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Research Article

Nuclear respiratory factor-1 and bioenergetics in tamoxifen-resistant breast cancer cells

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ABSTRACT

Acquired tamoxifen (TAM) resistance is a significant clinical problem in treating patients with estrogen receptor α (ERα)+ breast cancer. We reported that ERα increases nuclear respiratory factor-1 (NRF-1), which regulates nuclear-encoded mitochondrial gene transcription, in MCF-7 breast cancer cells and NRF-1 knockdown stimulates apoptosis. Whether NRF-1 and target gene expression is altered in endocrine resistant breast cancer cells is unknown. We measured NRF-1and metabolic features in a cell model of progressive TAM-resistance. NRF-1 and its target mitochondrial transcription factor A (TFAM) were higher in TAM-resistant LCC2 and LCC9 cells than TAM-sensitive MCF-7 cells. Using extracellular flux assays we observed that LCC1, LCC2, and LCC9 cells showed similar oxygen consumption rate (OCR), but lower mitochondrial reserve capacity which was correlated with lower Succinate Dehydrogenase Complex, Subunit B in LCC1 and LCC2 cells. Complex III activity was lower in LCC9 than MCF-7 cells. LCC1, LCC2, and LCC9 cells had higher basal extracellular acidification (ECAR), indicating higher aerobic glycolysis, relative to MCF-7 cells. Mitochondrial bioenergetic responses to estradiol and 4-hydroxytamoxifen were reduced in the endocrine-resistant cells compared to MCF-7 cells. These results suggest the acquisition of altered metabolic phenotypes in response to long term antiestrogen treatment may increase vulnerability to metabolic stress.

1. Introduction

Approximately 40% of breast cancer patients whose primary tumors express estrogen receptor α (ERα) and are initially responsive to endocrine therapies, *i.e.*, tamoxifen (TAM) or aromatase inhibitors (AI), relapse with acquired endocrine resistant disease progression [1]. Defining the mechanisms of TAM-resistance remains an important clinical issue for breast cancer patients. Although AIs have replaced TAM as the first-line treatment for postmenopausal women with ERα+ breast tumors, ten years of TAM is recommended as adjuvant therapy for ERα+ premenopausal breast cancer patients and for postmenopausal women who have relapsed on or cannot tolerate AI therapy [2]. There are thousands of breast cancer survivors who have received TAM as a mono-adjuvant therapy and are at unknown risk for developing TAM-resistant metastatic disease as a late recurrence, an emergence from dormancy [3]. TAM is a selective ER modulator (SERM) with agonist and antagonist activities mediated by ERα, ERβ, and G-protein coupled ER (GPER) [4]. Multiple mechanisms contribute to the evolution of cells resistant to the growth inhibiting, anti-estrogenic effects of TAM and AIs (reviewed in [4,5]).

Mitochondrial–nuclear crosstalk is critical for the maintenance of cellular homeostasis and is dysregulated in cancer [6]. Epithelial tumor cell metabolism is supported by fuel sources from cancer-associated fibroblasts and adipocytes [7]. Epithelial breast cancer cells have been suggested to have increases in mitochondrial number, anabolic function, oxidative phosphorylation (OXPHOS) [8], and nuclear respirator factor 1 (NRF-1) [9]. NRF-1 is a master regulator of nuclear-encoded mitochondrial gene transcription, including genes for mitochondrial bioenergetic function [10]. We reported that estradiol (E_2) stimulates NRF-1 transcription, which

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in turn, increases the transcription of its targets including the mitochondrial transcription factor TFAM (transcription factor, mitochondrial) in MCF-7 and T47D (both are ERα+/PR+, HER2-, luminal A [11]) breast cancer cells and mouse mammary gland [12,13].

MCF-7 cells chronically exposed to 25 μM H_2O_2 (simulating oxidative stress in a tumor) showed increased NRF-1 and TFAM expression, decreased ERα expression and increased colony-forming potential [14]. E_2 was reported to increase ROS thus activating AKT which phosphorylated and activated NRF-1 (p-NRF-1) and increased transcription of its cell cycle targets: *CDC2, PRC1, PCNA, cyclin B1*, and *CDC25C* in MCF-7 cells [15]. No one has evaluated NRF-1 expression or that of its targets involved in mitochondrial bioenergetics in TAM-resistant breast cancer cells.

A recent publication cataloged changes in the RNA levels of key genes in glycolysis, gluconeogenesis, glycogen synthesis and degradation, and the pentose phosphate pathway (PPP) in MCF-7 and BT-474 (ERα+, PR+, HER2+) breast cancer cell lines that had undergone EMT [16]. However, this study did not examine functional consequences of these changes in bioenergetic parameters. Studies in transgenic mouse mammary tumor virus (MMTV)–polyoma middle T (PyMT) mice with different mtDNA but identical nuclear DNA revealed that the mtDNA background directly affected primary tumorigenicity and metastatic efficiency, although the precise mechanism(s) are still unknown [17]. Overall, the contribution of metabolic reprogramming in the development of endocrine resistance in breast cancer is poorly understood.

The goals of this study were to evaluate the expression of NRF-1 and its target TFAM in TAM-resistant cells derived from MCF-7 cells and to identify and characterize bioenergetic differences of intact endocrine-sensitive *versus* TAM-resistant cells. We compared MCF-7 ERα+/PR+, HER2-, luminal A breast cancer cells with LCC1 (ERα+/PR+, HER2-; E_2-independent, TAM- and fulvestrant-sensitive), LCC2 (ERα+/PR+, HER2-; E_2-independent, TAM-resistant, fulvestrant-sensitive), and LCC9 (ERα+/PR+, HER2-; E_2-independent, TAM- and fulvestrant-resistant) breast cancer cell lines, which are derived from MCF-7 cells, as a cellular models of progression to endocrine-resistance [18]. Our results reveal increased NRF-1 and TFAM in endocrine-resistant cells as well differences in bioenergetic phenotypes in TAM-resistant breast cancer cells.

2. Materials and methods

2.1. Reagents and antibodies

17β-estradiol (E_2), 4-hydroxytamoxifen (4-OHT), Oligomycin A, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), Rotenone, Antimycin A were purchased from Sigma-Aldrich (St. Louis, MO., USA). Antibodies were purchased as follows: NRF-1, Rockland Immunochemicals, Inc (Pottstown, PA, USA); TFAM (DO1P), Abnova; total OXPHOS WB antibody cocktail (Abcam, Cambridge, MA, USA);α-tubulin, Neomarkers; β-actin, Sigma-Aldrich; GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA); UQCRC2 (PA530204, Thermo Fisher, Waltham, MA, USA).

2.2. Cell culture and treatments

MCF-7 and T47D cells were purchased from ATCC (Manassas, VA, USA), LCC2, and LCC9 cell lines were derived from MCF-7 cells by cultivation with the antiestrogens 4-hydroxytamoxifen, ICI 182,780 (Fulvestrant) and LY 117,018 respectively, and were graciously provided as a gift by Dr. Robert Clarke, Georgetown University [18]. LCC1 cells are derived from MCF-7 as E_2-independent, TAM-sensitive cells and were also a gift from Dr. Robert Clarke [19]. MCF-7, LCC1, LCC2, and LCC9 cells were maintained in IMEM (Cellgro, Manassas, VA, USA) containing 5% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA, USA) and 1% Penicillin/Streptomycin (Cellgro). T47D were grown in RPMI (Cellgro) containing 5% FBS and 25 nM insulin (Sigma). Where indicated, cells were treated 24 and 48 h with vehicle control (EtOH 0.001%), 10 nM E_2, or 100 nM 4-OHT. For other experiments, cells were grown in phenol red-free medium containing 5% dextran coated charcoal (DCC)-stripped FBS for 48 h prior to treatment as indicated.

2.3. Metabolic analysis with Seahorse XF24 extracellular flux analyzer

To measure mitochondrial bioenergetics profile in breast cancer cells, a Seahorse Bioscience XF24 Extracellular Flux Analyzer was used (Seahorse Bioscience, North Billerica, MA, USA). Cells were plated on XF24 plates for 24 h prior to assay. One hour prior to the assay, the cells were switched to DMEM assay media containing 2 mM Glutamax (ThermoFisher), 1 mM sodium pyruvate, 25 mM glucose , and 1.85 g/L NaCl (all from Sigma), pH 7.4, and maintained at 37 °C in an non-CO_2 incubator. Sensor cartridges were pre-incubated overnight in XF24 Calibrant solution (Seahorse Bioscience). After measurement of basal ECAR (extracellular acidification rate, mpH/min) and OCR (oxygen consumption rates, pMoles O_2/min), mitochondrial function was interrogated by the sequential injection of Oligomycin A (1.5 μM), FCCP (0.5 μM), and Antimycin A (10 μM) in combination with Rotenone (2 μM), as described previously [20]. This allowed for the calculation of ATP-linked O_2 consumption, proton leak, maximal respiratory capacity, reserve capacity and non-mitochondrial respiration [20]. Appropriate concentrations for oligomycin and FCCP were determined for each cell type ([21] and Supplementary Fig. 1). Antimycin A was injected at 10 μM to ensure complete inhibition of complex III. After each experiment, the protein concentrations in each well were measured by BioRad DC™ Protein Assay (BioRad, Hercules, CA, USA). All OCR and ECAR values were normalized to protein concentration. ATP-linked OCR, reserve capacity, proton leak, non-mitochondrial OCR, maximum mitochondrial capacity, glycolytic reserve, state apparent, respiratory control ratio (RCR) basal and RCRmax were calculated as described [21,22].

2.4. Mitochondrial:nuclear DNA ratios

Total DNA was isolated from untreated MCF-7 and T47D, cells. Quantitative, real time polymerase chain reaction (QRT-PCR) for mitochondrial DNA content was determined using SYBR Green ROX qPCR Mastermix (Qiagen, Valencia, CA, USA) for measuring the mitochondrial-encoded nicotinamide adenine dinucleotide dehydrogenases: *MTND1* and *MTND2*, neither of which show deletions, duplications, or mutations in studies in human diseases including breast cancer [23], and mitochondrial-encoded cytochrome c oxidase I *MTCO1*, which shows a low mutation rate in cancers, including breast tumors [24], relative to nuclear-encoded gene 18S rRNA [12,21]. Each sample was analyzed in triplicate in the Viia7 Real-Time PCR system (ThermoFisher). Values represent mean fold change ± SEM calculated from the equation $2^{-\Delta\Delta_{CT}}$ and normalized to EtOH values.

2.5. ADP/ATP ratio

The ADP/ATP ratio was measured using the ADP/ATP Ratio Assay Kit (catalog no. MAK135) from Sigma-Aldrich following the manufacturer's instructions. Briefly, cells were seeded (5000/well) in a 96-well, flat-bottom, black plate with clear bottoms (Corning-Costar, Tewksbury, MA, USA) and grown for 48 h in non-serum