Angiotensin II Plays a Critical Role in Alcohol-Induced Cardiac Nitrative Damage, Cell Death, Remodeling, and Cardiomyopathy in a PKC/NADPH Oxidase-Dependent Manner

Yi Tan, PhD[†‡] Xiaokun Li, MD, PhD[†] Sumanth D. Prabhu, MD[§] Kenneth R Brittian, BS[+], Qiang Chen, MD[‡] Xia Yin, MD[‡] Craig J. McClain, MD, PhD[*] Zhanxiang Zhou, PhD[+‖] Lu Cai, MD, PhD [†‡*]

Running title: Angiotensin II in alcoholic cardiomyopathy

Online appendexes : 1. Methods ; 2. Supplemental Figures and Figure Legends

Methods

Animals and alcohol feeding

Male wild type C57BL/6 mice for the first animal study and third SOD mimic animal study were obtained from Harlan Laboratories (Indianapolis, IN), and transgenic mice with AT1-knockout (AT1-KO) mice and C57BL/6 wild type (WT) control for the second study are from The Jackson Laboratory (Bar Harbor, Maine). Mice were treated according to experimental procedures approved by the Institutional Animal Care and Use Committee. For all animal studies on chronic alcohol exposure, 4-month old male mice were pair-fed a modified Lieber-DeCarli alcohol or isocaloric maltose dextrin control liquid diet for 2 months with a stepwise feeding procedure as described in our previous reports (1-2). The ethanol content (%, w/v) in the diet was 4.8 (34% of

total calories) at initiation, and gradually increased up to 5.4 (38% of total calories). The amount of food given to the pair-fed mice was matched to that of the alcohol-fed mice measured on the previous day. At the end of the experiments, after heart function (echocardiography) and blood pressure detection, mice were anesthetized, and hearts were harvested for protein, mRNA and histopathological examination.

For the first animal study, we only observed the cardiac effect of chronic alcohol consumption. Therefore, these mice did not receive any treatment, 8 mice for control and 11 mice for alcohol treatment group. The second animal study was to investigate effect of AT1-KO on alcohol-induced cardiac pathological changes. Therefore they did not receive further treatment, 8 mice for WT control, 12 mice WT alcohol treatment, 4 mice for AT1-KO control, 4 mice for AT1-KO alcohol treatment respectively. The third animal study, except for the alcohol feeding, these mice were simultaneously treated with SOD mimic MnTMPyP [Mn (111) tetrakis 1-methyl 4-pyridylporphyrin pentachloride, purchased from Sigma Chemical Co, St. Louis, MO] at 5 mg/kg body weight daily for 2 months, $n \geq 8$ for each group.

TUNEL staining

Apoptotic nuclei in the heart were examined by transferase mediated dUTP nick-end labeling (TUNEL) staining using ApopTag In Situ kit (Chemicon, Temecula, CA). Heart tissue was fixed in buffered neutral 10% formalin, dehydrated in graded alcohol series, embedded in paraffin, and sectioned at 4-5 μm. Deparaffinized and hydrated slides were used for TUNEL staining according to the manufacturer's instructions. TUNEL positive nuclei cells were counted under high magnification (20 X) in ten random fields for each of five slides from one mouse with at least

four mice (as indicated) each group, and presented as TUNEL positive nuclei per 10^6 cardiac cell nuclei.

Non-invasive blood pressure

Blood pressure (BP) was measured by tail-cuff manometry using a CODA^{TM} non-invasive BP monitoring system (Kent Scientific, Torrington, CT). The mice were restrained in a plastic tube restrainer. Occlusion and volume-pressure recording (VPR) cuffs were placed over the tail, and the mice were allowed to adapt to the restrainer for 5 minutes prior to starting BP measurement. After a 5 minute adaptation period, BP was measured for 10 acclimation cycles followed by 20 measurement cycles. Mice were warmed by heating pads during the acclimation cycles to ensure sufficient blood flow to the tail. The animals were monitored closely throughout the measurement protocol, and removed from restraint as soon as possible upon completing the measurement protocol (3). After three days of training for the BP measurement, formal measurements were performed and BP data were collected.

Cell cultures

H9c2 rat cardiac cell line was purchased from American Type Cell Collection (ATCC, Manassas, VA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). H9c2 cells in all the experiments were in passages between 3 and 15. The effects of different doses of ethanol (100-400 mmol/L) exposure for 24 h on cell death (the expression of cleaved caspase-3 and DNA fragmentation), nitrosative damage [3-nitrotyrosine (3-NT) expression] and NOX expression (subunit $p47^{phox}$ expression) were examined initially. In subsequent studies, exposure to ethanol at

200 mmol/L for 24 h was used. Alcohol-induced superoxide, nitric oxide and peroxynitrite accumulation, PKC-β1 and $p47^{phox}$ activation on cell death (cleaved caspase-3 expression) were defined with their corresponding inhibitors or scavengers respectively. Cells were pre-treated with 100 μmol/L peroxynitrite scavenger Urate, 100 μmol/L superoxide dismutase mimic MnTMPyP (Sigma Chemical Co, St. Louis, MO), 100 μmol/L NOX inhibitor apocynin [non-specifically inhibiting both NOX2 and NOX4 (4), Calbiochem, San Diego, CA], 100 μmol/L nitric oxide synthase inhibitor L-NAME (Sigma Chemical Co., St. Louis, MO), 1μmol/L PKCα/β1 specific inhibitor Go 6976 (Tocris Bioscience, Ellisville, Missouri) and 100 μmol/L angiotensin II type 1 receptor (AT1) blocker losartan (Sigma Chemical Co., St. Louis, MO) for 30 min and then continually exposed to alcohol for 24 h (5). In some experiments, cells were pre-treated with 100 μmol/L losartan or 1μmol/L Go 6976 for 30 min and then continually exposed to alcohol (200 mmol/L) for the indicated time durations to examine PKC-β1 expression and/or $p47^{phox}$ translocation, superoxide and peroxynitrite generation.

siRNA transfection

siRNAs specific for rat PKC-β1 (Accell SMART pool PRKCB1), along with non-targeting siRNA (Acell Non-targeting SiRNA) as negative control (Thermo Scientific Dharmacon RNAi Thechnologies, Lafayette, CO) were transfected into H9c2 cells for 48 h by DharmaFECT® transfection reagent (Thermo Scientific Dharmacon RNAi Thechnologies, Lafayette, CO) prior to alcohol (200 mmol/L) treatment. Transfection efficiency was assessed by western blot analysis for PKC-β1 protein expression. The effects of siRNA knockdown of PKC-β1 on the expression of $p47^{phox}$ and cleaved caspase-3 were assessed by western blot analysis at indicated time points.

Immunocytochemistry and immunofluorescence

For immunocytochemistry, paraffin sections (5 mm) from heart tissues were stained with goat anti-mouse fibronectin primary antibody (1:50) (Santa Cruz Biotechnology, CA) overnight at 4 °C. After washing, sections were incubated with horseradish peroxidase (HRP) conjugated secondary antibody against goat and developed with DAB (3,3-Diaminobenzidine) developing system (Vector Laboratories, Inc., CA), counterstained with hematoxylin and observed under light microscopy. For immunofluorescence, H9c2 cells were plated in a glass culture chamber (Nalge Nunc International, Rochester, NY) and exposed to alcohol (200 mmol/L) for 2 h with and without losartan (100 μmol/L) or Go 6976 (1 mmol/L). Methanol fixed cells were blocked with 10% normal donkey serum (Jackson ImmunoReserch Laboratories, Inc., West Grove, PA) for 1 h at room temperature and then incubated with primary antibodies (goat anti-$p47^{phox}$ at 1:50; rabbit anti-β-actin at 1:100, from Lifespan and Santa Cruz Biotechnology, CA, respectively). After washing, cells were incubated with secondary antibodies (Cy3-conjugated donkey anti-rabbit antibody at 1:200 dilution and FITC-conjugated donkey anti-goat antibody at 1:200 dilution, both from Jackson ImmunoReserch Laboratories, Inc., West Grove, PA), and then counterstained with 4 ,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma Chemical Co., St. Louis, MO) and mounted. Images were observed under a Nikon Eclipse E600 microscopy with X-Cite^{TM} 120 Fluorescence Illumination System.

Measurement of intracellular superoxide and peroxynitrite

Generation of superoxide (O_2^-) and peroxynitrite ($ONOO^-$) was measured using the fluorescent probes dihydroethidium (DHE) and dihydrorhodamine-123 (DHR-123) (Molecular Probe, Eugene, OR), respectively according to previous report (6). DHE, a nonfluorescent membrane-

permeant probe, interacts with $O_2^{\cdot -}$, leading to the liberation of membrane-impermeant ethidium cations that fluoresce on intercalating with nuclear DNA showing bright red fluoresce in nuclear. DHR-123 is oxidized by $ONOO^-$ to the highly fluorescent product rhodamine showing bright green in cytosol. H9c2 cells were plated in a glass culture chamber (Nalge Nunc International, Rochester, NY) and pretreated with and without apocynin (100 μmol/L), losartan (100 μmol/L) or Go 6976 (1 mmol/L) for 30 min and then continually exposed to alcohol for 2 h. DHE (1 μmol/L) or DHR-123 (10 μmol/L) were added for the last 60 min of incubation. After incubation, images were immediately observed under a Nikon Eclipse E600 microscopy with X-Cite™ 120 Fluorescence Illumination System.

Cell fractionation

H9c2 cells were stimulated with alcohol (100-400 mmol/L) for 2 h. In some experiments, cells were pre-exposed to losartan (100 μmol/L) or Go 6976 (1 μmol/L) for 30 min and then simultaneously exposed to alcohol (200 mmol/L) for 2 h. Cells were washed, scraped, and differentially centrifuged to obtain cell homogenate, membrane, and cytosolic fractions (7). $p47^{phox}$ expression and membrane translocation were assessed in membrane and/or cytosolic fractions respectively by Western blot.

Determination of histone-associated DNA fragmentation

To quantitatively determine the induction of apoptosis by alcohol in H9c2 cells, DNA fragmentation was measured using a Cell Death Detection ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. DNA fragmentation in alcohol-treated groups was compared with controls and expressed as the fold of control.

Real-time quantitative PCR analysis

Total RNA was extracted from heart tissues or H9c2 cells using RNA Stat-60 Reagent (IsoTex Diagnostics, Inc., Friendswood, Texas). RNA concentration and purity were quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). First strand complimentary DNA (cDNA) was synthesized from total RNA using a RNA PCR kit (Promega, Madison, WI), following the manufacturer's protocol. Primers (mouse AT1: Mm00616371_m1; mouse β-Actin: Mm00607939_s1; rat AT1: Rn02758772_s1*; rat β-Actin: Rn00667869_m1) for PCR were purchased from Applied Biosystems (Carlsbad, CA, USA). The house-keeping gene, β-actin, was used as a reference. Real time RT-PCR (qPCR) was carried out in a 20 μl reaction system that included the following: TaqMan Universal PCR Master Mix 10 μl, primers 1 μl, cDNA 6 μl, and ddH_2O 3 μl, and performed in duplicate for each sample in a ABI 7300 Real-Time PCR system (Life Technologies Corporation, Carlsbad, CA). The comparative cycle time (CT) method was used to determine fold differences between samples. The comparative CT method determines the amount of target, normalized to an endogenous reference (β-actin) and relative to a calibrator ($2^{-\Delta\Delta Ct}$).

Western blotting

Western blots were performed according to our previous studies (5,8-9). Briefly, heart tissues and harvested cells were homogenized or lysed in RIPA buffer (Santa Cruz Biotechnology, CA). Total protein was extracted and separated on 10% SDS-PAGE gels, and transferred to a nitrocellulose membrane (Bio-rad, Hercules, CA). The membrane was blocked with a 5% non-fat, dried milk for 1 h and them incubated overnight at 4 °C with the following antibodies: cleaved caspase-3 (1:1000, Cell Signaling Technology, Danvers, MA), 3-nitrotyrosine (3-NT, Chemicon,

Temecula, CA), PKC-β1 (1:1000), p-PKC-β1 (1:500), β-actin (1:1000), CTGF (1:1000), $p47^{phox}$ (1:1000) (Santa Cruz Biotechnology, CA), NOX1 and NOX4 (1:1000) (Novus Biologicals, CO). After three washes with Tris-buffered saline (pH 7.2) containing 0.05% Tween 20, membranes were incubated with the appropriate secondary antibodies for 1 h at room temperature. Antigen-antibody complexes were visualized with enhanced chemiluminescence. The expression of specific antigens were quantified using a ImageQuant 5.2 software (Molecular Dynamics, Inc., Sunnyvale, CA), the expression of β-actin or calnexin was used as loading control except where indicated, and the results were expressed as fold of control.

Enzyme immunoassay for Ang II

Extraction of peptides from heart tissues, plasma, H9c2 cells or culture medium was performed using a kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA) based on the instructions provided by the manufacturer. Briefly, heart tissue or H9c2 cells were boiled in 0.1 mol/L acetic acid for 20 min at 100 °C to destroy enzymes and then homogenized in an ice bath. The homogenate was centrifuged at 13,000 x g for 30 min at 4 °C. The supernatant was collected and the total protein concentration was determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA). Appropriate volume of the supernatant (about 1 ml), plasma (about 0.5 ml) or culture medium (about 25 ml from five 100 mm dishes) was mixed with buffer A (Phoenix Pharmaceuticals, Inc., Burlingame, CA) at the ratio of 1:3 respectively, and centrifuged at 1,000xg for 15 min at 4 °C. The supernatants were loaded onto the pre-treated C-18 SEP-COLUMN containing 200 mg of C18 (Phoenix Pharmaceuticals, Inc., Burlingame, CA) slowly washed with buffer A (3 ml, twice) without collection of the washouts. Then the peptides were slowly eluted with buffer B (3 ml, once) (Phoenix Pharmaceuticals, Inc., Burlingame, CA) and collected into a polystyrene tube.

The eluants were evaporated to dryness in a centrifugal concentrator and kept at -20 °C. Ang II levels in the dried samples from heart tissue, plasma, H9c2 lysate or culture medium were measured with the Ang II Enzyme Immunoassay Kit (SPI-BIO, Massy, France) according to the manufacture's instruction. Ang II concentration is expressed as pg/mg protein for heart tissue and H9c2 lysate or pg/ml for plasma and cell culture medium.

Sirius-red staining of collagen

Cardiac fibrosis was examined by Sirius-red staining of collagen as described in our previous studies (8-11). Briefly, Tissue sections of 5 μm were used for Sirius Red staining of collagen using 0.1% Sirius Red F3BA and 0.25% Fast Green FCF. The sections stained for Sirius Red then were assessed for the proportion of fibrosis (collagen) using a Nikon Eclipse E600 microscopy system as described in our previous study (9).

Echocardiography

Under sedation with tribromoethanol (0.25 mg/g IP), 2D, M-mode, and Doppler echocardiography were performed using a Philips SONOS 5500 high-performance ultrasound system and a 15-6L linear array (15 MHz) transducer (120 Hz frame rate) as previously described (12-14). Left ventricular (LV) systolic function was assessed by ejection fraction (EF, %).

References for supplemental materials

1. Zhou Z, Wang L, Song Z, et al. Zinc supplementation prevents alcoholic liver injury in mice through attenuation of oxidative stress. Am J Pathol 2005;166:1681-90.
2. Kang X, Zhong W, Liu J, et al. Zinc supplementation reverses alcohol-induced steatosis in mice through reactivating hepatocyte nuclear factor-4alpha and peroxisome proliferator-activated receptor-alpha. Hepatology 2009;50:1241-50.

3. Feng M, Deerhake ME, Keating R, et al. Genetic analysis of blood pressure in 8 mouse intercross populations. Hypertension 2009;54:802-9.
4. Rossary A, Arab K, Steghens JP. Polyunsaturated fatty acids modulate NOX 4 anion superoxide production in human fibroblasts. Biochem J 2007;406:77-83.
5. Zhou G, Li X, Hein DW, et al. Metallothionein suppresses angiotensin II-induced nicotinamide adenine dinucleotide phosphate oxidase activation, nitrosative stress, apoptosis, and pathological remodeling in the diabetic heart. J Am Coll Cardiol 2008;52:655-66.
6. Ichikawa T, Li J, Meyer CJ, et al. Dihydro-CDDO-trifluoroethyl amide (dh404), a novel Nrf2 activator, suppresses oxidative stress in cardiomyocytes. PLoS One 2009;4:e8391.
7. Touyz RM, Yao G, Schiffrin EL. c-Src induces phosphorylation and translocation of p47phox: role in superoxide generation by angiotensin II in human vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 2003;23:981-7.
8. Wang J, Song Y, Elsherif L, et al. Cardiac metallothionein induction plays the major role in the prevention of diabetic cardiomyopathy by zinc supplementation. Circulation 2006;113:544-54.
9. Cai L, Wang Y, Zhou G, et al. Attenuation by metallothionein of early cardiac cell death via suppression of mitochondrial oxidative stress results in a prevention of diabetic cardiomyopathy. J Am Coll Cardiol 2006;48:1688-97.
10. Song Y, Wang J, Li Y, et al. Cardiac metallothionein synthesis in streptozotocin-induced diabetic mice, and its protection against diabetes-induced cardiac injury. Am J Pathol 2005;167:17-26.
11. Wang Y, Feng W, Xue W, et al. Inactivation of GSK-3beta by metallothionein prevents diabetes-related changes in cardiac energy metabolism, inflammation, nitrosative damage, and remodeling. Diabetes 2009;58:1391-402.
12. Black RG, Jr., Guo Y, Ge ZD, et al. Gene dosage-dependent effects of cardiac-specific overexpression of the A3 adenosine receptor. Circ Res 2002;91:165-72.
13. Wang G, Hamid T, Keith RJ, et al. Cardioprotective and antiapoptotic effects of heme oxygenase-1 in the failing heart. Circulation 2010;121:1912-25.
14. Hamid T, Gu Y, Ortines RV, et al. Divergent tumor necrosis factor receptor-related remodeling responses in heart failure: role of nuclear factor-kappaB and inflammatory activation. Circulation 2009;119:1386-97.

Supplemental Figure and Figure Legend (see next page)

Fig S1

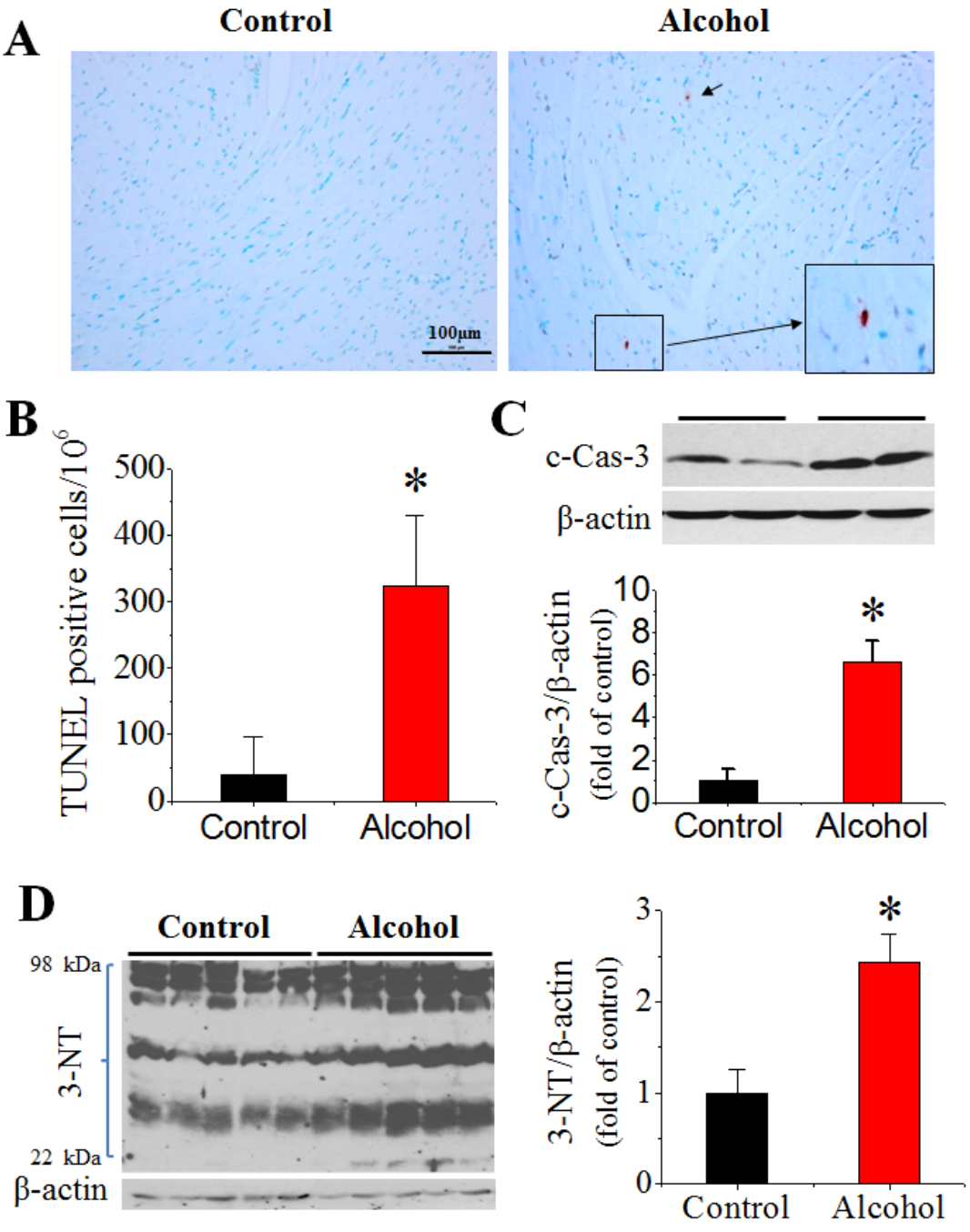

Figure S1. **Alcohol-induced cardiac cell death and nitrative damage *in vivo***. Mice were fed with alcohol for 2 months. Then heart tissues were collected and subjected to TUNEL staining for detecting apoptotic cells (A) with semi-quantitative analysis (B), Western blotting of cleaved-caspase-3 (c-Cas-3) (C), and 3-nitrotyrosin [3-NT, (D)], respectively (n=8 for control; n=11 for alcohol feeding). β-actin was used as loading control for Western blotting. *, $p<0.05$ vs control.

Fig S2

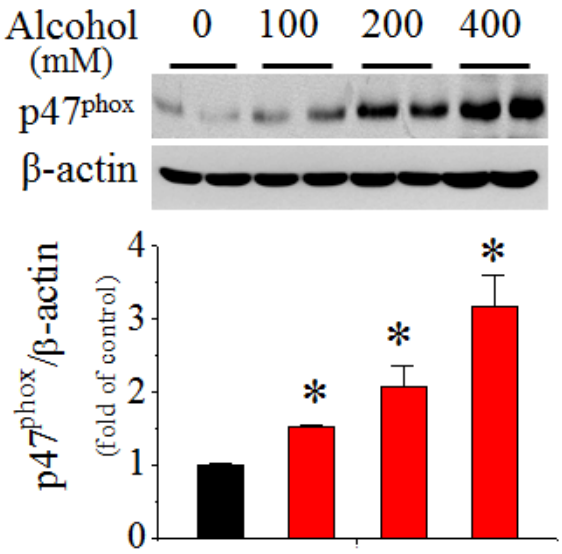

Figure S2. Alcohol-induced cardiac p47^{phox} upregulation *in vitro*. H9c2 cells were exposed to alcohol (100-400 mM) for 24 h, and then subjected to detect the expression of total p47^{phox} by Western blotting, β-actin was used as loading control. The presented data were from at least three independent experiments. *, $p<0.05$ vs control.

Fig S3

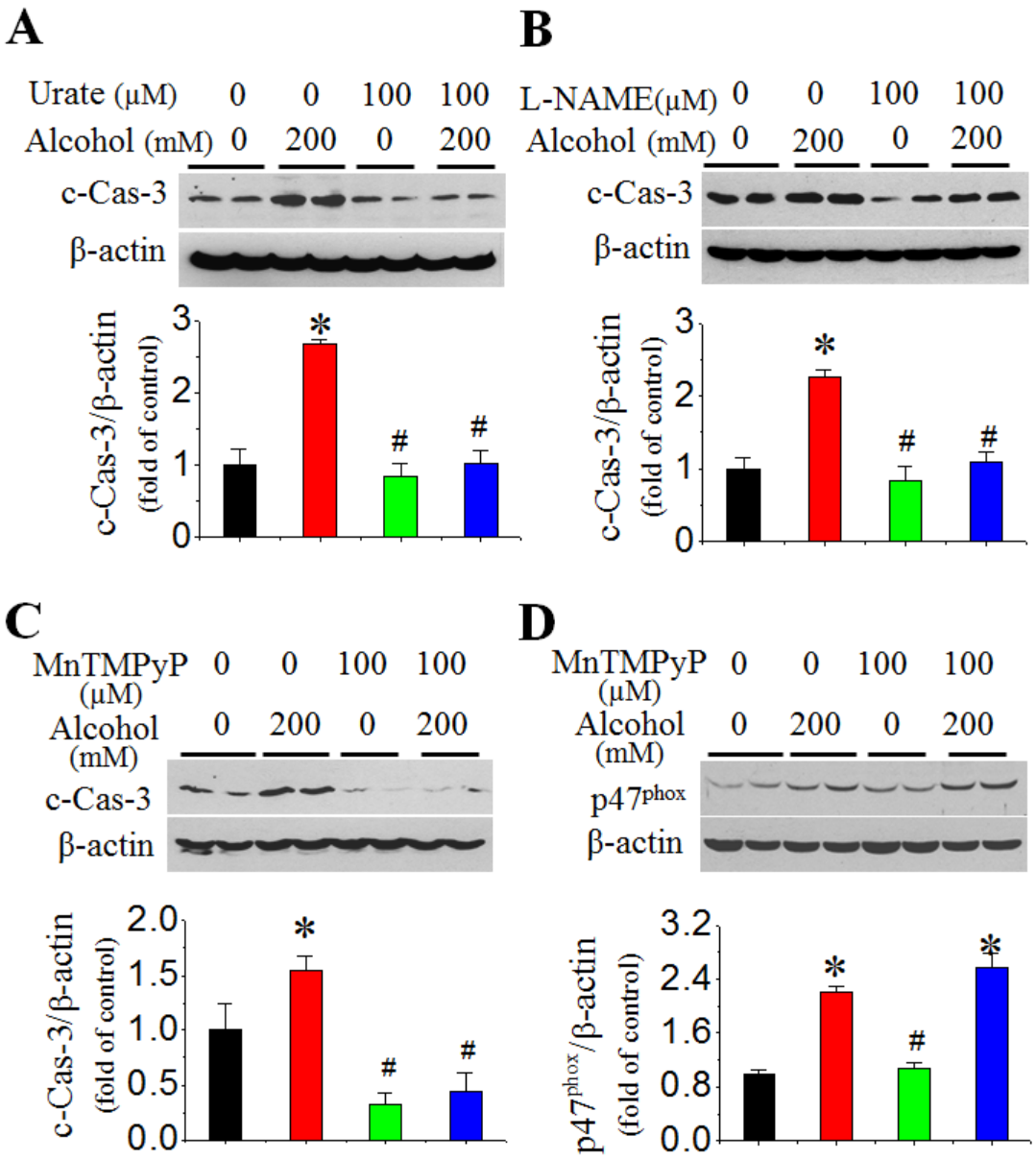

Figure S3. The causative role of nitrative stress in alcohol-induced cardiac cell death. H9c2 cells were treated with urate as peroxynitrite scavenger (A), L-NAME as NOS inhibitor (B), and MnTMPyP as superoxide dismutase mimic (C & D) at indicated concentrations for prior 30 min and co-exposure to alcohol at 200 mM for 24 h. The cleaved-caspase-3 (c-Cas-3) and $p47^{phox}$ expression were detected by Western blotting assay. The presented data were from at least three independent experiments. *, $p<0.05$ vs control; #, $p<0.05$ vs alcohol treatment.

Fig S4

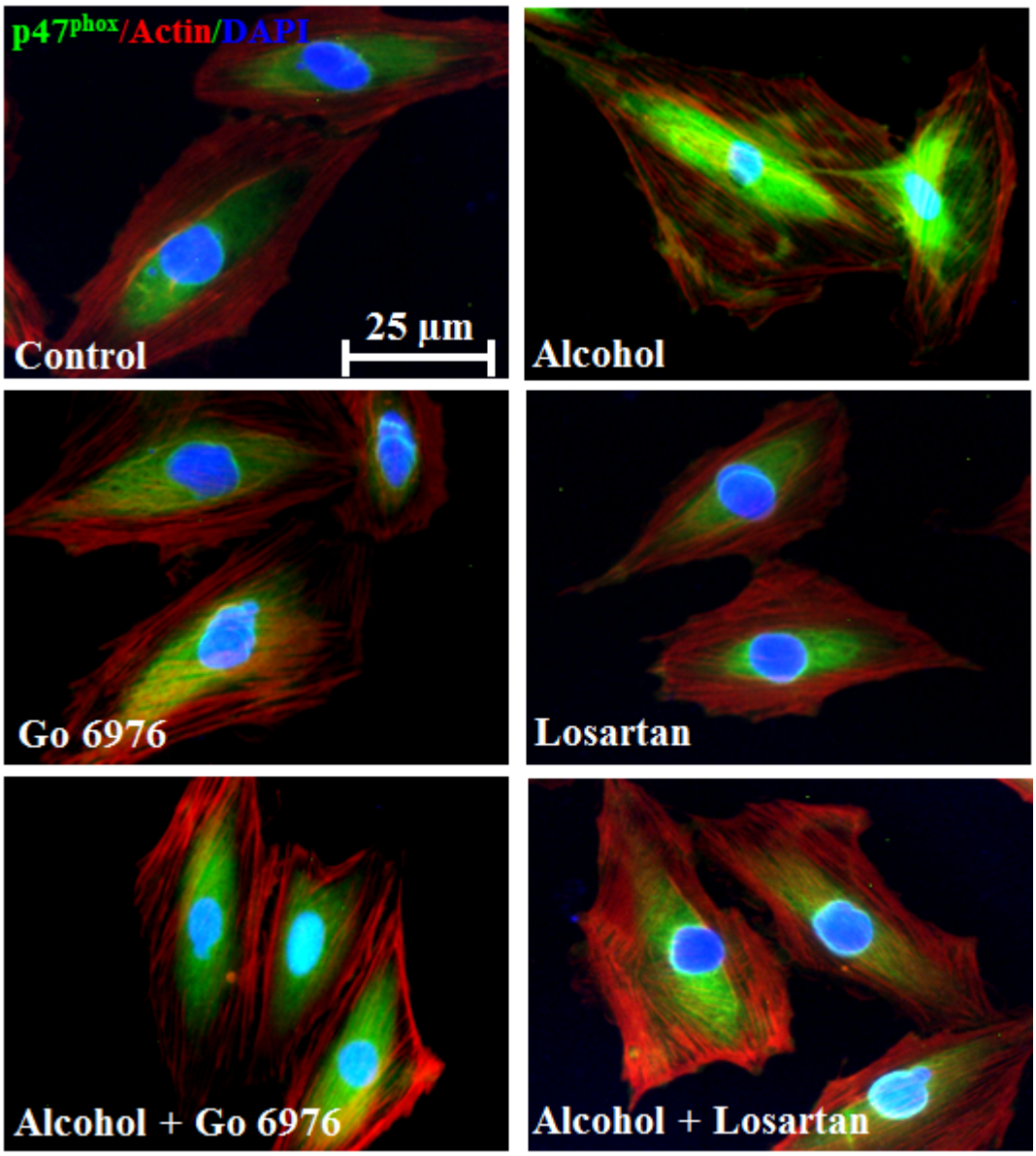

Figure S4. Alcohol-induced p47^{phox} translocation *in vitro*. H9c2 cells were given 30 min pre- and co-treatment of alcohol with PKCα/β1 inhibitor Go 6976 at 1 μM, or AT1 blocker losartan at 100 μM and then the translocation of p47^{phox} from cytosome to the membrane was visualized by immunefluorescent staining at 2 h. Green indicates p47^{phox}; Red indicates the cell skeletal structure protein (actin); Blue indicates nuclear (DAPI: 4',6-diamidino-2-phenylindole). The representative data were from at least three independent experiments.

Fig S5

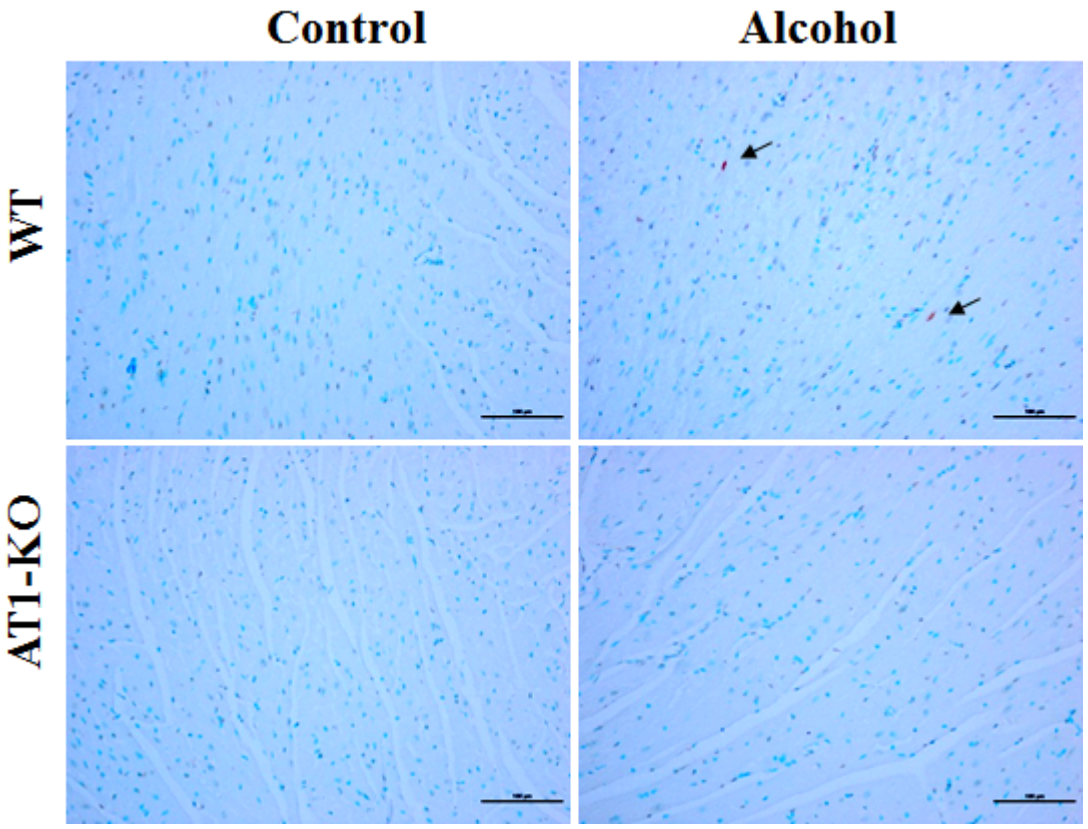

Figure S5. Cell death after alcohol feeding in the heart. AT1-KO and the wide-type (WT) mice were pair-fed and alcohol-fed for 2 months. Heart tissues were collected. Cardiac cell death was examined by TUNEL staining apoptotic cells and its quantitative analysis was presented in published Figure 5A. Chronic alcohol feeding significantly induced cardiac apoptosis in wild type mice, but AT1-KO mice are resistant to alcoholic cell death.

Fig S6

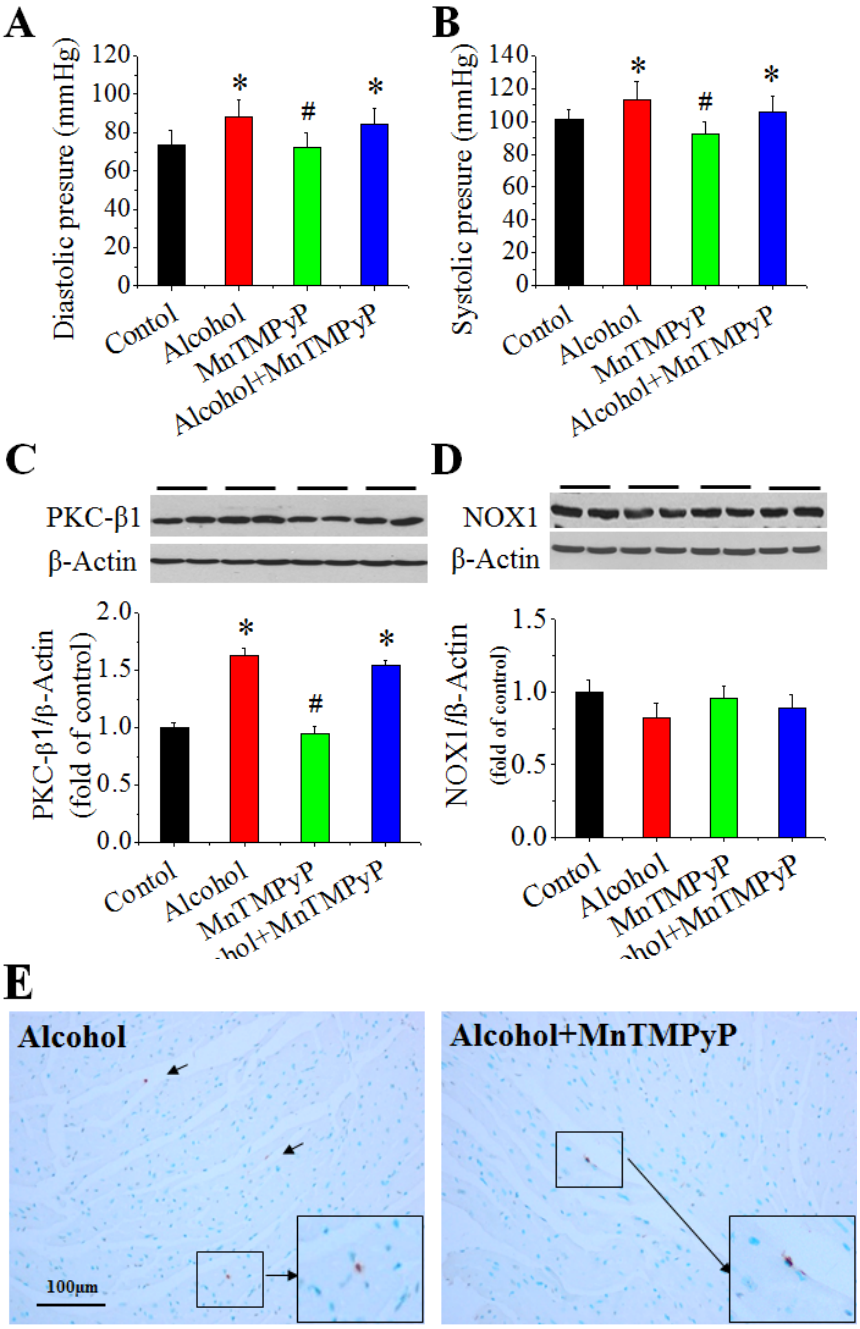

Figure S6 . Treatment of chronically alcohol-fed mice with MnTMPyP does not affect on blood pressure, and PKCβ1 and NOX1 expression but prevent alcoholic cell death. Pair-fed and alcohol-fed mice were treated with and without superoxide dismutase mimic manganese (III) tetrakis(1-methyl-4-pyridyl) porphyrin (MnTMPyP) at 5 mg/kg body weight, I.P. daily for 2 months. Diastolic (A) and systolic (B) blood pressures were measured using a CODATM non-invasive blood pressure monitoring system, and then heart tissues were collected to examine the expression of PKC-β1 (C) and NOX1 (D) by Western blotting assay (n=8 for each group). β-actin was used as loading control for Western blotting. *, $p<0.05$ vs control; #, $p<0.05$ vs alcohol treatment. Cardiac cell death was examined by TUNEL staining apoptotic cells (E) and its quantitative analysis was presented in published Figure 6E.