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Research Paper

Mitochondrial thiol modification by a targeted electrophile inhibits metabolism in breast adenocarcinoma cells by inhibiting enzyme activity and protein levels

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ABSTRACT

Many cancer cells follow an aberrant metabolic program to maintain energy for rapid cell proliferation. Metabolic reprogramming often involves the upregulation of glutaminolysis to generate reducing equivalents for the electron transport chain and amino acids for protein synthesis. Critical enzymes involved in metabolism possess a reactive thiolate group, which can be modified by certain oxidants. In the current study, we show that modification of mitochondrial protein thiols by a model compound, iodobutyl triphenylphosphonium (IBTP), decreased mitochondrial metabolism and ATP in MDA-MB 231 (MB231) breast adenocarcinoma cells up to 6 days after an initial 24 h treatment. Mitochondrial thiol modification also depressed oxygen consumption rates (OCR) in a dose-dependent manner to a greater extent than a non-thiol modifying analog, suggesting that thiol reactivity is an important factor in the inhibition of cancer cell metabolism. In non-tumorigenic MCF-10A cells, IBTP also decreased OCR; however the extracellular acidification rate was significantly increased at all but the highest concentration (10 μM) of IBTP indicating that thiol modification can have significantly different effects on bioenergetics in tumorigenic versus non-tumorigenic cells. ATP and other adenonucleotide levels were also decreased by thiol modification up to 6 days post-treatment, indicating a decreased overall energetic state in MB231 cells. Cellular proliferation of MB231 cells was also inhibited up to 6 days post-treatment with little change to cell viability. Targeted metabolomic analyses revealed that thiol modification caused depletion of both Krebs cycle and glutaminolysis intermediates. Further experiments revealed that the activity of the Krebs cycle enzyme, aconitase, was attenuated in response to thiol modification. Additionally, the inhibition of glutaminolysis corresponded to decreased glutaminase C (GAC) protein levels, although other protein levels were unaffected. This study demonstrates for the first time that mitochondrial thiol modification inhibits metabolism via inhibition of both aconitase and GAC in a breast cancer cell model.

Abbreviations: ACO, aconitase; BTPP, butyl triphenylphosphonium; DMEM/F12, Dulbecco's modified Eagle's medium/F12; ECAR, extracellular acidification rate; EtOH, ethanol; FBS, fetal bovine serum; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; GAC, glutaminase C; GAM, glutaminase isoform 3; IBTP, Iodobutyl triphenylphosphonium; KGA, kidney-type glutaminase; MB231, MDA-MB231; MitoE, mitochondria-targeted α-tocopherol; MitoQ, mitochondria-targeted ubiquinol; MRM, multiple reaction monitoring; NEM, N-ethylmaleimide; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; PIC, protease inhibitor cocktail; TBAHS, tetrabutylammonium hydrogen sulfate; TPP, triphenylphosphonium

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1. Introduction

One of the hallmarks of cancer cell transformation is the dysregulation of energetic pathways [1]. This process, known as "metabolic reprogramming," involves an increased reliance on glycolysis independent of oxygen levels to not only provide ATP, but also to provide intermediates for new lipids, amino and nucleic acids needed for rapid cell proliferation. In addition, upregulation of glutaminolysis is observed in many diverse cancer types including breast cancer, glioblastoma multiforme, and pancreatic cancer [2–7]. Glutaminolysis is an anaplerotic pathway that replenishes the Krebs cycle by the conversion of glutamine to α-ketoglutarate, thereby providing reducing equivalents to drive electron transport. Glutaminolysis is also important in providing intermediates necessary for the rapid synthesis of amino acids and glutathione [8,9]. In cancer cells, upregulation of glutaminolysis within the mitochondrion allows for generation of ATP with high efficiency, while also generating substrates required in protein synthesis serving as a complement to glycolysis for biomass synthesis and energy production [7].

Cells that are dependent on glutamine for proliferation are termed "glutamine addicted," and this feature presents an attractive therapeutic target, due to the fact that most normal non-tumorigenic cells are not glutamine addicted [10]. A key mediator of glutamine-addiction, the enzyme glutaminase C (GAC), is upregulated as a result of oncogenic gene transcription, and has recently become a widely-recognized target for the development of novel therapeutics [11–13]. However, current GAC inhibitors suffer from relatively low solubility, low-affinity and high toxicity [14–16], making novel inhibitors of glutaminolysis attractive for development. Of note, recent studies have also indicated that GAC possesses a reactive cysteine thiolate, which have been characterized as redox switches for other proteins involved in pathways such as metabolism and cellular proliferation [17,18].

In order to inhibit metabolism within the mitochondrion, it is necessary to design agents which not only enter the organelle, but an also inhibit one or more enzymes within the pathway. An effective method to target compounds to the mitochondrion is by coupling to a lipophilic cationic moiety, such as triphenylphosphonium (TPP), which allows the conjugated product to accumulate within the mitochondrion 100–500 fold based on mitochondrial membrane potential [19,20]. TPP has been used to target compounds such as doxorubicin, coenzyme Q, and electrophiles to the mitochondria to enhance cell death, protect against oxidative stress, or modulate antioxidant responses, respectively [21–23]. Iodobutyl triphenylphosphonium (IBTP) is a mitochondria-targeted electrophile which was originally developed as a research tool to probe the status of reduced thiols in isolated mitochondria, cell culture models, and isolated tissues from animal models of oxidative stress [24,25]. In a previous study, we reported that IBTP affected cellular function by inhibiting Nrf2-dependent antioxidant responses in endothelial cells [26]. More recently, we showed that IBTP inhibited overall metabolism in breast cancer cells after a short (4h) exposure, and also prevented cell migration and adhesion [27]. Due to the soft electrophilic nature of IBTP, this compound forms a covalent adduct with specific cysteinyl thiol groups of proteins, many of which play a central role in cell metabolism [24,25,28,29].

In this study, we sought to determine the mechanisms by which mitochondrial thiol modification inhibits metabolism in MDA-MB-231 (MB231) cells. MB231 cells represent a prototype of metabolically reprogrammed glutamine-dependent cancer cells. These "triple-negative" cells are characterized by lacking estrogen, progesterone, and Her2/neu receptors. In addition, these cells are rapidly proliferating, tumorigenic and metastatic, making them an ideal model for aggressive cancer cell types. Herein, we show that mitochondrial thiol modification blocks energy production by decreasing protein levels and activity, depleting Krebs cycle intermediates and inhibiting oxidative phosphorylation, ultimately decreasing ATP levels. Furthermore, our data suggest that likely mechanisms of IBTP inhibition of metabolism include decreasing enzyme activity and/or decreasing protein levels.

2. Experimental

2.1. Materials

All chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. IBTP and BTPP were prepared as previously described [24]. Anti-TPP antiserum was prepared as previously described [24]. Monoclonal [Abcam (Cambridge, MA), ab156876] or polyclonal [Proteintech (Chicago, IL), 23549-1-AP] anti-glutaminase antibodies were used as indicated in the. Other antibodies used were anti-β-actin [Cell Signaling (Beverly, MA], polyclonal anti-citrate synthetase [Abcam, ab96600], anti-complex IV subunit 2 [Life Technologies (Grand Island, NY); 20E8C12], affinity purified anti-aconitase (a generous gift from Dr. Scott Ballinger at UAB, prepared as described in [30]). The anti-aconitase antibody recognizes both mitochondrial and cytosolic aconitase (ACO2 and ACO1, respectively) isoforms in human breast cancer cells at distinguishable molecular weights (unpublished observations Smith, Zhou, Landar).

2.2. Cell culture and treatments

MDA-MB-231 (MB231) human breast adenocarcinoma cells were a generous gift from Dr. Danny Welch, and were originally obtained from ATCC (Manassas, VA). MB231 were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA). Cultures were maintained in 5% CO2 and humidified in a 37 °C incubator. Cells were plated at a density of 2×10^5 cells/well in 6-well plates and cultured for 48 h, unless otherwise indicated. The medium was changed to low FBS medium (0.5%) for 16 h prior to treatments. Cells were treated with EtOH vehicle, IBTP or BTPP in 2 mL of fresh medium at concentrations and times indicated in each experiment. MCF-10A human breast epithelial cells were a generous gift from Dr. Danny Welch, and were originally obtained from ATCC (Manassas, VA). MCF10A were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) (Mediatech, Manassas, VA) supplemented with BulletKit™ (Lonza; Basel Switzerland). Cultures were maintained in 5% CO2 and humidified in a 37 °C incubator. Cells were plated at a density of 2×10^5 cells/well in 6-well plates and cultured for 48h, unless otherwise indicated. The medium was changed to low supplement medium (0.5%) for 16h prior to treatments. Cells were treated with EtOH vehicle, IBTP or BTPP in 2 mL of fresh medium at concentrations and times indicated in each experiment.

2.3. Immunoblot analysis

Cells were washed with PBS and lysed in Lysis Buffer [10 mM Tris–HCl, pH 7.4, 1% Triton X-100 containing protease inhibitor cocktail (PIC; Roche)]. Soluble proteins were resolved using SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Protein levels were quantified using the method of DC-Lowry (Bio-Rad), and equivalent amounts of protein were loaded. Uniform protein loading was confirmed using Ponceau S staining of membranes and showed no significant differences in protein levels among samples. Membranes were blocked in 5% milk (w/v) in Tris Buffered Saline (pH 7.4) containing 0.05% Tween 20 (TBS-T), and