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PRE-CLINICAL RESEARCH

Angiotensin II Plays a Critical Role in Alcohol-Induced Cardiac Nitrative Damage, Cell Death, Remodeling, and Cardiomyopathy in a Protein Kinase C/Nicotinamide Adenine Dinucleotide Phosphate Oxidase–Dependent Manner

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Objectives The purpose of this study was to examine the cellular and molecular mechanisms underlying alcoholic cardiomyopathy.

Background The mechanism for alcoholic cardiomyopathy remains largely unknown.

Methods The chronic cardiac effects of alcohol were examined in mice feeding with alcohol or isocaloric control diet for 2 months. Signaling pathways of alcohol-induced cardiac cell death were examined in H9c2 cells.

Results Compared with controls, hearts from alcohol-fed mice exhibited increased apoptosis, along with significant nitrative damage, demonstrated by 3-nitrotyrosine abundance. Alcohol exposure to H9c2 cells induced apoptosis, accompanied by 3-nitrotyrosine accumulation and nicotinamide adenine dinucleotide phosphate oxidase (NOX) activation. Preincubation of H9c2 cells with urate (peroxynitrite scavenger), N^G-nitro-L-arginine methyl ester (a nitric oxide synthase inhibitor), manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin (a superoxide dismutase mimetic), and apocynin (NOX inhibitor) abrogated alcohol-induced apoptosis. Furthermore, alcohol exposure significantly increased the expression of angiotensin II and its type 1 receptor (AT1). A protein kinase C (PKC)-α/β1 inhibitor or PKC-β1 small interfering RNA and an AT1 blocker prevented alcohol-induced activation of NOX, and the AT1 blocker losartan significantly inhibited the expression of PKC-β1, indicating that alcohol-induced activation of NOX is mediated by PKC-β1 via AT1. To define the role of AT1-mediated PKC/NOX-derived superoxide generation in alcohol-induced cardiotoxicity, mice with knockout of the AT1 gene and wild-type mice were simultaneously treated with alcohol for 2 months. The knockout AT1 gene completely prevented cardiac nitrative damage, cell death, remodeling, and dysfunction. More importantly, pharmacological treatment of alcoholic mice with superoxide dismutase mimetic also significantly prevented cardiac nitrative damage, cell death, and remodeling.

Conclusions Alcohol-induced nitrative stress and apoptosis, which are mediated by angiotensin II interaction with AT1 and subsequent activation of a PKC-β1–dependent NOX pathway, are a causal factor in the development of alcoholic cardiomyopathy. (J Am Coll Cardiol 2012;59:1477–86) © 2012 by the American College of Cardiology Foundation

Regular heavy consumption of alcohol is associated with a nonischemic cardiomyopathy, termed alcoholic cardiomyopathy, that contributes to approximately one-fifth of all sudden cardiac death (1). Oxidative stress is considered responsible for alcoholic cardiomyopathy (2). However, how alcohol generates oxidative stress and how oxidative stress

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Abbreviations and Acronyms

3-NT = 3-nitrotyrosine
Ang II = angiotensin II
AT1 = angiotensin II type 1 receptor
AT1-KO = mice with knockout of angiotensin II type 1 receptor gene
LV = left ventricular
MnTMPyP = manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin
NOX = nicotinamide adenine dinucleotide phosphate oxidase
PKC-β1 = protein kinase C-β1
WT = wild-type

triggers the development of alcoholic cardiomyopathy has not been well defined.

Cell death, resulting from either necrosis or apoptosis, is an important component of the cardiomyopathic phenotype (3). Capasso et al. (4) found significant myocyte loss in the left ventricle of rats fed ethanol in drinking water for 8 months. Hearts from alcoholic patients with structural heart disease exhibited apoptotic indices similar to those from hypertensive donors (5), with greater Bax and Bcl-2 expression compared with hearts from control subjects. Moreover, alcoholic patients without structural heart damage only displayed higher Bax and Bcl-2 without apoptosis (5). Therefore, myocardial apoptosis occurs to a similar extent in heavy drinkers and long-standing hypertensive patients and is related to structural damage. However, how alcohol induces cardiac cell death requires further investigation.

Reactive oxygen and nitrogen species can be generated endogenously by specific enzymes (6). Nicotinamide adenine dinucleotide phosphate oxidase (NOX) generates superoxide through electron transfer from nicotinamide adenine dinucleotide phosphate to molecular oxygen. Seven NOX family members (i.e., NOX1 through 5 and Duox1 and 2) have been identified (7), of which NOX1, NOX2, and NOX4 are main isoforms expressed in cardiovascular cells. To date, both NOX2 and NOX4 were defined in cardiac myocytes (7). Each of these isoforms exists as a heterodimer with a lower molecular weight $p22^{phox}$ subunit and is predicted to be membrane bound, but 2 isoforms are also distinct from each other. NOX2 is normally quiescent and acutely activated by stimuli such as G protein–coupled receptor agonists (e.g., angiotensin II [Ang II], endothelin-1), growth factors, and cytokines in a tightly regulated process (7). NOX2 activation requires stimulus-induced membrane translocation of $p47^{phox}$ (i.e., formation of the active oxidase complex at the membrane) (8). We and others have demonstrated that activation of $p47^{phox}$ by diabetes and Ang II lead to significant cardiac oxidative damage and cell death and consequently results in cardiomyopathy (9,10). Unlike NOX2, NOX4 does not require additional regulatory subunits with a constitutive low-level activity and is regulated largely by changes in abundance (7). In addition to generating reactive oxygen species (11), NOX4 also protects the heart against oxidative damage under certain conditions (12).

A previous study implicated the possible involvement of Ang II in the development of alcoholic cardiomyopathy because simultaneous application of the Ang II type 1 receptor (AT1) blocker irbesartan significantly attenuated alcoholic inhibition of cardiac function (13); however, in this study, plasma Ang II levels and cardiac AT1 expression were significantly increased only in alcohol-treated dogs, but not in alcohol/irbesartan-treated dogs. Accordingly, this study resulted in several critical questions. 1) Why did the dogs in the alcohol group show significant increases in both plasma Ang II level and cardiac AT1 expression, but the dogs in the alcohol/irbesartan group did not? 2) Is the alcoholic increase in the plasma Ang II level and cardiac AT1 expression in the dogs in the alcoholic group really causative of alcoholic cardiomyopathy? 3) How did irbesartan prevent alcohol-induced cardiomyopathy if the dogs did not show increases in plasma Ang II level and cardiac AT1 expression? Therefore, this study actually did not support the involvement of Ang II/AT1 in the development of alcoholic cardiomyopathy.

Considering that NOX-mediated generation of superoxide and associated oxidative and nitrative stress play important roles in the development of various cardiomyopathies, we first investigated whether NOX activation and peroxynitrite formation are involved in alcohol-mediated cardiac cell death. Then we further investigated whether the nitrative stress and damage in the alcoholic heart is associated with an increase in systemic and cardiac Ang II and AT1. To these ends, mechanistic studies were conducted using long-term alcohol-fed mice and in vitro cultured H9c2 cardiomyocytes. We found that alcohol-induced cardiac cell death is triggered by nitrative stress generated from an Ang II-activated protein kinase C (PKC)-β1 and NOX-dependent pathway in vitro and in vivo. To define the causative role of Ang II in the alcoholic induction of cardiac cell death, transgenic mice with knockout of the AT1 gene (AT1-KO) were used, and to define the causative role of NOX-derived superoxide in the induction of cardiac nitrative damage and cell death, long-term alcohol-fed mice were simultaneously treated with superoxide dismutase mimetic to scavenge superoxide. Both animal models were completely resistant to alcoholic induction of cardiac nitrative damage and cell death and the development of cardiomyopathy. Therefore, this study provides, for the first time, direct evidence that Ang II plays a pivotal role in chronic alcohol consumption–induced cardiac nitrative damage, cell death, remodeling, and cardiomyopathy via a PKC/NOX-dependent pathway.

Methods

Details of the methods are provided in the Online Appendix. Briefly, 4-month-old male AT1-KO mice and the wild-type (WT) C57BL/6 mice were used and treated

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according to experimental procedures approved by the Institutional Animal Care and Use Committee. Mice were pair-fed a modified Lieber-DeCarli alcohol or isocaloric maltose dextrin control liquid diet for 2 months with a stepwise feeding procedure. Some alcohol-fed mice were simultaneously treated with superoxide dismutase mimetic manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP). At the end of experiments, blood pressure was measured by a tail-cuff monitoring system and heart function was evaluated by echocardiography. Then mice were killed to harvest the heart for protein, mRNA, and histopathologic examination.

H9c2 rat cardiac cells were exposed to different doses of alcohol (100 to 400 mmol/l) for 24 h. The effects of alcohol on apoptotic cell, nitrative damage (3-nitrotyrosine [3-NT] accumulation), and NOX expression and translocation (subunit $p47^{phox}$) were examined. Generation of superoxide ($O_2^{\cdot -}$) and peroxynitrite ($ONOO^-$) was measured using fluorescent probes dihydroethidium and dihydrorhodamine-123, respectively, according to a previous report (14). The role of

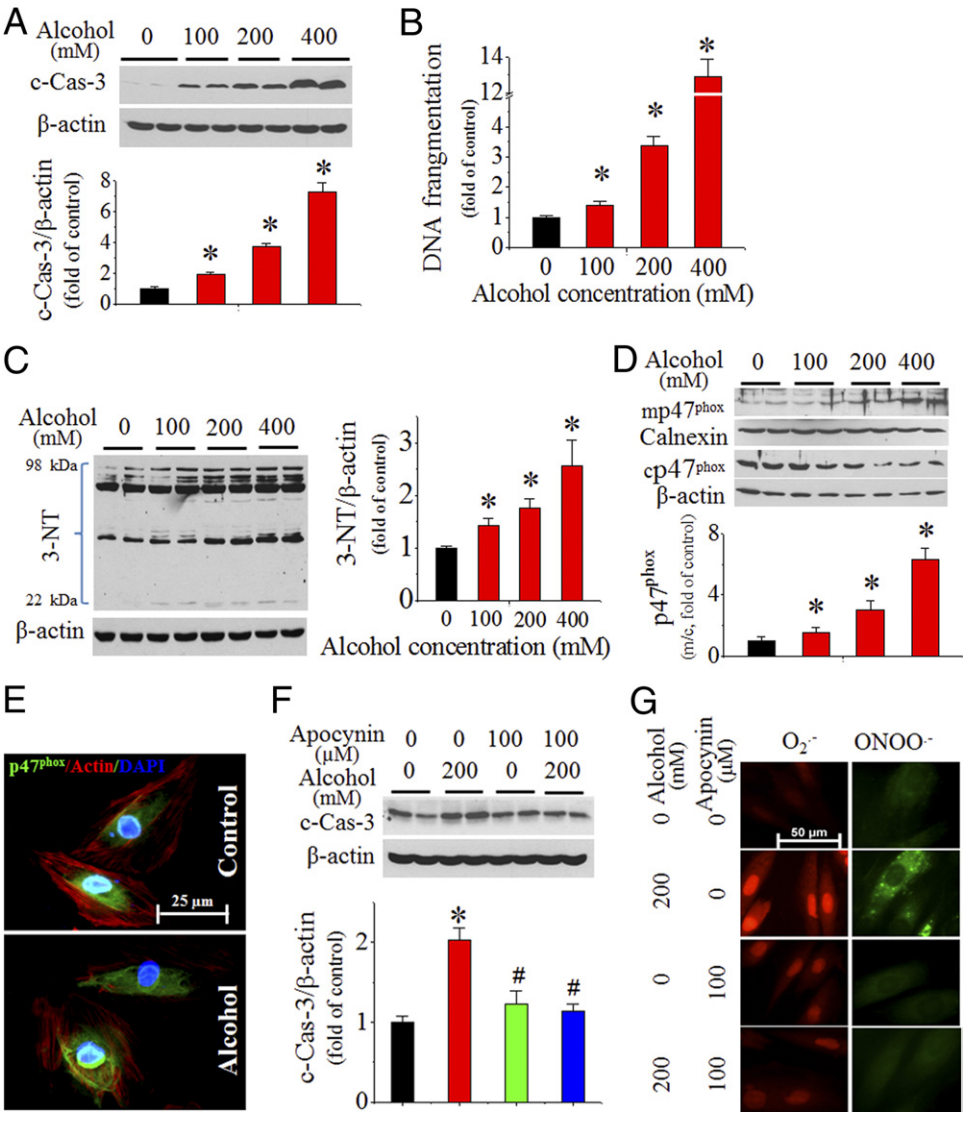

Figure 1 **Alcohol-Induced Cardiac Cell Death, Nitrative Damage, and NOX Activation In Vitro**

H9c2 cells exposed to alcohol (100 to 400 mM) for 24 h exhibited increased caspase-3 cleavage (c-Cas-3) **(A)**, DNA fragmentation **(B)**, and 3-nitrotyrosine (3-NT) accumulation **(C)**. Alcohol also activated $p47^{phox}$ at 2 h, shown by an increased ratio of membrane ($mp47^{phox}$) to cytosol ($cp47^{phox}$) (i.e., m/c) **(D)**, and membrane localization by immunofluorescent staining **(E)**. Pretreatment of H9c2 cells with the nicotinamide adenine dinucleotide phosphate oxidase (NOX) inhibitor apocynin 30 min before and during 24 h of alcohol (200 mM) exposure prevented alcohol-induced caspase-3 cleavage **(F)**, and superoxide ($O_2^{\cdot -}$) and peroxynitrite ($ONOO^{\cdot -}$) accumulation, detected by dihydroethidium and dihydrorhodamine-123 staining, respectively **(G)**. All in vitro data for Figures 1, 2, and 3 were from at least 3 independent experiments. *$p < 0.05$ vs. control. #$p < 0.05$ vs. alcohol treatment. DAPI = 4,6-diamino-2-phenylindole.

superoxide, nitric oxide, peroxynitrite, PKC-β1, and p47^{phox} activation in alcohol-induced caspase-3 cleavage was defined with corresponding inhibitors, scavengers, or small interfering RNA, respectively, as described previously (10).

Immunohistochemical staining was conducted, as described previously (10,15). AT1 mRNA expression was measured by real-time quantitative polymerase chain reaction. Western blot assay was performed for protein quantification, as described previously (10,15). Ang II levels in the plasma, cardiac tissue, H9c2 culture medium, and cell lysate were analyzed with enzyme immunoassay.

Statistical analysis. Data were collected from repeated experiments and presented as mean ± SD. For statistical analysis, 1-way analysis of variance was used with overall F test analysis for the significance of the analysis of variance. Then multiple comparisons were performed by the Bonferroni test with Origin 7.5 Lab data analysis and graphing software (OriginLab Corporation, Northampton, Massachusetts). Statistical significance was considered at $p < 0.05$.

Results

Alcohol induces cell death and nitrative damage in the heart. Apoptotic cell death in the heart of alcohol-fed mice, examined by transferase-mediated dUTP nick-end labeling staining (Online Fig. S1A and S1B) and Western blot of the cleaved caspase-3 (Online Fig. S1C), was significantly increased along with a significant increase in 3-NT modification of multiple proteins (22 to 98 kDa) (Online Fig. S1D) as nitrative damage.

Alcohol-induced cardiac cell death and nitrative damage are mediated by NOX activation. To define whether the cardiac cell death was a consequence of nitrative damage, H9c2 cells were directly exposed to alcohol in vitro for 24 h. A dose-dependent apoptotic effect of alcohol was

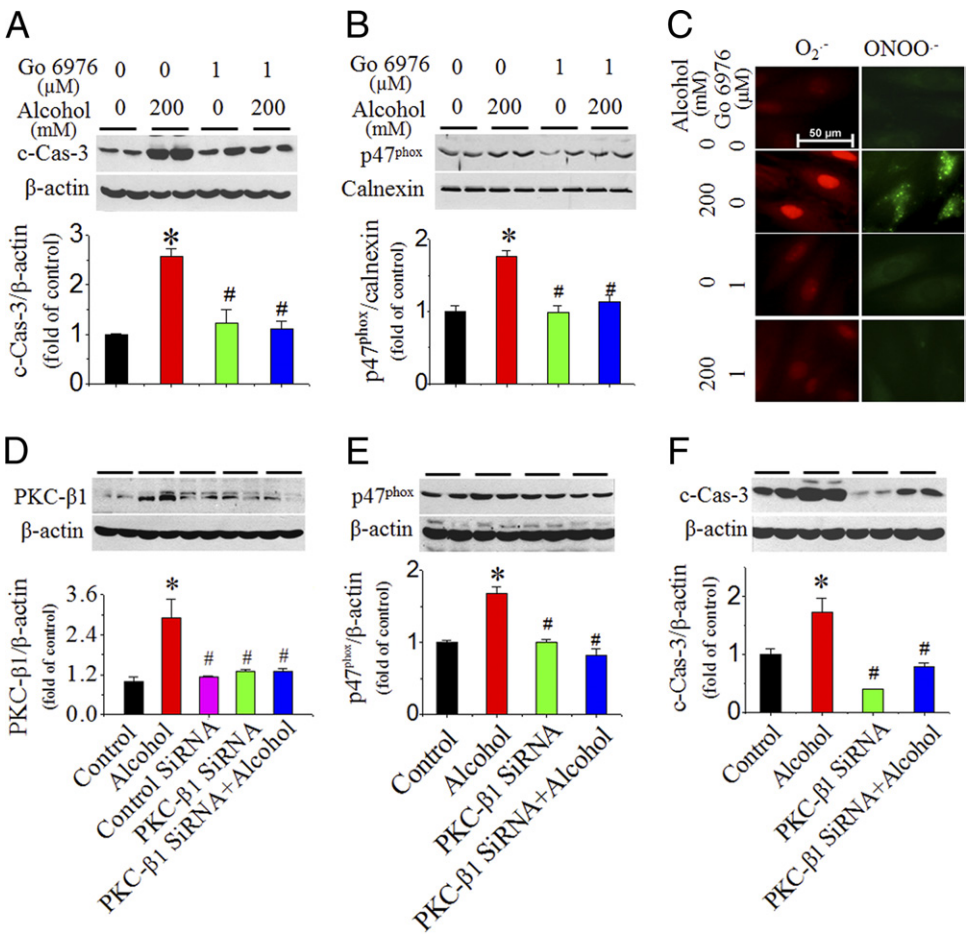

Figure 2 Alcohol Activation of NOX Is Mediated by PKC-β1

H9c2 cells were treated with protein kinase C (PKC)-α/β1 inhibitor (Go 6976) 30 min before and during alcohol exposure. c-Cas-3 at 24 h **(A)**, p47^{phox} activation **(B)**, and superoxide and peroxynitrite accumulation at 2 h **(C)** after alcohol exposure were detected. Knockdown of PKC-β1 expression with PKC-β1 small interfering RNA (SiRNA) in H9c2 cells with and without alcohol (200 mM) was confirmed **(D)** and completely attenuated alcohol induction of p47^{phox} expression at 2 h **(E)** and c-Cas-3 **(F)** at 24 h after alcohol exposure. β-Actin or calnexin was used as a loading control for Western blots. *$p < 0.05$ vs. control. #$p < 0.05$ vs. alcohol treatment. Abbreviations as in Figure 1.

found by examining caspase-3 cleavage (Fig. 1A) and DNA fragmentation (Fig. 1B). Alcohol-exposed H9c2 cells also exhibited augmented 3-NT modification of multiple proteins (Fig. 1C).

Next, we defined the role of NOX activation in these effects. First, alcohol exposure of H9c2 cells increased $p47^{phox}$ expression in a dose-dependent manner (Online Fig. S2). The ratio of $p47^{phox}$ expression in the cell membrane fraction to cytosolic fraction was also significantly increased in a dose-dependent manner by Western blot (Fig. 1D) and fluorescent staining for $p47^{phox}$ (Fig. 1E). Furthermore, H9c2 cells were treated with the NOX inhibitor apocynin at 100 μmol/l started 30 min before alcohol exposure. Apocynin significantly attenuated the caspase-3 activation

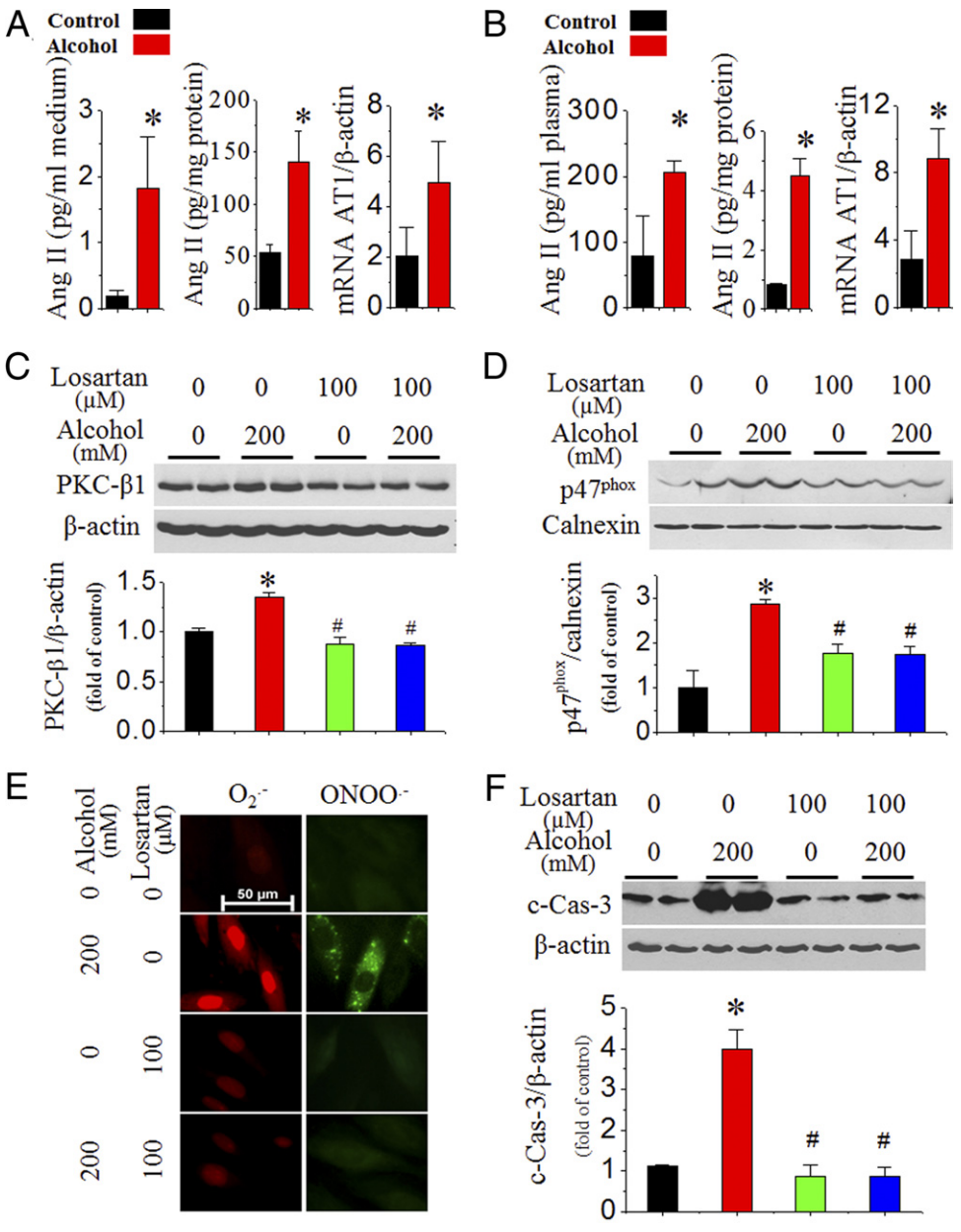

Figure 3 **Activation of PKC and NOX Is Mediated by Alcohol-Increased Ang II Contents and AT1 Expression**

Extracellular (in the culture medium) and intracellular (in the cell lysates) angiotensin II (Ang II) levels at 24 h and angiotensin II type 1 receptor (AT1) mRNA expression at 2 h after H9c2 cells exposed to alcohol (200 mM) were examined with enzyme-linked immunosorbent assay and quantitative polymerase chain reaction assay, respectively **(A)**. Ang II contents in the plasma and cardiac tissues and cardiac AT1 mRNA expression were also detected after mice ($n \geq 5$) were fed with alcohol for 2 months **(B)**. H9c2 cells were treated with the AT1-specific blocker losartan at 30 min before and during alcohol exposure. PKC-β1 expression **(C)**, $p47^{phox}$ membrane translocation **(D)**, and superoxide and peroxynitrite accumulation **(E)** were examined at 2 h after alcohol exposure, whereas caspase-3 cleavage **(F)** was examined at 24 h after alcohol exposure. β-actin or calnexin was used as loading control for Western blots. *$p < 0.05$ vs. control. #$p < 0.05$ vs. alcohol treatment. Abbreviations as in Figures 1 and 2.

(Fig. 1F) and the increased accumulation of superoxide and peroxynitrite in alcohol-treated cells (Fig. 1G), suggesting a causative role of NOX activation and nitrative stress in alcohol-mediated cell death.

We further defined the role of nitrative stress in alcohol-induced apoptosis because the peroxynitrite scavenger urate that was applied 30 min before 200 mmol/l alcohol exposure could completely attenuate caspase-3 activation (Online Fig. S3A). Alcohol-induced cell death was also abolished by pre-treatment with the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (Online Fig. S3B) or superoxide dismutase mimetic MnTMPyP (Online Fig. S3C).

Alcohol-induced NOX activation is mediated by PKC-β1. To define the role of PKC in alcohol-induced activation of NOX and subsequent apoptosis, we examined the effect of a specific PKC-α/β1 inhibitor (Go 6976) applied 30 min before alcohol exposure in H9c2 cells. Go 6976 significantly attenuated caspase-3 activation (Fig. 2A), NOX2 activation (shown by increased $p47^{phox}$ expression in membrane fraction [Fig. 2B] and scattering distribution of fluorescent staining of $p47^{phox}$ [Online Fig. S4]), and the accumulation of superoxide and peroxynitrite (Fig. 2C) in cells exposed to 200 mmol/l alcohol. Furthermore, the fact that inhibition of alcohol-induced PKC-β1 expression by its small interfering RNA (Fig. 2D) resulted in a complete abolishment of alcohol-induced $p47^{phox}$ expression (Fig. 2E), and caspase-3 activation (Fig. 2F) established the direct role of PKC-β1 in activation of NOX-mediated apoptosis.

Alcohol activation of PKC and NOX is AT1 dependent. In this study, whether alcohol exposure increases Ang II generation and AT1 receptor expression in cardiac cells was examined. Ang II levels in both cell lysate and the medium of H9c2 cells exposed to alcohol at 200 mmol/l for 24 h significantly increased relative to controls (Fig. 3A). Increased Ang II levels were accompanied by a significant increase in AT1 gene expression in alcohol-exposed cells (Fig. 3A). Furthermore, Ang II levels in the hearts and plasma and cardiac AT1 gene expression in mice fed alcohol for 2 months were also significantly increased (Fig. 3B).

Next, the study examined the effect of the AT1 blocker losartan on alcohol-induced PKC expression, NOX activation, and cell death. Pre-treatment of H9c2 cells with losartan (100 μmol/l) completely prevented alcohol-induced PKC-β1 up-regulation (Fig. 3C), $p47^{phox}$ membrane translocation (Fig. 3D, Online Fig. S4), superoxide and peroxynitrite accumulation (Fig. 3E), and cell death (Fig. 3F). Hence, Ang II interaction with AT1 was required for alcohol-induced nitrative stress and subsequent cell death mediated by PKC-β1and NOX activation.

Knockout AT1 gene prevents the cardiac nitrative stress, cell death, remodeling, and dysfunction in mice fed with chronic alcohol. To ensure that the in vitro finding described is applied to in vivo disease, AT1-KO and WT mice were pair-fed alcohol or control liquid diet for 2 months. Analysis of blood pressure showed that AT1-KO mice showed decreased blood pressure compared with WT mice at baseline (Table 1). Long-term alcohol feeding significantly increased plasma and cardiac Ang II levels in both strains of mice (Figs. 4A and 4B), but only significantly increased blood pressure in the WT mice and not in the AT1-KO mice (Table 1).

In WT mice, long-term alcohol feeding induced cardiac PKC-β1 expression (Fig. 4C) and activation (Fig. 4D), NOX up-regulation, including both $p47^{phox}$ (NOX2) (Fig. 4E) and NOX4 (Fig. 4F), and 3-NT accumulation (Fig. 4H). NOX1 expression was not significantly changed among groups in

Table 1 Biometric and Echocardiographic Parameters of Mice Fed an Alcohol Diet for 2 Months

Parameter	WT		AT1-KO	
	Control (n = 5)	Alcohol (n = 8)	Control (n = 4)	Alcohol (n = 4)
Body weight, g	34.0 ± 1.3	30.3 ± 2.1*	31.1 ± 0.8	31.7 ± 1.8
Heart weight, mg	124 ± 4	148 ± 11*†	114 ± 3	121 ± 8
Tibia length, cm	1.81 ± 0.05	1.82 ± 0.01	1.84 ± 0.05	1.83 ± 0.04
Heart weight/tibia length ratio, mg/cm	68.1 ± 2.1	81.4 ± 6.0*†	65.5 ± 4.2	62.6 ± 1.8
Diastolic blood pressure, mm Hg	68 ± 6	79 ± 5*†	53 ± 4	48 ± 2
Systolic blood pressure, mm Hg	95 ± 7	111 ± 9*†	76 ± 4	69 ± 3
Mean blood pressure, mm Hg	76 ± 6	89 ± 6*†	60 ± 3	55 ± 2
LV mass, mg	76 ± 15	103 ± 16	91 ± 25	80 ± 12
LV mass normalized by body weight, mg/g	2.4 ± 0.6	3.4 ± 0.5*	2.9 ± 0.8	2.6 ± 0.4
LVLD, mm	6.3 ± 0.4	6.7 ± 0.2*	6.1 ± 0.5	6.1 ± 0.2
LVALD, mm^2	17.9 ± 1.6	20.2 ± 1.7*	16.7 ± 1.7	17.4 ± 1.6
LVLS, mm	4.9 ± 0.3	5.5 ± 0.3*	4.8 ± 0.5	4.8 ± 0.5
LVALS, mm^2	8.0 ± 0.8	10.5 ± 1.4*†	8.0 ± 1.6	7.9 ± 1.5
Ejection fraction, %	75 ± 3	67 ± 4*	71 ± 5	74 ± 5

Values are mean ± SD. *$p < 0.05$ versus wild-type mice control. †$p < 0.05$ versus mice with knockout of angiotensin II type 1 receptor gene control.
AT1-KO = mice with knockout of angiotensin II type 1 receptor gene; LV = left ventricular; LVALD = left ventricular area of long axis (end-diastolic); LVALS = left ventricular area of long axis (end-systolic); LVLD = left ventricular long-axis diameter (end-diastolic); LVLS = left ventricular long-axis diameter (end-systolic); WT = wild-type.

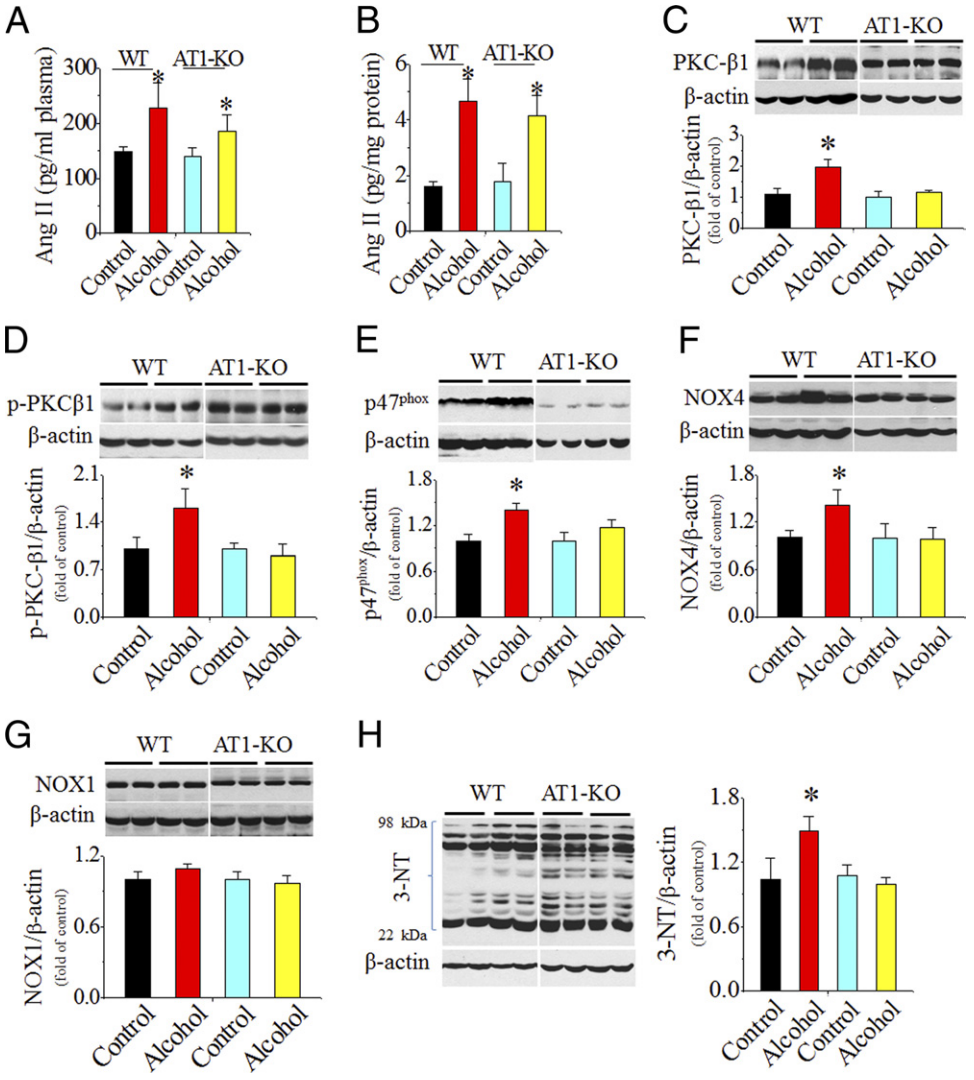

Figure 4 **Response of AT1-KO and WT Mice to Long-Term Alcohol Feeding**

Mice with knockout of AT1 receptor gene (AT1-KO) and wild-type (WT) mice were fed alcohol for 2 months ($n \geq 4$). Ang II levels in the plasma **(A)** and heart **(B)** were detected with enzyme-linked immunosorbent assay. Cardiac PKC-β1 expression **(C)** and activation **(D)**, NOX2 ($p47^{phox}$) **(E)**, NOX4 **(F)**, and NOX1 **(G)** expressions, and 3-NT accumulation **(H)** were examined with Western blots. *$p < 0.05$ vs. control. p-PKC-β1 = phospho-PKC-β1; other abbreviations as in Figures 1, 2, and 3.

both strains (Fig. 4G). Long-term alcohol feeding also significantly induced cardiac apoptosis (examined by transferase-mediated dUTP nick-end labeling staining [Fig. 5A, Online Fig. S5 for staining images] and caspase-3 activation [Fig. 5B]) and cardiac remodeling (shown by increased fibrosis with connective tissue growth factor expression [Fig. 5C] and Sirius red staining of collagen [Fig. 5D]). However, all of these pathogenic changes were not observed in alcohol-fed AT1-KO mice (Figs. 4C to 4H and 5A to 5D).

Echocardiographic and gravimetric evaluation (Table 1) revealed that alcohol feeding in WT mice induced left ventricular (LV) chamber enlargement (increased LV long-axis diameter and area at end-diastole and end-systole), mild LV systolic dysfunction (reduced ejection fraction), and chamber hypertrophy (increased heart weight/tibia length ratio and increased LV mass as determined by echocardiography and normalized for body weight). All these changes were observed only in the WT mice and not in AT1-KO mice.

Superoxide dismutase mimetic prevents alcohol-induced cardiac cell death and remodeling in mice. This study has established the role of Ang II/AT1 in the development of alcoholic cardiomyopathy. To further establish the role of NOX activation–mediated nitrative stress and damage in alcoholic cardiomyopathy, the pair-fed and alcohol-fed mice were intraperitoneally treated with the superoxide dismutase mimetic MnTMPyP at 5 mg/kg or vehicle daily for 2 months. Treatment with MnTMPyP did not change

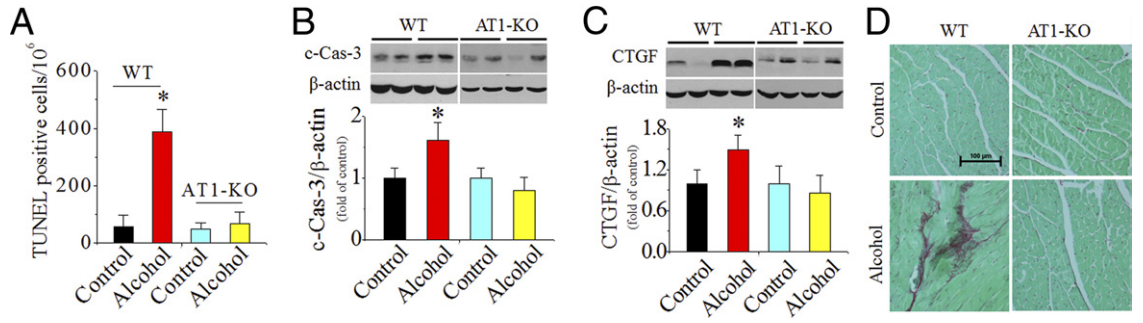

Figure 5 **AT1-KO Mice Are Resistant to Alcoholic Induction of Cardiac Cell Death and Remodeling**

Cardiac tissues were collected from mice described in Figure 4. Cardiac apoptotic cell death was detected with transferase-mediated dUTP nick-end labeling (TUNEL) staining **(A)** (representative images of transferase-mediated dUTP nick-end labeling staining are provided in Online Fig. S5) and caspase-3 cleavage **(B)**. Fibrotic response was examined by Western blot of connective tissue growth factor (CTGF) **(C)** and Sirius red staining of collagen **(D)**. *$p < 0.05$ vs. control. Abbreviations as in Figure 4.

alcohol-induced increases in systolic and diastolic blood pressure (Online Figs. S6A and S6B), cardiac PKC expression (Online Fig. S6C) and activation (Fig. 6A), and $p47^{phox}$ (Fig. 6B) and NOX4 (Fig. 6C) expressions. There was no change in these parameters is because they are upstream mediators of NOX-mediated superoxide generation. However, treatment with MnTMPyP significantly prevented alcohol-induced cardiac nitrative damage (Fig. 6D), cell death (shown by transferase-mediated dUTP nick-end labeling staining [Fig. 6E, Online Fig. S6E for staining images] and caspase-3 cleavage [Fig. 6F]), and remodeling (shown by increased Sirius red staining for collagen and immunohistochemical staining for fibronectin, respectively [Fig. 6G]).

Discussion

Cardiac apoptosis is a pivotal cause of various cardiomyopathies (3). Previous studies showed that apoptosis increases in the heart of animals and patients with long-term alcohol consumption (4,5). In an analogous manner, in the present study, we found that the induction of cardiac cell death in the hearts of alcohol-fed mice and cardiac cells exposed to alcohol in vitro and the prevention of cardiac nitrative damage and cell death in AT1-KO mice (Figs. 4 and 5). The prevention of the alcohol-induced cell death resulted in a significant prevention of cardiac remodeling (Fig. 5) and dysfunction (Table 1), further confirming the critical role of alcoholic cell death in the development of cardiomyopathy (Fig. 6H).

Oxidative and/or nitrative stress has been thought to play important roles in alcohol-induced cardiotoxicity. In animal models, both acute and chronic ingestion of alcohol increases cardiac lipid peroxidation and protein oxidation and reduces mitochondrial glutathione content, suggesting alcohol-induced oxidative stress (2,16). However, how alcohol induces intracellular oxidative stress that leads to cardiac cell death remains largely unknown (17).

A novel finding of the present study is that NOX-mediated superoxide generation and associated peroxynitrite formation play critical roles in alcohol-induced cardiac cell death. Specifically, the alcoholic cell death was prevented by suppression of NOX activation, inhibition of nitric oxide formation, and scavenging of either superoxide or peroxynitrite (Online Fig. S3). Although the role of NOX-associated peroxynitrite accumulation has been implicated in alcohol-induced hepatic cell damages (18), there was no evidence that peroxynitrite formation contributes to alcoholic cardiotoxicity. We directly measured the accumulation of superoxide and peroxynitrite in the cardiac cells exposed to alcohol (Fig. 1G). In vivo, MnTMPyP supplementation in conjunction with long-term alcohol feeding for 2 months in mice significantly attenuated alcohol-induced cardiac cell death and nitrative damage without altering blood pressure, PKC, and NOX expression and activation (Figs. 6A to 6C). This study further confirms the pivotal role of NOX-mediated superoxide in alcohol-induced cardiac nitrative damage, cell death, and remodeling (Fig. 6H).

The second novel finding is that alcohol activation of NOX and apoptosis is PKC-β1 dependent. In the heart, PKC activation is generally considered to be a protective response (19,20); however, cardioprotection is mainly attributed to the PKC-ε (19,20), whereas other isoforms of PKCs may have opposite effects (21). The prevention of alcohol-induced NOX activation and apoptosis by inhibition of PKC-α/β1 with its inhibitor (Figs. 2A to 2C) is consistent with previously revealed detrimental effects of PKC-α/β1 (21). Furthermore, we applied PKC-β1–specific small interfering RNA to clearly define the direct role of PKC-β1 in mediating NOX activation associated nitrative damage and cell death (Figs. 2E and 2F).

Although increased expression of AT1 was observed in the hearts of the dogs (13) and rats (22) with long-term alcohol feeding, it remained unclear whether increased cardiac Ang II and AT1 expression is the direct cause of alcoholic cardiomyopathy. In the present study, we demonstrate that direct exposure of H9c2 cardiac cells to alcohol augmented intracellular and extracellular Ang II levels and up-

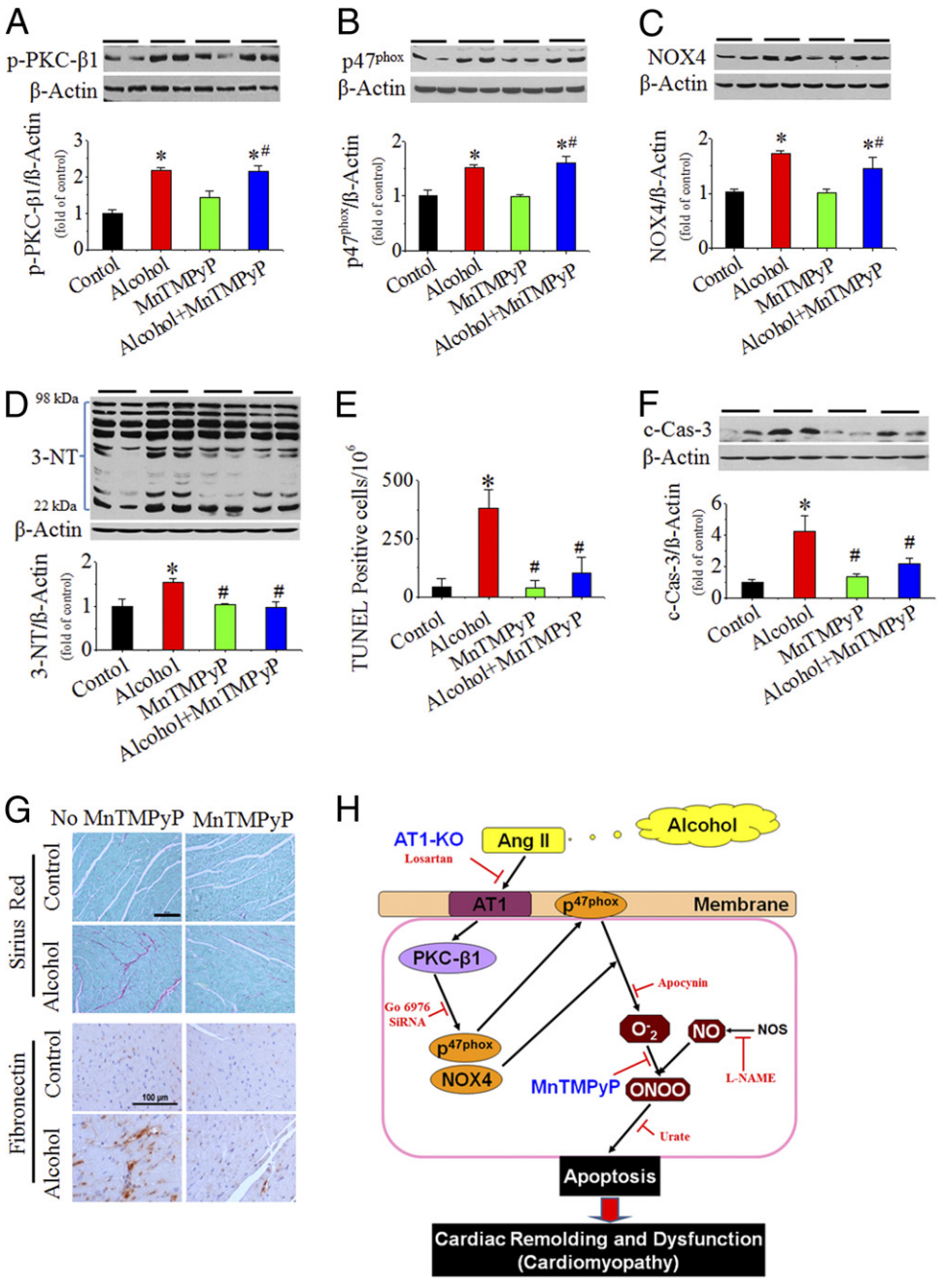

Figure 6 **Prevention of Cardiac Nitrative Damage and Cell Death in Mice by Scavenging Superoxide With MnTMPyP**

Pair-fed and alcohol-fed mice (n ≥ 8) were treated with and without the superoxide dismutase mimetic manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) at 5 mg/kg body weight daily for 2 months. Cardiac PKC-β1 activation **(A)**, NOX2 ($p47^{phox}$) **(B)** and NOX4 **(C)** expression, and 3-NT accumulation **(D)** were detected by Western blots. Cell death was examined by transferase-mediated dUTP nick-end labeling staining **(E)** (representative images of transferase-mediated dUTP nick-end labeling staining are provided in Online Fig. S6E) and Western blot of caspase-3 cleavage **(F)**. Cardiac remodeling was examined by Sirius red staining of collagen and immunohistochemical staining of fibronectin **(G)**. *$p < 0.05$ vs. control. #$p < 0.05$ vs. alcohol treatment. **(H)** A mechanistic illustration of cardiac nitrative damage and cell death mediated by the Ang II/AT1 axis in response to alcohol. The **blue font** indicates the in vivo experimental models. L-NAME = N^G-nitro-L-arginine methyl ester; NO = nitric oxide; NOS = nitric oxide synthase; other abbreviations as in Figures 1, 2, 3, and 4.

regulated Ang II AT1 receptor expression under an in vitro condition (Fig. 3A) and clarified the direct role of alcohol on the increases in cardiac Ang II levels and AT1 gene expression. More importantly, the present study provides direct evidence of the first time that AT1-KO mice are completely resistant to alcoholic induction of cardiac nitrative damage, cell death, and cardiomyopathy development (Figs. 4 and 5).

With regard to the mechanisms responsible for alcoholic induction of cardiac Ang II generation and AT1 expression, we do not have direct experimental evidence currently. However, several studies demonstrated the capability of cardiac cells to generate intracellular Ang II in response to various stresses (23,24). Intracellular increases in Ang II and AT1 expression in cardiac cells challenged by various stresses were related to p53 transcriptional function (23,24). Exposure to alcohol has been extensively documented to up-regulate cardiac p53 expression (25). Therefore, whether alcohol increases cardiac intracellular Ang II and AT1 expression via p53 activation needs further investigation. In addition, alcoholic tissue damage may be evident in the liver of these mice; therefore, whether an alcohol-induced increase in plasma Ang II is due to increased production of angiotensinogen in the liver is also an interesting topic for future studies.

Study limitation. There may be a limitation of the present study since we used a model with continual alcohol feeding that may not directly mimic human alcoholic situation.

Conclusions

In summary, we investigated the cellular and molecular mechanisms underlying alcohol-mediated cardiac cell apoptosis using a combination of in vivo and in vitro approaches with the following key findings: 1) cardiac cell death induced by alcohol is dependent on NOX activation and subsequent nitrative damage; 2) alcoholic activation of NOX is PKC-β1 dependent; 3) alcoholic exposure of cardiac cells increases intra- and extracellular Ang II levels and AT1 expression, which is required for alcohol activation of PKC- β1 and NOX; 4) AT1-KO mice or superoxide dismutase mimetic–treated mice are completely resistant to alcoholic induction of cardiac nitrative stress, cell death, remodeling, and dysfunction. These results indicate that alcohol-induced cardiac cell death, which is mediated by the interaction of Ang II with AT1 to activate PKC-β1–dependent activation of NOX with subsequent superoxide generation and nitrative damage, is a critical cause of alcoholic cardiomyopathy (Fig. 6H).

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Key Words: alcoholic cardiomyopathy ■ angiotensin II ■ cardiac cell death ■ losartan ■ oxidative and nitrative stress ■ protein kinase C.

APPENDIX

For an expanded Methods section and supplemental figures with legends, please see the online version of this article.