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Mitochondrial membrane cholesterol, the voltage dependent anion channel (VDAC), and the Warburg effect

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Abstract Normal cells of aerobic organisms synthesize the energy they require in the form of ATP via the process of oxidative phosphorylation. This complex system resides in the mitochondria of cells and utilizes oxygen to produce a majority of cellular ATP. However, in most tumors, especially those with elevated cholesterogenesis, there is an increased reliance on glycolysis for energy, even in conditions where oxygen is available. This aerobic glycolysis (the Warburg effect) has far reaching ramifications on the tumor itself and the cells that surround it. In this brief review, we will discuss how abnormally high membrane cholesterol levels can result in a subsequent deficiency of oxidative energy production in mitochondria from cultured Morris hepatoma cells (MH-7777). We have identified the voltage dependent anion channel (VDAC) as a necessary component of a protein complex involved in mitochondrial membrane cholesterol distribution and transport. Work in our laboratory demonstrates that the ability of VDAC to influence mitochondrial membrane cholesterol distribution may have implications on mitochondrial characteristics such as oxidative phosphorylation and induction of apoptosis, as well as the propensity of cancer cells to exhibit a glycolytic phenotype.

Keywords Mitochondrial membrane cholesterol · Voltage dependent anion channel · Warburg effect

It has been the better part of a century since Warburg described the predominance of the glycolytic phenotype of cancer cells (Warburg 1930; Racker 1972; Pedersen 2007). Recently, this idea has again become a focal point of current cancer biology and the field of cancer diagnostics, exhibited by the use of positron emission tomography (PET) scan imaging of the glucose analog ^{18}fluorodeoxyglucose used to visualize enhanced glucose uptake in tumors (Modica-Napolitano et al. 2007; Bos et al. 2002). The breadth of research into the Warburg effect has increased our understanding of this topic, but has not yet resolved the problem. In this article, we will discuss the contribution of the mitochondrion to the cancerous phenotype, in particular the role the voltage dependent anion channel (VDAC) and its interaction with mitochondrial membrane cholesterol will be detailed.

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Mitochondrial function and the glycolytic cancer cell

At first glance, the elevated glycolytic pathway of cancer cells appears to be a response to hypoxia due to the growth of the tumor surpassing the available vascular supplied oxygen (Mathupala et al. 2001). This is indeed true, but this does not explain the observation made by Warburg that cancer cells continue to show high glycolytic rates even when oxygen is sufficient for oxidative phosphorylation (OXPHOS). Although this is an intriguing metabolic problem, it is apparent that this leads to a benefit of the tumor because it has been suggested this characteristic can result in a survival and proliferation advantage of the tumor by providing protection from oxidative stress, a pathway to avoid apoptosis (Kondoh et al. 2007). An important player in enhanced glycolysis is the enzyme hexokinase (isoform II), the protein responsible for the first step of glycolysis, the conversion of glucose to glucose-6-phosphate. Hexokinase is over expressed in various tumors and contributes to

the increased glycolytic rate in the hypoxic environment and as the cancer cells proliferate (McEnery et al. 1993).

The metabolic fingerprint of cancer cells has recently been the focus of research involved in cancer therapeutics. For instance, the monocarboxylate transporter (MCT), the transporter of lactate across the cell membrane, has been studied as a possible target for an anti-cancer agent. As glycolysis increase, large amounts of lactate are transported out of the cell and blocking the MCT with a pharmacological agent (α-cyano-4-hydroxy cinnamic acid) can result in the intracellular accumulation of lactate and targeted destruction of the cancer cell (Mathupala et al. 2007). Another option that has showed great promise is the treatment of tumors with 3-bromopyruvate, an inhibitor of hexokinase that blocks the initial steps of glycolysis, targeting the tumor, but leaving healthy cells relatively unharmed (Pelicano et al. 2006). Other exciting developments include attenuation of lactate dehydrogenase expression (Fantin et al. 2006) and treatment of cancer cells with dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase (Bonnet et al. 2007). Still another example is the use of jasmonates, a class of plant stress response factors, to specifically target mitochondria in chronic lymphocytic cells (Rotem et al. 2005). These discoveries continue to enforce the importance of understanding the metabolic characteristics of the tumor in order to devise future treatment and prevention strategies.

Mitochondrial membrane cholesterol of cancer cells

The drastic reorganization of cellular and mitochondrial energy production in cancer cells from an oxidative form to a glycolytic form (Warburg effect) causes disruption and modulation of other biochemical pathways. For instance, the biological synthesis of cholesterol (cholesterogenesis) is vastly elevated in various cancer cells, specifically the hepatocellular carcinoma (hepatoma) (Mares-Perlman and Shrago 1988). It would seem logical that rapidly dividing cells would require increased lipid and cholesterol synthesis in order to produce new cell and organelle membranes; however the rate of cholesterogenesis in hepatoma cells is in abundance of what this would require (Coleman et al. 1997). This high cholesterogenesis results in abnormally elevated mitochondrial cholesterol levels (Pedersen 1978; Yu et al. 2005) and, as our laboratory reported, altered cholesterol ratios between the inner and outer mitochondrial membranes (Chan and Barbour 1983; Lau and Chan 1984; Campbell et al. 2002; Campbell and Chan 2007). This has a profound effect on the function of oxidative phosphorylation and may help answer the problem of altered bioenergetics observed in cancer cells.

High membrane cholesterol has been shown to alter the fluidity of biological membranes (Bathori et al. 1999; Epand 2006) and consequently detrimentally affect the function of mitochondria from hepatocellular carcinoma cells (Campbell and Chan 2007) and in other cell types as well (Yu et al. 2005). The mitochondrial activities such as ATP synthesis (ATP synthase), ATP/ADP exchange at the inner membrane (adenine nucleotide translocase, ANT [Barbour and Chan 1981]), and ATP/ADP and metabolite exchange at the outer membrane (VDAC) are affected by associated membrane composition.

Our laboratory has identified by a proteomic approach that VDAC from rat liver mitochondria has an important role in the altered cholesterol synthesis and transport in Morris hepatoma cells (Campbell and Chan 2007). VDAC can, under some circumstances, localize to the plasma membrane and associate with high cholesterol containing caveolae (De Pinto et al. 1993). In addition, the disruption of cholesterol rather than phospholipid was responsible for the release of VDAC from membranes in extraction experiments (Jancsik et al. 1988; Darbandi-Tonkabon et al. 2003). Most notably, in steroidogenic cells VDAC has been thought to associate with the peripheral type benzodiazepine receptor (PBR) and the steroidogenic acute regulatory protein (StAR), two cholesterol affinity proteins integral to the conversion of cholesterol to pregnenolone in steroid producing cells (Bogan et al. 2007). However, in newly conducted bioluminescence resonance energy transfer (BRET) experiments, the absence of StAR/PBR interactions has been reported (Tomiyama et al. 2006). Perhaps VDAC might act as an intermediate or conduit by which PBR and StAR can interact in steroidogenic cells. The role of a putative mitochondrial complex that would stimulate cholesterol transport has been recently proposed. This complex would include the interaction of VDAC, StAR, PBR, the translocator associated protein, and may involve the Iα regulator subunit of the cAMP-dependent protein kinase (Papadopoulos et al. 2007).

Membrane cholesterol, VDAC, and apoptosis

The mitochondrion has been explicitly linked to apoptosis by the release of cytochrome c as an apoptotic trigger through the pathway of mitochondrial outer membrane permeabilization (MOMP) through the formation of the permeability transition pore (PTP). This seemingly secondary role for the mitochondrion has been studied vigorously recently in an effort to diminish tumor cell growth and control the invasive nature of these cells (Dias and Bailly 2005). A group of pharmacological agents have been designed to alter the action of the apoptotic Bcl-2 protein family and increase the targeting of such proteins to the mitochondrion (Armstrong 2006). Another interesting development, particularly to our laboratory, is the recent link of cellular cholesterol levels and

apoptosis resistance in cancer cells. Statins, a class of HMG-coA reductase inhibitors that are commonly prescribed to individuals with high serum cholesterol levels, have been shown to activate the intrinsic mitochondrial pathway of apoptosis in myeloma and leukemia cells (Cafforio et al. 2005; Li et al. 2003). Because cholesterol elevation in mitochondria has an effect on mitochondrial function and has an impact on apoptosis, we propose that a link between mitochondrial cholesterol and apoptosis exists.

The process of MOMP occurs through the formation of a complex of proteins. The long accepted model of this complex (PTP) includes the proteins VDAC, the peripheral benzodiazepine receptor (PBR), creatine kinase, the adenine nucleotide translocase (ANT), and cyclophilin D (Green and Kroemer 2004). In this model, the VDAC 1 and 2 isoforms may serve as mitochondrial partners of the pro-apoptotic proteins Bax and Bak (Kroemer et al. 2007). In addition, VDAC is a necessary component for the process of MOMP either by direct interaction with Bax, or by binding with the inner membrane protein ANT to produce a membrane spanning channel that results in mitochondrial swelling and rupture along with subsequent cytochrome c release and apoptosis (Galluzzi and Kroemer 2007). This model has been hard to prove because of the logistical problems of purifying complexes that span both mitochondrial membranes, but remains the consensus. However, there is very new evidence that the process of MOMP may in fact occur independently of VDAC (Baines et al. 2007). The use of VDAC null mice, where one or two isoforms were deleted showed induction of the MOMP similar to that of normal cells in vitro. Also, these authors showed that knocking down the expression of all three VDAC isoforms through RNAi experiments could still result in MOMP induction. Although this work needs to be corroborated, it poses an interesting question about mitochondrially induced apoptosis. Perhaps the fact the VDAC deficient mice show signs of drastically deficient mitochondrial respiration and severely altered mitochondrial structure (Wu et al. 1999; Anflous-Pharayra et al. 2006; Sampson et al. 2001) result in an alternate pathway to MOMP. In this case, mitochondrial membrane complexes and biochemical environment may be grossly altered by the reduced membrane exchange (due to missing VDAC) and result in mitochondria that are in a state that is fragile and susceptible to MOMP induction. This recent VDAC absent model also fails to address the interaction between hexokinase and VDAC which provides a barrier to MOMP in cancerous cells (Mathupala et al. 2006).

We have shown that when the interaction between VDAC and hexokinase is interrupted there is an increase in induced mitochondrial swelling, a precursor to MOMP and apoptosis. Our lab and others have shown (Zaid et al. 2005; Campbell and Chan 2007), the glutamic acid (E) at position 72 is required for normal association of hexokinase with mitochondrial VDAC. Mutating this residue to glutamine (Q) removes the negative charge on the glutamate residue (at neutral pH) in favor of an uncharged amino group, which drastically reduces VDAC–HK interaction. Membrane topology models of VDAC that amino acid 72 is at the top end of an extra-mitochondrial loop which represents the site of hexokinase binding (Yehezkel et al. 2006; Colombini 2004). This makes possible a model where hexokinase, with its large molecular weight of 100kDa, binds to VDAC and buries the extra-mitochondrial ATP/

	Rat Liver	MH7777	MH7777 + MβC	MH7777 E72Q	MH7777 VG
P:O	1.5 (±0.15)	0.976 (±0.049)	1.28 (±0.16)	1.47 (±0.087)	1.17 (±0.065)

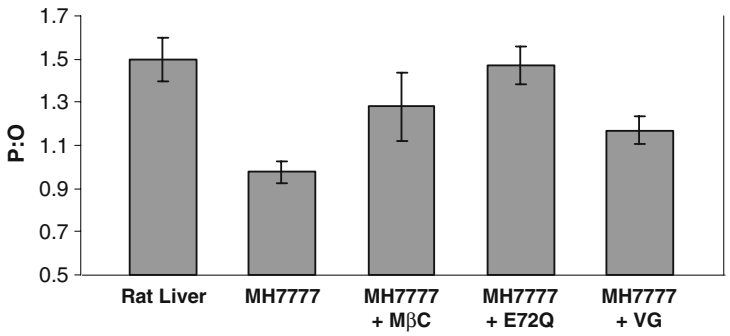

Fig. 1 Oxygen uptake of hepatoma mitochondria in response to succinate. Isolated mitochondria from rat liver served as the control and showed a P:O ratio (ATP per atom oxygen consumed) of 1.5 (±0.10). Mitochondria from the hepatoma cell line MH7777 exhibited a P:O ratio of 0.976 (±0.049), a reduction of 34.9%. Lowering membrane cholesterol by treatment of MH7777 mitochondria with methyl-β-cyclodextrin resulted in a P:O of 1.28 (±0.16). Transfection of MH7777 with E72Q-VDAC resulted in an increase of the oxidative capacity of the hepatoma mitochondria to a P:O ratio of 1.47 (±0.087). The MH7777+VG column represents the transfection of hepatoma mitochondria with the wild type VDAC. Results are reported as the mean plus S.E.M., $p<0.05$ (note: this figure appeared in Campbell and Chan 2007)

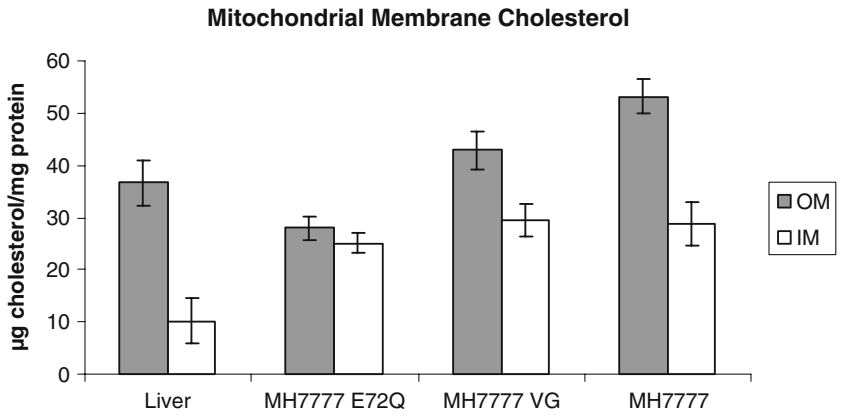

Fig. 2 Cholesterol determination of mitochondrial membranes. Transfection of MH7777 cells with E72Q VDAC resulted in a reduction of membrane cholesterol of 2 fold in the outer membrane (*OM*) and a marginal reduction in the inner membrane (*IM*). The MH7777+VG column represents the transfection of hepatoma mitochondria with the wild type VDAC. Any difference in total membrane protein between transfected and hepatoma cells was indiscernible (membrane cholesterol from the inner and outer fragments is reported in terms of microgram cholesterol per milligram membrane protein). Results are reported as the mean plus S.E.M., $p < 0.05$ (note: this figure appeared in Campbell and Chan 2007)

ADP binding site, rendering outer membrane ATP/ADP exchange difficult (Shanmugavadivu et al. 2007). This is normally not an issue in highly oxidative mitochondria but is exacerbated by the observation that hexokinase expression in the highly glycolytic tumor is drastically elevated (Goel et al. 2003). This E72Q VDAC mutation also results in an increase in OXPHOS activity (Fig. 1), and a reduction in mitochondrial membrane cholesterol (Fig. 2). The involvement of VDAC in three major mitochondrial functions (metabolite exchange, ATP production, and cholesterol distribution) can shed light on its role in the bioenergetics of cancer cells. As shown in Fig. 3, high cholesterol can reduce activity of membrane associated proteins and inhibit the metabolic function of VDAC. Also, in the tumor hexokinase activity increases in response to high glycolytic demand and its binding to VDAC is enhanced. This causes a MOMP resistant protein complex and blocks the VDAC mediated ATP/ADP exchange across the outer membrane due to the

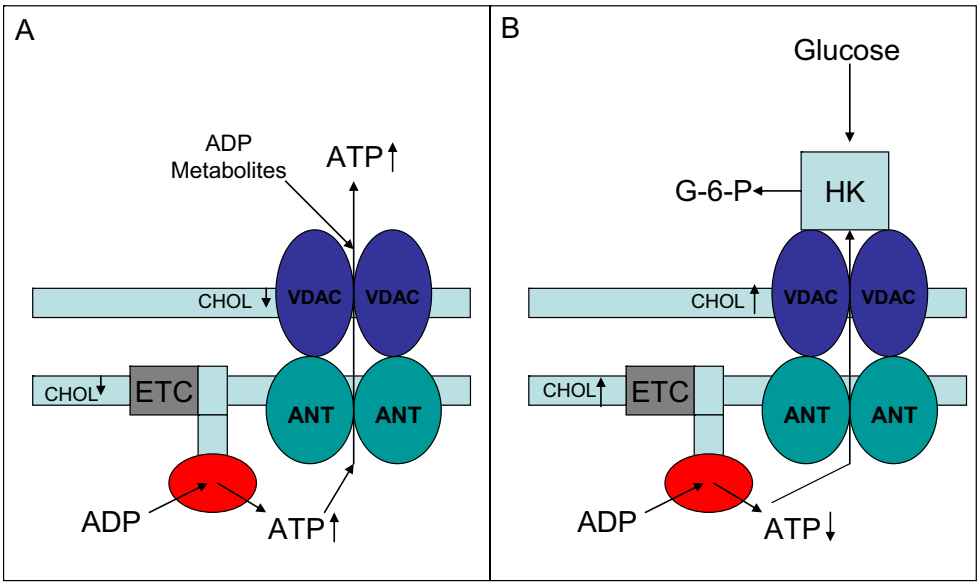

Fig. 3 Mitochondria membrane cholesterol and the effect on oxidative phosphorylation. **a** Normal metabolic state. Mitochondrial membrane cholesterol levels are in the normal range which results in optimal membrane associated protein activity. VDAC functions normally as a metabolite transporter and oxidative phosphorylation is producing ATP at high levels. **b** Aerobic glycolysis in the cancer cell (Warburg effect). Mitochondrial membrane cholesterol is elevated resulting in poor activity of the membrane associated proteins such as VDAC, ANT, and the ATP synthase complex. VDAC function is further debilitated by strong binding to HK II (which is up-regulated in cancer cells). Energy production is decreased and this is exacerbated by the preferential access of ATP by mitochondrially bound HK. Therefore, the dependency on glycolysis is perpetuated

preferential access of hexokinase to the VDAC supplied ATP. This, in turn creates a scenario where the cancer cell is primed for increased glycolytic activity and resistant to the MOMP induced mitochondrial apoptotic pathway. These two results increase the chance of tumor immortality and the perpetuation of the glycolytic phenotype. Reducing the mitochondrial membrane cholesterol or ablating the hexokinase–VDAC interaction can somewhat alleviate this problem by providing optimal membrane composition and increased OXPHOS activity.

Clearly, VDAC is integral to mitochondrial ATP production (OXPHOS) as a metabolite transporter, associated with the mitochondrial pathway to apoptosis, and involved in the distribution of cholesterol in the mitochondrial membranes. As we continue to learn about the metabolic phenotype of cancer cells, we are moving closer to devising safe, specific and effective treatments and prevention strategies to combat this debilitating disease.

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