erythropoietin or hemolysis might occur, and the iron derived from hemoglobin could accumulate in the spleen. Simultaneously, the plasma hepcidin levels increase, which could be associated with an acceleration of iron sequestration in the spleen and a decrease in plasma iron. The striking observation of this study was that a single HD session accelerated the release of iron from the spleen and increased plasma iron levels. These changes might be fully explained by the removal of hepcidin through HD (11).

Therefore, we conclude that in BNx, as well as in HD therapy, iron dynamically moves from hemoglobin and the plasma not only to the spleen but also to other organs. Further investigation is necessary to clarify whether hepcidin and proinflammatory cytokines affect the iron metabolism in tissues other than the spleen.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A.K., S.M., and N.A. performed experiments; T.K. provided conception and design of research; M.F. analyzed data; Y.O. and Y.H. drafted manuscript; Y.K. interpreted results of experiments; T.N. approved final version of manuscript.

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