THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 10, pp. 7692–7700, March 2, 2012

Ethanol Suppresses Ureagenesis in Rat Hepatocytes

*ROLE OF ACETALDEHYDE**

Received for publication, August 22, 2011, and in revised form, December 18, 2011 Published, JBC Papers in Press, January 6, 2012, DOI 10.1074/jbc.M111.293399

Ekhson L. Holmuhamedov[‡§¶1,2], **Christoph Czerny**[‖1], **Craig C. Beeson**[‡§], **and John J. Lemasters**[‡§**3]

From the [‡]*Center for Cell Death, Injury, and Regeneration, Departments of* [§]*Pharmaceutical and Biomedical Sciences and* [**]*Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425, the* [¶]*Center for Theoretical Problems of Physico-Chemical Pharmacology, Moscow, Russian Federation, and the* [‖]*Department of Trauma Surgery, J. W. Goethe University, Frankfurt am Main, Germany*

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.

We proposed previously that closure of voltage-dependent anion channels (VDAC) in the mitochondrial outer membrane after ethanol exposure leads to suppression of mitochondrial metabolite exchange. Because 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 O_2/min/10^6 cells (base line) to 29.4 ± 1.7 nmol O_2/min/10^6 cells in about 30 min. Ethanol (0–200 mM) suppressed extra respiration after ureagenic substrates (ureagenic respiration) by up to 51% but not base line 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 IC_{50} of 125 μM. AcAld also inhibited entry of 3-kDa rhodamine-conjugated dextran 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 membrane (MOM)[4] and mitochondrial inner membrane (MIM). Although the MIM transports metabolites via a variety of selective transporters (1–3), the MOM conducts hydrophilic solutes non-selectively via a channel called mitochondrial porin or the voltage-dependent anion channel (VDAC) (4, 5). The VDAC is the pathway across the 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. The 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 the MOM to hydrophilic metabolites (6, 9, 10). Ethanol undergoes two-step oxidation in the liver. The first step produces acetal-

* This work was supported, in whole or in part, by National Institutes of Health Grants DK073336 (to J. J. L.), DK37034 (to J. J. L.), AA017756 (to J. J. L.), and HL101240-01 (to E. L. H.). Imaging facilities for this research were supported, in part, by Cancer Center Support Grant P30 CA138313 (to the Hollings Cancer Center, Medical University of South Carolina).

[1] Both authors contributed equally to this work.

[2] Present address: Aurora Health Care, Aurora Medical Center; 3033 S. 27th St., Suite 201, Milwaukee, WI 53215. Tel.: 414-649-7589; E-mail: ekhson.holmuhamedov@aurora.org.

[3] To whom correspondence should be addressed: Center for Cell Death, Injury, and Regeneration, Departments of Pharmaceutical and Biomedical Sciences and Biochemistry and Molecular Biology, Medical University of South Carolina, 280 Calhoun St., MSC 140, QF213 Quadrangle Building, Charleston, SC 29425. Tel.: 843-792-2153; Fax: 843-792-8436; E-mail: JJLemasters@musc.edu.

[4] The abbreviations used are: MOM, mitochondrial outer membrane; MIM, mitochondrial inner membrane; VDAC, voltage dependent anion channel; AcAld, acetaldehyde; KRH, Krebs-Ringer-HEPES buffer; RhDex, rhodamine-conjugated dextran; MTG, MitoTracker Green FM; ICB, intracellular buffer; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; IMS, intermembrane space; MP, 4-methylpyrazole; ADH, alcohol dehydrogenase; DCE, *trans*-1,2-dichloroethylene; MPT, mitochondrial permeability transition; ALDH, aldehyde dehydrogenase; RhDex, 3-kDa rhodamine-conjugated dextran.

dehyde (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 the 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 the mitochondria and cytosol. In addition, ureagenesis is a highly energy-demanding process that requires hydrolysis of four equivalents of ATP (two each in the matrix and cytosol) per mol of urea formed (29). Overall, ureagenesis requires continuous exchange of hydrophilic metabolites between the cytosol and mitochondria, all of which must go through the VDAC. Thus, if the 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. We proposed recently that the 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 the 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- to 250-g overnight-fasted male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) by collagenase perfusion as described previously (33, 34). Hepatocytes were resuspended in Waymouth's MB-752/1 growth medium (Invitrogen) 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 (Nexcelcom 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/well) with 0.1% type-1 rat tail collagen dissolved in 0.1% acetic acid (Sigma). 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/well at a density of 10^5 cells/cm^2) and allowed to attach for 2 h in humidified 5% CO_2/95% 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% CO_2/95% 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 moved up and down to equilibrate oxygen), Wait (30 s, during which oxygen sensors descended into wells to form 7-μl measurement chambers above the plated hepatocytes), and 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 three times with 0.45 ml of Krebs-Ringer-HEPES buffer (KRH) containing 115 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM HEPES buffer (pH 7.4, with KOH) and supplemented with 27 mM $NaHCO_3$ (KRH-$NaHCO_3$). Hepatocytes were incubated for 30 min in a cell culture incubator in 5% CO_2/air before transferring to a Seahorse XF-24 extracellular flux analyzer. Injection ports were filled with 50 μl of KRH-$NaHCO_3$ supplemented with ureagenic substrates with or without ethanol or AcAld (port A) and inhibitors (port B) as specified. Respiratory rates were expressed as μmol O_2/min/10^6 cells or as percentage of base line. 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 (base-line 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 100 mM KCl, 1 mM NAD^+, 1 mM DTT, 100 mM 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 6.2×10^3 M/cm (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 (3×10^5 cells per dish) were incubated with 500 nM of MTG in Waymouth's media for 30 min at 37 °C in 5% CO_2/95% 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) 120 mM KCl, 20 mM NaCl, 2 mM KH_2PO_4, 2 mM $MgSO_4$, 1 mM EGTA, 10% dextran 100 kDa, 20 mM 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 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; 100% pure digitonin (molecular weight 1229.3) from Calbiochem; BSA (fraction IV), collagen (type I), dextran (100 kDa), glucose-6-phosphate dehydrogenase, hexokinase, aldehyde dehydrogenase, NAD^+, and other analytical grade chemicals from Sigma; HEPES and collagenase D from Roche Applied Science Biochemicals; Waymouth's medium MB-252/1 from Invitrogen; insulin from Squibb-Novo; and dexamethasone sodium phosphate from LyphoMed.

Statistics—Data are presented as mean ± S.E. Images shown are representative of three or more experiments. Statistical analysis was performed by Student's *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 O_2/min/10^6 cells under basal conditions (Fig. 1*A*). After addition of ureagenic substrates (3 mM NH_4Cl, 5 mM L-ornithine, and 5 mM L-lactate), respiration of cells increased to 29.4 ± 1.7 nmol O_2/min/10^6 cells within about 30 min (Fig. 1*A*). 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 O_2/min/10^6 cells (Fig. 1*B*). 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. 1*A*),

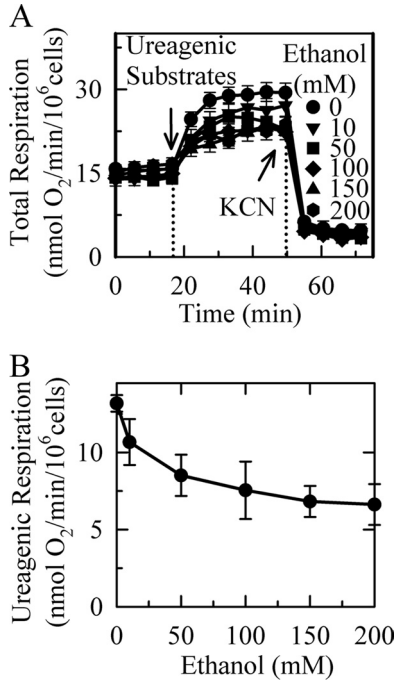

FIGURE 1. **Suppression of ureagenic respiration in cultured rat hepatocytes with ethanol.** *A*, respiratory rates were measured with a Seahorse XF24 extracellular flux analyzer from a representative experiment before and after sequential addition of ureagenic substrates (3 mM NH_4Cl, 5 mM ornithine, 5 mM lactate) and KCN (1 mM). As indicated, 0–200 mM ethanol was added simultaneously with the ureagenic substrates. Data are mean ± S.E. from four wells per treatment. *B*, steady-state rates of ureagenic respiration (total respiration minus basal respiration) are plotted *versus* ethanol concentration. Data are mean ± S.E. from six or more independent experiments of four wells per treatment.

and 200 mM ethanol decreased ureagenic respiration to 6.6 ± 1.3 nmol O_2/min/10^6 cells (*B*). 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/10^6 cells). After addition of ureagenic substrates, urea formation increased to 9.9 ± 1.4 nmol urea/10^6 cells (Fig. 2*A*). In these experiments, the total amount of ureagenic respiration averaged 12.1 ± 2.5 nmol O_2/10^6 so 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 four ATP consumed for each urea formed. Suppression of ureagenic respiration by ethanol led to a proportional inhibition of urea formation so that the ratio of urea formation to ureagenic respiration remained more or less constant (Fig. 2*B*). 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 pretreated 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. 3*A*, *Saline*). In contrast, when hepa-

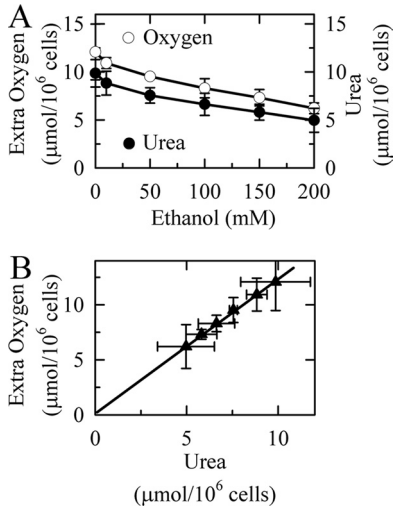

FIGURE 2. **Relationship of extra oxygen consumption to urea formation during ethanol-induced inhibition of ureagenesis.** Oxygen consumption and urea formation by cultured rat hepatocytes were measured for 33 min after addition of ureagenic substrates simultaneously with 0–200 mM ethanol, as described under "Experimental Procedures." *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. *B*, extra oxygen consumed is plotted *versus* urea formed at different ethanol doses. Data are mean ± S.E. from three independent experiments of four wells per treatment.

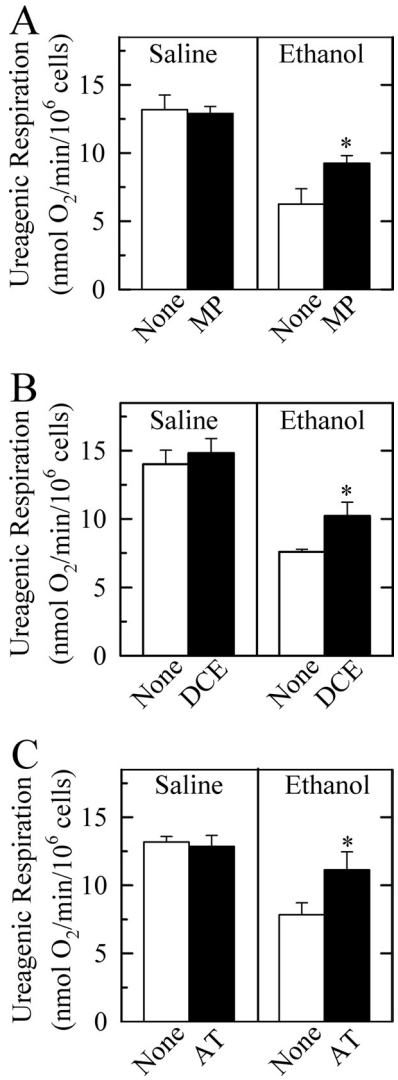

FIGURE 3. **Inhibition of individual ethanol oxidizing enzymes reverses suppression of ureagenic respiration with ethanol.** Hepatocytes were treated with 1 mM 4-MP (*A*), 5 mM t-DCE (*B*), 5 mM 3-AT (*C*), or vehicle (*None*), and ureagenic substrates with 100 mM ethanol or saline vehicle were added. Ureagenic respiration was measured as described in Fig. 1. Data are mean ± S.E. from three independent experiments of three or more wells per treatment. *, $p < 0.05$ *versus* none.

tocytes 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. 3*A*, *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. 3*B*, *Saline*). In 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. 3*B*, *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. 3*C*, *Saline*). Also like MP and DCE, AT partially reversed the inhibitory effect of 100 mM ethanol and attenuated ethanol-induced suppression of ureagenic respiration by 66% (Fig. 3*C*, *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 (10 μM), an inhibitor of ALDH (23), on ethanol-induced suppression of ureagenic respiration. In the absence of ethanol, phenethyl isothiocyanate did not inhibit ureagenic respiration (Fig. 4, *Saline*), but in the presence of subsequently added ethanol, phenethyl isothiocyanate enhanced inhibition of ureagenic respiration by 47% (*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. Because of 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

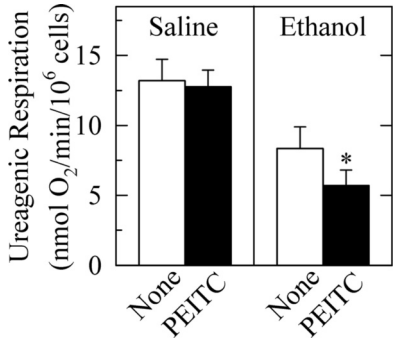

FIGURE 4. **Inhibition of aldehyde dehydrogenase enhances ethanol-mediated suppression of ureagenic respiration.** Hepatocytes were treated with 10 μM phenethyl isothiocyanate (*PEITC*) or vehicle (*None*) in the absence (*Saline*) or presence of 100 mM ethanol (*Ethanol*). Data are mean ± S.E. from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

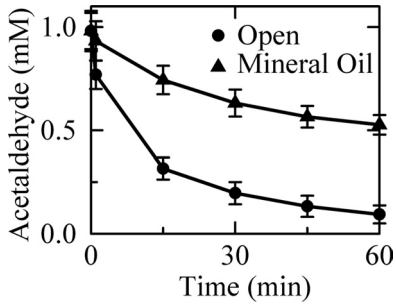

FIGURE 5. **Loss of acetaldehyde from incubation buffer in Seahorse plates.** Wells of 24-well Seahorse plates were filled with KRH-$NaHCO_3$ containing 1 mM acetaldehyde. Wells were left with open to the air (*Open*) or covered with 250 μl of mineral oil (*Mineral Oil*). At indicated times, AcAld concentration was determined as described under "Experimental Procedures." Data are mean ± S.E. from three independent experiments.

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* with *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. 6, *A* and *B*). The K_i for inhibition of ureagenic respiration was 125 μM (uncorrected for evaporative loss), as estimated from a Dixon plot (Fig. 6*B*, *inset*). In contrast to ureagenic respiration, 125 μM AcAld did not inhibit uncoupled respiration stimulated by 2,4-dinitrophenol (150 μM) (Fig. 6*C*, compare *Uncoupled* with *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

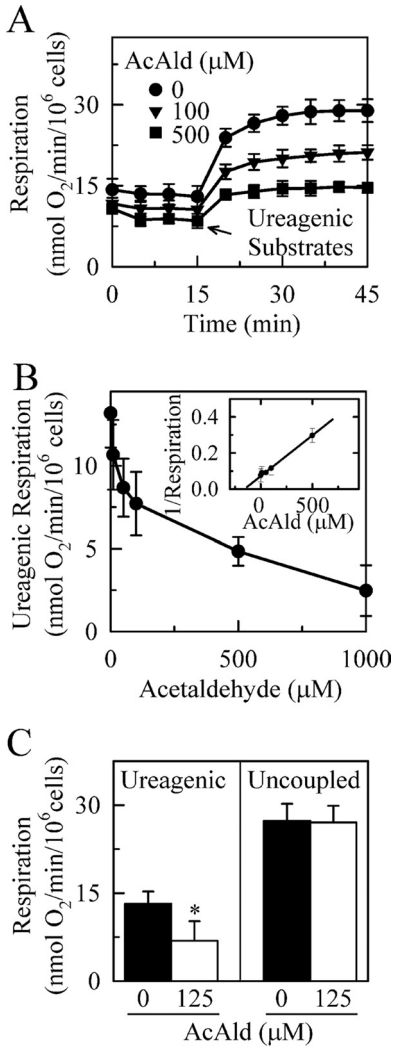

FIGURE 6. **Suppression of ureagenic respiration by acetaldehyde.** *A*, respiratory rates were measured before and after addition of ureagenic substrates (3 mM NH_4Cl, 5 mM ornithine, 5 mM lactate) in a representative experiment. As indicated, 0, 100, and 500 μM AcAld were added simultaneously with the ureagenic substrates. Data are mean ± S.E. from four wells per treatment. *B*, steady-state ureagenic respiration (total respiration minus basal respiration) is plotted *versus* AcAld concentration. *Inset*, a Dixon plot of the data. *C*, ureagenic and uncoupled respiration of hepatocytes were measured in the absence and presence of 125 μM AcAld. Data are mean ± S.E. from three independent experiments of three or more wells per treatment. *, $p < 0.05$ *versus* 0.

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. 7*A*, *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 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. 7*A*, *RhDex*), as shown previously (9). An MTG mask selectively outlining mitochondria was used to measure RhDex fluorescence only

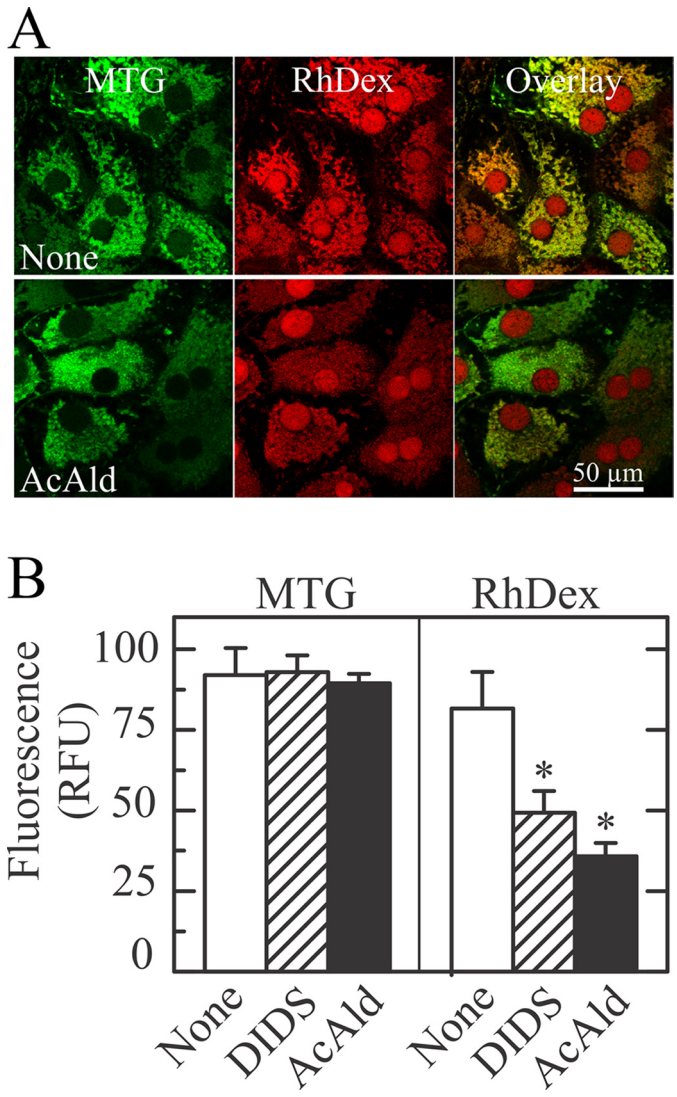

FIGURE 7. **Suppression of rhodamine dextran entry into the mitochondrial intermembrane space of permeabilized hepatocytes by acetaldehyde.** Hepatocytes were loaded with MTG; treated with vehicle, 500 μM acetaldehyde, or 300 μM DIDS; permeabilized with digitonin in the presence of RhDex; exposed to DIDS; and placed in RhDex-free ICB as described under "Experimental Procedures." *A*, green fluorescence of MTG-labeled mitochondria (*left column*), red fluorescence of RhDex (*center 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. *B*, mean intensities of MTG fluorescence and RhDex fluorescence colocalizing with MTG-labeled mitochondria are plotted for each group in *A*. Data are mean ± S.E. of 20 or more cells per treatment. *, $p < 0.05$ *versus* none.

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. 7, *A* and *B*, *AcAld*). Similarly, in hepatocytes pretreated with DIDS (300 μM, images not shown) to inhibit the VDAC before addition of RhDex, mitochondrial RhDex uptake was decreased by 40% (Fig. 7*B*, *DIDS*). In contrast, MTG fluorescence was not different between treatments (Fig. 7*B*). 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.

We hypothesized previously that ethanol-dependent impairment of mitochondrial function is initiated by closure of the VDAC in the MOM, which limits exchange of water-soluble metabolites into and out of mitochondria (6, 9, 10). This hypothesis was supported by data showing that the MOM became limiting after ethanol treatment for permeance of substrates for respiration in the 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 the 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 four 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. 2*B*).

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. 6*B*) Like ethanol, AcAld did not inhibit basal or uncoupled respiration by hepatocytes (Fig. 6*C*), 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 with 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, the VDAC is the only pathway known for uptake of solutes like 3-kDa RhDex across the MOM. Thus, our findings, taken together, support the conclusion that 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 the open/closed status of the VDAC regulates mitochondrial and cellular energetics through modulation of fluxes of hydrophilic metabolites across the 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 three 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. The 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 nonspecific MOM permeability. Because the VDAC is the only known pathway allowing transport of hydrophilic anionic metabolites across the 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 β 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.

Acknowledgments—We thank Drs. Wayne Rasband (National Institutes of Health, Bethesda, MD) and Lai Ding (Harvard Medical School, Harvard NeuroDiscovery Center (HNDC) Optical Imaging Core, Boston, MA) for help with writing ImageJ macros. We also thank Dr. Gregory L. Lovelace and Ms. Kemper Fagan for expert technical assistance.

REFERENCES

1. Kunji, E. R., and Crichton, P. G. (2010) Mitochondrial carriers function as monomers. *Biochim. Biophys. Acta* **1797,** 817–831
2. Palmieri, F., Pierri, C. L., De Grassi, A., Nunes-Nesi, A., and Fernie, A. R. (2011) Evolution, structure and function of mitochondrial carriers. A review with new insights. *Plant J.* **66,** 161–181
3. Pastorino, J. G., Hoek, J. B., and Shulga, N. (2005) Activation of glycogen synthase kinase 3β disrupts the binding of hexokinase II to mitochondria by phosphorylating voltage-dependent anion channel and potentiates chemotherapy-induced cytotoxicity. *Cancer Res.* **65,** 10545–10554
4. Colombini, M. (2004) *Mol. Cell. Biochem.* **256–257,** 107–115
5. Shoshan-Barmatz, V., De Pinto, V, Zweckstetter, M., Raviv, Z., Keinan, N., and Arbel, N. (2010) VDAC, a multi-functional mitochondrial protein regulating cell life and death. *Mol. Aspects Med.* **31,** 227–285
6. Lemasters, J. J., and Holmuhamedov, E. (2006) Voltage-dependent anion channel (VDAC) as mitochondrial governator. Thinking outside the box. *Biochim. Biophys. Acta* **1762,** 181–190
7. Rostovtseva, T. K., Tan, W., and Colombini, M. (2005) On the role of VDAC in apoptosis. Fact and fiction. *J. Bioenerg. Biomembr.* **37,** 129–142
8. Vander Heiden, M. G., Chandel, N. S., Li, X. X., Schumacker, P. T., Colombini, M., and Thompson, C. B. (2000) Outer mitochondrial membrane permeability can regulate coupled respiration and cell survival. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 4666–4671
9. Holmuhamedov, E., and Lemasters, J. J. (2009) Ethanol exposure decreases mitochondrial outer membrane permeability in cultured rat hepatocytes. *Arch. Biochem. Biophys.* **481,** 226–233
10. Deleted in proof
11. Cederbaum, A. I. (2006) Cytochrome P450 2E1-dependent oxidant stress and up-regulation of anti-oxidant defense in liver cells. *J. Gastroenterol. Hepatol.* **21,** S22–S25
12. Crabb, D. W., and Liangpunsakul, S. (2007) Acetaldehyde-generating enzyme systems. Roles of alcohol dehydrogenase, CYP2E1 and catalase, and speculations on the role of other enzymes and processes. *Novartis Found.*

Symp. **285,** 4–16; discussion 16–22, 198–199
13. Lakshman, R., Cederbaum, A. I., Hoek, J. B., Konishi, M., Koop, D., and Donohu, T. M. (2005) Use of CYP2E1-transfected human liver cell lines in elucidating the actions of ethanol. *Alcohol Clin. Exp. Res.* **29,** 1726–1734
14. Lieber, C. S. (2004) The discovery of the microsomal ethanol oxidizing system and its physiologic and pathologic role. *Drug Metab. Rev.* **36,** 511–529
15. Albano, E. (2006) Alcohol, oxidative stress and free radical damage. *Proc. Nutr. Soc.* **65,** 278–290
16. Bailey, S. M., and Cunningham, C. C. (2002) Contribution of mitochondria to oxidative stress associated with alcoholic liver disease. *Free Radic. Biol. Med.* **32,** 11–16
17. Fernández-Checa, J. C., Hirano, T., Tsukamoto, H., and Kaplowitz, N. (1993) Mitochondrial glutathione depletion in alcoholic liver disease. *Alcohol* **10,** 469–475
18. Fernández-Checa, J. C., Kaplowitz, N., Colell, A., and García-Ruiz, C. (1997) Oxidative stress and alcoholic liver disease. *Alcohol Health Res. World* **21,** 321–324
19. Kessova, I., and Cederbaum, A. I. (2003) CYP2E1. Biochemistry, toxicology, regulation and function in ethanol-induced liver injury. *Curr. Mol. Med.* **3,** 509–518
20. Lu, Y., and Cederbaum, A. I. (2008) CYP2E1 and oxidative liver injury by alcohol. *Free Radic. Biol. Med.* **44,** 723–738
21. Deitrich, R. A., Petersen, D., and Vasiliou, V. (2007) Removal of acetaldehyde from the body. *Novartis Found. Symp.* **285,** 23–40; discussion 40–51, 198–199
22. Kaplowitz, N. (2000) Mechanisms of liver cell injury. *J. Hepatol.* **32,** 39–47
23. Lindros, K. O., Badger, T., Ronis, M., Ingelman-Sundberg, M., and Koivusalo, M. (1995) Phenethyl isothiocyanate, a new dietary liver aldehyde dehydrogenase inhibitor. *J. Pharmacol. Exp. Ther.* **275,** 79–83
24. Marchitti, S. A., Brocker, C., Stagos, D., and Vasiliou, V. (2008) Non-P450 aldehyde oxidizing enzymes. The aldehyde dehydrogenase superfamily. *Expert Opin. Drug Metab. Toxicol.* **4,** 697–720
25. Tuma, D. J., and Casey, C. A. (2003) Dangerous byproducts of alcohol breakdown. Focus on adducts. *Alcohol Res. Health* **27,** 285–290
26. Caldovic, L., and Tuchman, M. (2003) *N*-acetylglutamate and its changing role through evolution. *Biochem. J.* **372,** 279–290
27. Davis, P. K., and Wu, G. (1998) Compartmentation and kinetics of urea cycle enzymes in porcine enterocytes. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **119,** 527–537
28. Hasselblatt, M., Krampe, H., Jacobs, S., Sindram, H., Armstrong, V. W., Hecker, M., and Ehrenreich, H. (2006) Arginine challenge unravels persistent disturbances of urea cycle and gluconeogenesis in abstinent alcoholics. *Alcohol* **41,** 372–378
29. Lanpher, B., Brunetti-Pierri, N., and Lee, B. (2006) Inborn errors of metabolism. The flux from Mendelian to complex diseases. *Nat. Rev. Genet.* **7,** 449–460
30. Withers, P. C. (1998) Urea. Diverse functions of a "waste" product. *Clin. Exp. Pharmacol. Physiol.* **25,** 722–727
31. Deleted in proof
32. Guide for the Care and Use of Laboratory Animals (1996) *Institute of Laboratory Research, Commission on Life Sciences, National Research Council*, The National Academies Press, Washington, D.C.
33. Qian, T., Nieminen, A. L., Herman, B., and Lemasters, J. J. (1997) Mitochondrial permeability transition in pH-dependent reperfusion injury to rat hepatocytes. *Am. J. Physiol.* **273,** C1783–C1792
34. Uchiyama, A., Kim, J. S., Kon, K., Jaeschke, H., Ikejima, K., Watanabe, S., and Lemasters, J. J. (2008) Translocation of iron from lysosomes into mitochondria is a key event during oxidative stress-induced hepatocellular injury. *Hepatology* **48,** 1644–1654
35. Gerencser, A. A., Neilson, A., Choi, S. W., Edman, U., Yadava, N., Oh, R. J., Ferrick, D. A., Nicholls, D. G., and Brand, M. D. (2009) Quantitative microplate-based respirometry with correction for oxygen diffusion. *Anal. Chem.* **81,** 6868–6878
36. Maughan, R. J. (1982) A simple, rapid method for the determination of glucose, lactate, pyruvate, alanine, 3-hydroxybutyrate and acetoacetate on a single 20-mul blood sample. *Clin. Chim. Acta* **122,** 231–240
37. Ronis, M. J., Korourian, S., Blackburn, M. L., Badeaux, J., and Badger, T. M. (2010) The role of ethanol metabolism in development of alcoholic steatohepatitis in the rat. *Alcohol* **44,** 157–169
38. Mathews, J. M., Etheridge, A. S., Raymer, J. H., Black, S. R., Pulliam, D. W., Jr., and Bucher, J. R. (1998) Selective inhibition of cytochrome P450 2E1 *in vivo* and *in vitro* with trans-1,2-dichloroethylene. *Chem. Res. Toxicol.* **11,** 778–785
39. Smith, P. S., Zhao, W., Spitz, D. R., and Robbins, M. E. (2007) Inhibiting catalase activity sensitizes 36B10 rat glioma cells to oxidative stress. *Free Radic. Biol. Med.* **42,** 787–797
40. Cunningham, C. C., and Bailey, S. M. (2001) Ethanol consumption and liver mitochondria function. *Biol. Signals Recept.* **10,** 271–282
41. Mantena, S. K., King, A. L., Andringa, K. K., Eccleston, H. B., and Bailey, S. M. (2008) Mitochondrial dysfunction and oxidative stress in the pathogenesis of alcohol- and obesity-induced fatty liver diseases. *Free Radic. Biol. Med.* **44,** 1259–1272
42. Sastre, J., Serviddio, G., Pereda, J., Minana, J. B., Arduini, A., Vendemiale, G., Poli, G., Pallardo, F. V., and Vina, J. (2007) Mitochondrial function in liver disease. *Front Biosci.* **12,** 1200–1209
43. Cahill, A., Cunningham, C. C., Adachi, M., Ishii, H., Bailey, S. M., Fromenty, B., and Davies, A. (2002) Effects of alcohol and oxidative stress on liver pathology. The role of the mitochondrion. *Alcohol Clin. Exp. Res.* **26,** 907–915
44. Grant, D. A. (1988) Genetic polymorphism of the alcohol metabolising enzymes as a basis for alcoholic liver disease. *Br. J. Addict.* **83,** 1255–1259
45. Hoek, J. B., Cahill, A., and Pastorino, J. G. (2002) Alcohol and mitochondria. A dysfunctional relationship. *Gastroenterology* **122,** 2049–2063
46. Reuben, A. (2006) Alcohol and the liver. *Curr. Opin. Gastroenterol.* **22,** 263–271
47. Tsukamoto, H. (1993) Oxidative stress, antioxidants, and alcoholic liver fibrogenesis. *Alcohol* **10,** 465–467
48. Koop, D. R. (1990) Inhibition of ethanol-inducible cytochrome P450IIE1 by 3-amino-1,2,4-triazole. *Chem. Res. Toxicol.* **3,** 377–383
49. Bradford, B. U., Seed, C. B., Handler, J. A., Forman, D. T., and Thurman, R. G. (1993) Evidence that catalase is a major pathway of ethanol oxidation *in vivo*. Dose-response studies in deer mice using methanol as a selective substrate. *Arch. Biochem. Biophys.* **303,** 172–176
50. Thurman, R. G., McKenna, W. R., Brentzel, H. J., Jr., and Hesse, S. (1975) Significant pathways of hepatic ethanol metabolism. *Fed. Proc.* **34,** 2075–2081
51. Bradford, B. U., and Rusyn, I. (2005) Swift increase in alcohol metabolism (SIAM). Understanding the phenomenon of hypermetabolism in liver. *Alcohol* **35,** 13–17
52. Lemasters, J. J. (2007) Modulation of mitochondrial membrane permeability in pathogenesis, autophagy and control of metabolism. *J. Gastroenterol. Hepatol.* **22,** S31-S37
53. Tsujimoto, Y., and Shimizu, S. (2007) Role of the mitochondrial membrane permeability transition in cell death. *Apoptosis* **12,** 835–840
54. Baines, C. P., Kaiser, R. A., Sheiko, T., Craigen, W. J., and Molkentin, J. D. (2007) Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat. Cell Biol.* **9,** 550–555
55. Madesh, M., and Hajnóczky, G. (2001) VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome c release. *J. Cell Biol.* **155,** 1003–1015
56. Rostovtseva, T. K., Sheldon, K. L., Hassanzadeh, E., Monge, C., Saks, V., Bezrukov, S. M., and Sackett, D. L. (2008) Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 18746–18751
57. Tikunov, A., Johnson, C. B., Pediaditakis, P., Markevich, N., Macdonald, J. M., Lemasters, J. J., and Holmuhamedov, E. (2010) Closure of VDAC causes oxidative stress and accelerates the $Ca^{(2+)}$-induced mitochondrial permeability transition in rat liver mitochondria. *Arch. Biochem. Biophys.* **495,** 174–181
58. Vander Heiden, M. G., Li, X. X., Gottleib, E., Hill, R. B., Thompson, C. B., and Colombini, M. (2001) Bcl-xL promotes the open configuration of the voltage-dependent anion channel and metabolite passage through the outer mitochondrial membrane. *J. Biol. Chem.* **276,** 19414–19419

59. Pediaditakis, P., Kim, J. S., He, L., Zhang, X., Graves, L. M., and Lemasters, J. J. (2010) Inhibition of the mitochondrial permeability transition by protein kinase A in rat liver mitochondria and hepatocytes. *Biochem. J.* **431,** 411–421
60. Maldonado, E. N., Patnaik, J., Mullins, M. R., and Lemasters, J. J. (2010) Free tubulin modulates mitochondrial membrane potential in cancer cells. *Cancer Res.* **70,** 10192–10201
61. Rostovtseva, T. K., and Bezrukov, S. M. (2008) VDAC regulation. Role of cytosolic proteins and mitochondrial lipids. *J. Bioenerg. Biomembr.* **40,** 163–170
62. Holmuhamedov, E. L., Lovelace, G., and Lemasters, J. J. (2011) *Biophys. J.* **100,** 460a