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Mitochondrial free fatty acid β-oxidation supports oxidative phosphorylation and proliferation in cancer cells

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ABSTRACT

Oxidative phosphorylation (OxPhos) is functional and sustains tumor proliferation in several cancer cell types. To establish whether mitochondrial β-oxidation of free fatty acids (FFAs) contributes to cancer OxPhos functioning, its protein contents and enzyme activities, as well as respiratory rates and electrical membrane potential ($\Delta\Psi m$) driven by FFA oxidation were assessed in rat AS-30D hepatoma and liver (RLM) mitochondria. Higher protein contents (1.4–3 times) of β-oxidation (CPT1, SCAD) as well as proteins and enzyme activities (1.7–13-times) of Krebs cycle (KC: ICD, 2OGDH, PDH, ME, GA), and respiratory chain (RC: COX) were determined in hepatoma mitochondria *vs.* RLM. Although increased cholesterol content (9-times *vs.* RLM) was determined in the hepatoma mitochondrial membranes, FFAs and other NAD-linked substrates were oxidized faster (1.6–6.6 times) by hepatoma mitochondria than RLM, maintaining similar $\Delta\Psi m$ values. The contents of β-oxidation, KC and RC enzymes were also assessed in cells. The mitochondrial enzyme levels in human cervix cancer HeLa and AS-30D cells were higher than those observed in rat hepatocytes whereas in human breast cancer biopsies, CPT1 and SCAD contents were lower than in human breast normal tissue. The presence of CPT1 and SCAD in AS-30D mitochondria and HeLa cells correlated with an active FFA utilization in HeLa cells. Furthermore, the β-oxidation inhibitor perhexiline blocked FFA utilization, OxPhos and proliferation in HeLa and other cancer cells. In conclusion, functional mitochondria supported by FFA β-oxidation are essential for the accelerated cancer cell proliferation and hence anti-β-oxidation therapeutics appears as an alternative promising approach to deter malignant tumor growth.

1. Introduction

Enhanced glycolysis is one of the most important cancer metabolic hallmarks (Cantor and Sabatini, 2012; Hanahan and Weinberg, 2011). It has been suggested that tumor cells permanently maintain an impaired oxidative phosphorylation (OxPhos) which promotes an increased glycolysis (Warburg, 1956). In consequence, OxPhos flux, and mitochondrial enzyme activities and contents are not usually determined in studies of cancer energy metabolism (Owens et al., 2011; Putignani et al., 2012). Several proposals on the possible mechanisms associated with the OxPhos impairment in cancer cells have emerged. Some of these proposed mechanisms are: mutations in the Krebs cycle enzymes (fumarate hydratase, succinate dehydrogenase and isocitrate dehydrogenases) (Xekouki and Stratakis, 2012; Yang et al., 2012); absence (Mayr et al., 2008) of one of the principal enzyme controlling

Abbreviations: AAT, aspartate aminotransferase; AcAc, acetoacetate; ANT, adenine nucleotide translocase; COX, cytochrome *c* oxidase; CPT1, carnitine palmitoyl transferase I; LCHAD, long-chain 3-hydroxyacyl CoA dehydrogenase; GA, glutaminase; GDH, glutamate dehydrogenase; Gln, glutamine; Glut, glutamate; ICD, isocitrate dehydrogenase; LDH, lactate dehydrogenase; Mal, malate; ME, malic enzyme; ND, NADH-ubiquinone oxidoreductase; OxPhos, oxidative phosphorylation; PDH, pyruvate dehydrogenase; Pyr, pyruvate; RLM, rat liver mitochondria; ROS, radical oxygen species; SCAD, short-chain acyl CoA dehydrogenase; SDH, succinate dehydrogenase; 2OG, 2-oxoglutarate; 2OGDH, 2-oxoglutarate dehydrogenase; β OHBut, β-hydroxybutyrate.

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tumor OxPhos. *i.e.* respiratory complex I (Rodríguez-Enríquez et al., 2000); and activation of several oncogenes (K-RAS), and transcription factors (HIF1-α), that may lead to lower OxPhos functioning (Chiaradonna et al., 2006). However, this view has been challenged by numerous recent studies demonstrating that in several metastatic cancers, OxPhos is the predominant ATP cellular supplier in strong preference over glycolysis (Moreno-Sánchez et al., 2007; Ralph et al., 2010; Zu and Guppy, 2004). Thus, active OxPhos and mitochondrial remodeling and ROS production have been associated with invasiveness onset (Zhao et al., 2013), metastatic and malignant phenotype acquisition (Sotgia et al., 2012; Ralph et al., 2015), cellular cycle activation and autophagy resistance (Salem et al., 2012), and other mechanisms implicated in drug resistance (Indran et al., 2011; Lu and Chao, 2012).

As a consequence of the misunderstanding on the role of OxPhos in cancer cells, scarcely any bioenergetics information has been reported using tumor mitochondria. In this regard, pioneer works (Moreadith and Lehninger, 1984; Rivera et al., 1988; Rodríguez-Enríquez et al., 2000) have shown that fast growing tumor cells oxidizes glutamine at high rates even when glucose is available (Fan et al., 2013; Guppy et al., 2002; Lazo, 1981; Reitzer et al., 1979). The oxidation of other substrates (ketone bodies, amino and imino-acids as well as free fatty acids) in isolated tumor mitochondria has not been extensively studied or contradictory results have been described (Cederbaum and Rubin, 1976; Ciapaite et al., 2011; Dietzen and Davis, 1993; Parlo and Coleman, 1984; Ralph et al., 2010; Rossignol et al., 2004).

Among the different mitochondrial pathways evaluated, the β-oxidation was of particular interest because it has been reported that the consumption of octanoyl-, myristoyl-, palmitoyl- and stearoyl-carnitine was significantly lower in AS-30D mitochondria than in RLM (Dietzen and Davis, 1993). In contrast, in human hepatocarcinoma HepG2 cells, β-oxidation is active and may replenish the mitochondrial acetyl-CoA pool required for OxPhos (Wong et al., 2004). High carnitine-acyl-transferase 1 (CPT1) mRNA contents have also been determined in ovary, colon, esophageal, prostate and colorectal carcinomas, as well as Zadjela hepatoma and K-RAS transformed fibroblasts (Alfonso et al., 2005; Capuano et al., 1997; Herrmann et al., 2003). Therefore, inhibition of β-oxidation has been suggested as potential target to diminish tumor growth (Samudio et al., 2010; Tirado-Vélez et al., 2012). However, for other β-oxidation enzymes such as acyl CoA dehydrogenase, the mRNA content was significantly decreased in colorectal carcinoma *vs.* colon normal tissue (Birkenkamp-Demtroder et al., 2002).

To characterize the mitochondrial β-oxidation in cancer cells, the first part of this work examined, in mitochondria isolated from AS-30D carcinoma grown in rats fed *ad libitum*, (i) the OxPhos rate and $\Delta\Psi m$ driven by FFAs comparing them to those driven by Krebs cycle intermediates, imino acids and ketone bodies; (ii) the β-oxidation and other OxPhos protein contents and activities; and (iii) the mitochondrial cholesterol content. In parallel, studies were also performed in mitochondria isolated from AS-30D carcinoma grown in 24h-fasted rats, in order to determine the changes in mitochondrial function and energy status induced by the nutritional state. For comparative purposes mitochondria isolated from rat liver (RLM), the organ from which the tumor originated, were also used (Orrick et al., 1973).

In the second part of the present study, mitochondrial β-oxidation was analyzed in intact tumor cells and human cancer biopsies (i) to resolve the existing discrepancies about the enzyme/transporter contents of this pathway; (ii) to establish whether this pathway is used for driving OxPhos and whether it has a functional role supporting tumor proliferation, which may help in the design of targeted strategies abolishing oxidative-type tumors; and (iii) to identify potential new metabolic tumor biomarkers.

2. Material and methods

2.1. Tumor cell and fibroblasts cultures

AS-30D tumor cells were propagated by intraperitoneal injection of $2–5 \times 10^8$ cells into female Wistar rats of 200–250 g weight. The cells were harvested, washed with Krebs–Ringer buffer and stored in ice at a density of $2–5 \times 10^8$ cells/mL until use as described previously (Rodríguez-Enríquez et al., 2001). Tumor cells from human carcinomas from cervix (HeLa), colon (Colo-205), lung (A-549) and breast (MCF-7, MDA-MB231, MDA-MB468) as well as 3T3 (mouse) and CCD25Lu (human) fibroblasts (American Type Culture Collection, ATCC; Rockville, MD, USA) were grown (1×10^7 cells/dish) in Dulbecco-MEM medium supplemented with 10% fetal bovine serum (Gibco; Rockville, MD, USA) *plus* 10,000 U penicillin/streptomycin (Sigma, Steinheim, Germany) and placed under a humidified atmosphere of 5% CO_2/95% air at 37 °C until 80–90% confluence was reached. The genotyping (National Institute of Genomic Medicine, INMEGEN, México) of the HeLa, Colo-205, A-549, MCF-7, MDA-MB231, MDA-MB468 and 3T3 cell lines used in the present study revealed genotypes identical to those of the original ATCC tumor clones.

2.2. Isolation of rat hepatocytes

Rat hepatocytes were isolated by the method of liver digestion using portal vein collagenase perfusion as previously described (Berry and Friend, 1969). Hepatocytes were resuspended in sterile phosphate-buffered saline (PBS), diluted (v/v) with an isosmotic Percoll solution (45 mL Percoll/4.5 mL of 10× Hank's balanced salt solution) and centrifuged at 800 rpm for 5 min. Cell viability ≥85% was estimated by trypan blue exclusion.

2.3. Human breast cancer biopsies

Five infiltrating ductal breast carcinoma samples were collected from female patients at Instituto Nacional de Cancerología, México, following the handling protocols approved by the Institutional Ethics Committee and supported by patient's informed consents according to the Declaration of Helsinki (Pacheco-Velázquez et al., 2014). For normal breast tissue, 5 samples were used as a control. Statistical analysis of human tumor and non-tumor samples was performed by using Student's *t*-test analyses as described elsewhere (Pacheco-Velázquez et al., 2014).

2.4. Isolation of mitochondria from rat AS-30D hepatoma and liver (RLM)

The digitonin permeabilization procedure was used to isolate AS-30D mitochondria (Moreadith and Fiskum, 1984). The final concentration of digitonin (Sigma Aldrich, CA, USA) used for plasma membrane solubilization was 10–40 μg/mg cellular protein (Rodríguez-Enríquez et al., 2001). The mitochondrial pellet was then washed with SHE (Sucrose 250 mM, HEPES 10 mM, EGTA 1 mM pH 7.4) buffer and incubated with 0.5% (w/v) fatty acid free-albumin and 1 mM ADP for 15 min at 4 °C before final centrifugation.

RLM were isolated as described previously (Moreno-Sánchez, 1985). Female Wistar rat (250–300 g) liver was extracted and homogenized in cold SHE buffer, pH 7.3. The cellular homogenate was centrifuged at 700 × *g* and 4 °C for 10 min. The supernatant of the first low-speed centrifugation was collected and further centrifuged at 7000 × *g*. The mitochondrial pellet was resuspended in cold SHE buffer and pre-incubated by 10 min in ice with 0.1% BSA *plus* 1 mM ADP with occasional stirring; subsequently, the mixture was diluted 20 times and centrifuged at 7000 × *g* and 4 °C for