Mitochondrion 29 (2016) 18–30

Contents lists available at ScienceDirect

Mitochondrion

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

Non-cytotoxic copper overload boosts mitochondrial energy metabolism to modulate cell proliferation and differentiation in the human erythroleukemic cell line K562

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ARTICLE INFO

Article history:
Received 2 October 2015
Received in revised form 15 March 2016
Accepted 14 April 2016
Available online 16 April 2016

Keywords:
Mitochondria
Bioenergetics
Mitochondrial dynamics
Copper
Erythropoiesis
Autophagy

ABSTRACT

Copper is integral to the mitochondrial respiratory complex IV and contributes to proliferation and differentiation, metabolic reprogramming and mitochondrial function. The K562 cell line was exposed to a non-cytotoxic copper overload to evaluate mitochondrial dynamics, function and cell fate. This induced higher rates of mitochondrial turnover given by an increase in mitochondrial fusion and fission events and in the autophagic flux. The appearance of smaller and condensed mitochondria was also observed. Bioenergetics activity included more respiratory complexes, higher oxygen consumption rate, superoxide production and ATP synthesis, with no decrease in membrane potential. Increased cell proliferation and inhibited differentiation also occurred. Non-cytotoxic copper levels can modify mitochondrial metabolism and cell fate, which could be used in cancer biology and regenerative medicine.

1. Introduction

The two metabolic pathways by which cells produce energy, in the form of adenosine triphosphate (ATP), are glycolysis and oxidative phosphorylation (OXPHOS). The latter, located in the inner mitochondrial membrane, is a copper dependent system due to two copper sites in the cytochrome c oxidase, or complex IV, of the respiratory chain (Kim et al., 2008; Turski and Thiele, 2009). Therefore, copper is an essential trace metal for cell physiology and life.

The physiological copper concentration of mammalian plasma is around 10 μmol/L (Hinks et al., 1983; Tapiero et al., 2003), and in hematopoietic cells, concentration is about 7 ng per million cells (Peled et al., 2002, 2005; Steinkühler et al., 1994). However, copper concentrations may vary according to diet, surgery, parenteral nutrition, and the levels of other mineral supplements such as zinc or iron (Doherty et al., 2011; Griffith et al., 2012; Willis et al., 2004). These variations could cause acquired copper overload or deficiency, statuses that cell-specifically affect metabolism and fate. Regarding copper overload, cell death occurs in the hepatic HepG2 cell line, lung A-549 cell line, and monocytes at 20 μM, 20–60 μM, and 80 μM of copper chloride ($CuCl_2$), respectively (Arnal et al., 2012; Wataha et al., 2000). In contrast, copper promotes cell proliferation in hepatic stellate cells at 100 μM (S.-Q. Xu et al., 2013), endothelial cells at 500 μM (Hu, 1998), and MEF cells at 10 μM (Itoh et al., 2008). Likewise, cell differentiation can be induced by copper, with hematopoietic progenitors differentiating into blood cells at 30 μM (Peled et al., 2002, 2005) and mesenchymal stem cells differentiating into osteoblasts at 50 μM (Rodríguez et al., 2002). While affected by copper, cell death, differentiation, and proliferation are regulated by mitochondria.

In cell death, mitochondria are involved in apoptosis and necrosis (Green and Reed, 1998; Javadov and Kuznetsov, 2013; Rasola and Bernardi, 2011). For cell proliferation and differentiation, mitochondria regulate the balance between the glycolytic and OXPHOS systems of energy production (Zhang et al., 2011, 2012). This balancing process is known as metabolic reprogramming and has been reported in erythropoietic cells (Elorza et al., 2008; Bustos et al., 2013), human pluripotent stem cells (Shyh-Chang et al., 2011; Zhang et al., 2011), and hematopoietic stem cells (Arranz et al., 2013; Romero-Moya et al., 2013). Furthermore, metabolic reprogramming is crucial for the uncontrolled proliferation of cancer cells as it allows these cells to avoid mitochondria-induced

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http://dx.doi.org/10.1016/j.mito.2016.04.005
1567-7249/

apoptosis (Bonnet et al., 2007) and to primarily rely on aerobic glycolysis in a process known as the Warburg effect (Warburg, 1956a,b).

As a prosthetic group of complex IV, copper might have a role in metabolic reprogramming. In fact, chronic exposure to elevated, but within normal, levels of copper in drinking water stimulates the proliferation of cancer cells in mice (Ishida et al., 2013), and prostate, breast, colon, lung, and brain cancers have high copper contents. The removal of copper through chelation therapies induces apoptosis in cancer cells, and this could be used as a potential treatment against cancer (Daniel et al., 2004, 2007; Tisato et al., 2009).

Mitochondria are sensitive to reactive oxygen species (ROS), which are mostly generated as a by-product of cellular respiration. Free copper ions inside mitochondria may also contribute to ROS production (Arnal et al., 2012; Barthel et al., 2007; Su et al., 2011). Exacerbated ROS production damages mitochondrial components, consequently generating dysfunctional mitochondrial units. Due to this, ROS are considered one of the causes for aging and for many neurological and metabolic diseases, including cancer, leukemia, and myelodysplastic syndromes (Arranz et al., 2013; Jitschin et al., 2014; Penta et al., 2001). However, at non-pathological levels, ROS may act as a second messenger in the promotion of cell proliferation and/or differentiation processes (Finkel, 2011; Maryanovich and Gross, 2013; Piccoli et al., 2013).

While the link between mitochondrial physiology, copper, and cell fate is well established, studies have not yet explored how copper overload modulates mitochondrial function to influence cell fate without inducing apoptosis or cell death. We hypothesized that non-cytotoxic copper overload modulates mitochondrial bioenergetics through ROS generation, thereby altering the balance between cell proliferation and differentiation. To test this, human erythroleukemic K562 cells were exposed to a non-cytotoxic copper concentration. Analysis showed increased mitochondrial oxidative metabolism promoted by *i*) copper-induced up-regulation of respiratory complexes and *ii*) higher rates of mitochondrial turnover stimulated by copper-induced mitochondrial ROS. This metabolic response could positively influence cell proliferation and inhibit cell differentiation in this cancer cell line.

2. Materials and methods

2.1. Cell lines

K562 cells were grown in a RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco-Invitrogen), 2% Pen/Strep, and 1% glutamine. To induce non-cytotoxic copper overload $CuCl_2$ (Sigma) was added to the culture medium, and effects were evaluated after 48 h. Where specified, cell cultures were also treated with the mitochondria-specific antioxidant manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (TBAP; EMD Millipore) at final concentration of 40 μM. To induce erythroid differentiation, K562 cells were cultured at an initial density of 100,000 cells/ml in a medium containing 0.9 mM of butyric acid (Sigma). Cell proliferation and mortality were assessed by Trypan blue staining, and erythroid differentiation was determined by benzidine staining. Cells were manually counted in a Neubauer chamber under a bright field microscope.

2.2. Flow cytometry

K562 cells (~500,000) were immunostained with fluorescein isothiocyanate-conjugated anti-CD71 (1:100) (BD Pharmingen) in a FACS buffer (PBS, 1% BSA) as previously described (Zhang, 2003). Flow cytometry was performed using an Attune Acoustic Focusing Cytometer (Life Technologies). FACS data analysis was performed with the FCS Express V3 program (De Novo Software).

2.3. Confocal microscopy

Cells were adhered to poly-lysine-coated coverslips, stained, and placed in a temperature-controlled and CO_2-supplemented chamber (Chamlide TM IC, Live Cell Instrument Inc.). The chambers were viewed under an Olympus Fluoview 1000 confocal microscope.

Changes in the mitochondrial membrane potential ($mt\Delta\Psi$) were determined using the potentiometric fluorescent probe tetramethylrhodamine-ethyl-ester (TMRE) (Life Technologies) in non-quenching mode. Mitochondria were stained with 10 nM of TMRE, and 50 μM of verapamil (Sigma) were also added to culture media. Z-series were acquired with a slice separation of 1 μm throughout the entire cell depth. Fluorescence intensity and morphometric mitochondria data were quantified with the Image J software (Molecular Devices).

2.4. Mitochondrial biomass

Mitochondria of K562 cells were stained with the membrane potential-independent probe Mitotracker Green (Life Technologies) according to the manufacturer's instructions. The cells were visualized under an epifluorescent microscope, and the acquired images were analyzed with the Image J software (Molecular Devices).

2.5. Oxygen consumption rate (OCR) and extracellular acidification

Oxygen consumption by K562 cells was measured with the XF24 Flux Analyzer (Seahorse Bioscience). Assays were performed as previously described (Bustos et al., 2013; Wu et al., 2006).

2.6. Western blot

Total protein samples for mitochondrial dynamics (MtDy), for the respiratory complexes and ATP synthase and for autophagy were fractionated in 12% polyacrilamide gel and transferred onto a polyvinyl difluoride membrane using a semidry transfer machine. The following antibodies were used: MFN2 (ab50843, Abcam), MFN1 (ab57602, Abcam), OPA1 (ab42364, Abcam), FIS1 (71498, Abcam), DRP1 (ab54038, Abcam), P62/SQSTM1 (ab56416, Abcam), Porin (ab15895, Abcam), Actin (ab8227, Abcam), GAPDH (ab8245, Abcam) and the total OXPHOS rodent WB antibody cocktail (MS604, Mitosciences).

2.7. Protein oxidation detection

Total protein oxidation was measured with the OxyBlot Detection Kit (Millipore) according to the manufacturer's instructions. This measurement is based on the detection of carbonyl groups that are introduced into protein side chains when proteins are exposed to oxidative stress.

2.8. Reactive oxygen species measurements

For cytosolic ROS measurements, cells were loaded with 5 μM of dihydroethidium (DHE; Invitrogen) for 20 min at 37 °C. For mitochondrial ROS, cells were loaded with 5 μM of Mitosox (Invitrogen), a mitochondrial superoxide indicator, for 10 min at 37 °C. Then, images were acquired by live confocal laser microscopy and analyzed with the Image J software (Molecular Devices).

2.9. ATP measurements

The ADP/ATP Ratio Assay Kit (ab6531, Bioluminescent, Abcam) was used to detect total ATP levels in K562 cells. Luminescence was read with a Sinergy H1 instrument (BioTek).