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Inhibition of Nrf2 alters cell stress induced by chronic iron exposure in human proximal tubular epithelial cells

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ABSTRACT

Iron can catalyze reactive oxygen species (ROS) formation, causing cellular injury. In systemic iron overload, renal tubular epithelial cells are luminally exposed to high iron levels due to glomerular filtration of increased circulating iron. Reports of tubular dysfunction and iron deposition in β-thalassemia major support an association between increased chronic iron exposure and renal tubular injury. In acute iron exposure, Nuclear factor-erythroid 2-related factor 2 (Nrf2) may protect from iron-induced injury, whereas chronic renal stress may lead to Nrf2 exhaustion. We studied the cytotoxic mechanisms of chronic iron exposure using human conditionally immortalized proximal tubular epithelial cells (ciPTECs). Long-term iron exposure resulted in iron accumulation, cytosolic ROS formation and increased *heme oxygenase 1 (HMOX-1)* mRNA expression (all $p < 0.001$). This was accompanied by nuclear translocation of Nrf2 and induction of its target protein NQO1, which both could be blocked by the Nrf2 inhibitor trigonelline. Interestingly, iron and trigonelline incubation reduced ROS production, but did not affect *HMOX-1* mRNA levels. Moreover, ferritin protein and *CHOP* mRNA expression were induced in combined iron and trigonelline incubated cells ($p < 0.05$). Together, these findings suggest that chronic iron exposure induces oxidative stress and that exhaustion of the antioxidant Nrf2 pathway may lead to renal injury.

1. Introduction

Iron is indispensable for life, but it can also be harmful by catalyzing reactive oxygen species (ROS) formation in the Fenton reaction (Koppenol, 1993). The human body carefully regulates iron uptake and storage, but has limited abilities to regulate iron excretion (Brissot and Loreal, 2016). As a result, increased intestinal iron uptake in hereditary hemochromatosis and increased body iron acquisition through frequent red blood cell transfusions in β-thalassemia syndromes have been shown to result in chronic systemic iron overload and subsequent organ damage (Brissot and Loreal, 2016). Patients with systemic iron overload suffer from elevated circulating iron levels, which are bound to the transport protein transferrin (transferrin-bound iron, TBI) (Brissot and Loreal, 2016). Once transferrin becomes largely saturated with iron, non-transferrin-bound iron (NTBI) can be detected (Brissot et al., 2012; de Swart et al., 2016). Although iron is tightly bound to transferrin in TBI, iron is only loosely bound to small molecules such as citrate, in NTBI (Brissot et al., 2012). As such, iron in NTBI is available for redox cycling and is, therefore, considered a toxic iron species (Brissot et al., 2012; Cabantchik, 2014). Circulating TBI and NTBI are filtered into the renal tubular lumen by the glomerulus (Thevenod and Wolff, 2016; Zhang et al., 2007; Norden et al., 2001). Subsequently, iron in the tubular lumen is suggested to be completely reabsorbed by renal tubular cells, since hardly any iron is present in urine of healthy volunteers (Rodriguez and Diaz, 1995; Green et al., 1968). As a result, in patients with elevated circulating iron levels, proximal tubular epithelial cells (PTECs) are chronically exposed to high and potentially harmful iron levels.

In patients with hereditary hemochromatosis (Ozkurt et al., 2014; Marble and Bailey, 1951; Rous, 1918; Okumura et al., 2002; Chmieliauskas et al., 2017) or β-thalassemia major (ElAlfy et al., 2018; Hashemieh et al., 2017), renal iron deposition has been observed. Moreover, renal dysfunction in patients with β-thalassemia major has been reported with increased urinary excretion of N-acetyl-β-D-glucosaminidase (NAG) and β-2-microglobulin, both markers for renal PTEC damage (ElAlfy et al., 2018; Hashemieh et al., 2017; Annayev et al., 2018; Quinn et al., 2011; Ahmadzadeh et al., 2011; Deveci et al., 2016). Combined, these observations suggest that chronic iron overload may cause increased iron accumulation in the kidney, and, as such, may lead to clinically relevant nephrotoxicity over time.

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The question remains, however, which exact molecular mechanisms are involved in renal tubular injury during chronic TBI and NTBI exposure. Previous animal and human studies suggest that chronically increased renal tubular iron exposure and injury in systemic iron overload depend on the balance between oxidative stress and anti-oxidative systems (Budak et al., 2014; Sponsel et al., 1996; Sheerin et al., 1999; Ansar et al., 2014; Gholampour et al., 2017; von Herbay et al., 1994; Ghone et al., 2008). The major cellular pathway that protects against oxidative injury has been shown to be coordinated by Nuclear factor-erythroid 2-related factor 2 (Nrf2) (Kerins and Ooi, 2017), which is reported to protect from short term iron-induced injury in PTECs. Nrf2 knockout mice showed increased PTEC injury and urinary excretion of NAG 24 h after a single ferric nitrilotriacetate (Fe-NTA) injection (Tanaka et al., 2008). In animal models of chronic kidney disease, Nrf2 activity exhausted over time despite continuous presence of oxidative stress (Aminzadeh et al., 2012; Kim and Vaziri, 2010; Kim et al., 2011). Therefore, we tested the hypothesis that Nrf2 exhaustion as a result of persistent oxidative stress underlies renal injury observed in chronic iron overload conditions.

To this end, we examined the intracellular effects of long-term iron overload exposure in human conditionally immortalized PTECs (ciPTECs) and the role of the Nrf2 pathway herein.

2. Methods

2.1. Cell culture

ciPTECs (clone T1, kindly provided by dr. M. Wilmer, Radboud university medical centre) (Jansen et al., 2014), were cultured using DMEM HAM's F-12 phenol red-free medium (Thermo Fisher Scientific) containing 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 36 ng/ml hydrocortisone, 10 ng/ml epithelial growth factor and 40 pg/ml triiodothyronine (all Sigma Aldrich), 10% (v/v) fetal calf serum (Greiner Bio-one), and 1% (v/v) penicillin-streptomycin (Thermo Fisher Scientific). Cells were cultured at 33 °C and 5% CO_2, and grown for 24 h at 33 °C and 5% CO_2 and 7 days at 37 °C and 5% CO_2 prior to experiments.

2.2. Iron exposure

Cells were exposed to 0–500 µM ferric citrate (FeC, Sigma Aldrich) for 72 h (toxicity array) or 48 h (all other experiments) with or without 1 µM trigonelline hydrochloride (Sigma Aldrich).). We calculated that already 100 µM FeC could saturate transferrin levels in fetal calf serum (Young and Garner, 1990) with iron and, as such, this would result in ciPTEC iron exposure containing both saturated TBI and NTBI. Cell pellets were collected and stored at −80 °C until analysis.

2.3. Protein isolation and immunoblotting

Cell pellets were lysed using RIPA buffer (0.15 M NaCl, 0.012 M Sodium Deoxycholate, 0.1% NP40, 0.1% SDS, 0.05 M Tris, pH 7.5, freshly supplemented with protease inhibitors (Complete mini, Roche)). Protein concentration was determined using the Pierce BCA assay kit according to the manufacturer's instructions (Thermo Fisher Scientific). Protein samples were separated on SDS-PAGE gels, transferred to nitrocellulose membrane and incubated with primary antibody overnight at 4 °C. After 1 h incubation at RT with secondary antibody, proteins were visualized on a LAS-3000 scanner for chemiluminescence (Transferrin receptor 1 (TfR1)) or Odyssey fluorescence scanner (all other proteins). Primary antibodies and dilutions are summarized in Supplementary Table 1.

2.4. Nucleus enrichment

Cells were resuspended in mild lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 270 mM sucrose, 0.1% NP-40, 20 mM Tris − HCl, pH 7.5, freshly supplemented with 1 mM DTT and protease inhibitors) and disrupted using a Douncer homogenizer. After centrifugation, pellets were incubated with RIPA lysis buffer and supernatant was collected as nuclear fraction.

2.5. RNA isolation and quantitative PCR

RNA isolation was performed using TRIzol™ (Thermo Fisher Scientific) according to the manufacturer's instructions. A reverse transcription reaction was performed with 1 µg RNA, 4 µl first strand buffer, 1 µl dNTPs (12.5 mM), 2.04 µl random primers, 2 µl DDT, 1 µl M-MLV (all Thermo Fisher Scientific) and 0.5 µl RNAsin (Promega Corporation). The PCR cycle existed of 20 °C for 10 min, 42 °C for 45 min and 95 °C for 10 min. Quantitative PCR was performed on a CFX96 (Bio-rad) using 4 µl cDNA (10 ng/ml), 10 µl SYBR Green Power master mix (Applied Biosystems) and 6 µl primer mix (containing 1 µM forward primer and reverse primer). The PCR protocol was as follows: 7 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C, and 10 min at 95 °C, with a measurement at the end of each cycle. Fold change values were calculated relative to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) using the ΔΔCt formula. Primers are summarized in Supplementary Table 2.

2.6. Toxicity array

The RT^2 Profiler PCR Array Human Stress & Toxicity Pathway Finder (Qiagen), containing 84 different genes and 5 housekeeping genes was used together with RT^2 SYBR Green qPCR Mastermix (Qiagen) according to the manufacturer's instructions.

2.7. Iron assessment

Intracellular iron level were determined using the chromogen bathophenanthroline as described (Torrance and Bothwell, 1968). Iron concentrations were calculated by comparison to a standard curve of ferrous sulfate and corrected for protein concentration.

2.8. Oxidative stress measurement

Cells were incubated with 10 µM 2′, 7′-dichlorodihydrofluorescein di-acetate (CM-H_2DCFDA, Thermo Fisher Scientific) and FeC for 1 h at 37 °C. Fluorescence was measured using a Victor X Multilabel Plate Reader (Perkin Elmer).

For oxidative stress staining, cells were incubated with 50 µM CellROX™ Green for 30 min at 37 °C, fixed with 4% paraformaldehyde, stained with DAPI (4′,6-diamidino-2-phenylindole, 300 µM, Thermo Fisher Scientific) and mounted. Images were taken using a Zeiss Apotome FL microscope and AxioVision software.

2.9. Statistical analysis

Data were statistically analyzed using GraphPad Prism 5.03 and presented as mean ± SEM. Results were analyzed by One-Way ANOVA with Dunnett's post test or Student's t-test, where appropriate. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Long term iron overload results in Nrf2 pathway activation

In ciPTECs, 48 h iron overload exposure significantly and concentration-dependently increased intracellular iron levels ($p < 0.05$ for 100 µM and 200 µM, $p < 0.001$ for 500 µM FeC compared to control) (Fig. 1a). High intracellular iron levels are known to decrease TfR1 and induce ferritin protein expression as a result of