Review

Voltage-dependent anion channel (VDAC) as mitochondrial governator—Thinking outside the box

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Abstract

Despite a detailed understanding of their metabolism, mitochondria often behave anomalously. In particular, global suppression of mitochondrial metabolism and metabolite exchange occurs in apoptosis, ischemia and anoxia, cytopathic hypoxia of sepsis and multiple organ failure, alcoholic liver disease, aerobic glycolysis in cancer cells (Warburg effect) and unstimulated pancreatic beta cells. Here, we propose that closure of voltage-dependent anion channels (VDAC) in the mitochondrial outer membrane accounts for global mitochondrial suppression. In anoxia, cytopathic hypoxia and ethanol treatment, reactive oxygen and nitrogen species, cytokines, kinase cascades and increased NADH act to inhibit VDAC conductance and promote selective oxidation of membrane-permeable respiratory substrates like short chain fatty acids and acetaldehyde. In cancer cells, highly expressed hexokinase binds to and inhibits VDAC to suppress mitochondrial function while stimulating glycolysis, but an escape mechanism intervenes when glucose-6-phosphate accumulates and dissociates hexokinase from VDAC. Similarly, glucokinase binds mitochondria of insulin-secreting beta cells, possibly blocking VDAC and suppressing mitochondrial function. We propose that glucose metabolism leads to glucose-6-phosphate-dependent unbinding of glucokinase, relief of VDAC inhibition, release of ATP from mitochondria and ATP-dependent insulin release. In support of the overall proposal, ethanol treatment of isolated rat hepatocytes inhibited mitochondrial respiration and accessibility to adenylate kinase in the intermembrane space, effects that were overcome by digitonin permeabilization of the outer membrane. Overall, these considerations suggest that VDAC is a dynamic regulator, or governator, of global mitochondrial function both in health and disease.
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1. Introduction

Mitochondria are quite possibly the most studied and best understood of organelles. Because structurally and functionally intact mitochondria can be isolated in large numbers from organs like heart, liver and brain, the molecular and mechanistic basis of most mitochondrial functions has been worked out in considerable detail. Best recognized of mitochondrial functions is oxidative phosphorylation, which links oxidation of various respiratory substrates to the synthesis of ATP from ADP and inorganic phosphate (Pi) [79]. Proton pumping by respiratory Complexes I, III and IV (NADH-ubiquinone oxidoreductase, ubiquinol-cytochrome *c* oxidoreductase and cytochrome *c* oxidase, respectively) creates an electrochemical gradient utilized by Complex V (ATP synthase) to synthesize ATP. Mitochondria also have their own genome that encodes 13 key proteins for this process, one or more each for Complexes I, III, IV and V [91]. The nucleus encodes the remainder of the ~1000 proteins in the mitochondrial proteome, which are imported into mitochondria from the cytosol [60,91]. Although perhaps less well known, mitochondria are also essential for other metabolic and signaling pathways, for example, ureagenesis, synthesis of cholesterol and heme, and signaling pathways leading to cell death. Virtually all these activities have been reconstituted in detail using isolated mitochondria alone or in combination with other isolated cellular elements.

2. Quirks in mitochondrial behavior

Mitochondria sometimes do not do quite what is expected of them. Although mitochondria are essential for aerobic multicellular life, mitochondria also play key roles in mechanisms of cell death. Mitochondrial dysfunction with resulting compromise of cellular ATP supply is often an essential event leading to necrotic cell death, also called oncosis or oncotic necrosis [58]. Additionally, programmed permeabilization of mitochondrial membranes releases proteins, such as cytochrome *c*, Smac/Diablo, apoptosis inducing factor and others, that activate the final and committed pathways to apoptosis [35,41,50]. Although the role of mitochondria in cell death is better understood, other anomalous and obscure mitochondrial behaviors are not, as the following examples illustrate.

2.1. Neahypoxia

A fundamental stress to aerobic cells is anoxia. Without oxygen, mitochondria cannot form ATP by oxidative phosphorylation. In anoxia/hypoxia, electrons accumulate in respiratory carriers (e.g., cytochromes) and cofactors (e.g., NADH) to create a reductive stress that promotes oxygen radical generation when molecular oxygen once again becomes available [33]. A series of papers almost two decades ago revealed that a global sealing to metabolite exchange occurs in mitochondria during anoxia [1,6,7]. This condition, called neahypoxia, is characterized by retention of the mitochondrial protonmotive force (pH gradient and membrane potential) despite the absence of energy input from electron flow and ATP hydrolysis. Similarly, anoxia inhibits mitochondrial exchange of Pi, ADP, ATP and several respiratory substrates. In hepatocytes exposed to cyanide and iodoacetate to inhibit respiration and glycolysis (a simulated hypoxia called chemical hypoxia), mitochondria retain membrane potential-indicating cationic fluorophores long after profound ATP depletion [51]. Membrane potential-indicating dyes are not released until a nonspecific permeabilization of the mitochondrial inner membrane occurs, presumably that associated with the mitochondrial permeability transition [94]. In hibernation also, a global membrane sealing to metabolite and ion exchange appears to occur [37].

2.2. Cytopathic hypoxia

Sepsis leading to multiple organ dysfunction syndrome impairs oxygen consumption by many tissues despite adequate oxygenation [23,28,29]. Suppression of oxygen uptake also occurs in cultured cells, such as Caco-2 human intestinal epithelial cells, after exposure to a cocktail of proinflammatory cytokines (TNFα, IL-1β, and IFN-γ) of the type that are released by activated macrophages during sepsis [44]. Various reports indicate inhibition of different mitochondrial enzymes, including pyruvate dehydrogenase, cytochrome *c* oxidase (Complex IV) and NADH-ubiquinone oxidoreductase (Complex I), as well as decreases of MTT reduction to formazan, a crude measure of mitochondrial respiratory activity [17,29,83, 87,90]. Thus, in cytopathic hypoxia as in neahypoxia, a type of global suppression of mitochondria metabolism seems to occur. Mechanisms to explain suppression of mitochondrial metabolism in cytopathic hypoxia include nitric oxide and peroxynitrite-dependent enzyme inhibition and NAD depletion due to activation of polyADP-ribose polymerase, an enzyme that not only repairs single strand breaks in DNA but can also cause NAD depletion if overactivated [18,28,44].

2.3. Swift increase of alcohol metabolism

Selective stimulation of mitochondrial metabolism in the liver also occurs by mechanisms that remain incompletely understood. After a single inebriating dose of ethanol, hepatic respiration increases and ethanol oxidation nearly doubles [16,40,84,85]. This swift increase of alcohol metabolism is adaptive and promotes ethanol oxidation to acetate, the major route of ethanol elimination and detoxification. Ethanol oxidation is a two-step process. Alcohol dehydrogenase in the cytosol and to a lesser extent cytochrome P450 in endoplasmic reticulum catalyzes the first oxidation step converting ethanol to acetaldehyde. The second oxidation step utilizes acetaldehyde dehydrogenase inside mitochondria to form acetate. The first step of ethanol metabolism transfers reducing equivalents (electrons) to NADH and thus requires cofactor supply in the form of NAD^+, which mitochondria provide through increased oxidation of NADH. Although increased mitochondrial respiration should, in theory, lead to increased ATP generation, alcohol treatment also causes a decline of ATP and activation of glycolysis resulting in glycogen depletion. Furthermore, fatty acid oxidation by mitochondria becomes inhibited, leading to rapid accumulation of neutral lipids within hepatocytes, a fatty change called steatosis [16,81].

Mitochondrial utilization of ethanol as a preferred fuel has the consequence of suppressing fat, carbohydrate and amino acid oxidation [16,27,57,81]. Ethanol exposure also causes intracellular oxidative stress and mitochondrial lipid peroxidation, leading to damage of mitochondrial DNA and decreases of mitochondria-encoded proteins, further hampering mitochondrial metabolic function [9,27,54]. Thus, ethanol stimulates mitochondrial oxidation of acetaldehyde but at the same time causes relatively global suppression of other mitochondrial activities, especially fatty acid oxidation. Interestingly, rats fed fat containing short to medium chain fatty acids have less steatosis after ethanol treatment than rats fed fat with long chain fatty acids, suggesting that mitochondria continue to metabolize shorter chain fatty acids after ethanol exposure [63,64].

2.4. Aerobic glycolysis in tumor cells

As recognized by Otto Warburg 75 years ago, malignant cancer cells typically display high rates of glycolysis even when fully oxygenated [31,92]. Ordinarily during aerobic metabolism, glycolysis is active only to the extent of providing

pyruvate for the Krebs cycle. Since so much more ATP is generated by aerobic mitochondrial metabolism of pyruvate compared to anaerobic metabolism of glucose to lactate, glycolytic pathways stimulated by anoxia/hypoxia are normally suppressed by oxygen. In cancer cells, high glycolytic rates and net formation of pyruvate and lactate persist despite adequate oxidation, a fact fully confirmed in cancer patients by techniques like positron emission tomography of the glucose analog [18]fluorodeoxyglucose [30]. The importance of aerobic glycolysis to tumor cell biology is still a matter of conjecture. Enhanced glycolytic capacity may be an advantage to tumors whose rapid growth often exceeds their blood and hence oxygen supply. Furthermore, glycolysis although less efficient may still produce ATP faster than oxidative phosphorylation, an advantage to rapidly growing cells. Alternatively, tumors may be adopting a dedifferentiated early developmental phenotype, since embryonic and early fetal tissues are strongly dependent on glycolysis rather than mitochondrial oxidative phosphorylation for their ATP supply [36].

Aerobic glycolysis in tumor cells is associated with increased expression of hexokinase and upregulation of glucose transport across the plasma membrane [19,32]. Although Warburg originally suggested that aerobic glycolysis in cancer cells might reflect defects in what we now know is mitochondrial oxidative phosphorylation, studies show that tumor mitochondria are fully functional with regards to respiration and ATP synthesis [62]. The mystery persists as to why such fully functional mitochondria inside tumor cells are nonetheless relatively inactive in terms of respiration and ATP generation.

2.5. Insulin secretion by beta cells

Mitochondria play an important and rather different role in regulation of insulin secretion by beta cells of pancreatic islets. Rather than simply producing ATP to meet cellular energetic needs, mitochondrial ATP production is a signal that leads ultimately to insulin release. In the current model, glucose enters the cytosol of beta cells via glucose transporters where glucokinase initiates glycolysis and the formation of pyruvate. Pyruvate then enters mitochondria to replenish Krebs cycle intermediates and fuel respiration, leading to increased mitochondrial polarization and ATP generation. Release of ATP by mitochondria into the cytosol increases the ATP/ADP ratio, which inhibits glibenclamide-sensitive K_{ATP} channels in the plasma membrane. As a consequence, plasmalemmal potential decreases, voltage sensitive Ca^{2+} channels open, cytosolic free Ca^{2+} increases, and Ca^{2+}-dependent exocytosis of insulin-containing secretory granules occurs [55]. The real glucose sensor in this scheme is glucokinase, a hexokinase isoform whose K_m of approximately 5 mM matches well the set point of blood glucose [59]. Other hexokinases with lower K_m are not expressed in beta cells.

Exogenous pyruvate, however, does not stimulate insulin secretion even at high concentrations [65]. Lack of stimulation is attributed to low expression of monocarboxylic acid transporters in the plasma membrane. However, more recent studies indicate that exogenous pyruvate does enter beta cells to alter cytosolic NADPH levels, but increases of mitochondrial NADH do not occur as would be expected if pyruvate entered mitochondria [73]. By contrast, methylpyruvate, a membrane permeant ester derivative that is de-esterified to pyruvate in both cytosol and mitochondria, is a potent insulin secretagogue. Methylpyruvate unlike pyruvate does increase mitochondrial NADH. The basis by which exogenous pyruvate is metabolized by cytosol but not mitochondria in beta cells is not known [73].

2.6. Global control of mitochondrial metabolism

These several examples point to a mechanism of global suppression of mitochondrial metabolism within the inner membrane and matrix. In many respects during this metabolic suppression, mitochondria behave as if the inner membrane–matrix compartment is sealed off from the rest of the cell. Although several mechanisms might act in concert to inhibit metabolite flux across the inner membrane, something may be occurring outside the inner membrane–matrix "box". Namely, a block of the permeability of the mitochondrial outer membrane could be sealing the mitochondrion and its metabolism from the rest of the cell. Normally, the outer membrane is freely permeable to solutes of molecular mass up to a about 5000 Da, although the cut-off point can be less for charged solutes. The voltage dependent anion channel (VDAC) in the outer membrane makes this permeability possible [22,77]. Could closure of VDAC then account for global suppression of mitochondrial metabolism observed in anoxia, cytopathic hypoxia, alcoholic fatty liver, the Warburg effect and unstimulated pancreatic beta cells?

3. VDAC and regulation of mitochondrial permeability

3.1. Properties of VDAC channels

VDAC is a highly conserved mitochondrial outer membrane protein. Yeast has one channel-forming VDAC isoform (plus a non-channel-forming isoform), and mice have three isoforms: VDAC1, VDAC2 and VDAC 3, each of a molecular mass of about 30 kDa (reviewed in [14,22,82]). Except for a relatively few membrane-permeant lipophilic compounds (*e.g.*, molecular oxygen, acetaldehyde, short chain fatty acids), all metabolites that enter and leave mitochondria must cross the mitochondrial outer membrane through VDAC. Each VDAC protein forms a barrel in the bilayer comprised of a transmembrane alpha helix and 13 transmembrane beta strands. This beta barrel encloses an aqueous channel of 2.5 to 3 nm in internal diameter, which in the open state allows passage of non-electrolytes up to ∼5 kDa in size. Similar models with additional beta strands have also been proposed [25].

As its name suggests, VDAC shows both ion selectivity and voltage dependence. In the open state, anions are favored over cations, but the selectivity is actually quite weak [21]. Both positive and negative membrane potentials close VDAC. The voltage effect is symmetrical, and half maximal closure occurs

at about ±50 mV. It remains a matter of conjecture as to whether membrane potential is a physiological regulator of VDAC conductance, since even in the closed state, VDAC conducts small cations, like K^+, Na^+ and Ca^{2+}, which tend to collapse electrical potentials forming across the outer membrane. However, a Donnan potential can still exist across the outer membrane consistent with a negative pH gradient recently measured with a pH-sensitive fluorescent protein [69]. By contrast, VDAC closure very effectively blocks movement of organic anions, including respiratory substrates, creatine phosphate, ATP, ADP and Pi [77]. VDAC closure, should it occur, would block most substrate supply for respiration and prevent exchange of ADP and Pi for ATP during oxidative phosphorylation.

4. Possible role of VDAC closure in pathologic states

4.1. VDAC closure in apoptosis

The assumption has generally been that VDAC is constantly open during metabolism. Recent data, however, suggest that VDAC has the ability to close and inhibit exchange of metabolites within intact cells [14,22,82]. In particular, VDAC closes early in the evolution of apoptosis with the consequence that mitochondria cannot release ATP or take up ADP, Pi and respiratory substrates from the cytosol [26,88]. *t*Bid, a proapoptotic Bcl2 member, closes VDAC, which may account for VDAC inhibition during apoptosis, at least in part [76]. In contrast, antiapoptotic Bcl2-XL prevents VDAC closure, which is consistent with the notion that VDAC opening is antiapoptotic [89]. VDAC also appears to be an anchoring point for pro- and antiapoptotic proteins and has been proposed to be part of the cytochrome *c* release channel in the outer membrane that forms in apoptosis (reviewed in [34,67,78,82,86]).

4.2. Anoxia, ischemia and cytopathic hypoxia

VDAC closure might also explain inhibition of metabolite movement during anoxia and the persistent suppression of mitochondrial function in cytopathic hypoxia during septic shock and multiple organ failure [23,28,29] (Fig. 1). A factor promoting VDAC closure may be NADH, since NADH increases markedly during anoxia and inhibits conductance of VDAC by up to 6-fold [49,89,95]. In Bcl-2 overexpressing mouse hearts, intracellular ATP hydrolysis and acidification is suppressed during ischemia, which has been proposed to be due to inhibition of VDAC conductance, adenine nucleotide translocase activity or the mitochondrial F_1F_0-ATPase (Complex V) [39]. The speculated VDAC closure during ischemia in Bcl2-overexpressing heart seems to contradict findings that Bcl-XL prevents VDAC closure. However, antiapoptotic family members like Bcl-XL can be converted to proapoptotic proteins by proteolytic processing, as might occur during ischemia [12].

In septic shock and multiple organ failure, suppression of mitochondrial oxygen consumption occurs despite adequate
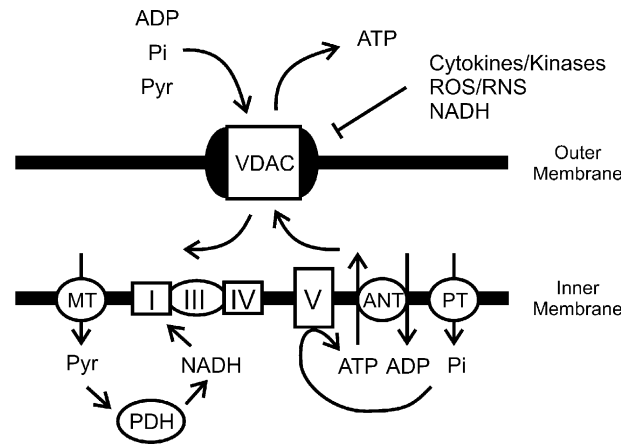

Fig. 1. Scheme of global suppression of mitochondrial metabolism by VDAC closure in anoxia and cytopathic hypoxia. ADP, Pi and pyruvate (Pyr) cross into the intermembrane space through VDAC and then through the inner membrane to the matrix via the adenine nucleotide transporter (ANT), the phosphate transporter (PT) and the monocarboxylate transporter (MT), respectively. Pyruvate dehydrogenase (PDH) oxidizes pyruvate to generate NADH, which is oxidized by the respiratory chain (Complexes I, III and IV) to generate a proton electrochemical gradient. ATP is then formed by Complex V and exits the mitochondria first through ANT and then VDAC. In anoxia and cytopathic hypoxia, NADH, reactive oxygen and nitrogen species (RNS and ROS) and cytokine-dependent kinase cascades are proposed to close VDAC and suppress both uptake and release of mitochondrial metabolites.

oxygenation, a condition of cytopathic hypoxia [23,28,29]. VDAC closure might also underlie for this suppression of mitochondrial metabolism. Reactive oxygen species (ROS), reactive nitrogen species (RNS) and proinflammatory cytokines all seem to contribute to the development of cytopathic hypoxia [28,44,83], but their role in modulating VDAC conductance is not known. In cultured Caco-2 human intestinal epithelial cells, cytokine stimulation alone leads to cytopathic hypoxia [44]. Cytokines initiate a variety of intracellular signaling events, including protein kinase cascades. In this regard, c-Raf kinase binds VDAC and blocks reconstitution of VDAC channels into planar bilayer membranes [47]. In addition, protein kinase C epsilon binds and phosphorylates VDAC, an effect causing inhibition of the mitochondrial permeability transition [10]. VDAC isoforms in guinea pig brain synaptosomes also undergo tyrosine phosphorylation in response to hypoxia [52]. Thus, phosphorylation may be an important regulator of VDAC function.

4.3. VDAC closure in the metabolic derangements induced by ethanol

Similar to cytopathic hypoxia, cytokines, ROS and RNS contribute to ethanol-induced cytotoxicity (reviewed in [4,71]). Ethanol ingestion increases gut permeability and promotes translocation of endotoxin and other bacterial cell wall products across the gut mucosa. Such products are then transported to the liver via the portal vein and stimulate cytokine release by Kupffer cells, the resident macrophages of liver. Knockout interventions show that this signaling pathway contributes to the development of both hepatic necroinflammatory changes and steatosis after ethanol. For example, TNF

receptor-1 knockout mice are protected against ethanol-induced hepatic steatosis [93].

Ethanol also seems to act directly on hepatocytes to promote ROS formation via cytochrome P450 2E1 and possibly other pathways (e.g., mitochondria, NADPH oxidase) [9,27,54,93]. Various antioxidants protect against ethanol-induced liver injury in a variety of in vitro and in vivo models [4]. Additionally, NO from inducible nitric oxide synthase (iNOS) contributes to ethanol-induced liver injury, since iNOS knock-out mice have less liver injury and steatosis than wild-type mice after ethanol treatment [5]. Thus, the same factors (cytokines, ROS, RNS) suppressing mitochondrial metabolism in cytopathic hypoxia may also be contributing to metabolic disruption after ethanol. Additionally, ethanol metabolism produces NADH, which inhibits VDAC conductance [49].

Ethanol ingestion decreases ATP, activates glycolysis, depletes glycogen, inhibits mitochondrial oxidation of medium and long chain fatty acids, promotes hepatic fat accumulation, and nearly doubles mitochondrial respiration in the liver [16]. A blockade of VDAC conductance would explain many of these metabolic disturbances (Fig. 2). ATP exchange for ADP and phosphate involves both the adenine nucleotide and phosphate transporters in the inner membrane and VDAC in the outer membrane. Thus, VDAC inhibition alone would suppress mitochondrial uptake of ADP and Pi for synthesis and release of ATP, an effect decreasing ATP/ADP ratios, stimulating glycolysis and depleting glycogen in the cytosol.

Similarly, VDAC blockade would suppress beta oxidation of medium and long chain fatty acids (Fig. 2). Beta oxidation of medium and long chain fatty acids requires entry of fatty acyl CoA esters through VDAC into the intermembrane space, formation of acyl carnitine by carnitine-palmytoyl transferase I, and movement of acylcarnitine across the inner membrane by acylcarnitine transferase (acylcarnitine shuttle) [11,16]. In the matrix space, fatty acylcarnitine is converted back to fatty acyl CoA by carnitine-palmytoyl transferase II, and the fatty acyl CoA then undergoes mitochondrial beta oxidation to yield NADH for oxidation by the respiratory chain. By inhibiting access of fatty acyl CoA to the acylcarnitine shuttle, VDAC closure blocks beta oxidation and promotes accumulation of medium and long chain fatty acids in the cytosol. Accumulation of these fatty acids as triglycerides might then lead to the fatty transformation of steatosis. By contrast, shorter chain fatty acids like octanoic acid cross mitochondrial membranes directly and independently of VDAC and the acylcarnitine shuttle. Mitochondrial oxidation of short chain fatty acids therefore is not dependent on VDAC. In this regard, it is noteworthy that rats fed ethanol and a diet containing fat with short and medium chain fatty acids do not develop steatosis in comparison to rats fed ethanol and long chain fat [64], an expected outcome if VDAC blockade was responsible for steatosis. A similar closure of VDAC may contribute to non-alcoholic fatty liver disease and the acute steatosis caused by a variety of other hepatotoxicants.

The first step of ethanol oxidation produces acetaldehyde in the cytosol. Acetaldehyde is uncharged and readily crosses mitochondrial membranes to be oxidized to acetate by acetaldehyde dehydrogenase with the production of NADH in the matrix space (Fig. 2). This NADH then feeds directly in the respiratory chain. The high potential of the acetaldehyde/acetate redox potential together with the inability to discharge the protonmotive gradient via ATP formation may then cause mitochondrial hyperpolarization, leading in turn to non-ohmic proton leaks and respiratory stimulation [66]. Activation of uncoupling proteins and the weak uncoupling effect of acetate may also contribute to increased respiratory
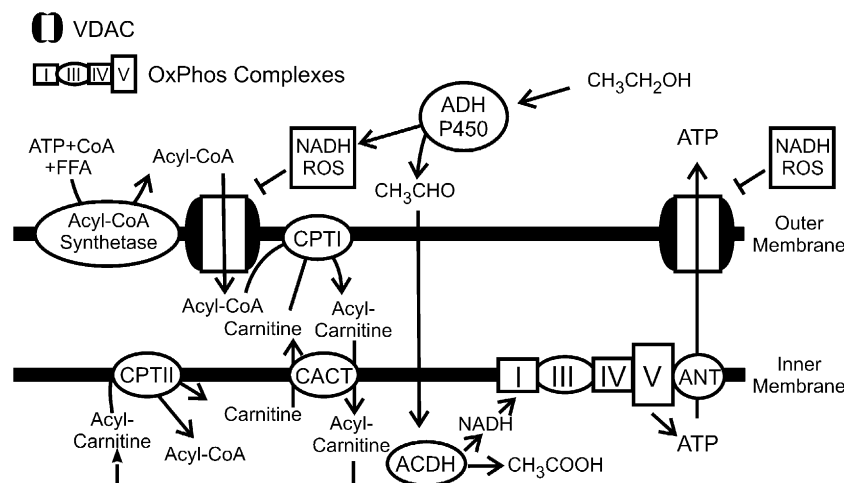

Fig. 2. Role of VDAC closure in ethanol-induced changes of hepatic fat metabolism. In oxidation of medium and long chain fatty acids, acyl-coenzyme A (acyl-CoA) is formed by acyl-CoA synthetase from ATP, CoA and free fatty acid (FFA). Acyl-CoA passes through VDAC into the intermembrane space, and carnitine-palmytoyl transferase I (CPTI) in the outer membrane converts the acyl moiety to acylcarnitine, which moves through the inner membrane into the matrix via carnitine-acetyl-carnitine transferase (CACT). On the inner surface of the matrix, acyl-CoA is regenerated by CPTII. Ethanol metabolism by alcohol dehydrogenase (ADH) and cytochrome P450 leads to formation of NADH and reactive oxygen species (ROS), which inhibit VDAC. VDAC inhibition prevents movement of acyl-CoA into the intermembrane space and operation of acylcarnitine-carnitine shuttle. Acetaldehyde, however, crosses membranes directly, allowing its metabolism to acetate by acetaldehyde dehydrogenase (ACD) in the matrix space. NADH formed by this reaction is oxidized by the respiratory chain. ATP formed by oxidative phosphorylation enters the intermembrane space via the adenine nucleotide transport (ANT) but cannot progress further into the cytosol through VDAC.

flux [46], consistent with a decrease in overall metabolic efficiency after ethanol ingestion as reported in human drinkers [42,53,68,81]. Together, accelerated respiration and selective oxidation of acetaldehyde would have the physiological function of removing and detoxifying ethanol. VDAC closure in this setting would promote acetaldehyde oxidation but at the expense of inhibited oxidation of long chain fatty acids, pyruvate and other polar anionic respiratory substrates. Blood lactate increases after ethanol [61], and pyruvate conversion to lactate by lactate dehydrogenase may help dispose of NADH produced by alcohol dehydrogenase in the cytosol (Fig. 2).

4.4. VDAC closure as a hexokinase-operated brake on mitochondrial metabolism in cancer cells

Aerobic glycolysis (Warburg effect) is a typical feature of cancer cell metabolism, which is characterized by high aerobic glycolysis, suppression of mitochondrial respiration and high expression of hexokinase [31,92]. Hexokinase, the first enzyme in the glycolytic pathway, binds to the mitochondrial outer membrane via a specific association with VDAC1, an effect proposed to give hexokinase selective access to ATP coming from mitochondria [3,15]. Glucose-6-phosphate, a product of the hexokinase reaction, antagonizes binding to VDAC and leads to release of hexokinase from mitochondria [75]. A recent report shows that hexokinase-I (one of 4 hexokinase isoforms, including glucokinase) acting through its N-terminal mitochondrial binding domain blocks conductance of rat liver mitochondrial VDAC reconstituted into lipid bilayers [8]. Hexokinase-I also blocks opening of high conductance mitochondrial permeability transition (MPT) pores in the mitochondrial inner membrane that are also reputed to contain VDAC [8]. Glucose-6-phosphate reverses blockade of VDAC conductance and protection against MPT pore opening by hexokinase-I. Furthermore, hexokinase-I overexpression protects against staurosporin-induced apoptosis in cultured cells. Since both VDAC and the MPT are implicated in the mitochondrial phase of apoptotic signaling, the authors of this work propose that hexokinase overexpression is a self-defense mechanism by cancer cells against apoptosis [8].

Hexokinase inhibition of VDAC would also put a brake on global mitochondrial metabolism. Inhibition of mitochondrial ATP production would then promote glycolysis, especially when hexokinase expression is increased. Glucose-6-phosphate, however, provides an escape mechanism. If glycolysis downstream to hexokinase becomes inhibited, glucose-6-phosphate would accumulate. Glucose-6-phosphate would then relieve the inhibition of VDAC by hexokinase and allow mitochondria to resume their normal aerobic metabolism, including uptake of ADP, Pi and respiratory substrates, oxidative phosphorylation, and release of ATP into the cytosol. These events provide a possible explanation for the Warburg effect; namely, hexokinase overexpression suppresses mitochondrial metabolism through VDAC blockade while simultaneously promoting glycolysis. Since not all VDAC is necessarily completely closed, the association of hexokinase I

with mitochondria may still give the enzyme preferential access to mitochondrial ATP.

Cancer cells still respire in the absence of glucose, indicating that VDAC blockade is relative rather than absolute. Respiration increases substantially after glucose addition, a phenomenon called the Pasteur effect [70]. Increased respiration is attributed to ADP generation by hexokinase and consequent stimulation of mitochondrial oxidative phosphorylation. Relief of inhibition of VDAC conductance by glucose 6-phosphate (also produced by hexokinase) may also stimulate respiration in the Pasteur effect. Following the Pasteur effect, respiration becomes inhibited, a phenomenon called the Crabtree effect. Because of trapping into glucose-6-phosphate and other sugar phosphates, Pi may become limiting for oxidative phosphorylation [45]. Increases of AMP and NADH and decreases of intracellular pH may also promote inhibition of respiration in the Crabtree effect [74], but a specific role of VDAC closure in the phenomenon is not known.

4.5. A VDAC brake suppressing mitochondrial metabolism in beta cells?

The purpose of this communication is to consider if VDAC can act as a global regulator of mitochondrial function and how such global regulation might play a role in physiology and pathophysiology. Another example where mitochondrial metabolism seems subject to global regulation is in insulin-secreting pancreatic beta cells. The question thus arises as to whether hexokinase-mediated regulation of VDAC conductance might explain how glucose stimulates mitochondrial ATP production and hence insulin release in beta cells. However, hexokinase in beta cells is exclusively glucokinase (hexokinase-IV) [59], and glucokinase in organs like liver does not bind to mitochondria and thus would be incapable of inhibiting VDAC [20]. Beta cells, however, express an alternatively spliced version of glucokinase [56]. Beta cell glucokinase has an additional N-terminal sequence that promotes glucokinase binding to both mitochondria and secretory granules [2]. If this glucokinase binding inhibited VDAC conductance in a fashion that was relieved by glucose-6-phosphate (or other downstream product of glycolysis), then release of a dynamic glucokinase brake on VDAC conductance could explain activation of mitochondrial metabolism by glucose. In support, a study employing fluorescence resonance energy transfer shows that glucokinase is released into the cytoplasm after glucose stimulation ([72] but see [2]). In the proposed scheme, glucose enters beta cells by glucose transporters and is metabolized to glucose-6-phosphate in the first step of glycolysis. Glucose-6-phosphate then acts to dissociate glucokinase from mitochondria and open VDAC, which permits mitochondrial uptake of ADP, Pi and respiratory substrates, formation of ATP, and release of ATP into the cytosol. Increased cytosolic ATP/ADP then inhibits K_{ATP} channels, which causes plasmalemmal depolarization, activation of Ca^{2+} channels and Ca^{2+}-dependent exocytosis of insulin granules. Exocytosis may be further promoted by glucose-6-phosphate-dependent glucokinase release from secretory granules. Such a mechanism would explain

why simple addition of high concentrations of respiratory substrates like pyruvate, lactate and long chain fatty acids do not promote insulin secretion. These compounds cannot pass through VDAC. However, permeable esters of respiratory substrates, like methylpyruvate, and short chain fatty acids, like octanoate, permeate mitochondrial membranes directly without the need for VDAC. These compounds do induce insulin secretion by beta cells [43,48], although in the postulated mechanism the resumption of respiration must somehow feed forward to open VDAC to ATP release, at least in part.

5. Assessment of VDAC activity in cells

The bulk of experimental work evaluating VDAC conductance has been in reconstituted systems, usually single channel recordings of VDAC inserted into planar phospholipid membranes. Reconstitution may separate VDAC from important regulatory factors. Recently, we sought to assess VDAC conductance of mitochondria in hepatocytes with and without exposure to ethanol in order to test the hypothesis that changes of VDAC conductance underlie ethanol-induced changes of mitochondrial metabolism [38].

5.1. Selective permeabilization of the plasma and mitochondrial outer membranes with digitonin

To assess how plasma membrane and mitochondrial outer membrane permeability influences mitochondrial oxidation of exogenous substrates by isolated rat hepatocytes, we first assessed how the non-ionic detergent digitonin, an agent that binds cholesterol to form aqueous pores [13,24,80], affected plasma membrane and outer membrane permeability. In a dose-dependent fashion, digitonin caused trypan blue nuclear labeling of hepatocytes in parallel with release of lactate dehydrogenase (LDH), a cytosolic enzyme. Maximum trypan blue labeling and LDH release occurred after 50 μg digitonin/10^6 cells (Fig. 3).
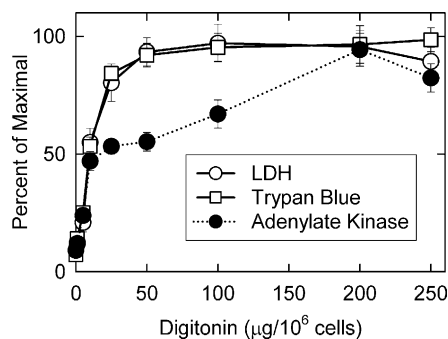
Fig. 3. Digitonin permeabilization of the plasma and mitochondrial outer membranes. Isolated rat hepatocytes (2×10^6 cells/ml) were incubated on ice for 10 min with 0–250 μg digitonin/10^6 cells plus 0.05% DMSO vehicle. Permeabilization of the plasma membrane was assessed by trypan blue nuclear labeling and expressed as the percentage of labeled cells. After centrifugation, lactate dehydrogenase and adenylate kinase were measured in the supernatant as a percentage of total activity in cellular lysates obtained with 0.5% Triton X-100. Graph shows means of triplicate measurements. After [38].
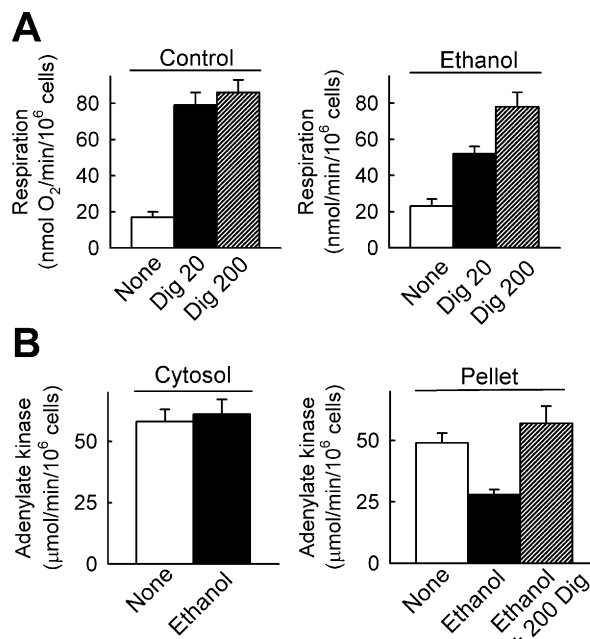
Fig. 4. Outer membrane permeabilization overcomes ethanol inhibition of respiration (A) and restores accessibility to AK (B) in rat hepatocytes. In A, aliquots of isolated hepatocytes (10^6 cells/ml) were incubated in isolation medium in the absence (Control) and presence (Ethanol) of 50 mM ethanol for 20 min at 37 °C. After centrifugation at $50 \times g$ for 2 min, pellets were resuspended in an intracellular buffer, and respiration supported by 5 mM succinate and 250 μM ADP in the presence of 0–200 μg digitonin/10^6 cells was measured. In B, ethanol-treated and untreated hepatocytes were incubated with 20 μg digitonin/10^6 cells, and adenylate kinase activity was measured in supernatants (cytosol) and pellets after centrifugation and in ethanol-treated pellets resuspended in 200 μg digitonin/10^6 cells. Graphs show means of triplicate measurements. After [38].

As digitonin increased to a concentration causing maximal trypan blue uptake and LDH release, about half of total cellular adenylate kinase was released (Fig. 3). Adenylate kinase is an enzyme with separate cytosolic and mitochondrial isoforms [80]. Adenylate kinase activity remaining with hepatocytes after 50 μg digitonin/10^6 cells reflected mitochondrial adenylate kinase located in the intermembrane space. Higher concentrations of digitonin (>200 μg/10^6 cells) released this adenylate kinase, indicating permeabilization of the mitochondrial outer membrane (Fig. 3). Simultaneously, cytochrome *c*, another intermembrane protein, was released as well (data not shown). Thus, digitonin dose-dependently permeabilized first the plasma membrane and then at higher concentrations the outer mitochondrial membrane of cultured hepatocytes in agreement with previous work [13,24,80].

5.2. Impairment by ethanol of mitochondrial metabolism in permeabilized hepatocytes

To examine the effect of ethanol on mitochondrial function in hepatocytes, we measured oxygen uptake (respiration) in the presence of exogenous succinate and ADP. In the absence of ethanol, respiration by hepatocyte suspensions was relatively low, but increased 4.5-fold after plasma membrane permeabilization with 50 μg digitonin/10^6 cells (Fig. 4A). This

observation illustrates that succinate and ADP stimulate mitochondrial respiration only if the plasma membrane is permeabilized. The reason is that succinate and ADP do not permeate the plasma membrane. More digitonin ($200 \ \mu g/10^6$) to permeabilize mitochondrial outer membranes did not increase respiration further. This finding shows that the mitochondrial outer membrane of normal hepatocytes is not a barrier to metabolism of succinate plus ADP.

After treatment with ethanol (50 mM), respiration by hepatocyte suspensions in the absence of digitonin increased moderately, possibly due to NADH generation by alcohol and acetaldehyde dehydrogenases (Fig. 4A). After permeabilization of the plasma membrane with low digitonin, however, respiration was inhibited after ethanol treatment in comparison to untreated hepatocytes. This inhibition was overcome after outer membrane permeabilization with higher digitonin (Fig. 4A). Similar inhibition by ethanol treatment of respiration in plasma membrane-permeabilized hepatocytes occurred when succinate was replaced with pyruvate plus malate (2.5 mM each) (data not shown). Again, inhibition was also overcome by high digitonin. These results seem to show that free diffusion of ADP and respiratory substrates through the outer mitochondrial membrane is impeded after ethanol treatment.

5.3. Impaired access to mitochondrial adenylate kinase after ethanol

We also measured the effect of ethanol treatment on accessibility of ADP to mitochondrial adenylate kinase in the intermembrane space. Adenylate kinase is an enzyme that catalyzes the reversible conversion of two molecules of ADP to AMP and ATP. To examine the accessibility of mitochondrial adenylate kinase to ADP, the plasma membranes of hepatocytes were permeabilized with low digitonin, and cytosolic adenylate kinase was separated from the permeabilized hepatocytes by centrifugation. Ethanol had virtually no effect on cytosolic adenylate kinase activity (Fig. 4B). By contrast, ethanol treatment inhibited mitochondrial adenylate kinase activity of plasma membrane-permeabilized hepatocytes by more than 50% (Fig. 4B). This inhibition of mitochondrial adenylate kinase was almost completely overcome by treating hepatocytes with more digitonin to permeabilize the mitochondrial outer membrane (Fig. 4B). Thus, ethanol treatment inhibited accessibility of ADP to adenylate kinase in the intermembrane space. This result also points to an ethanol-induced change of outer membrane permeability in support of the proposal that ethanol causes a decrease of VDAC conductance and global suppression of mitochondrial metabolism of polar compounds.

6. Conclusion

This overview of anomalous mitochondrial metabolism suggests that VDAC exerts global control over mitochondrial metabolism. In some instances, VDAC seems to act as a brake on mitochondrial metabolism, which could be beneficial to limit futile ATP hydrolysis during ischemia, anoxia and hibernation. Global suppression of mitochondrial oxidation of polar substrates would also foster selective oxidation of non-polar (bilayer-permeant) versus polar (bilayer-impermeant) substrates and toxins. In other instances, dynamic regulation of VDAC conductance by glucose-metabolizing enzymes may control insulin secretion in pancreatic beta cells and aerobic glycolysis in cancer cells. However, excessive VDAC closure may also promote apoptosis, cytopathic hypoxia, fatty liver disease and other pathophysiological changes. Preliminary experiments in isolated rat hepatocytes support the conclusion that ethanol treatment, at least, inhibits VDAC conductance as predicted. Overall, control of VDAC conductance appears both to up and down regulate mitochondrial function. Since VDAC is more than a simple brake on mitochondrial metabolism, we suggest that VDAC is a "governor"[1] of mitochondrial function. More than a simple governor that limits maximum velocity, VDAC as governor establishes set points for global mitochondrial activity that change in response to cellular needs and metabolic stresses. Future research will be needed to determine the merits of this hypothesis and the exact mechanisms by which VDAC conductance may be regulated in vivo by such factors as ROS, RNS, cytokines, NADH and protein phosphorylation.

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[1] Portions of this work were presented at the 49th Annual Meeting of the Biophysical Society, Long Beach, CA, February 12–16, 2005 [38], where the word governor is a title-in-jest for former actor Arnold Schwarzenegger, governor of California, best known for his role in motion pictures as 'The Terminator.'

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