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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 μM 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 μM) 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.
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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 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 consis-

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[1] *Abbreviations 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.

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tent 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 $CaCl_2$, 1 KH_2PO_4, 1.2 $MgSO_4$, 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 KH_2PO_4, 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 50*g* 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 $MgCl_2$, 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 O_2/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 $NaHCO_3$, 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%CO_2-95%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 μM), 64–76 kDa dextran (30 mg/ml), rotenone (1 μM), oligomycin (1 μg/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 μM 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