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Thyroid hormone differentially modulates Warburg phenotype in breast cancer cells

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ABSTRACT

Sustenance of cancer cells in vivo critically depends on a variety of genetic and metabolic adaptations. Aerobic glycolysis or Warburg effect has been a defining biochemical hallmark of transformed cells for more than five decades although a clear molecular basis of this observation is emerging only in recent years. In this study, we present our findings that thyroid hormone exerts its non-genomic and genomic actions in two model human breast cancer cell lines differentially. By laying a clear foundation for experimentally monitoring the Warburg phenotype in living cancer cells, we demonstrate that thyroid hormone-induced modulation of bioenergetic profiles in these two model cell lines depends on the degree of Warburg phenotype that they display. Further we also show that thyroid hormone can sensitize mitochondria in aggressive, triple-negative breast cancer cells favorably to increase the chemotherapeutic efficacy in these cells. Even though the role of thyroid hormone in modulating mitochondrial metabolism has been known, the current study accentuates the critical role it plays in modulating Warburg phenotype in breast cancer cells. The clinical significance of this finding is the possibility to devise strategies for metabolically modulating aggressive triple-negative tumors so as to enhance their chemosensitivity in vivo.

1. Introduction

Thyroid hormones, triiodothyronine (T3) and thyroxine (T4) are key endocrine regulators of metabolic rate in almost all tissues. Their primary mode of action is by binding to the thyroid hormone receptors in the nucleus and by influencing the transcription and expression patterns of target genes. A number of studies have enumerated the various roles of thyroid hormones in tissue growth, brain differentiation and in improving cardiac performance. From the mitochondrial point of view, T3 has been known to increase mitochondrial biogenesis and to enhance mitochondrial function in cells from patients with mtDNA defects [1–5]. Deregulation of direct T3 mitochondrial pathway as well as hypothyroidism in general has been directly implicated in decrease in mitochondrial mass and sarcopenia. T3 was further shown to regulate cytochrome c release in a membrane potential dependent manner [3,5,6]. Thyroid hormones also play a major role in the breast cancer etiology. Hypothyroidism has been recently shown to enhance tumor invasiveness and metastasis development [7]. Besides the genomic effects described above, thyroid hormone can also exert non-genomic effects independent of transcriptional activity [8,9]. Key developments in identifying the putative plasma membrane receptor for the thyroid hormones as well as in elucidating the downstream signaling cascade within cells are emerging only recently. In this paper, we report a connection between mitochondrial metabolism and thyroid hormone action in the context of modulating Warburg phenomenon in breast cancer cells. Originally proposed by Warburg in the 1950s, Warburg phenomenon pertains to the metabolic switch in cancer cells where the cells predominantly utilize the glycolytic pathway (“aerobic glycolysis”) even when oxygen is available (normoxia) [10–12]. Warburg originally hypothesized that aerobic glycolysis stems from mitochondrial dysfunction and this has been confirmed in most, if not all, cancer cells. It is still a matter of debate however, if mitochondrial dysfunction is a necessary pre-requisite for fulfilling Warburg hypothesis of glycolytic up-regulation. By means of high-resolution imaging, flow cytometry and biochemical assays, we show here that thyroid-hormone induced mitochondrial effects depend on the extent of Warburg phenotype displayed by the cancer cells and that mitochondrial dysfunction may not be a necessary condition for the observation of high degree of Warburg phenotype in breast cancer cells.

Abbreviations: T3, triiodothyronine; T4, thyroxine; 2NBDG, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose; DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; TMRM, tetramethyl rhodamine methyl ester; ROS, reactive oxygen species; CCD, charge-coupled device; NADH, nicotinamide adenosine dinucleotide (reduced); PKM2, pyruvate kinase M2 isoform; GLUT-1, glucose transporter isoform 1; HK, hexokinase; PGC-1, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; PI3K, phosphatidylinositol >3-kinases; WM, wortmannin (PI3K pathway inhibitor); PEPc, phosphoenol pyruvate.

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2. Materials and methods

2.1. Cells and reagents

MDA-MB-231 and MDA-MB-453 cells were originally from ATCC and were cultured in DMEM with low glucose (1 g/l) and 10% FBS and antibiotics. MDA231 and MDA435 are tumorigenic in nude mice whereas MDA453 cells are more differentiated-type that do not form tumors in vivo [13,14]. Tri-iodothyronine (T3) and the inhibitors (rotenone, antimycin A and wortmannin) were from Sigma–Aldrich.

2.2. Flow cytometry

Flow cytometry analysis of live cells was done in FACScan flow cytometer (BD Biosciences) with appropriate labeling mix in the cell population: 100 μM 2NBDG (Invitrogen; glucose uptake), 2.5 μM DCFDA (Invitrogen, ROS levels), 200 nM TMRM (Invitrogen, mitochondrial membrane potential). Glut-1 and HK-I levels in the cells were measured by FACS after additional paraformaldehyde fixation, permeabilization and antibody labeling steps using rabbit-anti-Glut 1 (Abcam, 1:100) and rabbit-anti-hexokinase I (Cell Signaling, 1:200) and Alexa-488 conjugated anti-rabbit secondary antibody (Invitrogen, 1:1000).

2.3. Live cell time-lapse imaging

Mitochondrial uptake of TMRM probe in live cells was monitored by plating the cells in Delta T chambers (Bioptechs) and imaging with an inverted epifluorescence microscope (Nikon TE2000, 20×, 0.75NA) fitted with a mercury arc lamp. A 14-bit cooled CCD camera coolSNAP (Roper Scientific) was used for imaging. Data acquisition and analysis were facilitated by QED Invivo software (Media Cybernetics). A typical time-lapse consisted of collecting images (acquisition time ~500 ms/image) every 20 s for about 40 min. Supporting high resolution images were obtained in a two-photon imaging system built around a Leica SP5 microscope platform and a Mai Tai femtosecond pulsed laser (SpectraPhysics).

2.4. Lactate measurements

Serum free medium (100 μl) was added to 24 h cultures of cells (12-well plates) and incubated for 1 h (37 °C, 5% CO_2) for measuring extracellular lactate generated using a commercial Lactate Plus analyzer (Sports Resource, USA).

2.5. Mitochondrial viability (MTT assay)

Twenty microliters of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma–Aldrich) was added to overnight cell cultures and then incubated for 3.5–4 h at 37 °C, 5% CO_2. Cell viability dependent absorbance of the dark blue formazon crystals was measured in a spectrophotometer (wavelength 570 nm).

2.6. Enzyme activity assay

Hexokinase catalyzes the first committed step in glycolysis (conversion of glucose to glucose-6-phosphate). Total hexokinase activity was monitored spectrophotometrically by measuring the rate of NADH generation in a coupled reaction with glucose-6-phosphate dehydrogenase as described [15]. Caspase-3 activity assay was performed using the calorimetric detection kit using appropriate inhibitors according to the manufacturer's instructions (Enzo Lifesciences, NY, USA).

2.7. Immunoblotting

Protein levels were measured by standard immunoblotting assay in SD–PAGE gel using appropriate antibodies for PKM2 (1:1000, Cell Signaling), GLUT-1 (1:400, Cell Signaling) and hexokinase-I (1:1000, Cell Signaling).

2.8. Oxygen consumption measurements

Mitochondrial oxygen consumption [pO_2] data were obtained with a clark-type oxygen microelectrode (Strathkelvin Instruments, Scotland) in a closed-cell respirometry design. The probe electrodes were calibrated with 5% sodiumthiosulfite solution (0% oxygen) and mammalian ringer solution (100% oxygen ~ 207 μmol/l) at 38 °C. All measurements were performed either in phosphate buffered saline or cell culture medium. After collecting basal respiration (in stirred medium) for about 10 min in the substrate-limiting conditions, 20 nM T3 was added through the groove in the microelectrode. Oxygen consumption rate was then calculated from the raw pO_2 data.

2.9. Statistics

Data presented are mean ± SE from at least three independent experiments unless otherwise mentioned. Statistical significance was estimated based on Student's t-test ($p < 0.05$).

3. Results

3.1. Defining experimental Warburg metrics in human breast cancer cells

In order to monitor the genomic and non-genomic effects of thyroid hormone on the Warburg phenotype in the two model breast cancer cell lines, it is imperative to define the key experimental metrics of this phenotype. Fig. 1 summarizes three parameters in MDA231 and MDA453 cell lines that critically define Warburg phenotype: (a) glucose uptake efficiency (b) lactate generation rate and (c) mitochondrial oxygen consumption rate. Glucose uptake rate in these two cell lines were quite different as probed by a fluorescent analog (2NBDG) of glucose (Fig. 1D). To explain the differences, we also compared the expression levels of two relevant proteins, Glut I and hexokinase I (HK-I), in these cells by FACS (Fig. 1A and B) and immunoblotting (data not shown). MDA231 cells displayed a higher expression of GLUT-1 and lower HK-I expression as compared with MDA453 cells. In addition to the HK-I expression in the cells, we also measured the enzymatic activity of hexokinase (Fig. 1C). In accordance with the HK1 expression levels, MDA453 cells also displayed the higher HK activity thereby explaining the 2NBDG uptake pattern. A measurement of 2NBDG uptake in the cells in the presence of 20 mM glucose as a competitive inhibitor (k_D of HK-I is ~20 mM) showed that MDA453 cells were more sensitive to changes in glucose availability. Despite this acute glucose dependence in these cells, the extracellular lactate generated in MDA 453 was about threefold lower than that in the aggressive breast cancer cell line MDA 231 (Fig. 1E). This could be due to the fact that the well-differentiated, MDA453 cells probably utilize mitochondrial pathway more efficiently and display lesser "*Warburg phenotype*" than MDA231 cells. This was further confirmed when we measured the oxygen consumption rate in these two cell lines (Fig. 1F) where MDA 453 cells displayed a significantly higher respiration rate as compared with the MDA231 cells. A key enzyme in the glycolytic complex that is frequently altered during tumorigenesis is the pyruvate kinase. The M2 isoform of this enzyme (PKM2) has been recently shown to be

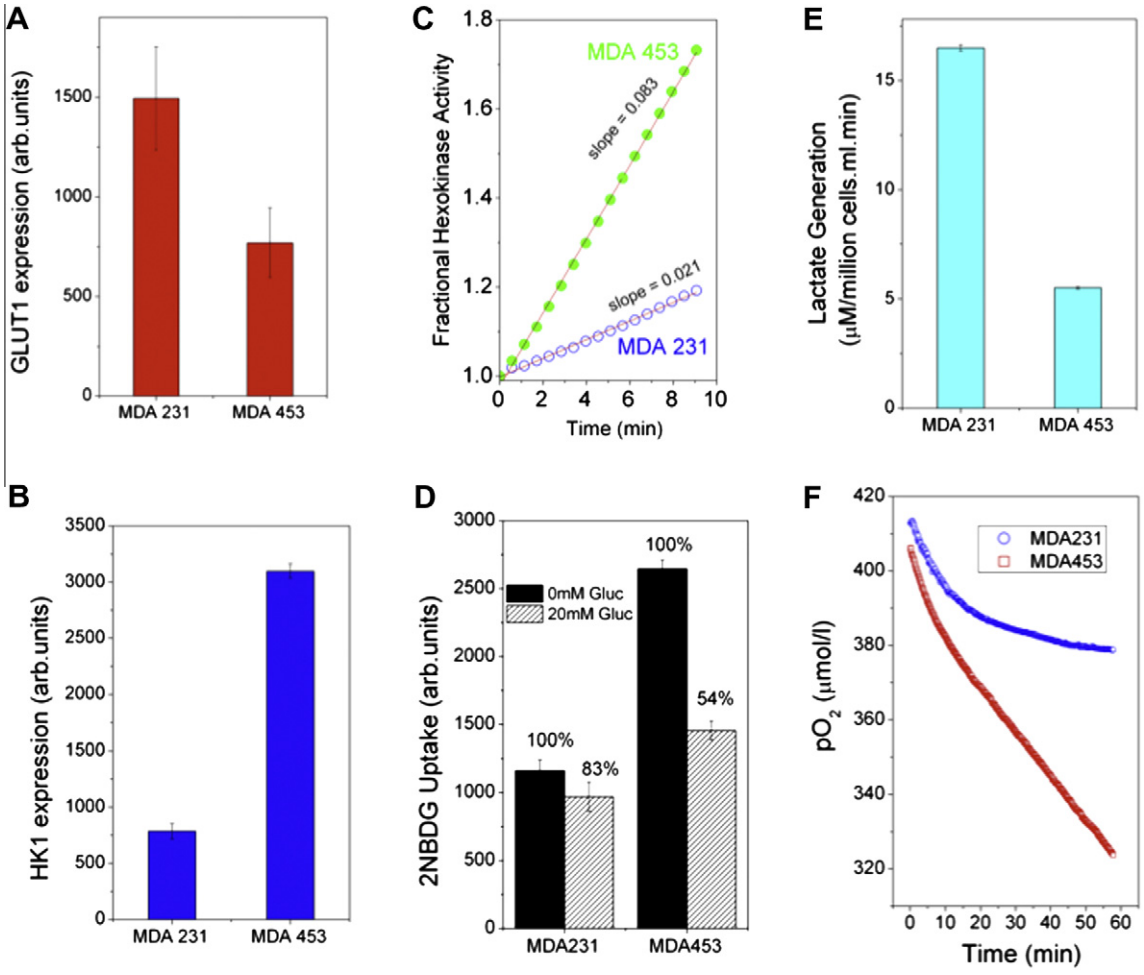

Fig. 1. Defining experimental metrics for monitoring Warburg phenotype in human breast cancer cells. (A and B) Basal expression levels of glucose transporter (GLUT 1) and hexokinase I (HK I) in MDA231 and MDA453 cell lines. Data (mean ± SE) from five independent flow cytometry experiments. (C) Hexokinase-I specific enzyme activity measured in the parental breast cancer cells by monitoring G-6-phosphate coupled generation of NADH absorbance (A) at 340 nm. Linear plots with corresponding slopes ($\alpha = \Delta A/\text{min}$) are shown for each cell line. See Section 2 for more details. (D) Glucose uptake in MDA231 and MDA453 cells as measured by flow cytometry using the fluorescent analog of glucose (2NBDG). Competitive inhibition with 20 mM glucose shows a pronounced dependence on glucose in MDA453 cells. The percentage values indicate the fluorescence changes with respect to control (no glucose) cells. (E) Also shown are the mean lactate generation ($n = 5$) in the two cell lines studied; and (F) comparison of mitochondrial oxygen consumption in MDA231 and MDA453 cell lines.

vital for sustaining Warburg phenotype in tumor cells as was verified in our earlier studies [Supplementary Fig. S.1]. Together these data set the basis of vastly different Warburg phenotype in the two cell lines under study.

3.2. T3 treatment elicits differential mitochondrial response in human breast cancer cells

We next investigated the typical time scales in which T3 could potentially elicit a change in mitochondrial metabolism. After equilibrating the cells with the TMRM probe for 20 min, three-dimensional time-lapse (xyzt) images were obtained by two-photon excitation microscopy. A single stimulus of 10 nM T3 was given after the first set of images and the subsequent membrane potential kinetics was measured for 30 min. As shown in Fig. 2B, T3 induced robust changes in mitochondrial membrane potential in both the cell lines studied. Flow cytometry measurements (cell population studies) under these conditions yielded similar results of T3 induced changes in mitochondrial membrane potential -sensitive fluorescence thereby confirming the results obtained from single cell imaging assays. Mitochondrial oxygen consumption data (Fig. 2C) further confirmed that T3 could directly influence mitochondrial metabolism in both the cell lines. Overall, the largest changes in mitochondrial response were observed within 60 min of T3 treatment [Fig. 2C and Supplementary Fig. S.4]. Among the cancer metabolism-specific signaling pathways, PI3K/Akt pathway has been known to promote aerobic glycolysis (Warburg effect) in cancer cells [10,16]. We hypothesized that the two breast cancer cells under investigation could manifest the differences in their Warburg phenotype in the corresponding thyroid hormone responses as well. The rationale behind this hypothesis is to confirm if T3 has any role in integrating mitochondrial responses and this key signaling pathway modulation in these cells. As demonstrated in Fig. 2B and C, T3 induced changes in mitochondrial membrane potential dependent fluorescence in live cells are indicative of real-time mitochondrial responses. Since the membrane-potential dependent accumulation of the TMRM probe depends on the basal mitochondrial metabolism, the rate of probe uptake in live cells could shed light on these subtle metabolic changes in real time. With this reasoning, we monitored the rate of TMRM uptake in the two breast cancer cells in the presence of PI3K inhibitor (100 nM wortmannin, WM) or 10 nM T3 or both. Fig. 2D and E shows the average kinetic profiles from three independent experiments. First, MDA231 cells displayed the most

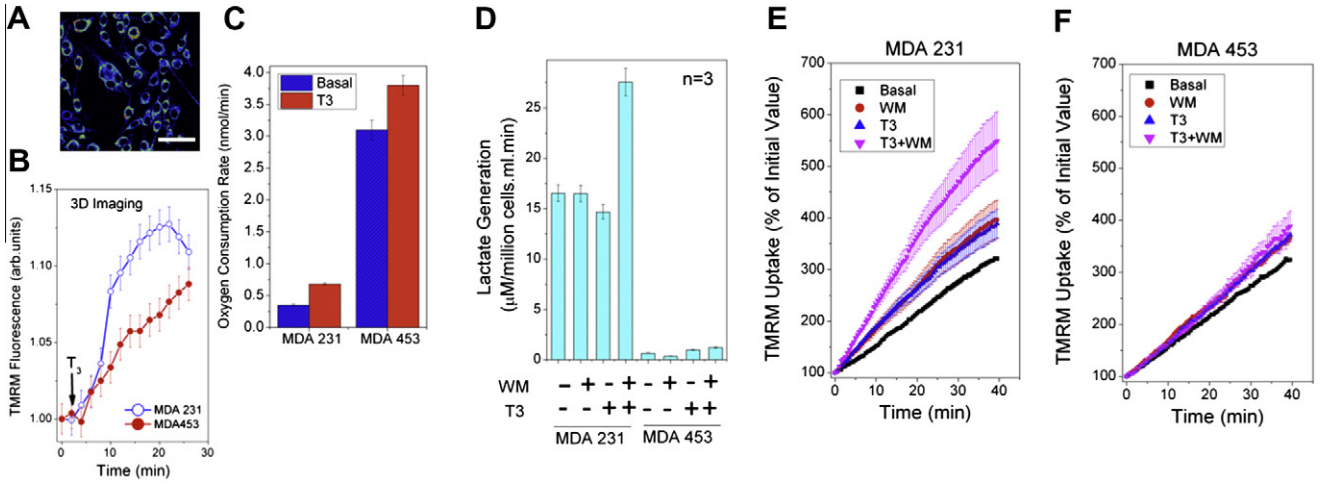

Fig. 2. T3-induced mitochondrial responses in breast cancer cells. Basal mitochondrial membrane potential as measured by three-dimensional (volumetric) multiphoton fluorescence imaging (A and B). Representative image of MDA231 cells shown in (A) reveals a clear mitochondrial localization of the TMRM probe thereby confirming that all fluorescence changes reported are of mitochondrial origin. Scale bar = 20 μm. After 20 min of TMRM incubation, 3D time-lapse imaging (xyzt) was carried out in these cells. Fractional change in membrane potential was measured after injecting a single stimulus of 10 nM T3 into the live cell chambers (B). Supporting population measurements by flow cytometry analysis of TMRM fluorescence in live cells indicate a clear difference in basal mitochondrial membrane potential in both the cell lines (data not shown). (C) Oxygen consumption measurements in live cells show a clear induction of mitochondrial respiration upon T3 stimulus (20 nM T3, 40 min) in MDA231 and MDA453 cells. PI3K pathway modulation of Warburg phenotype in MDA231 and 453 cells as probed by lactate generation measurements (D) and TMRM uptake kinetics (E and F). Cells were preincubated with either 100 nM wortmannin (WM, PI3K inhibitor) or 10 nM T3 or both as shown for 1 h before obtaining live cell time-lapse image data set. Images were analyzed by Imagej Software. Data shown are mean ± SE from three independent experiments.

robust changes in fluorescence during TMRM uptake in the presence of WM and T3. Interestingly, when the cells were treated with T3 and WM together, there was even further increase in the uptake rate thereby revealing T3-induced enhancement in mitochondrial function [Supplementary Table 1]. This feature was observed in glucose uptake kinetics rate (data not shown). On the other hand, MDA453 cells did not show this synergistic effect thereby suggesting that T3 has differential effect on modulating mitochondrial metabolism in breast cancer cells that have distinctly different Warburg phenotype.

3.3. Long-term T3 treatment enhances chemotherapy response in aggressive breast cancer cells by modulating mitochondrial biogenesis

Having confirmed that T3 could transiently (non-genomic) modulate mitochondrial metabolism in aggressive breast cancer cells, we then sought to monitor the long-term effects of T3 treatment in modulating the bio-energetic signatures in the two breast cancer cell lines. High resolution immunofluorescence imaging and immunoblotting revealed a significant increase in PKM2 levels upon T3 treatment in MDA231 cells and a modest increase in MDA453 cells (Fig. 3). As can be seen from these images, there are indications of both cytosolic and nuclear localization of PKM2 antigen in the cell lines. No attempts were made to stratify the image dataset for analysis of subcellular localization of PKM2 within the scope of this paper. PKM2 has been earlier shown to play a role in biosynthetic pathways by regulating key transcription factors [17]. In order to understand the implication of mitochondrial biogenesis in breast cancer cells that display Warburg effect, we sought to clarify the role of T3 in modulating mitochondrial metabolism and chemotherapeutic response in the two cell lines studied. The rationale for this experiment is to address two recent findings in the literature namely: (a) thyroid hormone increases mitochondrial metabolism by partial uncoupling of oxidative phosphorylation network [5] and (b) mitochondrial uncoupling induces chemoresistance in cancer cells [18]. In our experimental design, we monitored the effects of a classical chemotherapy agent, doxorubicin on the mitochondrial metabolism/caspase-3 activity in the two cancer cell lines in the presence/absence of 10 nM T3 (48 h treatment). We first confirmed that both MDA231 and MDA453 cell lines have similar levels of the thyroid hormone receptors by immunofluorescence studies [Supplementary Fig. S.2]. Fig. 4A shows the mitochondrial OxPhos complex protein subunit levels in the two cell lines after 48 h of doxorubicin treatment with or without T3. As can be seen, doxorubicin treatment increased the expression levels of almost all the complex subunits and T3 addition further accentuated this scenario in MDA231 cells. On the other hand MDA453 cells did show an opposite trend. In order to verify if the observed increase in OxPhos protein levels in MDA231 cells also correlated with the corresponding increase in mitochondrial mass, flow cytometry measurements were done using a mitochondrial-specific probe, mitotracker green (which accumulates in the mitochondria independent of mitochondrial membrane potential). Fig. 4B confirms that there is indeed a significant increase in mitochondrial biogenesis in MDA231 cells and a modest increase in MDA453 cells without significant change in cell proliferation index [Supplementary Fig. S.3]. Under the same conditions, concomitant increases in the net lactate generated and the reactive oxygen species levels were also observed only in MDA231 cells (Fig. 4C and D). Finally the cell viability measurements (Fig. 4E) and caspase-3 activity (Fig. 4F) revealed that T3 treatment only in MDA231 cells directly leads to an increased mitochondrial involvement in committing cells to caspase-dependent cell death in aggressive breast cancer cell lines that display larger Warburg effect.

4. Discussion

The major findings of this paper are: (a) thyroid hormone induces robust mitochondrial metabolism in the two model breast cancer cell lines studied; (b) non-genomic (short term; ~1 h) and genomic (long term, ~48 h) thyroid hormone effects seem to depend on the degree of Warburg phenotype displayed by these cell lines and (c) thyroid hormone exacerbates doxorubicin-induced cell death potency only in aggressive MDA231 cells but not in MDA453 cells. We first compared the effects of T3 in

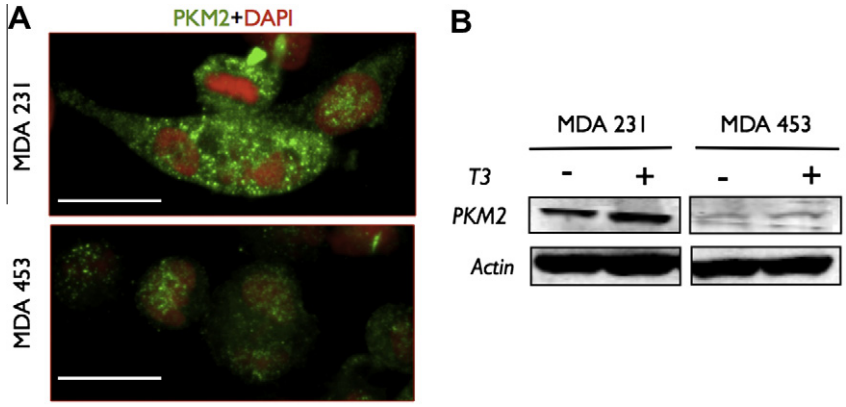

Fig. 3. T3-induced up-regulation of tumor-M2 isoform of pyruvate kinase (PKM2). (A) Representative single cell immunofluorescence images of MDA231 and MDA453 cells probed for pyruvate kinase M2 isoform (green), counterstained with nuclear DAPI (red). Scale bars = 10 μm. (B) Western blot measurements further confirm the expression of PKM2 and subsequent upregulation of PKM2 after 48 h of 10 nM T3 treatment in MDA231 cells. MDA453 cells showed a slight increase in PKM2 upon T3 treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

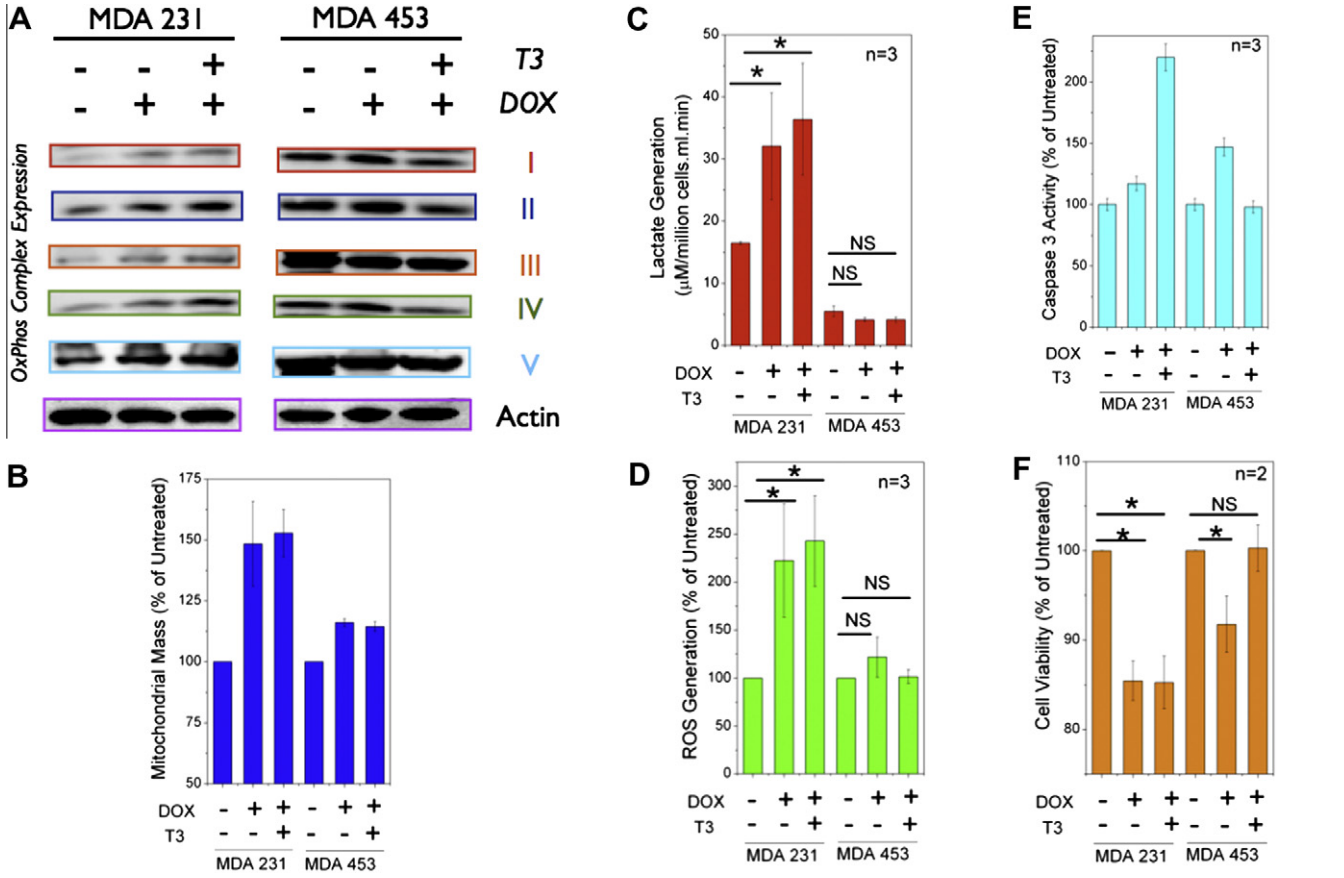

Fig. 4. Long-term treatment (48 h) with T3 sensitizes mitochondrial metabolism in aggressive cancer cells to enhance chemotherapeutic efficacy. (A) Expression levels of mitochondrial oxidative phosphorylation (OxPhos) complex subunits in MDA231 and MDA453 cells after 48 h of 50 nM doxorubicin (DOX) in the presence/absence of 10 nM T3. (B) Concomitant increase in mitochondrial mass was measured by labeling the cells with a mitotracker green dye and analyzed by FACScan as described in Section 2. Metabolic responses under the same conditions were monitored by measuring the extracellular lactate concentration (C) and reactive oxygen species levels during metabolism (D). Apoptosis rate was measured by monitoring caspase-3 activity (E) and cell viability by MTT assay (F). Data shown are mean ± SE from three independent experiments. Statistical significance was estimated for dataset with a difference in mean values is within $p < 0.05$ threshold. NS = not significant.

modulating the bioenergetic profiles by laying a foundation for monitoring the experimental manifestations of Warburg phenotype in breast cancer cells as exemplified by glucose uptake, lactate generation and mitochondrial oxygen consumption rate which define the Warburg effect in these two cell lines. Our observations that thyroid hormone elicits robust mitochondrial responses (oxygen consumption rate and mitochondrial membrane potential) within 45–60 min of stimulus are in accordance with the recent reports of non-genomic actions of thyroid hormones through putative membrane receptors [Supplementary Fig. S.4]. We further probed these non-genomic effects by monitoring the real-time uptake kinetics of mitochondrial membrane potential probe in live

cells (Fig. 2E and F). Refetoff and colleagues had recently demonstrated that cytosolic action of thyroid hormone in fibroblasts – by facilitating direct interaction between PI3K-regulatory subunit p85α and TRβ1 – could lead to the induction of glucose transporter GLUT1 as well lactate exporter MCT4 [8]. Both the breast cancer cell lines showed dependence on PI3K pathway inhibition (wortmannin) as revealed in an increase in extracellular lactate. However, only MDA231 cells showed a significant increase (~20%) in glucose uptake and a concomitant increase in membrane potential which again is in agreement with our interpretation of increase in glucose uptake in response to mitochondrial enhancement in these cells rather than mitochondrial dysfunction [19]. Warburg hypothesis in its original form, directly equates the increase in glycolytic activity with mitochondrial dysfunction [11]. Our findings uncover an alternative scenario where glycolytic activity could also arise from an increase in mitochondrial respiratory demand as observed in situations of "*substrate channeling*" where mitochondrially associated glycolytic enzymes up-regulate their activity in response to mitochondrial demand [19].

In the presence of a chemotherapy agent, doxorubicin, an increased mitochondrial biogenesis as evidenced by mitochondrial mass and the OxPhos complex expression as well as increased caspase-3 activity/decreased cell viability in MDA231 cells all point out to the scenario that thyroid hormone selectively sensitized the mitochondrial pathway to enhance chemotherapeutic efficacy in this cell line. Even though MDA231 and MDA453 cells express same levels of thyroid hormone receptors, it is interesting to note that only MDA231 cells showed such robust mitochondrial activity upon T3 treatment [Supplementary Fig. S.5]. The observed T3 -induced bioenergetic alterations (mitochondrial and glycolytic) in aggressive breast cancer cells could stem from either an increased mitochondrial biogenesis or by partial uncoupling as recently reported [18]. However, in contrast to this earlier study where partial uncoupling leads to chemoresistance, our data show that thyroid hormone acts in favor of improved chemosensitivity in aggressive cancer cells. This could be reasoned by the plausible enhancement in mitochondrial pathway which in turn triggers intrinsic apoptotic signals as evidenced by a concomitant increase in caspase-3 activity. Finally the observation that long-term treatment with T3 (48 h) could induce an up-regulation (~25%) of the glycolytic enzyme PKM2 in MDA231 cells can have two possible physiological consequences. As shown in a number of recent studies, the M2 isoform of the pyruvate kinase (mainly dimeric PKM2) is selectively overexpressed in cancer cells [17]. The biological significance of PKM2 in the context of Warburg phenotype in cancer cells is that the dimeric PKM2 can evade the phosphoenolpyruvate to pyruvate conversion step thereby allowing rapid proliferation of cancer cells. In the same context, thyroid hormone also has been shown to play an important role in mitochondrial biogenesis mediated by PGC-1α [3]. It is possible that these two effects could be acting in concert in aggressive cancer cells (MDA 231) displaying high Warburg effect but not in the less tumorigenic MDA453 cells owing to the absence of PKM2 in the latter. In conclusion, our data clearly show that thyroid hormone sensitizes mitochondrial metabolism in aggressive triple-negative breast cancer cells displaying high Warburg effect. Since there is no known, efficacious chemotherapy modality available for the tripe-negative breast cancers (as in MDA231 cells) to-date, it is imperative to devise alternative metabolic routes for improving the efficiency of tumor killing in these aggressive cancers. We believe our findings can render an alternative strategy for targeting chemosensitivity in hormone-responsive breast cancer cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.09.024.

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