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Time-dependent and ethanol-induced cardiac protection from ischemia mediated by mitochondrial translocation of εPKC and activation of aldehyde dehydrogenase 2

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Abstract

The cardioprotective effects of moderate alcohol consumption have been well documented in animal models and in humans. Protection afforded against ischemia and reperfusion injury (I/R) proceeds through an ischemic preconditioning-like mechanism involving the activation of epsilon protein kinase C (εPKC) and is dependent on the time and duration of ethanol treatment. However, the substrates of εPKC and the molecular mechanisms by which the enzyme protects the heart from oxidative damage induced by I/R are not fully described. Using an open-chest model of acute myocardial infarction *in vivo*, we find that intraperitoneal injection of ethanol (0.5 g/kg) 60 minutes prior to (but not 15 minutes prior to) a 30-minute transient ligation of the left anterior descending coronary artery reduced I/R-mediated injury by 57% (measured as a decrease of creatine phosphokinase release into the blood). Only under cardioprotective conditions, ethanol treatment resulted in the translocation of εPKC to cardiac mitochondria, where the enzyme bound aldehyde dehydrogenase-2 (ALDH2). ALDH2 is an intra-mitochondrial enzyme involved in the detoxification of toxic aldehydes such as 4-hydroxy-2-nonenal (4-HNE) and 4-HNE mediates oxidative damage, at least in part, by covalently modifying and inactivating proteins (by forming 4-HNE adducts). In hearts subjected to I/R after ethanol treatment, the levels of 4-HNE protein adducts were lower and JNK1/2 and ERK1/2 activities were diminished relative to the hearts from rats subjected to I/R in the absence of ethanol. Together, this work provides an insight into the mitochondrial-dependent basis of ethanol-induced and εPKC-mediated protection from cardiac ischemia, *in vivo*.

INTRODUCTION

The beneficial effects of moderate alcohol consumption on the heart have been well documented in both men and women [1–5]. While chronic moderate consumption of ethanol prior to and following an acute myocardial infarction (AMI) can improve patient prognosis [6,7], acute ethanol treatment may also significantly protect the heart from I/R injury [8,9]. In addition to changes in lipid levels and hemostatic factors [10,11], the protective effects of acute and chronic ethanol treatment proceed through direct preconditioning-like mechanisms within

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the cardiomyocyte [8,9,12,13]. Treatment of isolated cardiomyocytes or isolated hearts with 50 mM ethanol diminishes injury associated with prolonged ischemia and improves cardiac function [8,9,14]. However, other studies found no protection by acute ethanol treatment [15–17]. It is now evident that the acute cardioprotective effects of ethanol are largely dependent upon the timing and dose of ethanol treatment; ethanol administration directly before prolonged ischemia does not protect the heart from injury as shown in experimental models [18–20]. Downey and collaborators made the important finding that protection afforded by ischemic preconditioning is lost if ethanol is not washed out or sufficiently metabolized before the onset of ischemia [19], an effect that has been recently corroborated in humans [21].

The protective effects of ethanol administration require εPKC activation [8,14,22,23]. Additionally, we have determined through the use of *ex vivo* models of acute myocardial infarction that the protective effects of ethanol are due to a time-dependent mechanism involving δPKC-mediated adenosine release, resulting in εPKC activation [14,22]. However, protection afforded by the time-dependent activation of εPKC upon acute ethanol treatment has yet to be shown, *in vivo*. Treatment of isolated cardiomyocytes and isolated hearts, *ex vivo* with as little as 10 mM ethanol induces translocation of εPKC to the cellular particulate fraction [8,23,24]. Following translocation, εPKC interacts with its anchoring protein, εRACK (Receptor for Activated C-Kinase) and phosphorylates nearby substrates, conferring cardioprotection [23]. However, the protein target of εPKC following acute ethanol treatment is still unknown. Interestingly, several mitochondrial targets have been implicated for εPKC in ischemic preconditioning, including the mitochondrial ATP-sensitive K^+ channel ($mitoK_{ATP}$) [25,26], cytochrome-c oxidase (COIV) [27], and the permeability transition pore (MPTP) [28]. However, either conflicting evidence [9,19] or no evidence exists for a role of these targets in ethanol-mediated preconditioning and ethanol-stimulated mitochondrial localization of εPKC has not been reported.

Recently we found that εPKC activates the intra-mitochondrial enzyme ALDH2 in an *ex vivo* model of myocardial infarction, using the Langendorff preparation [29]. Using an *in vivo* model of acute myocardial infarction in rats, we show here that ethanol treatment 60 minutes prior to prolonged ischemia protected the heart from injury. This protection coincided with the translocation of εPKC to cardiac mitochondria, where it associated with the mitochondrial enzyme, aldehyde dehydrogenase-2 (ALDH2). These effects were dependent upon the timing of ethanol exposure and did not occur in the absence of I/R. Additionally, we show here that ethanol treatment prior to prolonged ischemia increased the activity of ALDH2 and decreased the formation of 4-hydroxy-2-nonenal (HNE)-protein adducts and activation of JNK1/2 and ERK1/2, all hallmarks of I/R injury. These data represent a novel protective mechanism by which ethanol increases the detoxification of cytotoxic aldehydes that accumulate during I/R *in vivo* by a mechanism involving the time-dependent εPKC-mediated activation of mitochondrial ALDH2.

MATERIALS and METHODS

In vivo model of left anterior descending coronary artery (LAD) ligation

Male Wistar rats (250–300g) were anesthetized by 3% isoflurane 15 or 60 minutes (indicated in text) after intraperitoneal (i.p.) injection of 0.5 g/kg ethanol. The surgical procedures used for left anterior descending coronary artery (LAD) ligation are based on a previously published protocol [30]. Briefly, animals were incubated, and ventilated with a Harvard rodent ventilator at a rate of 80 breaths per minute (5–15 mm Hg). Maintenance anesthesia was provided *via* 1% inhalational isoflurane and body temperature was maintained at 37°C using a rectal probe linked to a thermocoupled thermometer and an appropriate heating blanket. The heart was exposed by median sternotomy and control and ethanol-treated rats were subjected to a 10 minute period of stabilization, followed by a ligature being placed around the LAD coronary

artery, close to its origin from the aortic root. The normoxia control animals (sham) were exposed to the same procedure with no ligation. The free ends of the ligature were used to form a noose around a syringe plunger which was placed flat on the myocardium. Coronary occlusion was achieved by tightening the noose around the plunger for 30 minutes. Occlusion was determined by observation of immediate pallor of the left ventricular free wall and reflow was achieved by release of the ligature for 15 min. At the end of reperfusion, hearts were excised and flushed with 0.9% saline to remove blood.

For administration of ethanol, rats were mildly anesthetized with 3% isoflurane and 0.5g/kg ethanol was administered through intraperitoneal (i.p.) injection after which the animals were returned to their cages for either 15 or 60 minutes prior to surgery. Animals in the control groups received an injection of saline for the same durations prior to LAD occlusion. For measurement of humoral creatine phosphokinase (CPK), 0.45ml of blood was withdrawn into a 0.05ml heparin-primed syringe during the 15 minute reperfusion period at 5 min intervals *via* an apical punch of the left ventricle. Serum was separated by centrifugation at 5,000g for 5 minutes on a tabletop centrifuge and serum CPK values were determined using a CK-SL assay kit (Diagnostic Chemicals Ltd, Oxford, Connecticut). Background levels of CPK were taken prior to LAD occlusion and were subtracted from all CPK values and statistical analyses used are listed in the figure legends. All the animal protocols were approved by the Institutional Animal Care and Use Committees of Stanford University.

Western blot analysis

Following LAD occlusion and 15 minutes of reperfusion, the left ventricular free wall (without septum) was dissected away from the right ventricle and atria and homogenized in buffer A (210 mM mannitol, 70 mM sucrose, 5 mM MOPS and 1 mM EDTA). Tissue extract was centrifuged at 700g to pellet nuclei and unbroken cellular debris, followed by centrifugation at 10,000g to collect mitochondrial-enriched fractions. For εPKC translocation, mitochondrial fractions were resuspended in buffer A, analyzed by Western blot using antibodies against εPKC (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 dilution) and normalized to total cellular εPKC levels. For detection of HNE protein adducts in mitochondrial fractions, we used a specific antibody against the reductively stabilized HNE amino acid adducts (Calbiochem, Gibbstown, NJ; 1:1000 dilution). The levels of phosphorylated and unphosphorylated JNK, and Erk1/2 were analyzed using their respective antibodies (Cell Signaling, Danvers, MA) and mitochondrial purity was assessed with antibodies against the Na^+K^+ ATPase (Millipore, Billerica, MA), prohibitin (Santa Cruz Biotechnology), enolase (Santa Cruz Biotechnology,), and the ER targeting sequence KDEL (StressGen Corporation, Ann Arbor MI).

Immunoprecipitation analysis

For determination of ALDH2 and εPKC interactions, 700 μg of mitochondrial lysate protein was diluted into buffer A and immunoprecipitated with ALDH2 or εPKC-specific antibodies (Santa Cruz Biotechnology) (2 μg). After 3 hours of incubation, protein A/G agarose beads were added and incubated for an additional 2 hours before spinning down. Immunocomplexes were washed 3 times with buffer A and separated on an 8% SDS gel after which they were analyzed for the presence of associated proteins by Western blot using antibodies for εPKC, ALDH2 or εPKC (Santa Cruz Biotechnology) and visualized using a One-Step IP-Western kit (GenScript corp., Piscataway, NJ).

Enzymatic measurement of aldehyde dehydrogenase-2

Enzymatic activity of ALDH2 was determined spectrophotometrically by monitoring the reductive reaction of NAD^+ to NADH at A_{340nm}. The assays were carried out at 25°C in 50 mM sodium pyrophosphate buffer, pH=9.5. To this volume, 10 mM acetaldehyde and 200 μg of mitochondrial lysate protein isolated from the left ventricle of ethanol-treated or untreated

rat hearts that underwent LAD occlusion were added. To start the reaction, 2.5 mM NAD was added and the accumulation of NADH was monitored for 5 minutes with measurements being taken every 30 seconds. The reaction rates were recorded, compared to animals that were not treated with ethanol and expressed as % control.

Electron microscopic analysis of mitochondrial purity and integrity

Tissue was homogenized in buffer A as described above and fixed in 4% paraformaldehyde and 0.1% gluteraldehyde. The fixed material was sectioned by the Stanford Electron Microscopy Facility. Sections were taken between 75 and 80 nm, picked up on formvar/Carbon coated 75 mesh Ni grids and stained for 20 seconds in 1:1 saturated uracetate (~7.7%) in acetone followed by staining in 0.2% lead citrate for 3 to 4 minutes for contrast. Mitochondrial samples were observed in a JEOL 1230 transmission electron microscope at 80kV and photos were taken using a Gatan Multiscan 791 digital camera.

RESULTS

Occlusion of the left anterior descending coronary artery (LAD) results in ischemia, which subsequently leads to irreversible damage to the myocardium and tissue loss. We have previously shown in an *ex vivo* rat model of ischemia/reperfusion (I/R) injury that ethanol exposure prior to the ischemic event protects the heart from injury through an εPKC-dependent mechanism [8]. Very recently, we also found that activation of mitochondrial ALDH2 is required and sufficient to produce cardioprotection afforded by ethanol pretreatment, *ex vivo* [29]. In the current study, we first determined whether ethanol treatment in rats leads to cardioprotection from acute myocardial infarction, *in vivo*. As shown in Figure 1a, i.p. administration of 0.5 g/kg of ethanol 60 minutes prior to 30 minutes of LAD occlusion followed by 15 minutes of reperfusion resulted in a 57+/−9% decrease in creatine phosphokinase (CPK) release into the blood relative to rats that were injected with saline ($p<0.05$; n=7 animals/group). As seen in figure 1b, ethanol administration 60 minutes prior to ischemia significantly diminished the release of CPK over the 15 minutes of reperfusion as compared to controls (Fig. 1b, $p<0.05$; n=7). In agreement with the previous findings using *ex vivo* models [18,22,31], ethanol administration 15 minutes prior to LAD occlusion had no significant protective effects on the myocardium following reperfusion (Fig. 1a, n=4 animals/group).

Several studies demonstrated that treatment of whole hearts and isolated cardiomyocytes with ethanol results in the activation and translocation of εPKC to the particulate fraction of the cellular homogenate [8,14,22,23]. However, the subcellular localization and consequences of εPKC translocation had not been determined. Since under some conditions εPKC has been reported to be localized in the mitochondrial fraction [32,33], we isolated mitochondria from the left ventricle of hearts treated with 0.5 g/kg of ethanol 15 and 60 minutes prior to LAD ligation and performed Western blot analysis to determine the localization of εPKC. To confirm the purity of this mitochondrial preparation, we first evaluated the presence of contaminating fractions. In order to visualize mitochondrial proteins alongside total lysate proteins, we loaded equal amounts of mitochondrial and total lysate proteins, thereby concentrating the mitochondrial fraction. As seen in the top panel of Figure 2a, the mitochondrial fraction had minimal contamination of cytosolic proteins (enolase), plasma membrane proteins (Na^+K^+ATPase) and ER membrane proteins (KDEL). Furthermore, as evidenced by electron microscopy (Fig 2a, bottom panel), mitochondrial membrane integrity was maintained in this fraction.

We next found that cardioprotection induced by ethanol treatment 60 minutes prior to LAD occlusion correlated with translocation of εPKC to cardiac mitochondria, as compared with mitochondrial fractions isolated from rat hearts maintained under normoxic conditions or control hearts obtained from rats subjected to LAD ligation without ethanol pretreatment (Fig.

2b, c; n=7 animals/group). Furthermore, subfractionation experiments demonstrated that translocated εPKC was found to be associated with the inner mitochondrial membrane (data not shown). The total cellular levels of εPKC did not change by any of the treatments. Occlusion of the LAD alone (control hearts in Fig. 2b) did not result in translocation of εPKC, suggesting that ethanol treatment prior to the ischemic event is necessary for this εPKC translocation. In a recent publication, volatile anesthetics, such as sevoflurane, were shown to enhance ethanol-induced cardiac preconditioning through PKC activation [34]. Therefore, to determine the effect of the anesthetic used here, isoflurane, we injected control animals with 0.5 g/kg of ethanol followed 60 minutes later by an acute lethal dose of sodium pentobarbital. This study confirmed that isoflurane did not activate εPKC and that ethanol alone was not sufficient to induce translocation of εPKC (Fig. 2d; n=3 animals/group). Importantly, the lack of protection associated with ethanol treatment 15 minutes prior to LAD occlusion correlated with a lack of translocation of εPKC to the mitochondria (Fig. 2e, n=4 animals/group). These data suggest that translocation of εPKC may be dependent upon an ethanol-mediated priming event in the cytosol, which enables εPKC activation and translocation to cardiac mitochondria following I/R.

As seen in Figures 2b, d, e, two εPKC immunoreactive bands of 87 and 95 kDa are present in this fraction. To determine if these bands represent two different forms of εPKC, we subjected mitochondrial homogenate isolated from wild type and εPKC knockout mice to Western blot analysis. It was determined from these studies that the lower 87kDa band is a non-specific immunoreactive band and does not represent εPKC (insert Figure 2c).

We recently found that ALDH2 activation is required and sufficient to mediate cardioprotection from ischemic injury [29]. Since ethanol treatment protects the heart from injury and, in conjunction with I/R induces εPKC translocation to the mitochondria, we hypothesized that εPKC may increase ALDH2 activity to diminish the accumulation of toxic aldehydes, which damage the heart during I/R. Co-immunoprecipitation experiments conducted on mitochondria isolated from hearts subjected to I/R with and without ethanol showed that εPKC (95kDa) associated with ALDH2 (Fig 3a; n=4 animals/group) and ethanol treatment 60 minutes before I/R resulted in a 70% increase in ALDH2 activity (Fig. 3c; n=7 animals/group, $p<0.05$). There was no difference in ALDH2 activity between sham-treated rats and rats subjected to LAD ligation without ethanol treatment (control; insert figure 3c). Furthermore, ethanol treatment 15 minutes prior to I/R did not result in ALDH2 activation, correlating with our earlier findings that these conditions do not induce ethanol-mediated protection (Figure 3c; n=7 animals/group).

Ethanol is metabolized to acetaldehyde by alcohol dehydrogenase (ADH) and is further converted to acetate by the mitochondrial enzyme aldehyde dehydrogenase-2 (ALDH2). However, this mechanism is unlikely responsible for ethanol-mediated cardiac protection from I/R. During cardiac I/R, accumulation of the toxic aldehyde, 4-hydroxynonenal (4-HNE), has been proposed to be an indicator of oxidative damage and reperfusion injury [35–40]. We therefore determined whether the increased interaction with and activation of ALDH2 by εPKC affects 4-HNE levels. We found that the increase in enzymatic activity of ALDH2 (Fig. 3), was associated with a decrease in the formation of select HNE-protein adduct accumulation within the mitochondria (Figure 4, n=5 animals/group). These data suggest that the protective effects of ethanol may proceed through increased detoxification of the cytotoxic and highly reactive aldehyde, 4-HNE due to ALDH2 activation.

Pro-oxidants in the heart can lead to apoptosis *via* activation of JNK1/2 and ERK1/2 signaling pathways [41]. Additionally, 4-HNE was found to directly modify and activate JNK isoforms in hepatocytes and PC12 cells resulting in apoptosis [42,43]. In this study, we found that both cardiac isoforms of JNK1/2 (p46 and p54) and ERK1/2 (p42 and p44) were hyper-

phosphorylated after LAD occlusion, *in vivo* (Fig. 5a, b; n=3 animals/group). Intraperitoneal injection of 0.5g/kg of ethanol 60 minutes prior to I/R blocked this activation, suggesting that the cardioprotective effects of ethanol may proceed through εPKC-mediated activation of ALDH2 to diminish the accumulation of 4-HNE and downstream pro-death signaling.

DISCUSSION

In the current study, we have identified a mitochondrial target of εPKC and have elucidated a protective mechanism of ethanol-mediated preconditioning *in vivo*. Our results demonstrate for the first time that acute administration of ethanol 60 minutes prior to coronary occlusion results in εPKC translocation to cardiac mitochondria, ALDH2 and εPKC mitochondrial association, increased ALDH2 activity, diminished HNE-protein adduct formation and decreased pro-death signaling.

Many epidemiological studies in the past decade have demonstrated that chronic moderate consumption of ethanol decreases the risk of myocardial infarction [1–6]. We have previously found that acute ethanol exposure before ischemia is sufficient to protect the heart from damage [8]. Using an isozyme-selective inhibitor, we demonstrated that εPKC mediates the protective effects of ethanol in an adult rat heart *ex vivo* model of I/R and in isolated cardiac myocytes *in vitro* [8]. Several groups have shown in *ex vivo* models of I/R that ethanol-mediated protection is dependent upon the sustained activation of εPKC [9,24,44]. While it is clear that ethanol can induce the translocation of εPKC, it was not clear where the enzyme translocates to within the cell. Other cardioprotective phenomena utilizing different models have shown that protection is dependent upon εPKC activation [45–49]. Ohnuma *et al.* demonstrated that this cardioprotection coincided with εPKC translocation to the mitochondria [33] and we show here that similarly, ethanol administration 60 minutes prior to ischemia was sufficient to induce translocation of εPKC to cardiac mitochondria during I/R. We also show that the protective effects of ethanol and translocation of εPKC did not occur if ethanol was administered only 15 minutes prior to LAD ligation. These data further support our conclusion that the mitochondrial association of εPKC plays a critical role in cardioprotection.

Our findings further support the body of work suggesting that ethanol administration immediately prior to the ischemic period does not protect the heart from I/R-mediated injury [15,18–20,31]. Krenz *et al*. demonstrated that ethanol administrated immediately prior to ischemia did not reduce infarct size induced by I/R in a rabbit MI model [9,19]. In the same model, when ethanol was infused one hour prior to ischemia, infarct size was reduced. Ethanol is converted to acetaldehyde *via* alcohol dehydrogenase, which in turn is metabolized to acetic acid mainly by ALDH2 [50]. It is possible that the accumulating acetaldehyde competes for 4-HNE metabolism by ALDH2 allowing the accumulation of reactive 4-HNE thereby increasing 4-HNE-protein-adduct formation, and causing subsequent cellular injury.

We show here that εPKC translocation to the mitochondria was not induced by either ethanol or ischemia alone, supporting the hypothesis of a delayed two-part mechanism of εPKC activation and suggesting that a priming step is necessary for εPKC translocation and protection of the ischemic heart; here this priming was induced by ethanol. We further propose that in addition to this priming step, a second activation event occurring during ischemia is necessary for εPKC translocation to the mitochondria; ethanol treatment without a subsequent ischemic event did not induce mitochondrial translocation. Recently, εPKC has been shown to be activated by mild reactive oxygen species (ROS) [51]. We therefore propose that ROS, which is generated during ischemia [52] may oxidatively modify εPKC [53], resulting in its entry into the mitochondria in a mechanism that has yet to be determined. If the initial priming step does not occur and if sufficient time is not allowed for this second step to occur, εPKC cannot translocate into the mitochondria, and cardiac protection is not afforded.

Translocation of εPKC to cardiac mitochondria suggests that these organelles may play a role in ethanol-mediated cardioprotection. It is well established that mitochondria can regulate cardiac injury during ischemia and reperfusion through increased ROS generation [52,54,55], the release of pro-apoptotic molecules [56] and alterations in energy utilization [57,58]. Additionally, there are several mitochondrial targets of εPKC which protect the heart from I/R injury through mechanisms including ROS generation [59,60], opening of the mitochondrial permeability transition pore (MPTP) [61,62], regulation of the mitochondrial kATP channels (reviewed in [63,64]) and activation of mitochondrial ALDH2 [29]. One key mediator of cellular injury that accumulates during I/R is the toxic aldehyde 4-HNE [40]. Under physiological conditions, 4-HNE may act as a signaling molecule [65]. However, under conditions of oxidative stress, accumulated 4-HNE modifies and regulates enzymes involved in mitochondrial energy production [39], resulting in increased ROS generation[66], diminished protein degradation [35] and increased pro-apoptotic signaling [42,66]. Because of the reactivity of this and other aliphatic and aromatic aldehydes, the cell has developed mechanisms to detoxify these molecules [65]. In addition to glutathione-S transferase, aldehyde dehydrognesase-2 (ALDH2) is a mitochondrial enzyme that detoxifies 4-HNE and other toxic aldehydes, thereby diminishing cellular oxidative stress. Therefore, since 4-HNE accumulation during ischemia [37] can damage the heart through oxidative mechanisms and ethanol protects the heart through activation and translocation of εPKC to cardiac mitochondria, we hypothesize that εPKC might regulate ALDH2 activity to increase detoxification of 4-HNE, thereby conferring protection by diminishing oxidative stress. Interestingly, human cardiomyocytes in which ALDH2 is overexpressed are significantly protected from acetaldehyde-induced ROS accumulation and apoptosis [67], suggesting that activation of ALDH2 may be a therapeutic target to reduce ischemic damage to the heart. In a recent study, we identified a small molecule activator of ALDH2 (alda-1) that induces 60% reduction in infarct size, *in vivo*, an effect that is similar to the cardioprotection seen with ethanol here (Fig. 1). These data demonstrate that ALDH2 activation is required and sufficient to produce cardioprotection from ischemic injury. Together with the current study, these data suggest that εPKC translocates to cardiac mitochondria where it interacts with ALDH2, and phosphorylates and activates that enzyme to protect the heart from the injury induced by ischemia and reperfusion, *in vivo*. Ethanol-induced and εPKC-mediated activation of ALDH2 increase the metabolism of the reactive aldehyde, HNE, to HNA. This prevents HNE-protein adduct formation and diminishes pro-apoptotic signaling, thus improving cardiac function.

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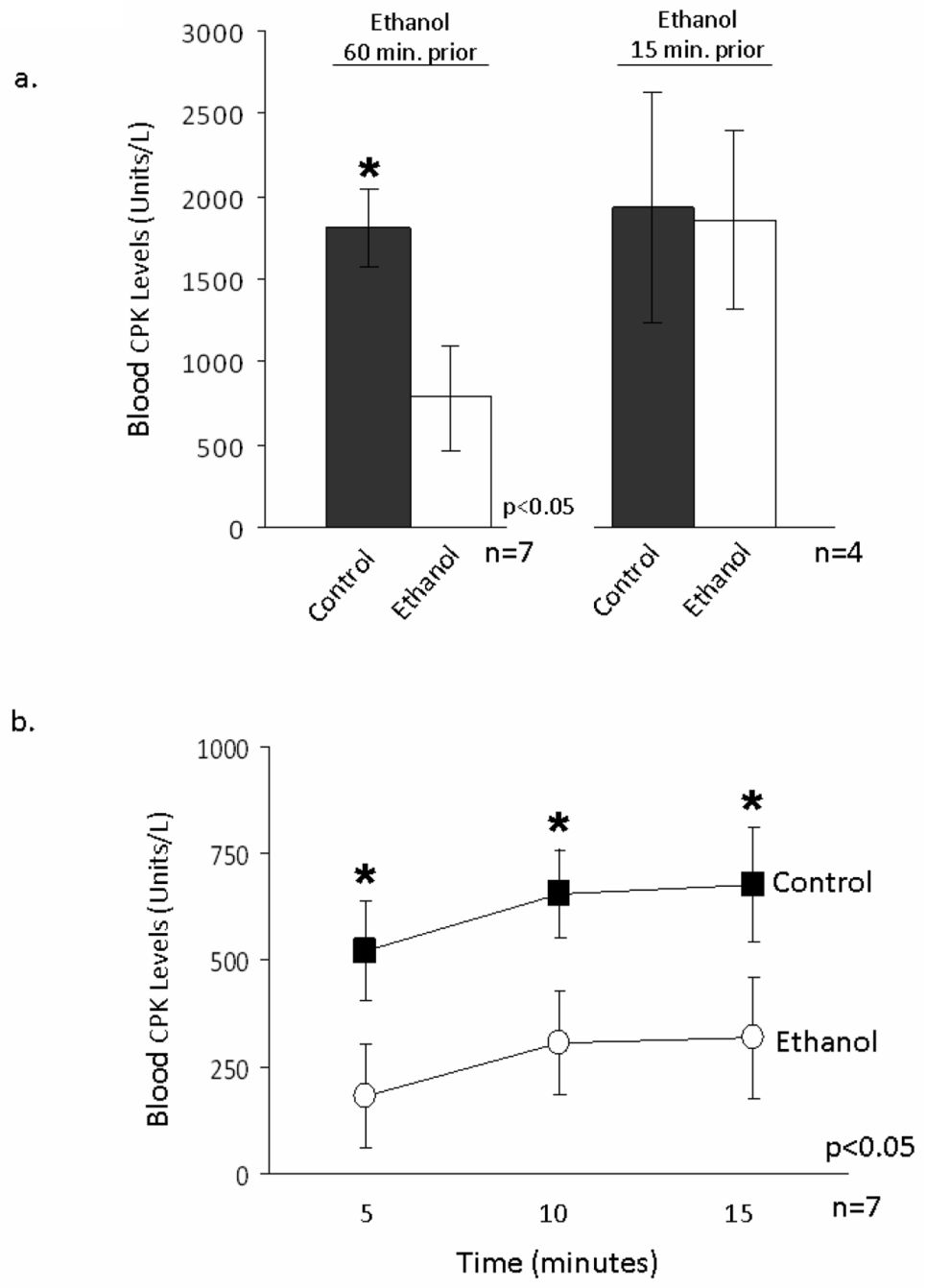
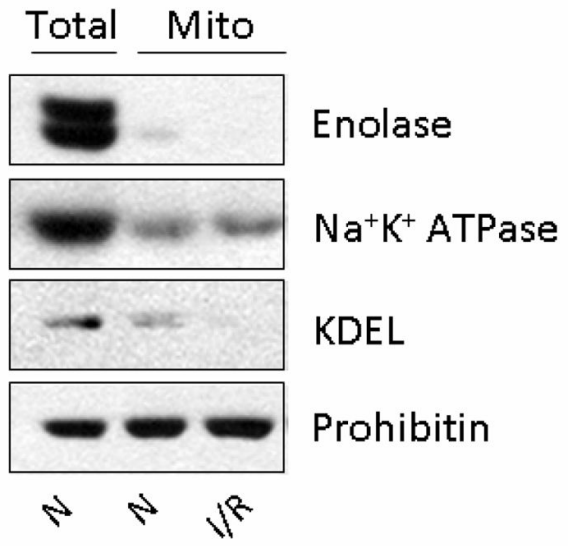


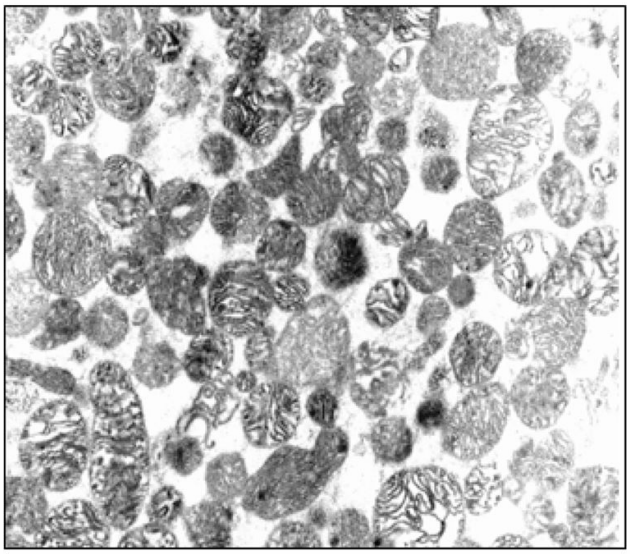
Figure 1. Ethanol decreases CPK release into the blood in an *in vivo* model of acute myocardial infarction

Rats were i.p. injected with 0.5 g/kg of ethanol 15 or 60 minutes prior to occlusion of the left anterior descending (LAD) coronary artery (ethanol treated) and compared to animals, which were injected with saline (control treated). Hearts were reperfused for 15 minutes by release of the ligature and humoral creatine phosphokinase (CPK) levels were monitored every five minutes in blood drawn through a cardiac apical punch. CPK released over the 15 minutes of reperfusion was summed together and expressed as % control. CPK values from sham animals that underwent surgery, but did not undergo coronary artery ligation were subtracted from the experimental groups. Comparisons between multiple groups were made using analysis of

variance (ANOVA) with a Newman-Keuls multiple comparison test and individual group comparisons were made with a student's t-test. The number of animals/group with standard error and statistical significance for all data are listed in the figures with a p-value of 0.05 being considered significant. (a) A significant difference in CPK release was seen with ethanol administration 60 minutes prior to ischemia but not in animals injected 15 minutes prior. (b) CPK release from animals injected with ethanol 60 minutes prior to occlusion were monitored every five minutes and expressed as blood CPK units/L. Ethanol significantly decreased CPK release relative to controls at each time point tested.

a.





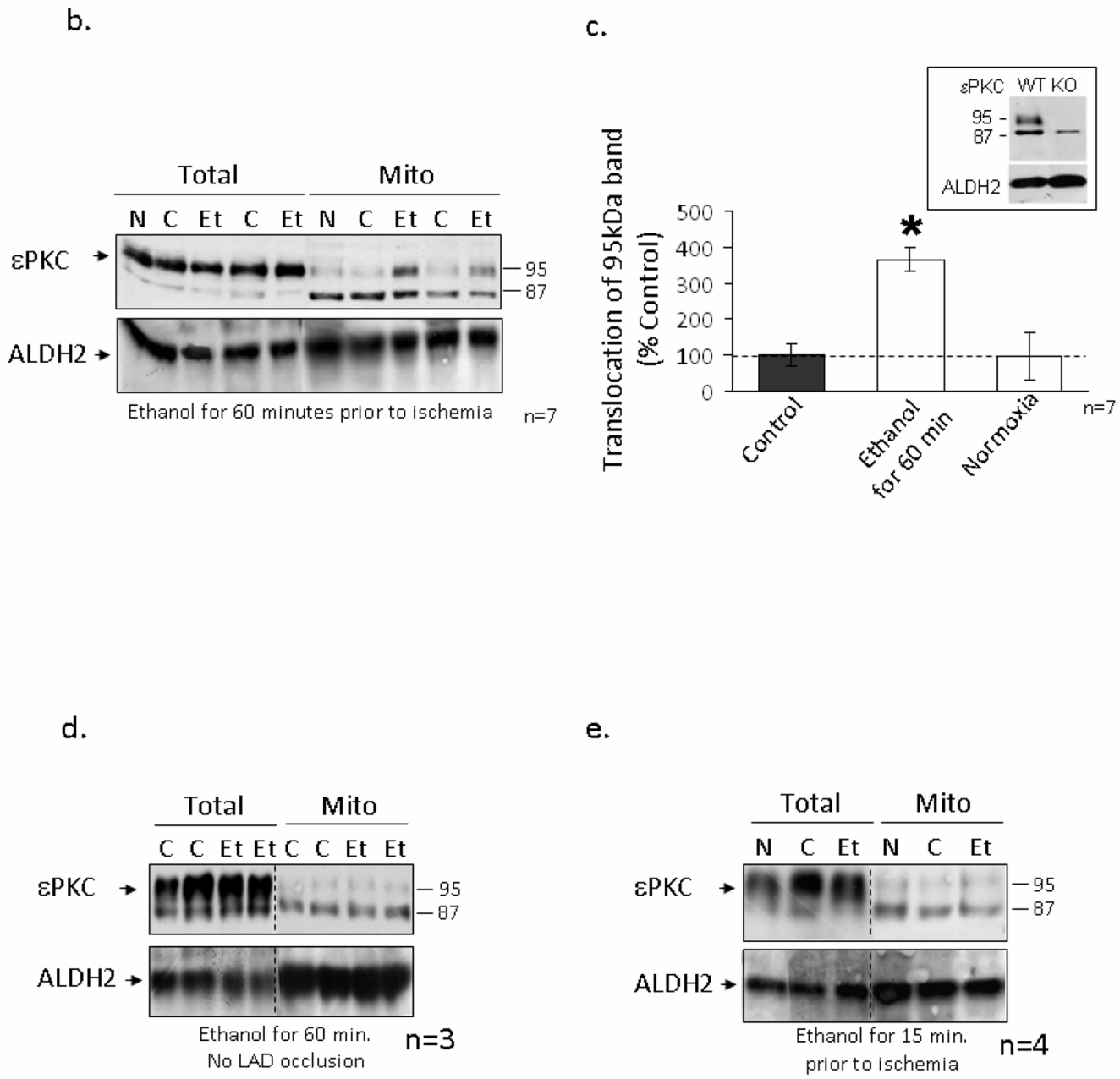


Figure 2. Ethanol-mediated protection is associated with εPKC translocation to cardiac mitochondria

Mitochondria were isolated from the left ventricles of animals that were treated with 0.5 g/kg of ethanol 15 and 60 minutes prior to LAD occlusion (Et) and compared to animals that were not treated with ethanol (C) and to animals that were not subjected to LAD occlusion (N). Following homogenization, mitochondrial lysates and total cellular lysates were analyzed by Western blot analysis utilizing an anti-εPKC antibody and equal loading was determined by monitoring the levels of mitochondrial ALDH2. (a) Electron micrographs illustrating the membrane integrity of isolated mitochondria. Additionally, the presence of mitochondrial protein (prohibitin), and the lack of cytosolic proteins (enolase), plasma membrane proteins (Na^+K^+ ATPase), and ER resident proteins (KDEL) illustrates the purity of our mitochondrial

preparation. (b) Data from two separate groups of animals treated with ethanol 60 minutes prior to LAD ligation are shown. Two different molecular weight forms of mitochondrial εPKC are marked with arrows. (c) Quantification of εPKC translocation to the mitochondria from 7 different experiments was done using NIH Image-J software, expressed as % control and significance was determined with a 2 way t-test (*= $p<0.05$). Significant changes were observed between the ethanol treated and control and normoxic hearts. (1c Insert) Hearts from wildtype and εPKC knock-out mice were isolated and homogenized for Western blot analysis. The lower 87kDa band is present in both wildtype and knock-out animals suggesting non-specific antibody recognition while the upper band represents εPKC. (d) Animals were subjected to ethanol treatment without LAD occlusion and data from two representative experiments are shown. (e) Representative data from 4 separate experiments from animals treated with ethanol 15 minutes prior to LAD ligation are shown.

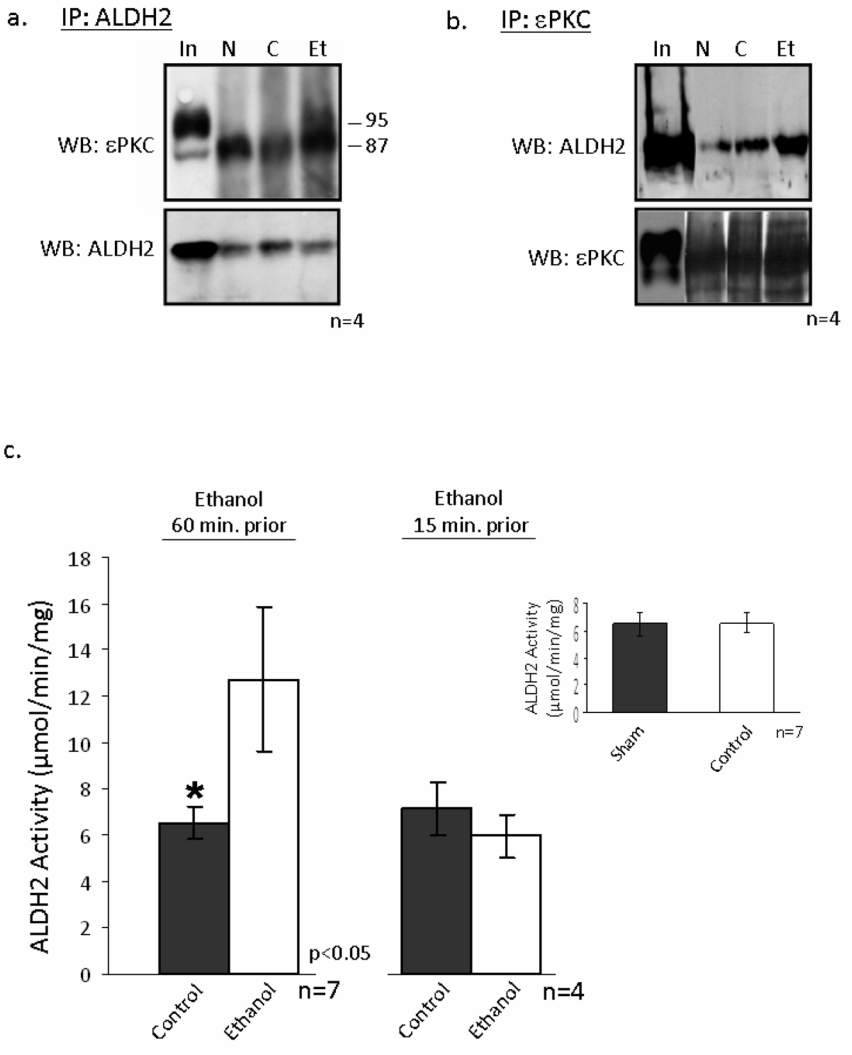


Figure 3. εPKC associates with mitochondrial ALDH2 following ethanol treatment

Mitochondrial protein was isolated from the left ventricles of animals that were administered 0.5 g/kg of ethanol 60 minutes prior to LAD occlusion (Et) and compared to animals that were not treated with ethanol (C) and animals which did not undergo LAD occlusion (N). (a) Following homogenization 700 μg of mitochondrial protein was immunoprecipitated with an anti-ALDH2 antibody and subjected to Western blot analysis with anti-εPKC. As a control, mitochondrial lysate was used (In). (B) Proteins were subjected to a reverse immunoprecipitation using the antibodies listed in the Figure. (C) ALDH2 activity was measured in mitochondrial fractions isolated from control and animals treated with ethanol 15 and 60 minutes prior to LAD occlusion and the results are expressed as μmole NADH produced/ minute/mg of protein of either 7 or 4 independent experiments, respectively. Differences in activity (as determined by a two way t-test) were observed between the control and ethanol groups of the animals injected with ethanol 60 minutes prior to ischemia (*= $p<0.05$) but not in animals treated 15 minutes prior. (Insert) There was no significant difference in ALDH2 activity between sham and control treated animals.

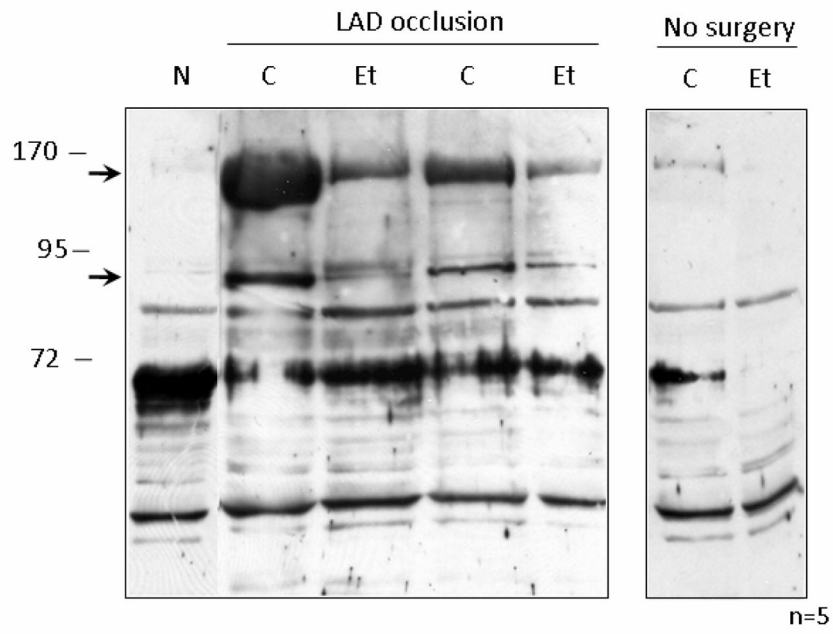


Figure 4. Ethanol treatment reduces HNE protein-adduct formation following I/R

Mitochondrial protein was isolated from the left ventricles of animals that were administered 0.5 g/kg of ethanol 60 minutes prior to LAD occlusion (Et) and compared to animals that were not treated with ethanol (C) and animals which did not undergo LAD occlusion (N). The right panel shows basal levels of HNE formation in cardiac mitochondria that were not subjected to surgery. Following homogenization, mitochondrial lysate was analyzed by Western blot utilizing antibodies which recognize HNE protein adducts. Two protein bands which showed a reduction upon in HNE-adduct formation upon ethanol treatment are denoted by arrows.

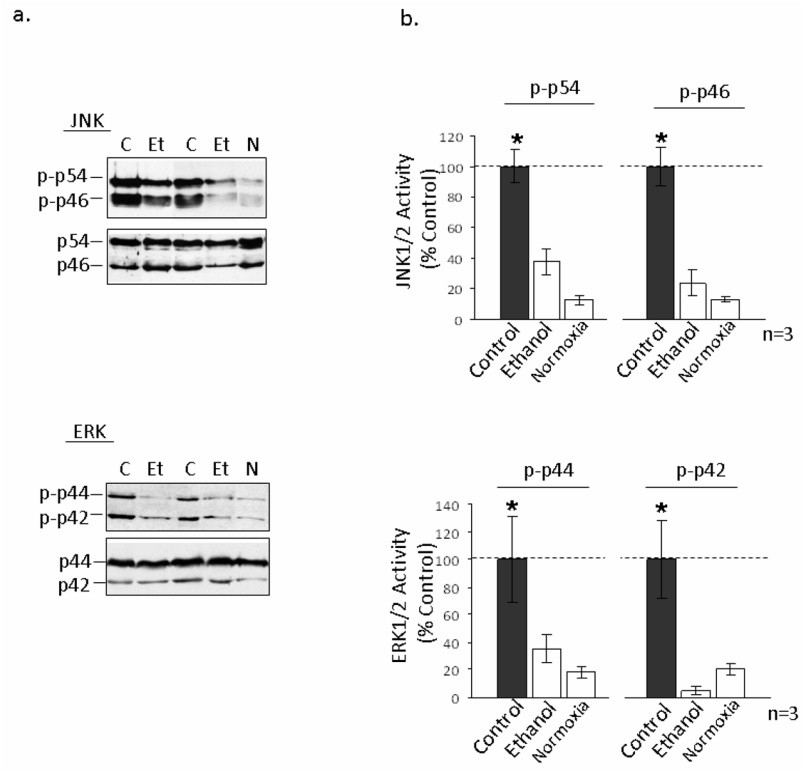


Figure 5. Ethanol treatment decreases activation of MAPK pathway signaling molecules

Mitochondrial protein was isolated from the left ventricles of animals that were administered 0.5 g/kg of ethanol 60 minutes prior to LAD occlusion (Et) and compared to animals that were not treated with ethanol (C) and animals which did not undergo LAD occlusion (N). (a) Following homogenization, cardiac total lysate from the left ventricle was analyzed by Western blot to determine the phosphorylation levels of JNK1/2 (p-p46 and p-p54), and Erk1/2 (p-p44 and p-p42). Data from two separate groups of experiments are shown. (B) Quantification of the data in panel a was done using NIH Image-J software, expressed as % control and significance was determined using a 2 way t-test.