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Intestinal over-expression of iron transporters induces iron overload in birds in captivity

Aslı Mete[a,*], Ruud Jalving[b], Bernard A. van Oost[b], Jaap E. van Dijk[a], Joannes J.M. Marx[c]

[a]*Department of Pathobiology, Division Pathology, Veterinary Medicine, The Netherlands*
[b]*Department of Animals, Science and Society, Utrecht University, The Netherlands*
[c]*Eijkman-Winkler Infection Center, University Medical Center Utrecht, The Netherlands*

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Abstract

Hereditary hemochromatosis (HH) is a frequent genetic disease of older subjects of northern European descent. It is characterized by increased iron absorption and severe iron overloading in parenchymal organs. A similar disturbance of iron metabolism occurs in specific animal species in captivity. To address the key features leading to high absorption and thus to iron overload in these animals, we have studied the two iron transport proteins DMT1 and Ireg1 in the best-known susceptible species, the mynah bird. Here, we show that these birds have a high expression of DMT1 in the duodenum and also a strikingly high expression of Ireg1 along the whole small intestine. We believe that the iron accumulation in susceptible species only occurs in captivity because of a genotypic adaptation to their natural environment, where contrary to captivity, dietary iron is hardly available. The Caucasian population carrying mutations leading to iron overload today may have also benefited from the genetic advantage of up-regulating iron transport millennia ago, when dietary iron was scarce.

Keywords: Hereditary hemochromatosis; Iron overload; Birds; DMT1; Ireg1

Introduction

Major climatological or cultural changes may benefit survival of the fittest species in terms of the ability to extract vital nutrients from available diets. Iron is a requisite nutrient for sustaining life for all organisms. Therefore, an obvious driving force for genetic selection is surviving severe iron deficiency at early age and during the reproduction period. When iron absorption is inappropriately increased for many years, however, iron overload with fatal consequences may occur.

Iron storage disease is encountered as a common disorder in specific animal species in captivity [1]. These animals develop iron overload primarily in the liver, regardless of their background and duration of captivity, and often die from its consequences. Among birds, postmortem diagnosis of iron overload in hepatocytes is frequently encountered in those that are mainly frugivorous. While the etiological aspects are constantly under discussion [2], the high prevalence has led to the development of preventive measures like adding tea leaves to their diets in order to inhibit the absorption of iron [3]. The mynah bird, a very common pet bird, is a prominent model of the susceptible species. Mynahs in the wild originally feed on a diet consisting of mainly fruits and sometimes insects, which are low in iron content. However, when confined to captivity, where they are given a commercial diet, they develop pathological iron accumulation in the liver. In our previous study, conducted to investigate the background of this susceptibility, we have observed that these birds have tremendously iron-loaded livers, can down-regulate uptake of iron to some extent, but still

* Corresponding author. Fax: +31 30 253 31 31.
E-mail address: a.mete@vet.uu.nl (A. Mete).

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absorb much more iron from the diet than non-susceptible, control animals [4].

In humans of Northern European origin, hereditary hemochromatosis (HH) caused by alterations in the HFE gene, one of the regulators of iron transport, is the most common autosomal recessive disorder [5]. Mutations in the HFE gene lead to disturbance of expression of other genes involved in the iron-sensing cascade in an indirect manner [6]. A major effect is on the expression of the two transport proteins regulating iron uptake and release by intestinal mucosal cells in mammals: DMT1 (Nramp2/DCT1) as the apical membrane iron importer [7] and Ireg1 (ferroportin/MTP) as the basolateral membrane iron exporter [8]. These two proteins are critically located and function in transporting optimal amounts of iron from dietary sources to the plasma. Studies in HH patients have shown that despite the iron overloading in parenchymal organs, dietary iron absorption is relatively high, and that DMT1 and Ireg1 in the intestines are expressed in inappropriately high levels with respect to body iron status [9–11].

In order to understand the molecular basis of the iron overload syndrome in susceptible species, we have investigated the gene expression pattern of DMT1 and Ireg1 in mynah birds in comparison to chickens, a non-susceptible bird species.

Materials and methods

Animals

We used material from 4 mynah birds and 4 chickens, all stabilized with respect to the dietary iron levels. The scheme of experiments is described elsewhere [12]. Sections of liver, kidney, and small intestines (corresponding to duodenum, jejunum, and ileum) were excised; pieces of each tissue were placed in liquid nitrogen for histology, RNA extraction, and protein work. A section of the liver was also taken for iron content measurements by atomic absorption spectrometry.

Histology and liver iron determinations

Histological examination was performed on 3-μm sections stained with hematoxylin and eosin and Perls' iron stain. Liver parts stored at −20°C were analyzed for iron content by atomic absorption spectrometry (Cobas Bio autoanalyzer, Roche, Basel, Switzerland).

Real-time PCR analysis

Total RNA was extracted with the use of TRIzol reagent (Life Technologies, Melbourne, Australia) as per the manufacturer's instructions. RNA integrity was confirmed using gel electrophoresis, and the concentration of each sample was determined by ultraviolet spectrophotometry. One microgram of the total RNA was reverse transcribed to cDNA using random hexamer primers (Invitrogen, CA, USA). Measurements of the efficiency of the conversion revealed amounts ranging from 800–980 ng cDNA/μl (NanoDrop 3.0, DE, USA). Samples were treated with DNAse prior to PCR amplifications.

Chicken EST sequences of DMT1 and Ireg1 (Genbank DMT1:BI393221 and Ireg1: BU305954) and chicken mRNA sequence of β-actin (Genbank L08165) were used to design primers and probes for both chicken and mynah samples. β-actin is used as the endogenous control (96% identity exists between the chicken and mynah sequences). Initial primers used for amplification and determination of mynah sequences were as follows: DMT1 forward 5′-ATTCGTGCTTCAGCTTCCG-3′, reverse 5′-GAGCCGATGACCTCCTGC-3′, Ireg1 forward 5′-TCTATGGACTGGTTGTGGC-3′, reverse 5′-GCCATCCATGGTATAAGGTC-3′, and β-actin forward 5′-GTGGGGCGCCCCAGGCACCA-3′, reverse 5′-TCCTTAATGTCACGCTCCATTTG-3′. The PCR reaction for DMT1 and Ireg1 consisted of denaturation at 94°C 2′, (94°C 30″, 55°C 30″, 72°C 1′) for 40 cycles and extension of 72°C for 7′. The annealing temperature for β-actin was 62°C. The obtained fragments were sequenced using the Big Dye terminator. Real-time PCR primers and Taqman probes (MGB probe, non-fluorescent quencher) (Applied Biosystems, CA, USA) were as follows: DMT1: chicken forward 5′-AGCAGCCCGATCACCGT-3′, reverse 5′-ATCGAGTCCGACCTGCAGTC-3′, mynah forward 5′-AGCAGCCCGATGACGGT-3′, reverse 5′-CGAGTCTGACCTGCAGTCTGG-3′, and probe for both species 6-FAM-CCCACAGCAGCTTG-MGB; Ireg1: chicken forward 5′-GATGCATTCTGAACAACCAAGGA-3′, mynah forward 5′-AGATGAATTCTGCACAATCAAGGA-3′, reverse and probe for both species 5′-GGAGACTGGGTGGACAAGAACTC-3′, and 6-FAM-TCTGGGCCACTTTGA-MGB, respectively; β-actin: forward 5′-CCCAAAGCCAACAGAGAGAAG-3′, reverse 5′-GGGACAGCACAGCCTGGAT-3′, and probe 6-FAM-GACACAGATCATGTTTGA-MGB. Reaction volume was 20 μl, consisting of 200 nM probe, 900 nM primers, and on average 90 ng template per reaction. Real-time PCR conditions were 95°C denaturation for 10′, then 40 cycles of 95°C 15″, 60°C 1′ (ABI Prism 7000 Sequence Detection System, Applied Biosystems).

The results were analyzed using the Comparative C_T (cycle threshold) method, where differences are determined by using the formula $2^{-\Delta\Delta CT}$ (Applied Biosystems, Users Bulletin #2). Standardizing the amplification signals of our genes of interest to the amount of endogenous control [β-actin (ΔC_T)] and a calibrator [kidney ($\Delta\Delta C_T$)] enabled us to make intra- and inter-species comparisons. To calculate the inter-species differences, the chicken values were used as calibrators, thus enabling us to compare the C_T values of the mynah to the chicken.