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Anemia upregulates lipocalin 2 in the liver and serum

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ABSTRACT

Lipocalin 2 (Lcn2), a mammalian protein that is expressed and secreted in various pathologic states, binds siderophores, which are high-affinity iron chelators. Besides its role in limiting iron availability to pathogens in the setting of bacterial infection, Lcn2:siderophore complexes can also deliver iron to cells. In this study, we examined Lcn2 regulation in the liver of mice in situations of increased iron utilization, namely, during anemia. Anemia induced by phlebotomy, iron deprivation, or phenylhydrazine treatment was associated with upregulation of Lcn2 gene expression in the liver and elevation of serum Lcn2 protein levels. We further explored the participation of several factors known to co-occur during anemia, including hypoxia, changes in iron levels, and erythropoietic drive, in the regulation of Lcn2 by anemia. We found that hypoxia, but not iron or erythropoietin, caused an induction of Lcn2 expression. The upregulation of Lcn2 levels by anemia and hypoxia, which is not directly mediated by iron or erythropoietin, suggests a possible physiological role for Lcn2 during increased iron utilization and mobilization from stores.

Introduction

Iron is an essential cofactor of numerous proteins and enzymes that are critical for cell proliferation, respiration, and signal transduction. However, free iron can also be toxic because of its capacity to induce the formation of dangerous free radicals. Thus, both systemic and cellular iron balance have to be tightly maintained. Biological systems have evolved various strategies to deal with the difficulty of acquiring iron, storing it and, at the same time, avoiding its toxic potential. Each of these tasks is accomplished via the coordinated regulation of specialized molecules involved in iron absorption, transport, cellular uptake and storage [1].

In normal situations, cellular iron uptake occurs through the transferrin (Tf)-dependent pathway, involving Tf binding to transferrin receptor-1 (TfR1) on the cell membrane and its internalization by receptor-mediated endocytosis [2]. However, in conditions of altered homeostasis, iron can also be delivered to cells by alternative mechanisms, which are Tf-independent [1].

More recently, an iron-delivery pathway was identified that involves a member of the lipocalin superfamily [3]: antimicrobial protein neutrophil gelatinase-associated lipocalin (NGAL) or lipocalin 2 (Lcn2) [4].

NGAL/Lcn2 was first identified as a 25-kDa glycoprotein associated with purified human neutrophil gelatinase [5]. Homologous proteins were also identified in the mouse (Lcn2/24p3/uterocalin) and rat (α_2-microglobulin-related protein/neu-related lipocalin) [6]. Lcn2 can bind catecholate-type bacterial ferric siderophores [7] (e.g. enterobactin), which are low molecular compounds that bind ferric iron and are able to acquire iron from mammalian iron-binding proteins, including Tf and lactoferrin [8]. Since Lcn2 binds enterobactin with higher affinity than the E. coli enterobactin transporter, it effectively interferes with bacterial iron uptake and, in fact, acts as a bacteriostatic agent [7].

In addition to its role as a bacteriostatic agent, Lcn2 has been shown to mediate a Tf-independent iron uptake pathway activated during kidney development [9]. The iron delivered to cells by Lcn2 is capable of regulating iron-dependent genes, such as ferritin and TfR1 [9,10], that are sensitive to cellular iron status [11], indicating that cells can utilize the iron provided by Lcn2.

While growing evidence supports the involvement of Lcn2 in a Tf-independent iron uptake pathway activated during early development, it remains unclear whether this pathway may become activated in response to disrupted iron homeostasis during adulthood.

In this study, we examined Lcn2 regulation in situations of disrupted iron homeostasis, namely, during anemia. We further explored separately the role of several factors known to co-occur during anemia, including hypoxia, changes in iron levels, and erythropoietic drive, in the regulation of Lcn2 by anemia.

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Materials and methods

Animals

All procedures were performed in accordance with guidelines of the Canadian Council on Animal Care and were approved by the Institutional Animal Care Committee of the Centre Hospitalier de l'Université de Montréal (CHUM). C57BL/6 female mice aged 3 or 8 weeks were purchased from Charles River Laboratories, Inc. (Wilmington, MA). All mice were kept under strict specific pathogen-free conditions.

Animal treatments

Control mice were fed a commercial diet containing approximately 226 mg of iron per kg (Teklad Global 18% protein rodent diet TD 2018, Harlan Teklad, Madison, WI). Dietary iron overload was produced by giving 8-week-old mice the same commercial diet supplemented with 25 g carbonyl iron (Sigma-Aldrich, St. Louis, MO) per kg (TD 02030) for 4 weeks. Iron deficiency was induced by feeding 3-week-old mice the same commercial diet deficient in iron containing less than 3 mg of iron per kg (TD 80396) for 9 weeks. All mice were 12 weeks old at the time of sacrifice.

To induce anemia through phlebotomy, 0.25 mL of blood was extracted by retro-orbital puncture from anesthetized mice. The procedure was repeated 24 h later, and the animals were sacrificed 16 h after the last phlebotomy.

Hemolytic anemia was produced by i.p. administration of 40 mg/kg body weight of phenylhydrazine (PHZ, Sigma-Aldrich), once daily for 4 days. The mice were sacrificed the following day.

Cobalt chloride ($CoCl_2$)-induced hypoxia was elicited by i.p. injection of 60 mg/kg of $CoCl_2$ (Sigma-Aldrich) dissolved in 0.9% saline. Control mice were injected with an equivalent volume of saline. All mice were sacrificed 24 h after injection.

Normobaric hypoxia was established by diluting ambient air with nitrogen in a specially ventilated chamber, in which N_2-enriched air supply was controlled with an O_2 sensor-driven inlet valve. The mice were maintained in the chamber and exposed to normobaric hypoxia (10% O_2) for 1, 3, and 5 days. An oxygen analyzer was used to monitor oxygen concentration in the hypoxic chamber. The control mice were kept under normoxia (room air) in the same room in which the hypoxic chamber was placed.

Erythropoiesis was induced by treating mice with 50 U of human biosynthetic erythropoietin-α (EPO) (epoetin alfa, Ortho Biotech, Bridgewater, NJ) dissolved in phosphate-buffered saline (PBS). The mice were injected i.p. daily for 4 days and were sacrificed on day 5. Control mice were similarly injected with an equivalent volume of PBS.

As a positive control for Lcn2 induction in the liver [12], mice were injected with lipopolysaccharide (LPS, *Escherichia coli* serotype 055: B5 — 1 mg/kg i.p., Sigma-Aldrich). Control mice were injected with an equivalent volume of saline solution. All animals were sacrificed 6 h after the injection.

Table 1
Erythroid parameters

	Treatments				
	Control ($n=6$)	Phlebotomy ($n=6$)	Iron deficiency ($n=6$)	Phenylhydrazin ($n=6$)	EPO ($n=8$)
Hb, g/dL	13.7±0.5	5.1±0.6**	12.4±0.5*	10.2±0.9**	15.0±0.5**
HCT, %	43.9±1.6	14.3±1.3**	38.1±1.2**	13.0±1.7**	52.3±1.7**

Data are presented as means±SD.
*$P<0.005$ compared to control mice.
**$P<0.0001$ compared to control mice.

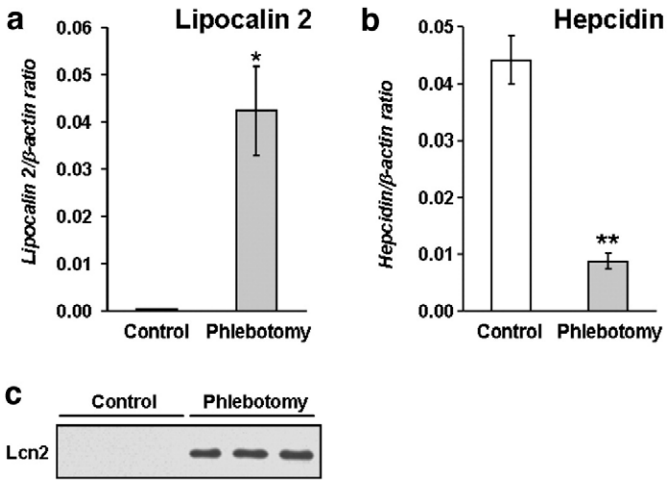

Fig. 1. Lipocalin 2 (Lcn2) expression is induced by acute anemia. Lcn2 (a) and hepcidin (b) mRNA levels measured in the liver of control and phlebotomized mice by real-time PCR. The results are presented as means±SEM, $n=6$ per group. Student's t test. *$P<0.001$ and **$P<0.0001$. (c) Lcn2 in serum as detected by Western blotting.

Hematological measurements and transferrin saturation

EDTA-treated blood samples were obtained by orbital puncture under anesthesia. Red blood cell (RBC) count, hemoglobin (Hb), hematocrit (HCT) and mean corpuscular volume (MCV) were measured in an ABC vet counter (ABX hématologie, Montpellier, France). Serum iron, total iron-binding capacity (TIBC) and transferrin saturation were assessed by colorimetric assay [13], with the Kodak Ektachem DT60 system (Johnson & Johnson, Ortho Clinical Diagnostics, Mississauga, ON, Canada).

Measurement of liver iron concentration

Liver iron concentration was assessed by acid digestion of tissue samples, followed by iron quantification with atomic absorption spectroscopy [13].

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Liver total RNA was isolated with Trizol reagent (Invitrogen, Burlington, ON, Canada), and RT was performed with the Thermoscript RT-PCR system (Invitrogen). Lcn2, hepcidin, and β-actin mRNA levels were measured by real-time PCR in a Rotor Gene 3000™ Real Time DNA Detection System (Montreal Biotech Inc., Kirkland, QC, Canada), with the QuantiTect SYBRGreen I PCR kit (Qiagen, Mississauga, ON, Canada) [14]. The primers were 5′-CCCATCTCTGCTCACTGTCC-3′ and 5′-TTTTTCTGGACCGCATTG-3′ for Lcn2, 5′-AGAGCTGCAGCCTTTGCAC-3′ and 5′-GAAGATGCAGATGGGGAAGT-3′ for hepcidin, and 5′-TGTTAC-CAACTGGGACGACA-3′ and 5′-GGTGTTGAAGGTCTCAAA-3′ for β-actin. Lcn2 and hepcidin expressions levels were normalized to the housekeeping gene β-actin.

Western blot analysis

1.5 μL of serum was boiled in loading buffer containing 4% sodium dodecyl sulfate (SDS), 20% glycerol, and bromophenol blue for 5 min. Proteins were resolved on 12% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Amersham Biosciences, Baie d'Urfé, Québec, Canada). The membranes were blocked with 6% nonfat dry milk solution and incubated with anti-sip24/Lcn2 antibody [12] (a gift from Dr. Marit Nilsen-Hamilton, Iowa State University). To detect immunocomplexes formed, peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., Mississauga,