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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 cells cultivated in 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/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 media 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 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 separated/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 Elvjehem 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 24%/18% interphase and washed once in isolation buffer without BSA (9000 ×*g*, 10 min at 4 °C). Rat liver mitochondria either pelleted on a 24%/18% Nycodenz® density gradient (Fig. 2) or accumulated at the interphase on a 33%/18% Nycodenz® density gradient. For the sake of comparability to mitochondria isolated from cell culture, mitochondria were retrieved from the 33%/18% 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) and Fluoresbrite® microspheres (diameter 0.94 μm, Polysciences Europe GmbH). 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), 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). 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 higher sensitivity

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setting in SSC-A quantitatively detected the NAO-stained mitochondria (SFig. 1 lower panel) and the Fluoresbrite® beads, a lower sensitivity setting did so for the TruCOUNT™ and the Fluoresbrite® beads (SFig. 1 upper panel). Thus, two consecutive measurements from the same sample were required.

Mitochondrial suspensions were diluted to a protein concentration of 0.2–0.6 μg/ml and stained with the fluorescent dye NAO (ex 488 nm, em 530 nm, final concentration 10 nM). A defined number of TruCOUNT™ beads were suspended in 500 μl isolation buffer. Pre-filtered (0.2 μm) isolation buffer was used throughout. For analyses by flow cytometry, typically around 100 μl TruCOUNT™ beads solution, 10 μl premixed Fluoresbrite® microspheres solution and 500 μl NAO-stained mitochondrial suspensions were combined. Data were analyzed with FlowJo software (Treestar).

In the first measurement, the concentration of Fluoresbrite® microspheres was calculated from the analyzed volume determined by the number of determined TruCOUNT™ beads. In the second measurement, the concentration of NAO-stained mitochondria was calculated from the analyzed volume determined by the number of determined Fluoresbrite® microspheres. The absolute number of mitochondria per mg protein was subsequently calculated from the concentration of NAO-stained mitochondria and the determined protein concentration of this mitochondrial suspension.

In control experiments the NAO staining efficiency and specificity for intact mitochondria was verified by measuring either unstained mitochondria or stained mitochondria before and after destruction by sonication (Fig. 3). As further control NAO stained lysosomes were analyzed (Fig. 3).

2.5. Mitochondrial citrate synthase activity

The activity of the mitochondrial citrate synthase was determined according to earlier reports (Saggerson and Carpenter, 1986; Williams et al., 1998). 280 μl of a solution containing 2.5% (w/v) Triton X-100, 100 μM 5,5′-dithiobis-(2-nitrobenzoic acid), 75 μg acetyl-CoA and 500 μM oxaloacetate was incubated at 37 °C. The reaction was started by adding 20 μg mitochondria and followed at 412 nm for 5 min. Citrate synthase activities were calculated from the linear slopes of the initial rates.

2.6. Mitochondrial complex II activity

Complex II activities were determined as described previously (Kiebish et al., 2008). 10 μg (rat liver and McA 7777) or 18 μg (rat liver) of mitochondrial protein were added to a buffer containing 25 mM K_2HPO_4, pH 7.4, 20 mM succinate, 2 mM KCN, 50 μM 2,6-dichloroindiphenol (DCIP), 2 μg/ml rotenone and 2 μg/ml antimycin. Reactions were started with 56 μM decylubiquinone and the DCIP reduction was monitored at 600 nm. Samples were measured with and without 500 μM thenoyltrifluoroacetone (TTFA). Specific activities were calculated by subtracting the slope with TTFA from the slope without TTFA. 5 μM or 2.5 μM of TTFA were used to determine the sensitivity of complex II from rat liver mitochondria compared to McA 7777 mitochondria.

2.7. Miscellaneous

Protein concentrations were determined by the Bradford assay (Bradford, 1976), and immunoblotting analyses were done with PVDF membranes (Towbin et al., 1979). Proper transfer was controlled by Ponceau red staining (SFig. 2). Antibodies were from Invitrogen (OxPhos Complex Kit with complex I subunit NDUFB8 and complex II 30 kDa subunit) and Novus Biologicals (mitochondrial citrate synthase). Quantification was done with ImageJ.

Electron microscopy of cells and therefrom isolated mitochondria was done as previously described (Zischka et al., 2008). Briefly, samples were fixed in 2.5% glutaraldehyde, and postfixation and prestaining were done with osmium tetroxide. After dehydration with ethanol and propylene oxide, samples were embedded in Epon. Ultrathin sections were stained with uranylacetate and lead citrate and examined with an EM 10 CR transmission electron microscope (Zeiss, Germany).

2.8. Statistics

Data were expressed as means ± SD. For each analysis the numbers of biological replicates are given in the respective figure legends or Table 1. Statistics were performed in Excel using t-test. Data were tested unpaired and two-tailed. Differences were denoted statistically significant with $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Results and discussion

3.1. The altered appearance of liver cancer mitochondria

In order to analyze liver cancer mitochondria for apparent structural alterations dependent on their cellular metabolic state, we initially chose the rat hepatocellular carcinoma cell line McA 7777. Cells were either grown in enriched glucose media (McA 7777 glucose) or in glucose free media enriched in galactose-glutamine (McA 7777 glutamine). The latter media forces the cells toward oxidative phosphorylation instead of glycolysis (Marroquin et al., 2007). As reference, rat liver mitochondria, the “gold standard” in mitochondrial research (Fuller and Arriaga, 2004), were employed.

In agreement with an earlier report (Mintz et al., 1967), *in situ*, a markedly different appearance in mitochondrial structure was observed on electron micrographs between rat liver mitochondria and hepatocellular carcinoma McA 7777 mitochondria (Fig. 1A–C). In comparison to mitochondria from healthy rat livers, McA 7777 mitochondria displayed less cristae and a less densely stained matrix, indicative of less structures (e.g., proteins) stained by the contrast agents. In agreement with our earlier report (Schmitt et al., 2013), these structural alterations were especially apparent in isolated mitochondria (Fig. 1D–E). Rat liver mitochondria displayed the typical condensed phenotype (Hackenbrock, 1966; Schnaitman et al., 1967) with triangle shaped well defined cristae (Fig. 1D), a minor inter-membrane space and an electron-dense matrix (Fig. 1D). As observed *in situ*, isolated mitochondria from McA 7777 cells grown under high glucose appeared less structured with widened cristae and a highly condensed matrix that seems to be reduced in quantity (Fig. 1E). Interestingly, isolated McA 7777 glutamine mitochondria appeared to be structurally similar to isolated reference rat liver mitochondria with less widened cristae, a higher matrix portion and small inter-membrane spaces (Fig. 1F). Thus, the hepatocellular carcinoma McA 7777 glucose mitochondria differ significantly in ultrastructure from their cellular origin, i.e., reference rat liver mitochondria, and this difference is dependent on the metabolism in these cancer cells (McA 7777 glucose vs. glutamine).

The lower appearance of highly-contrasted mitochondrial substructures in McA 7777 mitochondria compared to reference mitochondria indicated a substantial depletion in their protein content, as proteins are typical structures stained by the used contrast agents osmium tetroxide, uranylacetate and lead citrate (Mulisch and Welsch, 2010). Since the McA 7777 mitochondria appeared similar in size when compared to reference mitochondria (Fig. 1D–F), the assumed lower protein content would directly influence their physico-chemical properties. Indeed, upon further purification by discontinuous density gradient centrifugation, McA 7777 mitochondria demonstrated a lower buoyant density than reference mitochondria (Fig. 2). Whereas reference mitochondria completely passed the 24% Nycodenz® layer and pelleted at the bottom of the tube (30,000 rpm, 15 min, 4 °C), McA 7777 glucose mitochondria gathered at the 18/24% interface. Interestingly, McA 7777 glutamine mitochondria partially entered the 24% layer, demonstrating

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t1.1 **Table 1**
Summary of the mitochondrial quantifications by flow cytometry.

t1.2 Source	Isolation	Purity checked by electron microscopy	Yield [mg/g w.w.] [µ/6 × 10^6 cells]	10^9 mitochondria per mg protein	µg protein per 10^9 mitochondria
t1.3 Liver	Standard	$n = 6$	16.1 ± 6.1	8.1 ± 0.9 ($n = 3$)	124.6 ± 12.8
t1.4 Kidney	Standard	$n = 3$	3.9 ± 1.5	8.4 ± 1.0 ($n = 3$)	119.9 ± 12.8
t1.5 Heart	Standard	$n = 2$	1.2 ± 0.6	7.1 ± 0.7 ($n = 3$)	141.0 ± 15.4
t1.6 Brain	Standard	$n = 2$	0.4 ± 0.3	19.5 ± 4.0 ($n = 3$)	52.6 ± 10.3
t1.7 Liver	PCC	$n = 2$	2.3 ± 0.7	8.4 ± 1.5 ($n = 3$)	122.4 ± 23.0
t1.8 McA 7777 glucose	PCC	$n = 1$	19.1 ± 12.9	12.3 ± 1.9 ($n = 3$)	82.7 ± 12.0
t1.9 McA 7777 glutamine	PCC	$n = 4$	21.3 ± 10.6	11.1 ± 0.8 ($n = 3$)	90.4 ± 6.3
t1.10 H4IIE	PCC	$n = 1$	12.0 ± 4.3	12.9 ± 4.2 ($n = 3$)	82.9 ± 23.8

316 a buoyant density slightly higher than McA 7777 glucose but lower than
317 reference mitochondria (Fig. 2).

318 *3.2. The variable net protein content of mitochondria*

319 In order to quantitatively determine the net protein content of mito-
320 chondrial populations, we decided to develop means to count diverse
321 mitochondrial populations in suspensions of known protein concentra-
322 tion (SFig. 1). This approach required several prerequisites:
323 First, to accurately count large number of particles, we employed
324 flow cytometry and typically counted 100,000 events per measurement.

325 Second, in order to specifically determine the number of isolated
326 mitochondria in a given preparation, we aimed to distinguish them,
327 e.g., from contaminating particles in the isolation buffer, the flow cy-
328 tometry sheath fluid and from typical contaminations of mitochondrial
329 isolations, namely lysosomes. Despite the fact that pre-filtered solutions
330 were used (0.2 µm clearance), these solutions and the sheath fluid, how-
331 ever, still interfered with the light-scattering properties (forward and
332 sideward scatter) of isolated mitochondria (Fig. 3A vs. B, left panels).
333 Moreover, upon destruction of the mitochondria by sonication (Gallet
334 et al., 1995), the observed changes in light-scattering properties appeared
335 difficult to use to unequivocally distinguish mitochondrial debris from

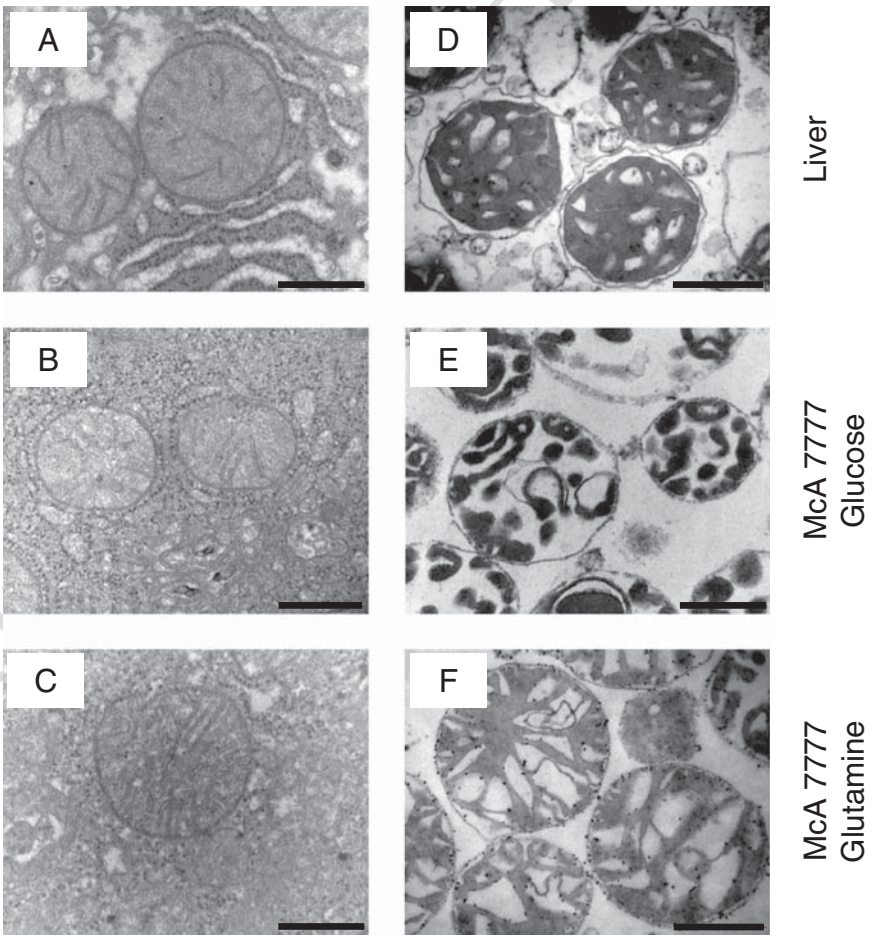

Fig. 1. Mitochondria, either *in situ* or isolated, from rat liver (A and D, respectively) markedly differ in their structure in comparison to mitochondria in (or isolated from) McA 7777 cells grown in glucose enriched medium (B and E, respectively) and McA 7777 cells grown in glucose-free medium supplemented with glutamine and galactose (C and F, respectively). Less cristae and a less densely stained matrix is apparent in McA 7777 mitochondria. Bars equal 500 nm. $n = 1$ (A), 7 (B), 2 (C), 6 (D), 9 (E) and 3 (F).

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intact mitochondria (Fig. 3B vs. D, left panels). Thus, the mitochondrial light scattering properties appeared to be of insufficient separation power for our purposes. In order to circumvent these difficulties, we therefore employed the fluorescent dye 10N-nonyl acridine orange (NAO) to specifically stain mitochondria. Maftah et al. have demonstrated that NAO stains mitochondria independently of the mitochondrial inner transmembrane potential in contrast to membrane potential dependent dyes like Rhodamine 123 (Maftah et al., 1989). This finding was confirmed for NAO labeled mitochondria in flow cytometry, demonstrating that NAO is a reliable mitochondrial marker even following strong mitochondrial depolarization (Mattiasson, 2004). As has been reported by Petit et al., the exceptional specificity of NAO for mitochondria is due to the high affinity of NAO for cardiolipin—two orders of magnitude higher than for other phospholipids (Petit et al., 1992)—which is solely present in the mitochondrial inner membranes of higher eukaryotic cells. Moreover, this staining has been demonstrated to be remarkably time stable. For example Ahmadzadeh et al. have demonstrated that NAO stained mitochondria, directly retrieved from pre-stained muscle cross sections, can be subsequently analyzed by capillary electrophoresis (Ahmadzadeh et al., 2004). Thus, NAO binding to mitochondria is highly specific, time stable, and independent of $\Delta\Psi$. Consequently, this dye has been employed successfully in mitochondrial analyses by flow cytometry (Mattiasson, 2004).

In agreement with these reports, NAO labeled mitochondria could clearly be distinguished by flow cytometry from unstained mitochondria, from isolation buffer containing NAO, and importantly, from mitochondrial debris obtained by sonication of NAO labeled mitochondria (Fig. 3C vs. B, A and D, right panels, respectively). Isolated lysosomes gave a markedly reduced NAO signal (Fig. 3E, right panel), validating the specificity of NAO for mitochondria. The slight amount of NAO positive signals in the chosen gate may be due to mitochondrial contaminations, as we have frequently encountered mitochondria in such lysosomal preparations (data not shown).

Third, in order to minimize subsequent calculation errors due to contaminating non-mitochondrial proteins, all mitochondrial populations to be analyzed were purified by density gradient centrifugation and representative samples were checked for comparable sample homogeneity by electron microscopy (Fig. 4). As can be seen on overview electron micrographs of such preparations, only minor amounts of non-mitochondrial contaminants are apparent in these samples. In addition, these preparations were largely devoid of mitochondrial debris, thus largely comprising intact, homogeneous mitochondrial populations (Fig. 4). It should be noted, however, that a possible drawback of this strategy, i.e., to count comparably pure and intact mitochondrial populations, may be a potential loss of mitochondrial heterogeneity present in the respective sources of isolation. While this is, however, true for all analyses of isolated mitochondria, the major aim of this study was to determine the average number of mitochondria in given populations (Fig. 4). We therefore have relied on "the standard purification strategy" for mitochondria that is density gradient centrifugation. To this end, density step gradients were adjusted such that a major part of the mitochondria gathered at the interface between two gradient steps. While this ensured a reasonable purification (Fig. 4), it also avoided a massive sample loss due to dilution. Thus, although we cannot completely exclude a potential loss of mitochondrial heterogeneity in our analyses, we refer here to the large body of mitochondria from "typical" isolates.

Fourth, to determine the exact volume of the mitochondrial suspensions that had been processed and quantified by the flow cytometer, we employed TruCOUNT™ beads (BD Biosciences). The number of TruCOUNT™ beads is precisely pre-determined by the manufacturer, and thus, solutions with known TruCOUNT™ bead concentrations can be generated. However, as described in the method section, due to difficulties to precisely determine NAO-stained mitochondria and TruCOUNT™ beads in a single measurement, we relied on two consecutive measurements instead, using Fluoresbrite® microspheres (Polysciences Europe GmbH) as a further internal standard. In the first measurement, the concentration of Fluoresbrite® microspheres was calculated from the analyzed volume specified by the number of determined TruCOUNT™ beads. In the second measurement, the concentration of NAO-stained mitochondria was calculated from the analyzed volume determined by the number of determined Fluoresbrite® microspheres. The absolute number of mitochondria per mg protein was subsequently calculated from the concentration of NAO-stained mitochondria and the determined protein concentration of this mitochondrial suspension (SFig. 1).

Using these settings, we determined the total number of mitochondria in suspensions with known protein content (Fig. 5). Irrespective of the isolation method used (standard in Fig. 5A, PCC in Fig. 5B) rat liver reference mitochondria contained around 8.1×10^9 mitochondria per mg protein (Table 1) or, in other terms, 10^9 rat liver mitochondria comprise around 125 μg protein (Table 1). This determined value is in excellent agreement with reported values obtained either by electron microscopy ($7.0–9.4 \times 10^9$), light microscopy (7.1×10^9) or Coulter counter ($2.0–9.1 \times 10^9$) (Gear and Bednarek, 1972; Schwerzmann

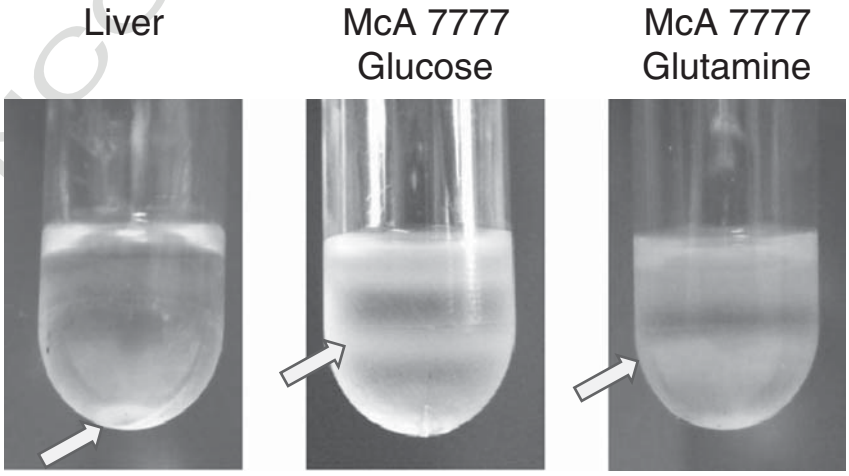

Fig. 2. Density gradient centrifugation unveils a higher density of isolated rat liver mitochondria compared to McA 7777 mitochondria, indicating an altered molecular composition. Isolated mitochondria from McA 7777 cells (grown in high glucose or glutamine) and from rat liver tissue were subjected to a discontinuous Nycodenz® density gradient and centrifuged for 15 min at 30,000 rpm at 4 °C. Whereas liver mitochondria gather at the tube bottom (left, arrow), McA 7777 mitochondria concentrate at the 18/24% interphase (middle and right, arrow). This feature was reproducibly observed throughout our mitochondrial isolations.

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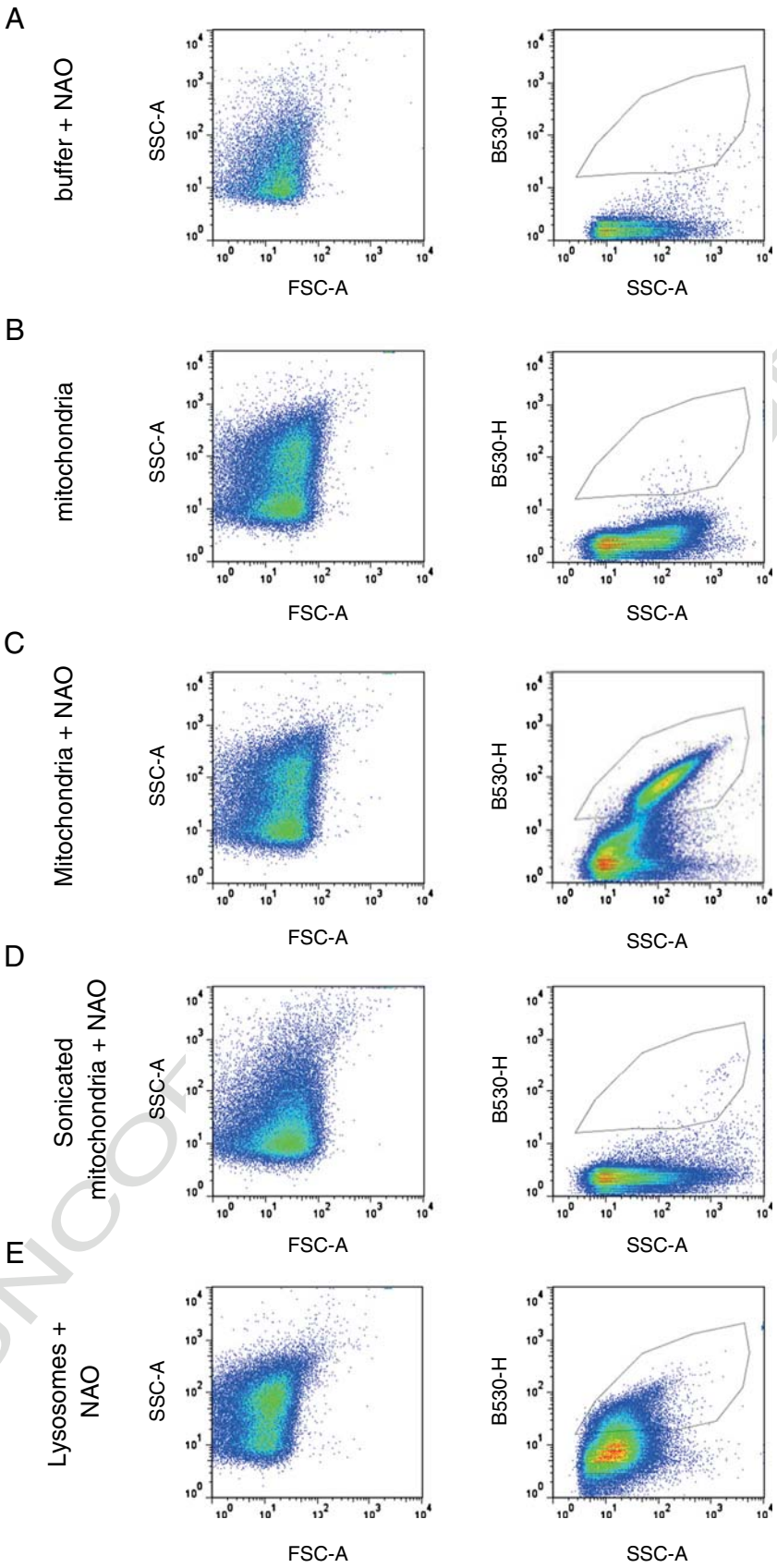

Fig. 3. Determination of NAO stained mitochondria by flow cytometry. Based on their light scattering properties, mitochondria cannot be unambiguously counted (left column). In contrast, however, NAO stained mitochondria form a distinct population which can clearly be detected by fluorescence at 530 nm and sideward scatter (SSC-A, right column C), and which were quantified. Upon subsequent sonication which destroys mitochondria, no NAO positive signals are apparent (right column, D). NAO stained lysosomal fractions only gave minor signals in the chosen "gate" (right column, E). Either 30,000 (buffer) or 100,000 (mitochondria or lysosomes) events were recorded ($n = 3$).

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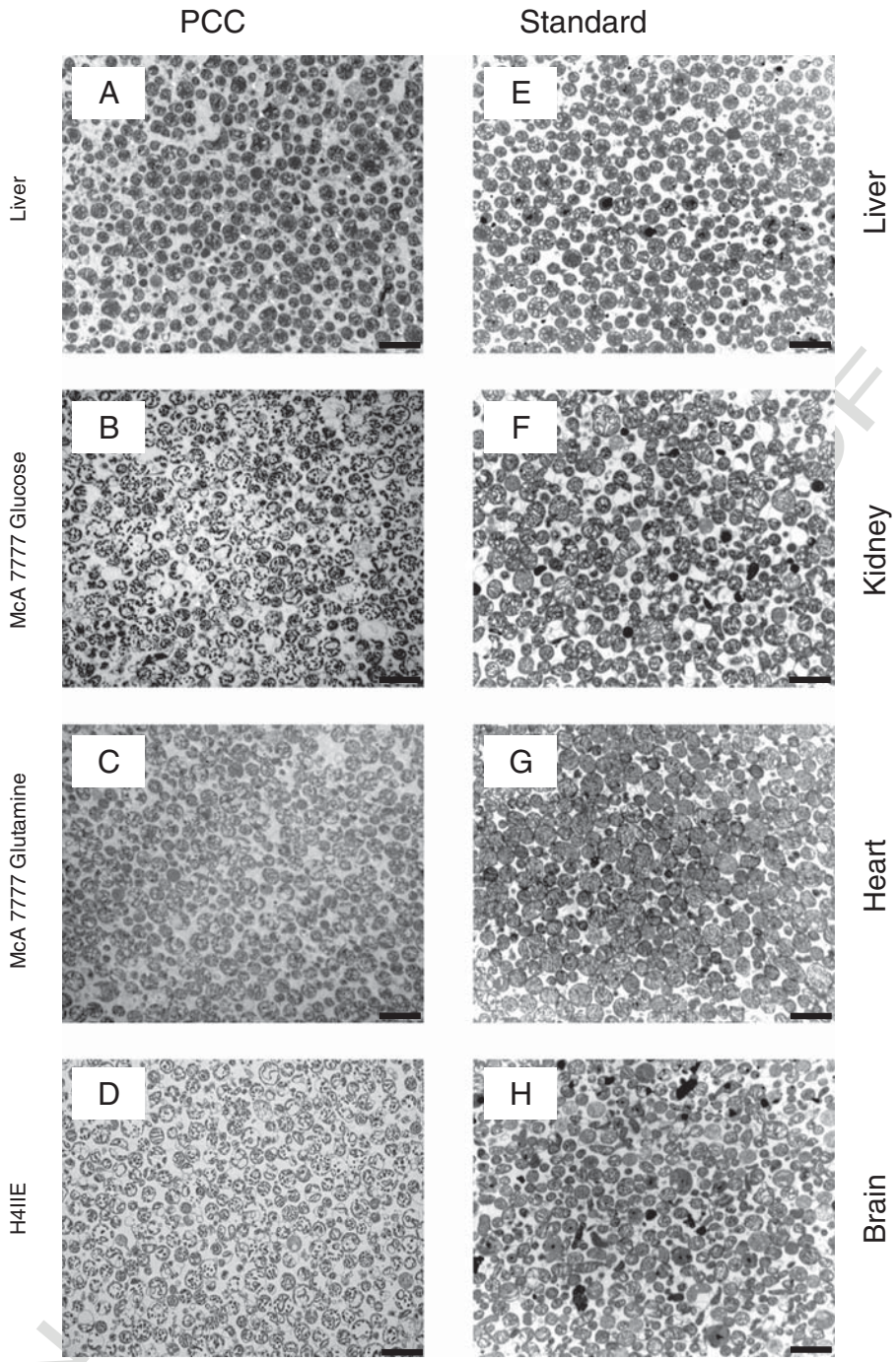

Fig. 4. Electron micrographs of density gradient purified mitochondria isolated either by PCC (left column) or standard techniques (right column). Sources of isolation were: (A, E) rat liver; (B, C) McA 7777 cells either grown in glucose or glutamine enriched media, respectively; (D) H4IIE cells; (F) rat kidney; (G) rat heart;(H) rat brain. Highly comparable sample homogeneities were observed. Bars equal 2 μm.

et al., 1986). This validates the herein described approach to accurately count mitochondria.

Furthermore, rat kidney and heart mitochondria demonstrated similar protein contents as rat liver mitochondria (Fig. 5A, Table 1). In contrast, however, mitochondria isolated from rat brain contained about twice the number of mitochondria per mg protein (Fig. 5A, Table 1). Despite the fact that the analyzed mitochondria were of comparable homogeneity after purification by Percoll density gradient (Fig. 4), we further validated the different protein content between rat liver and brain mitochondria by an additional Nycodenz® density gradient purification step ($n = 2$, data not shown).

While this difference in protein content between rat liver and brain mitochondria may be further substantiated in future experiments, we focused here on the comparison of rat liver mitochondria and mitochondria from rat HCC cell lines (Figs. 1 and 2). In fact, we determined 1.5 times more mitochondria per mg protein in HCC mitochondria compared to reference rat liver mitochondria (Fig. 5B, Table 1). In order to further validate this result, we measured the enzymatic activity of the

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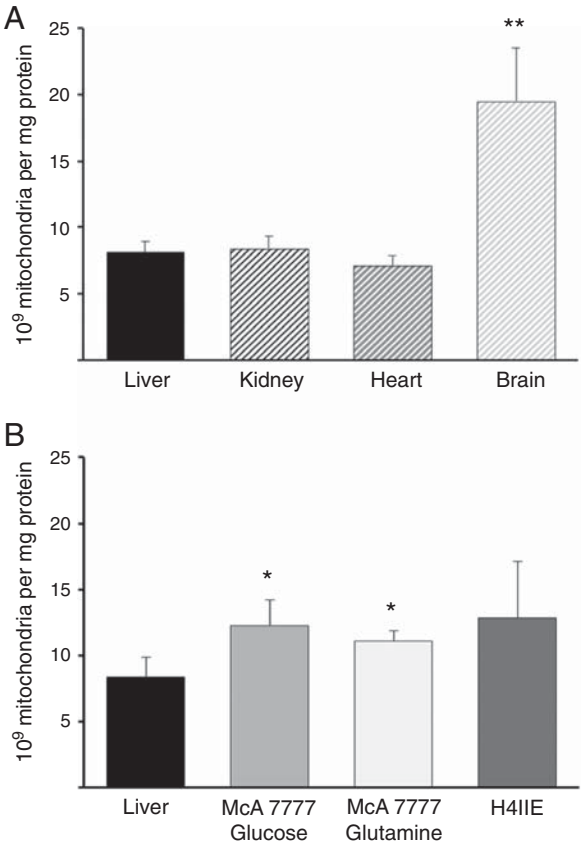

Fig. 5. Depending on the source of isolation, mitochondria may differ significantly in their protein content. A) Quantification of mitochondria from rat liver, kidney, heart and brain by flow cytometry. Similar numbers of mitochondria per mg protein were determined for liver, kidney and heart. For rat brain mitochondria, the determined number per mg protein was more than twofold higher ($n = 3$ for liver, kidney, heart or brain, respectively). B) Quantification of mitochondria by flow cytometry demonstrates an about 1.5 fold higher number of mitochondria per mg protein for isolated HCC mitochondria compared to rat liver mitochondria ($n = 3$ for rat liver, McA 7777 glucose, McA 7777 glutamine or H4IIE, respectively).

mitochondrial citrate synthase (CS) in McA 7777 and rat liver mitochondria. This "housekeeping" enzyme activity is frequently considered not to be subjected to fluctuations in pathological situations (Pallotti and Lenaz, 2007). Thus, CS activity is often used as a surrogate marker for mitochondrial content (Dalziel et al., 2005; Garrabou et al., 2007). Applying the same amount of protein, we determined a significantly higher activity for McA 7777 glucose and glutamine mitochondria (570 ± 282 and 514 ± 166 nmol/min/mg, respectively) compared to reference mitochondria (190 ± 60 nmol/min/mg). This CS activity which was about twice as high in McA 7777 mitochondria strengthens the above notion of a higher number of these HCC mitochondria per mg protein compared to reference rat liver mitochondria. Or expressed *vice versa*, McA 7777 mitochondria roughly have about half the protein content of reference rat liver mitochondria.

3.3. Mitochondrial comparisons: Normalization to mitochondrial number or protein amount?

Comparative analyses of isolated mitochondria are typically normalized to equal protein amounts. As an example, McA 7777 glucose and glutamine mitochondria slightly (but not significantly) differ in their protein content (Fig. 5B, Table 1). Thus, if equal protein amounts of these two mitochondrial populations are bio-analytically compared, around 10% more McA 7777 glucose mitochondria are analyzed than McA 7777 glutamine mitochondria. This deviation is probably of minor significance given the standard deviation of the determined mitochondrial numbers, which may result from minor gating discrepancies, inter-experimental mean variations of most comparative bio-analytical methods, and especially from the variations in protein quantification. The same holds true for mitochondria isolated from healthy rat liver, heart or kidney tissues (Fig. 5A, Table 1). It therefore seems reasonable to conclude that comparing these mitochondrial populations may either be normalized to equal protein amount or to equal mitochondrial number.

In contrast, however, rat liver and rat brain mitochondria or rat liver and mitochondria from the rat HCC cell line McA 7777 show significant differences in mitochondrial protein content (Fig. 5). Thus, upon comparing these mitochondrial populations, normalization either to protein amount or to absolute mitochondrial number could lead to conflicting results. In order to demonstrate this, we have chosen to exemplarily compare the abundance of the respiratory complexes I and II (CI or CII, respectively) in reference rat liver and McA 7777 glucose mitochondria (Fig. 6A, B). Immunoblotting analysis based on equal protein load revealed a significantly higher CI, CII, and citrate synthase abundance in McA 7777 compared to reference mitochondria (Fig. 6A). However, using the same mitochondrial preparations but analyzing an equal mitochondrial number, an almost equal CI and citrate synthase content but a significant depletion of CII was apparent in McA 7777 versus reference mitochondria (Fig. 6B). Thus, when normalizing to protein amounts CI and especially CII amounts, would be largely overestimated in McA 7777 mitochondria.

From these results, it does occur that McA 7777 glucose mitochondria have an impressively lower CII amount than reference mitochondria (Fig. 6B). In agreement, if normalized to an equal number of mitochondria, we determined a one third lower CII activity in McA 7777 mitochondria compared to reference mitochondria (23 vs. 38 nmol/min/10^9 mitochondria, Fig. 6D). In contrast, if normalized to the same protein amount, no CII activity differences were apparent between the two mitochondrial populations (Fig. 6C). The impact of the normalization type became even more apparent if the mitochondria were challenged with low doses of thenoyltrifluoroacetone (TTFA, 2.5 or 5 μM) (Fig. 6E and F). TTFA inhibits CII activity (Ramsay et al., 1981; Zhang et al., 2001). As McA 7777 mitochondria have a lower CII activity than reference mitochondria (Fig. 6D), inhibition of CII activity is significantly enhanced in McA 7777 mitochondria (Fig. 6F). This stronger inhibition was apparent upon normalization to absolute mitochondria number but not upon normalization to protein amount (Fig. 6F vs. E).

4. Conclusions

In this study we demonstrated significantly lower protein contents in rat brain mitochondria and mitochondria from the hepatocellular carcinoma cell line McA 7777 compared to reference rat liver mitochondria. Our results show that with regard to the comparison of mitochondrial populations the issue of normalization to either absolute mitochondrial number or to equal protein amount is of special concern. It appears that normalization solely to protein amount may arrive at misleading results.

Numerous studies have firmly established the outstanding adaptability of mitochondria to changes in their environment (i.e., nutritive conditions for OXPHOS vs. glycolysis, hypoxia vs. normoxia, etc.). Moreover, mitochondria differ in their biochemical properties and molecular composition depending on their origin (i.e., tissue vs. cell culture; species or tissue differences) and health status of the originating tissue (e.g., cancer tissue or cancer cells vs. healthy controls). Our results indicate that it is appropriate to consider other parameters than protein amount for normalization, if mitochondria from divergent origin or metabolic situations are compared. Citrate synthase activity has frequently been used in this respect. In fact, in our study a higher number of mitochondria coincided with an augmented citrate synthase activity and abundance. While in this case, this clearly argues for such

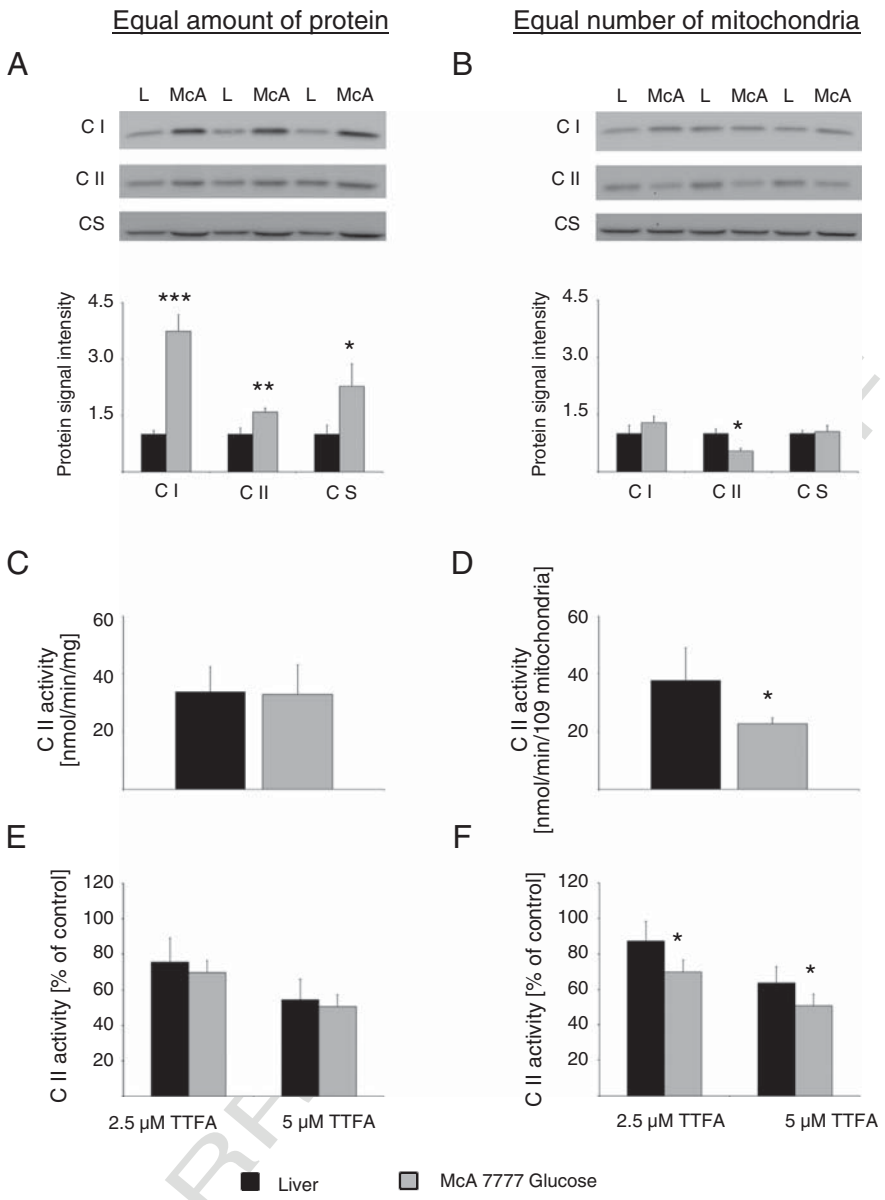

Fig. 6. Mitochondrial comparisons may arrive at conflicting results when normalized to either equal protein amount or to equal mitochondrial number. A and B) Quantitative immunoblotting analysis for abundance of complex I, complex II and mitochondrial citrate synthase (three independent isolations of rat liver and McA 7777 glucose mitochondria). A) equal amount of protein (8 µg), B) equal number of mitochondria (6.6×10^7). Protein signals were quantified with ImageJ ($n = 3$). C) and D) Enzymatic activity of mitochondrial complex II in mitochondrial suspensions from rat liver and McA 7777 glucose cells. Normalization was done either to an equal amount of mitochondrial protein (C) or to an equal mitochondrial number (D) ($n = 6, 4$ for liver or McA 7777, respectively). E) and F) Dose dependent inhibition of mitochondrial complex II activity by TTFA. A comparable sensitivity toward TTFA is observed for McA 7777 glucose mitochondria in comparison to rat liver mitochondria if equal amounts of protein were applied (E). This difference becomes significant if normalized to an equal number of mitochondria (F) (n = 6 or 4 for rat liver or McA 7777 glucose mitochondria, respectively).

an enzymatic normalization, other studies, however, have reported differing citrate synthase activities of mitochondria isolated from variant rat tissues (Saggerson and Carpenter, 1986). A further potential option for mitochondrial quantifications may be based on their mtDNA content. In fact, the ratio of mtDNA to nDNA may be used as an estimate for the number of mt-genomes per cell (Phillips et al., 2014). However, with respect to the quantification of isolated mitochondria, it needs to be stressed that the mtDNA content is not a non-regulated feature. In fact, mitochondria contain between 1 and 10 copies of mtDNA. Moreover, this number is highly dynamic and regulated in a cell-specific manner by mechanisms that are not completely understood (Phillips et al., 2014). Further, Veltri et al. have demonstrated that the mtDNA copy number per mitochondrion is organ-specific, with heart displaying the lowest mtDNA content expressed per g mitochondria followed by kidney and liver and brain with the highest mtDNA content (Veltri et al., 1990). Finally, a decrease in the mtDNA copy number is a common event in hepatocellular carcinomas, as over 60% of such tumors have a lower mtDNA copy number than their corresponding non-tumor liver tissue (Hsu et al., 2013). Consequently, counting mitochondria on the basis of their mtDNA would have to rely on the exactly predetermined average copy number of mtDNA present in the respective mitochondrial populations to be analyzed. Thus, it may be cumbersome to rely either on citrate synthase or potentially on mtDNA as a surrogate for mitochondrial quantifications. In contrast, to determine the absolute number of mitochondria in a given sample directly may be an advantageous alternative instead. The flow cytometry approach in our study

may help in this respect. It is fast (some minutes), requires low sample amounts, and is accurate due to the high number of counted events. This method, like other normalization parameters, relies on the accurate determination of the analyzed protein amounts. It therefore seems advisable to determine this parameter as precisely as possible.

The impact of a divergent normalization was exemplified by a comparative analysis of complex II from rat liver and McA 7777 glucose mitochondria. We observed a comparable activity of CII if normalization was based on equal protein content. However, if normalization was based on an equal number of mitochondria, we found a significantly lower activity of CII in the McA 7777 than in rat liver mitochondria. Furthermore, the sensitivity of CII against TTFA was significantly higher in McA 7777 than in rat liver mitochondria, which became apparent when the comparisons were normalized to mitochondrial number, but not, when normalized to equal protein amounts.

Finally, an increasing number of reports have suggested CII as a promising target for anti-cancer agents (recently reviewed in Kluckova et al., 2013). CII has a dual role, firstly, it oxidizes succinate to fumarate by its succinate dehydrogenase activity (SDH), and secondly, the resulting electrons are transferred to ubiquinone (UbQ), referred to as its SQR activity (Kluckova et al., 2013). Especially the inhibition of SQR, for example by a mitochondrially targeted analog of vitamin E succinate (MitoVES) but also TTFA, leads to an increase in superoxide production and ultimately cell death (Dong et al., 2011; Lemarie et al., 2011). Our data indicate a further important aspect in this emerging research topic which is a more specific reaction against liver cancer mitochondria by CII interference but to a lesser extent against normal liver mitochondria. We have found a significantly higher vulnerability of CII toward inhibition in McA7777 mitochondria compared to normal rat liver mitochondria. While these results need to be validated in mitochondria from further liver cancer cell lines, on a speculative note, this may open a therapeutic window for CII inhibitors as anti-cancer therapeutics against liver cancer mitochondria. It should be noted, however, that these data reflect the situation of isolated mitochondria and not within the respective cells. Interestingly, our first and preliminary results do indicate that the number of mitochondria is lower in McA7777 cells compared to primary hepatocytes, which would agree well with the reported depletion of the cellular mitochondrial content in liver carcinogenesis (Cuezva et al., 2002). Although this may decrease the CII activity at the tumor cell level even further, a correlation needs to be established of such a decreased CII activity and its phenotypic consequences. In fact, it has been reported that CII inhibition leads to cell death but on the contrary that tumor cells with a mutated CII could be very resistant to CII inhibitors (Kluckova et al., 2013; Lemarie and Grimm, 2011). Thus, it remains for future studies to investigate the potential impact of the lower CII activity in HCC mitochondria. Irrespective of these theoretical considerations, it appears that mitochondrial comparisons based on their equal absolute numbers may be an important approach in order to identify "true" molecular differences in mitochondrial populations.

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