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Ethanol exposure decreases mitochondrial outer membrane permeability in cultured rat hepatocytes

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Abstract

Mitochondrial metabolism depends on movement of hydrophilic metabolites through the mitochondrial outer membrane via the voltage-dependent anion channel (VDAC). Here we assessed VDAC permeability of intracellular mitochondria in cultured hepatocytes after plasma membrane permeabilization with 8 μ M digitonin. Blockade of VDAC with Koenig's polyanion inhibited uncoupled and ADP-stimulated respiration of permeabilized hepatocytes by 33% and 41%, respectively. Tenfold greater digitonin (80 μ M) relieved KPA-induced inhibition and also released cytochrome *c*, signifying mitochondrial outer membrane permeabilization. Acute ethanol exposure also decreased respiration and accessibility of mitochondrial adenylate kinase (AK) of permeabilized hepatocytes membranes by 40% and 32%, respectively. This inhibition was reversed by high digitonin. Outer membrane permeability was independently assessed by confocal microscopy from entrapment of 3 kDa tetramethylrhodamine-conjugated dextran (RhoDex) in mitochondria of mechanically permeabilized hepatocytes. Ethanol decreased RhoDex entrapment in mitochondria by 35% of that observed in control cells. Overall, these results demonstrate that acute ethanol exposure decreases mitochondrial outer membrane permeability most likely by inhibition of VDAC.

Keywords

Adenylate kinase; Ethanol; Hepatocyte; Mitochondria; Tetramethylrhodamine-conjugated dextran; VDAC

Introduction

Acute responses of liver to ethanol include compromised mitochondrial ATP generation, increased formation of reactive oxygen species (ROS)¹, lipid peroxidation and suppression of fatty acid oxidation [1–8]. Ethanol also causes a hypermetabolic state characterized by a swift increase in alcohol metabolism (SIAM), a near doubling of mitochondrial respiration and an

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¹Abbreviations used: AK, adenylate kinase; BSA, bovine serum albumin; DIDS, (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid); ICB, intracellular buffer; KPA, 10 kDa Koenig's polyanion; LDH, lactate dehydrogenase; MTG, MitoTracker Green FM; RhoDex, 3 kDa tetramethylrhodamine-conjugated dextran; ROS, reactive oxygen species; TMRM, tetramethylrhodamine methylester; VDAC, voltage dependent anion channel.

apparent uncoupling of mitochondrial oxidative phosphorylation [6–8]. Mitochondrial metabolism requires continuous exchange of substrates between the cytosol and the mitochondrial matrix. Such exchange is catalyzed by specific exchangers located within the inner membrane, including the adenine nucleotide transporter, the phosphate transporter, the dicarboxylic acid transporter, the carnitine–acetylcarnitine transporter and others [9,10].

By contrast, exchange of virtually all water soluble metabolites between the cytosol and the intermembrane space is widely accepted to occur principally through the voltage dependent anion channel (VDAC) of the mitochondrial outer membrane [11–17]. Other types of large channels are also described in the mitochondrial outer membrane, but these non-VDAC channels remain closed except when opened by proapoptotic and related signaling or are dedicated to specific functions, such as protein import (reviewed in [11,12]. VDAC is the only channel in the mitochondrial outer membrane yet identified that facilitates the exchange of small hydrophilic metabolites between the mitochondrial intermembrane space and the cytosol. Thus, changes of VDAC permeability could be important in global regulation of mitochondrial metabolism.

Although acute ethanol exposure induces mitochondrial dysfunction in hepatocytes, little is known about the molecular mechanism(s) underlying ethanol-mediated alterations of mitochondrial metabolism [1–8,18–20]. Recently, we proposed that VDAC closure could explain, at least in part, suppression of mitochondrial ATP generation and fatty acid oxidation after ethanol [21]. Here, we test this hypothesis to show that changes in the biochemical and permeability characteristics of mitochondria in rat hepatocytes exposed to a single dose of ethanol are consistent with decreased permeability of VDAC and restricted permeability of the outer membrane to hydrophilic mitochondrial metabolites.

Materials and methods

Hepatocyte isolation

Hepatocytes were isolated from 24-h-fasted male Sprague-Dawley rats (200–300 g) by collagenase digestion, as described previously [22]. Cell viability routinely exceeded 95% by trypan blue exclusion. Isolated hepatocytes were suspended in Krebs-Ringer-Hepes (KRH) buffer containing (in mM) 115 NaCl, 5 KCl, 2 CaCl₂, 1 KH₂PO₄, 1.2 MgSO₄, and 25 Hepes/NaOH, pH 7.35, supplemented with 0.2% bovine serum albumin (BSA) and stored on ice. Animal protocols were approved by the Institutional Care and Use Committee of the University of North Carolina at Chapel Hill.

Digitonin permeabilization

Freshly isolated hepatocytes (2×10^6 cells/ml) were suspended in ice-cold intracellular buffer (ICB) containing (in mM) 120 KCl, 10 NaCl, 1 KH₂PO₄, 2 Mg-ATP, 1 EGTA, and 20 Hepes/NaOH, pH 7.35, supplemented with protease inhibitors (pepstatin, antipain, leupeptin; 1 µg/ml each), oligomycin (5 µg/ml) and rotenone (10 µM), as described [21]. Digitonin (0–80 µM) was then added with continuous stirring. After 10 min, hepatocytes were centrifuged at 50g for 2 min, and the cells were resuspended in 50 ml of ICB (2×10^6 ml⁻¹) supplemented with 10 mg/ml BSA to remove non-bound digitonin. After a second centrifugation, pelleted cells were suspended in ICB (10^7 cells/ml) without BSA and stored on ice until use. In some experiments, cells were permeabilized with digitonin for 10 min, followed directly by measurements of trypan blue uptake, enzyme release or AK and respiratory activity. Efficiency of permeabilization was assessed using a trypan blue (0.2%) exclusion test [22].

Lactate dehydrogenase

Lactate dehydrogenase (LDH) activity was measured using a commercial kit (Sigma Chemical Co., St. Louis, MO) from pyruvate-dependent oxidation of NADH. Activity was expressed as nmol/min/ 10^6 cells or percentage of total cellular LDH activity measured in the presence of 0.05% Triton X-100.

Adenylate kinase

AK activity was measured from reduction of NADP⁺ utilizing hexokinase/glucose-6-phosphate dehydrogenase in the presence of glucose and ADP, as described [23]. Briefly, the reaction was initiated by addition of an aliquot of supernatant (cytosol) or pellet obtained from digitonin-treated hepatocytes to buffer containing (in mM) 100 potassium acetate, 20 glucose, 2 ADP, 4 MgCl₂, 2 NADP⁺, 1 EDTA, 1 dithiothreitol, 4.5 U/ml hexokinase, 2 U/ml glucose-6-phosphate dehydrogenase, and 20 Hepes/NaOH, pH 7.5. Activity was expressed as nmol/min/ 10^6 cells or percentage of total cellular AK activity measured in the presence of 0.05% Triton X-100.

Respiration

Oxygen consumption before and after treatment with digitonin was measured in ICB supplemented with succinate (5 mM) and cytochrome *c* (1 mg/ml) and not containing oligomycin and ATP using a Clark oxygen electrode (Oxygraph, Hansatech, CO). Respiration was expressed as nmol O₂/min/ 10^6 cells or percentage of maximal cellular respiration [24].

Western blot

Hepatocytes (2×10^6 cells/ml) were separated from incubation medium by centrifugation (14,000 rpm for 60 s). Aliquots of supernatants (50 µg protein) were resolved by SDS-PAGE (8–12%) and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) and immunoblotted for cytochrome *c* using an ECL Plus kit (Amersham Pharmacia Biotech, Piscataway, NJ), as described [25].

Cell culture

For imaging experiments, isolated hepatocytes in Waymouth's medium MB-752/1 (GIBCO, Grand Island, NY) supplemented with 2 mM L-glutamine, 27 mM NaHCO₃, 10% fetal calf serum, 100 nM insulin, 100 nM dexamethasone and 100 units of penicillin and streptomycin were plated on glass bottom culture dishes (MatTek, Ashland, MA) coated with 0.1% rat tail collagen type I at a density of 1.5×10^5 cells/ml and incubated overnight in 5% CO₂-95% air at 37 °C [21,22]. Cultured hepatocytes were used after 16–24 h of incubation.

Fluorescent labeling of mitochondria

Cultured hepatocytes were incubated with 500 nM of MTG in KRH for 60 min at 37 °C for covalent labeling of the mitochondrial inner membrane-matrix space [21,22] or with 100 nM of tetramethylrhodamine methylester (TMRM) in KRH for 60 min at 37 °C to monitor mitochondrial membrane potential [26].

Mechanical perturbation of the plasma membrane of cultured hepatocytes

Plasma membranes of hepatocytes plated on glass bottom Petri dishes were ruptured mechanically using a glass micropipette. With ICB, supplemented with protease inhibitors (pepstatin, antipain, leupeptin; 1 µg/ml each), oligomycin (5 µg/ml), rotenone (10 µM), succinate (5 mM) and Mg-ATP (2 mM) in both the micropipette and the surrounding medium, the micropipette was inserted and dragged across individual cells with a micromanipulator (Model MM-89, Narishige International USA, Inc., East Meadow, NY) to puncture and tear

the plasma membrane. The procedure was repeated for all cells in a microscope field. To compensate for the decrease of intracellular oncotic pressure from loss of cytosolic proteins, ICB for studies involving permeabilized hepatocytes was supplemented with 30 mg/ml of 64–76 kDa dextran (Sigma Chemical Co., St. Louis, MO) and 50 nM of TMRM.

Laser scanning confocal microscopy

MTG-labeled hepatocytes plated on glass bottom Petri dishes were placed on the stage of a LSM 510 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY). KRH was replaced with ICB buffer containing 3 kDa RhoDex (400 μ M), 64–76 kDa dextran (30 mg/ml), rotenone (1 μ M), oligomycin (1 μ g/ml) and succinate (5 mM). After equilibration, the plasma membranes of hepatocytes were mechanically ruptured using a glass micropipette and further incubated for 2 min to allow RhoDex to diffuse into intracellular compartments. The buffer was then replaced with the same buffer containing in addition 30 μ M DIDS, a blocker of VDAC. After 1 min, the medium was replaced with the same DIDS-containing ICB but without RhoDex. Colocalization of RhoDex with mitochondria was assessed from fluorescent confocal images of green-fluorescing MTG and red-fluorescing RhoDex using a 63 $\times$ N.A. 1.4 planapochromat oil immersion lens with pinholes set to 1.0 Airy unit in both the red and green channels. RhoDex and MTG were excited with 543-nm light from a HeNe laser and 488-nm light from an argon laser, respectively, with laser intensities attenuated to 0.05%. Emitted fluorescence was divided by a 545-nm dichroic reflector and passed through 500–530 nm band-pass and 560 nm long-pass barrier filters to measure green and red fluorescence, respectively.

Image analysis

To quantify RhoDex retention, green MTG-labeled mitochondria were outlined and segregated from the cytosol in the green channels of images using ImageJ 1.33 u (free download at <http://rsb.info.nih.gov/ij/download.html>). The outline of mitochondria was then copied into the red channels of the same images. Mean intensity of these mitochondrial regions in the red channels after subtraction of background was taken as a quantitative measure of mitochondrial RhoDex retention. Mitochondrial TMRM fluorescence of individual cells was calculated by subtracting mean fluorescence of nuclear pixels from mean cellular fluorescence and multiplying by the number of cellular pixels.

Materials

Reagents were obtained from the following sources: 3 kDa RhoDex, TMRM, DIDS and MTG from Molecular Probes (Eugene, OR); 100% pure digitonin (M.W. 1229.3) from Calbiochem (San Diego, CA); BSA (Fraction IV), hexokinase, glucose-6-phosphate dehydrogenase, type I collagen and dextran (64–76 kDa) from Sigma (St. Louis, MO); Hepes and collagenase D from Boehringer Mannheim Biochemicals (Indianapolis, IN); Waymouth's medium MB-252/1 from GIBCO Laboratories (Grand Island, NY); insulin from Squibb-Novo (Princeton, NJ); and dexamethasone sodium phosphate from LyphoMed (Rosemont, IL). Koenig's polyanion was the generous gift of Dr. Marco Colombini (University of Maryland). Other analytical grade chemicals were obtained from the usual commercial sources.

Statistics

Differences between groups were analyzed by the Student's *t*-test using $p < 0.05$ as the criterion of significance. Results were expressed as means $\pm$ SEM. When error bars are not present, they fall within the diameters of the symbols. Images and Western blots are representative of three or more experiments.

Results

Digitonin permeabilization of plasma and mitochondrial outer membranes of hepatocytes

Digitonin, a non-ionic detergent that forms pores in cholesterol-containing membranes [27], was used to permeabilize the plasma and mitochondrial outer membranes of rat hepatocytes. Isolated intact hepatocytes were suspended in intracellular buffer (ICB) and treated with 0–80 μM digitonin. Trypan blue labeling and LDH release increased in a dose-dependent manner (Fig. 1A). Trypan blue labeling occurred somewhat more rapidly than LDH release, suggesting that the size of digitonin-induced pores in the plasma membrane increased progressively with increasing concentration (Fig. 1A).

Adenylate kinase (AK) is present in both the cytosol and mitochondrial intermembrane space of hepatocytes [28]. After digitonin treatment, AK release was biphasic consistent with these two intracellular pools (Fig. 1A). Release of the first pool (~55% of total activity) was complete at 8 μM digitonin and paralleled trypan blue labeling and LDH release (98% and 95%, respectively), confirming that the first pool represented cytosolic AK (Fig. 1A). Due to differences in molecular weight and binding to cytosolic constituents, cytosolic AK may be released slightly earlier than LDH at 5 μM digitonin. Similarly, trypan blue uptake also occurs earlier. At 10–20 μM digitonin, 90% of LDH was released after 10 μM digitonin whereas AK release plateaued at ~50% (Fig. 1A). Since about half of adenylate kinase is in the mitochondrial intermembrane space, these results indicate that cytosolic LDH and cytosolic (non mitochondrial) AK are released at the same digitonin concentration. At the next lower concentration of digitonin (4 μM), relatively more of the cytosolic AK pool was released than LDH, consistent with the smaller molecular weight of cytosolic AK (21.7 kDa) [29] compared to LDH (140 kDa) [30]. The second pool of AK (~45% of activity) was released by higher digitonin, and essentially all AK activity was released by 80 μM digitonin (Fig. 1A). Release of the second AK pool coincided with release of cytochrome *c* (Fig. 1B). Thus, low digitonin (up to 20 μM) permeabilized the plasma membrane and released cytosolic AK and other cytosolic proteins like LDH, whereas high digitonin (80 μM) released mitochondrial AK and other intermembrane proteins like cytochrome *c*.

The effects of digitonin on mitochondria were evaluated in cultured hepatocytes loaded with tetramethylrhodamine methylester (TMRM), an indicator of mitochondrial membrane potential. Prior to digitonin, TMRM labeled round and oval mitochondria typical of healthy hepatocytes (Fig. 2A, Intact). After 8 μM digitonin in ICB, mitochondrial TMRM fluorescence decreased despite the inclusion of 50 nM TMRM to maintain the equilibrium distribution of the fluorophore (Fig. 2A, +Digitonin). Subsequent addition of succinate, a respiratory substrate, restored TMRM fluorescence (Fig. 2A, +Succinate), suggesting that loss of TMRM after digitonin permeabilization of the plasma membrane may have been due in part to release of endogenous respiratory substrates from the cytosol. After digitonin treatment and succinate supplementation, the morphology of TMRM-labeled mitochondria was essentially unchanged. The protonophoric uncoupler, 2,4-dinitrophenol (DNP), then greatly decreased TMRM fluorescence (Fig. 2A, +DNP). The mean intensity of TMRM fluorescence of these images changed from 49 ± 13 arbitrary units in intact cells to 30 ± 11 after digitonin permeabilization. Energization of mitochondria with succinate increased mean TMRM fluorescence to 46 ± 11 , which decreased to 15 ± 9 after depolarization with DNP ($n = 28$ cells from four isolations, $p < 0.05$). Thus, on average, relative mitochondrial TMRM fluorescence of individual cells changed from 100% before digitonin to $62 \pm 8\%$, $95 \pm 13\%$ and $31 \pm 4\%$ after sequential additions of digitonin (5 min), succinate (5 min) and DNP (5 min), respectively. These results showed that digitonin permeabilization of the plasma membranes of hepatocytes although releasing cytosol and endogenous mitochondrial substrates does not alter mitochondrial structure or the ability of mitochondria to maintain a membrane potential in the presence of exogenous respiratory substrate.

Respiration in permeabilized hepatocytes

Hepatocytes stored anaerobically on ice were added to ICB not containing oligomycin or ATP and supplemented with succinate and cytochrome *c* to replace cytochrome *c* that might leak from the intermembrane space after digitonin permeabilization. Subsequently, a rapid decrease of oxygen occurred that was followed by linear oxygen uptake at 7 ± 1 nmol O₂/min/10⁶ cells (Fig. 2B, Hepatocytes, and C, Intact). DNP increased this respiration to 14 ± 2 nmol O₂/min/10⁶ cells (Fig. 2B, DNP, and C, None). Digitonin (8 μM) further increased oxygen consumption to 110 ± 4 nmol O₂/min/10⁶ cells (Fig. 2B, Digitonin, and C, Dig 8), indicating that extracellular succinate was largely unavailable to intracellular mitochondria until permeabilization of the plasma membrane. Malonate (20 mM), an inhibitor of succinate dehydrogenase, decreased oxygen consumption, indicating that malonate was also available to mitochondria after digitonin permeabilization (Fig. 2B, Malonate). Similarly, low digitonin (8 μM) increased oxygen uptake by hepatocytes incubated with succinate plus ADP (data not shown). Respiratory stimulation by digitonin was dose-dependent and plateaued at ~8 μM (Fig. 2B, inset). Respiratory stimulation did not increase further at higher digitonin.

Inhibition of respiration by VDAC blockade

To assess the importance of VDAC in oxidative phosphorylation, we treated digitonin-permeabilized hepatocytes with 10 kDa Koenig's polyanion (KPA), an inhibitor of VDAC [14,15,31]. As shown in Fig. 3A, KPA inhibited DNP-stimulated, succinate-supported respiration of low digitonin (8 μM)-permeabilized hepatocytes by 33%. This inhibition was almost completely relieved by high digitonin (80 μM) (Fig. 3A). Similarly, KPA inhibited ADP-stimulated respiration by 41% in low digitonin-permeabilized hepatocytes (Fig. 3B). High digitonin again overcame KPA-inhibited respiration (Fig. 3B).

Suppression of respiration and accessibility to intermembrane adenylate kinase after ethanol treatment

To evaluate the effects of ethanol on respiration, isolated hepatocytes were incubated in culture medium with 50 mM ethanol for 20 min, centrifuged, resuspended in culture medium without ethanol, centrifuged again, and resuspended in ICB not containing oligomycin or ATP and supplemented with ADP and succinate. Respiration of ethanol-treated hepatocytes in the presence of ADP and succinate increased by 92% (23 ± 2 nmol O₂/min/10⁶ cells *vs* 12 ± 2 nmol O₂/min in untreated cells, Fig. 4). After permeabilization with low digitonin, respiration of control and ethanol-treated hepatocytes was 103 ± 2 and 62 ± 2 nmol O₂/min/10⁶ cells, respectively, showing a 40% inhibition of respiration by plasma membrane-permeabilized hepatocytes after ethanol exposure (Fig. 4A, Dig 8). This inhibition was reversed by high doses of digitonin, which restored respiration to 95 ± 4 nmol O₂/min/10⁶ cells in ethanol-treated hepatocytes, nearly the same as in untreated hepatocytes (Fig. 4, Dig 80). Thus, ethanol treatment suppressed respiration of hepatocytes permeabilized with low digitonin, and this inhibitory effect was overcome when mitochondrial outer membranes were permeabilized with high digitonin.

To assess further the effect of ethanol on outer membrane permeability, ethanol-treated and untreated hepatocytes were permeabilized with low digitonin, and AK activity was measured in the supernatant (cytosol) and pellet (permeabilized hepatocytes with intact mitochondria). Ethanol treatment did not alter cytosolic AK activity, which accounted for about 50% of total activity measured after Triton X-100 permeabilization (Fig. 5A). By contrast, mitochondrial AK activity in the pellet decreased from 42% of total activity in untreated cells to 24% after ethanol treatment (Fig. 5B, None *vs* Ethanol, Dig 8). Subsequent exposure of ethanol-treated hepatocytes to high digitonin restored mitochondrial AK activity to 48% of the total (Fig. 5B, Ethanol, Dig 80). Recovery of mitochondrial AK activity in ethanol-treated hepatocytes with high dose digitonin was associated with release of cytochrome *c*, confirming that high dose

digitonin permeabilized the mitochondrial outer membrane (data not shown). Thus, after ethanol treatment, the outer membrane became a barrier to the ingress and egress of substrate (ADP) and products (ATP and AMP) for the AK reaction in the intermembrane space.

Assessment of 3 kDa fluorescent dextran uptake into the intermembrane space by confocal microscopy

Cultured hepatocytes loaded with MitoTracker Green FM (MTG), a fluorescent probe that labels mitochondria, showed typical green fluorescent mitochondria by confocal microscopy (Fig. 6A). After replacing the incubation buffer with ICB containing 3 kDa RhoDex, individual hepatocytes were seen by negative contrast as non-fluorescent voids that were outlined and surrounded by the red fluorescence of RhoDex (not shown). A micropipette tip was then drawn across each individual cell in the microscopic field to rupture their plasma membranes and allow red-fluorescing RhoDex to enter the hepatocytes (Fig. 6B). In ruptured hepatocytes, mitochondria retained their typical morphology and were seen as green structures surrounded by the red fluorescence of RhoDex (Fig. 6B). Because 3 kDa RhoDex is below the size exclusion limit of VDAC [14,15,31], RhoDex permeates the mitochondrial outer membrane to enter the intermembrane space. RhoDex also entered nuclei, presumably through nuclear pores [23].

After 2 min to allow RhoDex entry into hepatocytes and the mitochondrial intermembrane space, DIDS, a non-selective inhibitor of VDAC [14,15,32], was added to close VDAC and entrap RhoDex within the intermembrane space. After exactly 1 min, the cells were washed with the ICB containing DIDS but no RhoDex, and fluorescent images of the washed hepatocytes were acquired after 5 min (Fig. 6C). After the final wash, RhoDex fluorescence disappeared entirely from the extracellular space and was largely removed from nuclei and cytoplasm while the MTG-labeled mitochondria remained virtually unchanged (Fig. 6D). Rare cells retained cytosolic RhoDex, indicating resealing of the plasma membrane, and were excluded from analysis.

Higher magnification of washed cells revealed retention of RhoDex in areas occupied by mitochondria (Fig. 6D and E). Red fluorescence in mitochondria was surrounded by non-fluorescent spaces, which showed that RhoDex was essentially completely washed from the cytosol. A smaller population of round structures that were not MTG-labeled mitochondria also retained bright RhoDex fluorescence (Fig. 6D–F, white arrows). These structures may be of endocytic origin. Uptake and retention of RhoDex by nuclei and in other vesicles (presumably of endocytic origin) was not affected by DIDS. Unruptured hepatocytes in adjacent fields also revealed RhoDex-labeled endocytic vesicles, but RhoDex uptake into the cytosol or mitochondria did not occur in unruptured cells (data not shown).

Inhibition of 3 kDa fluorescent dextran uptake into the intermembrane space after ethanol treatment

Hepatocytes were pretreated with ethanol, DIDS or no treatment and permeabilized in the presence RhoDex, as described above for Fig. 6. Images were then obtained after wash-out of unbound RhoDex with ICB supplemented with DIDS. Identification of RhoDex inside mitochondria was determined from red fluorescence that colocalized with MitoTracker green fluorescence, as described in Materials and methods. Ethanol pretreatment of hepatocytes decreased mitochondrial RhoDex entrapment (Fig. 7A, compare control and ethanol). On average, ethanol decreased mitochondrial RhoDex retention by 35% compared to untreated cells (Fig. 7B). These observations were in accord with our biochemical data and support the conclusion that ethanol treatment of hepatocytes decreases outer membrane permeability. Pretreatment of hepatocytes with the VDAC inhibitor, DIDS, decreased mitochondrial RhoDex uptake by 56% compared to control cells without affecting MTG retention or mitochondrial

morphology, consistent with the conclusion that RhoDex enters the intermembrane space through VDAC (Fig. 7B). Mitochondrial labeling with MTG showed some cell to cell variability, but consistent differences between ethanol-treated and untreated cells were not observed.

Discussion

The molecular mechanism(s) underlying the pathogenesis of alcohol-induced liver disease remains incompletely understood, but many factors converging on mitochondria may contribute to metabolic alterations and progressive liver injury [3–8]. Mitochondrial metabolic alterations after ethanol exposure include increased respiration and acetaldehyde oxidation, decreased fatty acid oxidation and ATP generation, glutathione depletion, and oxidative stress [1–8,29,30]. Normal mitochondrial metabolism requires continuous exchange of metabolites between the cytosol and the mitochondrial matrix. While the mitochondrial inner membrane contains a variety of specific exchangers for this purpose, transport of metabolites across the outer membrane does not involve specific transporters. Rather, hydrophilic mitochondrial metabolites cross the outer membrane and enter the intermembrane space principally via VDAC [12–16]. Although other large conductance channels have been described in the outer mitochondrial membrane (reviewed in [11,12]), these channels are generally closed until induction of apoptosis or are dedicated to protein import. Thus, the conductance state of VDAC could be important in global regulation of mitochondrial physiology and oxidative metabolism [12,20].

Recently, we hypothesized that VDAC closure contributes to suppression of mitochondrial function after alcohol exposure and in other pathophysiological and physiological settings, including anoxia/ischemia, cytopathic hypoxia and aerobic glycolysis (Warburg effect) in cancer [21]. The consequence of VDAC closure was proposed to be disruption of normal movement of metabolites (ADP, ATP, Pi, respiratory substrates, acylcarnitine) into and out of mitochondria, leading to global suppression of mitochondrial function. Here to test this hypothesis, we evaluated mitochondrial outer membrane permeability in normal and ethanol-stressed rat hepatocytes.

Mitochondrial functions were assessed after permeabilization of the plasma and mitochondrial outer membranes with different doses of digitonin. Selective plasma membrane permeabilization with low digitonin (8–20 μ M) was confirmed by LDH release and trypan blue uptake with retention of mitochondrial AK and cytochrome *c*, whereas higher digitonin (80 μ M) caused outer membrane permeabilization with release of cytochrome *c* and mitochondrial intermembrane AK (Fig. 1A and B). Higher digitonin did not cause inner membrane permeabilization, since mitochondrial membrane potential was maintained (Fig. 2A).

Ethanol treatment resulted in almost doubling of the rate of endogenous cellular respiration consistent with closure of VDAC (Fig. 4, compare A and B, None). At the same time, treatment of control and ethanol exposed cells with low digitonin resulted in greater respiration in control (~ 100 nmol/min/ 10^6 cells) as compared with ethanol exposed cells (~ 60 nmol/min/ 10^6 cells), demonstrating 40% suppression of the rate of respiration following ethanol exposure. The increased endogenous respiration may be an indication of partial uncoupling [5–8]. Metabolites of ethanol may persist after ethanol washout to explain the increase. Alternatively, an uncoupling mechanism may be activated that also persists after washout. High digitonin restored respiration of ethanol-treated hepatocytes to virtually the same rate as untreated hepatocytes (Fig. 4A and B). Similar inhibition of respiration after low and high digitonin was obtained using Koenig's anion, a specific VDAC inhibitor (Fig. 3).

Similarly, ethanol treatment inhibited the accessibility of mitochondrial AK to its substrates (Fig. 5). Although ethanol treatment had no effect on cytosolic AK activity, mitochondrial AK activity was 43% less after ethanol treatment than without treatment. High digitonin again restored mitochondrial AK activity after ethanol treatment to nearly the same levels as in untreated hepatocytes (Fig. 5A and B). These results are consistent with the conclusion that ethanol treatment causes a decrease of outer membrane permeability toward water soluble substances, most likely by inhibition of VDAC.

Decreased outer membrane permeability after ethanol was confirmed using confocal microscopy. To eliminate possible artifacts from use of digitonin, we mechanically disrupted the plasma membranes of hepatocytes using a glass micropipette (Fig. 6). Hepatocytes with disrupted plasma membranes were then incubated with 3 kDa RhoDex and treated with DIDS, a VDAC blocker, to entrap RhoDex in the intermembrane space. In comparison to untreated hepatocytes, mitochondrial RhoDex retention was 35% less in ethanol-treated hepatocytes, which was similar but not as great as the 56% inhibition of RhoDex uptake by DIDS pretreatment to block VDAC prior to RhoDex addition (Fig. 7). DIDS and other compounds known to inhibit VDAC do not completely block reconstituted VDAC channels [9,13–15,17, 31]. Thus, uptake and release of RhoDex from mitochondria was not completely inhibited by DIDS (Fig. 6). Accordingly, we evaluated retention of RhoDex fluorescence at exactly 300 s after wash-out of the dye, so that differences observed would not be due to time variations in the measurements. In experiments in which cells were permeabilized, loaded with RhoDex and washed in the absence of DIDS, RhoDex was rapidly released and little RhoDex was retained after 300 s (data not shown). The protocol used for RhoDex loading does not allow monitoring of the kinetics of RhoDex uptake. The time required to obtain a first image of the cell after RhoDex exposure is 30–40 s and the procedure of wash-out lasts about 30 s, thus making time resolution of this approach longer than 60 s.

Thus, both biochemical experiments and quantitative confocal microscopy supported the conclusion that ethanol treatment causes an impairment of outer membrane permeability to hydrophilic molecules. For the confocal microscopy experiments, Koenig's anion was not used because we had already exhausted our small supply and could not obtain more from either commercial or private sources. DIDS is a less specific inhibitor than Koenig's anion and has other effects, such as respiratory inhibition [30]. Thus, DIDS could only be used in experiments directly evaluating outer membrane permeability with 3 kDa RhoDex.

In untreated hepatocytes, we confirmed the general observation that outer membrane permeability is not rate-limiting for oxidative phosphorylation and other mitochondrial activities in cells under normal conditions. Because VDAC was not rate-limiting prior to ethanol treatment, suppression of respiration and AK activity due to decreased outer membrane permeability implies a proportionally much greater inhibition of VDAC permeability. However from the present data, we cannot quantify the exact extent to which VDAC conductance decreases after ethanol treatment.

Although ethanol exposure appears to close mitochondrial VDAC, the mechanisms regulating VDAC permeability are incompletely understood. Oxidation of ethanol in the cytosol and acetaldehyde in mitochondria leads to ROS formation and increased NADH [2–5], and high NADH and increased ROS can inhibit VDAC permeability [12,13,16]. Oxidative modification of VDAC and adduct formation with acetaldehyde might also inhibit VDAC activity. Since ethanol-dependent inhibition of outer membrane permeability persisted after ethanol washout, an effect on permeability mediated by covalent modification of VDAC seems more likely than regulation by NADH redox state. Future experiments will be needed to address these issues and determine whether adduct formation and/or protein kinases regulate VDAC activity after ethanol exposure. Recent observations that VDAC phosphorylation potentiates wortmanin-

induced apoptosis suggest that protein kinases may participate in ethanol-dependent regulation of VDAC [33–35].

Although VDAC is implicated in apoptotic signaling, neither apoptosis nor necrosis occurs to a significant extent in liver after acute ethanol treatment. Rather, ethanol decreases mitochondrial ATP production, increases alcohol metabolism and respiration, and induces hepatic steatosis [4–8,21,36–40]. VDAC closure helps explain many of these acute effects. Fat metabolism requires movement of fatty acylCoA through VDAC into the intermembrane space. Thus, VDAC closure would suppress fatty acid oxidation, leading to accumulation of lipids and steatosis. VDAC closure would also inhibit ATP release from mitochondria, leading to activation of glycolysis and a decrease of ATP. By inhibiting oxidation of lipid-impermeable respiratory substrates, VDAC closure would also promote selective oxidation of acetaldehyde, a toxic bilayer-permeant product of alcohol dehydrogenase. Ethanol oxidation in hepatocytes is associated with increased cytosolic NADH and inhibition of glycolysis. One of the possible mechanisms of NADH oxidation involves malate–aspartate shuttle [40–42], which mediates transfer of cytosolic redox equivalents into mitochondria for oxidation in respiratory chain. Closure of VDAC with ethanol might then result in blockage of substrate transport across the outer membrane into the intermembrane space and inhibit the malate–aspartate shuttle. To overcome the effect of closed VDAC on NADH-oxidizing efficiency of cell, the outer membrane may contain carriers for the malate–aspartate shuttle, analogous to the inner membrane. Future studies will be needed to address this and other possible mechanisms.

Although inhibition of VDAC permeability helps explain decreased mitochondrial ATP generation, steatosis and selective acetaldehyde oxidation after ethanol treatment, VDAC closure does not explain other ethanol-induced metabolic effects. *In vivo*, ethanol produces a hypermetabolic state characterized by increased hepatic alcohol metabolism and a near doubling of mitochondrial respiration (SIAM) [6–8]. SIAM is mediated, in part, by hormone-dependent changes and does not occur in isolated perfused livers or cultured hepatocytes. Respiratory stimulation without a corresponding increase of ATP production indicates mitochondrial uncoupling, consistent with a decrease in overall metabolic efficiency observed in human alcohol drinkers [43–46], but the mechanism for mitochondrial uncoupling remains uncertain. The acetaldehyde–acetate redox potential may cause mitochondrial hyperpolarization, non-ohmic proton leaks and respiratory stimulation [47]. Alternatively, activation of uncoupling proteins and weak uncoupling by acetate formed by aldehyde dehydrogenase may increase respiration [48]. Recently, mitochondrial K_{ATP} channel opening was reported in hyperlipidemic mice [49], and futile K^+ cycling after mitochondrial K_{ATP} channel opening could provide another mechanism of uncoupling after hepatic ethanol exposure.

VDAC inhibition and uncoupling may represent independent effects of ethanol that together promote accelerated respiration and selective oxidation of acetaldehyde. These changes are adaptive by effecting more rapid elimination ethanol and detoxification of acetaldehyde. VDAC closure promotes selective acetaldehyde oxidation but at the expense of inhibition of oxidation of other substrates, especially fatty acids. These metabolic effects reverse after ethanol elimination and are insufficient alone to cause substantial cell death. Nonetheless, VDAC closure and other ethanol-induced derangements, such as ROS formation, lipid peroxidation, glutathione depletion, and aldehyde-protein adduct formation and Kupffer cell activation, may combine as multiple “hits” culminating alcohol-induced hepatotoxicity, cell death and fibrosis [1–8,18,34,41,42].

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Fig. 1.

Digitonin-mediated release of cytosolic and mitochondrial enzymes from isolated rat hepatocytes. (A) Hepatocytes (2×10^6 cells/ml) were incubated at room temperature in ICB and treated with different concentrations of digitonin for 10 min. A portion of digitonin-treated cells was taken to assess trypan blue labeling (TB), and the rest was sedimented by centrifugation at 14,000 rpm for 60 s for enzymatic analysis of the supernatants. Data are means from 6 experiments. (B) Hepatocytes (2×10^6 cells/ml) treated with 0, 8 or 80 μ M of digitonin for 10 min in ICB were centrifuged, and supernatants were subjected to Western blotting for cytochrome *c*, as described in Materials and methods. Lanes are: first lane (far left), molecular weight markers (MWM); second, third and fourth lanes, supernatants after 0, 8 and 80 μ M digitonin; last lane (far right), 100 ng cytochrome *c*, (Cyt *c*). Representative Western blots are shown from three or more independent hepatocyte incubations yielding similar results.

Fig. 2.

Effect of digitonin treatment of hepatocytes on mitochondrial membrane potential and respiration. (A) Cultured hepatocytes were loaded with TMRM to visualize mitochondria, as described in materials and methods. Red fluorescence of TMRM was imaged by laser scanning confocal microscopy. Shown are representative images of a hepatocyte before (Intact) and after consecutive treatment with 8 μM digitonin (+Digitonin), 5 mM succinate (+Succinate) and 50 μM 2,4-dinitrophenol (+DNP). As indicated by uptake of TMRM, mitochondria of intact hepatocytes partially depolarized after digitonin (+Digitonin). Succinate restored mitochondrial polarization (+Succinate), but nearly complete depolarization occurred after dinitrophenol (+DNP). Images are representative from three independent experiments. (B) Representative tracing of oxygen uptake by isolated hepatocytes incubated in ICB supplemented with 5 mM succinate after sequential addition of 50 μM dinitrophenol (DNP), 8 μM digitonin and 20 mM malonate. Inset shows respiration as a function of digitonin concentration. Data are means from six experiments. (C) Respiration of isolated hepatocytes in the absence (Intact) and presence of dinitrophenol (DNP) either with intact (None) or digitonin-permeabilized plasma membranes (Dig 8, 8 μM). Oligomycin and ATP were omitted from ICB for respiration experiments. Data are means from six experiments.

Fig. 3.

Effects of Koenig's polyanion on DNP- and ADP-stimulated respiration in digitonin-permeabilized hepatocytes. Respiration of hepatocytes (2×10^6 cells/ml) was measured in ICB supplemented with 5 mM succinate. (A) DNP-stimulated respiration was measured after sequential addition of 8 μ M digitonin (Dig 8), 25 μ g/ml KPA (25 KPA), and 80 μ M digitonin (Dig 80). (B) ADP (500 μ M)-stimulated respiration was measured after sequential addition of 8 μ M digitonin (Dig 8), 25 μ g/ml KPA (25 KPA) and 80 μ M digitonin (Dig 80). Oligomycin and ATP were omitted from ICB for respiration experiments. Data are means $\pm$ SEM of at least six experiments. * $p < 0.05$ compared to Dig 8.

Fig. 4. Restoration of ethanol-inhibited respiration by high digitonin. (A) Respiration of untreated hepatocytes (2×10^6 cells/ml) was measured in ICB (without oligomycin and ATP), supplemented with 5 mM succinate and 500 μ M ADP after no addition (None) and after sequential addition of 8 μ M and 80 μ M digitonin (Dig 8 and Dig 80, respectively). (B) Respiration was measured as in A in hepatocytes exposed to 50 mM ethanol for 20 min. Data are means $\pm$ SEM of at least six experiments. * $p < 0.05$ compared to None; ** $p < 0.05$ compared to Dig 8.

Fig. 5.

Ethanol exposure limits accessibility to mitochondrial AK. Untreated and ethanol-treated hepatocytes (2×10^6 cells/ml) were incubated with digitonin (8 μ M) and centrifuged. AK activity was measured in the cytosol (A) and pellet (B), as described in Materials and methods. In (B) the pellet from low-digitonin treated, ethanol-treated hepatocytes was treated additionally with high digitonin (80 μ M). Data are means $\pm$ SEM of at least six experiments. * $p < 0.05$ compared to None. ** $p < 0.05$ compared to ethanol.

Fig. 6.

Mechanical permeabilization and RhoDex entrapment in cultured hepatocytes. Cultured hepatocytes were loaded with green-fluorescing MTG and incubated in ICB (A) RhoDex (400 μ M) was then added followed by mechanical perturbation using a micropipette. Afterwards, RhoDex penetrated the cytoplasm and nuclei (B) After 120 s, the medium was replaced with ICB containing both RhoDex and DIDS (30 μ M), the latter a VDAC inhibitor to entrap RhoDex in the mitochondrial intermembrane space. After another 60 s, the hepatocytes were washed with ICB containing only DIDS to remove unbound RhoDex. After another 300 s, images of MTG and RhoDex fluorescence were collected to identify RhoDex remaining within mitochondria (C) Higher magnification (lower panels) shows retention of red-fluorescing RhoDex (E) in MTG-labeled mitochondria (D) as evident in the overlay (F) White arrows identify strongly RhoDex-labeled structures that do not co-label with MTG.

Fig. 7. Decreased RhoDex entry into mitochondria after ethanol treatment. (A) Hepatocytes were pretreated with vehicle (c0ontrol), 50 mM ethanol, or 30 μ M DIDS and subjected to the RhoDex entrapment protocol described in Fig. 6. Ethanol and DIDS pretreatment decreased retention of red-fluorescing RhoDex. (B) Mean intensities of red fluorescence associated with mitochondria are plotted for the treatment groups in A. Data are means $\pm$ SEM of 20 or more cells per group from five or more hepatocyte isolations. * p < 0.05 compared to control.