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Activation of aldehyde dehydrogenase 2 (ALDH2) confers cardioprotection in protein kinase C epsilon (PKCε) knockout mice

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Abstract

Acute administration of ethanol can reduce cardiac ischemia/reperfusion injury. Previous studies demonstrated that the acute cytoprotective effect of ethanol on the myocardium is mediated by protein kinase C epsilon (PKCε). We recently identified aldehyde dehydrogenase 2 (ALDH2) as an PKCε substrate, whose activation is necessary and sufficient to confer cardioprotection *in vivo*. ALDH2 metabolizes cytotoxic reactive aldehydes, such as 4-hydroxy-2-nonenal (4-HNE), which accumulate during cardiac ischemia/reperfusion. Here, we used a combination of PKCε knockout mice and a direct activator of ALDH2, Alda-44, to further investigate the interplay between PKCε and ALDH2 in cardioprotection. We report that ethanol preconditioning requires PKCε, whereas direct activation of ALDH2 reduces infarct size in both wild type and PKCε knockout hearts. Our data suggest that ALDH2 is downstream of PKCε in ethanol preconditioning and that direct activation of ALDH2 can circumvent the requirement of PKCε to induce cytoprotection. We also report that in addition to ALDH2 activation, Alda-44 prevents 4-HNE induced inactivation of ALDH2 by reducing the formation of 4-HNE-ALDH2 protein adducts. Thus, Alda-44 promotes metabolism of cytotoxic reactive aldehydes that accumulate in ischemic myocardium. Taken together, our findings suggest that direct activation of ALDH2 may represent a method of harnessing the cardioprotective effect of ethanol without the side effects associated with alcohol consumption.

Introduction

Epidemiological studies have consistently shown beneficial effects of moderate consumption of ethanol in reducing ischemic heart disease^{1, 2}. Acute ethanol administration can also protect the heart and isolated cardiomyocytes from ischemic injury^{3–7}, although the acute cytoprotective effect of ethanol depends critically on the dose and timing of administration^{6, 8}. We found that the cardioprotective effect of ethanol requires activation of the epsilon isozyme of protein kinase C (PKCε)⁴ and recently identified mitochondrial aldehyde dehydrogenase 2 (ALDH2) as a PKCε substrate, whose activity strongly correlates with cardioprotection⁹. Acute ethanol administration, or treatment with the PKCε-selective

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Daria Mochly-Rosen is the founder and a shareholder of KAI Pharmaceuticals. However, none of the work was done in collaboration with or support from the company.

activator, ψ RACK, enhanced ALDH2 activity and reduced infarct size, whereas ALDH2 inhibition (with nitroglycerin-induced desensitization¹⁰, or inhibition with cyanamide) abolished ethanol-induced, PKC ϵ -mediated cardioprotection⁹. We also found that ethanol preconditioning results in mitochondrial translocation of PKC ϵ , increased ALDH2 activity and reduced cardiac injury, *in vivo*¹¹. Other recent studies have demonstrated that phosphorylation of ALDH2 is increased by nitrate preconditioning¹² and that increased phosphorylation status of ALDH2 correlates with increased resistance to ischemia-reperfusion associated with female gender¹³. Taken together, ALDH2 appears to be a critical enzyme in protecting the heart from ischemic injury.

ALDH2 is a mitochondrial enzyme and is part of the ethanol metabolic pathway, converting acetaldehyde (produced by alcohol dehydrogenase, ADH) to acetic acid. In addition, ALDH2 detoxifies other aromatic and aliphatic aldehydes, including 4-hydroxy-2-nonenal (4-HNE), which are produced during oxidative stress as a result of lipid peroxidation^{14, 15}. 4-HNE is a highly cytotoxic aldehyde that accumulates in the heart in response to IR^{16, 17} and forms protein adducts with cysteine, histidine and lysine residues *via* Michaelis addition, leading to inhibition of protein function^{15, 18}. ALDH2 catalyses oxidation of 4-HNE to the non-electrophilic and unreactive metabolite 4-hydroxynon-2-enoic acid (4-HNA)¹⁵. Thus, ALDH2-mediated detoxification of reactive aldehydes, represents a possible cytoprotective mechanism that enhances tissue survival in the heart during IR^{9, 11}.

Based on previous observations^{9, 11}, our current model predicts that ethanol activates PKC ϵ which phosphorylates and activates mitochondrial ALDH2, increasing the metabolism of 4-HNE and other reactive aldehydes, reducing cellular injury during IR. In the current study, we used PKC ϵ knockout mice to determine whether ethanol requires PKC ϵ activity to confer cytoprotection and used a novel activator of ALDH2 to determine whether direct pharmacological activation of ALDH2 can protect the heart in the absence of PKC ϵ .

Materials and Methods

Animals

Mutant mice lacking PKC ϵ were obtained from the laboratory of Dr. Robert Messing¹⁹. Male mice 12–16 weeks of age were used for the study. Animal care and husbandry procedures were in accordance with established institutional and National Institutes of Health guidelines.

Ex vivo model of myocardial infarction

An *ex vivo* model of cardiac ischemia reperfusion was used as described^{4, 20}. Excised mouse hearts were cannulated *via* the aorta and perfused on a Langendorff apparatus with oxygenated Krebs-Henseleit solution containing 118mM NaCl, 4.7mM KCl 1.8mM CaCl₂, 1.2mM MgSO₄, 25mM NaHCO₃, 1.2mM K₂HPO₄ and 5mM glucose (pH 7.4). Hearts were perfused at a constant flow rate of 2.5ml/min (at 37°C) and ischemia-reperfusion was induced by stopping flow and submerging the heart in Krebs-Henseleit at 37°C for a period of 30 min (global, no-flow ischemia) followed by a reperfusion period of 60 min. Coronary perfusate was collected every 5 mins during the first 30 min of reperfusion and creatine kinase (CK) release (CK U/L) assayed using a kit (Diagnostic Chemicals Ltd, Charlottetown, PE, Canada) as a measure of cardiac injury. For ethanol treatment, 50mM ethanol was applied following 10 min equilibration, for a period of 15 min, followed by 5 min washout, prior to ischemia. For Alda-44 treatment, 40 μ M Alda-44 was applied for 10 min prior to ischemia and for the first 10 min of reperfusion. Normoxic control hearts were subjected to 90 min perfusion in the absence of ischemia. Immediately after reperfusion, hearts were either immediately frozen for biochemical analysis (at –80°C) or were sliced into 4–5 transverse sections and incubated in 1% triphenyltetrazolium chloride (TTC) solution in phosphate buffered saline (PBS) for 10

min at 20°C in the dark. TTC stained sections were photographed using a digital camera and infarct size assessed by measuring the % infarct for each slice.

ALDH2 enzymatic activity assay

Enzymatic activity of either recombinant or mitochondrial ALDH2 was determined spectrophotometrically by monitoring the reductive reaction of NAD⁺ to NADH at 340 nm as previously described^{9, 11}. ALDH2 assays were carried out at 25°C in 50 mM sodium pyrophosphate buffer, pH=9.5. To this volume, 10 mM acetaldehyde and 400 µg of mitochondrial protein lysate were added. To start the reaction, 2.5 mM NAD was added and the accumulation of NADH was monitored for 5 min with measurements being taken every 30 s. ALDH2 reaction rates were expressed as µmol NADH/min/mg protein.

4-HNE- adduct formation on recombinant ALDH2

ALDH2 human recombinant protein (9µg) was incubated in 50mM sodium pyrophosphate in the absence and presence of Alda-44 (20 µM) for 5 min. This incubation was followed by the addition of 4-HNE (200 µM) (Sigma) for 5 min. The reaction was stopped by the addition of 5% perchloric acid to precipitate the proteins and the solution centrifuged at 100,000g for 10 min. Proteins were then re-suspended in loading buffer and ALDH2-HNE adducts visualized by Western blot using anti-HNE antibody (Calbiochem, CA). ALDH2 protein level was determined using an anti-ALDH2 antibody for control loading (Santa Cruz Biotechnology, CA).

Western Blotting

Mouse heart ventricles were dissected and homogenized using a Polytron homogenizer in 10x volume Lysis Buffer (0.1M Tris-HCl, pH 8.0, 10mM DTT, 20% glycerol, 1% Triton containing Sigma protease and phosphatase inhibitors, added 1:300 as per manufacturers instructions). Protein concentration was assessed by Bradford Assay. For western blot analysis, 20µg protein was separated on 10% SDS-PAGE gel and proteins transferred electrophoretically to nitrocellulose membranes. Membranes were then blocked for 1 hour in 5% milk in phosphate buffered saline containing 0.1% Tween 20 (PBS-T) before incubation with primary antibodies against PKCε, Enolase, GAPDH (Santa Cruz Antibodies) or SAPK/JNK (Cell Signaling Technology) or an antibody that binds to 4-HNE protein adducts (Calbiochem) at a concentration of 1:1000 then washed 3x in PBS-T before incubation with horseradish peroxidase-conjugated secondary antibody (1:2000) (Amersham). Protein levels were visualized by chemiluminescence (Pierce) and protein levels quantified by densitometry using Image-J software (NIH).

Statistical Analysis

Data are expressed as mean±S.E.M. Statistical analyses was performed using the Student's t-test (for comparisons between two groups) or 1-way analysis of variance (ANOVA) with a Bonferroni multiple comparisons post-hoc test (for comparisons between more than two groups). A probability value less than 0.05 was considered statistically significant.

Results

Alda-44 activates ALDH2 and prevents ALDH2 inhibition by 4-HNE

We previously reported N-(1,3-benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide (Alda-1) as an ALDH2 agonist⁹ that protects the rat heart from ischemia/reperfusion. We synthesized a more water soluble derivative of Alda-1, termed Alda-44, which was used in the current study. Alda-44 was indistinguishable from Alda-1 when tested *in vitro* and *ex vivo*. For example, Alda-44 (40µM) treatment resulted in a 65% increase in enzymatic activity of recombinant,

human ALDH2 (Fig. 1A, B n=4, $p<0.005$) which is comparable with that observed for Alda-1⁹. Furthermore, Alda-44 had no effect on the related isozymes ALDH3 or ALDH5, under the same conditions, demonstrating its selectivity for ALDH2 (Fig 1B).

In addition to activation of ALDH2, an interesting property of Alda-1 is the inhibition of 4-HNE-induced inactivation of ALDH2⁹. Similarly, treatment with 40 μ M Alda-44 prevented inhibition of ALDH2 induced by 200 μ M 4-HNE (Fig 1C, n=3, $p<0.005$). 4-HNE is thought to inhibit ALDH2 through protein adduct formation by covalent interaction (*via* Michaelis addition) at Cys302, located at the ALDH2 active site²¹. We therefore hypothesized that the mechanism by which Alda-44 prevents 4-HNE induced inactivation of ALDH2 is likely due to prevention of 4-HNE-ALDH2 adduct formation. We found that 40 μ M Alda-44 could prevent 4-HNE-ALDH2 adduct formation induced by incubation of recombinant human ALDH2 with 200 μ M 4-HNE (Fig 1D). Thus, the capacity of Alda-44 to maintain ALDH2 enzymatic activity in the presence of high concentrations of 4-HNE likely stems from its ability to protect the enzyme from 4-HNE adduct formation.

Alda-44 activates mitochondrial ALDH2 in PKC ϵ WT and KO mouse hearts

We found previously that cardioprotection conferred by PKC ϵ (activated by ethanol or by ψ εRACK) is mediated by phosphorylation and activation of ALDH2 by PKC ϵ ⁹. To determine whether PKC ϵ is required for ALDH2 activation by Alda-44, we performed the ALDH2 assay in cardiac mitochondria isolated from PKC ϵ KO and WT mice⁹. Alda-44 could activate ALDH2 equally in PKC ϵ WT (Fig 4A, C n=4, $p<0.05$) and PKC ϵ KO heart mitochondria (Fig 4B, C n=4, $p<0.05$). Thus, PKC ϵ is not required for direct activation of endogenous ALDH2 by Alda-44.

PKC ϵ KO hearts are not protected by ethanol but are protected by direct ALDH2 activation with Alda-44

Hearts isolated from PKC ϵ WT and KO mice were exposed to ischemia and reperfusion protocols (Fig 3) to determine whether PKC ϵ is essential for cardioprotection mediated by ethanol preconditioning or pharmacological activation of ALDH2. In WT and PKC ϵ KO hearts, exposure to 30 min ischemia and 60 minutes reperfusion induced similar infarction size ($61\% \pm 6\%$ in WT and $59\% \pm 5\%$ in PKC ϵ KO hearts, n=6) (Fig 4A, B) and CK release (5600 ± 1000 CK U/L in WT and 4900 ± 1400 in PKC ϵ KO hearts, n=6) (Fig 4C). These data were in agreement with our previous finding that IR induces a similar level of necrotic cell death in WT and PKC ϵ KO hearts²⁰. We next determined whether perfusion with 50mM ethanol for 15 mins followed by 5 mins washout prior to ischemia resulted in protection in PKC ϵ WT and KO hearts. In WT hearts, ethanol pretreatment resulted in a 46% reduction in infarct size ($33\% \pm 9$ in ethanol treated vs. $61\% \pm 6\%$ in IR control; n=5, $p<0.05$, Fig 4B) which was accompanied by significant reduction in CK release (2000 ± 1000 U/L in ethanol treated vs. 5600 ± 1000 CK U/L in control IR, n=5, $p<0.05$, Fig 4C). In PKC ϵ KO hearts, ethanol did not induce protection (infarct size was $55\% \pm 5\%$ and CK activity was 4300 ± 1100 U/L in PKC ϵ KO treated with ethanol vs. $59\% \pm 5\%$ and 4900 ± 1400 U/L in PKC ϵ KO IR alone, n=5, Fig 4B, C), suggesting that ethanol-mediated preconditioning requires PKC ϵ , in agreement with previous studies^{4, 9}. We next determined whether direct activation of ALDH2 protects PKC ϵ WT and KO hearts. We found that treatment with 40 μ M Alda-44, applied 10 min before ischemia and during the initial 10 min of reperfusion, significantly reduced infarct size and CK release in both WT and PKC ϵ KO hearts (Fig 4B, C). We also found that Alda-44 is protective when added at reperfusion alone (CK activity was 2100 ± 1000 U/L vs. 5600 ± 1000 CK U/L in WT control IR, n=6, $p<0.05$). Alda-44 treatment reduced infarct in PKC ϵ WT by 57% (infarct size after Alda-44 treatment was 26 ± 9 in PKC ϵ WT vs. $61\% \pm 6\%$ in untreated IR controls, n=5, $p<0.01$, Fig 4B) and PKC ϵ KO hearts by 43% (infarct size after Alda-44 treatment was 30.2 ± 8 vs. $59\% \pm 5\%$ in PKC ϵ KO hearts, n=5, $p<0.05$, Fig 4B).

Preservation of ALDH2 activity and reduced formation of 4-HNE protein adducts in PKCε WT and PKCε KO mice treated with Alda-44

4-HNE is a major, cytotoxic product of lipid peroxidation²² that accumulates in ischemic/reperfused myocardium^{16, 17}. 4-HNE protein adducts were therefore measured in WT and PKCε KO hearts exposed to IR in the absence and presence of Alda-44, using an antibody that recognizes proteins containing 4-HNE adducts at Cys, His and Lys residues¹⁶ (Fig 5A). In WT hearts, IR increased levels of 4-HNE protein adducts (normalized to GAPDH as an internal protein loading control) by 2 folds from 0.44 ± 0.04 in normoxia to 0.84 ± 0.10 after IR, which was reduced almost to control levels (to 0.46 ± 0.08) in the Alda-44-treated group ($n=4$, $p<0.05$). In PKCε KO hearts, the level of 4HNE adducts increased 2.4 folds, from 0.26 ± 0.08 in normoxia to 0.63 ± 0.03 after IR, which was reduced to 0.46 ± 0.03 in hearts treated with Alda-44 ($n=4$, $p<0.05$). There was no statistical difference in the levels of 4-HNE adducts between WT and PKCε KO hearts under basal conditions.

Because 4-HNE accumulation is associated with inactivation of ALDH2, we also measured ALDH2 activity in these samples (Fig 5B). Exposure to IR reduced ALDH2 activity in WT hearts from 3.1 ± 0.2 in normoxia to 2.1 ± 0.2 after IR ($n=4$, $p<0.05$) and in PKCε KO hearts ALDH2 activity was reduced from 3.4 ± 0.2 in normoxia to 2.1 ± 0.2 after IR ($n=4$, $p<0.05$). The IR-induced inactivation of ALDH2 was prevented by Alda-44 treatment in both WT ($n=4$, $p<0.05$) and PKCε hearts ($n=4$, $p<0.05$). Taken together, these results are in agreement with our *in vitro* data that 4-HNE induced inactivation of ALDH2 is prevented by Alda-44 and suggest that a cytoprotective benefit of Alda-44 is to prevent inactivation of ALDH2 thus maintaining detoxification of oxidative-stress induced reactive aldehydes during IR.

Treatment with Alda-44 reduces phosphorylation of JNK/SAPK induced by IR in PKCε WT and KO hearts

Having identified that ALDH2 activation protects the heart in the absence of PKCε, we sought to investigate whether enhanced ALDH2 activity could influence other signaling enzymes activated by ischemia reperfusion. The stress-activated protein kinase/cJun N-terminal kinase (SAPK/JNK) pathway is activated in cardiomyocytes following exposure to oxidative stress²³ and is thought to play a crucial role in induction of cardiomyocyte apoptosis following IR^{24, 25}. Under basal conditions, SAPK/JNK phosphorylation and activity status remain low, but are markedly increased within minutes of reperfusion of ischemic myocardium^{24, 26, 27}. Thus SAPK/JNK phosphorylation status is a sensitive marker of IR-induced cardiac injury. We determined SAPK/JNK phosphorylation in response to cardiac IR, in the absence and presence of 40μM Alda-44, in WT and PKCε KO mice. As shown previously^{24, 26}, IR induced a marked increase in phosphorylation of both the p46 SAPK/JNK and p54 SAPK/JNK isoforms (Fig 6B, C). The IR-induced increase in SAPK/JNK was observed in both PKCε WT and KO mice (Fig 6B, C, $n=3$, $p<0.05$) and was attenuated by Alda-44 treatment in both WT and PKCε KO hearts (Fig 6B, C $n=3$, $p<0.05$).

Discussion

In the present study we have demonstrated that direct activation of ALDH2 is sufficient to protect ischemic/reperfused myocardium in the absence of PKCε. These findings are in agreement with previous studies that ALDH2 plays a central role in mediating cytoprotection from cardiac ischemia/reperfusion^{9, 11–13}. These data also suggest that ALDH2 is downstream of PKCε in ethanol-induced cardioprotection and that this cytoprotective mechanism can be harnessed using ALDH2 activators, such as Alda-44. The study also provides further insight into the cytoprotective mechanism of Alda-44, demonstrating that Alda-44 can prevent 4-HNE-induced inactivation of ALDH2 by preventing the formation of 4HNE-ALDH2 protein adducts (summarized in Fig. 7).

Much of the cellular damage occurring during IR is predicted to be due to reactive oxygen species (ROS) production²⁸. IR-induced ROS accumulation leads to lipid peroxidation and the generation of reactive aldehydes including acetaldehyde²⁹ and 4-HNE^{16, 17, 30} which form protein adducts with cysteine, histidine and lysine residues *via* Michael's addition¹⁸ inhibiting protein function. 4-HNE, for example, inhibits key metabolic proteins such as GAPDH³¹, the Na/KATPase³² and the 20S proteasome³³ and is also an inducer of mitochondrial permeability transition³⁴. 4-HNE accumulates rapidly in the ischemic heart¹⁶, and has a considerably longer half-life than free radical species¹⁶, therefore, the efficient removal of cytotoxic aldehydes is likely to be of crucial importance for cell survival against ischemia/reperfusion injury. Glutathione (GSH) can scavenge 4-HNE in a reaction catalysed by glutathione-S-transferase (GST)¹⁵, however GSH levels fall rapidly during ischemia³⁵. 4-HNE is also metabolized by mitochondrial ALDH2 to the non-reactive metabolite, 4-HNA^{15, 30}. Thus, detoxification of reactive aldehydes, such as 4-HNE is a likely cytoprotective mechanism mediated by ALDH2 activation.

4-HNE also inactivates ALDH2 at high concentrations^{9, 21}, interacting covalently with Cys302, located at the ALDH2 active site²¹ and resulting in almost complete inhibition of the enzyme, *in vitro* (as shown in Fig 1C). It is therefore likely that the impaired 4-HNE metabolism³⁰ and accumulation of 4-HNE adducts¹⁶ observed during cardiac ischemia/reperfusion occurs as a dual consequence of increased generation of aldehydes from lipid peroxidation and impaired metabolism of these aldehydes due to 4-HNE-induced inactivation of ALDH2. Here, we demonstrate, that in addition to activation of ALDH2, Alda-44 prevents 4-HNE induced inactivation of ALDH2 *in vitro*, by reducing the formation of 4-HNE protein adducts on ALDH2 (Fig 1D). Furthermore, Alda-44 prevented IR-induced inhibition of ALDH2 in the heart, with a concomitant decrease in 4-HNE protein adduct formation. Thus, the mechanism of protection mediated by Alda-44 is likely to be due to a combination of increased ALDH2 activity and prevention of 4-HNE-mediated ALDH2 inactivation, resulting in reduced accumulation of cytotoxic aldehydes and enhanced preservation of ischemic myocardium.

Chronic, moderate alcohol consumption results in increased expression and activation of PKC ϵ ³⁶ and confers enhanced protection against myocardial ischemia³⁷. Acute ethanol treatment is also cardioprotective and is abolished by selective inhibition of PKC ϵ ³. Our data that ethanol is not protective in PKC ϵ knockout mice confirms the role of PKC ϵ in ethanol preconditioning. Recent studies by Slater *et al* have identified an ethanol binding site on the C1 domain of PKC ϵ , suggesting that ethanol mediates its action on PKC ϵ by direct binding to the enzyme³⁸. The direct cytoprotective effect of ethanol on the myocardium has been observed in a variety of species, however, other studies have shown a lack of ethanol-induced protection^{39, 40}. Furthermore, ethanol can abolish cytoprotection conferred by ischemic preconditioning if it is not sufficiently metabolized or washed out prior to the onset of ischemia^{6, 41}. Thus, the acute cardioprotective effect of ethanol depends critically on the dose of ethanol used and whether ethanol remains present during the ischemic period. The observation that the continued presence of ethanol blocks its own protection^{5, 6} and that of ischemic preconditioning^{5, 41} may perhaps be explained, at least in part, by the identification of ALDH2 as a key cytoprotective enzyme. Acetaldehyde is the primary product of ethanol metabolism and impairs cardiac excitation-contraction coupling, perturbs Ca²⁺ signaling and forms protein adducts⁴². Acetaldehyde is metabolized by ALDH2, and transgenic overexpression of ALDH2 rescues acetaldehyde-induced myocardial dysfunction^{43, 44}. It is likely that if ethanol is not sufficiently metabolized prior to ischemia, ethanol-derived acetaldehyde results in competitive inhibition of ALDH2 for 4-HNE metabolism. The dichotomous effect of ethanol in acute cardioprotection may therefore be a consequence of the beneficial effects of ethanol (mediated by PKC ϵ and ALDH2) being countered by the cytotoxic effects of acetaldehyde accumulation (resulting from ethanol metabolism). Direct activation

of ALDH2 with Alda-44 offers a means of harnessing the cytoprotective effect, without the associated cytotoxicity caused by acetaldehyde accumulation.

While 4-HNE induces necrosis through inhibition of key metabolic proteins, evidence indicates that 4-HNE can also induce programmed cell death through activation of SAPK/JNK^{45–49}. It is well established that SAPK/JNK becomes phosphorylated and activated immediately on reperfusion of ischemic myocardium^{24–26}. SAPK/JNK activation initiates apoptosis through the phosphorylation and activation of pro-apoptotic Bad⁵⁰, inactivation of pro-survival BCL-2⁵¹ and induction of cytochrome *c* release from cardiac mitochondria⁵². As 4-HNE can also increase SAPK/JNK phosphorylation and activity^{45–47}, IR-induced apoptosis may, at least in part, be mediated through 4-HNE. Our data that Alda-44 reduced IR-induced formation of 4-HNE protein adducts with a concomitant reduction in SAPK/JNK phosphorylation suggest that ALDH2 activators may reduce IR-induced apoptotic cell death, by reducing 4-HNE mediated activation of SAPK/JNK.

In summary, our data demonstrate that cardioprotection conferred by ethanol preconditioning is abolished in PKC ϵ KO mice, whereas direct activation of ALDH2 with Alda-44 can confer cardioprotection in the absence of PKC ϵ . Our data also provide mechanistic insight into the cytoprotective mechanism of Alda-44. We demonstrate that in addition to ALDH2 activation, Alda-44 prevents 4-HNE mediated inactivation of ALDH2. We provide evidence, *in vitro*, that the molecular basis for Alda-44 induced protection of ALDH2 is likely due to prevention of 4-HNE protein adducts on ALDH2. Thus, by preventing inactivation of ALDH2, Alda-44 maintains the detoxification of oxidative-stress induced reactive aldehydes, reducing ischemic injury (shown in Fig 7). In support of this hypothesis, we show that Alda-44 prevents ALDH2 inactivation, reduces accumulation of 4-HNE adducts and reduces SAPK/JNK phosphorylation induced by ischemia/reperfusion. The evidence supporting the beneficial effects of ethanol on the heart is abundant, however, because the cardioprotective effect of ethanol consumption must be countered by the cytotoxic effects of acetaldehyde, and the associated health risks (such as liver cirrhosis, cancer, alcohol-induced cardiomyopathy) ALDH2 activators, such as Alda-44, may therefore offer a means of exploiting this endogenous cytoprotective mechanism without the unwanted side effects of ethanol consumption.

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Figure 1. Alda-44 selectively activates ALDH2 and protects against ALDH2 inactivation by 4-HNE
A) Recombinant ALDH2 activity (rate of NADH production at 340nm) was recorded in the absence and presence of 40 μ M Alda-44 **B)** Alda-44 (40 μ M) increased recombinant ALDH2 activity by 65% but was without effect on the related enzymes, ALDH3 and ALDH5. **C)** Inactivation of recombinant ALDH2 by 200 μ M 4-HNE was prevented by 40 μ M Alda-44. **D)** Western blot analysis of 4-HNE protein adducts on human recombinant ALDH2. Treatment with 40 μ M Alda-44 prevented 4-HNE adduct formation on ALDH2 induced by incubation with 200 μ M 4-HNE. Data are expressed as mean S.E.M, n=4, *p<0.05 vs. control.

Figure 2. Alda-44 activates ALDH2 in cardiac mitochondria isolated from WT and PKC ϵ KO hearts

ALDH2 activity in purified mitochondria isolated from PKC ϵ WT hearts (**A**) and PKC ϵ KO hearts (**B**). **C**) Treatment with 40 μ M Alda-44 increased mitochondrial ALDH2 activity to a similar extent in WT and PKC ϵ cardiac mitochondria, suggesting that PKC ϵ is not required for Alda-44 activation of ALDH2. Data are expressed as mean $\pm$ S.E.M, n=4, *p<0.05 vs. control.

Group 1) IR Control

Group 2) Ethanol

Group 3) Alda-44

Timeline

Figure 3. Experimental protocols used for ischemia and reperfusion in isolated hearts

Hearts were exposed to 30 min global ischemia followed by 60 min of reperfusion or to normoxic perfusion for an equivalent duration. Ethanol (50mM) was applied for a period of 15 min, followed by 5 min washout, prior to ischemia. Alda-44 (40 μ M) was applied for 10 min prior to ischemia and for the first 10 min of reperfusion.

Figure 4. Effect of ethanol and Alda-44 on ischemia/reperfusion in WT and PKC ϵ KO hearts
A) Representative sections of PKC ϵ WT and PKC ϵ KO hearts showing viable tissue (red) and necrotic tissue (white) after ischemia reperfusion, with and without treatment with 50mM ethanol or 40 μ M Alda-44. Ethanol reduced infarct size (**B**) and CK release (**C**) in WT but not PKC ϵ KO hearts suggesting that ethanol preconditioning requires PKC ϵ . Alda-44 reduced necrotic cell death in both WT and in PKC ϵ KO hearts suggesting that Alda-44 confers protection in the absence of PKC ϵ . Data expressed as mean $\pm$ S.E.M, n=5, *p<0.05 vs. IR control.

Figure 5. Alda-44 preserves ALDH2 activity and reduces 4-HNE protein adducts induced by IR in WT and PKC ϵ KO hearts

A) 4-HNE protein adducts in hearts exposed to normoxia, or IR injury, in the absence or presence of Alda-44. Treatment with Alda-44 reduced IR-induced increase in 4-HNE protein adducts in both WT and PKC ϵ KO hearts **B)** ALDH2 activity in hearts exposed to normoxia or IR in the absence and presence of Alda-44. Treatment with Alda-44 prevented the IR-induced inhibition of ALDH2 in both WT and PKC ϵ KO hearts. Data expressed as mean S.E.M., n=4, *p<0.05 vs. IR control.

Figure 6. Effect of Alda-44 on SAPK/JNK phosphorylation in PKC ϵ WT and KO hearts

A) Western blot demonstrating absence of PKC ϵ protein in PKC ϵ KO hearts. **B)** Western blots of total and phosphorylated p46 and p54 SAPK/JNK and enolase in hearts exposed to normoxia or IR in the absence and presence of Alda-44. **C)** Quantification of p46 and p54 SAPK/JNK phosphorylation in hearts exposed to IR in the absence and presence of Alda-44. Data are expressed as mean $\pm$ S.E.M, n=3, *p<0.05 vs. IR.

Figure 7. Schematic diagram of cardioprotection conferred by ALDH2

Ischemia/reperfusion results in ROS generation, leading to the accumulation of reactive aldehydes, such as 4-HNE, formed due to ROS-induced lipid peroxidation. 4-HNE is oxidized by mitochondrial ALDH2 to the non-electrophilic metabolite, 4-HNA. However, at high concentrations, 4-HNE can also impair ALDH2 activity (red line). ALDH2 activity can be increased by ethanol preconditioning *via* PKC ϵ -mediated phosphorylation. Alternatively, ALDH2 can be activated directly by Alda-44, which also prevents 4-HNE-induced inhibition of ALDH2 (green arrow). Thus, the mechanism of protection mediated by Alda-44 is likely to be due to a combination of increased ALDH2 activity and prevention of 4-HNE-mediated ALDH2 inactivation, resulting in reduced 4-HNE accumulation during ischemia/reperfusion.