Chapter 5
Voltage-Dependent Anion Channels and Tubulin: Bioenergetic Controllers in Cancer Cells

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5.1 Introduction

5.1.1 Warburg Phenotype and Cell Proliferation

The interdependence between bioenergetics, catabolism, and anabolism differs in cancer and other proliferating cells compared to differentiated cells. A metabolic phenotype characterized by enhanced glycolysis and suppression of mitochondrial

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metabolism even in the presence of physiological levels of oxygen was first described by Otto Warburg in the early twentieth century (Warburg et al. 1927; Warburg 1956). Warburg also postulated that irreversible but not completely damaged respiration led to cancer. According to Warburg, cells compensate for lower energy production associated with damaged respiration by increasing the conversion of glucose to lactic acid (fermentation). Cells capable of increasing fermentation through successive divisions to compensate for defective respiration eventually become neoplastic (Warburg 1956). The lack of function of mitochondria in tumor tissues was challenged by Weinhouse and others demonstrating both high glycolysis and oxidative metabolism in cancer tissues (Weinhouse 1956). Since the early work of Warburg, several investigations showed active mitochondrial metabolism in cancer cells and their isolated mitochondria as determined by measurements of ATP generation, NADH production, and mitochondrial membrane potential ($\Delta\Psi$) among other functional parameters (Lim et al. 2011; Maldonado et al. 2010; Mathupala et al. 2010; Moreno-Sanchez et al. 2014; Nakashima et al. 1984; Pedersen 1978; Singleterry et al. 2014).

Although functional, the contribution of mitochondria to ATP generation in cancer cells through oxidative phosphorylation (OXPHOS) is lower compared to differentiated cells. Differentiated cells produce about 95% of total ATP by OXPHOS and the remaining 5% through aerobic glycolysis. By contrast in cancer and other proliferating cells, 20–90% of total ATP production derives from glycolysis with the remainder coming from mitochondrial oxidation of pyruvate, fatty acids, and glutamine (6, 11). Accordingly, tumor cells have increased uptake of glucose compared to differentiated cells. This glucose avidity of tumors can be used to diagnose primary tumors, recurrences, and metastases by positron emission tomography (PET) of the glucose analog ^{18}fluorodeoxyglucose (Zhu et al. 2011). Enhanced glycolysis in cancer cells is associated with a high rate of cell proliferation (Griguer et al. 2005; Guppy et al. 2002; Moreno-Sanchez et al. 2007; Scott et al. 2011). Nonetheless, bioenergetic profiles can be different among tumor types and even in cells from the same type of tumor. Subsets of cells with either high glycolysis or high levels of OXPHOS have been identified in gliomas and large B cell lymphomas (Beckner et al. 2005; Bouzier et al. 1998; Caro et al. 2012).

Incomplete breakdown of glucose through glycolysis generates only 2 moles of ATP per mole of glucose, whereas mitochondrial oxidation of the 2 moles of pyruvate generated from glucose to CO_2 and H_2O generates about an additional 31 moles of ATP taking into account currently accepted proton stoichiometries for respiration, ATP synthesis, ATP/ADP•Pi exchange, and the malate/aspartate shuttle, although actual ATP yields will be less due to proton leak and possible molecular "slippage" of the respiratory complexes (Brand 2005; Rich 2003; Rich and Marechal 2010; Walker 2013; Wikstrom et al. 2015). In cancer cells, lower efficiency of ATP generation by aerobic glycolysis appears to be offset by greater glycolytic rates (Locasale and Cantley 2010). It is also proposed that the ATP necessary for biosynthesis of macromolecules is lower than the energy requirements of basal cellular processes making unlikely that ATP generation is rate limiting in proliferating cells (Kilburn et al. 1969).

The metabolic requirements of cell division are not simply limited to energy generation. A dividing cell must double its biomass (lipids, proteins, and nucleic acids) before mitosis. This biosynthetic demand requires carbon backbones for the synthesis of new macromolecules. Full oxidation of glucose, glutamine, and fatty acids in mitochondria generates maximum ATP but not residual carbon backbones. By contrast, incomplete breakdown of glucose to lactate and possibly decreased mitochondrial degradation of glutamine and fatty acids provides precursors for biomass formation (Cairns 2015; DeBerardinis et al. 2008; Keibler et al. 2016; Liberti and Locasale 2016; Lunt and Vander Heiden 2011). Specifically, the by-products of glucose catabolism, glucose-6-phosphate, glyceraldehyde-3-phosphate, and 3-phosphoglycerate contribute to the synthesis of nucleotides, lipids, and amino acids, respectively. High glycolytic flux also increases NADPH production by the pentose phosphate pathway for reductive biosynthesis. Glutamine and other fuels also generate biosynthetic precursors in the Krebs cycle, including citrate for lipid biosynthesis and oxaloacetate and α-ketoglutarate for synthesis of nonessential amino acids (Fig. 5.1) (DeBerardinis and Cheng 2010). In addition, one-carbon metabolism, a set of reactions that transfer one carbon units from serine and glycine, plays an important role for de novo synthesis of purines and thymidylate during rapid tumor growth (Meiser and Vazquez 2016). In summary, the Warburg metabolic phenotype is a complex network of interrelated processes involving glycolysis and mitochondrial metabolism.

5.1.2 Cytosolic ATP/ADP Ratio: A Key to Sustain Glycolysis

Maximal mitochondrial oxidation of respiratory substrates, including pyruvate, fatty acyl-CoA, glutamine, and amino acids, by OXPHOS generates a maximum yield of ATP per mole of respiratory substrates and minimal residual carbon backbones. Newly synthesized ATP in the mitochondrial matrix is transported to the cytosol by the electrogenic adenine nucleotide translocator (ANT) because of the coupling to $\Delta\Psi$ of mitochondrial ATP^{-4} release for ADP^{-3} uptake. In differentiated cells with predominantly oxidative metabolism, cytosolic ATP/ADP ratios can be 50–100 times higher than in the mitochondrial matrix (Schwenke et al. 1981). A high cytosolic ATP/ADP ratio suppresses glycolysis through inhibition of phosphofructokinase-1 (PFK-1) although other mechanisms may be involved. ATP is a strong allosteric inhibitor, and ADP and AMP are activators of PFK-1 (Mor et al. 2011; Moreno-Sanchez et al. 2007). In cancer cells, suppression of mitochondrial metabolism contributes to a low cytosolic ATP/ADP ratio, which releases this brake on glycolysis. Recently, we demonstrated that closing of the voltage-dependent anion channels (VDAC) promoted by free tubulin limits ingress of respiratory substrates into mitochondria and limits ATP production, whereas replacement of electrogenic ATP/ADP exchange by ANT with a non-electrogenic exchange mechanism decreases cytosolic ATP/ADP

ratios. These two independent mechanisms contribute to suppress mitochondrial metabolism and to maintain a low cytosolic ATP/ADP ratio favoring aerobic glycolysis in cancer cells (Maldonado et al. 2013, 2016; Maldonado and Lemasters 2014).

5.2 VDAC Modulation of Cancer Bioenergetics

5.2.1 VDAC and the Warburg Phenotype

The bioenergetics of cancer cells depends on chemical reactions occurring in two functional, interconnected, and interdependent cellular compartments separated by the mitochondrial outer membrane (MOM) (Fig. 5.1). VDAC, the most abundant protein in the MOM, is the gateway through which most respiratory substrates, ADP, and Pi enter mitochondria and ATP exits. The subcellular localization of VDAC determines that the closing or the opening of the channels regulates the flux of metabolites that enter or leave mitochondria. Thus, VDAC is positioned to be a global controller or governator of mitochondrial metabolism and whole cellular bioenergetics (Lemasters and Holmuhamedov 2006; Maldonado et al. 2013; Maldonado and Lemasters 2012, 2014).

The influx of polar metabolites through VDAC is determined mostly by their charge and size (Colombini 1980, 2004). Metabolites that reach the intermembrane space are further transported to the matrix by numerous different transporters located in the mitochondrial inner membrane (MIM). Respiratory substrates in the matrix are catabolized in the Krebs cycle generating NADH and $FADH_2$ that enters the respiratory chain. The transfer of electrons from NADH and $FADH_2$ to the final acceptor O_2 produces proton translocation across MIM by Complexes I, III, and IV to generate a negative transmembrane $\Delta\Psi$ and positive ΔpH, the components of the proton motive force (Δp). Δp then drives ATP synthesis from ADP and Pi by Complex V (F_1F_O-ATP synthase) (Fig. 5.1).

Based on its role in metabolite exchange between mitochondria and the cytosol, VDAC is proposed to be a regulated governor or "governator" that limits global mitochondrial metabolism (Lemasters and Holmuhamedov 2006). Interactions with tubulin and possibly other proteins, such as hexokinase (Pastorino and Hoek 2003; Wolf et al. 2011), modulate the open/closed sate of VDAC. Single and double knockdown of the three different VDAC isoforms support this concept that VDAC serves as a master regulator of mitochondrial metabolism in cancer cells (Maldonado et al. 2013). Thus, VDAC regulation by free tubulin emerges as a mechanism to block or promote OXPHOS and indirectly regulate glycolysis through the cytosolic ATP/ADP ratio. Ultimately, disruption of VDAC-tubulin interactions may be a pharmacological target to increase mitochondrial metabolism in cancer cells and to revert Warburg metabolism.

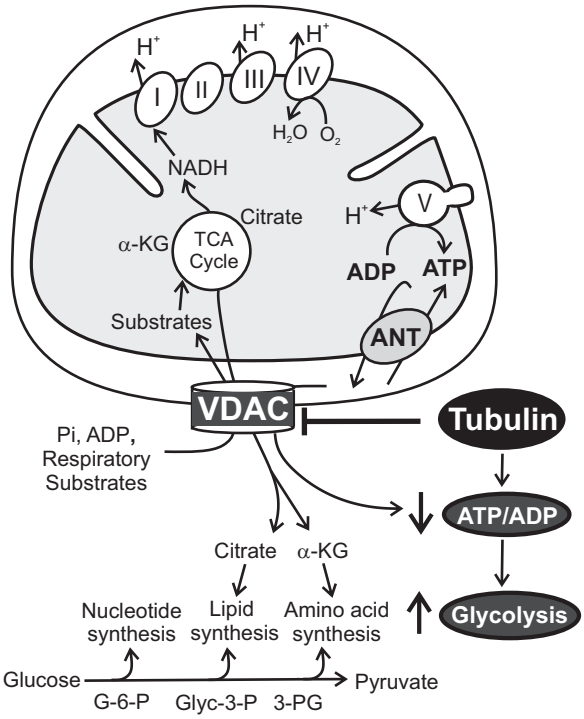

Fig. 5.1 VDAC in Warburg metabolism. Metabolites cross mitochondrial outer membranes through VDAC. Oxidation of respiratory substrates in the tricarboxylic acid cycle generates NADH and $FADH_2$, which feed into the respiratory chain (Complexes I–IV). Proton translocation by the respiratory chain across MIM generates $\Delta\Psi$. ATP is synthesized from ADP and Pi by the F_1F_O-ATP synthase (Complex V) driven by protons moving back across MIM into the matrix. Glucose-6-phosphate (G-6-P), glyceraldehyde 3-phosphate (Glyc-3-P), and 3-phosphoglycerate (3-PG) originating from the catabolism of glucose and intermediates of the Krebs cycle are used for synthesis of nucleotides, lipids, and amino acids. In cancer cells, high free tubulin blocks VDAC conductance, suppresses mitochondrial metabolism, and decreases cytosolic ATP/ADP to favor glycolysis. *α-KG* α-ketoglutarate; *MIM* Mitochondrial inner membrane

5.2.2 *VDAC Structure and Regulation of Mitochondrial Metabolism*

The three isoforms of VDAC present in all eukaryotic cells, VDAC1, VDAC2, and VDAC3, are encoded by separate genes. VDAC1 and VDAC2 are the main isoforms in most differentiated mammalian cells. The minor isoform VDAC3 is abundant only in testis (Sampson et al. 1997, 2001). In cancer cells VDAC1 and VDAC2 are also the major isoforms accounting for 90% of the total. The least abundant isoform, VDAC3, comprises the remaining 10% (De Pinto et al. 2010; Huang et al. 2014; Maldonado et al. 2013). Gating and selectivity of VDAC1 and VDAC2 are highly conserved among mammals (Blachly-Dyson and Forte 2001).

VDAC in humans and mice is a ~30 kDa protein enclosing an aqueous channel of ~3-nm internal diameter that allows the passage of molecules up to ~5 kDa (Colombini 1980, 2012; Song and Colombini 1996). In the closed state, the flux through VDAC of respiratory substrates, ATP, ADP, Pi, and other mostly anionic metabolites is blocked. Structural studies reveal that VDAC1 has a barrel configuration with staves formed by 19 β-strands (Hiller et al. 2010; Ujwal et al. 2008). An additional N-terminal sequence forms the only α-helical segment. The N-terminal helix appears to move to the center of the channel, blocking the passage of metabolites. Recently, a similar β barrel structure with 19 β-strands has been shown for VDAC2 from zebra fish (Schredelseker et al. 2014).

Because of its localization in the MOM and central role in mediating mitochondria-cytosol fluxes of metabolites, VDAC was initially considered constitutively open, but numerous studies show regulation by multiple factors, including hexokinase (Al Jamal 2005; Azoulay-Zohar et al. 2004; Nakashima et al. 1988), Bcl2 family members (Tsujimoto and Shimizu 2000), glutamate (Gincel et al. 2000), ethanol (Holmuhamedov and Lemasters 2009; Lemasters and Holmuhamedov 2006), and NADH (Zizi et al. 1994). VDAC phosphorylation by protein kinases, including glycogen synthase 3β (GSK3β), protein kinase A (PKA), and protein kinase C epsilon (PKCε), blocks or inhibits association of VDAC with other proteins, such as Bax and *t*Bid, and also regulates VDAC opening (Azoulay-Zohar et al. 2004; Baines et al. 2003; Das et al. 2008; Lee et al. 1994; Rostovtseva et al. 2004; Vander Heiden et al. 2000, 2001). PKA-dependent VDAC phosphorylation decreases VDAC conductance (Bera et al. 1995), whereas GSK3β-mediated VDAC2 phosphorylation induces VDAC opening (Das et al. 2008). Here, we will focus on the inhibitory effect of free tubulin on VDAC in cancer cells as a regulatory mechanism of VDAC opening (Maldonado et al. 2010, 2013; Palmieri and Pierri 2010).

5.3 VDAC-Tubulin Interaction

5.3.1 VDAC Inhibition by Free Tubulin

Mitochondrial $\Delta\Psi$ in cancer cells can be generated both by the respiratory chain and from hydrolysis of glycolytic ATP by the mitochondrial F_IF_O-ATPase working in reverse. Pharmacological interventions to destabilize microtubules with nocodazole and colchicine or stabilize microtubules with paclitaxel increase and decrease, respectively, cytosolic free tubulin. Such high and low cytosolic free tubulin promotes low and high mitochondrial $\Delta\Psi$, respectively (Maldonado et al. 2010). In nonproliferating cells like cultured rat hepatocytes, free tubulin is much lower compared to hepatoma cells, since nonproliferating hepatocytes do not need a reservoir of tubulin for spindle formation at mitosis. Thus, microtubule stabilization with paclitaxel does not increase $\Delta\Psi$ in hepatocytes, because free tubulin is already very low, whereas microtubule destabilization still increases tubulin and, in turn,

decreases ΔΨ. These findings imply that VDAC is indeed constitutively open in nonproliferating hepatocytes under normal incubation. By contrast, since paclitaxel increases and nocodazole/colchicine decreases ΔΨ in tumor cells, the conclusion can be made that VDAC is partially closed in tumor cells under the regulation of endogenous free tubulin (Maldonado et al. 2010). Negative modulation of ΔΨ by tubulin through VDAC closure is a mechanism that explains, at least in part, the suppression of mitochondrial metabolism in the Warburg phenotype. Our studies performed in intact cancer cells are in agreement with earlier work showing that heterodimeric αβ-tubulin closes VDAC inserted into lipid bilayers and decreases respiration in isolated brain mitochondria and permeabilized synaptosomes (Rostovtseva et al. 2008; Timohhina et al. 2009).

Knockdown studies of VDAC1, VDAC2, and VDAC3 in HepG2 cells further characterized the role of VDAC in mitochondrial metabolism in cancer cells. Single knockdown of each of the three VDAC isoforms, especially the minor isoform VDAC3, decreased mitochondrial ΔΨ, indicating that all VDAC isoforms contribute to ΔΨ formation. Knockdown of VDAC3 not only caused the greatest drop in ΔΨ but also decreased cellular ATP and ADP and the NAD(P)H/NAD(P)$^+$ ratio, suggesting that the VDAC3 contributed most to MOM permeability despite being the least abundant isoform (Maldonado et al. 2013). Double knockdown of VDAC isoforms in all possible combinations allowed determination of the response of each individual isoform to tubulin inhibition. All single and double knockdowns partially blocked suppression of ΔΨ induced by increased free tubulin (Maldonado et al. 2013). Further studies showed an almost identical voltage gating and response to dimeric αβ-tubulin of constitutive VDAC isolated from wild-type HepG2 cells compared to VDAC from heart and liver mitochondria. VDAC1 and VDAC2 isolated from double knockdown HepG2 cells inserted in lipid bilayers were almost equally sensitive to tubulin inhibition, whereas VDAC3 was insensitive even at tubulin concentrations fivefold higher than those used to inhibit VDAC1 and VDAC2 (Maldonado et al. 2013). The knockdown studies supported the conclusion that VDAC3, at least in HepG2 cells, is constitutively open, whereas VDAC1 and VDAC2 are totally or partially closed by free tubulin.

5.3.2 VDAC-Tubulin Influence on Warburg Metabolism During Cell Cycle

During the cell cycle, biosynthetic processes to generate a new cell occur during G1, S, and G2. Presumably, Warburg metabolism is maximal during these phases, and mitochondrial metabolism is suppressed. VDAC closing by a pool of constitutive free tubulin appears to contribute to mitochondrial suppression during these growth stages. Most of the cell cycle of cancer cells is composed by G1, S, and G2 phases. The actual cell division occurs during the M or mitotic phase lasting only about 30 min of a cell cycle lasting 30 h or more (Hahn et al. 2009). During mitosis,

energy demand increases sharply to support chromosome separation and cytokinesis. At this point, a Warburg metabolic phenotype may not be beneficial since all the new macromolecules have been already synthesized. Moreover, mitochondrial activation and full oxidation of respiratory substrates may be required to meet the ATP demands of cell division. A possible scenario is that as the spindle forms during prophase, the free tubulin pool decreases abruptly, releasing tubulin inhibition of VDAC. VDAC opening then promotes increased mitochondrial metabolism reverting the Warburg phenotype precisely when the energy demand is maximal. After mitosis, the pool of free tubulin increases again, and cells return to a high glycolytic, pro-proliferative phenotype during the non-mitotic stages of the cell cycle (Maldonado and Lemasters 2012).

5.3.3 Mitochondrial Contribution to Metabolic Heterogeneity in Tumors

The extent to which cancer cell metabolism is glycolytic or oxidative is not a permanent feature and is under epigenetic control. Tumor cells are metabolically flexible, and the relative contribution of OXPHOS can vary substantially over time depending on multiple factors, including availability to different fuels, proximity to newly formed vs. mature blood vessels, and the release of soluble factors such as lactate from neighboring cells, both cancerous and noncancerous. Hypoxia can decrease the OXPHOS flux depending on time of hypoxic exposure, cell type, and environmental conditions. In MCF-7 and HeLa cells that predominantly depend on OXPHOS for ATP supply, prolonged hypoxia increases glycolysis only in MCF-7 (Rodriguez-Enriquez et al. 2010). The respiratory chain of tumor cells can be fully functional at oxygen levels as low as 0.5%, which is biologically relevant because in solid tumors with heterogeneous perfusion, tumor cells exposed to 2% or less of oxygen can still produce ATP by OXPHOS.

Inadequate blood perfusion in rapidly growing tumors not only exposes cells to hypoxia but to a less frequently considered lower supply of nutrients such as glucose. The importance of nutrient availability on the bioenergetic profile of cancer cells is illustrated by the switch from aerobic glycolysis to OXPHOS in breast cancer cell lines and lymphoma cells cultured in glucose-free media (Robinson et al. 2012; Smolkova et al. 2010). Tumor cells also adapt to oxidize other substrates when glucose or glutamine are limited, including lactate, methionine, asparagine, leucine, arginine, cysteine, acetate, and even proteins and lipids from the environment (Chung et al. 2005; Clavell et al. 1986; Comerford et al. 2014; Commisso et al. 2013; Keenan and Chi 2015; Kennedy et al. 2013; Kreis et al. 1980; Mashimo et al. 2014; Scott et al. 2000; Sheen et al. 2011; Sonveaux et al. 2008). While glucose deprivation promotes a switch to oxidative metabolism, inhibition of Complex III by antimycin and Complex I by piericidin A triggers a compensatory increase in the uptake and consumption of glucose in myoblasts. Total cellular ATP production

before and after OXPHOS inhibition was similar indicating that the loss of ATP generation by OXPHOS was fully compensated by increased glycolytic ATP generation (Liemburg-Apers et al. 2015). This metabolic flexibility of tumors and the potential to switch from a predominantly glycolytic to an oxidative metabolism and vice versa underscore the importance of mechanisms like VDAC regulation that underlie these adaptive changes.

Most research efforts to target tumor metabolism have been directed toward inhibition of glycolysis (Doherty and Cleveland 2013; Pelicano et al. 2006). Only recently has mitochondrial metabolism emerged as a chemotherapeutic target (Bhat et al. 2015; Weinberg and Chandel 2015). Most approaches attempt to inhibit mitochondrial metabolism in cancer cells. The observation that the antidiabetic drug metformin decreased the prevalence of certain types of cancer triggered an interest in the role of mitochondrial inhibition as a mechanism to suppress abnormal cell proliferation (Giovannucci et al. 2010; Libby et al. 2009). Although metformin decreases OXPHOS by inhibiting Complex I of the respiratory chain, metformin also inhibits the mammalian target of rapamycin (mTOR), interferes with folate metabolism, and activates AMP kinase (AMPK) (Jara and Lopez-Munoz 2015). Other approaches to inhibit mitochondrial metabolism in various cancer cell models include etomoxir to inhibit carnitine *O*-palmitoyltransferase 1 and consequent mitochondrial fatty acid oxidation (leukemia), tigecycline to inhibit mitochondrial protein translation (leukemia), glutaminase inhibitors (breast cancer, lymphoma), and the compound VLX600 to inhibit OXPHOS (colon cancer) (Samudio et al. 2010; Skrtic et al. 2011; Wang et al. 2010; Zhang et al. 2014). By contrast, other anticancer, antiproliferative strategies attempt to promote mitochondrial metabolism. For example, the pyruvate analog dichloroacetate activates pyruvate dehydrogenase to increase mitochondrial metabolism, which promotes cell killing in several cancer cell lines and in some in vivo models (Sutendra and Michelakis 2013).

5.3.4 VDAC Opening: A Metabolic Switch

The relative closure of VDAC by free tubulin in cancer cells and the broad metabolic consequences of VDAC opening make VDAC-tubulin interaction a novel pharmacological target to revert the Warburg phenotype. Antagonizing the constitutive inhibition of VDAC by free tubulin would be expected to increase mitochondrial metabolism and to have an anti-Warburg effect. Our group reported the first antagonist of the inhibitory effect of free tubulin on VDAC, the small molecule erastin (Maldonado et al. 2013). Erastin selectively induces non-apoptotic cell death in human cells engineered to harbor small T oncoprotein and the oncogenic allele of HRAS, v-Ha-ras Harvey rat sarcoma viral oncogene homologue RAS^{v12} (Dolma et al. 2003). Erastin non-apoptotic-induced cell death is blocked by antioxidants, such as α-tocopherol, butylated hydroxytoluene, and desferal, but not by pan-caspase inhibitors (Dolma et al. 2003). Other cell lines harboring the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue (KRAS) and an activating V600E

mutation in v-raf-murine sarcoma viral oncogene homologue B1 (BRAF) are moderately sensitive to erastin. Erastin is proposed to bind to VDAC2 and VDAC3, leading to oxidative stress and cell death in cells with activated RAS-RAF-MEK signaling (Yagoda et al. 2007).

Erastin in wild-type HepG2 cells and other cell lines promotes mitochondrial hyperpolarization and prevents depolarization induced by microtubule destabilizers. In addition, erastin added after microtubule destabilizers restores mitochondrial $\Delta\Psi$, indicating that erastin prevents and reverts the inhibitory effect of free tubulin on VDAC (Maldonado et al. 2013). Erastin also completely blocks the inhibitory effect of free tubulin on VDAC conductance of wild-type VDAC from HepG2 cells inserted into planar lipid bilayers. Erastin alone did not modify the voltage dependence of VDAC closure, indicating that the effect of erastin was specific for tubulin-dependent inhibition of VDAC (Maldonado et al. 2013). Following the identification of erastin as a VDAC-tubulin antagonist, we identified a group of "erastin-like" compounds using a high-throughput cell-based screening. These erastin-like compounds were selected based on their capability of hyperpolarizing mitochondria in the presence of microtubule destabilizers (DeHart et al. 2015).

5.3.5 VDAC Opening-Related Effects in Cancer Cells

VDAC opening leads to three main biological effects: increased mitochondrial metabolism, decreased glycolysis, and increased formation of reactive oxygen species (ROS). After VDAC opening, flux of pyruvate, fatty acids, and other metabolic substrates into mitochondria fuels the tricarboxylic acid cycle to produce NADH that enters the electron transport chain. Increased mitochondrial $\Delta\Psi$ and increased reduction of respiratory chain components lead to superoxide anion ($O_2\bullet^-$) generation (Chance et al. 1979; Suski et al. 2012). Quantitatively mitochondria are the most important source of ROS, with Complex III (Site III_{Qo}), Complex I (Site I_Q), and Complex II (Site II_F) being the main ROS-producing sites out of seven major mitochondrial sites (Chen et al. 2003; Quinlan et al. 2012; Tribble et al. 1988), (Skulachev 1996). $O_2\bullet^-$ formed at Complexes I and II is released to the matrix, whereas $O_2\bullet^-$ generated at Complex III is released in large part to the intermembrane space and hence to the cytosol through VDAC (Brand 2010; Han et al. 2003; Muller et al. 2004). $O_2\bullet^-$ is rapidly converted to H_2O_2 by superoxide dismutases located in the mitochondrial matrix (manganese-containing enzyme MnSOD or SOD2) and the cytosol (copper-and-zinc-containing enzyme Cu, ZnSOD, or SOD1) (Fridovich 1997). H_2O_2, the least reactive of ROS, diffuses across membranes and is a cell signaling molecule that does not necessarily disrupt redox homeostasis (Morgan et al. 2011; Veal et al. 2007). For example, H_2O_2 modulates the pro-survival HIF-1 and MAP/ERK, PI3K/akt/mTOR pathways that favor tumorigenesis and metastasis (Clerkin et al. 2008; Giles 2006; Ushio-Fukai and Nakamura 2008). Alternatively, H_2O_2 can accept an electron from free and loosely bound Fe^{2+} to form the highly

reactive hydroxyl radical (OH•) by the Fenton reaction. $O_2^{\bullet-}$ and especially the highly reactive OH• are damaging for cells.

VDAC opening promotes mitochondrial ROS formation by increasing mitochondrial $\Delta\Psi$ and the reduction of the respiratory chain. Continued ROS production eventually overcomes the antioxidant capacity of cancer cells leading to cytotoxicity. Opening of VDAC by antagonism of the inhibitory effect of free tubulin on VDAC selectively affects cancer and other proliferating cells, since free tubulin is low and does not inhibit VDAC in differentiated cells (Maldonado et al. 2010; Maldonado and Lemasters 2012). In cancer cells, ROS can be cytostatic, favor tumor growth, or be cytotoxic (Marengo et al. 2016; Panieri and Santoro 2016; Sullivan and Chandel 2014). Although basal levels of ROS are higher in cancer cells compared to differentiated cells, these higher ROS levels are compensated by the higher content of scavenging enzymes and antioxidants, including glutathione-linked enzymes that reduce protein disulfide bonds, catalase that converts H_2O_2 to H_2O and O_2, and SODs (Liou and Storz 2010; Panieri and Santoro 2016; Sullivan and Chandel 2014; Venditti et al. 2013). Oxidative stress is reported to induce cancer cell cycle arrest, senescence, apoptosis, or necrosis (Liou and Storz 2010). Chemotherapeutic agents including cisplatin, adriamycin, the anthracyclines doxorubicin, epirubicin, and daunorubicin among others promote oxidative stress and depletion of the antioxidant capacity of tumor cells leading to a tumoricidal effect (Conklin 2004; Faber et al. 1995; Ladner et al. 1989; Weijl et al. 1998).

The effects of mitochondrially generated ROS on cellular structures depend on the specific ROS. The lifetimes of H_2O_2 and $O_2^{\bullet-}$ allow them to react both with mitochondria and extramitochondrial structures. By contrast, OH• is so reactive that its effects are almost completely restricted to mitochondria. Both $O_2^{\bullet-}$ and OH• inactivate mitochondrial proteins, including ATP synthase, NADH oxidase, and NADH dehydrogenase (Zhang et al. 1990). Beyond proteins, ROS damage mitochondrial DNA and lipids in the MIM. Cardiolipin, a MIM phospholipid rich in polyunsaturated fatty, is peroxidized by ROS, and peroxidized cardiolipin is considered an early event in apoptosis (Schenkel and Bakovic 2014). Cytosolic ROS, in turn, activate members of the MAPK family of serine/threonine kinases, especially c-Jun N-terminal kinase (JNK), the extracellular signal-regulated kinase (ERK 1/ERK 2), and p38 whose signaling can cause mitochondrial dysfunction (Kamata et al. 2005; Son et al. 2011).

5.3.6 A Metabolic Double Hit: Anti-Warburg Effect and Oxidative Stress

Heterogeneity of metabolism among cells within a tumor is a complicating factor for the success of cancer chemotherapy (Dang 2012; Eason and Sadanandam 2016; Gerlinger et al. 2012; Yun et al. 2012). However, nearly all cancer cells display some level of enhanced glycolysis, suggesting some degree of contribution of VDAC closure to suppression of mitochondrial metabolism (Griguer et al. 2005;

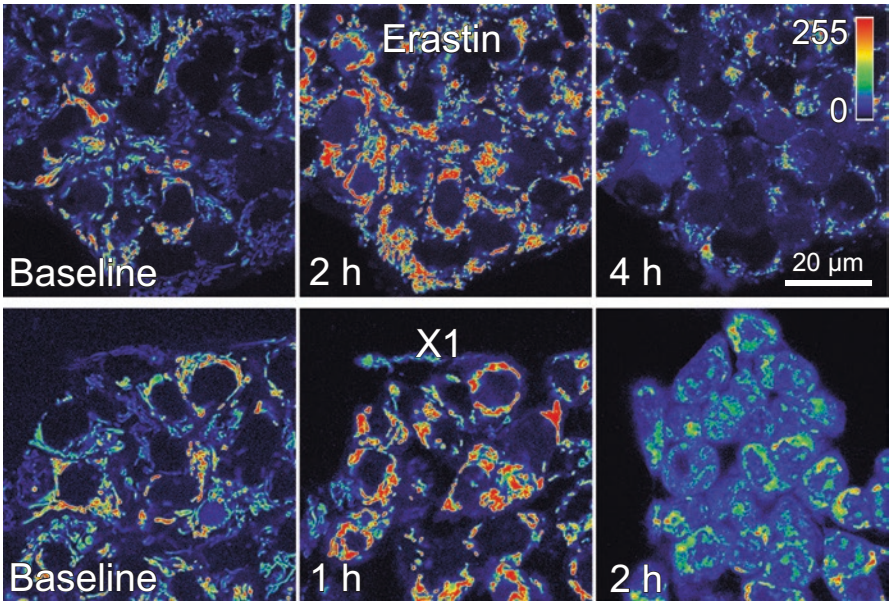

Fig. 5.2 Erastin and X1-dependent mitochondrial dysfunction. Initial mitochondrial hyperpolarization induced by erastin (*center upper panel*) and X1 (*center lower panel*) was followed by mitochondrial depolarization indicative of mitochondrial dysfunction (*right upper and lower panels*)

Guppy et al. 2002; Moreno-Sanchez et al. 2007; Scott et al. 2011). Antagonism of the inhibitory effect of tubulin on VDAC triggers two distinct and nearly simultaneous effects: (1) activation of OXPHOS with consequent decrease of glycolysis (anti-Warburg effect) and (2) an increase in ROS formation leading to oxidative stress. The antiproliferative effect of derepression of mitochondrial function (anti-Warburg effect) may be quantitatively more important in highly glycolytic tumors, whereas oxidative stress may cause tumoristatic and tumoricidal effects on a more broad population of cells.

The VDAC-tubulin antagonist erastin and erastin-like compounds cause mitochondrial hyperpolarization followed by mitochondrial depolarization indicative of mitochondrial dysfunction in human hepatocarcinoma cells (Fig. 5.2). The initial increase in $\Delta\Psi$ is just in advance of the increase in ROS generation, whereas subsequent JNK activation precedes mitochondrial dysfunction. A lead erastin-like compound identified by small molecule screening also decreases glycolysis as evidenced by a decrease in lactate release (DeHart 2015). The combination of reversal of Warburg metabolism and oxidative stress by the lead compound causes cell death to human hepatocarcinoma cell lines in culture and to xenografted Huh7 hepatocarcinoma cells (DeHart et al. 2015). Thus, erastin and lead erastin-like compound by causing "two hits" of anti-Warburg metabolism and promotion of oxidative stress represent a potential new class of cancer chemotherapeutic agents (Fig. 5.3).

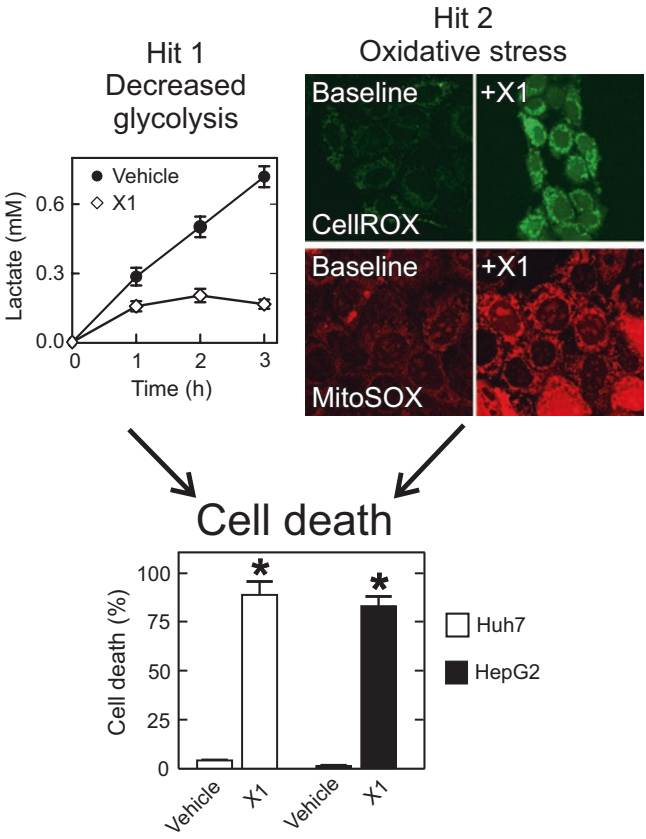

Fig. 5.3 Mechanisms to promote cell death after VDAC opening. X1 decreased lactate release by 80% in Huh7 cells (anti-Warburg effect, Hit 1). Lead compound X1 also increased fluorescence of the cellular ROS indicator CellROX *green* and the mitochondrial superoxide anion indicator MitoSOX *red* in Huh7 cells (oxidative stress, Hit 2). The two-hit mechanism led to over 90% cell death in Huh7 cells and over 80% in HepG2 cells

5.4 Concluding Remarks

VDAC-tubulin interaction in cancer cells is a global bioenergetic controller. Drug-induced VDAC opening increases mitochondrial metabolism and decreases glycolysis. Opening of the VDAC switch triggers two "hits" – an anti-Warburg effect that promotes a nonproliferative metabolic phenotype and an increase in ROS formation leading to mitochondrial dysfunction and cell death. ROS may be lethal for some cells and sublethal for others, whereas the anti-Warburg effect will decrease or stop cell proliferation. In summary, VDAC-tubulin is a new pharmacological target to turn a pro-proliferative into a nonproliferative phenotype and to induce oxidative death to cancer cells.

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