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ATP/ADP Ratio, the Missed Connection between Mitochondria and the Warburg Effect

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Abstract

Non-proliferating cells generate the bulk of cellular ATP by fully oxidizing respiratory substrates in mitochondria. Respiratory substrates cross the mitochondrial outer membrane through only one channel, the voltage dependent anion channel (VDAC). Once in the matrix, respiratory substrates are oxidized in the tricarboxylic acid cycle to generate mostly NADH that is further oxidized in the respiratory chain to generate a proton motive force comprised mainly of membrane potential ($\Delta\Psi$) to synthesize ATP. Mitochondrial $\Delta\Psi$ then drives release of ATP^{-4} from the matrix in exchange for ADP^{-3} in the cytosol via the adenine nucleotide translocator (ANT) located in the mitochondrial inner membrane. Thus, mitochondrial function in non-proliferating cells drives a high cytosolic ATP/ADP ratio, essential to inhibit glycolysis. By contrast, the bioenergetics of the Warburg phenotype of proliferating cells is characterized by enhanced aerobic glycolysis and suppression of mitochondrial metabolism. Suppressed mitochondrial function leads to lower production of mitochondrial ATP and hence lower cytosolic ATP/ADP ratios that favor enhanced glycolysis. Thus, cytosolic ATP/ADP ratio is a key feature that determines if cell metabolism is predominantly oxidative or glycolytic. Here, we describe two novel mechanisms to explain the suppression of mitochondrial metabolism in cancer cells: the relative closure of VDAC by free

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tubulin and inactivation of ANT. Both mechanisms contribute to low ATP/ADP ratios that activate glycolysis.

Keywords

ANT; ATP/ADP ratio; cancer cells; glycolysis; mitochondria; oxidative phosphorylation; VDAC; Warburg

Introduction

Cellular bioenergetics differs between proliferating and non-proliferating cells. Cancer and other proliferating cells display enhanced aerobic glycolysis even in the presence of physiological oxygen (Harvey, et al., 2002;Warburg, 1956). This phenomenon was first described by Otto Warburg in the early 20th century and causes fully aerobic tumors to produce net lactic acid (Warburg, et al., 1927). Lactic acid is usually an end product of anaerobic glycolysis in the cytosol. In aerobic non-proliferating cells, pyruvate generated by glycolysis is not converted to lactic acid but is instead diverted to mitochondria and oxidized to CO_2 and H_2O by the tricarboxylic acid cycle and the respiratory chain. Lactic acid generation by non-proliferating tissues generally indicates hypoxia. Thus, lactic acid production by aerobic tumors signifies a fundamentally altered metabolism, including suppressed pyruvate oxidation by mitochondria. Indeed, Warburg originally postulated that tumor cells had a defect in what we now call oxidative phosphorylation.

A possible advantage of Warburg metabolism to proliferating cells is that glycolysis furnishes carbon backbones for biomass formation and reductive biosynthesis, whereas oxidative phosphorylation converts glucose and other glycolytic substrates completely to CO_2 and H_2O (Vander Heiden, et al., 2009;DeBerardinis, et al., 2008). A similar phenomenon occurs in yeast. Grown on a fermentable (glycolytic) substrate like glucose, yeast aerobically generate lactic acid as the end product of glycolysis, which is further metabolized to ethanol. When the fermentable substrate is exhausted or replaced with a non-fermentable substrate like glycerol, a pause in growth occurs as new proteins are expressed, including enzymes of oxidative phosphorylation (diauxic shift) (Galdieri, et al., 2010;Vivier, et al., 1997). Cell proliferation then resumes, but growth is never as rapid as when supported by a fermentable substrate. In a broth, selection is for the most rapidly proliferating cells, and if yeast could grow faster on a non-fermentable substrate, then they would. Arguably then, the Warburg metabolic phenotype favoring more rapid cellular proliferation is why we have beer.

Metabolite exchange across mitochondrial membranes

Mitochondrial ATP synthesis involves oxidation of pyruvate, glutamine, fatty acids and other respiratory substrates by enzymes of the tricarboxylic acid cycle in the mitochondrial matrix to produce mostly NADH, which is then oxidized by the respiratory chain to generate the protonmotive force (Δp) that drives ATP synthesis from ADP and Pi by the reversible F_1F_O-ATP synthase (Fig. 1). Δp is comprised of a negative-inside membrane potential ($\Delta\Psi$) and an alkaline-inside pH gradient (ΔpH) by the relationship (in mV): $\Delta p = \Delta\Psi - 59\Delta pH$. In

mammalian cardiomyocytes and hepatocytes, $\Delta\Psi$ is in the range of -120 mV to -150 mV and ΔpH is 0.6-0.8 pH units (Brand and Nicholls, 2011;Chacon, et al., 1994;Emaus, et al., 1986;Hoek, et al., 1980;Nicholls and Ferguson, 2013;Petit, et al., 1990;Rottenberg, 1975;Santo-Domingo and Demaurex, 2012;Zahrebelski, et al., 1995;Mitchell, 2011). Newly synthetized mitochondrial ATP^{4-} in the matrix then exchanges for cytosolic ADP^{3-} via the adenine nucleotide transporter (ANT), and OH^- exchanges for Pi^- via the phosphate transporter (PT). Both ANT and PT are located in the mitochondrial inner membrane. Similarly, various respiratory substrates cross the inner membrane via separate dedicated carriers. By contrast, movement of hydrophilic anionic metabolites across the mitochondrial outer membrane occurs exclusively via a single channel - the voltage dependent anion channel (VDAC) (Fig. 1). This review addresses altered roles of VDAC and adenine nucleotide exchange in mitochondrial ATP release to the cytosol of proliferating tumor cells.

ANT catalyzes an electrogenic molecule for molecule exchange of ATP^{4-} for ADP^{3-}. Because of the highly negative mitochondrial membrane potential ($\Delta\Psi$), ANT acts as a secondary active transport system that pumps ATP^{4-} out and ADP^{3-} into mitochondria. Consequently, ATP/ADP ratios can become 50 to 100 times higher in the cytosol than in the mitochondrial matrix in cells with active aerobic mitochondrial metabolism, such as neurons, cardiomyocytes and hepatocytes (Klingenberg, 2008). High cytosolic ATP/ADP ratios as a consequence of aerobic mitochondrial metabolism and electrogenic ANT exchange suppress glycolysis through inhibition of phosphofructokinase-1 among other possible mechanisms (Fig. 1) (Hers and Van, 1982;Mor, et al., 2011). However, when mitochondrial ATP synthesis becomes compromised as during hypoxia/ischemia, ATP/ADP ratios drop dramatically, which markedly stimulates glycolysis to generate ATP anaerobically.

In proliferating cells, low ATP/ADP ratios are necessary to maintain enhanced glycolysis, which can only occur if mitochondrial metabolism is suppressed or altered. Although considerable research has been devoted to understanding the upregulation of genes and enzymes involved in the glycolytic pathway of cancer cells, much less is known about the basis for mitochondrial metabolic changes in the Warburg effect. Moreover, the ATP/ADP ratio has not been considered a key component in the Warburg metabolism of proliferating cells.

Here, we review evidence that relative closure of VDAC in cancer cells limits access of respiratory substrates and ADP to the matrix, thus decreasing ATP synthesis. We also discuss recent findings that ANT in cancer cells does not exchange ATP for ADP as occurs in non-proliferating cells. Rather, ATP appears to move non-electrogenically via another exchanger, possibly the ATP-Mg/Pi carrier (Joyal and Aprille, 1992;Fiermonte, et al., 2004;Palmieri, 2012). Suppressed mitochondrial function by VDAC closure combined with non-electrogenic ATP-ADP exchange acts to maintain a lower ATP/ADP ratio that is stimulatory of glycolysis. Thus, changes of activity of both VDAC and ANT contribute the aerobic glycolytic Warburg metabolic phenotype of cancer cells.

Energy conversion in non-proliferating cells

In non-proliferating cells, most ATP is formed in the mitochondrial matrix, a highly regulated microenvironment limited by the mitochondrial inner membrane and separated from the cytosol. Mitochondria oxidize respiratory substrates and produce ATP in response to the demand imposed by ATP-consuming reactions in the cytosol. Hence, mitochondria couple an input and an output. The input comprises oxidizable substrates (pyruvate, glutamine, fatty acids and other respiratory substrates), O_2, ADP and Pi. Since O_2 is the final electron acceptor of the respiratory chain, O_2 consumption inhibitable by respiratory chain inhibitors (*e.g.*, cyanide, myxothiazol, rotenone) is a quantitative measurement of mitochondrial respiration in intact cells. In vertebrates, blood flow through a closed circulation fine tunes delivery of O_2 and nutrients to match dynamically the energy and metabolic demands of tissues.

The output of mitochondrial energy conversion is ATP, and the free energy made available by ATP hydrolysis to ADP and Pi is the phosphorylation potential ($\Delta Gp = \Delta G_P°' + RT \ln([ATP]/[ADP][Pi])$, where $\Delta G_P°$ is the standard free energy change of ATP hydrolysis). Since $\Delta\Psi$ drives mitochondrial ATP-ADP exchange via the electrogenic ANT and ΔpH drives OH^--Pi exchange by the PT, ΔG_P in the cytosol becomes amplified relative to the mitochondrial matrix by up to the energetic equivalent of Δp (15-20 kJ/mol). By generating a 33% greater energetic punch to ATP hydrolysis, such ΔG_P amplification represents an important advantage of mitochondrial metabolism compared to that of prokaryotes (Lemasters, 1981). This higher ΔG_P also exerts a strong brake on glycolysis.

Energy conversion in cancer cells

The ATP yield per mole of glucose is much lower for glycolysis compared to mitochondrial oxidative phosphorylation (2 moles of ATP vs. ~35 moles of ATP per mole of glucose, respectively). The lower efficiency of ATP generation by glycolysis is offset in cancer cells by increased expression of enzymes involved in the glucose catabolism. Although Warburg proposed that mitochondria are damaged in cancer cells and even that mitochondrial damage might be the origin of cancer (Warburg, 1956), numerous studies document that mitochondria isolated from tumor cells can catalyze oxidative phosphorylation effectively, as assessed by $\Delta\Psi$ formation, respiratory control ratios and activity of respiratory chain components (Nakashima, et al., 1984;Mathupala, et al., 2010a;Singleterry, et al., 2014). Mitochondria also contribute to some extent to ATP generation in cancer cells with the relative contribution differing between cell lines but being consistently much lower than in non-proliferating cells (Griguer, et al., 2005;Moreno-Sanchez, et al., 2007;Zu and Guppy, 2004). Overall, glycolysis contributes to 50 to 70% of total ATP production in cancer cells with the remainder contributed by mitochondrial oxidation of pyruvate, glutamine and fatty acids (DeBerardinis, et al., 2008;Vander Heiden, et al., 2009; Mathupala, et al., 2010).

Voltage dependent anion channel and Warburg metabolism

Metabolites that enter and leave mitochondria for oxidative phosphorylation and other matrix reactions must cross both mitochondrial membranes. Non-polar compounds like oxygen and short chain fatty acids are bilayer-permeant and cross both mitochondrial membranes by diffusion. For polar metabolites, numerous specific transporters in the inner

membrane facilitate transport into and out of the matrix space. By contrast, movement of polar metabolites across the outer membrane occurs through one common channel, VDAC.

VDAC, first discovered from *Paramecium aurelia* and found in all eukaryotic cells, is the most abundant protein in the mitochondrial outer membrane (Sampson, et al., 1997). VDAC in humans and mice comprises three isoforms, VDAC1, VDAC2 and VDAC3, with a molecular mass of approximately 30 kDa and a high degree of sequence homology (Blachly-Dyson and Forte, 2001;Colombini, 2004). VDAC1 and VDAC2 are the most abundant isoforms in most tissues and tumors, except for testis where VDAC3 is most abundant (Sampson, et al., 2001).

As determined by NMR and X-ray crystallography, VDAC1 forms barrels in the lipid bilayer comprised of 19 beta strands, but this non-native structure is disputed by a model suggesting that functional VDAC forms only 13 beta-strands (Bayrhuber, et al., 2008;Colombini, 2009;Hiller, et al., 2008;Ujwal, et al., 2008). Recently, the structure of VDAC2 was resolved showing a similar 19-strand beta barrel (Schredelseker, et al., 2014). The wall of the beta barrel of about 1 nm in thickness surrounds an aqueous channel with an internal diameter or 2.5 nm in the open state and about 1.8 nm in the closed state. An N-terminal alpha-helix lies inside the pore parallel to the membrane plane, which is important for regulation of the flux of metabolites through the channel (Choudhary, et al., 2010;Mannella, 1998;Teijido, et al., 2012). In the open state, solutes up to ~5 kDA can permeate freely through VDAC (Colombini, 1980;Colombini, et al., 1987). In the closed state, most anionic metabolites, including respiratory substrates, creatine phosphate, adenine nucleotides and Pi, cannot cross through VDAC, although small ions like K^+, Na^+, Ca^{+2} and Cl^- remain permeant (Tan and Colombini, 2007). Since VDAC is the only channel allowing flux of metabolites through the mitochondrial outer membrane, its conductance can control mitochondrial metabolism globally and modulate ATP delivery to the cytosol (Lemasters and Holmuhamedov, 2006). Thus, VDAC opening and closing correspondingly increase and decrease mitochondrial energy conversion. In this way, relative closure of VDAC limits mitochondrial oxidative phosphorylation and lowers cytosolic ATP/ADP ratios to favor the aerobic glycolysis of the Warburg phenomenon, which is the metabolic signature of both normal proliferating cells and malignant cells.

VDAC is gated by voltage with half maximal closure at ±50 mV. Whether $\Delta\Psi$ closes VDAC in intact cells is not clear. A report of a ΔpH across the outer membrane implies a Donnan potential of ~40 mV, which might be enough to gate VDAC (Porcelli, et al., 2005). Donnan potentials depend on the asymmetrical distribution of non-permeant charged molecules, mainly proteins, and the magnitude of any Donnan potential forming is controversial because charged macromolecules reside on both sides of the outer membrane. Other factors also regulate VDAC conductance, including glutamate, protein kinase A, glycogen synthase 3β, hexokinase II, NADH, acetaldehyde, bcl_2 family members, ethanol and free tubulin (Azoulay-Zohar, et al., 2004;Das, et al., 2008;Gincel, et al., 2000;Lee, et al., 1994;Rostovtseva, et al., 2008;Vander Heiden, et al., 2000;Vander Heiden, et al., 2001; Holmuhamedov, et al., 2012;Lemasters, et al., 2012).

Voltage dependent anion channel and mitochondrial metabolism in tumor cells

Mitochondrial ΔΨ is an indicator of mitochondrial metabolism both in proliferating and non-proliferating cells. ΔΨ formation depends on respiration or, alternatively, can be supported by hydrolysis of ATP by the mitochondrial F_1F_O-ATP synthase acting in reverse (Maldonado, et al., 2010). For example during anoxia, ischemia or respiratory inhibition, ΔΨ can be maintained as long as glycolysis can provide ATP (Nieminen, et al., 1994;Zhang and Lemasters, 2013). To collapse mitochondrial ΔΨ, respiration and ATP supply to mitochondria must be inhibited simultaneously, as with myxothiazol (Complex III respiratory inhibitor) and oligomycin (ATP synthase inhibitor) (Maldonado, et al., 2010).

Free tubulin is an important endogenous regulator of VDAC that induces VDAC closure both in VDAC inserted into lipid bilayers and in isolated mitochondria (Rostovtseva, et al., 2008). In cancer cells such as HepG2 human hepatoma cells, free to polymerized tubulin ratios are high compared to non-transformed hepatocytes. Ratios of free tubulin to polymerized tubulin can be manipulated experimentally with microtubule destabilizers (colchicine, nocodazole) and stabilizers (paclitaxel) to show that mitochondrial ΔΨ in tumor cells increases and decreases as free tubulin decreases and increases. By contrast in hepatocytes with already low free tubulin, microtubule stabilization does not increase ΔΨ, whereas microtubule destabilization to increase free tubulin causes a decline of ΔΨ. Overall, ΔΨ inversely correlates with free to polymerized tubulin ratios. These findings support the conclusion that inhibition of VDAC conductance by free tubulin suppresses ΔΨ formation in intact tumor cells. In non-transformed cells with low free tubulin, such as hepatocytes but presumably other highly aerobic cell types as well, VDAC is constitutively open but may be closed by increased free tubulin (Maldonado, et al., 2010). Tubulin control of VDAC conductance is further regulated by protein kinases. For example, protein kinase A agonists promote VDAC closure and a decrease of ΔΨ, whereas antagonists decrease tubulin-dependent VDAC closure and the depolarizing effects of high tubulin in intact cells (Maldonado, et al., 2010;Sheldon, et al., 2011).

Importantly, VDAC conductance is not completely shut down in tumor cells. Single and double siRNA knockdowns of the three VDAC isoforms in all possible combinations reveal that all VDAC isoforms contribute to maintenance of mitochondrial ΔΨ but that VDAC3 contributes to the greatest extent. As shown in Fig. 2, VDAC3 knockdown decreases uptake of the ΔΨ-indicating fluorophore, tetramethylrhodamine methylester (TMRM), by ~80%. VDAC3 knockdown also decreases by a third the blue autofluorescence of mitochondrial NAD(P)H, a measure of respiratory substrate delivery to mitochondria. Total cellular ATP also decreases by half after VDAC3 knockdown (Fig. 2). Because ADP increases after VDAC3 knockdown (not shown), ATP/ADP decreases even more. These findings illustrate the importance of VDAC, especially VDAC3, in modulating mitochondrial bioenergetics status (Maldonado, et al., 2013).

When specific VDAC isoforms are inserted into planar lipid bilayers, free tubulin inhibits conductance of VDAC1 and VDAC2 but not that of VDAC3 (Maldonado, et al., 2013). This observation accounts for why in tumor cells VDAC3 is most important of the three isoforms for ΔΨ formation, because high free tubulin inhibits VDAC1 and VDAC2 but not VDAC3. Although VDAC3 is the least abundant isoform, its resistance to inhibition by tubulin makes

VDAC3 the most important isoform for ΔΨ formation and other indices of mitochondrial function in unperturbed tumor cells. Nonetheless when tubulin decreases, VDAC1 and VDAC2 conductance increases to upregulate ΔΨ and other aspects of mitochondrial metabolism. Interestingly, erastin, a compound that interacts with VDAC (Yagoda, et al., 2007), blocks and reverses mitochondrial depolarization after microtubule destabilizers in intact cells. This effect is accounted for by the fact that erastin antagonizes tubulin-induced VDAC blockage in planar bilayers. Overall, free tubulin inhibits VDAC1/2 and limits mitochondrial metabolism in tumor cells, thus decreasing ATP/ADP and contributing to the Warburg phenomenon (Fig. 1). Reversal of tubulin-dependent VDAC inhibition by erastin antagonizes Warburg metabolism and restores non-Warburg oxidative mitochondrial metabolism (Maldonado, et al., 2013).

An intriguing question concerns the broader, biologic role of tubulin-dependent inhibition of VDAC. In yeast, aerobic glycolysis supports a higher rate of cell proliferation than aerobic oxidative phosphorylation. Moreover, rapidly dividing cells must maintain a free tubulin reserve for spindle formation at metaphase. Thus, high free tubulin is characteristic of cell proliferation. Although Warburg-type aerobic glycolysis fosters greater proliferation and biomass formation, at mitosis the energy-demanding events of chromosome separation and cytokinesis create an acute need for ATP. As the spindle apparatus forms in prophase, microtubules assemble and free tubulin decreases. Consequently, VDAC inhibition by tubulin is relieved, which turns on aerobic ATP-generating mitochondrial metabolism. In this way, rapidly proliferating cells may transiently suspend Warburg-type aerobic glycolysis in favor of oxidative phosphorylation to meet the uniquely high bioenergetic demands of cells at metaphase. Afterwards, as microtubules of the spindle apparatus depolymerize during telophase, free tubulin again increases and Warburg metabolism becomes restored (Lemasters, et al., 2012).

Adenine nucleotide translocator

Approximately 20 carriers mediate flux of anionic metabolites through the mitochondrial inner membrane whose activities are well characterized in isolated mitochondria and/or reconstituted liposomes (Palmieri, 2012). Most carriers belong to the SLC25 family of nuclear-encoded transporters, also known as the mitochondrial carrier family (Walker and Runswick, 1993). ADP/ATP transport through the inner membrane is catalyzed by ANT, the most abundant inner membrane protein on a molar basis accounting for about 10% of the total. Exchange of ADP and ATP between the matrix and intermembrane space is highly selective and occurs on a 1:1 molar ratio that maintains the adenine nucleotide pool in the matrix constant. ANT translocates specifically free ADP^{-3} and ATP^{-4}. AMP and Mg^{+2} complexes of ADP and ATP are not transported by ANT (Klingenberg, 1989;Klingenberg, 2008).

ANT is an electrogenic transporter that exchanges ADP^{-3} for ATP^{-4}. As a consequence during each cycle of ATP release and ADP uptake, one negative charge is expelled from the matrix to the cytosol. Thus, the negative mitochondrial ΔΨ drives ATP release and ADP uptake such that ATP/ADP and ΔGp are greater in the cytosol than the matrix (Kawamata, et al., 2010;Klingenberg, 2008). In humans, ANT has 4 isoforms, ANT1 through ANT4, that

are encoded by different nuclear genes (Palmieri, 2012). The tissue distribution of the isoforms differs from tissue to tissue: ANT1 is expressed in skeletal muscles, heart and brain, ANT2 is expressed mainly in liver and in proliferating tissues, and ANT3 is ubiquitous and expressed at low levels (Stepien, et al., 1992;Chevrollier, et al., 2005). ANT4, recently discovered in humans, is found mainly in liver, testis and brain (Dolce, et al., 2005).

ANT2 expressed in proliferating cells, such as lymphocytes and tumor cells, is considered a marker of cell proliferation, and most non proliferating tissues with the exception of liver have low or very low expression of ANT2 (Barath, et al., 1999;Battini, et al., 1987). In tumor cell lines originating from colon (HT29), breast (MCF7) and liver (HepG2), ANT2 mRNA is more abundant than ANT1 (Giraud, et al., 1998). Increased ANT2 expression is also reported for cancers of the bladder, thyroid gland, lung, ovary, breast and testis (Le, et al., 2006).

In yeast, two isoforms of ANT, AAC1 and AAC2, are expressed only under aerobic conditions, whereas AAC3, the equivalent of ANT2 in mammalian cells, is expressed exclusively during anaerobiosis (Kolarov, et al., 1990;Lawson and Douglas, 1988). AAC3 is essential to maintain yeast cell proliferation on a fermentable substrate, which led to the hypothesis that the AAC3 isoform imports glycolytic ATP into mitochondria to support the anabolic functions of mitochondria (Drgon, et al., 1991). In mice, ANT2 deficiency is embryonically lethal, whereas ANT1 disruption causes mitochondrial myopathy in viable offspring (Kokoszka, et al., 2004).

Adenine nucleotide translocator and mitochondrial membrane potential

Carboxyatractyloside and bongkrekic acid inhibit mitochondrial ATP/ADP exchange by binding respectively to different inhibitory sites on the intermembranous and matrix sides of ANT. Both ANT inhibitors block ADP-stimulated respiration in isolated mitochondria similarly to oligomycin, the F_1F_O-ATP synthase inhibitor. In intact rat hepatocytes, respiratory inhibition by myxothiazol only slightly decreases $\Delta\Psi$, measured by TMRM uptake. Maintenance of $\Delta\Psi$ despite respiratory inhibition is due to ATP hydrolysis by the F_1F_O-ATP synthase working in reverse, since subsequent oligomycin addition leads to complete collapse of $\Delta\Psi$. Similarly, carboxyatractyloside and bongkrekic acid collapse $\Delta\Psi$ in myxothiazol-treated hepatocytes, signifying that cytosolic ATP enters mitochondria by ANT to be hydrolyzed by the F_1F_O-ATP synthase (Maldonado et al., 2013).

In HepG2 cells and A549 human lung cancer cells, myxothiazol also slightly decreases $\Delta\Psi$, and subsequent oligomycin collapses $\Delta\Psi$ (Fig. 3 and 4, and not shown). In marked contrast to hepatocytes, however, carboxyatractyloside and bongkrekic acid fail to collapse $\Delta\Psi$ (Fig. 3 and not shown). Nonetheless, subsequent oligomycin does lead to full depolarization (Fig. 3). Similarly, 2-deoxyglucose, a glycolytic inhibitor, added after myxothiazol alone, myxothiazol plus carboxyatractyloside or myxothiazol plus bongkrekic acid leads to $\Delta\Psi$ collapse (Fig. 4 and not shown). These results show that although mitochondrial hydrolysis of glycolytic ATP can maintain $\Delta\Psi$, entry of glycolytic ATP into mitochondria occurs by a pathway other than ANT, possibly through the ATP-Mg/Pi carrier (Joyal and Aprille, 1992;Palmieri, 2012).

A similar pattern is observed from measurements of oxygen uptake. In hepatocytes, oligomycin, carboxyatractyloside and bongkrekic acid each decrease respiration to a comparable extent, signifying inhibition of respiration-linked mitochondrial ATP synthesis and the release of such ATP to the cytosol. By contrast in tumor cell lines, carboxyatractyloside and bongkrekic acid do not inhibit respiration, unlike oligomycin which does. Thus, although ANT2 is abundant in cancer cells, it is inactive and not the principal ATP transporter responsible for mitochondrial uptake of glycolytic ATP required to maintain mitochondrial $\Delta\Psi$ after respiratory inhibition (Maldonado, et al., 2009;Maldonado, et al, 2013b). Nonetheless, ATP does gain entry into tumor mitochondria by a yet unidentified alternative carrier, possibly via the electroneutral ATP-Mg/Pi carrier (Joyal and Aprille, 1992;Fiermonte, et al., 2004). Overall, the substitution of an electrogenic pathway of ATP/ADP exchange (ANT) for a non-electrogenic pathway (possibly the ATP-Mg/Pi carrier) would mean loss of ΔG_P amplification. The resultant lower cytosolic ATP/ADP ratios then favor Warburg-type aerobic glycolysis (Fig. 1).

Conclusion

The ATP/ADP ratio has been neglected as an important regulator of glycolysis in cancer cells despite the importance of ATP/ADP in the control of both glycolysis and oxidative phosphorylation in non-transformed cells. Enhanced aerobic glycolysis confers an anabolic advantage for proliferating cells but can only be sustained if cytosolic ATP/ADP ratios are lower than in non-proliferating cells. Beyond upregulation of genes and enzymes of the glycolytic pathway, mitochondrial ATP production is likely a major factor controlling glycolysis. High ATP/ADP ratios in aerobic non-proliferating cells block glycolysis even in the presence of increased expression of transporters and enzymes involved in glycolysis. In this topical review, we describe two novel mechanisms that contribute to mitochondrial suppression and low ATP/ADP ratios in proliferating cells. The first mechanism is the relative closure of VDAC by the high free tubulin levels characteristic of proliferating cells. Such VDAC closure exerts a global suppression of mitochondrial metabolism. The second mechanism is the loss of function of electrogenic ANT2 and its apparent replacement by a non-electrogenic ATP-ADP exchange pathway, the ATP-Pi/Mg carrier (Fig. 1). The roles of VDAC and ANT in producing Warburg metabolism in cancer cells makes these two proteins potential targets for the development of a new generation of anti-Warburg anti-cancer drugs.

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HIGHLIGHTS

- VDAC mediates flux of metabolites across the mitochondrial outer membrane
- Electrogenic ANT exchanges matrix ATP for cytosolic ADP across the inner membrane
- High cytosolic ATP/ADP generated by oxidative phosphorylation inhibits glycolysis
- VDAC closure and ANT inactivation lead to low ATP/ADP in proliferating cells
- In cancer cells, low ATP/ADP favors the aerobic glycolytic Warburg phenotype

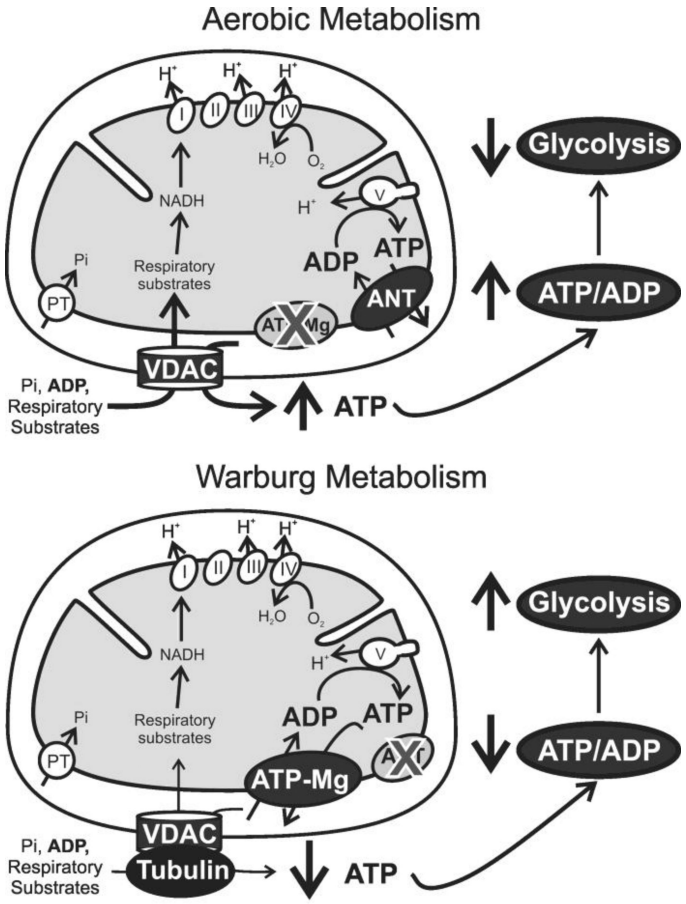

Fig. 1. VDAC closure and inactivation of ANT suppress mitochondrial metabolism and activate glycolysis in the Warburg phenomenon

Respiratory substrates, ADP and Pi first cross mitochondrial outer membranes via VDAC and then mitochondrial inner membranes via individual transporters, including the ANT. Respiratory substrates generate mostly NADH, which feeds into the respiratory chain (Complexes I-IV). Electron transfer leads to proton translocation from the matrix into the intermembrane space, generating Δp as oxygen is reduced to water. Protons return into the matrix through the F_1-F_0-ATP synthase (Complex V) driving synthesis of ATP from ADP and Pi. In aerobic metabolism by non-proliferating differentiated cells (top scheme), newly synthesized ATP exchanges for ADP via ANT and subsequently moves into the cytosol through VDAC. A strongly negative mitochondrial ΔΨ drives ANT-mediated outward electrogenic exchange of ATP^{-4} for inwardly directed ADP^{-3}, which increases cytosolic relative to mitochondrial ATP/ADP ratios by ~100-fold. In proliferating cells (bottom scheme), high free tubulin causes a relative blockade of VDAC conductance. In addition, eletrogenic ATP/ADP exchange by ANT becomes inactivated and is replaced by electroneutral ATP/ADP exchange likely mediated by the ATP-Mg/Pi carrier. Relative VDAC closure and loss of ANT function together produce global suppression of mitochondrial metabolism and decrease cytosolic ATP/ADP ratios that promote Warburg-type aerobic glycolysis.

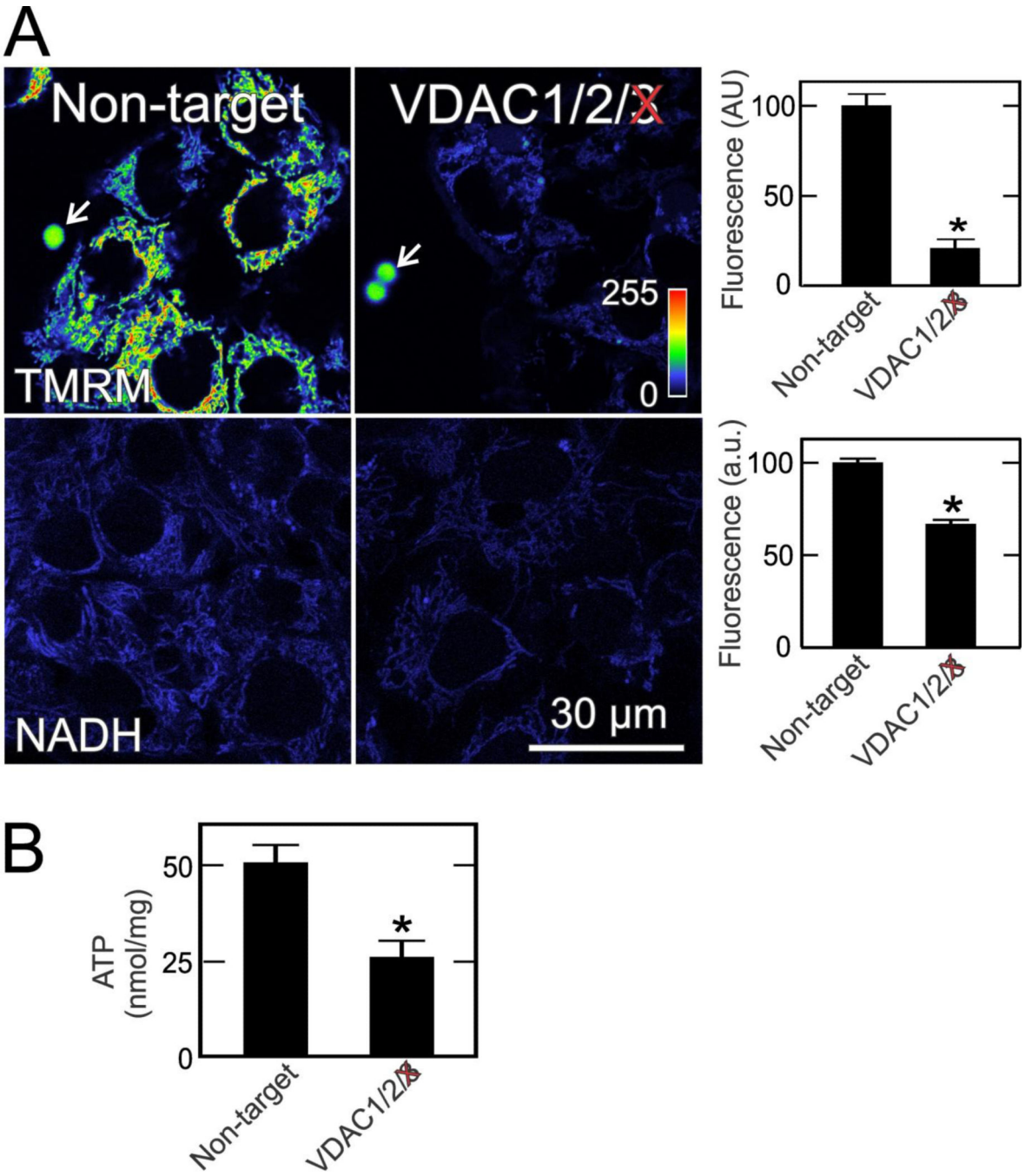

Fig 2. VDAC3 knockdown decreases mitochondrial membrane potential, NADH and ATP in HepG2 cells

In **A,** HepG2 hepatoma cells were transfected with non-target siRNA and siRNA against VDAC3. After 48 h, ΔΨ-indicating TMRM and mitochondrial NAD(P)H-indicating blue autofluorescence were imaged by confocal and multiphoton microscopy, respectively. Note the decrease of TMRM fluorescence and autofluorescence after VDAC3 knockdown, which is quantified in the right panels. Arrows identify 4-μm fiduciary fluorescent beads. In **B**, total cellular ATP is shown under the same conditions. *, $p<0.05$. Adapted from (Maldonado, et al., 2013).

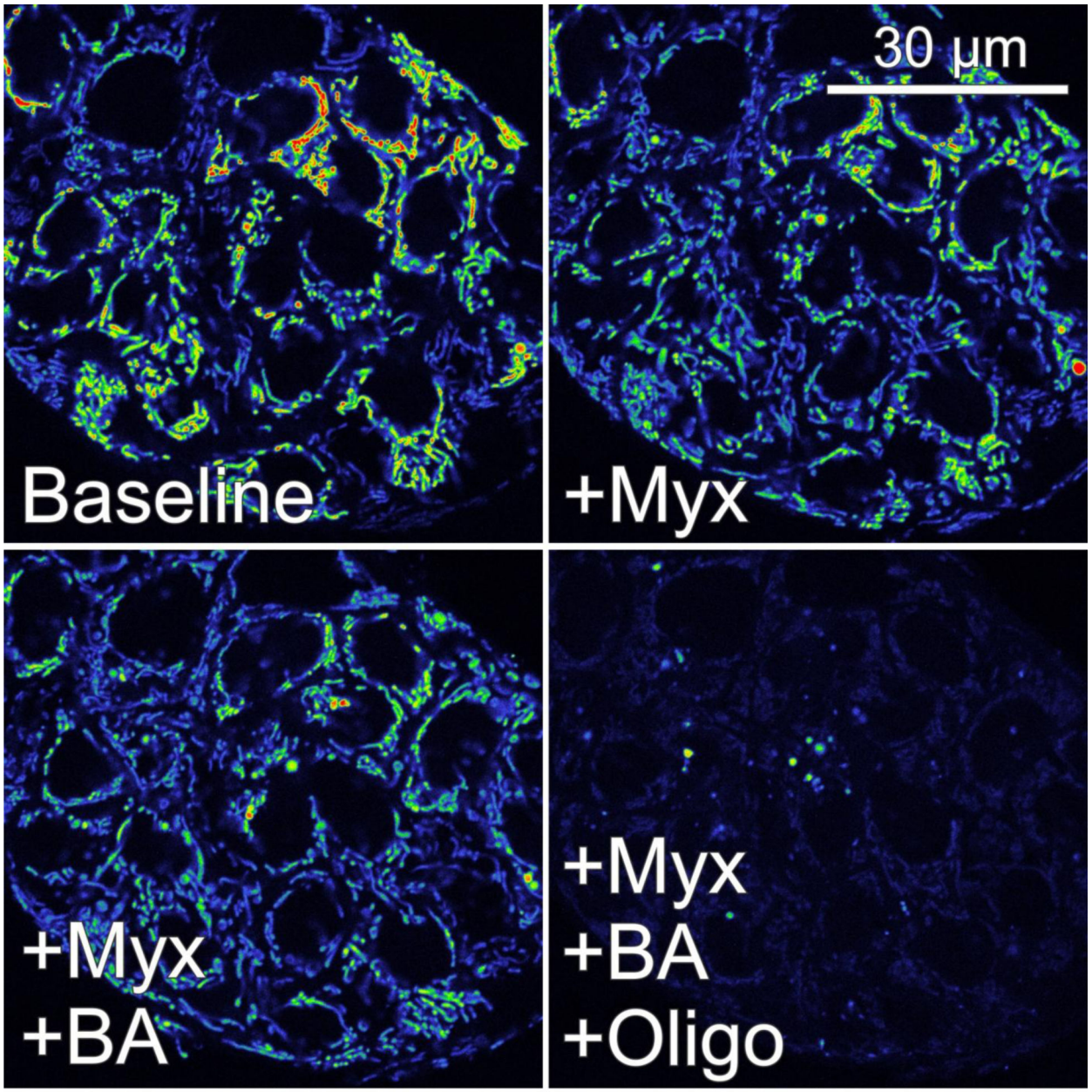

Fig. 3. ANT-independent ATP hydrolysis sustains mitochondrial membrane potential in HepG2 hepatoma cells after respiratory inhibition

HepG2 cells were loaded with TMRM, as described in Fig. 2. Note a small of decrease of TMRM fluorescence after myxothiazol (Myx, 10 μM). Persisting mitochondrial TMRM uptake indicates that ATP hydrolysis supports ΔΨ formation during respiratory inhibition (compare right and left upper panels). Subsequent bongkrekic acid (BA, 5 μM), an ANT inhibitor, does not further decrease ΔΨ, indicating that ATP supply to mitochondria is independent of ANT (bottom left panel). Subsequent oligomycin (Oligo, 10 μg/ml) collapses ΔΨ, confirming that ANT-independent ATP entry into mitochondria maintains ΔΨ after respiratory inhibition (right bottom panel). Additions are 30 min apart. Adapted from (Maldonado, et al., 2013).

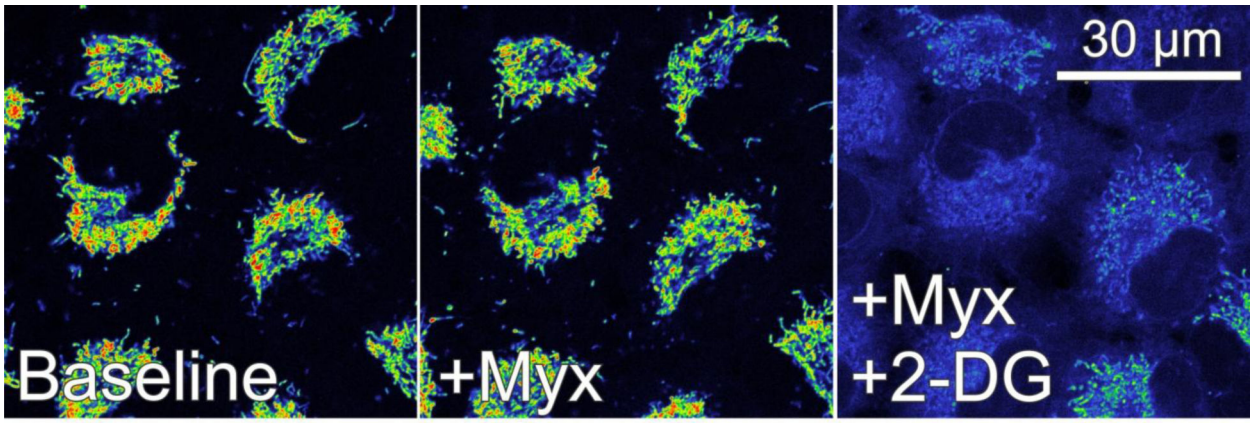

Fig. 4. Glycolytic ATP supports mitochondrial membrane potential after respiratory inhibition in A549 cells

A549 lung cancer cells were loaded with TMRM, as described in Fig. 2. Note that myxothiazol (Myx) slightly decreases TMRM fluorescence similarly to HepG2 cells (compare left and center panel). Subsequent 2-deoxyglucose (2-DG, 50 mM), a glycolytic inhibitor, collapses $\Delta\Psi$ virtually completely, indicating that mitochondrial hydrolysis of glycolytic ATP supports mitochondrial $\Delta\Psi$ formation after respiratory inhibition.