Redox Biology 17 (2018) 432–439

Contents lists available at ScienceDirect

Redox Biology

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

Research Paper

Ceruloplasmin and hephaestin jointly protect the exocrine pancreas against oxidative damage by facilitating iron efflux

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ARTICLE INFO

Keywords:
Multicopper ferroxidase
Hephaestin
Ceruloplasmin
Pancreas
Oxidative damage
Iron efflux

ABSTRACT

Little is known about the iron efflux from the pancreas, but it is likely that multicopper ferroxidases (MCFs) are involved in this process. We thus used hephaestin (*Heph*) and ceruloplasmin (*Cp*) single-knockout mice and *Heph/Cp* double-knockout mice to investigate the roles of MCFs in pancreatic iron homeostasis. We found that both HEPH and CP were expressed in the mouse pancreas, and that ablation of either MCF had limited effect on the pancreatic iron levels. However, ablation of both MCFs together led to extensive pancreatic iron deposition and severe oxidative damage. Perls' Prussian blue staining revealed that this iron deposition was predominantly in the exocrine pancreas, while the islets were spared. Consistent with these results, plasma lipase and trypsin were elevated in *Heph/Cp* knockout mice, indicating damage to the exocrine pancreas, while insulin secretion was not affected. These data indicate that HEPH and CP play mutually compensatory roles in facilitating iron efflux from the exocrine pancreas, and show that MCFs are able to protect the pancreas against iron-induced oxidative damage.

1. Introduction

Iron is a redox active metal that can exist in the ferrous or ferric state, and it is essential for many basic physiological processes [1]. However, excess ferrous iron can generate toxic reactive oxygen species (ROS) that can damage proteins, lipids, and DNA [2]. The safe handling of iron in the body is maintained by a complex interaction among multiple iron-binding proteins, transporters, receptors, ferroxidases, and ferrireductases [3,4]. Disruption of this finely-tuned system can lead to iron overload or iron deficiency and associated adverse effects [2,4].

Multicopper ferroxidases (MCFs) are known to facilitate cellular iron efflux in conjunction with the membrane ferrous iron exporter ferroportin1 (FPN1), by oxidizing ferrous iron to the ferric state [1]. Three MCFs, namely ceruloplasmin (CP), hephaestin (HEPH), and zyklopen, have been identified in vertebrates [1,5]. Despite their similar function, these MCFs vary widely in tissue distribution. HEPH is expressed most strongly in the small intestine and, accordingly, mice with global *Heph* knockout display severe iron accumulation in duodenal enterocytes [6]. However, HEPH is also expressed in human pancreatic β-cells [7] and a range of other tissues. CP is largely recognized as a soluble serum protein which is secreted by the liver, but it has also been found as a glycosylphosphatidylinositol (GPI)-linked protein in astrocytes and multiple organs [8,9]. Individuals with mutations in the *CP* gene (aceruloplasminemia) display significant iron deposition in the liver, glial cells, and, interestingly, in the pancreas [10].

The pancreas can easily become iron-overloaded. Patients with hereditary hemochromatosis, a common inherited disorder characterized by excessive dietary iron absorption and iron accumulation in many tissues, show most extensive iron deposition in the liver, heart, and pancreas [11]. Furthermore, iron accumulation in the exocrine pancreas has been found in a series genetically engineered mouse models that are characterized by systemic iron loading, including hypotransferrinaemic mice, and bone morphogenetic protein 6-, hemojuvelin-, and hepcidin-deficient strains [12–15]. These studies suggest that the pancreas is very active in taking up iron from the plasma. They also imply that the pancreas needs an equally efficient iron efflux

Abbreviations: MCF, multicopper ferroxidase; HEPH, hephaestin; CP, ceruloplasmin; ROS, reactive oxygen species; FPN1, ferroportin1; GPI, glycosylphosphatidylinositol; KO, knockout; WT, wild-type; PBS, phosphate buffered saline; IRE, iron responsive element; SOD, superoxide dismutase; GPx, glutathione peroxidase; MDA, malondialdehyde

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https://doi.org/10.1016/j.redox.2018.05.013
Received 13 May 2018; Received in revised form 23 May 2018; Accepted 28 May 2018
Available online 31 May 2018

mechanism to balance iron levels and protect itself against oxidative damage. Although FPN1 is expressed in human pancreatic islets and ductal epithelial cells [7] and in mouse pancreatic islets [16], the mechanism of iron efflux from the pancreas has not been investigated. Mice both lacking *Cp* and carrying a mutation in the *Heph* gene have iron loading in the pancreas [17], suggesting that CP and HEPH might both be involved in pancreatic iron efflux. In this study, we used *Heph* and *Cp* single-knockout (KO) mice and *Heph/Cp* double-knockout mice to examine the role of these MCFs in the pancreas.

2. Materials and methods

2.1. Mouse models

All animal studies were carried out in accordance with NIH guidelines. All mice used in this study were on a C57BL/6J genetic background. *Heph* KO, *Cp* KO, and wild-type (WT) mice used in this study were obtained from the laboratory of Gregory Anderson (QIMR Berghofer Medical Research Institute) as previously described [18]. *Heph* KO mice were bred to *Cp* KO mice to generate homozygous *Heph/Cp* KO mice. The mice were allowed unlimited access to a standard rodent diet containing approximately 180 mg/kg iron. All mice were bred and maintained at the Medical School of Nanjing University and the studies were approved by the Institutional Animal Care and Use Committee of Nanjing University [18]. Male mice at 6 months of age were used for all experiments.

2.2. Tissue collection and processing

Male mice were euthanized at six months of age. Blood was collected by cardiac puncture and the body was perfused with phosphate buffered saline (PBS) via the heart. The pancreas was quickly removed and a piece of the pancreatic tail was immediately fixed with isoamyl alcohol for later examination by transmission electron microscopy. Whole blood was centrifuged to provide plasma. Tissue samples were snap frozen in liquid nitrogen and then stored at − 80 °C until they were required for RNA, protein, and iron concentration analyses. In addition, four mice of each genotype were perfused via the heart, first with PBS and then with 4% paraformaldehyde. The collected tissues were fixed in 4% paraformaldehyde solution for later histological analysis.

2.3. Histology

Perls' Prussian blue staining was performed as previously described [6]. TUNEL staining (Cat#11684817910, Roche) was performed following the manufacturer's protocol. For CD68 and insulin staining, paraffin-embedded pancreas sections were deparaffinized in xylenes, rehydrated in a series of ethanol rinses from 100% to 70% ethanol, then washed in distilled water. Antigen retrieval was performed at 95 °C for 30 min. Sections were allowed to cool slowly, washed in distilled water, and incubated in 3% H_2O_2 for 25 min. Subsequently the sections were blocked in blocking buffer containing 3% BSA and 0.1% Tween-20 in PBS (PBST), at room temperature for 30 min. Sections were stained with the primary antibody overnight at 4 °C. Then, excess antibody was removed and sections were washed 3 times with PBST for 5 min each. Sections were incubated with the secondary antibody for 50 min at room temperature. After extensive washing with PBST (three times for 5 min), the sections were washed for 5 min in PBS and incubated with diaminobenzidine substrate (Cat#K5007, Dako) to visualize the antibody. Finally, the sections were dehydrated, counterstained with hematoxylin and mounted with xylene mounting media.

For CD68 staining, a rabbit polyclonal antibody against CD68 (Cat#ab125212, Abcam; diluted 1:2000 in blocking buffer) and a horseradish peroxidase conjugated goat anti-rabbit secondary antibody (Cat#G1210-2-A, Servicebio; diluted 1:1000 in blocking buffer) were used. For insulin staining, a mouse monoclonal antibody against insulin (Cat#ab6995, Abcam; diluted 1:100 in blocking buffer) and a horseradish peroxidase conjugated goat anti-mouse secondary antibody (Cat#BA1050, Boster; diluted 1:1000 in blocking buffer) were used.

2.4. Measurement of tissue non-heme iron levels

The concentration of non-heme iron was measured using a microtiter plate reader as previously described [6].

2.5. Transmission electron microscopy

Samples of the pancreas were fixed with isoamyl alcohol and delivered to the Department of Pathology, Nanjing General Hospital for further processing. Ultrathin sections (80 nm) were cut onto copper grids, treated with the contrast agents 2% uranyl acetate and lead citrate, and examined in a JEM-1011 microscope (JEOL Ltd., Japan) with an accelerating voltage of 100 keV. Photomicrographs were analyzed with respect to the intracellular insulin granules by a histologist who was experienced in transmission electron microscopy.

2.6. Western blotting

Protein lysates were prepared and subjected to western blot analysis as previously described [19]. The following primary antibodies were used: anti-CP (rabbit polyclonal antibody; 1:1000; Cat#AP7340a, Abgent), anti-HEPH (rabbit polyclonal antibody raised against an N-terminus oligopeptide of HEPH; 1:1000) [20], anti-ferritin light chain (mouse monoclonal antibody; 1:1000; Cat#sc-74513, Santa Cruz Biotechnology), and anti-β-tubulin (mouse monoclonal antibody; 1:5000; Cat#M20005, Abmart, Shanghai, China). The levels of individual protein bands were quantified using ImageJ software (NIH) following densitometry.

2.7. Total RNA extraction and quantitative real-time PCR analysis

Total RNA from the tissues was isolated and reverse transcribed as previously described [18]. Real-time PCR was performed using FastStart Universal SYBR Green Master (Rox) (Cat#04913914001, Roche Applied Science) in an Applied Biosystems 7300 Real-Time PCR System machine (Life Technologies, Shanghai, China) as per the manufacturer's instructions. The levels of mRNA were normalized to that of the housekeeping gene *GAPDH*. The primer sequences used are listed in Table S1.

2.8. Lipase and trypsin measurements

Snap-frozen plasma was used to determine lipase and trypsin levels spectrophotometrically using a lipase assay kit (Cat#A054, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and a trypsin assay kit (Cat#A080-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) respectively. For both analytes, data were collected using a microplate reader (Molecular Devices).

2.9. Analysis of oxidative stress-related markers

Lysates of snap-frozen pancreas were used to determine total superoxide dismutase (SOD) activity, total glutathione peroxidase (GPx) activity, and malonaldehyde (MDA) concentration with the following kits: Total Superoxide Dismutase Assay Kit with WST-8 (Cat#S0101; Beyotime Biotechnology); Total Glutathione Peroxidase Assay Kit (Cat#S0058; Beyotime Biotechnology); and Lipid Peroxidation MDA Assay Kit (Cat#S0131; Beyotime Biotechnology). Levels of carbonylated protein in pancreatic homogenates were measured using a Protein Carbonyl Colorimetric Assay Kit (Cat#10005020; Cayman Chemical). For all four analytes, data were collected using a microplate reader