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AMP-Dependent Kinase and Autophagic Flux are Involved in Aldehyde Dehydrogenase 2-Offered Protection against Cardiac Toxicity of Ethanol

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Abstract

Mitochondrial aldehyde dehydrogenase-2 (ALDH2) alleviates ethanol toxicity although the precise mechanism is unclear. This study was designed to evaluate the effect of ALDH2 on ethanol-induced myocardial damage with a focus on autophagy. Wild-type FVB and transgenic mice overexpressing ALDH2 were challenged with ethanol (3 g/kg/d, i.p.) for 3 days and cardiac mechanical function was assessed using the echocardiographic and IonOptix systems. Western blot analysis was used to evaluate essential autophagy markers, Akt and AMPK and their downstream signaling mTOR. Ethanol challenge altered cardiac geometry and function evidenced by enlarged ventricular end systolic and diastolic diameters, decreased cell shortening and intracellular Ca^{2+} rise, prolonged relengthening and intracellular Ca^{2+} decay, as well as reduced SERCA Ca^{2+} uptake, the effects of which were mitigated by ALDH2. Ethanol challenge facilitated myocardial autophagy as evidenced by enhanced expression of Beclin, ATG7 and LC3B II, as well as mTOR dephosphorylation, which was alleviated by ALDH2. Ethanol challenge-induced cardiac defect and apoptosis were reversed by the ALDH-2 agonist Alda-1, the autophagy inhibitor 3-MA, and the AMPK inhibitor compound C whereas the autophagy inducer rapamycin and the AMPK activator AICAR mimicked or exacerbated ethanol-induced cell injury. Ethanol promoted or suppressed phosphorylation of AMPK and Akt, respectively, in FVB but not ALDH2 murine hearts. Moreover, AICAR nullified Alda-1-induced protection against ethanol-triggered autophagic and functional changes. Ethanol increased GFP-LC3 puncta in H9c2 cells, the effect of which was ablated by Alda-1 and 3-MA. Lysosomal inhibition using bafilomycin A1, E64D and pepstatin A obliterated Alda-1- but not ethanol-induced responses in GFP-LC3 puncta. Our results suggested that ALDH2 protects against ethanol toxicity through altered Akt and AMPK signaling and regulation of autophagic flux.

Keywords

Ethanol; ALDH2; myocardial dysfunction; autophagy; autophagy flux; Akt; AMPK

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INTRODUCTION

Binge drinking has been demonstrated to predispose myocardium to pathological changes including myofibrillar disruption and compromised ventricular contractility ultimately leading to alcoholic cardiomyopathy [1-3]. Despite the intense effort to manage alcoholic cardiomyopathy, effective clinical remedies have been somewhat dismal to cope with the devastating myopathic changes following binge drinking. Recent findings from our lab have indicated a protective role of the mitochondrial isoform of aldehyde dehydrogenase (ALDH), ALDH2, in alcohol-induced cardiac injury [3-5]. ALDH2 is known for its role in the metabolism of the ethanol metabolite acetaldehyde. In addition, ALDH2 serves as a critical metabolic enzyme in the detoxification of other reactive aldehydes such as 4-hydroxy-2-nonenal (4-HNE) and conversion of nitroglycerin (from glyceryl trinitrate [GTN]) to 1,2-glyceryl dinitrate (1,2-GDN) [6-9]. ALDH2 has been recently shown to consistently reduce ischemic and reperfusion arrhythmias injury to the heart [10-12], suggesting its novel cardioprotective role. However, the precise mechanism of action underlying ALDH2-mediated cardioprotection is still elusive. Very recent findings from our lab revealed that ALDH2 protects against ischemia-reperfusion injury possibly through regulation of autophagy in the heart [12, 13]. Nonetheless, whether autophagy plays a role in ALDH2-offered protection against alcoholic cardiomyopathy is still unknown.

Autophagy is a process of intracellular bulk degradation in which cytosolic proteins and organelles are sequestered into the double-membrane vesicles autophagosomes to be fused with lysosomes, forming autophagolysosomes for degradation by lysosomal enzymes [14, 15]. Given that autophagy may serve as a rather critical player in cell survival and damage in myopathic hearts [16], our present study was designed to examine the role of autophagy in ALDH2-offered cardioprotection against acute ethanol toxicity using our unique murine model of ALDH2 overexpression. We examined cell signaling pathways involved in the regulation of autophagy, in particular AMP-dependent protein kinase (AMPK) and Akt, two main kinases governing myocardial survival and function [12]. It has been shown that activation of AMPK may promote whereas activation of Akt suppresses autophagy [12, 13, 17]. In addition, AMPK has been shown to facilitate autophagy through its inhibitory effect on Akt and its downstream signal molecular mammalian target of rapamycin (mTOR) [17-19]. Considering our recent observation of over-activated AMPK signaling following acute ethanol challenge [20], it is intriguing to examine the role of AMPK in ethanol challenge-induced changes in autophagy, if any, and ultimately, the ethanol-induced myocardial geometric and functional alterations. In an effort to determine if ethanol- or ALDH2-induced autophagic responses, if any, were due to changes in early or late stages of autophagy pathway, the autophagy flux (i.e., fusion of autophagosome with lysosomes to form autophagolysosomes and their degradation) was determined by measuring LC3-II levels in the absence (steady-state autophagosomes) or the presence (cumulative autophagosomes) of a mixture of lysosomal inhibitors [14].

METHODS

Experimental animals and acute ethanol challenge

All animal procedures were approved by our institutional Animal Care and Use Committee at the University of Wyoming (Laramie, WY). The ALDH2 transgenic mouse line was produced using the chicken β -actin promoter as described previously [5]. In brief, five to six month-old adult male ALDH2 overexpression transgenic mice and FVB wild-type mice were housed in a temperature-controlled room under a 12hr/12hr-light/dark with access to tap water *ad libitum*. For acute ethanol challenge, FVB and ALDH2 mice were injected with ethanol (3 g/kg, i.p. for 3 days) [20] and were used for experimentation 24 hrs after the last ethanol challenge.

Echocardiographic assessment

Cardiac geometry and function were evaluated in anesthetized (Avertin 2.5%, 10 μ l/g body weight, i.p.) mice using the 2-D guided M-mode echocardiography (Sonos 5500) equipped with a 15-6 MHz linear transducer. Left ventricular (LV) anterior and posterior wall dimensions during diastole and systole were recorded from three consecutive cycles in M-mode using methods adopted by the American Society of Echocardiography. Fractional shortening was calculated from LV end-diastolic [21] and LV end-systolic (ESD) diameters using the equation (EDD-ESD)/EDD. Heart rates were averaged over 10 cardiac cycles [5].

Cardiomyocyte isolation and in vitro drug treatment

After ketamine/xylazine (ketamine 80 mg/kg and xylazine 12 mg/kg, i.p.) sedation, hearts were removed and perfused with KHB buffer containing (in mM): 118 NaCl, 4.7 KCl, 1.2 MgSO_4 , 1.2 KH_2PO_4 , 25 NaHCO_3 , 10 HEPES and 11.1 glucose. Hearts were digested with 223 U/ml Liberase (Roche, 1988476) for 20 min. Left ventricles were removed and minced before being filtered. Cardiomyocyte yield was roughly 75% which was unaffected by ethanol or ALDH2 transgene. Only rod-shaped cardiomyocytes with clear edges were used for mechanical evaluation [5]. To examine the effect of autophagy on ethanol or ALDH2-induced cardiac contractile response, cardiomyocytes from FVB mice were treated with ethanol (240 mg/dl) at 37°C for 4-5 hrs in the absence or presence of the ALDH2 activator Alda-1 (20 μ M, Calbiochem, 126920) [10], the autophagy inducer rapamycin (5 μ M, EMD Chemicals, 553210) [22] or the autophagy inhibitor 3-methyladenine (3-MA, 10 mM, Sigma, M9281) [23] prior to mechanical or biochemical assessment. To assess the role of AMPK in ethanol-induced cardiomyocyte contractile response, cardiomyocytes were treated with ethanol (240 mg/dl) at 37°C for 4-5 hrs in the absence or presence of the AMPK agonist 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR, 500 μ M, Sigma, A9978) [24], the AMPK inhibitor compound C (10 μ M, Sigma, P5499) [20] or Alda-1 (20 μ M) prior to the assessment of mechanical function and autophagy.

Cell shortening and relengthening

Mechanical properties of cardiomyocytes were evaluated utilizing a SoftEdge MyoCam® system (IonOptix Corporation) [5]. Briefly, cardiomyocytes were visualized under an inverted microscope (Olympus, IX-70) and were stimulated with a voltage frequency of 0.5 Hz. The myocyte being observed was shown on a computer monitor using an IonOptix MyoCam camera. IonOptix SoftEdge software was utilized to capture cell shortening and relengthening changes. The indices considered were peak shortening amplitude (PS), time-to-peak shortening (TPS), time-to-90% relengthening (TR_{90}), maximal velocity of shortening and relengthening ($\pm \text{dL/dt}$).

Intracellular Ca^{2+} transients

A cohort of myocytes was loaded with fura-2/AM (0.5 μ M, Molecular Probe, F1221) for 10 min and fluorescence intensity were recorded with a dual-excitation fluorescence photomultiplier system (IonOptix). Myocytes were placed onto an Olympus IX-70 inverted microscope and imaged through a Fluor $\times$ 40 oil objective. Cells were exposed to light emitted by a 75W lamp and passed through either a 360 or a 380 nm filter, while being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480-520 nm and qualitative change in fura-2 fluorescence intensity (FFI) was inferred from FFI ratio at the two wavelengths (360/380). Fluorescence decay time was calculated as an indicator of intracellular Ca^{2+} clearing [5].

SERCA activity measured by $^{45}\text{Ca}^{2+}$ uptake

Cardiomyocytes were sonicated and solubilized in a Tris-sucrose homogenization buffer consisting of 30 mM Tris-HCl, 8% sucrose, 1 mM PMSF and 2 mM dithiothreitol, pH 7.1. To determine SERCA-dependent Ca^{2+} uptake, samples were treated with and without the SERCA inhibitor thapsigargin (10 μM , Sigma, T9033) for 15 min. The difference between the two readings was deemed the thapsigargin-sensitive uptake through SERCA. Uptake was initiated by the addition of an aliquot of supernatant to a solution consisting of (in mM) 100 KCl, 5 NaN_3 , 6 MgCl_2 , 0.15 EGTA, 0.12 CaCl_2 , 30 Tris-HCl pH 7.0, 10 oxalate, 2 ATP and 1 μCi $^{45}\text{CaCl}_2$ at 37°C. Aliquots of samples were injected onto glass filters on a suction manifold and washed 3 times. Filters were then removed from the manifold, placed in scintillation fluid and counted. SERCA activity was expressed as cpm/mg protein [5].

Separation of cytosolic and mitochondrial fractions

Ventricles were minced and homogenized by Polytron in the ice-cold MSE buffer [220 mM mannitol, 70 mM sucrose, 2 mM EGTA, 5 mM 3-(4-morpholino) propane sulfonic acid (MOPS), pH 7.4, 0.2% bovine serum albumin (BSA) and a protease inhibitor cocktail containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), E-64, bestatin, leupeptin, aprotinin, and EDTA obtained from Sigma Chemicals (St. Louis, MO)]. The homogenates were centrifuged at $600 \times g$ for 10 min to remove unbroken tissues and nuclei, and the supernatants were centrifuged at $3000 \times g$ for 10 min to pellet mitochondria. The supernatants were further centrifuged for 30 min at $100,000 \times g$ to obtain cytosolic fraction. The mitochondrial pellet was dissolved in a lysis buffer and centrifuged at $10,000 \times g$ for 30 min at 4°C to yield a soluble protein. Fifty μg of the mitochondrial or cytosolic protein was separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and was analyzed by western blot using the ALDH2 antibody [25].

Western blot

Ventricular tissues were homogenized and sonicated in a lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS and 1% protease inhibitor cocktail. Equal amounts (30 μg protein) of proteins were separated on 10% or 15% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad) and were then transferred electrophoretically to nitrocellulose membranes. The membranes were blocked with 5% milk in Tris-buffered saline (TBS) before overnight incubation at 4°C with the anti-ALDH2 (1:1,000, provided by Dr. Henry Weiner from Purdue University, West Lafayette, IN), the anti-Bcl-1 (1:1,000, Cell Signaling, 3738), anti-Akt (1:1,000, Cell Signaling, 9272), anti-pAkt (Ser473, 1:500, Cell Signaling, 9271), anti-AMPK (1:1,000, Cell Signaling, 2532), anti-pAMPK (Thr172, 1:500, Cell Signaling 2535), anti-mTOR (1:1,000, Cell Signaling, 2972), anti-pmTOR (Ser2448, 1:1,000, Cell Signaling, 2971), and anti-LC3B (1:500, Cell Signaling, 2775) antibodies. Membranes were then incubated for 1 hr with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000). After immunoblotting, films were scanned and the intensity of immunoblot bands was detected with a Bio-Rad Calibrated Densitometer. GAPDH was used as the loading control [5]. To assess the role of AMPK and ALDH2 on acute ethanol exposure-induced autophagy, cardiomyocytes were treated with the AMPK activator AICAR (500 μM) or the ALDH2 activator Alda-1 (20 μM) in the presence or absence of ethanol (240 mg/dl) for 4-5 hrs.

Caspase-3 assay

Caspase-3 is an enzyme activated during induction of apoptosis. Caspase-3 activity was determined according to published method [5]. In brief, myocytes were lysed in 100 μl of ice-cold cell lysis buffer (50 mM HEPES, 0.1% CHAPS, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% NP40). Following cell lysis, 70 μl reaction buffer and 20 μl caspase-3

colorimetric substrate (Ac-DEVD-*p*-nitroanilide) were added to cell lysate and incubated for 1 hr at 37°C, during which time, caspase enzyme in the sample was allowed to cleave the chromophore pNA from its substrate molecule. Absorbency was detected at 405 nm with caspase-3 activity being proportional to the color reaction. Caspase-3 activity was expressed as picomoles of *p*-nitroanilide released per micrograms of protein per minute.

LC3B-GFP-adenovirus production and infection in H9C2 cells

H9c2 cells, a clonal cell line derived from fetal rat hearts, were purchased from American Type Culture Collection (ATCC, Manassas, VA). Due to the technical difficulty of viral transfection in murine cardiomyocytes, H9c2 cells were used to assess the autophagy using GFP fluorescence. In brief, cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY) and 1% penicillin and streptomycin and maintained in 95% air and 5% CO₂ at 37°C. Cells were grown to 80% confluence prior to usage. Adenovirus containing GFP-LC3 construct (kindly provided by Dr. Cindy Miranti from Van Andel Institute, Grand Rapids, MI) was propagated using HEK293 cell line. Cells were then transfected with GFP-LC3 adenovirus as described previously in our laboratory [26]. Upon plaque formation, infected cells were collected, washed with PBS, resuspended in culture medium and lysed by 3 cycles of freeze-thaw (37°C). Cell debris was collected by centrifugation and aliquots of supernatant with viral particles were stored at -80°C. Adenovirus was purified using an Adeno-X Maxi purification kit (Clontech Laboratories, Inc. Mountain View, CA). H9c2 cells were grown to confluence on Lab-Tek chamber slide. Cells were then infected at an MOI of 2 with adenoviruses expressing GFP-LC3 fusion protein. Medium was replaced with fresh DMEM after 6 hrs. Twenty four hrs later, cells were visualized for autophagy using fluorescence microscopy [26].

Quantification of the GFP-LC3

H9c2 cells transfected with GFP-LC3 adenovirus were treated with or without ethanol (240 mg/dl) at 37°C for 4 hrs in the absence or presence of Alda-1 (20 μM) [10] or 3-MA (10 mM) [23]. Rapamycin (5 μM) was used as the positive control [22]. To evaluate autophagy flux, GFP-LC3 positive cells were evaluated in ethanol- or Alda-1-treated cells incubated with (cumulative autophagosomes) or without (steady-state autophagosomes) of a mixture of lysosomal inhibitors [bafilomycin A1 (50 nM), E64D (2.5 μg/ml) and pepstatin A methyl ester (5 μg/ml)] [14]. The cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature followed by three times washing with PBS. Cover slips were mounted on the slides using Vecta mount™ AQ-aqueous mounting medium (Vector Laboratories, Inc, Burlingame, CA). For autophagy assessment, cells were visualized at 40× magnification using a Olympus BX51 fluorescence microscope (Olympus America, Inc, Melville, NY) and percentage of GFP-LC3 positive cells showing numerous GFP-LC3 puncta (> 10 dots/cell) were scored as described previously [27]. A minimum of 300-400 cells were scored in at least three independent experiments.

Statistical analysis

Data were expressed as Mean ± SEM. Statistical significance ($p < 0.05$) for each variable was estimated by analysis of variance (ANOVA) followed by Tukey's test for *post hoc* analysis.

RESULTS

Expression of ALDH2 in the cytosolic and membrane fractions in myocardium from FVB and ALDH2 mice challenged with alcohol

Our data shown in Fig. 1 displayed that ALDH2 enzyme was predominantly expressed in the mitochondria as opposed to cytosol. Expression of ALDH2 was overtly higher in mitochondrial but not cytosolic fraction from ALDH2 transgenic mouse hearts, validating the transgenic model. Expression of ALDH2 (cytosolic or mitochondrial) was not affected by acute ethanol challenge.

Echocardiographic properties of FVB and ALDH2 mice challenged with alcohol

Acute ethanol challenge did not affect body weight. Heart rate and LV wall thickness were comparable among all groups. Although LV EDD and ESD parameters were both enlarged by acute ethanol exposure, fractional shortening remained unchanged. Interestingly, ALDH2 rescued acute ethanol exposure-induced increase in ESD and EDD without eliciting any effect on myocardial geometry itself (Fig. 2). These data suggest that ALDH2 transgene is capable of alleviating acute ethanol exposure-induced myocardial remodeling.

Effect of acute ethanol exposure on cardiomyocyte mechanics and intracellular Ca^{2+} handling in FVB and ALDH2 mice

Our further assessment of cardiomyocyte mechanics revealed that acute ethanol treatment significantly depressed peak shortening and $\pm \text{dL/dt}$ as well as prolonged TR_{90} without affecting TPS. Although ALDH2 itself failed to alter these mechanical indices, it significantly attenuated acute ethanol exposure-induced cardiomyocyte mechanical abnormalities (Fig. 3). To evaluate if intracellular Ca^{2+} handling plays an essential role in the ethanol and/or ALDH2-elicited cardiac contractile responses, intracellular Ca^{2+} transient and SERCA function assessed by $^{45}\text{Ca}^{2+}$ uptake were measured in cardiomyocytes from FVB or ALDH2 mice treated with or without ethanol. Data shown in Fig. 4 depicted depressed intracellular Ca^{2+} rise in response to electrical stimulus (ΔFFI), dampened $^{45}\text{Ca}^{2+}$ uptake and reduced intracellular Ca^{2+} decay along with an unchanged baseline intracellular Ca^{2+} following acute ethanol challenge. Similar to its effect on myocardial contraction, ALDH2 negated ethanol-induced changes in ΔFFI , intracellular Ca^{2+} decay and $^{45}\text{Ca}^{2+}$ uptake without eliciting any notable effect on intracellular Ca^{2+} properties in the absence of ethanol challenge (Fig. 4).

Effect of ALDH2 on ethanol-induced changes in protein markers for autophagy

Recent evidence has indicated a role of autophagy in ethanol-induced tissue damage [28]. Our data revealed that acute ethanol challenge upregulated expression of several key autophagic markers including Beclin-1, LC3B II (absolute or the ratio of LC3B II-to-LC3B I) and ATG7, the effect of which was alleviated by ALDH2 (Fig. 5A-F). These data favored a possible role of autophagy in the ethanol- and ALDH2-elicited cardiac contractile response.

Effect of autophagosome inhibition on ethanol-induced cardiomyocyte mechanical defect

To further examine the causal relationship between autophagy and mechanical response following acute ethanol toxicity in the heart, acute ethanol-induced cardiomyocyte mechanical dysfunction was examined *in vitro* in the absence or presence of the autophagosome formation inhibitor 3-MA. Short-term incubation of cardiomyocytes with ethanol (240 mg/dl) led to significantly depressed peak shortening and $\pm \text{dL/dt}$ as well as prolonged TR_{90} without affecting TPS, reminiscent of the *in vivo* effects of ethanol cardiac toxicity. Although 3-MA did not exert any mechanical effect by itself, it obliterated ethanol

exposure-induced cardiomyocyte mechanical abnormalities (Fig. 5G-L), suggesting a role of autophagy in ethanol-induced cardiac contractile dysfunction.

Effect of ALDH2 and ALDH2 agonist on ethanol-induced mTOR expression and autophagy induction or ALDH2 agonist-elicited cardiomyocyte mechanical response

Phosphorylation of mTOR, a key signaling molecule for autophagy, displays a reciprocal relationship with autophagy [29]. Our results revealed that acute ethanol exposure significantly dampened mTOR phosphorylation (either absolute or normalized value) in FVB but not ALDH2 myocardium. Likewise, *in vitro* treatment (4 hrs) of Alda-1 also reversed ethanol exposure-induced dephosphorylation of mTOR. ALDH2 transgene or the ALDH2 agonist itself did not affect the phosphorylation of mTOR. In addition, neither ethanol nor ALDH2 or Alda-1 altered the expression of pan mTOR (Fig. 6A-C). These data are in accordance with the possible role of autophagy in ethanol-induced cardiac toxicity and ALDH2 (or Alda-1)-exerted protection. We further examined the impact of the autophagy inducer rapamycin on ethanol-induced cardiomyocyte survival and mechanical function. Data shown in Fig. 6D-I revealed that rapamycin depressed $\pm$ dL/dt without affecting apoptosis (shown as the caspase-3 activity), peak shortening, TPS and TR₉₀. Interestingly, rapamycin augmented the ethanol-induced cardiomyocyte apoptosis and mechanical defects ($\pm$ dL/dt and TR₉₀) although autophagy induction failed to alter the pattern of ethanol-induced responses in peak shortening and TPS. Last but not least, Alda-1 ablated ethanol-induced cardiomyocyte apoptosis and mechanical defects without eliciting any effect by itself.

Effect of ALDH2 and Alda-1 on ethanol-induced change in Akt and AMPK

To examine the potential signaling pathways involved in ethanol and/or ALDH2-elicited cardiac autophagic and mechanical response, we examined levels of the key cardiac surviving factor Akt and the cardiac energy fuel sensor AMPK, two essential regulators for autophagy [12, 13, 17]. Our results indicated that acute ethanol treatment markedly elevated the expression of pan and phosphorylated AMPK associated with an unchanged pAMPK-to-AMPK ratio in FVB mice. To the contrary, ethanol exposure suppressed phosphorylation of Akt (both absolute and normalized) without affecting the expression of pan Akt. While ALDH2 itself failed to affect the expression of pan or phosphorylated AMPK and Akt, it effectively reconciled acute ethanol exposure-induced change in pan or phosphorylated AMPK and Akt. Similarly, *in vitro* treatment of Alda-1 also reversed ethanol-induced changes in phosphorylation of AMPK and Akt. Alda-1 itself failed to affect the expression of pan or phosphorylated AMPK and Akt (Fig. 7). These data suggest that ALDH2 may compensate for ethanol-induced changes in Akt and AMPK signaling.

Role of AMPK signaling in acute ethanol-induced autophagy

Activation of AMPK has been shown to promote autophagy through inhibition of Akt and mTOR [13]. To examine if AMPK plays a pivotal role in ethanol-elicited cytotoxicity and ALDH2-offered protective effects in autophagy and mechanical function, cardiomyocytes from normal FVB mice were incubated with ethanol (240 mg/dl) with Alda-1 (20 μ M) in the absence or presence of the AMPK activator AICAR (500 μ M) or the AMPK inhibitor compound C (10 μ M) for 4-5 hrs. Evaluation of the autophagic markers showed that Alda-1 and compound C significantly alleviated or mitigated ethanol-induced elevation in LC3B II expression (absolute or LC3B II-to-LC3B I ratio), in a manner reminiscent of the *in vivo* findings from ALDH2 mice. AICAR itself significantly enhanced the expression of LC3B II (absolute or the LC3B II-to-LC3B I ratio). Interestingly, the Alda-1-elicited beneficial effect against ethanol was nullified by AMPK activation with AICAR. Alda-1 alone affected the expression of LC3B II while none of the chemicals tested altered the expression of LC3B I. Consistent with the autophagic data, Alda-1 and compound C effectively rescued ethanol-

induced cardiomyocyte dysfunction including depressed peak shortening and $\pm$ dL/dt and prolonged TR₉₀ without affecting rest cell length and TPS. AICAR itself compromised the cardiomyocyte contractile function including decreased peak shortening and $\pm$ dL/dt without affecting TPS and TR₉₀. More importantly, AICAR nullified Alda-1-elicited beneficial mechanical responses against ethanol toxicity (Fig. 8). These data depicted a likely role of AMPK in ALDH2-elicited cardioprotection against the ethanol-induced mechanical and autophagic responses.

Effect of lysosomal inhibitors, Alda-1 and autophagosome formation inhibition on ethanol-induced autophagy in H9c2 cells

To determine if ethanol exposure-induced autophagy was due to changes in early or late stages of autophagy pathway, H9c2 myoblasts were transfected with an adenovirus expressing the GFP-LC3 fusion protein for 24 hrs prior to the exposure of ethanol (240 mg/dl) for 4 hrs in the absence or presence of mixed lysosomal inhibitors (bafilomycin A1, E64D and pepstatin A methyl ester) [14] to inhibit autophagy flux (formation of autophagolysosomes and lysosomal degradation). Data shown in Fig. 9 revealed that lysosomal inhibition did not alter the ethanol-induced autophagy shown as GFP-LC3 puncta, indicating a role of lysosomal inhibition in ethanol-induced autophagic response. The lysosomal inhibitors themselves displayed a tendency to increase GFP-LC3 puncta also no statistical significance was reached. To consolidate the effect of Alda-1 on ethanol-induced autophagy, the GFP-LC3 transfected H9c2 myoblasts were exposed to ethanol (240 mg/dl) for 4 hrs in the absence or presence of Alda-1 (20 μ M) or 3-MA (10 mM). Our data revealed that Alda-1 abolished ethanol-induced rise in GFP-LC3 puncta, the effect of which was negated by lysosomal inhibitors. The autophagosome formation inhibitor 3-MA also mitigated ethanol-induced GFP-LC3 puncta formation, as expected. Neither Alda-1 nor 3-MA significantly affected GFP-LC3 puncta in the absence of ethanol. The autophagy inducer rapamycin (5 μ M) was employed as a positive control for GFP-LC3 puncta formation. These findings suggest that ethanol exposure-induced autophagy may be more likely due to inhibition of lysosomal autophagic flux as oppose to induction of autophagosome formation. On the other hand, the Alda-1-offered beneficial effect against ethanol-induced autophagy may be associated with stimulation of autophagy flux.

DISCUSSION

The major findings of this study indicated that ALDH2 exerts protective effects against ethanol cardiotoxicity through inhibition of an AMPK-dependent autophagy. Our data indicated that acute ethanol exposure-induced change in cardiac mechanical and autophagic responses may be linked to elevated expression of pan and phosphorylated AMPK, dampened activation of Akt and its downstream signaling molecule mTOR. ALDH2 and its agonist Alda-1 are likely to offer their cardioprotection by reversing ethanol-induced changes in AMPK, Akt and mTOR, *en route* to suppressed autophagy and cardiac mechanical dysfunction. Furthermore, possible involvement of autophagy in ethanol-induced cardiomyocyte dysfunction was confirmed by pharmacological modulation of autophagy using 3-MA and rapamycin. Measurement of GFP-LC3 positive puncta in the absence (steady-state autophagosomes) or the presence (cumulative autophagosomes) of a mixture of lysosomal inhibitors [14] revealed that ethanol exposure-induced autophagy may be more likely due to an inhibition of the late-stage autophagic flux as oppose to induction of the early-stage autophagosome formation. The ALDH2-offered cardioprotection was demonstrated by the ALDH2 agonist Alda-1, testifying to the therapeutic value of this enzyme. Interestingly, the Alda-1-elicited beneficial effect against ethanol-induced changes in autophagy and mechanical function was mitigated by the AMPK activator AICAR. In addition, the ethanol-promoted GFP-LC3 puncta was mitigated by Alda-1 in H9c2 cells,

consolidating a beneficial role of ALDH2 against ethanol-induced autophagy. The fact that lysosomal inhibitors abrogated Alda-1-induced response in GFP-LC3 puncta following ethanol exposure suggests a likely role of autophagy flux stimulation (which should decrease the LC3B II levels) in ALDH2-offered cardioprotection. These observations, along with the finding that AMPK inhibition using compound C obliterated the ethanol-induced autophagy and mechanical defects in cardiomyocytes, support a pivotal role of AMPK in ethanol and/or ALDH2-induced cardiac contractile responses. Taken together, these findings revealed that ALDH2 may be protective against acute ethanol exposure-induced cardiac toxicity via regulation of autophagy flux, inhibition of AMPK-dependent autophagy and mechanical dysfunction.

Binge drinking is known to result in myocardial abnormalities and cardiac mortalities [1, 30]. Overt cardiac damage may be seen following ethanol ingestion at 90 to 100 g/d (~1.5 g/kg) in humans [1]. In this study, we used an ethanol dosage at 3 g/kg which closely resembles the state of heavy ethanol intake (given the doubled ED₅₀ in rodents). Our data revealed that acute ethanol exposure triggered cardiomyocyte contractile defects including reduced peak shortening, $\pm$ dL/dt and prolonged TR₉₀. Our echocardiographic evaluation depicted enlarged LV ESD and LV EDD, indicating initiation of geometric remodeling in our acute setting of ethanol treatment. These findings are somewhat in agreement with the previous findings of myopathic alteration following ethanol exposure featured by compromised myocardial contractility [1, 3, 31]. A number of theories have been put forward regarding acute ethanol exposure-induced cardiac toxicity including lipid peroxidation, oxidative damage [2], mitochondrial dysfunction [3], and altered membrane properties [32]. To expand on this list, intracellular Ca²⁺ homeostasis was interrupted in response to acute ethanol challenge manifested as depressed intracellular Ca²⁺ rise in response to electrical stimulus (Δ FFI), dampened ⁴⁵Ca²⁺ uptake and reduced intracellular Ca²⁺ decay, suggesting an essential role of intracellular Ca²⁺ dysregulation in acute ethanol toxicity in the heart. Although our earlier studies demonstrated that overexpression of ALDH2 alleviates alcohol and acetaldehyde-induced cell injury both *in vivo* and *in vitro* [3, 4, 7, 9], little is known in regard to the precise mechanism involved in ALDH2-offered protection against acute ethanol toxicity. Data from our present observation revealed that ALDH2 counteracts cardiac toxicity of ethanol (cardiac function, cardiac geometry and intracellular Ca²⁺ handling) possibly associated with the regulation of autophagy in particular autophagic flux. This is supported by the findings from the pharmacological regulators of autophagy, lysosomal autophagic flux and the ALDH2 agonist Alda-1. In particular, the autophagy inhibitor 3-MA and the AMPK inhibitor compound C (which should suppress autophagy [33]) mitigated whereas the autophagy inducer rapamycin exacerbated ethanol-induced cardiomyocyte toxicity. Moreover, lysosomal inhibition did not alter the ethanol-induced GFP-LC3 puncta formation, indicating a role of inhibition of autophagy flux (fusion and degradation of autophagolysosomes) in ethanol-induced autophagic response. Static levels of LC3B II may render possible misinterpretation for autophagy as LC3B II levels may increase, decrease or remain unchanged in the setting of autophagic induction [34]. Therefore it is pertinent to measure LC3B II levels in the presence and absence of lysosomal inhibitors to block the degradation of LC3B II through autophagic flux [14, 34]. Moreover, our data depicted that Alda-1 reversed ethanol-induced autophagy, in line with its beneficial effect against ethanol-induced cardiac mechanical defect. Interestingly, the mixed lysosomal inhibitors reversed the Alda-1-offered suppression of GFP-LC3 puncta following ethanol exposure. These findings suggest that Alda-1 (or ALDH2) may offset ethanol-induced autophagy through, at least in part, stimulation of the late-stage autophagic flux mechanism.

Although our current data favor the notion that ALDH2 stimulates autophagic flux (to reduce the LC3B II levels), the precise mechanism of action behind the mitochondrial

enzyme-offered reversal against ethanol-induced autophagy is still elusive. It is plausible to speculate that ALDH2 inhibits ethanol-induced autophagy via inhibition and activation of, respectively, AMPK and mTOR. This is supported by our findings that ALDH2 and Alda-1 nullified ethanol-induced AMPK activation and mTOR inhibition. Our data further depicted that the ethanol-induced Akt dephosphorylation may be reversed by ALDH2 and its agonist Alda-1, suggesting a role of Akt in autophagy regulation. Akt and AMPK are essential regulators for cardiac autophagy, survival, energy metabolism and contractile function [35-37]. Our earlier study indicated that ethanol-induced cardiomyocyte contractile dysfunction is associated with reduced Akt activity and an enhanced AMPK activation [20, 37]. Consistently, data from our current study also revealed depressed Akt phosphorylation associated with enhanced AMPK phosphorylation in conjunction with cardiac dysfunction and autophagy following acute ethanol challenge. Akt is known to turn on mTOR leading to suppression of autophagy [12]. On the other hand, AMPK is an essential regulator for myocardial survival, contractile function and autophagy either independently or indirectly through Akt or mTOR [35, 36]. Therefore, mTOR may serve as a converging point for ALDH2 and Alda-1-mediated autophagic regulation through the Akt and AMPK signaling. Our results demonstrate that ethanol-induced AMPK activation with suppressed phosphorylation of Akt and mTOR is reversed by ALDH2 overexpression and Alda-1. These findings favor a role of the Akt and AMPK signaling interplay in the regulation of ALDH2-elicited autophagic responses in alcoholism. Likewise, *in vitro* ethanol exposure-induced autophagy (LC3BII or LC3B II-to-LC3B I ratio) and cardiomyocyte contractile dysfunction were ablated or attenuated by Alda-1 although such effects were ablated by the AMPK activator AICAR. This is consistent with the finding that AMPK inhibition mitigated ethanol-induced autophagic and mechanical changes in cardiomyocytes. These observations favor the notion that ALDH2 may inhibit phosphorylation of AMPK *en route* to de-inhibition of mTOR signaling, thus favoring suppression of ethanol-induced autophagy. Our data revealed that ALDH2 may rescue ethanol-induced loss of mTOR phosphorylation through its upstream signaling molecule Akt, resulting in an Akt-dependent inhibition of autophagy such as via its downstream FoxO transcription factors [38]. Evidence from our lab revealed that ALDH2 significantly attenuated ethanol-induced decrease in Akt-stimulated phosphorylation of Foxo3 [3], favoring inhibition of autophagy [38]. Such dual regulatory machinery involving AMPK and Akt may underscore the homeostatic machinery for ALDH2 and Alda-1-elicited cardioprotection against ethanol toxicity. Our recent report has demonstrated a somewhat similar dual regulatory mechanism for AMPK and Akt in ALDH2-elicited cardioprotection against ischemia-reperfusion injury [12, 13]. This is consistent with the finding that elevation of Alda-1 activity may protect against ischemic heart and arrhythmic damage [10, 11]. Nonetheless, given the notion that AMPK may regulate mTOR and autophagy via an Akt-dependent mechanism [17, 18], further scrutiny is warranted to better illustrate the role of Akt in the ethanol-initiated, AMPK-dependent regulation of autophagy and subsequently, changes in myocardial geometry and function. In addition, given that ethanol-induced increase in the LC3B II-to-LC3B I ratio may be resulted from potential lysosomal damage following ethanol exposure, future work on lysosomal enzymes and autophagic flux is in need for ethanol- and/or ALDH2-induced regulation of autophagy.

In summary, findings from our study have provided rather convincing evidence that ALDH2 rescues acute ethanol exposure-induced cardiac mechanical defect, intracellular Ca^{2+} dysregulation and apoptosis through regulation of autophagy and autophagy flux, leading to preserved phosphorylation of Akt and mTOR as well as suppressed activation of AMPK. Further study is warranted to unveil the impact of ALDH2 on autophagy in the more clinically relevant condition of chronic alcohol ingestion-induced onset and progression of alcoholic cardiomyopathy.

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Research highlights

- In this study we examined the protective mechanism of ALDH2 after alcohol intake;
- ALDH2 was found to rescue alcohol-induced cardiac dysfunction and remodeling;
- The beneficial effect of ALDH2 was mediated through Akt and AMPK signaling;
- ALDH2 exerted its protection through regulation of autophagic flux;

Fig. 1. Effect of acute ethanol exposure on cytosolic and mitochondrial ALDH2 expression in FVB and ALDH2 mouse hearts. Inset: Representative gel blots of cytosolic and mitochondrial ALDH2 expression (GAPDH as loading control) using specific antibodies. Mean $\pm$ SEM, $n = 4 - 6$ mice per group, * $p < 0.05$ vs. FVB group.

Fig. 2. Effect of acute ethanol exposure on echocardiographic indices in FVB and ALDH2 mice. (A) Representative echocardiographic images; (B) Body weight; (C) heart rate; (D) LV EDD; (E) LV ESD; (F) LV wall Thickness; and (G) Fractional shortening. Mean $\pm$ SEM, $n = 6 - 11$ mice per group, * $p < 0.05$ vs. FVB group, # $p < 0.05$ vs. FVB-Ethanol group.

Fig. 3. Effect of acute ethanol exposure on cell shortening in cardiomyocytes from FVB and ALDH2 mice. (A) resting cell length; (B) peak cell shortening (normalized to cell length); (C) maximal velocity of shortening (+ dL/dt); (D) maximal velocity of relengthening (-dL/dt); (E) time-to-peak shortening (TPS); and (F) time-to-90% relengthening (TR₉₀). Mean $\pm$ SEM, n = 54-58 cells from 3 mice per group, *p < 0.05 vs. FVB group, # p < 0.05 vs. FVB-Ethanol group.

Fig. 4.

Effect of acute ethanol exposure on intracellular Ca²⁺ handling in cardiomyocytes from FVB and ALDH2 mice. (A) baseline fura-2 fluorescence intensity (FFI); (B) rise in FFI (ΔFFI) in response to electrical stimuli; (C) intracellular Ca²⁺ decay rate; and (D) intracellular Ca²⁺ uptake rate measured by ⁴⁵Ca²⁺ uptake. Mean ± SEM, n = 41-42 cells (n = 11 – 13 for panel D) from 3 mice per group, *p < 0.05 vs. FVB group, # p < 0.05 vs. FVB-Ethanol group.

Fig. 5.

Effect of ALDH2 overexpression on acute ethanol exposure-induced change in autophagy markers in myocardium from FVB and ALDH2 transgenic mice and impact of the autophagy inhibitor 3-MA on acute ethanol exposure-induced cardiomyocyte contractile defects. Panels A-F depict acute ethanol exposure-induced change in the autophagy markers in myocardium from FVB and ALDH2 transgenic mice. (A) Representative gel bolts depicting protein expression of autophagic proteins using specific antibodies, GAPDH was used as the loading control for normalization and a second loading control β -actin was used since GAPDH serves as a substrate for autophagy; (B) Beclin expression; (C) Atg 7 expression; (D) LC3B II expression; (E) LC3B I expression; and (F) LC3B II-to-LC3B I ratio. Mean $\pm$ SEM, $n = 3-4$ mice per group, * $p < 0.05$ vs. FVB group, # $p < 0.05$ vs. FVB-Ethanol group. Panels G-L depict cardiomyocyte mechanical properties in freshly isolated cardiomyocytes from normal FVB mice incubated with ethanol (240 mg/dL) in the presence or absence of 3-MA (10 mM) for 3-4 hrs. (G) Resting cell length; (H) Peak shortening (normalized to cell length); (I) Maximal velocity of shortening (+ dL/dt); (J) Maximal velocity of relengthening (- dL/dt); (K) Time-to-peak shortening (TPS); and (L) Time-

to-90% relengthening (TR₉₀). Mean $\pm$ SEM, n = 46 - 53 cells from 3 mice per group, * p < 0.05 vs. Control group, # p < 0.05 vs. Ethanol group.

Fig. 6. Effect of ALDH2 overexpression (*in vivo*) and the ALDH2 agonist Alda-1 (*in vitro*) on ethanol exposure-induced phosphorylation of mTOR in myocardium and effect of the autophagy inducer rapamycin and Alda-1 on acute ethanol exposure-induced cardiomyocyte apoptosis and contractile defects. (A) pmTOR expression; (B) mTOR expression; and (C) pmTOR-to-mTOR ratio. A cohort of cardiomyocytes from FVB mice were challenge with ethanol (240 mg/dl) for 4 hrs in the absence or presence of Alda-1 (20 μ M). Mean $\pm$ SEM, $n = 3-5$ mice or isolations per group, * $p < 0.05$ vs. FVB group, # $p < 0.05$ vs. FVB-Ethanol group. Panels D-I depict apoptosis and mechanical function in cardiomyocytes from FVB mice exposed to ethanol (240 mg/dL) in the absence or presence rapamycin (5 μ M) or Alda-1 (20 μ M) for 3-4 hrs. (D) Caspase-3 activity; (E) Peak shortening (normalized to cell length); (F) Maximal velocity of shortening (+ dL/dt); (G) Maximal velocity of relengthening (- dL/dt); (H) Time-to-peak shortening (TPS); and (I) Time-to-90% relengthening (TR₉₀). Mean $\pm$ SEM, $n = 4$ (panel D) or 44 - 58 cells from 3 mice (panel E-I), * $p < 0.05$ vs. Control group, # $p < 0.05$ vs. Ethanol group.

Fig. 7. Effect of acute ethanol exposure on expression of pan and phosphorylated Akt and AMPK in myocardium from FVB and ALDH2 transgenic mice. A cohort of cardiomyocytes from FVB mice were challenge with ethanol (240 mg/dl) *in vitro* for 3-4 hrs in the absence or presence of Alda-1 (20 μ M). (A) AMPK expression; (B) Akt expression; (C) pAMPK expression; (D) pAkt expression; (E) pAMPK-to-AMPK ratio; and (F) pAkt-to-Akt ratio. Insets: Representative gel blots of pan and phosphorylated Akt and AMPK (GAPDH as loading control) using specific antibodies. Mean $\pm$ SEM, n = 3-5 mice or isolations per group, * p < 0.05 vs. FVB group, # p < 0.05 vs. FVB-Ethanol group.

Fig. 8.

Effect of the AMPK activator AICAR, the ALDH2 agonist Alda-1 and the AMPK inhibitor compound C on ethanol-induced autophagy and cardiomyocyte contractile defects. Freshly isolated cardiomyocytes from normal FVB mice were incubated with AICAR (500 μM), Alda-1 (20 μM), compound C (10 μM) or both AICA and Alda-1 in the presence or absence of ethanol (240 mg/dL) for 4-5 hrs. (A) LC3B II expression; (B) LC3B I expression; (C) LC3B II-to-LC3B I ratio; (D) Resting cell length; (E) Peak shortening (normalized to resting cell length); (F) Maximal velocity of shortening (+ dL/dt); (G) Maximal velocity of relengthening (- dL/dt); (H) Time-to-peak shortening (TPS); and (I) Time-to-90% relengthening (TR₉₀). Mean $\pm$ SEM, $n = 4-5$ mice per group (Panels A-C) or 51-58 cells from 4 mice (Panels D-I), * $p < 0.05$ vs. Control group, # $p < 0.05$ vs. Ethanol group, † $p < 0.05$ vs. Alda-1-Ethanol group.

Fig. 9.

Effect of inhibition of lysosomal enzyme and autophagosome formation on acute ethanol exposure and Alda-1-induced autophagy in H9c2 myoblasts. H9c2 cells were transfected with adenovirus for 24 hrs to express the GFP-LC3 fusion protein. Cells were then exposed to ethanol (240 mg/dl) for 4 hrs in the absence or presence of the ALDH2 agonist Alda-1 (20 μ M), the autophagosome formation inhibitor 3-MA (10 mM) or the mixture of the cell-permeable lysosomal inhibitors bafilomycin A1 (50 nM), E64D (2.5 μ g/ml) and pepstatin A methyl ester (5 μ g/ml). The autophagy inducer rapamycin (5 μ M) was used as the positive control. DAPI staining was used for identification of nucleus. A-J: Representative images (GFP, DAPI and Merged) depicting GFP-LC3 puncta in H9c2 cells following ethanol exposure with or without Alda-1, 3-MA or lysosomal inhibitors; K: Percentage of cells with autophagosomes. Cells with 10 or more punctate spots were scored as positive for autophagosomes. Mean $\pm$ SEM, n = 300 - 400 cells per group from 3 independent experiments, * $p < 0.05$ vs. control group, # $p < 0.05$ vs. Ethanol group, † $p < 0.05$ vs. Alda-1-ethanol group.