Moderate alcohol consumption induces sustained cardiac protection by activating PKC-ε and Akt

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CORONARY HEART DISEASE is the leading cause of death in the United States (2). Existing therapies, including aspirin, heparin, β-adrenergic receptor antagonists, and platelet GP IIb/IIIa receptor antagonists improve prognosis associated with acute coronary syndromes. However, patients with these disorders remain at high risk of reinfarction or death for months and would benefit from novel cardioprotective strategies during this period (2). Numerous pharmacological agents protect the heart acutely against ischemia-reperfusion injury and reduce the severity of myocardial infarction (18). The utility of these agents is limited by a brief duration of action and loss of efficacy with repeated use (45). Currently, few experimental models exist in which protection against myocardial reperfusion injury can be maintained for weeks to months.

Moderate alcohol consumption has been shown to reduce coronary heart disease in some epidemiological studies (9, 29, 40). Canine (31) and rodent (25, 26, 47) models confirm the benefits observed in human studies and they exhibit sustained cardioprotection for 10 mo or longer (47) with continued oral administration of alcohol. Chronic ethanol (EtOH) feeding of guinea pigs (27) upregulates protein kinase C (PKC)ε, a signal transduction molecule required for the cardioprotective effects of acute ischemic preconditioning (38). EtOH activates ATP-sensitive K+ channels, putative end effectors of cardioprotection (31, 47). Therefore, animal models of moderate alcohol consumption may be useful for identification of novel therapeutic targets for sustained protection against coronary heart disease in humans.

In the present study, inbred C57BL/6 mice were fed 18% EtOH (vol/vol) in drinking water for 12 wk and developed substantial cardioprotection, measured as increased contractile recovery and decreased creatine kinase (CK) release during reperfusion. Chronic EtOH feeding also increased expression and activation of PKC-ε and Akt, intracellular kinases closely linked to cardiac growth and survival (8, 23, 38, 44). Importantly, inhibition of PKC-ε with an isozyme-selective translocation antagonist (3, 13, 39) blocked cardioprotection and increases in Akt activity induced by moderate alcohol consumption. Our results strongly support low-level expression of activated PKC-ε as a cause of increased resistance to reperfusion injury and suggest that therapeutic agents can be developed to produce sustained cardioprotection in humans.

METHODS

All experimental protocols were reviewed and approved by the Animal Care Subcommittee of the San Francisco Veterans Affairs Medical Center. All protocols conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the “Guiding Principles in the Care and Use of Animals” of the American Physiological Society.

Animals. Male C57BL/6 mice were purchased from Charles River Laboratories (Hollister, CA) and divided ran-
domly into an alcohol consumption group (EtOH) and an age-matched control group (control). All mice received standard rodent chow and water ad libitum. Beginning at the age of 2 mo, EtOH-fed mice received 2.5% EtOH (vol/vol) for 1 wk to acclimate to drinking alcohol. EtOH mice were fed 5% EtOH in drinking water during the second week, 10% EtOH during the third week, and 18% EtOH for 12 wk. To investigate whether cardioprotection persisted after cessation of EtOH dosing, EtOH solutions were removed from cages of selected mice (EtOH withdrawn) 16 h before death. Alcohol concentrations in venous blood were measured using a commercial kit (Sigma; St. Louis, MO).

Isolated isovolumic mouse heart preparation. Mice were heparinized (1,000 U/kg ip) and anesthetized with pentobarbital sodium (60 mg/kg ip). Hearts were excised, washed in cold arresting solution composed of 120 mmol/l NaCl and 30 mmol/l KCl, and cannulated via the aorta (14). Hearts were paced at 6 Hz with the use of platinum-tipped electrodes connected to a stimulus generator (Grass Instruments; Quincy, MA) and perfused at 70 mmHg on a modified Langendorff apparatus using Krebs-Henseleit solution containing (in mmol/l) 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 2.0 KH2PO4, 24 NaHCO3, 5.5 glucose, 5.0 Na pyruvate, and 0.5 EDTA (14). Left ventricular (LV) developed pressure (LVDP = LV systolic pressure – LV end-diastolic pressure (LVEDP)) was measured (Gould Electronics; Hayward, CA) with a micromanometer (Millar Instruments; Houston, TX) passed into a polyvinylchloride balloon within the LV cavity. Balloon volume was adjusted with water to preset the LVEDP at 10 mmHg. Coronary flow was measured by collecting effluent from the right ventricular outflow tract.

Peptide synthesis. Tat-PKC-ε antagonist peptide (YGRKKRRQRRR-EAVSLKPT) and Tat-PKC-ε scrambled antagonist peptide (YGRKKRKRRQRRR-LSETKPAV) were synthesized at the University of California at San Francisco Biomolecular Resource Center by 9-fluorenylmethoxycarbonyl (FMOC) chemistry using an Applied Biosystems 431A peptide synthesizer (13, 14, 39). Peptides were purified (>95%) by preparative reverse-phase high-performance liquid chromatography. Purity was confirmed by electrospray mass spectrometry.

Experimental protocol. After baseline hemodynamic parameters were recorded during a 20-min equilibration period, all mouse hearts were subjected to 20-min global ischemia and 30-min reperfusion (Fig. 1). Random control and EtOH hearts were pretreated with chelerythrine chloride (10 μmol/l) as a 5-min infusion or with PKC-ε antagonist peptide (5 μmol/l) as a 20-min infusion before ischemia-reperfusion with no washout period (Fig. 1).

Western analysis of PKC translocation. Left ventricles not subjected to ischemia-reperfusion were homogenized as described (13, 14). Samples of the 100,000-g supernatant and Triton X-100 extracted pellet fractions were adjusted for protein content, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose. PKC isozyme distribution among fractions was determined using selective primary antibodies (Transduction Laboratories; Lexington, KY) and enhanced chemiluminescence detection (Amersham; Piscataway, NJ). PKC immunoreactive bands were quantitated using NIH Image software. In separate experiments, whole cell lysates of EtOH and control hearts were subjected to SDS-PAGE, and transferred to nitrocellulose. Activated PKC-ε was detected using phosphorylation state-dependent primary antibodies (Upstate Biotechnology; Lake Placid, NY).

Measurement of Akt expression and kinase activity. Tissue lysates were subjected to SDS-PAGE and transferred to nitrocellulose. Akt expression was determined using phosphorylation state independent primary antibodies (New England Biolabs; Beverly, MA) and enhanced chemiluminescence detection reagents (Amersham). Akt kinase activity was measured using a commercially available kit (New England Biolabs). Briefly, hearts from control and EtOH mice

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**Fig. 1. Schematic illustration of the experimental protocol.** Control: hearts isolated from age-matched mice. Ethanol (EtOH): hearts from ethanol-fed mice. EtOH + Chel: hearts from EtOH mice pretreated with chelerythrine. EtOH + εV1-2: hearts from ethanol-fed mice pretreated with protein kinase C (PKCε) antagonist peptide. Ethanol withdrawn: ethanol removed 16 h before death.
were homogenized in cell lysis buffer containing (in mmol/l) 20 Tris-HCl (pH 7.5), 150 NaCl, 1 EDTA, 1 EGTA, 2.5 sodium pyrophosphate, 1 β-glycerolphosphate, 1 Na3VO4, and 1 phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin and 1% Triton X-100. Supernatants were immunoprecipitated overnight with Akt antibodies crosslinked to agarose hydrazide beads. Pellets were resuspended in kinase buffer containing (in mmol/l) 25 Tris-HCl (pH 7.5), 5 β-glycerolphosphate, 2 dithiothreitol, 0.1 Na3VO4, and 10 MgCl2. The kinase assay was initiated by addition of 200 μmol/l ATP and 1 μg glycogen synthase kinase (GSK)-3α fusion protein. After incubation for 30 min at 30°C, reaction mixtures were boiled in sample buffer, subjected to SDS-PAGE, and transferred to nitrocellulose. Relative Akt kinase activities in LV lysates in sample buffer, subjected to SDS-PAGE, and transferred to nitrocellulose were determined using phosphorylation state-dependent GSK-3α/β primary antibodies provided with the assay kit.

Measurement of CK release. Coronary effluent was collected throughout the reperfusion period. CK release was measured by enzyme spectrophotometric methods using a commercially available kit (Sigma). Values were corrected for coronary flow and heart weight.

Statistical analysis. Results are reported as means ± SE. Comparisons between groups were made using one-way ANOVA or repeated-measures ANOVA as indicated. Differences were confirmed with the use of a Bonferroni post hoc test. P < 0.05 was considered significant.

RESULTS

Moderate alcohol consumption does not alter body weight, heart weight, or baseline cardiac function. In the present study, male inbred C57BL/6 mice fed 18% EtOH for 12 wk were found to have blood alcohol concentrations of ~5 mmol/l (24.1 ± 2.6 mg/dl, n = 18). Withdrawal of EtOH from drinking water 16 h before death reduced blood alcohol concentrations to 0.7 mmol/l (3.3 ± 1.3 mg/dl, n = 6). Body weights of EtOH-fed mice were similar to those of age-matched controls (34.3 ± 0.9 vs. 36.5 ± 1.1 g for control), as were wet heart weights (154 ± 4 vs. 164 ± 5 mg for control). As shown in Table 1, EtOH feeding had no effect on baseline LVDP (97 ± 4 vs. 98 ± 4 mmHg for control) or coronary flow (3.7 ± 0.2 vs. 3.6 ± 0.02 ml/min for control).

EtOH feeding causes sustained improvement of cardiac contractile recovery during reperfusion. As shown in Table 1 and Fig. 2A, LVDPs in hearts isolated from mice fed 18% EtOH for 12 wk were greater than in control hearts during reperfusion. LVDP recovered to 68 ± 8 mmHg in the EtOH group versus only 33 ± 8 mmHg in the control group (n = 10, P < 0.05). Improvements in contractile recovery were still evident when EtOH was withdrawn from drinking water 16 h before ischemia-reperfusion. LVDP recovered to 67 ± 6 mmHg in the EtOH withdrawn group (n = 6, P < 0.05 vs. control). As shown in Fig. 3A, prior EtOH exposure blunted the pathological rise in LVEDP during reperfusion (21 ± 4 vs. 37 ± 3 mmHg for control; n = 10, P < 0.05). Improvements in LVEDP were also evident in the EtOH withdrawn group (19 ± 3 mmHg; n = 6, P < 0.05 vs. control). Therefore, moderate alcohol consumption induces sustained protection against ischemia-reperfusion injury in mouse hearts that persists for at

Table 1. Summary of hemodynamic data

<table>
<thead>
<tr>
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<th>Baseline</th>
<th>30-min Reperfusion</th>
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<tr>
<td></td>
<td>LVDP, mmHg</td>
<td>LVEDP, mmHg</td>
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<tr>
<td>Control</td>
<td>98 ± 4</td>
<td>10 ± 3.6 ± 0.2</td>
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<tr>
<td>EtOH</td>
<td>97 ± 4</td>
<td>10 ± 3.7 ± 0.2</td>
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<tr>
<td>EtOH + Chel</td>
<td>96 ± 5</td>
<td>10 ± 3.7 ± 0.1</td>
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<tr>
<td>EtOH + eV1-2</td>
<td>95 ± 2</td>
<td>10 ± 3.5 ± 0.1</td>
</tr>
<tr>
<td>EtOH withdrawn</td>
<td>98 ± 5</td>
<td>10 ± 3.5 ± 0.2</td>
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Values are means ± SE; n = 8–10 mice per treatment group. LVDP, left ventricular developed pressure; LVEDP, LV end-diastolic pressure; CF, coronary flow; Chel, chelerythrine; EtOH, ethanol. Baseline LVEDP was preset at 10 mmHg. *P < 0.05 vs. control, EtOH + Chel, and EtOH + eV1-2; †P < 0.05 vs. control, EtOH + Chel, and EtOH + eV1-2.
Acute PKC inhibition with chelerythrine blocks sustained cardioprotection. When we developed our model of moderate alcohol consumption, one specific aim was to determine whether cardioprotective signaling pathways activated by acute preconditioning (18) contribute to sustained resistance to reperfusion injury. In the present study, we added the nonisoenzyme-selective PKC inhibitor chelerythrine chloride (10 μmol/l) to coronary perfusate for 5 min before global ischemia. Chelerythrine pretreatment had no effect on baseline contractile function or coronary flow (data not shown) but blocked EtOH-mediated improvement of LV contractile recovery during reperfusion. As shown in Table 1 and Fig. 2B, LVDP recovered to only 43 ± 9 mmHg in the EtOH + Chel group [n = 8, P = not significant (NS) vs. control]. As shown in Table 1 and Fig. 3, chelerythrine pretreatment blocked improvement of LVDP during reperfusion (37 ± 7 vs. 37 ± 3 mmHg for control; n = 8, P = NS) and increased CK release. Therefore, PKC inhibition blocks enhanced resistance to reperfusion injury in hearts from EtOH-fed mice.

Moderate alcohol consumption causes low-level cardiac expression of activated PKC-ε. Substantial experimental evidence suggests that PKC-ε isoform activation is necessary for the cardioprotective effects of many forms of acute ischemic and pharmacological preconditioning (38). Localization of PKC isoforms to cellular particulate fractions is one recognized sign of activation (19). In the present study, we measured PKC isoform expression in left ventricles isolated from control and EtOH-fed mice with the use of Western blot analysis. As shown in Fig. 4A, EtOH feeding more than doubled total LV expression of PKC-ε and increased activated PKC-ε localized to particulate fractions (65 ± 6 vs. 34 ± 4 density units for control; n = 6, P < 0.05).

In contrast, chronic EtOH feeding had no effect on either relative or absolute subcellular distributions of PKC-ε.

Improvement of contractile recovery correlates with reduction of myocardial injury. We measured CK activity in coronary effluent to test whether moderate alcohol consumption improves contractile recovery by preventing cardiac myocyte injury and loss of membrane integrity. As shown in Fig. 3B, prior EtOH exposure reduced CK release during reperfusion (0.26 ± 0.04 vs. 0.51 ± 0.08 U·min⁻¹·g wet wt⁻¹ for control; n = 10, P < 0.05). Reduction of CK release was also evident after EtOH withdrawal (0.30 ± 0.02 U·min⁻¹·g wet wt⁻¹; n = 6, P < 0.05 vs. control). Therefore, EtOH-induced improvement of cardiac contractile recovery during reperfusion correlates with reduction of myocardial injury, and cardioprotection persists for at least 16 h despite negligible serum EtOH concentrations at death.

![Fig. 3. Chronic EtOH feeding reduces cardiac ischemia-reperfusion injury. A: LV end-diastolic pressure (LVEDP) was measured during reperfusion in 5 groups of hearts (n = 8–10 each). Pathological elevation of LVEDP was reduced in hearts from ethanol-fed mice. PKC inhibition blocked ethanol-mediated cardioprotection (P = NS vs. control). B: creatine kinase (CK) release was measured during reperfusion in 5 groups of hearts (n = 8–10 each). CK release was reduced in hearts from ethanol-fed mice, and PKC inhibition blocked ethanol-mediated cardioprotection. *P < 0.05 vs. control; **P < 0.05 vs. control.](image)

Fig. 4. Moderate alcohol consumption increases PKC-ε protein expression and activation. A: Western blots of soluble and particulate fractions from control and EtOH hearts were probed with PKC isoform-selective antibodies. Each lane represents equal protein loading of fractions prepared from single hearts. Results are representative of two independent experiments (n = 6 per group). B: Western blots of LV lysates from control and EtOH hearts were probed with phosphorylation state-dependent antibodies against the COOH-terminal hydrophobic priming site of PKC-ε (Ser729). Each lane represents equal protein loading of lysates prepared from single hearts. Results are representative of two independent experiments (n = 6 per group).
PKCa, -ß, -δ, or -λ (data not shown), the other PKC isozymes present in adult mouse myocardium (6, 37).

PKC isozyme conformation and function are also regulated by phosphorylation of molecules at activation loop, COOH-terminal tail, and COOH-terminal hydrophobic priming sites (32). Phosphorylation of the COOH-terminal hydrophobic priming site (Ser729) of PKC-ε increases kinase activity, whereas absence of phosphorylation at this priming site reduces kinase activity (33). In the present study, we measured phosphorylation of Ser729 of PKC-ε in LV lysates prepared from EtOH-fed mice and age-matched controls. As shown in Fig. 4B, chronic EtOH feeding more than doubled the expression of phosphorylated PKC-ε (46 ± 3 vs. 19 ± 4 density units for control; n = 6, P < 0.01). Because in vivo phosphorylation of PKC-ε at its priming sites requires diacylglycerol and the coordinated activation of 3-phosphoinositide-dependent kinase (PDK-1), mammalian target of rapamycin, and intracellular kinases responsible for phosphorylation of Ser729 (33), these results strongly support chronic activation of cardiac PKC-ε in response to moderate alcohol consumption.

Selective inhibition of PKC-ε translocation blocks sustained cardioprotection. Increasing evidence suggests that many of the biological actions of chelerythrine may be mediated by mechanisms other than PKC inhibition. For example, Yu et al. (46) demonstrated that chelerythrine activates extracellular mitogen-activated protein kinase-1 (MEKK1)- and mitogen-activated protein kinase kinase-4 (MEKK4)-dependent p38 and c-Jun NH₂-terminal kinase pathways in HeLa cells without inhibiting PKC. Because of concerns regarding the non-PKC effects of chelerythrine in our model, we also perfused hearts from control and EtOH-fed mice with a PKC inhibitor peptide that selectively disrupts binding of activated PKC-ε to anchoring proteins, or receptors for activated C kinase (RACKs) (13, 14, 39). Chen et al. (3) recently used isozyme-selective peptide inhibitors of PKC function in adult rat cardiac myocytes and ex vivo rat heart models to study opposing actions of PKCβs and PKC-ε in ischemia-reperfusion injury and cardioprotection. Importantly, these investigators found protein transduction an efficient means for delivery of bioactive peptides into intact myocardium and confirmed that these agents block PKC isozyme function by inhibiting translocation and not by altering enzymatic activity (3).

In the present study, we added a peptide inhibitor of PKC-ε translocation termed εV1–2 linked to an amino acid sequence derived from the protein transduction domain of human immunodeficiency virus (HIV) recombinant protein Tat (3, 13, 14) to coronary perfusate (5 μmol/l) for 20 min before global ischemia. Tat-PKC-ε antagonist peptide had no effect on baseline contractile function or coronary flow but blocked EtOH-mediated improvement of LV contractile recovery during reperfusion. As shown in Table 1 and Fig. 2B, LVDP recovered to only 32 ± 4 mmHg in the EtOH + V1–2ε group (n = 8, P = NS vs. control). As shown in Table 1 and Fig. 3, pretreatment with Tat-PKC-ε antagonist peptide blocked improvement of LVEDP during reperfusion (34 ± 3 vs. 37 ± 3 mmHg for control; n = 8, P = NS) and increased CK release. Tat-PKC-ε scrambled antagonist peptide (3, 13, 14) had no effect on LV contractile recovery or CK release during reperfusion. Therefore, selective disruption of protein-protein interactions between activated PKC-ε and RACKs also blocks sustained cardioprotection induced by moderate alcohol consumption.

Cardiac Akt expression and kinase activity increase during chronic EtOH feeding. There is current intense interest regarding the role of Akt signaling in cardioprotection. Studies using gene therapy techniques suggest that increased Akt kinase activity is sufficient to improve LV contractile recovery after transient ischemia. For example, Matsui et al. (23) observed that in vivo gene transfer of a constitutively active Akt mutant into rat heart restores regional wall thickening and maximal rates of LV pressure rise and fall after ischemia-reperfusion to levels seen in sham-operated rats. Increased Akt activity may also preserve cardiac function during periods of oxidative stress by reducing myocyte apoptosis. For example, Fujiy et al. found that in vivo gene transfer of constitutively active Akt into mouse heart reduces cardiac myocyte apoptosis after ischemia-reperfusion (8). Conversely, Yamashita et al. (44) used insulin-like growth factor-1 overexpressing (Igf-1/v–) transgenic mice to demonstrate that reperfusion-mediated activation of Akt is required for resistance to myocyte apoptosis.

In the present study, we measured Akt protein expression in LV lysates prepared from control and EtOH hearts with the use of Western blot analysis. As shown in Fig. 5A, EtOH feeding increased cardiac Akt levels threefold (111 ± 17 density units vs. 39 ± 5 density units for control; n = 6, P < 0.01). We measured cardiac Akt activity by subjecting immunoprecipitates obtained from LV lysates using Akt antibodies to an in vitro kinase assay, including the Akt-selective substrate GSK-3α (5). As shown in Fig. 5B, phosphorylation of GSK-3α was greater in samples from EtOH hearts than in control samples (65 ± 11 vs. 12 ± 1 density units for control; n = 6, P < 0.01), indicating a concomitant increase in Akt kinase activity. Therefore, alcohol consumption upregulates expression and function of the cardioprotective protein Akt, an effect that may account in part for improvement of contractile recovery and reduction of CK release observed during reperfusion.

Selective inhibition of PKC-ε translocation blocks increased Akt kinase activity. The cellular mechanisms through which increased expression of PKC-ε leads to sustained protection against ischemia-reperfusion injury have not been fully explored. Proteomic analysis of cardiac lysates from PKC-ε transgenic mice developed by Ping et al. (35) revealed that active PKC-ε physically associates with >30 different proteins localized to multiple subcellular compartments within cardiac myocytes, including Akt. Importantly, those investigators found that doubling of PKC-ε activity produces resistance to ischemia-reperfusion injury and greater...
translocation termed H9280. In vivo hearts with a peptide inhibitor of PKC-ε attenuations as in our model. They did not determine n for control; P < 0.05). As shown in Fig. 6A, bottom, removal of ethanol did not reduce phosphorylation of the COOH-terminal hydrophobic priming site (Ser729) of PKC-ε (126 ± 6 vs. 35 ± 10 density units for control; n = 4, P < 0.05).

Western analysis of EtOH withdrawn hearts (Fig. 6B, top) revealed continued elevation of Akt expression (88 ± 4 vs. 57 ± 5 density units for control; n = 4, P < 0.05). Increased Akt kinase activity (Fig. 6B, bottom) was also evident in EtOH withdrawn hearts (86 ± 6 vs. 52 ± 4 density units for control; n = 4, P < 0.05). Therefore, both cardioprotection (Figs. 2 and 3) and myocardial activation of PKC-ε and Akt persist for at least 16 h after removal of EtOH from drinking water. These results suggest that once enhanced resistance to reperfusion injury is established, less frequent EtOH dosing may be sufficient to maintain the cardioprotective effects of moderate alcohol consumption.

than fivefold increase in cardiac Akt expression, responses comparable to those observed when native PKC-ε is activated by exposure to moderate EtOH concentrations as in our model. They did not determine whether activated PKC-ε acutely modulates cardiac Akt signaling.

In the present study, we examined potential functional interactions between PKC-ε and Akt by perfusing ex vivo hearts with a peptide inhibitor of PKC-ε translocation termed εV1–2 linked to an amino acid sequence derived from the protein transduction domain of HIV Tat (3, 13, 14). We measured Akt activity by subjecting immunoprecipitates obtained from LV lysates using immobilized Akt antibodies to an in vitro kinase assay with the Akt-selective substrate GSK-3α. As shown in Fig. 5C, pretreatment with Tat-PKC-ε antagonist peptide blocked EtOH-mediated increases in Akt kinase activity (45 ± 4 vs. 51 ± 15 density units for control; n = 6, P = NS). Tat-PKC-ε scrambled antagonist peptide (3, 13, 14) had no effect on phosphorylation of Akt-selective substrate. These results strongly support acute PKC-ε modulation of Akt activity in mouse heart and suggest that Akt functions as a downstream effector of EtOH-induced resistance to myocardial ischemia-reperfusion injury.

Activation of PKC-ε and Akt persists after cessation of chronic EtOH feeding. We removed EtOH from drinking water before experiments in one group (EtOH withdrawn) to determine how long cardioprotective signaling persists once serum EtOH concentrations fall to negligible levels. As shown in Fig. 6A, top, activated PKC-ε localized to particulate fractions of the mouse heart remained elevated 16 h after EtOH withdrawal (159 ± 8 vs. 99 ± 10 density units for control; n = 4, P < 0.05). As shown in Fig. 6A, bottom, removal of ethanol did not reduce phosphorylation of the COOH-terminal hydrophobic priming site (Ser729) of PKC-ε (126 ± 6 vs. 35 ± 10 density units for control; n = 4, P < 0.05).

Western analysis of EtOH withdrawn hearts (Fig. 6B, top) revealed continued elevation of Akt expression (88 ± 4 vs. 57 ± 5 density units for control; n = 4, P < 0.05). Increased Akt kinase activity (Fig. 6B, bottom) was also evident in EtOH withdrawn hearts (86 ± 6 vs. 52 ± 4 density units for control; n = 4, P < 0.05). Therefore, both cardioprotection (Figs. 2 and 3) and myocardial activation of PKC-ε and Akt persist for at least 16 h after removal of EtOH from drinking water. These results suggest that once enhanced resistance to reperfusion injury is established, less frequent EtOH dosing may be sufficient to maintain the cardioprotective effects of moderate alcohol consumption.
DISCUSSION

The principal findings of this study are that moderate alcohol consumption induces substantial resistance to ischemia-reperfusion injury in the mouse heart and that low-level PKC-ε activation is required for cardioprotection. Our work is the first to demonstrate EtOH-mediated cardioprotection in a mouse model and to implicate Akt as a possible downstream mediator of the beneficial effects of EtOH on myocardial function. The kinase signaling pathways induced by moderate alcohol consumption that increase resistance to reperfusion injury has not been fully explored. Miyamae et al. (27) observed sustained cardioprotection in guinea pigs fed 15% EtOH-derived calories for 8 wk. Those investigators demonstrated chronic activation of PKC-ε in cardiac myocytes isolated from EtOH-fed mice and found that pretreatment of ex vivo hearts with chelerythrine blocks resistance to ischemia-reperfusion injury (27). However, they did not examine the effects of more selective modulators of PKC function on ischemia-reperfusion injury and did not identify effector molecules contributing to cardioprotection.

In the present study, we observed that consumption of 18% EtOH (vol/vol) by C57BL/6 mice for 12 wk produced blood alcohol concentrations of 24 ± 3 mg/dl (5 mmol/l). The minimum blood alcohol concentration associated with intoxication in humans is ~40 mg/dl or 8 mmol/l or 0.04% (12). The minimum blood alcohol concentration associated with intoxication in mice is ~150 mg/dl or 30 mmol/l or 0.15%, as measured using the moving belt test or similar assay of ataxia (11). Therefore, mice in the present study were not intoxicated by criteria established for either species. Importantly, body weights and wet heart weight-to-body weight ratios after 12 wk were the same in EtOH-fed mice and age-matched controls. These data support the hypothesis that the cardiovascular benefits of moderate alcohol consumption are not a consequence of altered nutritional status or cardiac hypertrophy, confounding effects that may develop during heavy alcohol consumption (15, 26).

Our investigation revealed that moderate alcohol consumption selectively upregulated expression of PKC-ε in mouse hearts and increased activated PKC-ε localized to particulate fractions (Fig. 4A). Modest increases in PKC-ε expression and activation are now known to produce cardioprotective effects over time. For example, low-level cardiac expression of active PKC-ε in transgenic mice developed by Ping et al. (35) and low-level activation of PKC-ε by cardiac expression of agonist peptide in transgenic mice developed by Dorn et al. (7) were recently shown to prevent injury after transient myocardial ischemia. Therefore, our model of moderate alcohol consumption provides a third line of evidence supporting the hypothesis that low-level cardiac expression of activated PKC-ε increases resistance to ischemia-reperfusion injury in the context of normal baseline myocardial physiology.

We also found that chronic EtOH feeding increased phosphorylation of the COOH-terminal hydrophobic priming site (Ser729) of PKC-ε in mouse hearts (Fig. 4B). In vivo regulation of PKC function by allosteric modulators and anchoring proteins is thought to be dependent on phosphorylation of PKC molecules at 1) activation loop, 2) COOH-terminal turn, and 3) COOH-terminal hydrophobic priming sites (33). Studies performed by the Parker Laboratory at the Imperial Cancer Research Fund suggest differences in priming phosphorylations that activate classical and novel PKC isozymes. First, the COOH-terminal hydrophobic priming site of PKC-ε is not autophosphorylated because PKC inhibitors such as bisindolylmaleimide I do not block its phosphorylation (32). Ser729 of PKC-ε may instead be phosphorylated by an atypical PKC isozyme controlled by the mammalian target of rapamycin. Second, although novel PKC isozyme priming sites modulate conformation and localization, phosphorylation of Ser729 of PKC-ε also increases kinase function and is strongly indicative of activation (33).

We did not measure in vitro kinase activity of cardiac PKC-ε because technical considerations limit the utility of this approach for the aims of the present study. Immunoprecipitation steps remove natural substrates, scaffolding proteins, and other regulators of PKC isozyme signaling normally present in the subcellular compartments of cardiac myocytes. Also, pharmacological reagents such as phorbol esters that are used to drive in vitro phosphorylation reactions (34) provoke extensive PKC activation unlikely to mimic activation induced by diacylglycerol under physiological conditions. Because in vivo phosphorylation of PKC-ε at its priming sites requires diacylglycerol and activation of PDK-1, mammalian target of rapamycin, and the intracellular kinase(s) responsible for phosphorylation of Ser729 (32, 33), our results provide independent confirmation that PKC-ε activation develops in response to moderate alcohol consumption.

Chelerythrine chloride has been used in numerous investigations of acute ischemic and pharmacological preconditioning to test whether PKC activation is required for cardioprotection (38). In the present study, we found that chelerythrine pretreatment of ex vivo mouse hearts blocked EtOH-mediated improvement of cardiac contractile recovery and CK release during reperfusion (Figs. 2 and 3). However, many of the biological actions of chelerythrine may be mediated by mechanisms other than PKC inhibition. For example, Lee et al. (22) observed that chelerythrine obtained from commercial sources causes minimal inhibition of PKC activity in purified brain preparations. More recently, Yu et al. (46) demonstrated that chelerythrine activates MEKK1- and MKK4-dependent p38 and c-Jun NH2-terminal kinase pathways in HeLa cells without inhibiting PKC. Given the nature of these reports, we were concerned that the experiments using chelerythrine to elucidate cellular mechanisms that contribute to EtOH-mediated cardioprotection might be confounded by non-PKC effects.
Accordingly, we used protein transduction to introduce an eight-amino acid peptide (39) that inhibits binding of activated PKC-ε to RACKs into ex vivo hearts from EtOH-fed mice. We (13) previously employed this PKC-ε-selective inhibitor termed εV1-2 in a neonatal rat cardiac myocyte culture model of hypoxic preconditioning to demonstrate that PKC-ε translocation is required for protection from cellular injury. Chen et al. (3) recently used isozyme-selective peptide inhibitors of PKC function in ex vivo rat hearts to study the opposing actions of PKCδ and PKC-ε in ischemia-reperfusion injury. Importantly, those investigators found protein transduction effective for delivery of bioactive peptides into intact myocardium and confirmed that these agents block PKC function by inhibiting isozyme translocation and not by altering enzymatic activity (3). In the present study, Tat-PKC-ε antagonist peptide blocked EtOH-mediated improvement of cardiac contractile recovery and CK release during reperfusion (Figs. 2 and 3). Because disruption of protein-protein interactions between PKC-ε and RACKs inhibits the beneficial effects of moderate alcohol consumption, our results provide independent confirmation that PKC-ε activation is necessary for sustained cardioprotection.

The cellular mechanisms through which myocardial expression of activated PKC-ε increases resistance to ischemia-reperfusion injury are incompletely understood. Proteomic analysis of cardiac lysates from PKC-ε transgenic mice developed by Ping et al. (35) revealed that active PKC-ε physically associates with >30 different proteins localized to multiple subcellular compartments within cardiac myocytes, including the cardioprotective protein Akt. In the present study, we found that moderate alcohol consumption increased Akt protein expression and kinase activity in mouse hearts (Fig. 5). We plan to test the hypothesis that PKC-ε signaling is required for EtOH-mediated up-regulation of Akt protein expression in future experiments using PKC-ε knockout mice (17). Design considerations that complicate such an investigation include differences in genetic background (1), variability in neurobehavioral effects of EtOH (16, 30), and compensatory changes in other proteins triggered by disruption of the PKC-ε gene (10, 28).

In the present study, we explored functional interactions between activated PKC-ε and Akt by treating ex vivo hearts with Tat-PKC-ε antagonist peptide and then subjecting immunoprecipitated Akt to an in vitro kinase assay employing the Akt-selective substrate GSK-3α. As shown in Fig. 5C, selective inhibition of PKC-ε translocation and function blocked expected increases in Akt kinase activity induced by moderate alcohol consumption. Using CHO cell and L6 myotube cultures, Matsumoto et al. (24) established that kinase-deficient mutants of PKC-ε interfered with phosphoinositide-dependent kinase (PDK-1) phosphorylation and activation of Akt downstream of phosphatidylinositol 3-kinase (PI3). Those investigators (24) proposed a model of insulin-activated signaling in which PKC-ε phosphorylates an unidentified substrate important for interactions between PDK-1 and Akt and kinase-deficient mutants of PKC-ε exert a dominant negative effect on endogenous PKC-ε. Using ex vivo rat hearts, Tong et al. (42) observed that the PKC activator 1,2-dioctanoyl-sn-glycerol improved contractile recovery during reperfusion via mechanisms downstream of PI3-kinase but did not increase Akt phosphorylation. Those investigators proposed a model of preconditioning-induced signaling in which PI3-kinase activates PKC-ε, Akt, and endothelial nitric oxide synthase in heart. However, they did not test whether PKC inhibition blocks Akt phosphorylation during ischemic preconditioning.

Contemporary models of cardiovascular kinase function place less emphasis on traditional linear pathways in favor of networks or modules that acknowledge contributions of anchoring proteins, allosteric modulators, substrates, and other kinases to the regulation of intracellular signaling (43). Thus it is possible that acute Akt modulation of PKC-ε activity occurs in the heart. We cannot test this reciprocal relationship between the two kinases directly in our model because of the present lack of availability of pharmacological inhibitors of Akt function. In future experiments beyond the scope of the present investigation, we plan to study the effects of PI3-kinase inhibition on EtOH-mediated cardioprotection using wortmannin and LY-294002 (43). However, because PDK-1 activates both Akt and PKC, PI3-kinase inhibition is not an optimal approach to determine whether Akt is upstream or downstream of PKC or to measure the relative importance of the two kinases in development of resistance to cardiac reperfusion injury.

The cellular mechanisms through which Akt increases resistance to ischemia-reperfusion injury are under intense investigation. Although Akt activation has been shown to reduce myocyte apoptosis in models of transient ischemia (8, 44), mouse hearts in the present study were subjected to ischemia-reperfusion insufficient to produce apoptosis. Akt kinase function may also improve contractile recovery through mechanisms unrelated to its anti-apoptotic effects. For example, Matsui et al. (23) observed that expression of constitutively active Akt in rat cardiac myocytes preserved contractile function and calcium handling during hypoxia. Importantly, we found that resistance to reperfusion injury (Figs. 2 and 3) and activation of cardioprotective signaling pathways (Fig. 6) persist as serum EtOH concentrations fall to negligible levels. Parekh et al. (33) postulated that phosphorylation of kinase priming sites causes accumulation of phosphatase-resistant molecules for minutes to hours that buffer against changes in the extracellular environment. Our results support roles for PKC-ε and possibly Akt as “amplitude controls” (33) of cardioprotective signaling and suggest that less frequent EtOH dosing may be sufficient to maintain the cardiac benefits of moderate alcohol consumption.

In summary, we developed a mouse model of moderate alcohol consumption that exhibits protection against cardiac ischemia-reperfusion injury for at least
12 wk with continued administration of EtOH. We established that EtOH feeding causes cardiac expression of activated PKC-ε and that PKC inhibition blocks sustained cardioprotection. We identified Akt as a downstream mediator of resistance to myocardial injury and found that cardioprotective signaling persists after EtOH solutions are withdrawn. The present study elucidates the effects of moderate alcohol consumption on myocardial physiology and highlights potential therapeutic targets for protection against coronary heart disease that do not require EtOH ingestion, an issue arising from concerns regarding adverse effects on other organ systems in humans. This study was supported by National Institutes of Health Grant AA-11135. M. O. Gray was the recipient of an Advanced Research Career Development award from the Department of Veterans Affairs.

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