## Selective Inhibition of NLRP3 inflammasome Protects against Acute Ethanol-Induced Cardiotoxicity through a FBXL2-Dependent Manner

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Original Article

Selective inhibition of the NLRP3 inflammasome protects against acute ethanol-induced cardiotoxicity in an FBXL2-dependent manner

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Running head: NLRP3 and alcoholic heart diseases

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Abstract
Binge drinking exerts cardiac toxicity through various mechanisms, including oxidative stress and inflammation. NLRP3 inflammasomes possess both pro- and anti-inflammatory properties, although the role of NLRP3 in ethanol-induced cardiotoxicity remains unknown. This study was designed to examine the role of NLRP3 inflammasomes in acute ethanol cardiotoxicity and the underlying mechanisms of action. Nine- to twelve-week-old adult male C57BL/6 mice were administered ethanol (1.5 g/kg, twice daily, i.p.) for 3 days. A cohort of control and ethanol-challenged mice were treated with the NLRP3 inhibitor MCC950 (10 mg/kg/d, i.p., days 1 and 3). Myocardial geometry and function were monitored using echocardiography and cardiomyocyte edge-detection techniques. Levels of NLRP3 inflammasome, mitophagy and apoptosis were evaluated using Western blot and immunofluorescence techniques. Acute ethanol challenge resulted in abnormally higher cardiac systolic function, in conjunction with deteriorated cardiac diastolic function and cardiomyocyte contractile function. Levels of NLRP3 inflammasome and apoptosis were elevated, and mitophagy flux was blocked (elevated Pink1-Parkin and LC3B along with diminished p62 and Rab7) in mice receiving acute ethanol challenge. Although MCC950 did not elicit a notable effect on myocardial function, apoptosis or inflammasome activation in the absence of ethanol exposure, it effectively rescued acute ethanol cardiotoxicity, as manifested by restored myocardial and cardiomyocyte functional homeostasis, suppressed NLRP3 inflammasome activation and apoptosis, and improved mitophagy flux. Our data further suggested that FBXL2, an E3 ubiquitin ligase associated with mitochondrial homeostasis and mitophagy, was destabilized due to proteasomal degradation of caspase-1 by ethanol-induced hyperactivation of NLRP3-caspase-1 inflammasome signaling, resulting in mitochondrial injury and apoptosis. These findings denote a role for NLRP3 inflammasomes in acute ethanol exposure-induced cardiotoxicity in an FBXL2-dependent manner and the therapeutic promise of targeting NLRP3 for acute ethanol cardiotoxicity.

Key words: ethanol, cardiotoxicity, NLRP3 inflammasomes, mitophagy

Introduction
Binge drinking is associated with overtly elevated cardiovascular morbidity and mortality in abstainers and heavy drinkers [1-5]. In general, chronic heavy alcohol consumption (> 90 g/day for 5+ years) provokes the etiology of alcoholic cardiomyopathy, which mainly presents as left ventricular (LV) dilation, interstitial fibrosis, and compromised cardiac output, ultimately prompting heart failure [6-8]. Although a plethora of machineries have been proposed for the pathogenesis of alcoholic myopathy, including ethanol- and metabolite-evoked toxicity, buildup of oxidative stress and fatty acid ethyl esters, the main culprit responsible for alcoholic cardiotoxicity remains elusive [9, 10]. For patients with evident alcoholic heart injury, little curative remedy is available other than symptomatic and supportive care. Not surprisingly, abstinence from alcohol use is perceived as the most effective therapeutic option for alcoholic organ damage, including alcoholic cardiomyopathy, resulting in the recovery of myocardial function in the realm of alcohol abuse or alcoholism [11, 12]. Nevertheless, such a manoeuvre is unlikely to be enforceable and practical for those drinkers who are addicted to alcohol, largely due to compliance issues. To retard the progression of alcoholic cardiomyopathy, it is pertinent to identify more effective approaches to intervene in acute alcohol intake-induced cardiotoxicity.

Accumulating evidence has indicated a unique role for inflammation in ethanol-induced organ damage, including cardiotoxicity [13-16]. The main pathologic processes of inflammation encompass tissue metamorphosis, exudation, and proliferation, each constituting a complex molecular network of action. In addition to inflammatory factors, inflammasomes are gaining some recent attention due to their regulatory role in inflammation and cell fate. Inflammasomes belong to a group of multiprotein intracellular pattern recognition receptors (PRRs) that are recognized by a plethora of exogenous pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs), resulting in the release of proinflammatory cytokines, namely, IL-1β and IL-18 [17]. Nod-like receptor pyrin domain-containing 3 (NLRP3), which consists of the adapter protein apoptosis-associated speck-like protein (ASC), NLRP3, and pro-caspase-1, represents an important tissue damage sensor that triggers sterile inflammation. Upon NLRP3 assembly, inactive pro-caspase-1 residing within the inflammasome complex is autoprocessed to active caspase-1, prompting maturation of pro-interleukin-1β (pro-IL-1β) or pro-IL-18 to form IL-1β or IL-18 [18, 19].
MCC950 is a newly developed small molecule inhibitor that selectively suppresses NLRP3 inflammasome activation and reduces IL-1β or IL-18 production, thus inhibiting inflammation [20]. However, whether MCC950 has any effect on acute alcohol-induced cardiotoxicity remains unknown.

It was reported that mitochondrial permeabilization, indicative of mitochondrial damage, occurred as a consequence of NLRP3 activation [21]. Similarly, NLRP3 deficiency protected mitochondria from depolarization in hypoxic renal tubular cells [22]. Activation of NLRP3 inflammasomes then promotes caspase-1-mediated mitochondrial injury, resulting in disruption and permeabilization of mitochondrial membranes and the ultimate collapse of the mitochondrial network [23]. Among the machineries governing mitochondrial quality, mitophagy serves a cytoprotective role to remove long-lived and/or impaired mitochondria to preserve mitochondrial homeostasis [24]. Upon the onset of mitophagy, injured mitochondria may be identified and degraded through autophagy [24, 25]. PINK1/Parkin represents one important nonreceptor-mediated mitophagy machinery. With mitochondrial membrane depolarization, PINK1 is recruited onto the mitochondrial outer membrane to phosphorylate the E3 ubiquitin ligase Parkin. Through the interaction with PINK1, Parkin ubiquitinates various mitochondrial proteins through its E3 ligase capacity to solicit p62 to be bridged with LC3, resulting in the induction of mitophagy [24, 26]. Accumulating evidence has suggested that ethanol administration may interrupt mitophagy to evoke cellular damage [13, 27-29].

Ample evidence has denoted a unique role for multimeric ubiquitin ligase SCF complexes, which are composed of SKP1, CUL1, and F-box proteins, in human diseases [30, 31]. Among various F-box proteins, the E3 ubiquitin ligase FBXL2 [F-box and LLR (leucine-rich repeats) containing protein 2] controls SCF specificity through its interaction with SCF substrates for ubiquitylation and later proteasomal degradation [30, 31]. Recent findings from our group and others revealed that the mitophagy receptor FUNDC1 regulates mitochondrial Ca^{2+} handling through interaction with FBXL2 to drive degradation of the inositol 1,4,5-trisphosphate receptor IP3R3 [32, 33]. More evidence has indicated that the NLRP3 component cleaved caspase-1 may directly cleave E3 ubiquitin ligases, including Parkin and NEDD4 [34, 35], although the involvement of the E3 ubiquitin ligase FBXL2 remains elusive. To this end, our current study was performed to evaluate the impact of the NLRP3 inhibitor MCC950 on acute
ethanol-evoked cardiotoxicity and the possible mechanisms involved. Our results suggested that FBXL2 was destabilized due to proteasomal degradation of caspase-1 in the face of hyperactive NLRP3-caspase-1 inflammasome signaling following binge drinking, resulting in mitochondrial injury and apoptosis.

**Materials and Methods**

**Animal procedures**

All animal procedures described here received approval from the institutional Animal Care and Use Committee at Zhongshan Hospital Fudan University (Shanghai, China). In brief, adult male C57/BL6J mice aged 9-12 weeks (25~30 g) were randomly divided into four groups: control (CON), ethyl alcohol (EtOH), MCC950 (MCC950), and alcohol plus MCC950 (EtOH-MCC950) (12-14 mice per group). Mice received acute ethanol challenge twice a day (intraperitoneal injection, i.p., 1.5 g/kg) for three consecutive days. MCC950 was intraperitoneally injected 1 hour before ethanol injection at a dose of 10 mg/kg/d (on day 1 and day 3).

**Echocardiographic assessment**

Cardiac geometry and function were evaluated in anaesthetized (ketamine 80 mg/kg and xylazine 12 mg/kg, i.p.) mice 24 hours after the final ethanol challenge using 2-dimensional echocardiography (Vevo 2100, FUJIFILM VisualSonics, Toronto, ON, Canada) equipped with a 22-55 MHz linear transducer (MS550D, FUJIFILM VisualSonics, Toronto, ON, Canada). Hearts were first imaged in parasternal long-axis view (2-D) prior to M-mode visualization. Left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD) and left ventricular ejection fraction (LVEF) were recorded [36]. Diastolic function was evaluated using transmitral Doppler flow E and A velocities, tissue Doppler imaging E’ velocities, and E-wave deceleration time. All recordings were averaged in 5 consecutive cardiac cycles by experienced technicians in a blind manner [36].

**Isolation of cardiomyocytes and cell shortening recording**

Heart was removed under anaesthesia. An EDTA buffer and perfusion buffer were delivered
into the left ventricles before the perfusion of type II and IV collagenase to digest the heart.

Left ventricles were dispersed and pulled into 1-mm³ pieces using forceps to dissociate cardiomyocytes. Ca²⁺ reintroduction was conducted to restore extracellular Ca²⁺ back to 1.2 mM. A yield of at least 80% rod-shaped cells was deemed successful. The mechanical properties of cardiomyocytes were evaluated using an IonOptixTM system (IonOptix, Milton, MA) to record peak shortening (PS), maximal velocities of shortening/relengthening (± dL/dt), time-to-PS (TPS) and time-to-90% relengthening (TR₉₀) at 0.5 Hz [37].

**Intracellular Ca²⁺ transients**

Cardiomyocytes were incubated with fura-2/AM (0.5 μM for 10 min) prior to assessment of fluorescence intensity using a dual-excitation fluorescence photomultiplier system (IonOptix). Cells were exposed to light emitted by a 75-W lamp and passed through either a 360- or a 380-nm filter (with emissions at 480-520 nm). Changes in fura-2 fluorescence intensity (FFI) were inferred from FFI readings at 360- and 380-nm wavelengths. The fluorescence decay time was derived to estimate intracellular Ca²⁺ clearance [37].

**Histological staining**

Heart tissues were stained with hematoxylin and eosin (H&E) (Sigma-Aldrich, St. Louis, MO, USA) and Sirius red (Serviciebio Technology, Wuhan, China) as described previously [38, 39]. High-resolution images of cardiomyocytes (400x) or red-stained collagen fibres (200x) were collected using a Leica DM750 microscope (Heidelberg, Germany) and analysed using ImageJ software.

**Transmission electron microscopy (TEM)**

The ultrastructure of the heart was assessed using TEM. Following fixation in PIPES-buffered formaldehyde-glutaraldehyde, the myocardium was trimmed into 1-mm³ blocks. Myocardial blocks were rinsed with a PIPES buffer along with 2% sucrose (pH 7.4) before further processing in PIPES buffered 1% OsO₄ along with 2% sucrose and 1.5% K₂Fe(CN)₆·3H₂O overnight. Tissue blocks were dehydrated and enclosed in Epon/Araldite. An RMC-MTXL ultramicrotome and a Diatome diamond knife were employed to generate thin sections prior to
incubation with lead citrate and uranyl acetate. Micrographic images were captured under a
Hitachi 7500 transmission electron microscope (Hitachi, Tokyo, Japan) [40].

**TUNEL staining**

Cardiomyocyte apoptosis was evaluated using TUNEL staining with an In Situ Cell Death
Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). Following fixation in
paraformaldehyde, samples were incubated with permeabilizing solution for 30 min prior to
incubation with TUNEL reaction mixture for 1 h at 37°C. Morphological evaluation was
carried out using a Leica DM1000 microscope (Heidelberg, Germany). Nine microscopy image
fields were randomly chosen to evaluate > 100 cells for apoptosis assessment [41].

**Detection of superoxide (O$_2^-$)**

Mitochondrial O$_2^-$ was measured using dihydroethidium (DHE). Cardiomyocytes were loaded
with 5 μM DHE (Invitrogen, Thermo Fisher Scientific, Waltham, USA) for 30 min at 37 °C
prior to rinsing with PBS buffer. Olympus FV3000 laser confocal microscope (Tokyo, Japan)
was utilized to monitor the fluorescence of O$_2^-$ production with ImageJ software [32].

**ELISA of TNF-α, IL-6, IL-1β, IL-18, creatine kinase (CK) and lactate dehydrogenase (LDH)**

Serum levels of TNF-α, IL-6, IL-1β and IL-18 were detected using ELISA kits (R&D Systems,
USA) per the manufacturer’s instructions. Serum levels of CK and LDH were evaluated using
colorimetric assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) per the
manufacturer’s instructions [42].

**Real time-polymerase chain reaction (real time-PCR)**

Total RNA was harvested from cells or tissues using TRIzol Reagent (Thermo Fisher Scientific,
Waltham, USA) along with an RNeasy Total RNA Isolation Kit (Qiagen, Hilden, Germany).
Isolated RNA was then reverse-transcribed courtesy of an iScript cDNA Synthesis Kit (Takara
BIO, Otsu, Japan). Real-time PCR was conducted using iQ SYBR Green Supermix (Bio-Rad
Laboratories, Hercules, USA) and CFX96TM Real-Time System (Bio-Rad Laboratories). The
PCR reaction protocol was as follows: 3 min at 95°C, then 10 sec at 95°C, 30 seconds at 56°C for 40 cycles, and finally increment 0.5°C from 55°C to 95°C for 30 seconds. The mRNA relative expression was calculated using the ∆∆CT Method. All primers were obtained from GenePharma Corporation (Shanghai, China) with sequence information: NLRP3-F, 5’-TGAAGAGTGTGATCTGCGGAAAC-3’ and NLRP3-R, 5’-GAAAGTCACTGGCTGGAGCTGT-3’; β-actin-F, 5’-AGACCTTCAACACCCCCAG-3’ and βactin-R, 5’-CAGATTTCTCCTCAGC-3’.

Detection of mitochondrial membrane potential (MMP)

A commercially available MMP kit (C2006, Beyotime Biotechnology) was employed. In short, isolated cardiomyocytes were incubated with the MMP fluorescence dye JC-1 at 37°C for 20 min. Cells were then rinsed and maintained in DMEM (Gibco, Grand Island, NY, USA). Images were captured using an Olympus FV3000 laser confocal microscope (Tokyo, Japan). The aggregate-to-monomer (red/green) fluorescence ratio was calculated to reflect the MMP level. Approximately 20-30 fields from 3 independent experiments per group were used. At least 40-50 cells per group were counted per image field [43].

Neonatal cardiomyocyte (NCM) isolation and siRNA silencing

Neonatal mice were sterilized with ethanol prior to removal of hearts. Hearts were then cut into small pieces followed by digestion with 0.25% trypsin. Upon tissue defragmentation, samples were centrifuged at 800 g for 10 min, and pellets containing cells were resuspended in DMEM supplemented with fetal bovine serum (20%) with penicillin and streptomycin prior to being plated in a culture dish for 1 h. NCM cells were plated in a confocal plate precoated with 1% gelatin and were further cultured for 48 h at 37°C under 95% O₂ and 5% CO₂ [44]. Cells were subsequently transfected with NLRP3 siRNA (siRNA-1: sense 5’-GGUUCUGAGCUCCAACCAUTT-3’, antisense: 5’-AUGGUUGGAGCUGACCAACTT-3’; siRNA-2: sense 5’-GGUCUCUUCUCAAGUCUAATT-3’, antisense 5’-UUAGACUUGAGAAGACCTT-3’; and siRNA-3: sense 5’-CCAACUGGUCAAGGCAUTT-3’, antisense 5’-AUGCUCCUUGGACGUUGTT-3’) or siRNA-NC (Negative Control; sense 5’-UUCUCGAACGUGUCACGUTT-3’, antisense
5’-ACGUGACACGUUCGGAGAATT-3’) in DMEM. Forty-eight hours later, neonatal cardiomyocytes were exposed to ethanol (160 mM), MCC950 (20 μM), caspase-1 inhibitor AC-YVAD-CMK (50 μM), FBXL2 activator BC-1258 (10 μM), or FBXL2 inhibitor GGTI-2418 (10 μM) for an additional 8 hours prior to biochemical assessment.

**Immunofluorescence analysis**
Cardiomyocytes were first fixed in 4% paraformaldehyde. Following rinsing with PBS, cells were treated with goat serum containing 0.3% Triton X-100 for 1 h before an overnight incubation at 4°C with anti-NLRP3 (1:50; Abcam), anti-caspase-1 (1:200; Santa Cruz Biotechnology), anti-COXIV (1:200; Cell Signaling Technology), or anti-LC3B (1:200; Cell Signaling Technology) antibodies. Cells were then treated with the fluorescence-conjugated secondary antibody (Proteintech, USA) for 1 h. Cells were stained with DAPI containing anti-fade reagent. Micrographs (10 fields from 3 independent experiments per group) were generated through an Olympus laser confocal microscope and fluorescence colocalization (Pearson correlation and Mander coefficient) was analysed using ImageJ via the Coloc 2 modality.

**Western blot analysis**
Samples were homogenized and sonicated in RIPA lysis buffer (Beyotime Biotechnology) containing protease inhibitor cocktail (1%). Protein levels were assessed using the Bradford assay. Approximately 20-30 μg of protein was loaded and separated by 15% SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes. Membranes were incubated in 5% nonfat milk in Tris-buffered saline buffer. After blocking, membranes were incubated overnight at 4°C with secondary antibody (1:5000) prior to densitometry using a ChemiDoc Image device (Bio-Rad). GAPDH or β-actin was used as a loading control [45].

**Regents and antibodies**
Drugs such as MCC950 (Sigma-Aldrich), caspase-1 inhibitor AC-YVAD-CMK (Sigma-Aldrich), FBXL2 activator BC-1258 (Sigma-Aldrich), and FBXL2 inhibitor GGTI-2418 (MedChem Express, New Jersey, USA) were applied to mice or cardiomyocytes. Anti-NLRP3
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(1:1000; Cell Signaling Technology), anti-caspase-1 p20 antibody (1:100; Santa Cruz Biotechnology), anti-IL-1β (1:1000; Cell Signaling Technology), anti-IL-18 (1:1000; Cell Signaling Technology), anti-Pink1 (1:1000; Cell Signaling Technology), anti-p62 (1:1000; Cell Signaling Technology), anti-LC3B (1:1000; Cell Signaling Technology), anti-Rab7 (1:1000; Cell Signaling Technology), anti-Bax (1:500; Cell Signaling Technology), anti-Bcl-2 (1:500; Cell Signaling), anti-FBXL2 (1:2000; Novus Biologicals, USA), anti-GAPDH (1:1000; Cell Signaling), anti-β-actin (1:5000; KangChen Biotech, Shanghai, China), and anti-rabbit or anti-mouse secondary antibody (1:5000; Proteintech, USA) were used in western blot analysis.

Statistical analysis

Statistical parameters are expressed as the mean ± SEM. Multiple groups were compared using one-way ANOVA comparisons tests (Prism 8 software; GraphPad, La Jolla, USA). *P* < 0.05 was considered statistically significant.

Results

**MCC950 attenuated ethanol-induced cardiac dysfunction in mice**

Following acute ethanol challenge or MCC950 challenge, the survival rate and echocardiographic function were determined (Figure 1A–N). No mortality was noted in the control and MCC950 alone groups (data not shown). Ethanol challenge significantly lowered survival, the effect of which was greatly attenuated by MCC950 (*P* < 0.01) (Figure 1B). Echocardiographic findings revealed that ejection fraction (EF) and fractional shortening (FS) were surprisingly higher in the EtOH group than in the CON group. In addition, ethanol challenge overtly promoted cardiac hypertrophy, increased systolic ventricular septal thickness (IVSs), diastolic ventricular septal thickness (IVSd) and diastolic posterior wall thickness of the left ventricle (LVPWd) (*P* < 0.05), displayed a trend of increased systolic posterior wall thickness of the left ventricle (LVPWs) (*P* > 0.05) and significantly decreased left ventricular end-systolic diameter (LVESD) and left ventricular end-diastolic diameter (LVEDD) (Figure 1F,G). Although MCC950 itself did not produce any notable echocardiographic effect, it rescued ethanol-induced changes in echocardiographic indices, including ejection fraction and...
fractional shortening. Assessment of echocardiographic diastolic function also showed that
EtOH overtly decreased the E/A value (< 1) and increased the E/E’ value, indicating diastolic
dysfunction, the effect of which was alleviated by MCC950 with little effect from MCC950
alone (Figure 1L–N). These data denoted a cardioprotective role of NLRP3 inflammasome
inhibitor MCC950 against ethanol-induced cardiac dysfunction in mice.

**MCC950 abrogates cardiomyocyte mechanical and intracellular Ca\(^{2+}\) handling
derangement triggered by acute ethanol challenge**

Acute ethanol challenge compromised cardiomyocyte contractile function, including decreased
peak shortening, maximal velocities of shortening (+ dL/dt) and relengthening (-dL/dt), as well
as prolonged TR\(_{90}\), the effect of which was mitigated by MCC950 with little effect from
MCC950 itself (Figure 2A–G). To discern possible mechanisms of action behind the benefits
of MCC950 against acute ethanol toxicity in the heart, intracellular Ca\(^{2+}\) handling was
examined using the Fura-2 fluorescent technique. Our data revealed that acute ethanol
challenge significantly depressed the electrically induced rise in intracellular Ca\(^{2+}\) (ΔFFI)
without affecting baseline intracellular Ca\(^{2+}\) and intracellular Ca\(^{2+}\) decay, the effect of which
was reversed by MCC950 with little effect from MCC950 itself (Figure 2H–J). These data
indicated that NLRP3 inflammasome inhibition rescued acute ethanol insult-induced
cardiomyocyte mechanical and intracellular Ca\(^{2+}\) handling derangement.

**MCC950 improved histological changes with acute ethanol challenge**

Acute ethanol treatment promoted cardiac hypertrophy and interstitial fibrosis, the effects of
which were ablated by MCC950. In particular, HE staining showed that the cross-sectional
area of cardiomyocytes was overtly increased in the ethanol group in comparison with the CON
group, the effect of which was reversed by MCC950. Sirius scarlet staining displayed overt
myocardial interstitial fibrosis in the EtOH group in comparison with the CON group, the effect
of which was reconciled by MCC 950. Finally, MCC950 failed to affect myocardial histology
in the absence of ethanol challenge (Figure 3A, B, D, E). These data denoted a cardioprotective
role of NLRP3 inflammasome inhibition against ethanol-induced histological changes in mice.
MCC950 attenuated acute ethanol-induced superoxide release and apoptosis

To detect superoxide levels, mouse cardiomyocytes were isolated, and DHE fluorescence intensity was determined. As shown in Figure 3C, F, superoxide levels were significantly increased in isolated cardiomyocytes from the EtOH group. Although MCC950 itself did not have any impact on DHE staining in the absence of ethanol challenge, it overtly attenuated the ethanol-induced increase in DHE fluorescence intensity. TUNEL staining of myocardial tissue displayed more abundant TUNEL-positive cells in the EtOH group than in the CON group, the effect of which was negated by MCC950, with little effect from MCC950 itself (Figure 3G, L). Consistent with the TUNEL data, assessment of apoptotic protein markers using western blot noted higher Bax levels along with downregulated Bcl2 in the ethanol group compared with the CON group, the effect of which was nullified by MCC950 with little effect from MCC950 itself (Figure 3H‒K). These data indicated that NLRP3 inflammasome inhibition attenuated superoxide release and apoptosis in ethanol-treated mice.

MCC950 attenuated acute ethanol-induced inflammation and cardiac injury by inhibiting NLRP3 inflammasome activation

The specific NLRP3 inflammasome inhibitor MCC950 rescued ethanol-induced increases in serum levels of TNF-α, IL-6, IL-1β, and IL-18 as well as markers for cardiac injury CK and LDH and protein expression of NLRP3 inflammasome-related signaling components, including NLRP3, caspase-1, IL-1β and IL-18 (Figure 4A‒K). In particular, MCC950 also significantly reduced ethanol-evoked elevation in the colocalization levels of NLRP3 and caspase-1 (Figure 4L‒M). These data suggested that NLRP3 inflammasome activation and inflammatory cytokine release played an important role in acute ethanol-induced cardiotoxicity.

MCC950 attenuates ethanol challenge-induced changes in Parkin-mediated mitophagy

TEM was conducted to analyse changes in mitochondrial morphology and mitophagy. Based on transmission electron microscopy, no difference was found in the myocardial ultrastructure between the CON and MCC950 groups, and the mitochondria and myocardial fibres were arranged normally. EtOH decreased the area of mitochondria while increasing the number of mitochondria and the number of mitochondrial autophagosomes. Interestingly, these anomalies
were effectively reversed by MCC950 (Figure 5G-I,K). Western blot analysis revealed that EtOH increased the levels of PINK1, Parkin, LC3B and p62 and reduced the level of Rab7, and these effects were reversed by MCC950 (Figure 5A–F). Findings from confocal microscopy revealed higher colocalization of COX IV and LC3B in the EtOH group (indicating facilitated mitophagy), and the effect was abolished by MCC950 (Figure 5J, L). These data denoted a role of NLRP3 inflammasomes in the regulation of mitophagy and thus mitochondrial integrity in acute ethanol-induced cardiotoxicity.

**Ethanol induces interruption of mitophagy flux via an NLRP3/caspase-1/FBXL2-dependent mechanism**

The NLRP3 inflammasome component cleaved caspase-1 is known to cleave E3 ubiquitin ligases such as Parkin and NEDD4 [23, 34, 35]. In this context, we explored the possible involvement of FBXL2 in ethanol toxicity and NLRP3 inhibition-evoked cardiac responses. As shown in Figure 6A,B, ethanol reduced the levels of FBXL2, the effect of which was reversed by MCC950. Following NLRP3 silencing with siRNA-1 (efficiency displayed in Supplementary Figure S1, which was also verified using western blot analysis) or caspase-1 inhibition using AC-YVAD-CMK, acute ethanol failed to downregulate FBXL2 and upregulate cleaved caspase-1, indicating an essential role for NLRP3/caspase-1 in ethanol-induced changes in FBXL2 (Figure 6C–I). Our further findings revealed that the NLRP3 inflammasome inhibitor MCC950 alleviated ethanol-induced cardiomyocyte contractile derangements, including decreased peak shortening, maximal velocity of shortening (+dL/dt) and relengthening (−dL/dt) with unchanged duration of shortening and relengthening, the effects of which were reversed by the FBXL2 inhibitor GGti-2418. This is in line with the observation that the FBXL2 activator BC-1258 nullified ethanol-induced cardiomyocyte contractile defects. Neither GGti2418 nor BC-1258 had any notable effect on cardiomyocyte mechanics themselves (Figure 7A–F). Moreover, NLRP3 knockdown rescued ethanol-induced mitochondrial membrane potential collapse and colocalization of COX IV and LC3B, the effects of which were ablated and mimicked by the FBXL2 inhibitor GGti-2418 and FBXL2 activator BC-1258, respectively (Figure 8A–D). These results indicated that ethanol challenge evoked compromised cardiomyocyte mitochondrial and contractile function likely in an
NLRP3/caspase-1/FBXL2-dependent manner.

Discussion

The salient findings from our study indicated that inhibition of NLRP3 inflammasomes using MCC950 rescued acute ethanol insult-induced cardiac dysfunction manifested by NLRP3 inflammasome activation, inflammation, apoptosis, myocardial fibrosis, and contractile dysfunction. Furthermore, our results suggested interrupted mitophagy flux in acute ethanol challenge-induced cardiotoxicity, the effect of which was reversed by MCC950. In addition, our results provide evidence for the first time of a role for the NLRP3/caspase-1/FBXL2 signaling axis in MCC950-mediated protection against acute alcohol toxicity. These findings revealed an important role for NLRP3 inflammasome-mediated regulation of mitophagy in ethanol cardiotoxicity, providing therapeutic promise for NLRP3 in ethanol-induced cardiotoxicity.

Pronounced myocardial damage has been shown following binge ethanol intake (90 to 100 g/d or ~1.5 g/kg in humans [37, 46, 47]. The ethanol dosage used in our current study (3 g/kg) should mimic heavy ethanol binge intake (considering the much higher ED50 in rodents) [37]. Our data revealed overt changes in cardiac geometry, interstitial fibrosis, contractile and intracellular Ca\textsuperscript{2+} properties (decreased LVESD and LVEDD, increased LV septal and wall thickness, elevated LV fractional shortening and ejection fraction, decreased E/A ratio, elevated E/E’ ratio, dampened cardiomyocyte peak shortening, ±dL/dt and intracellular Ca\textsuperscript{2+} release as well as prolonged TP\textsubscript{90} following acute ethanol challenge), somewhat consistent with our earlier reports [37, 46, 47]. Interestingly, inhibition of NLRP3 using MCC950 reversed ethanol cardiotoxicity evaluated by cardiomyocyte cross-sectional area, interstitial fibrosis, echocardiographic and cardiomyocyte contractile function. Diastolic function appeared to be compromised, as evidenced by changes in the E/A and E/E’ ratios. Meanwhile, ethanol challenge evoked cardiomyopathy indistinguishable from dilated cardiomyopathy.

Echocardiography noted cardiac hypertrophy (wall and septal thickness) following ethanol challenge, suggesting that acute ethanol insult not only results in dilated cardiomyopathy but also triggers compensatory hypertrophy of the ventricular wall early on in ethanol insult, which may support increases in fractional shortening and ejection fraction. Cardiomyocyte
mechanical measurement indicated that acute ethanol exposure compromised cardiomyocyte contractile function, suggesting that abnormally higher EF and FS did not bring benefits but increased the burden on the heart. Rises in fractional shortening and ejection fraction in conjunction with elevated chamber size and wall or septal thickness following acute ethanol challenge are somewhat unexpected, as alcohol abuse may trigger a compensatory increase in cardiac contractile force early on following insults possibly related to hemodynamic changes. With regard to the higher echocardiographic contractility following acute ethanol intake, several scenarios may be considered. First, acute exposure to the main ethanol metabolite acetaldehyde evokes a biphasic dose-dependent effect on cardiac contractility[48]. Elevated contractility with a low dose of acetaldehyde may be related to sympathetic activation, while the direct action of acetaldehyde on cardiomyocytes may suppress contractility [49]. Our short-term ethanol exposure may cause a transient effect from ethanol metabolism, thus resulting in higher fractional shortening and ejection fraction in conjunction with decreased chamber size and elevated wall or septal thickness. Hematoxylin-eosin (HE) and Sirius scarlet staining revealed that ethanol induced cardiomyocyte swelling and interstitial fibrosis (the subtle but significant effect may be due to the acute nature of the study) contributing to myocardial remodelling. Interestingly, these ethanol-evoked cardiac geometric and functional changes were effectively reversed by MCC950 treatment. Meanwhile, MCC950 itself failed to affect general biometric and mechanical properties in the heart, indicating minimal contribution of NLRP3 to cardiac homeostasis in physiological settings.

Inflammation represents a significant cardiovascular risk factor and participates in the pathogenesis of cardiovascular disease, including ethanol-induced cardiotoxicity [13, 50-52]. In addition to classical inflammatory factors, inflammasomes have been shown to play an important role in inflammation. NLRP3, as a broad sensor of disturbed cell homeostasis, can be activated downstream of a plethora of pathological stimuli and is assembled into NLRP3 inflammasomes composed of NLRP3 protein, adapter protein apoptosis-associated speck-like protein (ASC), and pro-caspase-1. Once assembled, inactive pro-caspase-1 in the inflammasome complex is autoprocessed to active caspase-1, which subsequently induces maturation of pro-interleukin-1β (pro-IL-1β) or pro-IL-18 to their active forms IL-1β or IL-18 to regulate inflammation [53]. In our hands, MCC950 rescued ethanol-induced increases in
serum levels of TNF-α, IL-6, IL-1β, IL-18, CK and LDH, protein levels of NLRP3 inflammasome-associated signaling components, including NLRP3, caspase-1, IL-1β and IL-18, and colocalization levels of NLRP3 and caspase-1. These findings denote a role for NLRP3 inflammasome activation and inflammation in ethanol cardiotoxicity, the effects of which were rescued by MCC950. Upon NLRP3 inflammasome activation, the mature proinflammatory cytokines IL-1β and IL-18 are produced, contributing to myocardial damage and pathological remodelling. MCC950 inhibited NLRP3 inflammasome activation and blocked overactivation of inflammatory responses, thus attenuating ethanol-induced cardiac anomalies. Our data also supported that MCC950 alleviated ethanol challenge-evoked ROS production and apoptosis, indicating a role for oxidative stress and apoptosis in MCC950- and ethanol-induced changes in cardiac geometry and mechanical function. The pro-oxidant effect of ethanol depends on the induction of a major isoform of the cytochrome P450 family CYP2E1, which has been reported to lead to the formation of ROS [54, 55]. With elevated oxidative stress, cardiac apoptosis usually ensues in the face of ethanol challenge [13, 56]. ROS production is triggered by NLRP3 activation [57].

The derangement of mitophagy, a conservative machinery for mitochondrial quality control through the disposal and recycling of injured mitochondria, is evident in alcoholic organ injury, including cardiomyopathy. Earlier evidence noted mitochondrial injury and myocardial inflammation following alcohol intake. Both oxidative stress and NLRP3 inflammasome activation can induce injury to mitochondria, which can be removed through mitophagy [58, 59]. Our data revealed elevated levels of mitophagy and autophagy protein markers, including PINK1, Parkin, LC3B, and p62, along with decreased levels of Rab7, which seems to denote interrupted mitophagy flux in acute ethanol-induced cardiotoxicity, the effect of which was ameliorated by MCC950 treatment. Deranged Pink1-Parkin-mediated mitophagy is in line with mitochondrial ultrastructural damage and apoptosis in acute ethanol cardiotoxicity. These data suggest a role of NLRP3 inflammasomes in the regulation of mitophagy and thus mitochondrial integrity in acute ethanol-induced cardiotoxicity.

Our data revealed activated NLRP3 in conjunction with activated caspase-1 and loss of the E3 ligase FBXL2 in ethanol-challenged hearts. The NLRP3-caspase-1 cascade compromises cardiac homeostasis in pathological settings such as diabetes mellitus. Our data noted that
inhibition of caspase-1 using AC-YVAD-CMK counters ethanol-induced loss of FBXL2 levels. Consistently, stimulation of FBXL2 rescued ethanol-induced cardiomyocyte contractile dysfunction and collapse of mitochondrial membrane potential in a manner reminiscent of NLRP3 inhibition or silencing. Likewise, disruption of FBXL2 cytosolic localization using GGti-2418 nullified NLRP3 inhibition (or silencing)-induced protective effects against ethanol-induced cardiomyocyte mechanical and mitochondrial derangements. It may be speculated that FBXL2 was destabilized due to proteasomal degradation of caspase-1 in the face of hyperactive NLRP3-caspase-1 inflammasome signaling, resulting in mitochondrial injury and apoptosis. Several reports have demonstrated that cleaved caspase-1 can cleave E3 ubiquitin ligases such as Parkin and NEDD4 [23, 34, 35]. In this context, we explored the role of the E3 ubiquitin ligase FBXL2 in NLRP3 inflammasome-associated cleaved caspase-1 activation, mitochondrial injury and apoptosis in the face of acute ethanol toxicity.

**Experimental limitations:** Several limitations exist for our study. First and foremost, it appears that both apoptosis and pyroptosis exist in the setting of acute ethanol toxicity. The precise interplay between the two cell death domains as well as other forms of cell death domains remains unknown and deserves further study. Second, only male mice were used in our study given the existence of sex differences in alcoholism [60]. Therefore, sex differences in acute ethanol toxicity may affect the experimental outcome.

In conclusion, our study provided convincing evidence for the first time that NLRP3 inflammasome inhibition using MCC950 rescued ethanol-induced myocardial geometric and functional anomalies via an NLRP3-caspase-1-FBXL2-mediated mechanism. This is supported by the finding that stimulation of FBXL2 protected against ethanol-induced cardiac anomalies, while inhibition of FBXL2 disengaged NLRP3-induced beneficial effects against ethanol challenge. These findings favored the notion that NLRP3 and FBXL2 may serve as possible targets for drug development for patients with alcoholism and alcoholic heart diseases. Further study is warranted to unveil the precise mechanism behind ethanol-evoked NLRP3 activation, regulation of F-box protein and mitophagy in a clinically relevant setting of alcoholic heart diseases.
CONFLICT OF INTEREST: None declared.

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DATA AVAILABILITY: All original experimental data will be made available with a request to the corresponding author (J. Ren)
References


[17] W.T. He, H. Wan, L. Hu, P. Chen, X. Wang, Z. Huang, Z.H. Yang, C.Q. Zhong, J. Han,


FUNDC1 interacts with FBXL2 to govern mitochondrial integrity and cardiac function through an IP3R3-dependent manner in obesity, Science advances, 6 (2020) eabc8561.


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FIGURE LEGENDS

Figure 1. Effect of MCC950 on acute ethanol exposure-induced echocardiographic changes  
Adult C57BL/6 mice were challenged with ethanol (1.5 g/kg, twice daily, i.p.) for 3 days while receiving MCC950 (10 mg/kg/d, i.p., days 1 and 3) prior to echocardiographic assessment. (A) Representative echocardiographic images from all 4 mouse groups. (B) Animal survival curve (n=20 mice/group). (C) Heart rate. (D) Ejection fraction (EF). (E) Fractional shortening (FS). (F) LV end systolic diameter (LVESD). (G) LV end diastolic diameter (LVEDD). (H) Septal thickness in systole. (I) Septal thickness in diastole. (J) LV posterior wall thickness in systole (LVPWs). (K) LV posterior wall thickness in diastole (LVPWd). n=8, 8, 7 and 7 for respective groups. (L) E/A ratio. (M) E/E’ ratio. (N) Representative Doppler image depicting E/A wave. n=8, 7, 7 and 7 for respective groups. Scale bar = 50 μm. Data were represented as the mean ± SEM. *P < 0.05 vs control (CON) group; #P < 0.05 vs EtOH group.

Figure 2. Contractile and intracellular Ca^{2+} handling properties of cardiomyocytes from treated mice  
C57BL/6 mice were challenged with ethanol (1.5 g/kg, twice daily, i.p.) for 3 days while receiving MCC950 (10 mg/kg/d, i.p., days 1 and 3). (A) Representative light microscopy images of isolated cardiomyocytes. (B) Resting cell length. (C) Peak shortening (PS). (D) Maximal velocity of shortening (+ dL/dt); (E) Maximal velocity of relengthening (-dL/dt). (F) Time-to-PS (TPS). (G) Time-to-90% relengthening (TR_{90}). n = 64 cells from 2 mice per group. (H) Resting fura-2 fluorescence intensity (Baseline FFI). (I) Electrically stimulated rise in FFI (ΔFFI). (J) Intracellular Ca^{2+} decay rate. n = 35 cells from 2 mice per group. Scale bar = 50 μm. Data were represented as the mean ± SEM. *P < 0.05 vs control (CON) group; #P < 0.05 vs EtOH group.

Figure 3. Influence of MCC950 on acute ethanol exposure-induced changes in myocardial morphology, oxidative stress (O_2^-) and apoptosis  
(A–C) Representative H&E staining (A, scale bar =100 μm), Sirius red staining (B, scale bar =100 μm) and DHE staining (C, scale bar =50 μm) depicting cardiomyocyte cross-sectional area, interstitial fibrosis and oxidative stress. (D) Pooled cardiomyocyte cross-sectional area. n=3. (E) Pooled Sirius Red staining. n=6. (F)
Pooled DHE staining. \( n = 5 \). (G) Representative apoptosis using TUNEL/DAPI staining. Scale bar: 50 \( \mu \)m. \( n = 5 \). (H) Representative immunoblots depicting levels of apoptotic protein markers Bax and Bcl2 using specific antibodies (\( \beta \)-actin as loading controls). (I) Bax level (\( n = 6 \)). (J) Bcl2 level (\( n = 6 \)). (K) Bax-to-Bcl-2 ratio (\( n = 6 \)). (L) Pooled cardiomyocyte apoptosis using percentage of TUNEL positive cells. Data were represented as the mean \( \pm \) SEM. *\( P < 0.05 \) vs control (CON) group; #\( P < 0.05 \) vs EtOH group.

Figure 4. Influence of MCC950 on acute ethanol exposure-induced changes in circulating inflammatory markers and NLRP3 components (A) Serum TNF-\( \alpha \) level. (B) Serum IL-6 level. (C) Serum IL-1\( \beta \) level. (D) Serum IL-18 level. (E) Serum CK level. (F) Serum LDH level. (G) NLRP3 level. (H) Cleaved Caspase-1 level. (I) Representative immunoblots depicting levels of NLRP3, IL-18, cleaved Caspase-1 and cleaved IL-1\( \beta \) levels using specific antibodies. (J) Cleaved IL-1\( \beta \) level. (K) IL-18 level. (L) Representative fluorescence image depicting overlap between NLRP3 and caspase-1. Scale bar =10 \( \mu \)m. (M) Pearson’s correlation coefficient representing overlap between NLRP3 and caspase-1. Data were represented as the mean \( \pm \) SEM. \( n = 10 \) for panels A–F and \( n = 6 \) for panels G–M. *\( P < 0.05 \) vs control (CON) group; #\( P < 0.05 \) vs EtOH group.

Figure 5. Influence of MCC950 on acute ethanol exposure-induced changes in autophagy and mitophagy protein markers (A) Representative immunoblots depicting levels of PINK1, Parkin, p62, LC3B and Rab7 using specific antibodies. (B) PINK1. (C) Parkin. (D) p62. (E) LC3B. (F) Rab7. (G) Representative TEM ultrastructural micrographs; H: Mitochondrial area. (I) Mitochondrial number. (J) Fluorescence imaging depicting mitophagy using colocalization between COXIV and LC3B. Scale bar =10 \( \mu \)m. (K) Mean number of autophagosomes. (L) Pearson’s coefficient of COXIV and LC3B. Data were represented as the mean \( \pm \) SEM. \( n = 6 \) per group. *\( P < 0.05 \) vs control (CON) group; #\( P < 0.05 \) vs EtOH group.

Figure 6. Influence of MCC950 on acute ethanol exposure-induced changes in FBXL2 and NLRP3 levels and effect of NLRP3 silencing or AC-YVAD-CMK on acute ethanol exposure-induced changes in cleaved caspase-1 and FBXL2 (A) Representative
immunoblots depicting levels of FBXL2 using specific antibody in control and ethanol mice treated with or without MCC950 (20 μM). (B) Pooled FBXL2 level. (C) Representative immunoblots depicting levels of NLRP3, cleaved caspase-1, and FBXL2 using specific antibodies in control and ethanol-challenged cells with or without NLRP3 silencing. (D) Pooled NLRP3 level. (E) cleaved caspase-1 level. (F) Pooled FBXL2 level. (G) Representative immunoblots depicting levels of cleaved caspase-1 and FBXL2 using specific antibodies in control and ethanol-challenged cells with or without AC-YVAD-CMK (50 μM) treatment. (H) Pooled cleaved caspase-1 level. (I) Pooled FBXL2 level. Data were represented as the mean ± SEM. *P < 0.05 vs control (CON) group; †P < 0.05 vs EtOH group.

Figure 7. Influence of FBXL2 activation and FBXL2 colocalization inhibition on MCC950-mediated protection against acute ethanol exposure-induced defects in cardiomyocyte contractile function Adult murine cardiomyocytes were exposed to ethanol (160 mM) in the presence or absence of the FBXL2 activator BC-1258 (10 μM) or the FBXL2 colocalization inhibitor GGTi-2418 (10 μM) prior to assessment of cardiomyocyte function. (A) Resting cell length. (B) Peak shortening (normalized to cell length). (C) Maximal velocity of shortening (+ dL/dt). (D) Maximal velocity of relengthening (- dL/dt). (E) Time-to-peak shortening (TPS). (F) Time to 90% relengthening (TR90). n=37 cells from 2 mice/group. Data were represented as the mean ± SEM. *P < 0.05 vs control (CON) group; †P < 0.05 vs EtOH group.

Figure 8. Influence of FBXL2 activation and FBXL2 colocalization inhibition on NLRP3 silencing-offered protection against acute ethanol exposure-induced changes in mitochondrial membrane potential and mitochondrial LC3B puncta Neonatal rat cardiomyocytes were exposed to ethanol (160 mM) in the presence or absence of the FBXL2 activator BC-1258 (10 μM) or the FBXL2 colocalization inhibitor GGTi-2418 (10 μM) prior to assessment of mitochondrial membrane potential and LC3B puncta formation and colocalization in mitochondria (using COXIV as a marker). (A) Representative JC-1 fluorescence images depicting the effect of BC-1258 and GGTi-2418 on NLRP3 silencing-
mediated protection against acute ethanol exposure-induced collapse of mitochondrial membrane potential. CCCP was used as a positive control. Scale bar = 100 μm. (B) Representative LC3B puncta and COXIV fluorescence images depicting the effect of BC-1258 and GGTi-2418 on NLRP3 silencing-offered protection against acute ethanol exposure-induced mitochondrial autophagosome formation. Scale bar =10 μm. (C) Pooled data of JC-1 using the aggregate-to-monomer ratio. (D) Pooled data depicting colocalization of LC3B and COXIV. n =6. Data were represented as the mean ± SEM. *P < 0.05 vs control (CON) group; #P<0.05 vs EtOH group; †P < 0.05 vs EtOH-siNLRP3 group.
Fig 1: Effect of MCC950 on acute ethanol exposure-induced echocardiographic changes. Adult C57BL/6 mice were challenged with ethanol (1.5 g/kg, twice daily, i.p.) for 3 days while receiving MCC950 (10 mg/kg/d, i.p., day 1 and 3) prior to echocardiographic assessment. A: Representative echocardiographic images from all 4 mouse groups; B: Animal survival curve (n=20 mice/group); C: Heart rate; D: Ejection fraction (EF); E: Fractional shortening (FS); F: LV end systolic diameter (LVESD); G: LV end diastolic diameter (LVEDD); H: Septal thickness in systole; I: Septal thickness in diastole; J: LV posterior wall thickness in systole (LVPWs); K: LV posterior wall thickness in diastole (LVPWd), n=8, 8, 7 and 7 for respective groups; L: E/A ratio; M: E/E' ratio; and N: Representative Doppler image depicting E/A wave, n=8, 7, 7 and 7 for respective groups. Scale bar = 50 μm. Mean ± SEM; *P < 0.05 vs. control (CON) group; #P < 0.05 vs. EtOH group.
Figure 2: Contractile and intracellular Ca2+ handling properties of cardiomyocytes from C57BL/6 mice challenged with ethanol (1.5 g/kg, twice daily, i.p.) for 3 days while receiving MCC950 (10 mg/kg/d, i.p., day 1 and 3). A: Representative light microscopic images of isolated cardiomyocytes; B: Resting cell length; C: Peak shortening (PS); D: Maximal velocity of shortening (+ dL/dt); E: Maximal velocity of relengthening (- dL/dt); F: Time-to-PS (TPS); and G: Time-to-90% relengthening (TR90), n = 64 cells from 2 mice per group; H: Resting fura-2 fluorescence intensity (Baseline FFI); I: Electrically-stimulated rise in FFI (ΔFFI); and J: Intracellular Ca2+ decay rate, n = 35 cells from 2 mice per group. Scale bar = 50 μm. Mean +/- SEM. *P < 0.05 vs. control (CON) group, #P < 0.05 vs. EtOH group.
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49x61mm (600 x 600 DPI)
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49x65mm (600 x 600 DPI)
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Fig. 7: Influence of FBXL2 activation and FBXL2 colocalization inhibition on MCC950-offered protection against acute ethanol exposure-induced defects in cardiomyocyte contractile function. Adult murine cardiomyocytes were exposed to ethanol (160 mM) in the presence or absence of FBXL2 activator BC-1258 (10 μM), or the FBXL2 colocalization inhibitor GGTi-2418 (10 μM) prior to assessment of cardiomyocyte function. A: Resting cell length; B: Peak shortening (normalized to cell length); C: Maximal velocity of shortening (+ dL/dt); D: Maximal velocity of relengthening (- dL/dt); E: Time-to-peak shortening (TPS); and F: Time-to-90% relengthening (TR90), n=37 cells from 2 mice/group; Mean ± SEM, *P < 0.05 vs. control (CON) group, # P < 0.05 vs. EtOH group, †P < 0.05 vs. EtOH-MCC950 group.

49x62mm (600 x 600 DPI)
Fig. 8: Influence of FBXL2 activation and FBXL2 colocalization inhibition on NLRP3 silencing-offered protection against acute ethanol exposure-induced change in mitochondrial membrane potential and mitochondrial LC3B puncta. Neonatal rat cardiomyocytes were exposed to ethanol (160 mM) in the presence or absence of FBXL2 activator BC-1258 (10 μM), or the FBXL2 colocalization inhibitor GGTi-2418 (10 μM) prior to assessment of mitochondrial membrane potential and LC3B puncta formation and colocalization in mitochondria (using COXIV as a marker). A: Representative JC-1 fluorescence images depicting the effect of BC-1258 and GGTi-2418 on NLRP3 silencing-offered protection against acute ethanol exposure-induced collapse in mitochondrial membrane potential. CCCP was used as a positive control, Scale bar = 100 μm; B: Representative LC3B puncta and COXIV fluorescence images depicting the effect of BC-1258 and GGTi-2418 on NLRP3 silencing-offered protection against acute ethanol exposure-induced mitochondrial autophagosome formation, Scale bar = 10 μm; C: Pooled data of JC-1 using the aggregate-to-monomer ratio; and D: Pooled data depicting colocalization of LC3B and COXIV. n = 6, Mean ± SEM; *P < 0.05 vs. control (CON) group, #P < 0.05 vs. EtOH group, †P < 0.05 vs. EtOH-siNLRP3 group.
Supplemental Materials
Selective Inhibition of NLRP3 inflammasome Protects against Acute Ethanol-Induced Cardiotoxicity through a FBXL2-Dependent Manner

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Running head: NLRP3 and alcoholic heart diseases

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Supplemental Figure 1: NLRP3 mRNA expression of neonatal rat cardiomyocytes transfected with NLRP3 siRNA. Mean ± SEM, n = 3 per group; *P < 0.05 vs. control (CON) and NLRP3 siRNA-NC group; #P < 0.05 vs. NLRP3 siRNA-1 group.

NLRP3 siRNA-1:
F: 5’-GGUUCUGAGCUCCAACCAUTT-3’
R: 5’-AUGGUUGGAGCUCAGAACCTT-3’

NLRP3 siRNA-2:
F: 5’-GGUCUCUUCUCAAGUCUAATT-3’
R: 5’-UUAGACUUGAGAAGAGACCTT-3’

NLRP3 siRNA-3:
F: 5’-CCAACUGGUCAAGGAGCAUTT-3’
R: 5’-AUGCUCCUUGACCAGUUGGTT-3’

NLRP3 siRNA-NC (Negative Control):
F: 5’-UUCUCCGAACGUGUCACGUTT-3’
R: 5’-ACGUGACACGUUCGAGAATT-3’.
NLRP3 inflammasomes possess both pro- and anti-inflammatory properties, although the role of NLRP3 in ethanol-induced cardiotoxicity remains unknown. This study was designed to examine the role of NLRP3 inflammasomes in acute ethanol cardiotoxicity and the underlying mechanisms of action.

1. Inhibition of NLRP3 inflammasome using MCC950 rescues against acute ethanol exposure-induced myocardial geometric and functional anomalies.

2. MCC950 attenuates ethanol challenge-induced change in Parkin-mediated mitophagy.

3. Ethanol induces interruption of mitophagy flux through a NLRP3/caspase1/FBXL2-dependent mechanism, which can be reversed by MCC950.

4. These findings reveal an important role for NLRP3 inflammasome-mediated regulation of mitophagy in ethanol cardiotoxicity, providing therapeutic promises for NLRP3 in ethanol-induced cardiotoxicity.
Scheme of NLRP3 inhibition-induced cardioprotection in acute ethanol toxicity

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