Biochemical and Biophysical Research Communications 490 (2017) 1210–1214

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Diurnal variations in iron concentrations and expression of genes involved in iron absorption and metabolism in pigs

Yiming Zhang [a, b], Dan Wan [a, *], Xihong Zhou [a], Ciming Long [a], Xin Wu [a, **], Lan Li [a], Liuqin He [a], Pan Huang [a], Shuai Chen [a], Bie Tan [a], Yulong Yin [a, b, ***]

[a] Key Laboratory of Agro-Ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Chinese Academy of Sciences, National Engineering Laboratory for Pollution Control and Waste Utilization in Livestock and Poultry Production, Hunan Provincial Engineering Research Center for Healthy Livestock and Poultry Production, Scientific Observing and Experimental Station of Animal Nutrition and Feed Science in South-Central, Ministry of Agriculture, Changsha, Hunan 410125, China
[b] Animal Nutrition and Human Health Laboratory, School of Life Sciences, Hunan Normal University, Changsha, Hunan 410125, China

ARTICLE INFO

Article history:
Received 22 June 2017
Accepted 30 June 2017
Available online 1 July 2017

Keywords:
Iron
Up take
Homeostasis
Diurnal variation
Pig

ABSTRACT

Diurnal variations in serum iron levels have been well documented in clinical studies, and serum iron is an important diagnostic index for iron-deficiency anemia. However, the underlying mechanism of dynamic iron regulation in response to the circadian rhythm is still unclear. In this study, we investigated daily variations in iron status in the plasma and liver of pigs. The transcripts encoding key factors involved in iron uptake and homeostasis were evaluated. The results showed that iron levels in the plasma and liver exhibited diurnal rhythms. Diurnal variations were also observed in transcript levels of divalent metal transporter 1 (*DMT1*), membrane-associated ferric reductase 1 (*DCYTB*), and transferrin receptor (*TfR*) in the duodenum and jejunum, as well as hepcidin (*HAMP*) and *TfR* in the liver. Moreover, the results showed a network in which diurnal variations in systemic iron levels were tightly regulated by hepcidin and Tf/TfR via *DCYTB* and *DMT1*. These findings provide new insights into circadian iron homeostasis regulation. The diurnal variations in serum iron levels may also have pathophysiological implications for clinical diagnostics related to iron deficiency anemia in pigs.

1. Introduction

Iron is a microelement that is essential for various physiological functions, including electron transfer, gene regulation, oxygen transport and storage, energy metabolism, and redox reactions [1,2], but can be toxic when present in excess [3]. The systemic iron concentration is a key factor determining the risk of anemia [4], heart failure [5], premature birth [6], low birth weight in infants [7,8], and delayed maturation in children [9].

In the diet, iron is present as heme or non-heme, the latter of which is frequently present in animal feeds. Iron enters the body primarily via duodenal enterocytes, where the ferric iron is reduced by membrane-associated ferric reductase 1 (*DCYTB*) and transported via divalent metal transporter (*DMT1*) [1,10]. Most iron is delivered to cells bound to plasma transferrin (Tf) under the regulation of the liver-derived peptide hepcidin (HAMP). Consequently, Tf-bound iron is delivered into the interior of the cell via TfR1 through receptor-mediated endocytosis [3].

Clinical studies have shown that serum iron (SI), total iron binding capacity (TIBC), and hepcidin and ferroportin concentrations fluctuate with the diurnal cycle [11–14]. However, no reports have shown the daily rhythms of iron levels in the liver or the gene transcription involved in iron uptake and homeostasis regulation. Because plasma iron levels can be used to monitor iron deficiency and overload [15], investigating the diurnal expression of genes involved in the regulation of iron uptake and homeostasis would be helpful for devising methods to prevent iron deficiency and overload. The pig model, as a major mammalian model for human studies because of its similarities in size, physiology, sleep-wake rhythms, organ development, and disease progression, allows for

* Corresponding author. Institute of Subtropical Agriculture, Chinese Academy of Sciences, Yuanda (Second) Road 644, Changsha, Hunan 410125, China.
** Corresponding author. Institute of Subtropical Agriculture, Chinese Academy of Sciences, Yuanda (Second) Road 644, Changsha, Hunan 410125, China.
*** Corresponding author. Institute of Subtropical Agriculture, Chinese Academy of Sciences, Yuanda (Second) Road 644, Changsha, Hunan 410125, China.
E-mail addresses: w.dan@isa.ac.cn (D. Wan), wuxin@isa.ac.cn (X. Wu), yinyulong@isa.ac.cn (Y. Yin).

http://dx.doi.org/10.1016/j.bbrc.2017.06.187
0006-291X/

deliberately timed studies and collection of repeated peripheral samples and tissues [16]. Thus, in the present study, we evaluated whether iron content in the plasma and liver displayed diurnal variations in piglets and assessed changes in the diurnal expression patterns of genes related to the regulation of iron uptake and homeostasis.

2. Materials and methods

2.1. Animals, feeding, and sampling

The animal experiment was conducted according to the animal welfare requirements and approved by the Animal Protocol Review Committee of the Institute of Subtropical Agriculture, Chinese Academy of Science (Changsha, China).

Piglets (Landrace × Yorkshire × Duroc) were weaned at 28 days of age in good health and housed individually under a 12:12-h light-dark cycle (lights on at 06:00 and lights off at 18:00), with free access to water. The composition of the experimental diet was presented in our pervious study [17]. The detected iron level in feed was 159 mg/kg feed by ICP-OES. After a 7-day adaption period, all piglets were fed a corn-soybean based diet at 08:00, 12:00, and 17:00 for 21 days. Pigs were fed ad libitum for 30 min every time, and the remaining food was removed.

Sixty pigs with an average body weight (BW) of 16.5 ± 0.23 kg were selected at 56 days of age for sample collection at 03:00 (Clo3) and 07:00 (Clo7), 11:00 (Clo11), 15:00 (Clo15), 19:00 (Clo19), and 23:00 (Clo23). At each time point, 10 pigs were chosen for blood collection by jugular puncture. Four pigs were then anaesthetized with Zoletil (15 mg tiletamine/kg BW, 15 mg zolazepam/kg BW, i.m.) and bled by exsanguination. The liver, duodenum, and jejunum samples were then collected within 30 min at each time point.

2.2. Iron status analysis

SI content was assayed using a Cobas C311 analyzer (Roche Diagnostics, Rotkreuz, Switzerland), according to the manufacturer's instructions. Liver iron levels were determined by inductively coupled plasma optical emission spectrometry (ICP-OES; ICP 720 ES; Agilent, USA). Liver samples (5.00 ± 0.20 g) were weighed in triplicate and subjected to acid digestion using a mixture of nitric and perchloric acids following heating (80 °C for 60 min; 120 °C for 30 min; and 180 °C for 30 min). The samples were dried at 260 °C and redissolved in 5 mL of 1% HNO_3. Samples were then transferred to a 25-mL volumetric flask and diluted with 1% HNO_3. Subsequently, samples were filtered and subjected to ICP analyses for confirmation with standard references, and the precision of the analytical method was calculated as the relative standard deviation of metal concentrations in the digests of the same sample (6.45%).

2.3. Gene expression analysis

Total RNA was isolated from liquid nitrogen-frozen and ground tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Primers were designed with Primer 5.0 (Table 1). Real-time polymerase chain reaction (PCR) was performed, and the results were calculated as previously described [16].

2.4. Statistical analysis

The data were presented as mean values. All statistical analyses were performed using SPSS 17.0 software. Differences among treatments were evaluated using one-way analysis of variance followed by least significant difference (LSD) tests, considering the time point as the fixed effect and the animal as the randomized effect. These data were presented as means ± standard errors of the means (SEMs). Differences in mean values were considered significant when the *P* value was less than 0.05.

Table 1
Primer sequences used for RT-PCR.

Gene	Primer sequences (5′–3′)
β-Actin (Reference gene)	F: TGCGGGACATCAAGGAGAAG
	R: AGTTGAAGGTGGTCTCGTGG
Tf	F: GCGGGTTTGGTATTTGAGGC
	R: GGTTTGTGGATTATCTTTCTGCCC
TfR1	F: GGCTGTATTCTGCTCGTGGA
	R: AGCCAGAGCCCCAGAAGATA
TfR2	F: GTGATGGAGACCCCCTTGTG
	R: GCCCATTATGAAAGGCGCTG
HAMP	F: GTCGCAAAGCAATCTGTGGG
	R: GTTGGAGGCAGCCGGAATAA
DMT1	F: GCAGGTGGTTGACGTCTGTA
	R: CACGCCCCCTTTGTAGATGT
DCYTB	F: CTGTCCGTGATCTTCACCCTC
	R: ACCCTGCGTGGATGGATTTC

Tf = transferrin; *TfR1* = transferrin receptor 1; *TfR2* = transferrin receptor 2; *HAMP* = hepcidin; *DMT1* = divalent metal transporter 1; *DCYTB* = membrane-associated ferric reductase CYBRD1.

3. Results and discussion

3.1. Diurnal variations in iron status in the serum and liver

Several clinical studies have demonstrated that serum iron levels display rhythms dependent on the time of day [11–14,18–21]. However, the diurnal variations in SI are still unclear because of inconsistencies in published research. In the present study, SI levels reached a peak at Clo15 in the afternoon and reached a nadir at Clo7 in the morning (Fig. 1A). This result was similar to the findings of Statland et al. [18], who found that SI levels were significantly higher at 14:00 than at 08:00 and 11:00. Moreover, liver iron levels have been shown to exhibit rhythms dependent on the time of day. In our study, liver iron levels peaked at Clo7 in the morning and showed a nadir at Clo23 at night (Fig. 1B). Although significant diurnal variations in calcium, zinc, and copper levels were found in human serum [22], diurnal variations in calcium, zinc, and copper were not found in the serum or liver (Fig. S1).

3.2. Diurnal variations in the expression of genes related to non-heme iron uptake

We found significant oscillations in *DMT1* (Fig. 2A, B) and *DCYTB* (Fig. 2C, D) expression in the duodenum and jejunum. The diurnal transcript levels of *DMT1* and *DCYTB* in the duodenum and jejunum were similar. Peaks of *DMT1* transcripts appeared at Clo11 and Clo23 and nadirs appeared at Clo7 and Clo19. Concurrently, peaks of *DCYTB* transcripts appeared at Clo11 in the duodenum and at Clo11 and Clo19 in the jejunum and ileum. Previous reports have shown that *DMT1* transcripts are mainly regulated by the post-transcriptional iron regulatory protein (IRP)/iron response element (IRE) system under high-iron conditions [23]. Our results showed that the nadir time of *DMT1* transcript levels appeared after the peak time of SI levels, consistent with a study by Lymboussaki [23]. In addition to *DMT1* transcription, diurnal variations in *DCYTB* were also likely to be post-transcriptionally regulated by SI concentrations since *DMT1* and *DCYTB* exhibited similar trends under the circadian rhythm. However, no significant daily variations in *DMT1* and *DCYTB* transcription were found in the liver (data not shown).