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Why to compare absolute numbers of mitochondria

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ABSTRACT

Prompted by pronounced structural differences between rat liver and rat hepatocellular carcinoma mitochondria, we suspected these mitochondrial populations to differ massively in their molecular composition. Aiming to reveal these mitochondrial differences, we came across the issue on how to normalize such comparisons and decided to focus on the absolute number of mitochondria. To this end, fluorescently stained mitochondria were quantified by flow cytometry. For rat liver mitochondria, this approach resulted in mitochondrial protein contents comparable to earlier reports using alternative methods. We determined similar protein contents for rat liver, heart and kidney mitochondria. In contrast, however, lower protein contents were determined for rat brain mitochondria and for mitochondria from the rat hepatocellular carcinoma cell line McA 7777. This result challenges mitochondrial comparisons that rely on equal protein amounts as a typical normalization method. Exemplarily, we therefore compared the activity and susceptibility toward inhibition of complex II of rat liver and hepatocellular carcinoma mitochondria and obtained significant discrepancies by either normalizing to protein amount or to absolute mitochondrial number. Importantly, the latter normalization, in contrast to the former, demonstrated a lower complex II activity and higher susceptibility toward inhibition in hepatocellular carcinoma mitochondria compared to liver mitochondria. These findings demonstrate that solely normalizing to protein amount may obscure essential molecular differences between mitochondrial populations.

1. Introduction

Mitochondria are key integrators of cell death decisions (Green and Kroemer, 2004). While augmented mitochondria-dependent cell death is a major obstacle in neuro-degenerative disorders (Lin and Beal, 2006; Winklhofer and Haass, 2010), avoidance of cell death is a hallmark of cancer (Hanahan and Weinberg, 2000). Consequently, the identification of specific mitochondrial targets to either protect or destroy mitochondria is a central aim in biomedical research. Typically, the identification of such targets is achieved by comparing mitochondria isolated from healthy controls to mitochondria from diseased tissues. These comparative biochemical analyses, e.g., by proteomics, immuno-blotting or enzymatic measurements, are mostly normalized to an equal amount of mitochondrial protein. While this practice surely is generally useful, it does, however, obscure information of changes in the net amount of mitochondrial protein (Gear and Bednarek, 1972).

Throughout the eukaryotic kingdom the overall cellular protein concentrations are remarkably comparable (280 mg/ml in yeast, 260 mg/ml in rat muscle, and 310 mg/ml in rat liver Brown, 1991). Nevertheless, especially extensive changes of the cellular environment directly impact on the cellular metabolism and change the intracellular protein composition. With regards to mitochondria, massive adaptations in their total number, their morphology, protein composition and protein amount may occur (Cuezva et al., 2002; Ernster and Schatz, 1981; Hackenbrock, 1966, 1968a, 1968b; Hostetler et al., 1976; Morton et al., 1976; Rossignol et al., 2004). One of the most impressive examples for this adaptability has been described by the group of Gottfried Schatz. If baker's yeast is grown under anaerobic conditions they form poorly differentiated thread-like "promitochondria" (Criddle and Schatz, 1969; Plattner and Schatz, 1969). Promitochondria have a dramatically changed enzymatic composition compared to "normal" mitochondria (Criddle and Schatz, 1969) and transform to mitochondria when the cells are back-shifted to aerobic conditions (Plattner et al. 1970). Moreover, in the presence of oxygen, yeast switch from respiro-fermentative to respiratory metabolism simply upon change of the nutritive carbon source (Dejean et al., 2002). This simulated "diauxic shift" is associated with tremendous mitochondrial adaptations regarding their protein composition and structure (DeRisi et al., 1997; Zischka et al., 2006). While these findings refer to yeast, they are, however, transferable to higher eukaryotic cells. For example, a pre-clinical test to evaluate

Abbreviations: CI, respiratory complex I; CII, respiratory complex II; CS, citrate synthase; HCC, hepatocellular carcinoma; NAO, 10N-nonyl acridine orange; PCC, pump controlled cell rupture system; TTFA, thenoyltrifluoroacetone.

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mitochondrial drug toxicity uses HepG2 cells either grown on glucose (with ATP derived mostly via cytosolic glycolysis) or galactose and glutamine (Marroquin et al., 2007). The latter condition forces mitochondria to oxidative phosphorylation (OXPHOS) as the net ATP yield with galactose via glycolysis is zero (Dykens and Will, 2007). Cells grown in galactose-glutamine media become susceptible to mitochondrial toxicants, e.g., a wide variety of drugs that impair and/or uncouple OXPHOS (Dykens and Will, 2007).

As HepG2s are hepatocellular carcinoma cells (HCC), this test setting demonstrates their profound metabolic adaptability, which is characteristic for most cancer types. It further demonstrates that metabolic changes directly impact on the molecular composition of mitochondria (Galluzzi et al., 2010; Gogvadze et al., 2008, 2009). Besides the metabolic changes and adaptations in cancer cells, important metabolic differences and metabolite preferences do also exist in different healthy tissues of our body (Löffler and Petrides, 1990). Whereas brain tissue relies on glucose as the major metabolite, liver, especially in the postresorption phase, relies on fatty acids (Löffler and Petrides, 1990). Consistent with these metabolic preferences, marked differences in the molecular composition of the respective mitochondrial populations are known (Mootha et al., 2003; Veltri et al., 1990; Vijayasarathy et al., 1998). But what about fundamental biochemical parameters like the net protein content of mitochondria? Do mitochondria from cell culture (typically cancer cells) differ in this aspect from mitochondria in tissue, i.e., from their healthy cellular origins? Moreover, what about this parameter in mitochondria from other healthy tissues? Evidently, a pronounced difference in the overall protein content of mitochondria from different sources would challenge the validity of potential proteinaceous targets or differences identified by comparisons solely based on equal mitochondrial protein amount. Over- and underestimations of the true amount of such proteins per mitochondrion would result. Moreover, discrepancies in the effectiveness of mitochondrially targeted drugs in cultured cells and *in vivo* testing may arise simply from the fact that the metabolic situation, and consequently the net amount of the proteins to be targeted, differ between cells in culture and solid tissues.

In order to substantiate these theoretical considerations, we determined the net protein content of mitochondria isolated from four different rat tissues, i.e., liver, heart, kidney and brain. Further, rat liver mitochondria were compared to mitochondria isolated from two rat HCC cell lines, one of which was grown under two different metabolic conditions. Isolated mitochondria were fluorescently labeled and quantified by flow cytometry. Our results demonstrate a significant decrease in net protein amount in brain mitochondria and in mitochondria from the rat HCC cell line McA 7777 when compared to healthy rat liver mitochondria. Exemplified by the protein amount of two complexes of the respiratory chain, we further show that such comparisons arrive at conflicting results when based on either equal protein amount, or on an equal number of mitochondria.

2. Methods

2.1. Animals

Rats (heterozygous LPP strain provided by Jimo Borjigin, Michigan, USA) were housed under the guidelines for the care and use of laboratory animals at the Helmholtz Center Munich.

2.2. Cell culture

Rat hepatocellular carcinoma cells (McA 7777, H4IIE) were obtained from ATTC, USA and cultured in high glucose (4.5 mg/l) DMEM (Sigma-Aldrich, Germany) with 1% glutamate (GlutaMAX™, Gibco, UK). McA 7777 were alternatively grown in glucose-free DMEM supplemented with 10 mM galactose, 2% glutamate (GlutaMAX™, Gibco, UK) and 1 mM sodium pyruvate (PAA, Austria). Media were supplemented with 10% FCS (Biochrom, Germany) and 1% penicillin/streptomycin (Gibco, UK). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. For mitochondria isolation, cells were trypsinized and singularized with a syringe. Only cell suspensions with more than 80% vitality were used for the isolation of mitochondria.

2.3. Isolation and purification of mitochondria

2.3.1. Standard isolation

Mitochondria from rat liver, kidney, heart and brain tissue were isolated essentially as described earlier (Petit et al., 1998), with slight adaptations depending on the respective tissue source. Briefly, fresh rat liver, kidney and brain tissues were homogenized with a motor driven Elvehjem glass Teflon potter (5–6 strokes, 800 rpm) in isolation buffer with 0.1% BSA on ice. Heart tissue was minced with scissors and a razor blade and homogenized with a hand driven glass/glass potter (three strokes). The homogenate was cleared from debris and nuclei two times by centrifugation at 800 ×*g* (10 min at 4 °C). Liver, kidney and heart mitochondria were pelleted at 9000 ×*g* (10 min at 4 °C), brain mitochondria at 20,000 ×*g* (10 min at 4 °C) and further purified by discontinuous Percoll™ density gradient centrifugation, followed by two washing steps (9000 ×*g*, 10 min at 4 °C) in isolation buffer without BSA.

2.3.2. PCC isolation

Mitochondria from cultured cells and rat liver tissue were isolated by a semi-automated, pump-controlled cell rupture system (PCC) as previously described (Schmitt et al., 2013). Briefly, cell suspensions at concentrations of 5–7 × 10^6 cells/ml were pumped three times through the PCC (clearance 6–10 μm, flow rate 700 μl/min). 30–40 mg rat liver tissue/ml isolation buffer (300 mM sucrose, 5 mM TES, 200 μM EGTA, pH 7.2, without BSA) was pumped once through the PCC (clearance 18 μm, flow rate 700 μl/min). The homogenate was centrifuged at 800 ×*g* (4 °C) to remove nuclei and cell debris and mitochondria were pelleted at 9000 ×*g*. For purification, mitochondria were loaded on a Nycodenz® density gradient (24%/18% or 33%/18% for McA 7777 and H4IIE or for rat liver, respectively) and centrifuged at 30,000 rpm for 15 min at 4 °C in a Beckman ultracentrifuge (rotor SW 55.Ti). McA 7777 and H4IIE mitochondria were collected at the 18%/24% interphase and washed once in isolation buffer without BSA (9000 ×*g*, 10 min at 4 °C). Rat liver mitochondria either pelleted on a 18%/24% Nycodenz® density gradient (Fig. 2) or accumulated at the interphase on a 18%/33% Nycodenz® density gradient. For the sake of comparability to mitochondria isolated from cell culture, mitochondria were retrieved from the 18%/33% interphase and washed once in isolation buffer without BSA (9000 ×*g*, 10 min at 4 °C).

2.4. Quantification of mitochondria by flow cytometry

Supplementary Fig. 1 outlines the employed quantification procedure.

In order to distinguish mitochondria by flow cytometry from other particles, we stained them with 10N-nonyl acridine orange (NAO). To determine the exact number of mitochondria per volume, we relied on two internal standards, TruCOUNT™ beads (BD Biosciences, USA) and Fluoresbrite® microspheres (diameter 0.94 μm, Polysciences Europe GmbH, Germany). Both beads and microspheres can be distinguished in flow cytometry by fluorescence at 530 nm and sideward scatter (SSC-A) from NAO-stained mitochondria (SFig. 1 upper panel). The number of TruCOUNT™ beads is precisely pre-determined by the manufacturer (BD Biosciences, USA), and thus, solutions with known TruCOUNT™ bead concentrations can be generated. The large difference in size as well as in optical density of TruCOUNT beads and mitochondria prevented to record both with the same gain settings in sideward scatter (SSC-A) in our flow cytometer (LSRII, BD Biosciences, USA). Sideward scatter as a trigger signal is necessary to clearly separate mitochondria from other particles and intrinsic instrumental noise signals. Therefore a second internal standard for volume determination had to be introduced, the smaller Fluoresbrite® beads. While a