Theriogenology 103 (2017) 90–97

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Expression of hepcidin and ferroportin in full term placenta of pregnant cows

Sante Roperto [a, *], Valeria Russo [a], Chiara Urraro [a], Anna Cutarelli [b], Antonella Perillo [c], Francesca De Falco [a], Franco Roperto [d]

[a] Dipartimento di Medicina Veterinaria e Delle Produzioni Animali, Università di Napoli Federico II, Napoli, Italy
[b] Istituto Zooprofilattico Sperimentale Del Mezzogiorno, Portici (NA), Italy
[c] Dipartimento di Medicina Veterinaria, Università di Bari "Aldo Moro", Bari, Italy
[d] Dipartimento di Biologia, Università di Napoli Federico II, Napoli, Italy

ARTICLE INFO

Article history:
Received 31 January 2017
Received in revised form
4 May 2017
Accepted 25 July 2017
Available online 27 July 2017

Keywords:
Hepcidin
Ferroportin
Iron homeostasis
Placenta
Cattle

ABSTRACT

Hepcidin (HEP) and ferroportin (FPN) play a central role in systemic iron homeostasis. The HEP/FPN axis controls both extracellular iron concentration and total body iron levels. HEP is synthesized mainly by hepatocytes and controls the absorption of dietary iron and the distribution of iron to the various cell types; its synthesis is regulated by both iron and innate immunity. FPN is a membrane protein and the major exporter of iron from mammalian cells, including iron recycling macrophages, iron absorbing duodenal enterocytes, and iron storing hepatocytes. HEP limits the pool of extracellular iron by binding FPN and mediating its degradation, thus preventing its release from intracellular sources. Here we investigated, for the first time, the molecular and morphological expression of HEP and FPN in placenta of pregnant cows at term. Their expression has been evaluated investigating their mRNAs by reverse transcriptase PCR (RT-PCR). Sequencing of related amplicons revealed a 100% identity with HEP and FPN sequences from *Bos taurus* as reported in the GeneBank (mRNASequence ID: NM_001114508.2 and ID: NM_001077970.1, respectively). HEP and FPN proteins have also been revealed by Western blot analysis and immunohistochemistry. The strongest immunoreactivity for both proteins was observed in the cytoplasm of the trophoblastic cells of the villi and the caruncular crypts of the placentome. Hep mRNA was more representative in caruncular rather cotyledonar areas; on the contrary, Fpn mRNA was more expressed in cotyledonar rather than in caruncular areas. Transcripts of ferritin, transferrin and its receptor have been also documented by real time RT-PCR.

HEP and FPN placental proteins may play a dual role. HEP/FPN axis seems to have a central role in infections, with microorganisms within macrophages or that survive in the bloodstream or other cellular spaces. In addition, HEP may be responsible for iron flux regulation as a molecular bridge for iron trafficking and response to infection. FPN may also have a significant role for embryonic development, growth and organogenesis.

1. Introduction

Hepcidin (HEP) is the key peptide which regulates systemic iron homeostasis in vertebrates [1]. HEP is synthesized and secreted by hepatocytes, free circulates in the blood plasma with the exception of weak binding to albumin and α2-macroglobulin and is filtered by the kidneys [2,3]. HEP is also part of the innate immune system and is strongly induced during inflammation and infections [1,4]. HEP expression appears to be modulated by multiple endocrine stimuli including testosterone, growth factors such as platelet-derived growth factor BB, and gluconeogenesis [5,6] but these pathophysiological mechanisms are poorly understood [7].

HEP was first described as a cationic antimicrobial peptide with microbicidal properties against many microorganisms in vitro [8,9]. During bacterial infection, hepatic HEP expression is strongly upregulated via the induction of interleukin 6 (IL-6), which results in rapid reduction of serum iron levels [1,10]. It has been also suggested that HEP may play an important role in parasitic, fungal

* Corresponding author.
E-mail address: sante.roperto@unina.it (S. Roperto).

http://dx.doi.org/10.1016/j.theriogenology.2017.07.031
0093-691X/

and viral infections [11,12].

In addition to hepatocytes, many cell types express low levels of HEP such as monocytes and macrophages [13–15], syncytiotrophoblast cells of the placenta [16], gastric parietal cells [17], and adipose tissue cells [18].

In veterinary medicine, searches from genome databases allowed Hep sequences to be detected in cattle, pig, dog, cat [19]. Furthermore, identification and characterization of Hep gene has been performed in the liver of sheep [20], horse [21], buffalo [22]. Lower levels of HEP were also found to be expressed in different ovine and equine tissues such as abomasum, bladder, bone marrow, duodenum, cerebellum, cerebral cortex, cervical spinal cord, hearth, kidney, lung, lymph nodes, pancreas, spleen, skeletal muscle, stomach [20,21]. Bovine, ovine and bubaline HEP amino acid sequences have a strong identity [20,22]. Unlike other antimicrobial peptides, HEP sequences are believed to be quite similar among various mammalian species [23,24].

Ferroportin (FPN) is the only known mammalian iron exporter protein and it is believed to be central to iron homeostasis at the systemic level [25]. FPN is essential for iron transport among cell types, and playing a role in both prenatal and postnatal iron metabolism [26,27].

In the duodenum, FPN facilitates iron transport from the enterocytes to the circulation. In the macrophage, iron released from the breakdown of heme is transported out of the cell to the plasma via FPN. Accordingly, FPN is highly expressed in the membrane of mature duodenal enterocytes, hepatocytes and in macrophages recycling iron from breakdown of aged red blood cells [28,29].

FPN is also found in heart, placenta, lung, kidney, all organs not apparently involved in systemic iron homeostasis. The precise role in these tissues has not been yet clarified [25,28].

FPN-mediated iron transport is highly regulated by the HEP antimicrobial peptide hormone. HEP regulates iron acquisition causing internalization and degradation of FPN [30].

During fetal development, placenta actively transfers iron from the mother to the fetus [31,32]. It is believed that placenta may function as storage compartment for iron not transferred to plasma [31]. Some heme and non-heme trafficking proteins are found to be expressed in the placenta; however, the role, regulation and functions of these proteins in the placenta are largely uncharacterized [33].

Regarding placental HEP and FPN, there are limited data about their role in the regulation of placental uptake of maternally ingested heme and non-heme Fe sources [33] and the role of HEP and FPN in iron transport across the placenta is still poorly understood [32].

To our knowledge, HEP and FPN expression has not been previously investigated in the placenta of domestic animals.

Here we describe some molecular and morphological findings of the expression of HEP and FPN in pregnant cows' full term placenta.

2. Materials and methods

2.1. Animals

Placentomes from twelve pregnant cows were collected immediately after delivery. They were longitudinally cut into two halves, with one of them being immediately fixed in 10% neutral buffered formalin for light microscopy. The contralateral half was frozen at −80 °C for biomolecular analyses.

2.2. Western blot analysis

Placentome samples were lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 400 mM NaCl, 1 mM EDTA, 2 mM PMSF, 1.7 mg/ml Aprotinin, 50 mM NaF, and 1 mM sodium orthovanadate). HepG2 human hepatoma cell line was utilized as control both for HEP and FPN. Samples were clarified by centrifugation, separated by SDS-PAGE (15% for HEP and 10% for FPN) and transferred onto nitrocellulose membranes (GE Heatlhcare, UK). Membranes were blocked with TBST (Tris buffered saline: 20 mM Tris, 500 mM NaCl, pH 7,5 and 0.1% Tween 20) containing 5% no fat dry milk for 1 h at room temperature, being subsequently incubated overnight at 4 °C with primary antibodies to HEP (rabbit polyclonal antibody - Biorbyt Ltd, CA, USA), FPN (rabbit polyclonal antibody - Biorbyt Ltd, CA, USA) and actin (mouse monoclonal antibody - Santa Cruz Biotechnology, TX, USA). Membranes were washed three times with TBST, incubated for 1 h at room temperature with goat anti-rabbit (Bio-Rad, CA,USA) and goat anti-mouse (Bio-Rad, CA, USA) HRP-conjugated secondary antibodies diluted at 1:2000 in TBST, and washed three times with TBST. Immunoreactive bands were detected using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, TX, USA) and ChemiDoc XRS Plus (Bio-Rad, CA, USA). Images were acquired with Image Lab Software version 2.0.1 (Bio-Rad, CA, USA).

2.3. Reverse transcriptase (RT)-PCR

Total RNA was extracted from the bovine placentome by RNeasy Mini Kit (Qiagen TM, ME, DE). 1 μg of the total RNA was used to generate the first strand of cDNA by the QuantiTect Reverse Transcription Kit (Qiagen TM, ME, DE), in according to the manufacturer's instructions. PCR was performed with a specific primer set designed by the Primer3 online tool for hepcidin (HAMP gene), ferroportin (SLC40A1 gene), ferritin-heavy chain (FTH1 gene), transferrin (TF gene) and transferrin receptor (TFRC gene). The same procedure was used to process control liver samples from healthy cattle from abattoirs. The following primers were used: **HAMP** forward 5′-TCCTTGTCCTGCTCAGCCTG -3′, reverse 5′-CAGCAGAAGATGCAGATGGGAA-3′; **SLC40A1** forward 5′- GCTGTGGTTTCATTTCGGCA-3′, reverse 5′- GCTTGGCTCTTGCTCATGT -3′; **TF** forward 5′ -GCTGTGGTCTCACGGAAAGA -3′, reverse 5′- GCTCTGACGTAGTCATCCCC-3′; **TFRC** forward 5′- GGAGTGGTGGAGACTTTGGA-3′, reverse 5′- TGTCCCGATACAGAGACAGC-3′; **FTH1** forward 5′-TGCAATGGAATGTGCGCTGT-3′, reverse 5′ CCCAGGGTGTGCTTGTCAAA-3′. Conditions for PCR were: 94 °C for 5 min, 40 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30s.

2.4. Real time RT-PCR

Placentome samples were obtained both from cotyledonar (fetal) and caruncular (maternal) components. The expression of Hep and Fpn in both samples was evaluated by real time RT-PCR. To perform real time RT-PCR analysis, total RNA and cDNA from placentome samples were produced as previously described. Real-time RT-PCR was carried on a Bio Rad CFX Connect™ Real Time PCR Detection System (Bio Rad Hercules, CA, USA) using iTaq Universal SYBR® Green Supermix (Bio Rad Hercules, CA, USA). Each reaction was set in triplicate and the primers used for HEP and Ferroportin were the same of RT-PCR. The thermal profile for the PCR was 95 °C for 10 min, 40 cycles of 94 °C for 15 s, 58 °C for 30 s, followed by melting curve. The relative quantification (RQ) was calculated by using CFX Manager™ software, based on the equation $RQ = 2^{-\Delta\Delta Cq}$, where Cq is the quantification cycle to detect fluorescence. Cq data were normalized to the reference β-actin gene (forward: 5′- TAGCACAGGCCTCTCGCCTTCGT-3′, reverse 5′-GCACATGCCGGAGCCGTTGT-3′).