Biochimica et Biophysica Acta 1807 (2011) 726–734

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Inhibition of fatty acid oxidation by etomoxir impairs NADPH production and increases reactive oxygen species resulting in ATP depletion and cell death in human glioblastoma cells ☆

Lisa S. Pike [a], Amy L. Smift [a], Nicole J. Croteau [b], David A. Ferrick [a], Min Wu [a,*]

[a] Seahorse Bioscience, North Billerica, MA 01862, USA
[b] Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA

ARTICLE INFO

Article history:
Received 30 June 2010
Received in revised form 22 October 2010
Accepted 27 October 2010
Available online 12 November 2010

Keywords:
Fatty acid oxidation
NADPH
ROS
Oxidative stress
Mitochondrial dysfunction

ABSTRACT

Normal differentiated cells rely primarily on mitochondrial oxidative phosphorylation to produce adenosine triphosphate (ATP) to maintain their viability and functions by using three major bioenergetic fuels: glucose, glutamine and fatty acids. Many cancer cells, however, rely on aerobic glycolysis for their growth and survival, and recent studies indicate that some cancer cells depend on glutamine as well. This altered metabolism in cancers occurs through oncogene activation or loss of tumor suppressor genes in multiple signaling pathways, including the phosphoinositide 3-kinase and Myc pathways. Relatively little is known, however, about the role of fatty acids as a bioenergetic fuel in growth and survival of cancer cells. Here, we report that human glioblastoma SF188 cells oxidize fatty acids and that inhibition of fatty acid β-oxidation by etomoxir, a carnitine palmitoyltransferase 1 inhibitor, markedly reduces cellular ATP levels and viability. We also found that inhibition of fatty acid oxidation decreases nicotinamide adenine dinucleotide phosphate (NADPH) levels and the reduced glutathione (GSH) content and elevates intracellular reactive oxygen species. These results suggest that modulation of fatty acid oxidation controls the NADPH level. In the presence of reactive oxygen species scavenger tiron, however, ATP depletion is prevented without restoring fatty acid oxidation. This suggests that oxidative stress may lead to bioenergetic failure and cell death. Our work provides evidence that mitochondrial fatty acid oxidation may provide NADPH for defense against oxidative stress and prevent ATP loss and cell death. This article is part of a Special Issue entitled: Bioenergetics of Cancer.

1. Introduction

Otto Warburg [1] first described the high rate of glucose conversion to lactate in cancer cells, compared to their normal counterparts, despite the presence of ample oxygen, known as the Warburg effect, more than 80 years ago. It is now recognized that this altered cellular metabolism is a result of oncogenic transformation and has emerged as a hallmark of cell transformation to a cancer phenotype [2,3]. Genetic alterations in multiple signaling pathways, predominantly the PI3K–AKT pathway and Myc mediated pathways, allow cancer cells to constitutively take up and metabolize glucose and glutamine in excess of their bioenergetic and biosynthetic needs [3–5].

In normal differentiated cells, mitochondrial respiration burns glucose, fatty acids and amino acids from dietary sources with oxygen, through oxidative phosphorylation, to produce usable energy in the form of ATP. Mitochondrial oxygen consumption reflects both the activities of the electron transport chain (ETC) and the tricarboxylate (TCA) cycle within the mitochondria. It also reflects the availability and types of bioenergetic substrates supplied from the cytosol, that is, the cellular context in which mitochondria function. Unlike mitochondria in normal differentiated cells which function as primarily as bioenergetic organelle, in addition to producing ATP, mitochondria of cancer cells as well as proliferating cells are also biosynthetic organelles for lipid and amino acid production, both of which are needed to support cellular replication [4]. Bioenergetic and biosynthetic activities are coordinated through the shared TCA cycle in the mitochondria. Bioenergetically, the TCA cycle extracts energy from substrates into the reducing equivalent of NADH, which is then oxidized in the electron transport chain, reducing molecular oxygen to water and fueling oxidative phosphorylation for ATP production. Biosynthetically, TCA cycle intermediaries such as citrate and malate exit the cycle to provide precursors for lipid and amino acid biosynthesis resulting in a truncated TCA cycle. ATP production and biosynthesis of building blocks required to sustain cellular function and cell viability are functionally coordinated by interlocking regulatory mechanisms that control the rates of both the electron transport (oxygen consumption) and the TCA cycle. In addition, the

☆ This article is part of a Special Issue entitled: Bioenergetics of Cancer.

* Corresponding author. Tel.: +1 978 671 1655; fax: +1 978 671 1611.
E-mail address: mwu@seahorsebio.com (M. Wu).

0005-2728/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2010.10.022

TCA cycle can also produce the reducing equivalent NADPH, a potent non-enzymatic antioxidant in the mitochondria that is crucial to cell viability [3].

During mitochondrial respiration, electrons leak out of the electron transport chain and generate ROS. ROS include sequential intermediates generated by univalent reductions of molecular oxygen through stepwise electron transfer from the superoxide radical (O_2^-) to hydrogen peroxide (H_2O_2) and then the hydroxyl radical (OH), which can finally be reduced to water. ROS production increases when excessive nutrients become available to the cells, and these ROS can either promote cell proliferation or potentially cause oxidative damage.

Fatty acids are synthesized from excessive dietary glucose and stored as lipids (triglyceride) in the cell, predominantly in adipose and liver tissues. They can be released into the blood stream and taken up and utilized by other tissues, including skeletal and cardiac muscles where fatty acids are degraded in the mitochondria by β-oxidation and oxidative phosphorylation to produce ATP. In contrast to normal cells, *de novo* fatty acid synthesis occurs at high rates in tumor tissues [6,7].

In this study, we investigated the role of fatty acid oxidation in cancer cell metabolism, growth and survival. We found that an essential function of fatty acid oxidation in cancer cells is to provide the reducing equivalent NADPH to maintain the antioxidant system against oxidative stress and thereby promote cell viability.

2. Material and methods

2.1. Reagents

Etomoxir, FCCP, dihydroethidine, tiron, sodium palmitate, glucose and L-glutamine were obtained from Sigma (St. Louis, MO, USA). Oligomycin was obtained from EMD (San Diego, CA, USA). Fatty acid ultra-free bovine serum albumin (BSA) was obtained from Roch Diagostics (Indianapolis, IN, USA). All compounds were prepared according to the manufacturers' instructions.

2.2. Cell line and cell culture

Human SF188 glioblastoma cells were obtained from the University of California at San Francisco Brain Tissue Bank. The cells were initially maintained MEM containing 5.5 mM glucose and 2 mM glutamine (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) as recommended. When we adapted them into DMEM (Invitrogen) containing 25 mM glucose and 6 mM L-glutamine supplemented with 10% FBS, the cells adopted a much more rapid proliferation rate and a much more oxidative metabolic phenotype, which will be published elsewhere. The rapid growing and oxidative SF188 cells were used in this study. C2C12 myoblast was obtained from American Type Culture Collection (Manassas, VA 20108). They were maintained in DMEM containing 25 mM glucose, 4 mM L-glutamine and 1 mM pyruvate supplemented with 10% FBS. Both cell lines were maintained in a humidified incubator at 37 °C/10% CO_2 until they reached ~70%–80% confluence and subcultured into T flasks or plated on microplates for assays. Growth medium was refreshed every two days.

C2C12 myoblasts were differentiated into myocytes prior to an assay. They were plated at 1.0×10^4 cells/well and incubated for 48 hours prior to switching to differentiation medium, which was DMEM supplement with 1% horse serum, for 3 days allowing myocyte differentiation.

2.3. Measurement of cellular oxygen consumption rate and extracellular acidification rate using the XF Extracellular Flux Analyzer

Cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were used to monitor fatty acid oxidation and glycolysis in real time when the appropriate substrates were added to or included in assay medium as indicated in the relevant experiments. Details of assaying glucose, glutamine and fatty acid metabolism will be published elsewhere.

Oxygen consumption rate and extracellular acidification rate measurements were performed using the Seahorse XF24 Extracellular Flux Analyzer as described [8]. SF188 cells were plated in XF24 V7 cell culture plates (Seahorse Bioscience, North Billerica, MA, USA) at 2.0×10^4 cells/well and incubated for 24 hours in a 37 °C/10% CO_2 incubator. Cells were equilibrated with bicarbonate-free low buffered DMEM medium without any supplement or supplemented with glucose and/or glutamine as indicated in each experiment and incubated in a 37 °C non CO_2 incubator for 45 minutes immediately before XF assay. Substrates or perturbation compounds were prepared in the identical assay medium as in the corresponding well and were injected from the reagent ports automatically to the wells at the time as indicated.

2.4. Preparation of palmitate–BSA conjugates

Palmitate was conjugated to BSA when both components were solubilized. Briefly, odium palmitate was solubilized in 150 mM sodium chloride by heating up to 65 °C in a water bath. BSA was dissolved in phosphate buffered saline (PBS) (Invitrogen) and warmed up to 37 °C. with continuous stirring. Solubilized palmitate was added to BSA at 37 °C with continuous stirring. The conjugated palmitate–BSA was aliquoted and stored at −20 °C. Palmitate–BSA conjugate was used to assess oxidation of exogenous fatty acid. Detailed protocol will be published elsewhere.

2.5. Proliferation assay

The number of viable cells per well were determined after trypan blue staining. Cells were plated at 1×10^5 cells/well in 12-well tissue culture plate. After 24, 48 or 72 hours, cells were trypsinized and suspended in medium. Cells were counted using a ViCell Counter (Beckman Coulter, Fullerton, CA, USA).

2.6. Cellular ATP measurement

Luminescence was used to determine the relative ATP level of cells exposure to etomoxir. Cells were plated in white 96 well tissue culture microplates at 2×10^4 cells/well and incubated in 37 °C/10% CO_2 incubator for 24 hours. The cells were exposed to etomoxir in bicarbonate-free DMEM medium containing 25 mM glucose and 6 mM glutamine for 45 minutes in a 37 °C non CO_2 incubator before being assayed with CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA). Luminescent intensity from each well was measured using a FLUOstar Optima plate reader (BMG Labtech, Durham, NC, USA).

2.7. Calcein AM cell viability assay

The relative viability was determined using the Calcein AM assay. Cells were plated in black 96 well tissue culture microplates at 2×10^4 cells/well and incubated in the 37 °C incubator for 24 hours in parallel with the cells for ATP assay. Cells were treated with etomoxir in the identical assay medium for the ATP assay for 45 minutes, and the relative viabilities were analyzed using the cell-permeable fluorochrome calcein AM (Invitrogen). Cells were incubated with1 μM Calcein AM for 30 minutes before analysis on a FLUOstar Optima plate reader.

2.8. Measurement of intracellular ROS

Fluorescence image analysis was used to determine the relative levels of ROS in response to etomoxir. Cells were harvested and

suspended at 1×10^6 cells/ml in phosphate buffered saline (PBS) (Invitrogen) supplemented with 25 mM glutamine and 6 mM L-glutamine. They were and treated with 1 mM etomoxir for 25 minutes. The relative levels of intracellular ROS were analyzed using the cell-permeable superoxide-sensitive fluorochrome dihydroethidine. Cells were incubated with dihydroethidine (10 μM) for 15 minutes at 37 °C before analysis using a BD FACSCanto II flow cytometry (BD Biosciences, San Jose, CA, USA).

2.9. Cellular GSH assay

GSH sensing luminescence was used to determine the relative glutathione level of cells exposed to etomoxir. Cells were plated in white 96 well tissue culture microplates at 4×10^3 cells/well and incubated in 37 °C/10% CO_2 incubator for 24 hours. The cells were exposed to etomoxir in bicarbonate-free DMEM medium containing 25 mM glucose and 6 mM glutamine for 30 minutes in a 37 °C non CO_2 incubator and the relative levels of glutathione were analyzed using GSH-Glo™ Glutathione Assay (Promega). Luminescent intensity of each sample was measured using a FLUOstar Optima plate reader.

2.10. Measurement of cellular NADPH

NADPH sensing fluorescence was used to determine NADPH level of cells treated with etomoxir. Cells were harvested and suspended at 4×10^6 in DMEM supplemented with 25 mM glutamine and 6 mM L-glutamine. They were treated with etomoxir and lysed before NADPH detection. NADPH cycling reaction was performed using an Amplite™ Fluorimetric NADPH Assay (AAT Bioquest, Sunnyvale, CA, USA) before analysis on a FLUOstar Optima plate reader.

2.11. Statistical analysis

Data are presented as mean ± SD. Statistical analysis was performed using Student's *T*-test, with $p<0.05$ considered significant.

3. Results

3.1. Fatty acid oxidation in SF188 glioblastoma cells

Fatty acids taken up from extracellular environment or released from intracellular lipid storage in the cytoplasm of normal cells can enter the mitochondria for β-oxidation. Fatty acids are converted to Acyl CoA, which is then transported into the mitochondria by the long chain fatty acid transporter CPT-1. Once inside the mitochondria, Acyl CoA is degraded to Acetyl CoA via β-oxidation. Acetyl CoA enters the TCA cycle where it is further broken down to CO_2, producing the reducing equivalent NADH which fuels oxidative phosphorylation or the reducing equivalent NADPH (Fig. 1A). Early studies indicate that cancer cells can take up and oxidize exogenous fatty acids from the extracellular environment [9,10]. Whether cancer cells perform oxidation of endogenous fatty acids, which they synthesize at a high rate, remains unclear. Monitoring cellular oxygen consumption provides an alternative and easy means to assess oxidation of both endogenous and exogenous fatty acids. Oxidation of exogenous fatty acid can be assessed by monitoring cellular oxygen consumption upon addition of fatty acid such as palmitate. We observed increased OCR of the cells in medium containing 5.5 mM glucose and 0.5 mM carnitine upon addition of palmitate (200 μM), which was blunted by subsequent etomoxir addition, suggesting that they can take up and oxidize exogenous fatty acids (data not shown). Importantly, oxidation of intracellular (endogenous) fatty acids by cells can be determined by monitoring the OCR of cells in medium containing no exogenous glucose or glutamine and by using specific inhibitors of fatty acid oxidation.

To determine if SF188 cells oxidize endogenous fatty acid, we first determined the oxygen consumption rate of the cells in medium containing no exogenous substrate (glucose or glutamine). We found that these cells consumed significant amounts of oxygen, ~250 pmol/min, (Fig. 2A), in the absence of exogenous substrate suggesting oxidation of endogenous substrate. Supplementing glutamine in assay medium, regardless of presence or absence of glucose, raised the OCR by about 150 pmol/min, again confirming that glutamine is oxidized by the cells (Fig. 1B).

Transport of long chain fatty acids into mitochondria through CPT-1 on the mitochondrial outer membrane is the main controlling step of fatty acid oxidation. Therefore, blocking the transport of fatty acids into the mitochondria (in the absence of exogenous substrates) should decrease the oxygen consumption rate of the cells if endogenous fatty acid is oxidized. This can be accomplished with etomoxir, which binds irreversibly to the CPT-1 transporter and inhibits fatty acid oxidation. To confirm that oxidation of endogenous fatty acid contributed to the observed oxygen consumption rate in the absence of exogenous substrates, we assessed whether etomoxir

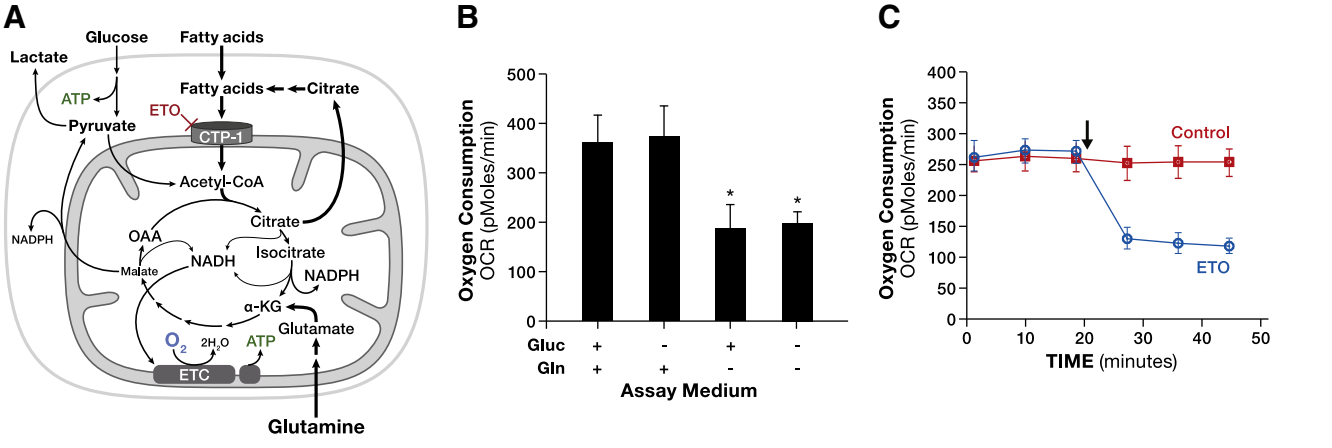

Fig. 1. Human glioblastoma SF188 oxidizes endogenous fatty acid. Oxidation of endogenous fatty acid was determined by measuring oxygen consumption rate using XF24 Extracellular Flux Analyzer. A. Illustration of fatty acid oxidation and synthesis in mitochondria. Fatty acids enter the mitochondria for β-oxidation to Acetyl CoA. Acetyl CoA enters the TCA cycle generating NADH that is oxidized by the ETC consuming oxygen or producing NADPH as indicated. Endogenous fatty acids can be synthesized *de novo* from glucose or glutamine. Glucose is converted to pyruvate via a series of catabolic reactions. Pyruvate is either converted to lactate or enters mitochondria to be converted to Acetyl CoA. Acetyl CoA enters the TCA cycle and exit as citrate, which is then exported to cytosol for the synthesis of fatty acids. Glutamine enters the TCA cycle after conversion to α-ketoglutarate (α-KG) and exits as malate and citrate into the cytosol for fatty acid synthesis. B. Glucose (25 mM) plus glutamine (6 mM), glutamine (6 mM), glucose (25 mM) or no substrate were supplemented in assay medium. C. Etomoxir (ETO, 1 mM) was added to the cells after baseline measurement in assay medium containing no exogenously added glucose or glutamine. The arrows indicate the time when ETO was added to the cells.

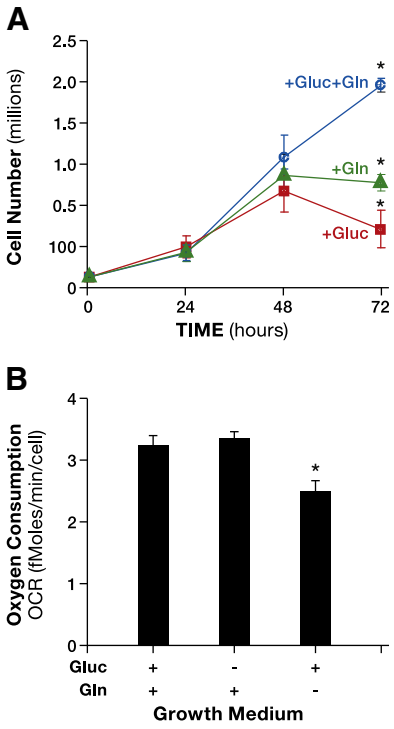

Fig. 2. Glutamine as the carbon source for endogenous fatty acids. A. Glutamine is required for cell growth and survival. Cells were cultured in DMEM medium supplemented with glucose (25 mM) plus glutamine (6 mM), glucose (25 mM) or glutamine (6 mM) in a 12-well microplate, and the number of viable cells under each condition was determined at 24, 48 and 72 hours post plating. Mean ± SD. ($n = 3$; $^*p < 0.05$). B. Eliminating glutamine from growth medium (glucose only) for 48 hours led to decreased endogenous fatty acid oxidation in these cells. Cells were cultured in DMEM medium supplemented with glucose (25 mM) plus glutamine (6 mM), glucose (25 mM) or glutamine (6 mM) in an XF microplate for 48 hours. The growth medium was replaced with assay medium containing no glucose or glutamine before measuring oxygen consumption rate. The OCRs were normalized against the number of viable cells being measured. Mean ± SD. ($n = 3$, $p < 0.001$).

would decrease the OCR. Etomoxir (1 mM) was added to the cells in the medium containing no glucose and no glutamine, and the oxygen consumption by the cells was monitored. As shown in Fig. 2B, etomoxir addition lowered the OCR to about 50% of the control. This confirmed that the cells indeed respired on endogenous fatty acids, accounting for up to 1/3 of total cellular oxygen consumption when glutamine was present. These data show that SF188 cells oxidize both endogenous and exogenous fatty acids.

3.2. Glutamine as the carbon source for the synthesis of endogenous fatty acid

Glucose-derived carbon is considered the primary source for *de novo* fatty acid synthesis. The body converts excess dietary glucose to fatty acids and store them as intracellular lipids in adipocytes as well as hepatocytes. SF188 cells have been shown to depend on glutamine for their growth and survival [11,12]. In addition to glucose, a fraction of glutamine also contributes to fatty acid synthesis. First, we determined cell proliferation and viability of SF188 cells grown in medium containing glucose only, glutamine only or both glucose and glutamine over a 72 hours period of time. Cell viability was monitored every 24 hours and the number of viable cells was determined using ViCell after removing the cells from the microplates. The growth of the cells over this 72 hour period was shown in Fig. 2A. The number of viable cells and the viability of cells under all three growth conditions were similar within 48 hours post plating, and significant differences in growth and viability were not observed until 72 hours. When glucose was eliminated from the medium (glutamine only), cell proliferation slowed significantly compared to the control cells at 72 hours; however, the cell viability was similar to the control. This suggests that glucose contributes to cell growth but is not required for cell survival and glutamine is sufficient to sustain cell growth and viability. In contrast, when glutamine was eliminated from the growth medium (glucose only), massive cell death was observed at 72 hours (data not shown) resulting in fewer viable cells (Fig. 2A) suggesting that glutamine is necessary for cell survival. This result is consistent with glutamine addiction by SF188 cells reported previously [12]. The number of viable cells and the viability of cells under all three growth conditions were similar at 48 hours post plating.

Next, we examined the oxidation of endogenous fatty acid of cells under these three growth medium conditions for 48 hours when they display similar viability and growth rate. Cells were plated in medium containing glucose only, glutamine only or both glucose and glutamine and cultured for 48 hours in XF cell culture plates. The growth medium was replaced with assay medium containing no exogenous substrate before their oxygen consumption rates were measured. Cells were removed from the microplate after the assay by trypsin digestion and the number of viable cells was determined. The OCRs of the cells under each condition were normalized using the number of cells in each well. As shown in Fig. 2B, when glutamine was eliminated from the growth medium (glucose only), the oxygen consumption rate of the cells was significantly lower compared to that of the cells grown in control medium. Interestingly, the oxygen consumption rate of cells grown in medium containing no glucose did not differ significantly from the control cells suggesting that glucose may not contribute significantly to the intracellular fatty acids that flux into mitochondria for oxidation and/or that the presence of glucose inhibits oxidative phosphorylation (the Crabtree effect). Fatty acid oxidation was confirmed again with the addition of etomoxir to the cells. We found that the oxygen consumption rate was reduced to a similar extent compared to the baseline rate of each group of cells before etomoxir addition (data not shown) confirming the occurrence of endogenous fatty acid oxidation under all three growth conditions.

The decreased endogenous fatty acid oxidation in cells grown in the absence of glutamine (glucose only) for 48 hours can be interpreted as that the lowered endogenous fatty acid content in the cells may lead to the reduced flux of fatty acids for β-oxidation in the mitochondria and/or the Crabtree effect. The flow of carbon from glutamine to fatty acids integrated with oxidation of endogenous and exogenous fatty acids is illustrated in Fig. 1A. These results provide indirect evidence suggesting that glutamine is involved in the *de novo* synthesis of fatty acids which feed into mitochondrial β-oxidation in SF188 cells.

3.3. Inhibition of fatty acid oxidation causes acute cellular ATP depletion and massive cell death within 18 hours

Having established that SF188 cells oxidize fatty acids, both endogenous and exogenous, we then investigated whether they depend on fatty acid for their growth and survival. We took the approach of pharmacologically inhibiting fatty acid oxidation using etomoxir. Cells were treated with 1 mM etomoxir, the concentration that causes maximal inhibition of fatty acid oxidation as determined by acute exposure, for 18 hours in the standard growth medium, and viability was examined microscopically and the number viable cells were determined using ViCell after removing the cells from each well. Oligomycin, an ATP synthase inhibitor, abolishes oxidative phosphorylation and inhibits mitochondrial ATP production. The oligomycin concentration (0.5 μM) that induces maximal decrease of OCR of the cells during an acute exposure, was used as a control for inhibition of mitochondrial ATP synthesis in the same experiment. We found that etomoxir treatment induced massive cell death as observed microscopically and reduced the number of viable cells to ~30% of the control. This suggests that fatty acid oxidation is required for cancer

cells survival. Oligomycin treatment only slightly reduced the cell number (Fig. 3A) but did not have a significant effect on the cell viability (data not shown) suggesting that inhibition of oxidative phosphorylation can be compensated for by an increase in glycolysis to sustain cellular ATP level and cell viability in vitro where glucose supply is ample.

We then investigated whether inhibition of fatty acid oxidation impairs cellular ATP production, which might ultimately lead to cell death, by measuring ATP levels in cells treated with etomoxir or oligomycin. Fatty acid oxidation can produce ATP through mitochondrial oxidative phosphorylation, a process that is inhibited by the ATP synthase inhibitor oligomycin. The cells were treated with etomoxir (1 mM) and oligomycin (0.5 μM) respectively, for 45 minutes, prior to ATP level measurement. As shown in Fig. 3B, the ATP level of the etomoxir treated cells declined to approximately 50% of the control cells. However, oligomycin treatment did not affect the ATP level significantly because of the compensatory increase in glycolysis as we have shown previously [8]. The cells treated under both conditions remained viable as determined by Calcein AM viability staining (Fig. 3B).

We have shown previously that the ATP levels of cancer cells are maintained during oligomycin treatment [8] as a result of a compensatory increase in glycolysis rate in response to the increased energy demand. We explored whether etomoxir inhibition of fatty acid oxidation by the cells led to a compensatory increase in glycolysis as does oligomycin by monitoring their ECAR during their exposure to etomoxir or oligomycin. We observed an increase in glycolysis rate of the cells in response to etomoxir although to a lesser extent than that of oligomycin (Fig. 3C), suggesting that the ATP depletion by etomoxir could not be completely accounted for by a moderate compensatory increase in the glycolysis. These findings raised the possibility that fatty acid oxidation may have additional roles beyond ATP synthesis that indirectly impact cellular energy metabolism and viability.

3.4. NADPH deficiency induced by inhibition of fatty acid oxidation results in oxidative stress and ATP depletion

Oxidative stress can promote tumorigenesis, cell proliferation or cell death depending on the magnitude of stress and cellular context [13,14]. It has been previously shown that cancer cells exhibit elevated ROS [15,16] and can adapt their antioxidant system to survive the increased oxidative stress. Excessive ROS can kill cancer cells as well [17]. We suspected that etomoxir might kill the cells by inducing severe oxidative stress. To test this, we analyzed intracellular ROS levels etomoxir treated cells. Superoxide was measured by dihydroethidine staining of the cells followed by flow cytometry analysis. Cells were harvested and suspended in PBS supplemented with 25 mM glucose and 6 mM glutamine and then treated with etomoxir (1 mM) for 30 minutes before dihydroethidine staining. We first analyzed the ROS level of SF188 cells and detected a substantial increase in fluorescence intensity. This suggests a significant endogenous superoxide level present in SF188 control cells (Fig. 4A). Etomoxir (1 mM) treatment of the cells resulted in a large increase in fluorescence intensity compared to the control untreated cells indicating a greatly increased superoxide level (Fig. 4B). This finding suggests that inhibition of fatty acid oxidation results in vastly elevated ROS and therefore oxidative stress.

Increased intracellular ROS can result from increased production of and/or a decreased elimination of ROS in cells. We, therefore, investigated the antioxidant system which eliminates intracellular ROS in SF188 cells. GSH in mitochondria and cytosol is a vital antioxidant that eliminates ROS and defends cells against oxidative stress and cell death [18,19]. The reducing equivalent NADPH is required to regenerate GSH from the oxidized form G-S-S-G, particularly in mitochondria, maintaining the GSH level [20,21]. We tested the hypothesis that fatty acid oxidation in SF188 could provide the substrate for NADPH production to maintain the GSH antioxidant system. First, we evaluated the effect of etomoxir on the GSH level of the cells. The cells plated in a microplate were treated with etomoxir for 30 minutes in medium supplemented with 25 mM glucose and 6 mM glutamine and their GSH levels were measured by a luminescent GSH assay. We observed that GSH level declines starting at 0.75 mM etomoxir and 1 mM etomoxir decreased the GSH level to approximately 50% relative to the control cells (Fig. 5A). This suggests that inhibition of fatty acid oxidation compromised the GSH antioxidant system of the cells.

Next, we determined the NADPH level in the cells treated with etomoxir. The cells were harvested and suspended in medium containing 25 mM glucose and 6 mM glutamine, then were treated with etomoxir for 30 minutes and lysed for NADPH detection. The NADPH level of each sample was determined using a fluorescent NADPH assay. We found that the NADPH level declined beginning at 0.75 mM etomoxir and to about 50% of the control at 1 mM etomoxir (Fig. 5B) mirroring the etomoxir-induced decrease in GSH level in the cells. This suggests that fatty acid oxidation can produce NADPH, thereby providing the reducing equivalents to regenerate GSH in the cells. The parallel declines of GSH and NADPH levels caused by etomoxir treatment provide direct evidence to support the hypothesis that oxidation of endogenous fatty acid can produce NADPH that may protect cells from oxidative stress, ATP depletion and cell death. Given that inhibition to fatty acid oxidation decreases NADPH, we also sought to test whether the converse is true. Cells were pre-incubated in DMEM containing palmitate–BSA for 1 hour before they were harvested and lysed for NADPH detection. We found that NADPH level increased by ~20% when palmitate–BSA was fed to the cells (Fig. 5C), suggesting exogenously added fatty acids boosted cellular NADPH

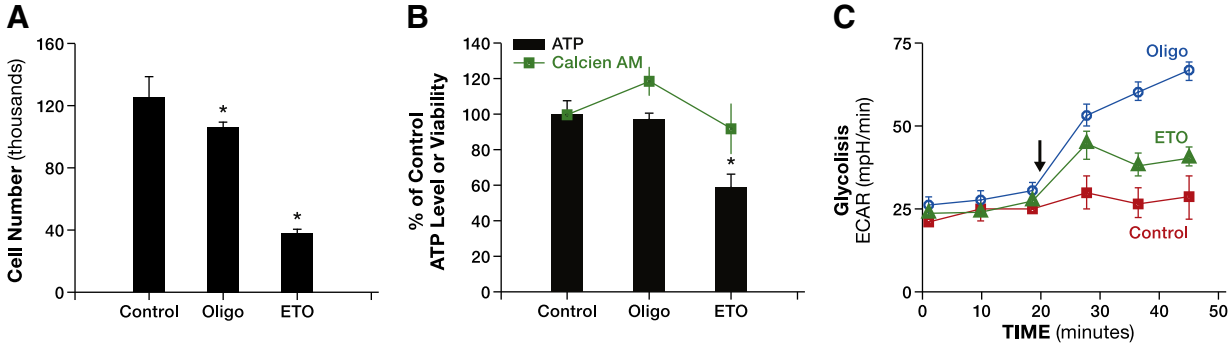

Fig. 3. Inhibition of fatty acid oxidation by etomoxir causes cell death and ATP depletion. A. The number of viable cells was determined after treatment with etomoxir (1 mM) and oligomycin (0.5 μM) for 18 hours. Mean ± SD. ($n = 3$; $p < 0.01$). B. ATP levels of cells treated with etomoxir (1 mM) and oligomycin (0.5 μM) for 45 minutes were determined using a luminescent ATP assay. Mean ± SD. ($n = 3$; $p < 0.001$). Cell viability was determined with Calcein AM staining. C. Glycolysis responses to etomoxir or oligomycin (Oligo) were determined by monitoring ECAR using XF24 Extracellular Flux Analyzer. Arrow indicates the time when each compound was added.

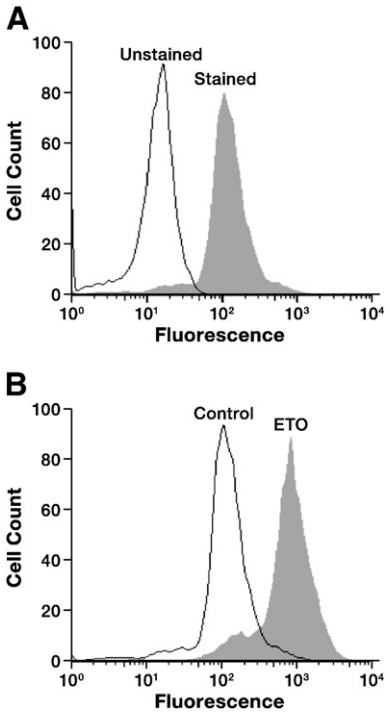

Fig. 4. Inhibition of fatty acid oxidation elevated intracellular ROS level. A. Intracellular superoxide level of SF188 cells was measured by dihydroethidine staining (shaded histogram). Unstained cells were the control (open histogram). B. Intracellular superoxide level was measured by dihydroethidine staining the cells after treatment with 1 mM etomoxir (shaded histogram) and control (open histogram).

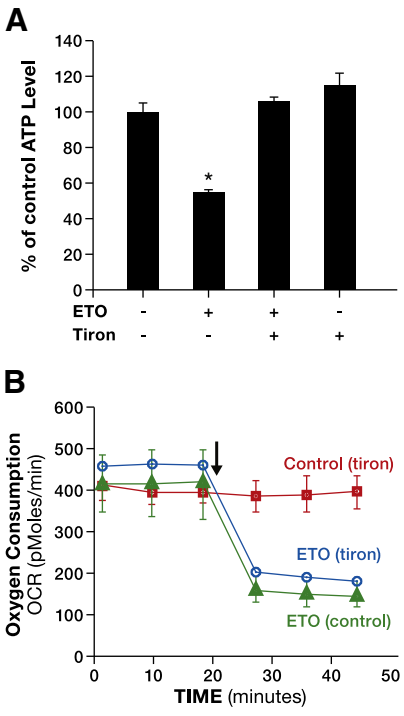

Fig. 6. Superoxide scavenger tiron rescues cellular ATP level but does not rescue fatty acid oxidation. A. Cellular ATP levels were determined after treatment with etomoxir (0.75 mM), tiron (1 mM) and etomoxir in combination with tiron for 45 minutes. Mean ± SD. ($n = 3$; $p < 0.001$). B. Cellular oxygen consumption rate in response to etomoxir (0.75 mM) was measured in the presence or absence of tiron (1 mM) in the medium. Arrow indicates the time when each compound was added.

level. This result provides direct evidence that fatty acid oxidation leads to NADPH production and suggests that modulation of fatty acid oxidation can control the NADPH level of the cells.

Since inhibition of fatty acid oxidation impairs the endogenous antioxidant system causing ATP depletion and cell death as we observed (section 3.3), we reasoned that supplementing the cells with exogenous antioxidant during etomoxir treatment might restore the antioxidant system and prevent ATP loss. We used tiron, a superoxide scavenger, for this purpose. The cells were treated with 0.75 mM etomoxir, 1 mM tiron and a combination of etomoxir and tiron for 45 minutes prior to ATP measurement. As shown in Fig. 6A, ATP level was maintained in cells treated with tiron and etomoxir while in cells treated with etomoxir, ATP level was reduced to ~60% of the control, suggesting that tiron protected the cells from ATP depletion. Oxidative damage to energy generation systems may, therefore, be the primary cause of cellular ATP depletion which can be prevented by tiron. We also tested the antioxidant N-acetyl cysteine (NAC), a precursor of glutathione, in preventing etomoxir-induced ATP depletion. In this case, we did not find a significant effect (data not shown), suggesting GSH loss most likely occurred due to an impaired ability to regenerate GSH rather than an insufficient supply of GSH precursor.

It can be argued that tiron may exert its protective effect by restoring fatty acid oxidation. To exclude this possibility, we examined the effect of tiron on etomoxir-induced inhibition of fatty acid oxidation. Cellular oxygen consumption rate of the cells was monitored in the medium containing 1 mM tiron before and after 0.75 mM etomoxir was added. We did not find any significant difference between OCR of the cells in response to etomoxir in the presence and absence of tiron (Fig. 6B). In other words, fatty acid oxidation remained suppressed by etomoxir in the presence of tiron while the ATP level was restored, strongly suggesting that the etomoxir-induced NADPH decline and the resulting antioxidant

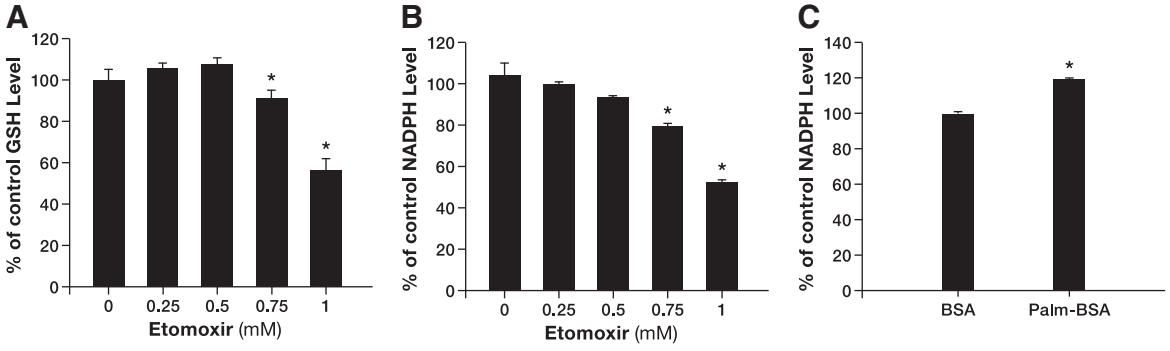

Fig. 5. Inhibition of fatty acid oxidation causes depletion of GSH and NADPH level is modulated by fatty acid oxidation. A. GSH level was determined after treating cells with etomoxir for 30 minutes using a luminescent GSH assay. Mean ± SD. ($n = 3$; $p = 0.05$). B. NADPH level was determined in cell lysate after treating the cells in suspension with etomoxir for 30 minutes using a fluorescent NADPH assay. Mean ± SD. ($n = 3$; $p < 0.05$). C. NADPH level was determined in cell lysate after feeding the cells with palmitate–BSA (palm–BSA) for 45 minutes. Cells fed with BSA was the control. Mean ± SD. ($n = 3$; $p < 0.01$).

system deficiency is the primary cause of ATP depletion. Therefore, these results lend further support to our hypothesis that oxidative stress resulting from NADPH deficiency causes ATP depletion.

Finally, we sought to test whether tiron could protect the cells from etomoxir-induced death, similar to its ability to prevent ATP depletion. Cells were treated with 0.75 mM etomoxir, 1 mM tiron and a combination of etomoxir and tiron for 18 hours, and the number of viable cells was determined. In contrast to the observed protection against ATP loss, we observed minimal protection from etomoxir-induced cell death by tiron compared to the control cells without tiron (data not shown). This suggests that the impairment of the antioxidant defense system that resulted from prolonged exposure to etomoxir was extremely severe and the cell survival over an extended period of time could not be rescued by tiron alone.

3.5. Etomoxir partially suppresses mitochondrial electron transport system

It has reported that etomoxir increases oxidative stress in HepG2 cells [23], as we learned after the completion of this work. This raises the possibility that etomoxir might have additional effects on mitochondria in addition to blocking the transport of fatty acids. We assessed whether etomoxir inhibits the mitochondrial electron transport by measuring maximal respiration capacity using mitochondrial uncoupler FCCP in the presence or absence of etomoxir in the presence of saturating concentrations of glucose, glutamine pyruvate. The rationale of the experimental design is as follows. FCCP uncouples electron transport from oxidative phosphorylation, allowing maximal rate of electron transport under the condition of unlimited electron supply (saturating substrate concentration), which is reflected by maximal cellular oxygen consumption. If a compound, for example etomoxir, inhibits the ETC, the FCCP-stimulated increase in OCR would be less than that in the absence of an ETC inhibitor. We used oligomycin, an inhibitor of ATP synthase but not the ETC, as a negative control in the experiment; the FCCP-stimulated OCR increase following oligomycin reflects maximal mitochondrial respired capacity.

SF188 cells and C2C12 myocytes were used to test the effect of etomoxir on the ETC. The cells were first exposed to etomoxir (0.5 mM) or oligomycin (1 μM) followed by FCCP (0.3 μM) in assay medium containing 25 mM glucose, 6 mM (SF188) or 4 mM (C2C12) glutamine and 1 mM pyruvate, which saturate the ETC as determined in separate experiments (data not shown). Fig. 7 showed that FCCP-stimulated OCR increase following etomoxir addition is significantly lower than after oligomycin addition in both SF188 cells and C2C12 myocytes. The smaller than expected OCR increase suggests that etomoxir may restrict the electron transport. We also used complex I inhibitor rotenone as a positive control for ETC inhibition and found that FCCP completely failed to stimulate OCR increase after rotenone addition (data not shown) in both SF188 and C2C12 myocytes. These results suggest that etomoxir, in addition to blocking fatty acid transport into mitochondria, may partially inhibit the electron transport chain. Hence, the observed etomoxir effect on SF188 could be the sum of inhibition of fatty acid oxidation and inhibition of the ETC.

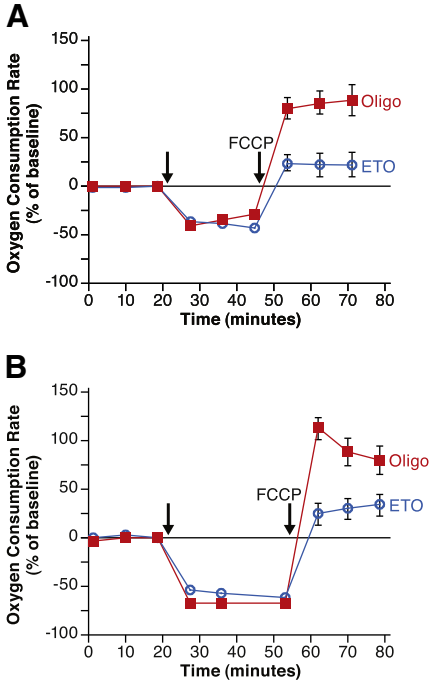

Fig. 7. Etomoxir partially suppresses mitochondrial electron transport chain. Etomoxir's effect on the ETC was determined by sequential addition of etomoxir (0.5 mM) and FCCP (0.3 μM) in SF188 cells (A) and C2C12 myocytes (B) in assay medium containing glucose (25 mM), glutamine (6 mM for SF188 or 4 mM for C2C12) and pyruvate (1 mM). A 0.3 μM FCCP is the concentration that stimulates maximal oxygen consumption in both cell types determined in previous experiments. Mean ± SD, ($n = 4$). Arrow indicates the time when each compound was added to cells in each well.

4. Discussion

While moderate ROS levels promote cellular transformation and cell proliferation, severe oxidative stress leads to cell death. Intracellular ROS levels are controlled and balanced between ROS production and elimination. The elimination of ROS is performed by the antioxidant system, of which GSH is a central component [18,19]. GSH requires constant replenishment, and NADPH is essential in the regeneration of GSH in the cellular defense against oxidative stress to promote cancer cell growth and survival [20–22]. It is thought that cellular NADPH for reductive biosynthesis is derived mainly from glucose flux through the pentose phosphate pathway and glutamine flux through the malic enzyme [4]. Our results suggest that NADPH production fueled by mitochondrial fatty acid oxidation provides the cells with an additional mechanism for producing the quantities of NADPH needed to meet the demands of defense against oxidative damage and cell death. This idea is further supported by our observation that tiron prevents ATP depletion even though fatty acid oxidation is not restored.

The fact that NADPH decrease occurs as a result of fatty acid oxidation inhibition, even in the presence of glutamine and glucose, suggests that NADPH production powered by these nutrients might either be insufficient or has a minimal impact on maintaining the antioxidant system. Yuneve et al. [24] reported that glutamine depletion-induced cell death in Myc-expressing human cells does not involve an increase in ROS levels. Furthermore, oxidative stress is not involved in apoptosis induced by glutamine depletion in those cells, that is, tiron and NAC do not prevent cell death. These findings could be interpreted as lack of glutamine involvement in antioxidant defense of the cells. The present study supports and extends these findings by demonstrating that fatty acid oxidation is a major source of NADPH for defense against oxidative stress. Our results, therefore, support a model in which fatty acids, rather than glutamine, serve as the critical source of NADPH, particularly in the mitochondria, against oxidative stress in rapidly proliferating Myc-dependent cells such as SF188 where the oxygen consumption rate and ROS generation are especially high. One explanation for glutamine's lack of prominence in antioxidant defense could be that nearly all carbons derived from glutamine exit a truncated TCA cycle and are exported into the cytoplasm for fatty acid synthesis by two possible mechanisms. Glutamine-derived malate and citrate (as reductive power and building blocks) could either exit a forward-running truncated TCA cycle prior to their conversion to isocitrate (Fig. 1A) [2] or exit a backward-running TCA from α-KG to citrate as reportedly occurs in brown adipocytes [25]. In other word, glutamine conversion to

isocitrate in the TCA cycle might be minimal. Additional experiments would be required to verify either scenario.

How could oxidative stress induced by inhibition of fatty acid oxidation cause acute cellular ATP depletion? Mitochondrial ATP is produced by oxidative phosphorylation and substrate level phosphorylation in the TCA cycle. TCA cycle enzymes such as aconitase and α-ketoglutarate dehydrogenase (α-KDH) as well as the ETC complexes are sensitive to ROS inactivation [26,27]. Mitochondria not only generate ROS but are also major targets of ROS-induced oxidative damage leading to severe harm to the TCA cycle enzymes and the ETC complexes. Severe damages to the bioenergetic machinery would result in a sharp decline in the mitochondrial ATP level, which is also required for continuous glucose phosphorylation by mitochondria-bound hexokinase in cancer cells [28]. Limited mitochondrial ATP production would, therefore, impair glycolytic ATP synthesis. Disabling both mitochondrial and glycolytic ATP production would cause a catastrophic depletion of total cellular ATP and rapid cell death. Tiron may temporarily protect key mitochondrial TCA cycle enzymes and ETC complexes from ROS-induced inactivation, allowing continuous flux of NADH and ATP synthesis to proceed using glutamine present in the medium while fatty acid oxidation is suppressed. However, the data present here do not allow us to explain why tiron did not prevent etomoxir-induced cell death. It is well-known that excessive ROS can modify mitochondrial proteins that are involved in cell death including the mitochondrial permeability transition pore components voltage-dependent anion channel (VDAC) and/or the adenine nucleotide translocase (ANT), and cardiolipin triggering cell death [29]. It is conceivable that greatly increased intracellular ROS that accumulates over hours of exposure to etomoxir damages these proteins so severely that tiron alone cannot overcome it.

Although our study did not directly address the biochemical pathways through which fatty acid oxidation fuels NADPH production, two clues suggest that mitochondrial rather than cytosolic NADPH production might be involved. Firstly, fatty acid oxidation occurs within mitochondria. Secondly, tiron eliminates the superoxide radical O_2^- [30,31], which is the first ROS generated in the mitochondrial sequence. Therefore, tiron might eliminate ROS early to minimize oxidative damage to the bioenergetics machinery and thus prevent ATP loss induced by fatty acid oxidation inhibition. While three enzymes contribute to cytosolic NADPH production in the cell, only one enzyme, $NADP^+$-dependent isocitrate dehydrogenase 2 (IDH2), can produce NADPH within the mitochondria. A number of recent studies indicate that mitochondrial IDH2 is essential in supplying the NADPH needed to defend cancer cells against mitochondrial oxidative damage and promote cell survival [20–22]. For example, Jo et al. [16] has demonstrated that decreased expression of IDH2 (IDPm) markedly elevates the ROS generation, lipid peroxidation, DNA fragmentation and mitochondrial damage with a significant reduction in ATP level. However, genetic inactivation of NADPH-producing enzymes such as IDH2 is still required to clearly identify whether IDH2 is the responsible enzyme and whether the cytosolic IDH1 (IDPc) is also involved in our model system.

Several lines of evidence indicate that intracellular lipid catabolism is important for cancer cell growth and survival. First, monoacylglycerol lipase, which catalyzes the release of fatty acid from intracellular lipid, promotes tumor growth and survival [32]. Inhibition of this enzyme reverses the process. Second, inhibition of fatty acid oxidation reportedly sensitizes human leukemia cells to apoptosis induction by BcL2 inhibitors [33]. Finally, fatty acid oxidation has been shown to be associated with resistance to chemotherapeutic agents and radiation and insensitivity to oxidative stress [34]. Our results—that fatty acid oxidation produces NADPH to protect cells from oxidative stress and death—are consistent with the importance of fatty acid catabolism in promoting cancer cell growth and survival. We suggest that this fatty acid oxidation-fueled NADPH production in protecting cells from oxidative stress may be a common attribute of those rapidly proliferating and highly oxidative cancer cells that results from metabolic reprogramming by genetic alterations including Myc amplification. However, the present data do not allow us to discern the relative contribution of each component, inhibition of fatty acid oxidation or inhibition of electron transport, to the observed cellular effects in SF188 cells. Genetic inactivation of CPT-1 is required to address this.

In conclusion, our studies suggest that fatty acid oxidation may provide a source of NADPH for regeneration of the GSH antioxidant system and protects the cancer cells from oxidative damage, ATP depletion and death. Our work also underscores the importance of integrated mitochondrial metabolism, oxidative phosphorylation, intermediary metabolism and redox balance in cancer cell growth and survival.

5. Disclosure statement

LS Pike, AL Smift, DA Ferrick and M Wu are employees of Seahorse Bioscience at the time of this work.

Acknowledgments

We would like to thank Dr. Martin Sattler for his helpful comments on the manuscript, Daniel Fitzpatrick for the assistance with the graphic design and Cyd Wong for the assistance with the weekend cell culture.

References

[1] O. Warburg, On the origin of cancer cells, Science 123 (1956) 309–314.
[2] R.J. DeBerardinis, J.J. Lum, G. Hatzivassiliou, C.B. Thompson, The biology of cancer: metabolic reprogramming fuels cell growth and proliferation, Cell Metab. 7 (2008) 11–20.
[3] M.G. Vander Heiden, L.C. Cantley, C.B. Thompson, Understanding the Warburg effect: the metabolic requirements of cell proliferation, Science 324 (2009) 1029–1033.
[4] R.J. Deberardinis, N. Sayed, D. Ditsworth, C.B. Thompson, Brick by brick: metabolism and tumor cell growth, Curr. Opin. Genet. Dev. 18 (2008) 54–61.
[5] C.V. Dang, Rethinking the Warburg effect with Myc micromanaging glutamine metabolism, Cancer Res. 70 (2010) 859–862.
[6] F.P. Kuhajda, Fatty-acid synthase and human cancer: new perspectives on its role in tumor biology, Nutrition 16 (2000) 202–208.
[7] G. Medes, A. Thomas, S. Weinhouse, Metabolism of neoplastic tissue. IV. A study of lipid synthesis in neoplastic tissue slices in vitro, Cancer Res. 13 (1953) 27–29.
[8] M. Wu, A. Neilson, A.L. Swift, R. Moran, J. Tamagnine, D. Parslow, S. Armistead, K. Lemire, J. Orrell, J. Teich, S. Chomicz, D.A. Ferrick, Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells, Am. J. Physiol. Cell Physiol. 292 (2007) C125–36.
[9] S. Weinhouse, R.H. Milington, C.E. Wenner, Metabolism of neoplastic tissue. I. The oxidation of carbohydrate and fatty acids in transplanted tumors, Cancer Res. 11 (1951) 845–850.
[10] S. Weinhouse, On respiratory impairment in cancer cells, Science 124 (1956) 267–269.
[11] R.J. DeBerardinis, A. Mancuso, E. Daikhin, I. Nissim, M. Yudkoff, S. Wehrli, C.B. Thompson, Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis, Proc. Natl Acad. Sci. USA 104 (2007) 19345–19350.
[12] D.R. Wise, R.J. DeBerardinis, A. Mancuso, N. Sayed, X.Y. Zhang, H.K. Pfeiffer, I. Nissim, E. Daikhin, M. Yudkoff, S.B. McMahon, C.B. Thompson, Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction, Proc. Natl Acad. Sci. USA 105 (2008) 18782–18787.
[13] D. Trachootham, W. Lu, M.A. Ogasawara, R.D. Nilsa, P. Huang, Redox regulation of cell survival, Antioxid. Redox Signal. 10 (2008) 1343–1374.
[14] D. Trachootham Test, Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat. Rev. Drug Discov. 8 (2009) 579–591.
[15] T.P. Szatrowski, C.F. Nathan, Production of large amounts of hydrogen peroxide by human tumor cells, Cancer Res. 51 (1991) 794–798.
[16] S. Toyokuni, K. Okamoto, J. Yodoi, H. Hiai, Persistent oxidative stress in cancer, FEBS Lett. 358 (1995) 1–3.
[17] D. Trachootham, Y. Zhou, H. Zhang, Y. Demizu, Z. Chen, H. Pelicano, P.J. Chiao, G. Achanta, R.B. Arlinghaus, J. Liu, P. Huang, Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate, Cancer Cell 10 (2006) 241–252.
[18] A. Meister, M.E. Anderson, Glutathione, Annu. Rev. Biochem. 52 (1983) 711–760.

[19] M. Marí, A. Morales, A. Colell, C. García-Ruiz, J.C. Fernández-Checa, Mitochondrial glutathione, a key survival antioxidant, Antioxid. Redox Signal. 11 (2009) 2685–2700.
[20] S.H. Jo, M.K. Son, H.J. Koh, S.M. Lee, I.H. Song, Y.O. Kim, Y.S. Lee, K.S. Jeong, W.B. Kim, J.W. Park, B.J. Song, T.L. Huh, Control of mitochondrial redox balance and cellular defense against oxidative damage by mitochondrial NADP$^+$-dependent isocitrate dehydrogenase, J. Biol. Chem. 276 (2001) 16168-1617.
[21] I.S. Kil, S.Y. Kim, S.J. Lee, J.W. Park, Small interfering RNA-mediated silencing of mitochondrial NADP+-dependent isocitrate dehydrogenase enhances the sensitivity of HeLa cells toward tumor necrosis factor-alpha and anticancer drugs, Free Radic. Biol. Med. 43 (2007) 1197–1207.
[22] I.S. Kil, K.H. Chung, J.W. Park, Silencing of mitochondrial NADP+-dependent isocitrate dehydrogenase gene enhances selenite-induced apoptosis, Free Radic. Res. 44 (2010) 332–339.
[23] C.L. Merrill, H. Ni, L.W. Yoon, M.A. Tirmenstein, P. Narayanan, G.R. Benavides, M.J. Easton, D.R. Creech, C.X. Hu, D.C. McFarland, L.M. Hahn LM, H.C. Thomas, K.T. Morgan, Etomoxir-induced oxidative stress in HepG2 cells detected by differential gene expression is confirmed biochemically, Toxicol. Sci. 68 (2002) 93–101.
[24] M. Yuneva, N. Zamboni, P. Oefner, R. Sachidanandam, Y. Lazebnik, Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells, J. Cell Biol. 178 (2007) 93–105.
[25] H. Yoo, M.R. Antoniewicz, G. Stephanopoulos, J.K. Kelleher, Quantifying reductive carboxylation flux of glutamine to lipid in a brown adipocyte cell line, J. Biol. Chem. 283 (2008) 20621–20627.
[26] L. Tretter, V. Adam-Vizi, Inhibition of Krebs cycle enzymes by hydrogen peroxide: a key role of [alpha]-ketoglutarate dehydrogenase in limiting NADH production under oxidative stress, J. Neurosci. 20 (2000) 8972–8979.
[27] Y. Zhang, O. Marcillat, C. Giulivi, L. Ernster, K.J. Davies, The oxidative inactivation of mitochondrial electron transport chain components and ATPase, J. Biol. Chem. 265 (1990) 16330–16336.
[28] E. Bustamante, P.L. Pedersen, High aerobic glycolysis of rat hepatoma cells in culture: role of mitochondrial hexokinase, Proc. Natl Acad. Sci. USA 74 (1977) 3735–3739.
[29] S. Orrenius, V. Gogvadze, B. Zhivotovsky, Mitochondrial oxidative stress: implications for cell death, Annu. Rev. Pharmacol. Toxicol. 47 (2007) 143–183.
[30] J. Yamada, S. Yoshimura, H. Yamakawa, M. Sawada, M. Nakagawa, S. Hara, Y. Kaku, T. Iwama, T. Naganawa, Y. Banno, S. Nakashima, N. Sakai, Cell permeable ROS scavengers, Tiron and Tempol, rescue PC12 cell death caused by pyrogallol or hypoxia/reoxygenation, Neurosci. Res. 45 (2003) 1–8.
[31] J.B. Mitchell, A. Samuni, M.C. Krishna, W.G. DeGraff, M.S. Ahn, U. Samuni, A. Russo, Biologically active metal-independent superoxide dismutase mimics, Biochemistry 29 (1990) 2802–2807.
[32] D.K. Nomura, J.Z. Long, S. Niessen, H.S. Hoover, S.W. Ng, B.F. Cravatt, Monoacylglycerol lipase regulates a fatty acid network that promotes cancer pathogenesis, Cell 140 (2010) 49–61.
[33] I. Samudio, R. Harmancey, M. Fiegl, H. Kantarjian, M. Konopleva, B. Korchin, K. Kaluarachchi, W. Bornmann, S. Duvvuri, H. Taegtmeyer, M. Andreeff, Pharmacologic inhibition of fatty acid oxidation sensitizes human leukemia cells to apoptosis induction, J. Clin. Invest. 120 (2010) 142–156.
[34] M.E. Harper, A. Antoniou, E. Villalobos-Menuey, A. Russo, R. Trauger, M. Vendemelio, A. George, R. Bartholomew, D. Carlo, A. Shaikh, J. Kupperman, E.W. Newell, I.A. Bespalov, S.S. Wallace, Y. Liu, J.R. Rogers, G.L. Gibbs, J.L. Leahy, R.E. Camley, R. Melamede, M.K. Newell, Characterization of a novel metabolic strategy used by drug-resistant tumor cells, FASEB J. 16 (2002) 1550–1557.