Mitochondrial VDAC closure in rat hepatocytes oxidizing ethanol

Ethanol Suppresses Ureagenesis in Rat Hepatocytes: Role of Acetaldehyde*

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Background: Ethanol oxidation alters mitochondrial metabolism in rat hepatocytes.

Results: Ethanol via acetaldehyde formation closes mitochondrial voltage dependent anions channels (VDAC) and decreases outer membrane permeability.

Conclusion: Acetaldehyde, derived from ethanol oxidation, suppresses ureagenesis by inhibiting exchange of mitochondrial substrates across the outer membrane.

Significance: Acetaldehyde-mediated VDAC closure is an early adaptive event in ethanol metabolism that may contribute to alcoholic liver disease.

SUMMARY

Previously, we proposed that closure of voltage dependent anion channels (VDAC) in the mitochondrial outer membrane after ethanol exposure leads to suppression of mitochondrial metabolite exchange. Since ureagenesis requires extensive mitochondrial metabolite exchange, we characterized the effect of ethanol and its metabolite, acetaldehyde (AcAld), on total and ureagenic respiration in cultured rat hepatocytes. Ureagenic substrates increased cellular respiration from 15.8 ± 0.9 nmol O₂/min/10⁶ cells (baseline) to 29.4 ± 1.7 nmol O₂/min/10⁶ cells in about 30 min. Ethanol (0 - 200 mM) suppressed extra respiration after ureagenic substrates (ureagenic respiration) by up to 51% but not baseline respiration. Urea formation also declined proportionately.

Inhibition of alcohol dehydrogenase, cytochrome P450 2E1 and catalase with 4-methylpyrazole, trans-1,2-dichloroethylene and 3-amino-1,2,3-triazole restored ethanol-suppressed ureagenic respiration by 46%, 37% and 66%, respectively. By contrast, inhibition of aldehyde dehydrogenase with phenethyl isothiocyanate increased the inhibitory effect of ethanol on ureagenic respiration by an additional 60%. AcAld, an intermediate product of ethanol oxidation, suppressed ureagenic respiration with an apparent IC₅₀ of 125 μM. AcAld also inhibited entry of 3 kDa RhDex in the...
mitochondrial intermembrane space of digitonin-permeabilized hepatocytes, indicative of VDAC closure. In conclusion, AcAld, derived from ethanol metabolism, suppresses ureagenesis in hepatocytes mediated by closure of VDAC.

Balanced and coordinated functioning of hepatocytes depends on optimized exchange of metabolites between mitochondria and the cytosol, in particular the continuous exchange of hydrophilic metabolites like ATP, ADP, inorganic phosphate and respiratory substrates. To enter the mitochondrial matrix, where most mitochondrial metabolism occurs, all metabolites must cross two separate barriers - the mitochondrial outer and inner membranes (MOM and MIM). While MIM transports metabolites via a variety of selective transporters (1-3), MOM conducts hydrophilic solutes non-selectively via a channel called mitochondrial porin or the voltage-dependent anion channel (VDAC) (4,5). VDAC is the pathway across MOM for entry and exit of virtually all water soluble metabolites involved in oxidative phosphorylation and related metabolism (4-8). Exceptions are hydrophobic metabolites, such as molecular oxygen and short chain fatty acids that directly permeate biological membranes. VDAC is often considered to be constitutively open, and its potential importance in regulating mitochondrial metabolism has been largely ignored.

Previously, we showed that ethanol suppresses mitochondrial metabolism in permeabilized hepatocytes, an effect attributed to inhibition of VDAC and decreased permeability of MOM to hydrophilic metabolites (6,9,10). Ethanol undergoes two-step oxidation in the liver. The first step produces acetaldehyde (AcAld) catalyzed principally by alcohol dehydrogenase (ADH) in the cytosol and also by cytochrome P450 2E1 in the endoplasmic reticulum and catalase in peroxisomes (11-14). Oxidation of ethanol via cytochrome P450 2E1 pathway is associated with generation of reactive oxygen species, oxidative stress and lipid peroxidation (11,13,15-20). AcAld is further oxidized to acetate by aldehyde dehydrogenases, predominantly by aldehyde dehydrogenase-2 (ALDH2) in the mitochondrial matrix (11,12,21-25).

In hepatocytes, ureagenesis comprises a sequence of five biochemical reactions distributed between two intracellular compartments – the mitochondrial matrix and cytosol (26-30). The compartmental separation of reactions of the urea cycle requires exchange of ornithine and citrulline, major substrates of the urea cycle, between mitochondria and cytosol. In addition, ureagenesis is a highly energy-demanding process that requires hydrolysis of 4 equivalents of ATP (2 each in the matrix and cytosol) per mol of urea formed (29). Overall, ureagenesis requires continuous exchange of hydrophilic metabolites between cytosol and mitochondria, all of which must go through VDAC. Thus, if VDAC were to adopt a more closed state, ureagenesis would likely be one of the first metabolic processes affected.

AcAld, the toxic intermediate of ethanol metabolism, is a small neutral compound that does not require carriers or channels to cross mitochondrial membranes and enter the matrix from the cytosol. Recently we proposed that VDAC closes in response to ethanol (6,9,10,31). VDAC closure suppresses oxidation of respiratory substrates competing with AcAld to promote selective and more rapid AcAld oxidation. Here, we demonstrate that ethanol dose-dependently suppresses ureagenesis in cultured rat hepatocytes and that AcAld mediates this inhibitory effect apparently by closing VDAC and decreasing MOM permeability.

**EXPERIMENTAL PROCEDURES**

*Animals.* All animal protocols were approved by the Institutional Animal Care and Use Committee in accordance with recommendations published in the Guide for the Care and Use of Laboratory Animals, National Academic Press, Washington DC, 1996 (32).

*Hepatocyte isolation and culture.* Hepatocytes were isolated from 200-250 g overnight-fasted male Sprague-Dawley rats (Charles River, Wilmington, MA) by collagenase perfusion, as described previously (33,34). Hepatocytes were resuspended in Waymouth’s MB-752/1 growth medium (GIBCO, Grand Island, NY)
supplemented with 10% fetal bovine serum, 100 nM insulin (Squibb-Novo, Princeton, NJ), 100 nM dexamethasone (LyphoMed, Rosemont, IL), 100 units/mL penicillin, and 100 µg/mL streptomycin. Viability of cells was typically greater than 90%, as determined using a Cellometer Vision Cell Counter (Nexcelom Biosciences, Lawrence, MA).

**Measurement of hepatocyte respiration.** The rate of oxygen consumption (respiration) was measured in hepatocytes plated on Seahorse 24-well V7-plates (Seahorse Biosciences, Billerica, MA). Prior to plating cells, wells were coated (20 µl per well) with 0.1% Type-1 rat-tail collagen dissolved in 0.1% acetic acid (Sigma, St. Louis, MO). After drying, wells were rinsed once with 0.5 ml of warm Waymouth’s medium. Aliquots of 0.1 ml of isolated hepatocyte suspension were transferred to each well (3 × 10^4 cells per well at a density of 10^5 cells per cm²) and allowed to attach for 2 h in humidified 5% CO₂, 95% air at 37°C. Subsequently, the wells were supplemented with 0.4 ml of warm complete growth medium and the hepatocytes were incubated for 16-18 h in humidified 5% CO₂/95% air at 37°C.

Rates of oxygen consumption (respiration) were measured in a Seahorse Bioscience XF-24 Extracellular Flux Analyzer using 24-well plate sensor cartridges. The parameters of the measurement cycle were adjusted to avoid anoxia and were set as followings: Mix (4 min, during which the sensor plate moves up and down to equilibrate oxygen); Wait (30 sec, during which oxygen sensors descend into wells to form 7-µl measurement chambers above the plated hepatocytes); Measure (1 min, 10 measurements of oxygen concentration to calculate respiratory rates) (35). Respiratory rates were calculated using Seahorse XF-24 software and the Direct ACOS Fast Algorithm with Continuous Averaging, as described (35). All incubations and measurements were done at 37°C. Prior to measurement, hepatocytes were rinsed 3 times with 0.45 ml of Krebs-Ringer-HEPES buffer containing (in mM): 115 NaCl, 5 KCl, 1 CaCl₂, 1 KH₂PO₄, 1.2 MgSO₄, 25 HEPES buffer (pH 7.4 with KOH) and supplemented with 27 NaHCO₃ (KRH-NaHCO₃). Hepatocytes were incubated for 30 min in a cell culture incubator in 5% CO₂/air before transferring to a Seahorse XF-24 extracellular flux analyzer. Injection ports were filled with 50 µL of KRH-NaHCO₃ supplemented with ureagenic substrates with or without ethanol or AcAld (Port A) and inhibitors (Port B), as specified. Respiratory rates were expressed as µmol O₂/min/10⁶ cells or as percentage of baseline. Oxygen consumed by hepatocytes during ureagenesis was determined by an area under the curve calculation with subtraction of oxygen consumed in the absence of ureagenic substrates (baseline respiration).

**Measurement of urea synthesis.** Urea in dried aliquots of incubation medium was determined using a QuantiChrom Urea Assay Kit according to the manufacturer’s instructions (Bioassay System, Hayward CA).

**Measurement of acetaldehyde.** Aliquots of incubation medium were placed in airtight vials, and AcAld was measured by ALDH enzymatic assay in reaction buffer containing (in mM): 100 KCl, 1 NAD⁺, 1 DTT, 100 Tris-HCl, and 0.05 units/ml ALDH, pH 8.0, 37°C. After 60 min, NADH produced stoichiometrically by ALDH-dependent oxidation of AcAld was measured spectrophotometrically using an NADH extinction coefficient 6.2 x 10³ M⁻¹·cm⁻¹ (36).

**Measurement of entrapment of 3 kDa fluorescent dextran conjugated with tetramethylrhodamine.** Uptake and retention of 3 kDa fluorescent tetramethylrhodamine-conjugated dextran molecules (RhDex) by mitochondria of permeabilized cultured hepatocytes was monitored using confocal microscopy, as described (9) with minor modification. Hepatocytes cultured on glass-bottomed MatTek dishes (3 x 10⁵ cells per dish) were incubated with 500 nM of MTG in Waymouth’s media for 30 min at 37°C in 5% CO₂, 95% air to label mitochondria. The MTG-labeled hepatocytes were then placed on the stage of a laser scanning confocal microscope (LSM 510 META, Carl Zeiss, Thornwood, NY) inside of a temperature controlled environmental chamber (37°C). After 2 min, buffer was removed and replaced with intracellular buffer (ICB, in mM: 120 KCl, 20 NaCl, 2 KH₂PO₄, 2 MgSO₄, 1 EGTA, 10% dextran 100 kDa, 20 HEPES/NaOH, pH 7.35, with protease inhibitors [pepstatin, antipain, leupeptin; 1 µg/ml each], oligomycin [5 µg/ml], rotenone [10 µM] and
succinate [5 mM]) plus digitonin (10 μM) to permeabilize the plasma membrane. After another 2 min, cells were washed once with 2 ml of ICB lacking digitonin and further incubated in 2 ml of ICB containing 3 kDa RhDex (400 μM). After 5 min, ICB/RhDex buffer was replaced with 2 ml of identical buffer supplemented with 4', 4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, 300 μM), a blocker of VDAC. After another 5 min, ICB/RhDex/DIDS was removed, and cells were washed once with 2 ml of ICB/DIDS without RhDex. Images of hepatocytes were then collected after 5 min. In this protocol, RhDex entered the IMS of digitonin-permeabilized hepatocytes and was subsequently “locked in” by DIDS.

Confocal images of green-fluorescing MTG and red-fluorescing RhDex were collected at each step and analyzed with ImageJ 1.43u (http://rsb.info.nih.gov/ij/download.html) using a customized macro algorithm. Briefly, the macro used green MTG fluorescence of mitochondria to create a “mask”, which then was used to measure the intensity of red RhDex fluorescence only in areas inside mitochondria, a procedure excluding cytosolic and nuclear fluorescence outside the mask. Mean intensity of red RhDex fluorescence in MTG-labeled mitochondrial regions was taken as a measure of RhDex content in mitochondria.

Materials. DIDS, MTG and 3 kDa RhDex were obtained from Invitrogen (Carlsbad, CA); 100% pure digitonin (M.W. 1229.3) from Calbiochem (San Diego, CA); BSA (Fraction IV), collagen (type I), dextran (100 kDa), glucose-6-phosphate dehydrogenase, hexokinase, aldehyde dehydrogenase, NAD+ and other analytical grade chemicals were obtained 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-Novob (Princeton, NJ); and dexamethasone sodium phosphate from LyphoMed (Rosemont, IL).

Statistics. Data are presented as means ± S.E.M. Images shown are representative of three or more experiments. Statistical analysis was performed by Student t-test or analysis of variance using P<0.05 as the criterion of significance.

RESULTS

Ethanol suppresses ureagenic respiration of cultured hepatocytes. Respiration by cultured rat hepatocytes was measured in a Seahorse XF24 Extracellular Flux Analyzer and averaged 15.8 ± 0.9 nmol O₂/min/10⁶ cells under basal conditions (Fig. 1A). After addition of ureagenic substrates (3 mM NH₄Cl, 5 mM L-ornithine and 5 mM L-lactate), respiration of cells increased to 29.4 ± 1.7 nmol O₂/min/10⁶ cells within about 30 min (Fig. 1A). Ureagenic respiration of hepatocytes, defined as the difference between total respiration after addition of ureagenic substrates and basal respiration, increased to 13.6 ± 0.5 nmol O₂/min/10⁶ cells (Fig. 1B). To investigate the effect of ethanol on ureagenic respiration, ethanol was added simultaneously with ureagenic substrates. Under these conditions, ethanol dose-dependently suppressed ureagenic respiration (Fig. 1A), and 200 mM ethanol decreased ureagenic respiration to 6.6 ± 1.3 nmol O₂/min/10⁶ cells (Fig. 1B). Ethanol added to cultured hepatocytes in the absence of ureagenic substrates had no effect on basal respiration (data not shown).

Ethanol suppresses urea formation in cultured hepatocytes. We also measured urea formation during measurements of respiration. In the absence of ureagenic substrates, urea formation was below the limits of detection (<0.2 nmol urea/10⁶ cells). After addition of ureagenic substrates, urea formation increased to 9.9 ± 1.4 nmol urea/10⁶ cells (Fig. 2A). In these experiments, the total amount of ureagenic respiration averaged 12.1 ± 2.5 nmol O₂/10⁶, such that the stoichiometry of urea formed to oxygen atoms consumed was 0.4, which is equivalent to an ATP/O ratio of 1.6 assuming 4 ATP consumed for each urea formed. Suppression of ureagenic respiration by ethanol led to a proportional inhibition of urea formation such that the ratio of urea formation to ureagenic respiration remained more or less constant (Fig. 2B). Thus, ethanol suppressed both ureagenic respiration and urea formation but did not alter the energetic efficiency of ureagenesis.

Contribution of cytosolic alcohol dehydrogenase to ethanol-induced suppression of ureagenic respiration. To assess the role of ethanol metabolism via ADH in ethanol-induced suppression of ureagenic respiration,
hepatocytes were pre-treated before ethanol addition with 4-methylpyrazole (MP, 1 mM), an ADH inhibitor (37). In the absence of ethanol, MP did not alter ureagenic respiration (Fig. 3A, saline). By contrast when hepatocytes were subsequently treated with ethanol (100 mM), MP partially protected against ethanol-mediated suppression of ureagenic respiration and attenuated suppression by 46% after 100 mM ethanol (Fig. 3A, ethanol).

Contribution of cytochrome P450 2E1 to ethanol-mediated suppression of ureagenic respiration. To assess the importance of ethanol metabolism via CYP450 2E1 in ethanol-induced suppression of ureagenic respiration, hepatocytes were pretreated with trans-1,2-dichloroethylene (DCE, 5 mM), a CYP450 2E1 inhibitor (38), before ethanol addition. DCE did not affect ureagenic respiration in the absence of ethanol (Fig. 3B, saline). By contrast after subsequent ethanol addition, DCE partially abrogated the inhibitory effect of 100 mM ethanol on ureagenic respiration and attenuated ethanol-induced suppression by 37% (Fig. 3B, ethanol).

Contribution of catalase to ethanol-induced suppression of ureagenic respiration. To assess the role of catalase in ethanol-induced suppression of ureagenic respiration, hepatocytes were pretreated with 3-amino-1,2,4-triazole (AT, 5 mM), a catalase inhibitor (39) in the absence of ethanol. Like MP and DCE, AT had no effect on ureagenic respiration in the absence of ethanol (Fig. 3C, saline). Also like MP and DCE, AT partially reversed the inhibitory effect of 100 mM ethanol on ureagenic respiration and attenuated ethanol-induced suppression by 66% (Fig. 3C, ethanol).

Inhibition of aldehyde dehydrogenase enhances ethanol-induced suppression of ureagenic respiration. AcAld, the product of the first step of ethanol oxidation, is further metabolized to acetate predominantly by mitochondrial ALDH2. Our finding that inhibitors of ADH, CYP450 2E1 and catalase abrogated, at least in part, ethanol-induced suppression of ureagenic respiration suggested that AcAld might be mediating ureagenic inhibition. Accordingly, we assessed the effect of phenethyl isothiocyanate (PEITC, 10 µM), an inhibitor of ALDH (23), on ethanol-induced suppression of ureagenic respiration. In the absence of ethanol, PEITC did not inhibit ureagenic respiration (Fig. 4, saline), but in the presence of subsequently added ethanol, PEITC enhanced inhibition of ureagenic respiration by 47% (Fig. 4, ethanol).

Acetaldehyde alone suppresses ureagenic respiration of cultured hepatocytes. Abrogation of ethanol-induced suppression of ureagenic respiration by ADH, CYP450 2E1 and catalase inhibition and enhancement by ALDH inhibition supports the conclusion that AcAld produced during ethanol oxidation mediates suppression of ureagenic respiration. Accordingly, we investigated the effect of directly added AcAld on ureagenic respiration. Due to the high volatility of AcAld, we layered mineral oil (250 µl) over the incubation medium in the Seahorse plates to minimize evaporation of AcAld. Mineral oil did not interfere with measurements of oxygen consumption (data not shown) but substantially decreased the rate of loss of AcAld from >90% in open wells after 60 min to 40-45% in wells covered with mineral oil (Fig. 5, compare open versus mineral oil). The effect of added AcAld on ureagenic respiration was then evaluated using the same protocol as for ethanol, except that the incubation medium bathing hepatocytes in each well of Seahorse plate was covered with 250 µl of mineral oil. In these measurements, AcAld caused dose-dependent inhibition of ureagenic respiration (Fig. 6A and B). The K_i for inhibition of ureagenic respiration was 125 µM (uncorrected for evaporative loss), as estimated from a Dixon plot (Fig. 6B, inset). In contrast to ureagenic respiration, 125 µM AcAld did not inhibit uncoupled respiration stimulated by 2,4-dinitrophenol (DNP, 150 µM) (Fig. 6C, panel uncoupled, compare to ureagenic). To determine whether AcAld increases after ethanol to a level that could account for ethanol-dependent suppression of ureagenic respiration, we measured AcAld in the medium of cultured hepatocytes exposed to ethanol for 90 min. After 60 min of incubation with 50 mM ethanol, AcAld in the incubation buffer increased to 44.6 ± 0.8 µM (n=2).

Acetaldehyde suppresses entry of fluorescent 3 kDa rhodamine-conjugated...
dextran into the intermembrane space of mitochondria of digitonin-permeabilized hepatocytes. Previously, we showed that ethanol inhibits entry of 3 kDa RhDex into the IMS of MTG-labeled mitochondria in permeabilized hepatocytes (9). Here, we used a similar approach to study the effect of AcAld on RhDex entry into the IMS. Intracellular mitochondria were first labeled with MTG (Fig. 7A, MTG), and then hepatocytes were treated with digitonin to permeabilize the plasma membrane. As digitonin was washed out, RhDex was added, and the cells were treated with DIDS to entrap RhDex inside the IMS. Lastly, the permeabilized cells were washed to remove excess of RhDex. In fluorescence images collected at the end of this procedure, much red-fluorescing RhDex colocalized with green-fluorescing MTG, indicating that 3 kDa RhDex was entrapped in the mitochondrial IMS. RhDex also accumulated in nuclei and in small vesicles of non-mitochondrial origin (Fig. 7A, RhDex), as shown previously (9). An MTG mask selectively outlining mitochondria was used to measure RhDex fluorescence only in mitochondria and to exclude cytosolic and nuclear fluorescence outside of the mask. In comparison to untreated hepatocytes, pretreatment with AcAld (500 μM) decreased RhDex uptake by 56% (Fig. 7A and Fig. 7B, AcAld). Similarly, in hepatocytes pretreated with DIDS (300 μM, images not shown) to inhibit VDAC before addition of RhDex, mitochondrial RhDex uptake was decreased by 40% (Fig. 7B, DIDS). By contrast, MTG fluorescence was not different between treatments (Fig. 7B). In these confocal imaging experiments, a layer of oil could not be used, and the medium remained open to the atmosphere. Thus, actual AcAld concentration was less due to evaporative loss.

DISCUSSION

Many factors contributing to progressive loss of liver function in alcoholic liver disease converge at the level of mitochondrial dysfunction and disturbed mitochondrial metabolism (17,18,40-42). Ethanol exposure compromises mitochondrial ATP synthesis, produces steatosis, increases oxidative stress and suppresses endogenous liver specific functions (11,14,16,18-22,25,37,42-47). Although, these ethanol-induced disruptions of mitochondrial metabolism are well documented, the underlying mechanism(s) of the pathogenesis of alcohol-induced liver disease remains incompletely understood.

Previously, we hypothesized that ethanol-dependent impairment of mitochondrial function is initiated by closure of VDAC in MOM, which limits exchange of water soluble metabolites into and out of mitochondria (6,9,10). This hypothesis was supported by data showing that MOM became limiting after ethanol treatment for permeance of substrates for respiration in MIM and adenylate kinase in the IMS. Moreover, ethanol decreased permeance of 3 kDa RhDex into the IMS. Here, we sought to determine whether VDAC closure was directly caused by ethanol or indirectly via an ethanol metabolite, such as AcAld, using ureagenesis as an indirect measure of metabolite flux through VDAC. Inhibitor studies implicated that AcAld was responsible for ethanol-dependent inhibition of ureagenic respiration, a conclusion that we confirmed and extended by showing that exogenously added AcAld also inhibited ureagenic respiration and, in addition, decreased MOM permeability as assessed directly by RhDex permeance into the IMS.

If ethanol causes VDAC closure, then mitochondrial processes heavily reliant on metabolite exchange with the cytosol should be most sensitive to suppression by ethanol. Urea formation is one such process, requiring exchange of ornithine for citrulline, mitochondrial release of ATP in exchange for ADP and phosphate, and uptake of respiratory substrates to support synthesis of 4 ATP for each urea formed. In agreement with expectations of the VDAC hypothesis, ethanol suppressed ureagenesis by cultured rat hepatocytes as shown by decreased respiration and urea formation in the presence of ureagenic substrates. Ethanol did not inhibit basal and uncoupled respiration. Likewise, ethanol did not decrease the efficiency of ureagenesis as determined from the ratio of urea formed to ureagenic oxygen consumed (Fig. 2B).

Inhibitor studies indicated that AcAld formed by ethanol metabolism was causing suppression of ureagenic respiration. Inhibition
of ADH, CYP450 2E1 and catalase, enzymes that metabolize ethanol to AcAld, decreased ethanol-induced suppression of ureagenesis (Fig. 3). Catalase inhibition with AT abrogated ethanol-induced suppression of ureagenic respiration to a greater extent than inhibition of ADH or CYP450 (Fig. 3). However, AT also inhibits CYP450, especially at high concentrations (10-50 mM), and thus the greater extent of abrogation with AT may reflect the combined effects of catalase and CYP450 inhibition (48). Additionally, ethanol metabolism switches from predominantly ADH-mediated at low concentrations of ethanol (7–12 mM) to more predominantly catalase-mediated at higher doses (>25 mM), which may also contribute to the greater effectiveness of AT in reversing ethanol-induced suppression of ureagenesis (49-51).

In contrast to inhibition of ADH, CYP450 2E1 and catalase, inhibition of ALDH, the enzyme that oxidizes AcAld to acetate, enhanced suppression of ureagenesis (Fig. 4). Moreover, AcAld alone dose-dependently suppressed ureagenic respiration with an IC$_{50}$ of 125 μM as estimated by Dixon plot (Fig. 6B). Like ethanol, AcAld did not inhibit basal or uncoupled respiration by hepatocytes (Fig. 6C), indicating that AcAld was not directly inhibiting mitochondrial respiratory chain activity. Although the concentration of AcAld at its intracellular sites of generation is difficult to assess, measurements of AcAld accumulation in the incubation buffer showed that AcAld increased to 45 μM after 60 min incubation with 50 mM ethanol. In consideration that actual intracellular AcAld concentration is likely to be even higher, this AcAld concentration generated by hepatocytes from added ethanol is comparable to concentrations of exogenous AcAld that directly produced an equivalent suppression of ureagenic respiration (Fig. 6).

AcAld also suppressed entrapment of 3 kDa RhDex into the mitochondrial IMS of permeabilized hepatocytes (Fig. 7), which was similar to the decreased RhDex permeance observed after ethanol treatment in our earlier study (9). In mitochondria from liver and other organs, VDAC is the only known pathway allowing transport of hydrophilic anionic metabolites across MOM. Thus, our findings taken together support the conclusion the ethanol metabolism to AcAld leads to VDAC closure and suppression of mitochondrial metabolism like ureagenesis that requires movement of hydrophilic metabolites between mitochondria and the cytosol.

VDAC was long considered to be a component of mitochondrial permeability transition (MPT) pores (5,52,53). However, recent observations show that VDAC isoforms are not essential for MPT pore formation or induction of MPT-dependent cell death (54). However, growing evidence indicates that open/closed status of VDAC regulates mitochondrial and cellular energetics through modulation of fluxes of hydrophilic metabolites across MOM, including toxic superoxide that can form as a result of disordered mitochondrial respiration (4,5,7,8,55-58). Restriction of exchange of vital metabolites between the mitochondrial IMS and cytosol and hence regulation of overall mitochondrial metabolism signifies a role of VDAC as a “governator” of mitochondrial function (6).

Although AcAld appears to close mitochondrial VDAC, the molecular mechanism by which AcAld decreases VDAC permeability remains to be determined. VDAC exists as 3 isoforms in liver mitochondria – VDAC1, 2 and 3, and AcAld-induced VDAC closure may be isoform specific, which could explain why ureagenic respiration and RhDex uptake into the IMS are not completely inhibited by AcAld and ethanol. VDAC is also the target of several kinases whose inhibition or activation after AcAld might mediate VDAC closure (3,45,59). Additionally, free tubulin promotes VDAC closure, and ethanol treatment leads to tubulin acetylation and microtubular depolymerization (56,60,61). These and other mechanisms of AcAld-dependent VDAC closure will be the subject of future study.

In conclusion, our findings show that ethanol suppresses ureagenesis in cultured rat hepatocytes, an effect mediated by AcAld and associated with a decrease in non-specific MOM permeability. Since VDAC is the only known pathway allowing transport of hydrophilic anionic metabolites across MOM, decreased MOM permeability in hepatocytes oxidizing ethanol is most likely due to a decrease of VDAC conductance. Such VDAC closure is
adaptive in promoting selective oxidation and detoxification of AcAld, but after repeated ethanol challenge to the liver VDAC closure may become an early hit in the multihit pathogenesis of alcoholic liver disease. Ethanol causes oxidative stresses via CYP450 2E1 metabolism, and other aldehydes formed from lipid peroxidation may also suppress VDAC function. Indeed, preliminary data indicate that malondialdehyde and 4-hydroxynonenal, products of beta scission in lipid peroxidation, suppress ureagenesis (62). Thus, aldehyde-induced VDAC closure and mitochondrial dysfunction may contribute to other liver diseases in which oxidative stress is an important factor.

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FOOTNOTES

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The abbreviations used are: AcAld, acetaldehyde; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CYP450 2E1, cytochrome P450 2E1; DIDS, 4,4’-diisothiocyanatostilbene-2,2’-disulfonic acid; DCE, trans-1,2-dichloroethylene; DTT, 1,4-dithio-D-threitol; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; IMM, mitochondrial outer membrane; IMS, intermembrane space; ICB, intracellular buffer; 4-MP, 4-methylpyrazole; MTG, MitoTracker Green FM; MPT, mitochondrial permeability transition; 4-NHE, 4-hydroxynonenal; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; MPT, mitochondrial permeability transition; RFU, relative fluorescence unit; ROS, reactive oxygen species; RhDex, 3 kDa rhodamine conjugated dextran; VDAC, voltage dependent anion channel.

FIGURE LEGENDS

FIGURE 1. Suppression of ureagenic respiration in cultured rat hepatocytes with ethanol. In A, respiratory rates were measured with a Seahorse XF24 Extracellular Flux Analyzer from a representative experiment before and after sequential addition of ureagenic substrates (3 mM NH₄Cl, 5 mM ornithine, 5 mM lactate) and KCN (1 mM). As indicated, 0 - 200 mM ethanol was added simultaneously with the ureagenic substrates. Data are means ± S.E.M. from 4 wells per treatment. In B, steady state rates of ureagenic respiration (total respiration minus basal respiration) are plotted versus ethanol concentration. Data are means ± S.E.M. from 6 or more independent experiments of 4 wells per treatment.

FIGURE 2. Relationship of extra oxygen consumption to urea formation during ethanol-induced inhibition of ureagenesis. Oxygen consumption and urea formation by cultured rat hepatocytes were measured for 33 min after addition of ureagenic substrates simultaneously with 0-200 mM ethanol, as described in MATERIALS AND METHODS. In A, extra oxygen consumption (total oxygen consumed after addition of ureagenic substrates minus basal oxygen consumption) (left axis) and urea formation (right axis) are plotted versus ethanol. In B, extra oxygen consumed is plotted versus urea formed at different ethanol doses. Data are means ± S.E.M. from 3 independent experiments of 4 wells per treatment.
FIGURE 3. Inhibition of individual ethanol oxidizing enzymes reverses suppression of ureagenic respiration with ethanol. Hepatocytes were treated with 1 mM 4-MP (A), 5 mM t-DCE (B), 5 mM 3-AT (C) or vehicle (None), and ureagenic substrates with 100 mM ethanol or saline vehicle were added. Ureagenic respiration was measured as described in Fig. 1. Data are means ± S.E.M. from 3 independent experiments of 3 or more wells per treatment. *, p<0.05 vs. None.

FIGURE 4. Inhibition of aldehyde dehydrogenase enhances ethanol-mediated suppression of ureagenic respiration. Hepatocytes were treated with 10 μM PEITC or vehicle (None) in the absence (Saline) or presence of 100 mM ethanol (Ethanol). Data are means ± S.E.M. from 3 independent experiments of 3 or more wells per treatment. *, p<0.05 vs. None.

FIGURE 5. Loss of acetaldehyde from incubation buffer in Seahorse plates. Wells of 24-well Seahorse plates were filled with KRH-NaHCO₃ containing 1 mM acetaldehyde. Wells were left with open to the air (Open) or covered with 250 μl of mineral oil (Mineral Oil). At indicated times, AcAld concentration was determined as described in MATERIALS AND METHODS. Data are means ± S.E.M. from 3 independent experiments.

FIGURE 6. Suppression of ureagenic respiration by acetaldehyde. In A, respiratory rates were measured before and after addition of ureagenic substrates (3 mM NH₄Cl, 5 mM ornithine, 5 mM lactate) in a representative experiment. As indicated, 0, 10 and 500 μM AcAld were added simultaneously with the ureagenic substrates. Data are means ± S.E.M. from 4 wells per treatment. In B, steady state ureagenic respiration (total respiration minus basal respiration) is plotted versus AcAld concentration. Inset is a Dixon plot of the data. In C, ureagenic and uncoupled respiration of hepatocytes were measured in the absence and presence of 125 μM AcAld. Data are means ± S.E.M. from 3 independent experiments of 3 or more wells per treatment. *, p<0.05 vs. 0.

FIGURE 7. Suppression of rhodamine-dextran entry into the mitochondrial intermembrane space of permeabilized hepatocytes by acetaldehyde. Hepatocytes were loaded with MTG, treated with vehicle, 500 μM acetaldehyde or 300 μM DIDS, permeabilized with digitonin in the presence of RhDex, exposed to DIDS and placed in RhDex-free ICB, as described in MATERIALS AND METHODS. Panel A shows green fluorescence of MTG-labeled mitochondria (left column), red fluorescence of RhDex (middle column) and the overlay MTG and RhDex fluorescence (right column). Note colocalization of MTG and RhDex fluorescence after vehicle treatment that is decreased after acetaldehyde treatment. In B, mean intensities of MTG fluorescence and RhDex fluorescence colocalizing with MTG-labeled mitochondria are plotted for each group in A. Data are means ± S.E.M. of 20 or more cells per treatment. *, p<0.05 vs. None.
Figure 2

[A] Oxygen and Urea levels in ethanol concentrations.

[B] Extra Oxygen levels in urea concentrations.
Figure 3

A

Ureagenic Respiration
(nmol O₂/min/10⁶ cells)

Saline | Ethanol
None | MP

B

Ureagenic Respiration
(nmol O₂/min/10⁶ cells)

Saline | Ethanol
None | DCE

C

Ureagenic Respiration
(nmol O₂/min/10⁶ cells)

Saline | Ethanol
None | AT

*
Figure 4

The figure shows a bar chart comparing ureagenic respiration (nmol O₂/min/10⁶ cells) under different conditions. The x-axis represents the presence or absence of PEITC and the y-axis represents the respiration rate.

- **Saline**
  - None: No PEITC
  - PEITC: With PEITC

- **Ethanol**
  - None: No PEITC
  - PEITC: With PEITC

The chart indicates a significant decrease in respiration rate with PEITC treatment in the Ethanol condition compared to the Saline condition.

* indicates a statistically significant difference.
Figure 5

Graph showing the concentration of Acetaldehyde (mM) over time (min) for two conditions: Open and Mineral Oil.
Figure 6

A

[Graph showing respiration (nmol O₂/min/10⁶ cells) against time (min) for different concentrations of AcAld (µM).]

B

[Graph showing ureagenic respiration (nmol O₂/min/10⁶ cells) against Acetaldehyde (µM) with a inset showing a linear relationship between AcAld (µM) and respiration.]

C

[Graph comparing ureagenic and uncoupled respiration (nmol O₂/min/10⁶ cells) for different concentrations of AcAld (µM).]
Figure 7

A

B

Fluorescence (RFU)

MTG RhDex

None DIDS AcAld None DIDS AcAld
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