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Cardioprotection induced by a brief exposure to acetaldehyde: role of aldehyde dehydrogenase 2

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Aims We previously demonstrated that acute ethanol administration protects the heart from ischaemia/reperfusion (I/R) injury thorough activation of aldehyde dehydrogenase 2 (ALDH2). Here, we characterized the role of acetaldehyde, an intermediate product from ethanol metabolism, and its metabolizing enzyme, ALDH2, in an *ex vivo* model of cardiac I/R injury.

Methods and results We used a combination of homozygous knock-in mice (ALDH2*2), carrying the human inactivating point mutation ALDH2 (E487K), and a direct activator of ALDH2, Alda-1, to investigate the cardiac effect of acetaldehyde. The ALDH2*2 mice have impaired acetaldehyde clearance, recapitulating the human phenotype. Yet, we found a similar infarct size in wild type (WT) and ALDH2*2 mice. Similar to ethanol-induced preconditioning, pre-treatment with 50 μM acetaldehyde increased ALDH2 activity and reduced cardiac injury in hearts of WT mice without affecting cardiac acetaldehyde levels. However, acetaldehyde pre-treatment of hearts of ALDH2*2 mice resulted in a three-fold increase in cardiac acetaldehyde levels and exacerbated I/R injury. Therefore, exogenous acetaldehyde appears to have a bimodal effect in I/R, depending on the ALDH2 genotype. Further supporting an ALDH2 role in cardiac preconditioning, pharmacological ALDH2 inhibition abolished ethanol-induced cardioprotection in hearts of WT mice, whereas a selective activator, Alda-1, protected ALDH2*2 against ethanol-induced cardiotoxicity. Finally, either genetic or pharmacological inhibition of ALDH2 mitigated ischaemic preconditioning.

Conclusion Taken together, our findings suggest that low levels of acetaldehyde are cardioprotective whereas high levels are damaging in an *ex vivo* model of I/R injury and that ALDH2 is a major, but not the only, regulator of cardiac acetaldehyde levels and protection from I/R.

Keywords Ischaemia-reperfusion injury • Alcohol consumption • Mitochondria • Heart attack • Cardiovascular disease

1. Introduction

Humans are exposed to acetaldehyde generated from a wide variety of sources, including endogenous metabolism, food, beverages, and environment. Acetaldehyde is highly reactivity, has short half-life, diffuses readily throughout the body and forms either unstable (Schiff base) or stable (N-ethyllysine residues) adducts with proteins.[1,2] It is therefore likely that acetaldehyde regulates both physiological and pathological processes according to its concentration and location.

Excessive ethanol consumption and the likely increase in acetaldehyde levels are associated with increased risk for alcoholic cardiomyopathy.[3] Furthermore, even moderate alcohol consumption correlated with subclinical adverse effects on cardiac systolic functions in subjects carrying variants of alcohol metabolizing genes that result in higher acetaldehyde levels.[4] However, neither the direct effects of acetaldehyde in cardiac ischaemia/reperfusion (I/R) injury nor its involvement in ethanol-induced cardioprotection have been examined.

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Aldehyde dehydrogenase 2 (ALDH2) is a mitochondrial enzyme, which is highly expressed in the heart. It plays a major role in the detoxification of acetaldehyde produced by alcohol dehydrogenase during ethanol metabolism.[5] In addition, ALDH2 metabolizes other aldehydes generated during oxidative stress, such as the lipid peroxidation product, 4-hydroxynonenal.[6,7] Thus, ALDH2-mediated detoxification of reactive aldehydes in general and acetaldehyde in particular, has been suggested to be a mechanism that increases tissue viability upon stress.[8–11]

Indeed, individuals carrying a point mutation in the ALDH2 gene (ALDH2*2) are more susceptible to cardiovascular disease.[4,12] This variant, found in nearly 35–50% of East Asians,[13] results in a reduction in the ALDH2 enzymatic activity by ~70 and 98% in heterozygotes and homozygotes, respectively[14]; therefore maximizing the unpleasant effect of acetaldehyde accumulation after alcohol intake, including flushing, cardiac palpitation, headache and nausea.[15]

Here, we set out to determine the role of the ALDH2 substrate, acetaldehyde, in an *ex vivo* model of cardiac I/R injury. It is known that acetaldehyde, the main product of ethanol metabolism, is a reactive molecule that can modify proteins through adduction.[16,17] This protein modification impair its activity and/or stability and therefore, likely affect cardiac physiology and pathology.[18,19] To determine the effect acetaldehyde in the heart, we compared response to ischaemic insult of hearts from wildtype (WT) mice and from homozygous ALDH2*2 mice, carrying the inactivating E487K point mutation in ALDH2,[20] identical to the mutation found in nearly 540 million East Asians.[13]

2. Methods

2.1 Animals

This study was conducted in accordance with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation (www.cobea.org.br) and conform the NIH guidelines (Guide for the care and use of laboratory animals). The animal care and protocols in this study were reviewed and approved by the Ethical Committee of the Institute of Biomedical Sciences at University of São Paulo (2014/25). A cohort of 4-month-old male WT C57BL/6 J and homozygous ALDH2*2 mice was selected for the study. Knock-in mouse (ALDH2*2) carrying the human inactivating point mutation ALDH2 (E487K) with a C57BL/6 J background were generated by homologous recombination as previously described in[20] ALDH2*2 mice are an ideal representation of the human ALDH2*2 carriers and can serve as an experimental model to reflect the true nature of the genetic defect, the ALDH2 deficiency.

2.2 I/R injury

An *ex vivo* model of MI was used as described elsewhere.[21] Briefly, animals were heparinized (2000 U/kg IP) and then anaesthetized with Ketamine (100 mg/kg IP) and Xylazine (10 mg/kg IP). The hearts were rapidly excised, cannulated *via* the aorta and perfused on a Langendorff apparatus with oxygenated Krebs-Henseleit buffer (118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM dextrose, and 1.8 mM $CaCl_2$, pH 7.4). Hearts were perfused at a constant flow rate of 2.5 mL/min at 37°C. After 10 min of stabilization, I/R was induced by stopping the flow and submerging the heart in Krebs-Henseleit buffer at 37°C for 35 min (global, no-flow ischaemia) followed by 60 min of reperfusion. Ethanol treatment: 50 mM Ethanol was applied following 10 min equilibration for 15 min, followed by 5 min washout, prior to ischaemia as previously described in.[22] ALDH2 inhibitor treatment: 20 μM CVT-10216 [a selective ALDH2 inhibitor,[23] Acme Bioscience, Palo Alto, CA] was applied for 10 min prior to ischaemia and for the first 10 min of reperfusion. Ethanol and CVT-10216 treatment: 20 μM CVT-10216 was applied for 10 min, followed by 15 min of 50 mM ethanol, followed by 5 min washout, prior to ischaemia, and 20 μM CVT-10216 was applied for the first 10 min of reperfusion. Ethanol and ALDH2 selective activator [Alda-1[11]] treatment: 50 mM ethanol was applied following 10 min equilibration for 15 min, followed by 10 min of 20 μM Alda-1, followed by 5 min washout, prior to ischaemia, and 20 μM Alda-1 was applied for the first 10 min of reperfusion. Acetaldehyde treatment: 50 μM acetaldehyde was applied following 10 min equilibration for 15 min, followed by 5 min washout, prior to ischaemia. Ischaemic preconditioning (IPC): three bouts of 5 min of ischaemia and 5 min of reperfusion were applied prior to a sustained period of ischaemia in the presence or absence of 20 μM CVT-10216.

2.3 Tissue sampling

At the end of the reperfusion period, fresh isolated hearts were sliced into 1-mm thick transverse sections and incubated with triphenyltetrazolium chloride solution (TTC, 1% in phosphate buffer, pH 7.4) at 37°C for 8 min in the dark, then fixed in 4% formalin. TTC-stained sections were scanned and infarct size assessed by measured the % infarct for each slide.

Another set of hearts was prepared to isolate mitochondrial-enriched fraction as described elsewhere.[24] Briefly, fresh isolated whole hearts from various treatment protocols were minced and homogenized in isolation buffer (300 mM sucrose, 10 mM HEPES, 2 mM EGTA, pH 7.2, 4°C) containing 0.1 mg/mL of Type I protease to release mitochondria from within muscle fibres and later washed in the same buffer in the presence of 1 mg/mL bovine serum albumin. The suspension was homogenized in a 4 mL tissue grinder and centrifuged at 950*g* for 5 min. The resulting supernatant was centrifuged at 9500*g* for 10 min. The mitochondrial pellet was washed, resuspended in isolation buffer and submitted to a new centrifugation (9500*g* for 10 min). The mitochondrial pellet was washed and the final pellet was resuspended in a minimal volume of isolation buffer. ALDH2 activity was measured in fresh isolated mitochondria.

2.4 LDH activity assay

Coronary effluent was collected every 5 min during the first 15 min of reperfusion to determine lactate dehydrogenase (LDH) release. For each assay, the effluent was combined with Sodium pyruvate (30 mM), NADH (6.6 mM), and Tris-HCl (0.2 M, pH 7.3), in a final volume of 200 μL. LDH activity was determined by a decrease in absorbance at 340 nm for 10 min at 25°C, resulting from the oxidation of NADH. We measured the cumulated LDH release over 15 min of reperfusion.

2.5 ALDH2 enzymatic activity assay

Enzymatic activity of mitochondrial ALDH2 was determined spectrophotometrically by monitoring the reductive reaction of NAD^+ to NADH at 340 nm as previously described in.[11,25] ALDH2 assays were carried out at 25°C in 50 mM sodium pyrophosphate buffer, pH 9.5. To this volume, 300 μM acetaldehyde and 160 μg of mitochondrial protein lysate isolated from fresh hearts were added. To start the reaction, 2.5 mM NAD was added and the accumulation of NADH was monitored for 10 min with measurements being taken every 30 s.

2.6 Acetaldehyde quantification

Isolated whole hearts were homogenized in lysis buffer (210 mM Mannitol, 70 mM Sucrose, 5 mM MOPS, 1 mM EDTA, and 1% Triton); 0.2% butylated hydroxytoluene (in Ethanol) was added and samples were incubated on ice for 1 h and centrifuged at 3500 rpm for 10 min. Ethanol was added to the supernatant and incubated on ice for 40 min. Next, samples were centrifuged at 13 500 rpm at 4°C for 10 min and a mix with 2, 4-dinitrophenylhydrazine [DNPH—20 mM in 1.0 M HCl (in Ethanol)] and HNE-$_{d11}$-DNPH internal standard (0.2 nmol/μL) was added to the supernatant. Samples were then incubated for 2 h at 37°C under agitation (500 rpm) and extracted with a mixture of dichloromethane (500 μL) and miliQ water (300 μL). The organic phase was collected and the solvent evaporated at room temperature under stirring. The residue was solubilized in 500 μL acetonitrile and 20 uL were injected into the HPLC-ESI-MS/MS system.

Analyses in the negative mode were carried out on an API 4000 QTRAP mass spectrometer (AB Sciex). An Agilent HPLC system consisting of autosampler (Agilent 1200 High Performance), automated switching valve, pumps (Agilent 1200 Binary pump SL and 1200 Isocratic pump SL) and a detector (Agilent 1200 DAD G1315C) were controlled by the Analyst 1.4.2 software. The analytical column Luna C18 (250 × 4.6 mm i.d., 5 μm; Phenomenex) was kept at 35°C in an Agilent 1200 G1216B column oven. The gradient used for analyte elution started with 30% acetonitrile with 0.1 mM ammonium acetate and 70% 0.1 mM ammonium acetate in water, and increased to 100% acetonitrile with 0.1 mM ammonium acetate in 25 min. The acetaldehyde derivative was detected by selected reaction monitoring. Signals from ions at m/z values of 222.9/158.0 and 222.9/162.8 for acetaldehyde-DNPH transitions; and 346.1/166.7 and 346.1/162.8 for HNE-d_{11}-DNPH were monitored for quantification and structural confirmation. Briefly, all samples containing internal standard (0.1 nmol) were injected into the HPLC/ESI/MSMS system and the transitions were monitored with a dwell time of 100 ms. For each compound, the declustering voltage, entrance potential, collision energy, and cell exit were optimized. All other parameters of the mass spectrometer were adjusted for acquisition of the best transitions for each aldehyde derivative. The Turbo Ion Spray voltage was kept at -3500 V, the curtain gas at 10 psi and the nebulizer and auxiliary gas at 70 psi. The temperature was set to 650°C, and the collision cell nitrogen pressure was adjusted to medium.

2.7 Cell culture

H9c2 cells were cultured in Dulbecco's modified Eagle medium, supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin at 37°C in 5% CO_2 in 95% air. H9c2 cells were differentiated towards a cardiac phenotype by culturing them in low serum medium (1% FBS) in the presence of retinoic acid for 7 days. Simulation

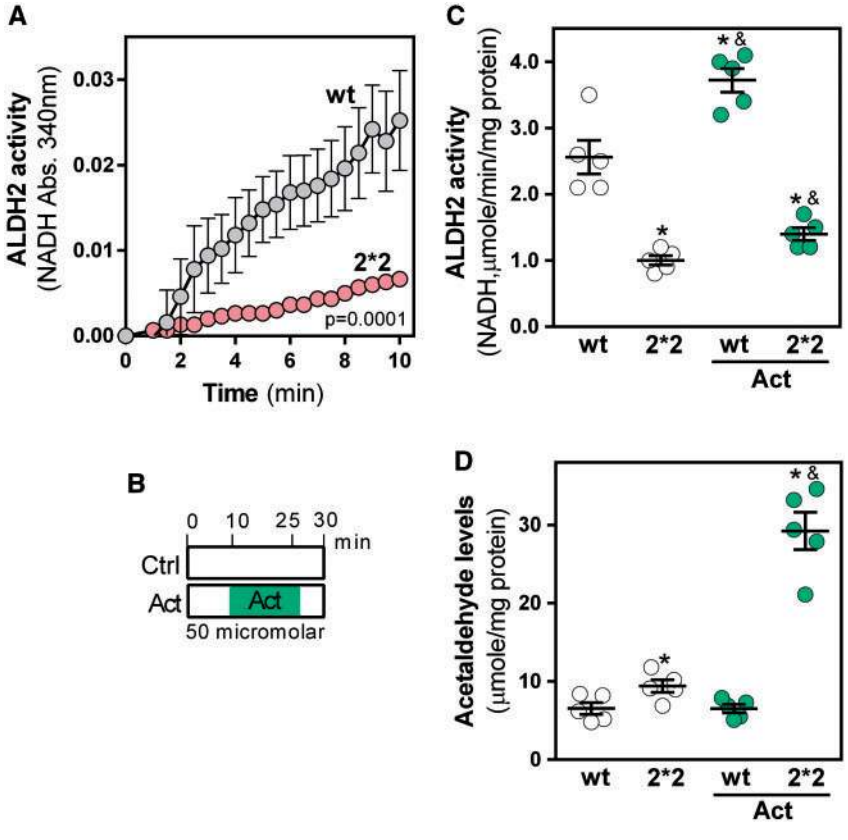

Figure 1 ALDH2 activity and acetaldehyde levels in hearts carrying ALDH2*2 variant. Cardiac ALDH2 catalytic activity (*A*, baseline) in WT (grey circles) and ALDH2*2 (2*2, red circles) mice. (*B*) Experimental protocol: After 10 min of pre-equilibration, acetaldehyde (50 μM) was applied for a period of 15 min, followed by 5 min washout in hearts isolated from WT and ALDH2*2 mice. (*C*) Cardiac ALDH2 activity and (*D*) acetaldehyde levels in WT and 2*2 *ex vivo* hearts with and without acetaldehyde pre-treatment. Data are expressed as mean ± S.E.M. Individual data are presented as open circles (n = 5 per condition). *P < 0.05 vs. WT; &P < 0.05 vs. WT Act.

of I/R injury was performed using hypoxic pouches as previously described in.[26] Briefly, after differentiation cells were exposed to 6 h of hypoxia followed by 2 h of reoxygenation. Protein kinase C (PKC) epsilon translocation to mitochondria was inhibited by the isozyme-specific translocation inhibitor, ϵV1-2 peptide,[27] at final concentration of 1 μM in cultured cells throughout I/R-induced injury.

2.8 Statistical analysis

Data are presented as mean +S.E.M. Data normality was assessed through Shapiro–Wilk's test. Two-way repeated measures analysis of variance (ANOVA) was used to analyse data presented in *Figure 1A*. Two-way ANOVA was used to analyse data presented in *Figures 1B* and *C*, *2–5*. Whenever significant *F*-values were obtained, Duncan's adjustment was used for multiple comparison purposes. Linear regression was used to assess the association between infarct size and acetaldehyde levels in *Figure 6A*. GraphPad Prism Statistics was used for the analysis, and statistical significance was considered achieved when the value of *P* was < 0.05.

3. Results

To confirm that the mutant ALDH2*2 mice mimic the human phenotype,[14] we measured the ALDH2 activity. Similar to human carrying the ALDH2*2 variant,[14] cardiac ALDH2 catalytic activity was significantly lower in ALDH2*2 mice as compared with WT controls (*Figure 1A*), reflecting the loss of catalytic function of the mutant enzyme. We therefore measured cardiac acetaldehyde levels [the main ALDH2 substrate] in WT and ALDH2*2 mice. Under baseline, cardiac acetaldehyde levels in WT mice were ∼6 μM and in ALDH2*2 ∼11 μM (see Supplementary material online, *Figure S1A*).

Next, we isolated and perfused hearts from WT and ALDH2*2 mice. After 10 min of equilibration, WT and ALDH2*2 isolated hearts were

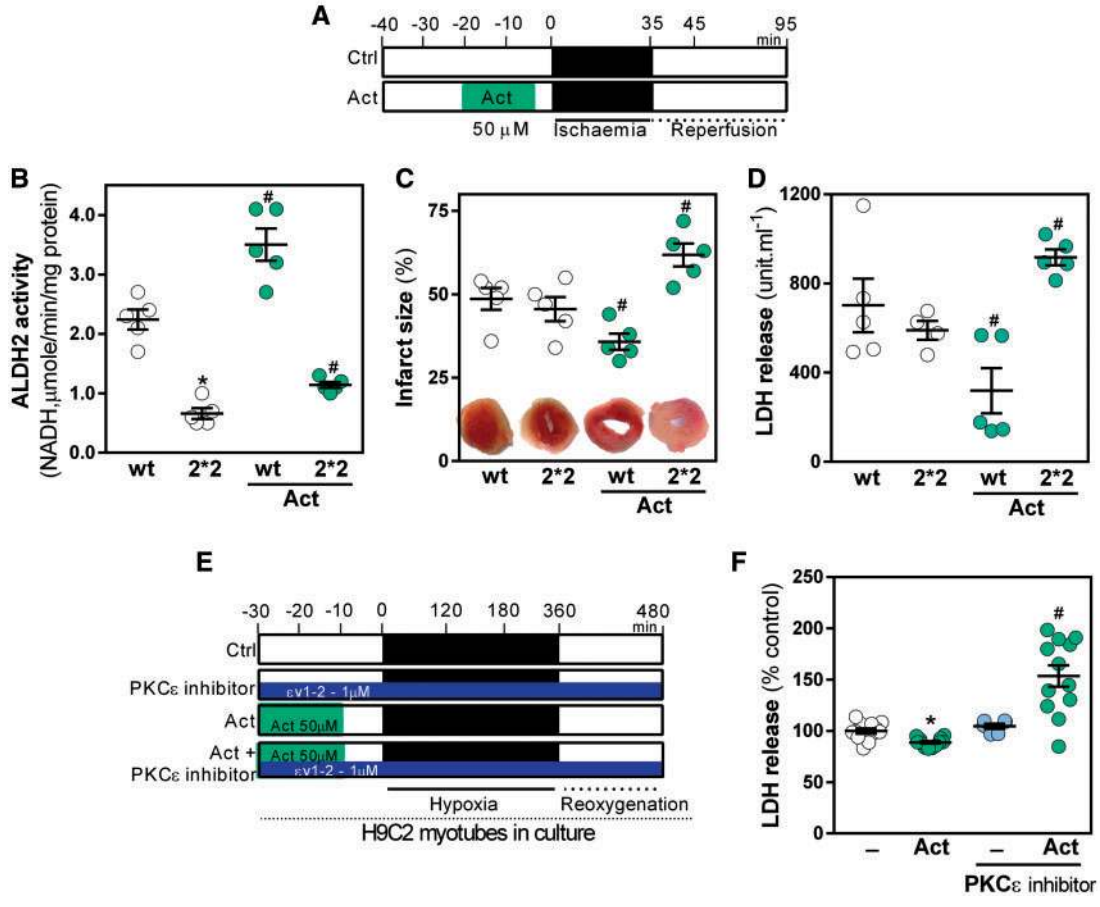

Figure 2 *Ex vivo* acetaldehyde preconditioning is lost in hearts carrying ALDH2*2 variant. (*A*) Experimental protocols used for ischaemia-reperfusion injury and acetaldehyde preconditioning in *ex vivo* hearts. Briefly, hearts were exposed to 35 min global ischaemia followed by 60 min of reperfusion. Acetaldehyde (Act-50 μM, green) was applied for a period of 15 min, followed by 5 min washout, prior to ischaemia. (*B*) Cardiac ALDH2 activity, (*C*) infarct size (representative images and quantification), and (*D*) LDH release in WT and ALDH2*2 (2*2) hearts exposed to protocols described in panel (*A*). Data are expressed as mean ± S.E.M. Individual data are presented as open circles (n = 5 per condition). *P < 0.05 vs. WT; #p < 0.05 vs. WT and 2*2. (*E*) Experimental protocols used for hypoxia-reoxygenation injury, acetaldehyde preconditioning and ϵV1-2 treatment (a selective inhibitor of ϵPKC,[22] ↓PKCϵ) in H9c2 cells. Briefly, cells were exposed to 6 h of hypoxia followed by 2 h of reoxygenation. Act (50 μM, green) was applied for a period of 20 min, followed by 10 min washout, prior to hypoxia. ϵV1-2 (1 μM, blue) was applied for a period of 30 min prior to hypoxia, until the end of the protocol. (*F*) LDH release in H9c2 cells exposed to protocols described in panel (*E*). Data are expressed as mean ± S.E.M. Individual data are presented as open circles (n = 6–12, 1 replicate). *P < 0.05 vs. Ctrl; #P < 0.05 vs. other groups.

treated with acetaldehyde (50 μM) for a period of 15 min, followed by 5 min washout (*Figure 1B*). This acetaldehyde concentration is similar to the blood acetaldehyde levels found in individuals carrying the inactive ALDH2*2 variant after consuming a moderate dose of alcohol.[28] Acetaldehyde treatment increased ALDH2 activity by about 46% in hearts of WT mice (*Figure 1C*). This increase was sufficient to maintain cardiac acetaldehyde levels close to the baseline (seen in WT hearts without treatment, *Figure 1D*). In contrast, hearts of mice carrying ALDH2*2 treated with the same amounts of acetaldehyde, exhibited only a mild activation of ALDH2, and more than two-fold increase in acetaldehyde levels (*Figure 1C* and *D*).

We previously reported that inflammation and increased oxidative stress correlate with increased acetaldehyde levels.[20] However, whether acetaldehyde levels increase also in the heart following ischaemia and reperfusion-induced oxidative stress has not been determined. Thirty minutes ischaemia followed by an hour of reperfusion brought up the levels of acetaldehyde in WT hearts by 78%, similar to the levels of ALDH2*2 hearts under normoxic conditions (see Supplementary material online, *Figure S1A*). However, unexpectedly, acetaldehyde levels in hearts of ALDH2*2 mice did not increase by I/R relative to basal levels (see Supplementary material online, *Figure S1A*). Correlated with acetaldehyde levels, hearts from WT and ALDH2 knock-in animals with reduced ALDH2 activity had the same infarct size after I/R (*Figure 2A–C*).

Next, we determined the impact of a 15-min pre-treatment with 50 μM acetaldehyde followed by a 5-min washing on cardiac I/R injury (*Figure 2A*). As observed in hearts in the absence of ischaemic protocol (*Figure 1B*), acetaldehyde pre-treatment to hearts of WT mice was sufficient to increase ALDH2 activity in the ischaemic heart by about 60% and induce cardioprotection [demonstrated by a reduction in both infarct size and LDH release] as compared with untreated controls (*Figure 2B–D*). In contrast, acetaldehyde pre-treatment to hearts of ALDH2*2 mice showed that although acetaldehyde pre-treatment caused an ~50% increase in ALDH2 activity, this was insufficient to provide protection; myocardial infarct size and LDH release significantly increased by 40% relative to untreated hearts (*Figure 2B–D*).

We next determined the molecular mechanisms involved in acetaldehyde-induced cardioprotection seen in WT. We previously demonstrated that epsilon PKC (εPKC) phosphorylation-induced activation of ALDH2 is critical to induce ethanol preconditioning.[8,11] Therefore, we evaluated whether acetaldehyde preconditioning shares the same pathway. For that, we exposed H9C2 myotubes to acetaldehyde and/or εV1-2 (a selective inhibitor of εPKC)[22] prior to hypoxia-reoxygenation (*Figure 2E*) and found that inhibition of εPKC was sufficient to abolish acetaldehyde-induced protection in H9C2 myotubes in culture; rather, injury following pre-treatment with acetaldehyde increased by 50% (*Figure 2F*). These findings indicate that protection by acetaldehyde pre-treatment is mediated by activation of εPKC in H9C2 myotubes.

Considering that acetaldehyde is an intermediate of ethanol metabolism, we next set out to determine whether acetaldehyde levels are associated with the cardiac effects of ethanol. For that, we measured cardiac acetaldehyde levels in two opposite conditions: ethanol-induced

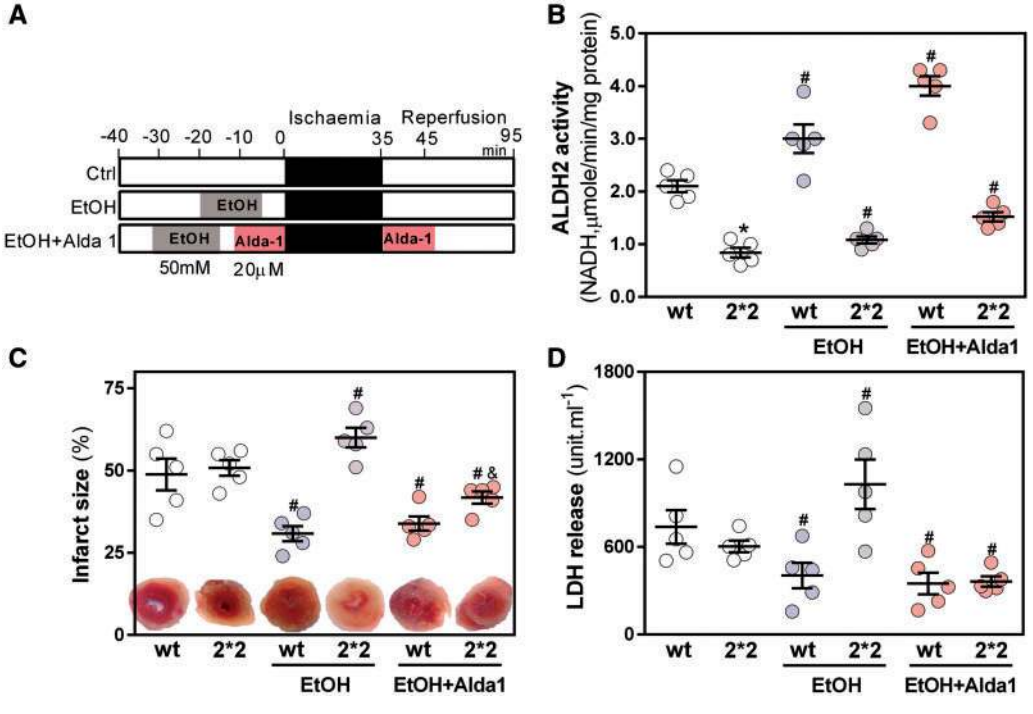

Figure 3 *Ex vivo* ethanol preconditioning is lost in hearts carrying ALDH2*2 variant. (*A*) Experimental protocols used for ischaemia-reperfusion injury, ethanol preconditioning and Alda-1 treatment in *ex vivo* hearts. Briefly, after 10 min of pre-equilibration, hearts were exposed to 35 min global ischaemia followed by 60 min of reperfusion. Ethanol (EtOH, 50 mM, grey) was applied for a period of 15 min, followed by 5 min washout, prior to ischaemia; Alda-1 (20 μM, pink) was applied for 10 min prior to ischaemia and for the first 10 min of reperfusion. Cardiac ALDH2 catalytic activity (*B*), infarct size (*C*, representative images and quantification) and LDH release (*D*) in WT and ALDH2*2 (2*2) hearts exposed to protocols described in panel (*A*). Data are expressed as mean ± S.E.M. Individual data are presented as open circles ($n = 5$ per condition). *$P < 0.05$ vs. WT; #$P < 0.05$ vs. WT and 2*2; & $P < 0.05$ vs. 2*2 EtOH.

cardioprotection and ethanol-induced cardiotoxicity. Ethanol-induced cardioprotection was associated with reduced acetaldehyde levels of about 70% in hearts of WT mice as compared with untreated I/R WT group (see Supplementary material online, *Figure S1A*). Under ethanol-induced cardiotoxicity in ALDH2*2, there was a 70% increase in cardiac acetaldehyde levels (see Supplementary material online, *Figure S1A*). Of interest, acute pharmacological ALDH2 activation using Alda-1 abolished the accumulation of acetaldehyde in hearts isolated from ALDH2*2 mice exposed to ethanol, bringing it close to WT levels (see Supplementary material online, *Figure S1A*).

Next, to determine whether the reduced acetaldehyde metabolism seen in ALDH2*2 variant affects ethanol-mediated cardioprotection, we exposed hearts isolated from WT and ALDH2*2 mice to ethanol prior to I/R injury (*Figure 3A*). Perfusion with ethanol (50 μM) for 15 min followed by 5 min washout prior to I/R increased ALDH2 activity without affecting protein levels (*Figures 3B* and see Supplementary material online, *Figure S1B*). As expected, [9] increased ALDH2 activity was associated with a ∼40% reduction in both infarct size and necrotic cell death, measured by LDH release (*Figure 3C* and *D*). This concentration of ethanol was chosen because it is similar to the blood ethanol levels found after consuming >3 units of any type of alcoholic beverages.[28]

In contrast, ethanol pre-treatment of hearts isolated from ALDH2*2 exhibited a very mild increase in ALDH2 activity as compared with ALDH2*2 I/R control group, to levels that were still only 50% of WT control hearts (*Figure 3B*). This insufficient improvement in ALDH2 activity of the hearts of ALDH2*2 mice was correlated with an ∼20% increased infarct size and higher cardiac LDH release (*Figure 3C* and *D*). These results suggest that in contrast to the benefit of ethanol as a preconditioning agent in WT hearts, ethanol pre-treatment induced further cardiac I/R injury in ALDH2*2.

Next, we determined whether the re-establishment of cardiac acetaldehyde metabolism by using an ALDH2 small molecule activator developed by our group (Alda-1, for aldehyde dehydrogenase activator[7,11]) minimizes ethanol-induced increase in cardiac injury following I/R in ALDH2*2. For that, Alda-1 (20 μM) was applied for 10 min after ethanol treatment and again, during the first 10 min of reperfusion (*Figure 3A*). Alda-1 treatment caused additional increase in ALDH2 activity in hearts isolated from both WT and ALDH2*2 mice treated with ethanol and subjected to I/R as compared with their ethanol controls, respectively (*Figure 3B*). This incremental effect of Alda-1 on ALDH2 activity correlated with a reduction in infarct size, cardiac LDH release and mitochondrial H_2O_2 release in hearts of ALDH2*2 mice subjected to I/R and treated with ethanol (*Figures 3C* and *D* and see Supplementary material online, *Figure S1C*). These findings suggest that pharmacological ALDH2 activation promotes a shift from ethanol-induced cardiotoxicity to ethanol-induced cardioprotection in hearts isolated from mice carrying

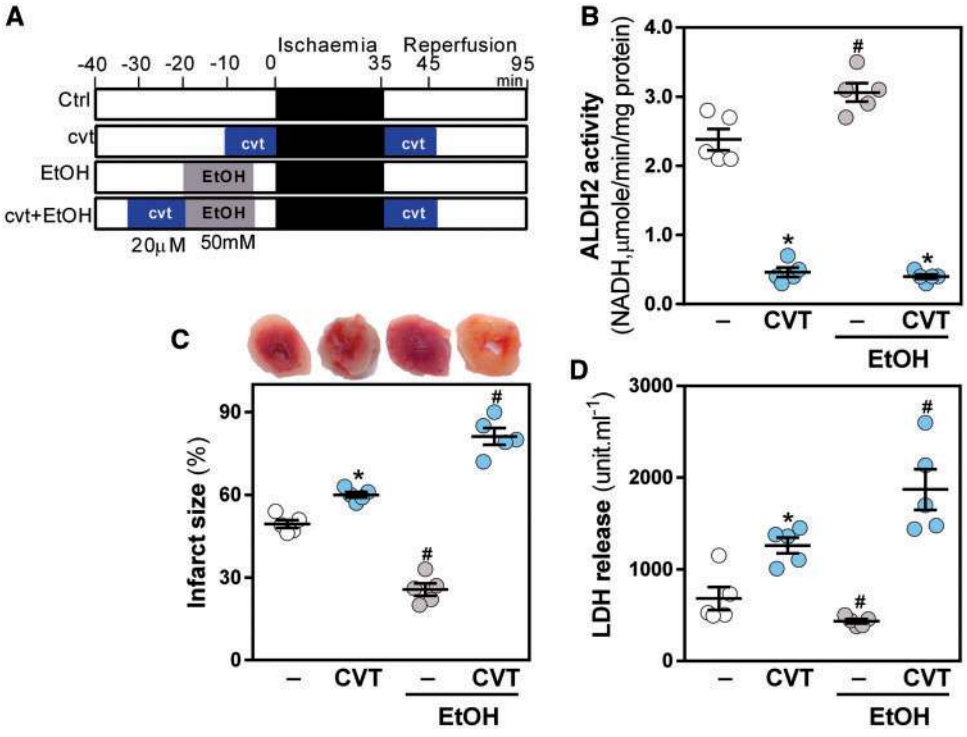

Figure 4 Pharmacological ALDH2 inhibition exacerbates myocardial infarction and abolishes ethanol preconditioning in WT hearts. (*A*) Experimental protocols used for ischaemia-reperfusion injury, ethanol preconditioning and CVT-10216 treatment in *ex vivo* hearts. Hearts were exposed to 35 min global ischaemia followed by 60 min of reperfusion. CVT-10216 (20 μM, ALDH2 inhibitor, blue) was applied for 10 min prior to ischaemia and for the first 10 min of reperfusion. Ethanol (EtOH, 50 mM, gray) was applied for a period of 15 min, followed by 5 min washout, prior ischaemia. For CVT-10216 and ethanol protocol, CVT-10216 was applied for 10 min, followed by EtOH for 15 min and 5 min washout prior to ischaemia. CVT-10216 was applied again for the first 10 min of reperfusion. (*B*) Cardiac ALDH2 activity, (*C*) infarct size (representative images and quantification) and (*D*) LDH release in WT hearts exposed to protocols described in panel (*A*). Data are expressed as mean ± SEM. Individual data are presented as open circles (n = 5 per condition). *$P < 0.05$ vs. Ctrl; #$P < 0.05$ vs. Ctrl and CVT-10216.

ALDH2*2 variant and subjected to I/R. This protective effect may be due to reduced ALDH2 inactivation by its substrate, acetaldehyde; we previously found that aldehydes interact with cysteine residues at the catalytic site of the enzyme and because Alda-1 shields these residues, substrate-induced ALDH2 inactivation is inhibited.[11]

Oxygen consumption in isolated cardiac mitochondria, mitochondrial protein levels (NDUFA9, IF1, and SOD2) and myocardial perfusion pressure were unchanged among conditions (see Supplementary material online, *Figure S1D–I*). In WT, combination treatment with Alda-1 and ethanol had an incremental effect in ALDH2 activity (*Figure 3B*), but no change in cardioprotection, when compared with ethanol-treated hearts alone (*Figure 3C* and *D*).

Considering that pharmacological ALDH2 activation protects hearts of mice carrying the ALDH2*2 variant from ethanol-induced cardiotoxicity, we sought to determine whether acute pharmacological ALDH2 inhibition, using CVT-10216, is sufficient to induce ethanol cardiotoxicity in heart of WT mice (*Figure 4A*). This strategy was also used to exclude possible compensatory mechanisms activated in the ALDH2*2 mice by other genes involved in detoxification from damaging agents.[29] Treatment of hearts isolated from WT mice with CVT-10216 (20 μM), applied 10 min prior to ischaemia and during the initial 10 min of reperfusion, resulted in 80% decrease in ALDH2 activity (*Figure 4B*) and a 2.3-fold increase in cardiac acetaldehyde levels compared with control hearts (see Supplementary material online, *Figure S1A*). This excessive accumulation of toxic acetaldehyde during acute ALDH2 inhibition was sufficient to increase infarct size and cardiac LDH release compared with I/R control group (*Figure 4C* and *D*). When applied after ethanol treatment and prior to I/R, CVT-10216 abolished ethanol-mediated increased ALDH2 activity and significantly increased infarct size by an ~three-fold and LDH release in WT (*Figure 4C* and *D*). These results suggest that acute disruption of acetaldehyde metabolism not only abolishes ethanol-induced cardioprotection, but rather exacerbates ethanol-induced cardiotoxicity, similar to what we found in hearts isolated from ALDH2*2 mice (*Figure 3*).

Since ALDH2 activation is involved in acetaldehyde- (*Figure 2*), ethanol- (*Figure 3*)[8,11] and isoflurane-mediated preconditioning,[30] we set out to determine the levels of acetaldehyde during IPC as well as to evaluate the contribution of ALDH2 to this process. Three bouts of 5 min ischaemia and 5 min reperfusion were sufficient to increase ALDH2 activity without affecting acetaldehyde levels in hearts of WT and ALDH2*2 mice (*Figure 5A–C*). Note that acetaldehyde levels maintained elevated in hearts of ALDH2*2 mice exposed to IPC compared with control (*Figure 5C*).

IPC-induced ALDH2 activation correlated with reduced infarct size, LDH release (*Figure 5E–G*) and cardiac acetaldehyde levels (see Supplementary material online, *Figure S1A*) in hearts from WT but not in hearts from ALDH2*2 mice. Finally, to test the involvement of acetaldehyde metabolism on IPC, hearts isolated from WT mice were treated

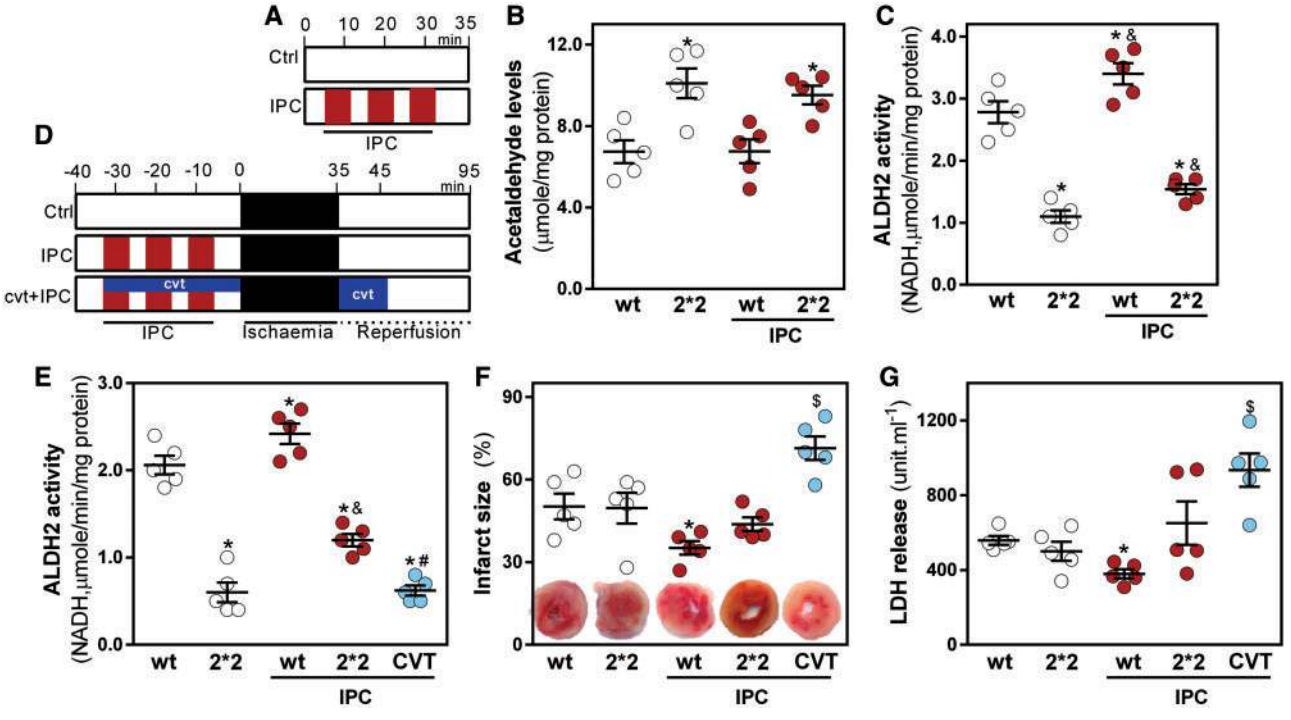

Figure 5 ALDH2*2 variant mitigates *ex vivo* IPC. (*A*) Experimental protocol used for IPC [3 bouts of 5 min ischaemia followed by 5 min reperfusion, red]. (*B*) Cardiac acetaldehyde levels, and (*C*) ALDH2 activity in WT and ALDH2*2 (2*2) hearts exposed to protocol described in panel (*A*). (*D*) Experimental protocols used for IPC followed by ischaemia-reperfusion injury in isolated hearts. Briefly, hearts were exposed to 35 min global ischaemia followed by 60 min of reperfusion. IPC was performed by 3 bouts of 5 min ischaemia followed by 5 min reperfusion, in the presence or absence of CVT-10216. CVT-10216 (20 μM, ALDH2 inhibitor, blue) was applied for 30 min prior to ischaemia and for the first 10 min of reperfusion. (*E*) Cardiac ALDH2 activity, (*F*) infarct size (representative images and quantification), and (*G*) LDH release in WT and 2*2 hearts exposed to protocols described in panel (*D*). Data are expressed as mean ± S.E.M. Individual data are presented as open circles ($n = 5$ per condition). *$P < 0.05$ vs. WT; &$P < 0.05$ vs. 2*2; #$P < 0.05$ vs. IPC; \$$P < 0.05$ vs. other groups.

with 20 μM CVT-10216 during the IPC period and for the first 10 min of reperfusion (*Figure 5D*). CVT-10216 treatment not only abolished IPC-induced cardioprotection, but resulted in a further increase in both infarct size and necrotic cell death (LDH release) (*Figure 5E–G*). Taken together, our data demonstrate a positive correlation between cardiac infarct size and acetaldehyde levels in hearts exposed to global (*ex vivo*) I/R injury (*Figure 6A*).

4. Discussion

Despite the well-known impact of ethanol intake on cardiovascular system, the molecular mechanisms involved in its cardioprotective and cardiotoxic effects are not completely understood. Here we show, for the first time, that acetaldehyde, the main product of ethanol metabolism, plays a critical role in this scenario. We demonstrate that acetaldehyde levels not only correlate well with IR damage but acetaldehyde actually regulates infarct size in a bimodal fashion, when adding exogenously as a pre-conditioning agents, with a major contribution of ALDH2 activity (*Figure 6B*).

Previous studies have demonstrated the deleterious cardiac effect of acetaldehyde accumulation due to either alcohol dehydrogenase over-expression or genetic ALDH2 disruption; therefore resulting in alcoholic cardiomyopathy.[31,32] Here, we show that 50 μM of acetaldehyde pre-treatment followed by a 5-min washout prior to I/R injury is sufficient to induce cardioprotection in hearts of WT mice (or acetaldehyde preconditioning). However, the same pre-treatment with acetaldehyde exacerbates I/R injury in hearts with impaired acetaldehyde metabolism from ALDH2 deficient mice, ALDH2*2. These findings indicate a bimodal effect of acetaldehyde in I/R injury. Supporting an ALDH2 mechanism for such bimodal response, ALDH2 activation using Alda-1 promotes a shift from acetaldehyde-induced cardiotoxicity to acetaldehyde-induced cardioprotection in hearts of ALDH2*2 mice subjected to I/R and pharmacological ALDH2 inhibition in WT hearts shifts acetaldehyde-induced cardioprotection to cardiotoxicity.

The bimodal effect of acetaldehyde in cardiac physiology can be explained by its ability to accumulate inside the cell and form either unstable (Schiff base) or stable (N-ethyllysine residues) adducts with proteins; thus affecting protein activity and stability.[1,2] Acetaldehyde has also been linked to renin-angiotensin system activation through cardiac mast cell degranulation, which has a critical impact in cardiac physiology.[18] However, further studies are required to better understand the role of acetaldehyde in cardiac physiology and pathology. Here we show that, similar to ethanol preconditioning,[8,11] acetaldehyde-induced cardioprotection requires εPKC translocation to mitochondria and activation of ALDH2.

ALDH2 is a tetrameric enzyme responsible for the oxidization of aldehydic substrates, including those generated during oxidative stress such as 4-hydroxy-2-nonenal.[7] We have previously found that the inability of ALDH2 to remove endogenous cytotoxic aldehydes accumulated during oxidative stress is involved in a variety of pathological conditions such as nitroglycerin-induced increased cardiac damage,[9,33] myocardial infarction,[6] heart failure,[7] hypertension,[34] and inflammatory pain.[20] Moreover, direct activation of ALDH2 using Alda-1, a small molecule allosteric activator developed by our group,[11] is sufficient to increase the clearance of cytotoxic aldehydes and mitigates their pathological effects.[6,7,9,20] Similarly, ALDH2 knockout mice are more susceptible to

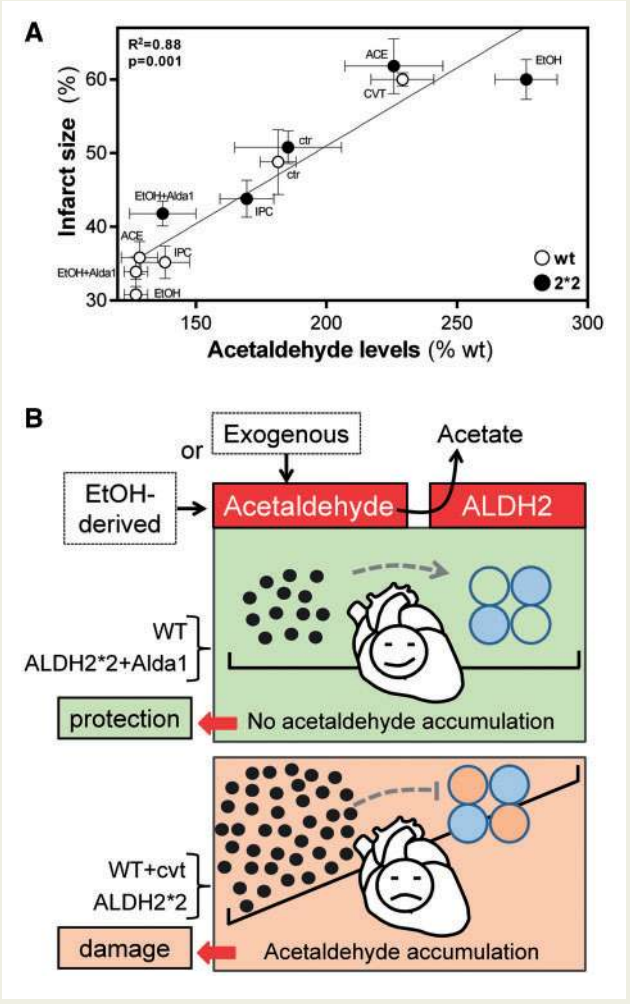

Figure 6 Acetaldehyde levels display a positive correlation with infarct size. (*A*) Linear regression analysis between cardiac acetaldehyde levels and infarct size in WT (open circles) and ALDH2*2 (2*2, filled circles) hearts exposed to *ex vivo* global ischaemia-reperfusion protocol. A subset of hearts exposed to ischaemia-reperfusion was preconditioned with Ethanol (EtOH, 50 mM), EtOH (50 mM) + Alda1 (ALDH2 activator, 20 μM), acetaldehyde (Act, 50 μM), IPC (3 bouts of 5 min ischaemia followed by 5 min reperfusion) or pharmacologically inhibited by CVT-10216 (20 μM, ALDH2 inhibitor). Data are expressed as mean ± SEM ($n = 5$ per condition). (*B*) Proposed model for cardiac acetaldehyde preconditioning in WT and ALDH2*2 mice. Ethanol is metabolized by alcohol dehydrogenase (ADH) to generate acetaldehyde. Acetaldehyde is then metabolized by the mitochondrial ALDH2 to acetate. Either administration of exogenous acetaldehyde or its endogenous production during ethanol metabolism induces cardioprotection through ALDH2 activation and faster acetaldehyde oxidation in hearts carrying the ALDH2*2 variant treated with the ALDH2 activator (Alda-1) or untreated WT hearts. This process results in a mild acetaldehyde accumulation compared with control hearts. Oppositely, the same amount of acetaldehyde increases infarct size in hearts carrying the ALDH2*2 variant or WT hearts treated with the ALDH2 inhibitor (CVT-10216). This phenotype is followed by a severe accumulation of cardiac acetaldehyde.

stress-induced injury and ALDH2 overexpressing mice are more protected from stress.[29]

Our findings suggest that even moderate consumption of alcoholic beverages (50 mM ethanol is equivalent to about three standard drinks according to NIAAA[35]) may cause cardiotoxicity in about 540 million people carrying the ALDH2*2 variant due to their reduced ability to metabolize acetaldehyde.[4,36] Because of the reactions to acetaldehyde, individuals carrying the ALDH2*2 variant are expected to consume lower amount of alcoholic beverages and have reduced likelihood of heavy drinking compared with other individuals. However, the increased cultural acceptance of alcohol consumption has contributed to a progressive increase in the annually rate per capita of alcohol consumption among individual carrying ALDH2*2 variant.[37] Once they consume more ethanol, the interaction between ethanol and the ALDH2*2 variant will likely increase their risk for cardiovascular diseases[4,12] and damage from acute myocardial infarction (the current study). In fact, we previously demonstrated that induced pluripotent stem cell-derived cardiomyocytes from patients carrying the ALDH2*2 variant are more susceptible to ischaemic injury when compared with WT controls.[10]

Unexpectedly, we found that hearts excised from ALDH2*2 knock-in mice with impaired acetaldehyde metabolism have the same ischaemic damage compared with WT littermates. This lack of difference could be because the damage generated in an *ex vivo* model of I/R (30'/60') was too severe; therefore dampening the potential difference between ALDH2*2 and WT hearts. Perhaps, a shorter period of ischaemia would be sufficient to detect the cardiac contribution of the ALDH2*2 mutation.

Overall, using a combination of pharmacological and genetic approaches, our study demonstrates that acetaldehyde levels have a positive correlation with the cardiac outcome after I/R injury (*Figure 6A*). In addition, acetaldehyde levels and its metabolizing enzyme, ALDH2, dictate whether acute ethanol/acetaldehyde plays beneficial or detrimental role in ischaemic hearts, and the use of selective ALDH2 activators, such as Alda-1, can rescue ALDH2*2 hearts from ethanol metabolism-induced cardiotoxicity (*Figure 6B*). Therefore, the maintenance of active acetaldehyde metabolism thorough ALDH2 is critical to promote cardioprotection.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: D.M.-R. and C.-HC hold patents related to Alda and ALDH2 now licenced to Foresee. However, they do not hold stocks of the company and none of this research was supported by the company. W.Y. is Chief Scientific Officer of Foresee Pharmaceuticals and co-inventor of patents that licenced to Foresee. Foresee is developing ALDH2 agonist in the treatment of peripheral artery disease (PAD). The other authors declare no conflict of interest.

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