Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Transcription factor NRF2 protects mice against dietary iron-induced liver injury by preventing hepatocytic cell death

Sandro Silva-Gomes[1], Ana G. Santos[1], Carolina Caldas[1], Cátia M. Silva[2], João V. Neves[1], Joanne Lopes[3], Fátima Carneiro[3,4], Pedro N. Rodrigues[1,5], Tiago L. Duarte[1,*]

[1]*Iron and Innate Immunity Group, Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Porto, Portugal;* [2]*Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Porto, Portugal;* [3]*Department of Pathology, Centro Hospitalar de São João, Porto, Portugal;* [4]*Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP) and Medical Faculty of University of Porto, Porto, Portugal;* [5]*Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Porto, Portugal*

Background & Aims: The liver, being the major site of iron storage, is particularly exposed to the toxic effects of iron. Transcription factor NRF2 is critical for protecting the liver against disease by activating the transcription of genes encoding detoxification/antioxidant enzymes. We aimed to determine if the NRF2 pathway plays a significant role in the protection against hepatic iron overload.

Methods: Wild-type and *Nrf2*$^{-/-}$ mouse primary hepatocytes were incubated with ferric ammonium citrate. Wild-type and *Nrf2*$^{-/-}$ mice were fed standard rodent chow or iron-rich diet for 2 weeks, with or without daily injection of the antioxidant mito-TEMPOL.

Results: In mouse hepatocytes, iron induced the nuclear translocation of NRF2 and the expression of cytoprotective genes in an NRF2-dependent manner. Moreover, *Nrf2*$^{-/-}$ hepatocytes were highly susceptible to iron-induced cell death. Wild-type and *Nrf2*$^{-/-}$ mice fed iron-rich diet accumulated similar amounts of iron in the liver and were equally able to increase the expression of hepatic hepcidin and ferritin. Nevertheless, in *Nrf2*-null mice the iron loading resulted in progressive liver injury, ranging from mild confluent necrosis to severe necroinflammatory lesions. Hepatocytic cell death was associated with gross ultrastructural damage to the mitochondria. Notably, liver injury was prevented in iron-fed animals that received mito-TEMPOL.

Conclusions: NRF2 protects the mouse liver against the toxicity of dietary iron overload by preventing hepatocytic cell death. We identify NRF2 as a potential modifier of liver disease in iron overload pathology and show the beneficial effect of the antioxidant mito-TEMPOL in a mouse model of dietary iron-induced liver injury.

Keywords: Hepatocyte; Hemochromatosis; Antioxidant; Mito-TEMPOL; 8-Hydroxy-2′-deoxyguanosine; Necrosis.

Received 11 January 2013; received in revised form 21 August 2013; accepted 2 September 2013; available online 7 September 2013

* Corresponding author. Address: Instituto de Biologia Molecular e Celular, Rua do Campo Alegre 823, 4150-180 Porto, Portugal. Tel.: +351 226074900; fax: +351 226099157.

E-mail address: tduarte@ibmc.up.pt (T.L. Duarte).

Abbreviations: HH, hereditary hemochromatosis; ARE, antioxidant response element; FAC, ferric ammonium citrate; AC, ammonium citrate; PI, propidium iodide; hOGG1, human 8-hydroxyguanine DNA glycosylase 1; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ROS, reactive oxygen species; MPT, mitochondrial permeability transition.

Introduction

Iron is a component of several metalloproteins involved in crucial metabolic processes such as oxygen sensing and transport, energy metabolism and DNA synthesis. However, iron in excess is detrimental, as it can catalyze the formation of damaging radical species via Fenton-type reactions [1]. In normal conditions, iron toxicity is prevented by the binding of extracellular iron in the plasma to transferrin and by storage of intracellular iron in a protein with high storage capacity, ferritin [2]. In addition, plasma iron levels are tightly regulated by the action of the peptide hormone hepcidin. Hepcidin, which is mostly secreted in the liver, promotes the degradation of the iron exporter ferroportin expressed on the surface of iron-releasing cells, thus reducing intestinal iron absorption and its mobilization from hepatic stores, and promoting iron retention within erythrophagocytosing macrophages [2]. Nevertheless, mutations in the genes encoding hepcidin (*Hamp*) or other key regulatory proteins like HFE, hemojuvelin or transferrin receptor 2 result in inappropriately low hepcidin levels and lead to the development of Hereditary Hemochromatosis (HH), an autosomal recessive disorder that is characterized by excessive absorption of dietary iron and its deposition in the parenchymal cells of the liver and other body organs [3]. The liver, in particular, has the capacity to accumulate a great amount of iron and is thus particularly exposed to its toxic effects [4]. Cellular injury is presumably caused by oxygen radical-mediated damage to cellular organelles, leading to hepatocyte death. The HH disease penetrance is variable though. Whilst the chronic accumulation of iron in the organism *per se* is reasonably well tolerated in most individuals, some HH patients develop liver fibrosis and cirrhosis, and in some cases primary hepatocellular carcinoma [3]. In rodent models, long-term iron

supplementation *per se* fails to induce significant liver damage [5,6], suggesting that under normal conditions organisms activate mechanisms that allow an adaptation to increased oxidative insult.

Transcription factor Nuclear factor-erythroid 2-related factor 2 (*Nfe2l2*/*Nrf2*) is a bZIP redox-sensitive transcription factor that regulates the transcriptional induction of a battery of antioxidant response element (ARE)-containing genes in response to cellular stresses. There is growing evidence that NRF2 is an important modifier of diseases involving oxidative stress (e.g. inflammatory and neurodegenerative diseases, and cancer) [7] and the liver of *Nrf2*$^{-/-}$ mice is reportedly more susceptible to oxidative and electrophilic stress [8]. However, the protective role of NRF2 in iron overload disease remains undetermined. Here, we investigated whether NRF2 is critical for the protection of liver parenchymal cells against iron toxicity *in vitro* and *in vivo*.

Materials and methods

Animals and experimental design

C57BL/6J mice and *Nrf2*$^{-/-}$ mice on a C57BL/6J background [9] (Riken, Japan) were housed and bred at the 'Instituto de Biologia Molecular e Celular' animal facility. Male mice (16 week-old) were fed standard rodent chow (Global rodent diet, Harlan Laboratories, Barcelona, Spain) or iron-rich diet (Global rodent diet supplemented with 2.0% carbonyl iron) for 2 weeks. In a subsequent experiment, *Nrf2*$^{-/-}$ animals were maintained on the iron-rich diet for the same time period and injected daily with either (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-xoethyl)triphenylphosphonium chloride monohydrate (mito-TEMPOL, Santa Cruz Biotechnology, Heidelberg, Germany) (10 mg/kg intraperitoneally) or saline (control), starting from the day before the start of dietary supplementation. At the end of the dietary treatment, mice were anaesthetized, blood was collected by retro-orbital bleeding, and the animals were sacrificed for organ collection. Since the saline injections in *Nrf2*$^{-/-}$ animals on iron-rich diet had no effect on any of the parameters assessed when comparing with *Nrf2*$^{-/-}$ animals on the same diet, samples from both groups were pooled for statistical analysis.

Cell cultures

Primary hepatocyte cultures from C57BL/6 (*Nrf2*$^{+/+}$) and *Nrf2*$^{-/-}$ mice were prepared as described in Supplementary data.

For a full description of Materials and methods, see Supplementary data.

Results

NRF2 activation protects mouse hepatocytes from iron toxicity

Under oxidative stress, NRF2 escapes proteasomal degradation and is translocated to the nucleus, where it activates the transcription of a battery of ARE-containing cytoprotective genes [7]. The activation of NRF2 by iron was investigated in primary hepatocyte cultures derived from wild-type and *Nrf2*$^{-/-}$ mice that were incubated with ferric ammonium citrate (FAC), a source of inorganic iron that is rapidly taken up by cultured cells and mimics the non-transferrin-bound iron found in the plasma of hemochromatosis patients [10,11]. By immunofluorescence, we show an increase of NRF2 in the nuclei of wild-type hepatocytes at the end of 17 h of incubation with FAC (1.71 μg/ml) (Fig. 1A). We also monitored the mRNA expression of three prototypical NRF2 target genes in hepatocytes exposed to FAC. In basal conditions, wild-type hepatocytes expressed higher levels of *Nqo1*, *Gclc*, and *Gsta1* than *Nrf2*$^{-/-}$ cells. Incubation with FAC for 24 h significantly increased the steady-state levels of these genes in wild-type hepatocytes but not in *Nrf2*$^{-/-}$ cells, showing that NRF2 is required for their transcriptional activation by iron (Fig. 1B).

After demonstrating that NRF2 is activated by iron in wild-type hepatocytes, we assessed whether *Nrf2*$^{-/-}$ hepatocytes were more susceptible to iron toxicity. Cells were incubated with increasing concentrations of FAC or ammonium citrate (AC) as control for 24 h and their viability was assessed by the MTT assay, which measures the formation of a water-insoluble MTT formazan by mitochondrial dehydrogenases of living cells. As depicted in Fig. 1C, AC had no effect on the activity of hepatocytic mitochondrial dehydrogenases in either wild-type or *Nrf2*$^{-/-}$ cells. While wild-type hepatocytes were resistant to all concentrations of FAC, a dramatic, dose-dependent reduction in the activity of mitochondrial dehydrogenases was observed in *Nrf2*$^{-/-}$ hepatocytes. In the latter, FAC treatment caused morphological alterations that included rounding up of hepatocytes (Supplementary Fig. 1). We then performed a propidium iodide (PI) uptake assay that measures the loss of cytoplasmic membrane integrity in dead cells. Incubation with FAC (1.714 μg/ml) caused a significant increase in the number of PI-positive nuclei in *Nrf2*$^{-/-}$ hepatocytes but not their wild-type counterparts (Fig. 1D). We inspected the nuclear morphology of PI-positive cells for the presence of apoptotic bodies. As a positive control, we incubated *Nrf2*$^{-/-}$ cells with a concentration of staurosporine that induces apoptosis in mouse primary hepatocytes [12]. Staurosporine-treated cells displayed chromatin margination and condensation, two typical features of apoptotic cells. In *Nrf2*$^{-/-}$ hepatocytes incubated with FAC, however, we did not observe any nuclei with the typical features of apoptosis (Fig. 1E), which suggests that iron kills *Nrf2*$^{-/-}$ hepatocytes by primary necrosis rather than apoptosis.

Finally, we investigated the formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a product of oxidatively damaged DNA formed by hydroxyl radical or singlet oxygen [13], in mouse hepatocytes incubated with iron. DNA damage was measured using the alkaline comet assay as human 8-hydroxyguanine DNA glycosylase 1 (hOGG1)-sensitive sites. hOGG1 is considered to detect 8-OHdG in cell culture experiments with great specificity [14]. Fig. 1F shows the level of hOGG1 sensitive sites in hepatocyte cultures after 24 h exposure to FAC. Notably, iron increased hOGG1-sensitive sites exclusively in *Nrf2*$^{-/-}$ hepatocytes, which suggests that the latter are more susceptible to iron-induced oxidative stress.

NRF2 protects mice against dietary iron-induced liver injury

C57BL/6 (wild-type) and *Nrf2*$^{-/-}$ mice were either fed standard rodent chow or a diet containing 2.0% carbonyl iron (iron-rich diet) *ad libitum* for 2 weeks. No significant differences in the relative liver weight were found. The levels of non-heme liver iron in animals of both strains under standard diet were not significantly different either. Feeding carbonyl iron increased the hepatic iron stores, transferrin saturation and serum iron levels significantly, irrespective of the *Nrf2* status (Table 1). Significant increases in hepatic total ferritin levels (Fig. 2A) and in the steady-state mRNA levels of *Hamp* and *Hamp2* were also observed in both wild-type and *Nrf2*$^{-/-}$ mice fed iron-rich diet (Fig. 2B). We conclude that the lack of *Nrf2* had no impact on the hepatic iron loading or on the ability of the liver to respond by activating key regulators of iron homeostasis.