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Alda-1 attenuation of binge alcohol-caused atrial arrhythmias through a novel mechanism of suppressed c-Jun N-terminal Kinase-2 activity

Jiajie Yan[a], **Saugat Khanal**[a], **Yuanyuan Cao**[a], **Nikola Ricchiuti**[a], **Alma Nani**[c], **S.R. Wayne Chen**[b], **Michael Fill**[c], **Dan J. Bare**[a], **Xun Ai**[a,*]

[a]Department of Physiology and Cell Biology, College of Medicine/Wexner Medical Center, The Ohio State University, Columbus, OH, USA

[b]Department of Physiology and Pharmacology, The Libin Cardiovascular Institute, University of Calgary, Calgary, Canada

[c]Department of Physiology & Biophysics, Rush University Medical Center, Chicago, IL, USA

Abstract

Holiday Heart Syndrome (HHS) is caused by excessive binge alcohol consumption, and atrial fibrillation (AF) is the most common arrhythmia among HHS patients. AF is associated with substantial morbidity and mortality, making its prevention and treatment of high clinical interest. This study defines the anti-AF action of Alda-1 (an established cardioprotective agent) and the underlying mechanisms of the action in our well-characterized HHS and cellular models. We found that Alda-1 effectively eliminated binge alcohol-evoked Ca^{2+} triggered activities (Ca^{2+} waves, prolonged Ca^{2+} transient diastolic decay) and arrhythmia inducibility in intact mouse atria. We then demonstrated that alcohol impaired human RyR2 channels (isolated from organ donors' hearts). The functional role of alcohol-caused RyR2 channel dysfunction in Ca^{2+} triggered arrhythmic activities was evidenced in a unique transgenic mouse model with a loss-of-function mutation ($RyR2^{E4872Q+/-}$). Alda-1 is known to activate aldehyde dehydrogenase 2 (ALDH2), a key enzyme in alcohol detoxification. However, we found an increased level of ALDH2 and a preserved normal balance of pro- *vs* anti-apoptotic signaling in binge alcohol exposed hearts and H9c2 differentiated myocytes, which suggests that the link of alcohol-ALDH2-apoptosis is unlikely to be a key factor leading to binge alcohol-evoked arrhythmogenicity. We have previously reported that binge alcohol-activated stress response kinase JNK2 causatively drives

*Corresponding author at: 333 W. 10th Avenue, Columbus, Ohio 43210, USA. ai.87@osu.edu (X. Ai).

CRediT authorship contribution statement

Jiajie Yan: Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Saugat Khanal:** Writing – original draft, Validation, Investigation, Data curation. **Yuanyuan Cao:** Validation, Investigation, Data curation. **Nikola Ricchiuti:** Validation, Investigation, Formal analysis, Data curation. **Alma Nani:** Validation, Investigation, Data curation. **S.R. Wayne Chen:** Writing – review & editing, Resources, Methodology. **Michael Fill:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Dan J. Bare:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Xun Ai:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yjmcc.2024.10.003.

Ca^{2+}-triggered atrial arrhythmogenicity. Here, we found that JNK2-specific inhibition in either isolated human RyR2 channels or intact mouse atria abolished alcohol-evoked RyR2 channel dysfunction and Ca^{2+} triggered arrhythmic activities, suggesting a strong alcohol-JNK2-RyR2 interaction in atrial arrhythmogenicity. Furthermore, we revealed, for the first time, that Alda-1 suppresses JNK2 (but not JNK1) enzyme activity independently of ALDH2, which in turn alleviates binge alcohol-evoked Ca^{2+} triggered atrial arrhythmogenesis. Our findings provide novel mechanistic insights into the anti-arrhythmic action of Alda-1 and suggest that Alda-1 represents a potential preventative agent for AF management for HHS patients.

Keywords

Holiday heart syndrome; Atrial arrhythmias; c-Jun N-terminal kinase-2; Alda-1; Cardiac protection; Calcium transients

1. Introduction

About 61 million Americans are binge drinkers according to a Centers for Disease Control and Prevention (CDC) report. [1–3] For years, much effort has been made for the advocation of alcohol abstinence including the growing nation-wide prevention efforts by the CDC. Unfortunately, there continues to be a rise in the prevalence of heavy binge drinking. [4–6]

Holiday Heart Syndrome (HHS) is a well-recognized clinical issue caused by excessive holiday binge alcohol drinking (consuming large amounts of alcohol over a brief period) during holidays and weekends. [3,7–11] This has also been shown to cause heart rhythm disorders even in patients without any history of cardiac diseases. [3,7,12] HHS patients presenting with cardiac arrhythmias (AF is the most common type) often appear in emergency rooms around 12–36 h after the cessation of their heavy binge alcohol exposure. Interestingly, by this time, their blood alcohol concentration has already returned to baseline levels, suggesting HHS AF is unlikely due to an acute toxic alcohol effect but rather due to cardiac remodeling caused by alcohol. Aldehyde dehydrogenase 2 (ALDH2) is a mitochondrial enzyme responsible for the detoxification of the acetaldehyde that is produced during ethanol consumption. [13–16]. It has been reported that ALDH2 is cardioprotective through its anti-apoptotic action in myocardial ischemia/reperfusion injury and injury-associated arrhythmias as well as other cardiac pathologies like alcoholic cardiomyopathy and heart failure. [1–3] Alda-1 (N-1,3-benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide) is a known agonist of ALDH2. However, whether Alda-1 has a protective effect on HHS caused AF remains unknown.

Our pilot studies suggest that Alda-1 prevented alcohol-evoked atrial arrhythmogenicity in intact HHS hearts. However, we surprisingly observed that increased atrial arrhythmogenicity was associated with increased ALDH2 along with unchanged apoptotic signaling in the binge alcohol exposed hearts and myocytes. Thus, mechanisms other than ALDH2-suppressed cellular apoptosis are suggested to be involved in Alda-1 attenuated binge alcohol-evoked AF risk. We recently detailed the causative role of cardiac stress-response kinase JNK2 (c-Jun N terminal kinase type 2) in AF pathogenesis in HHS hearts. Specifically, JNK2 activation leads to diastolic sarcoplasmic reticulum (SR) Ca^{2+}

handling dysfunction and ultimately to triggered arrhythmogenesis. [11,14,17] We thus investigated the role of Alda-1 in alcohol-evoked JNK2 activity and JNK2-driven triggered Ca^{2+} activities *via* sensitized RyR2 channels.

In the current studies, we first assessed the effect of Alda-1 on binge alcohol-evoked atrial arrhythmogenicity using a combination of electrophysiological and biochemical approaches including *ex vivo* and *in vivo* atrial arrhythmia induction, confocal intravital atrial Ca^{2+} imaging in intact hearts, single human RyR2 channel recording, and biochemistry assays in binge alcohol-exposed differentiated H9c2 myocytes as well as in our well-characterized HHS mouse models. [11,14,15] HHS mice exhibit heart phenotypes 24 h after the cessation of the last dose of four repetitive binge alcohol-exposures to mimic a holiday drinking pattern in humans. [11,14,15] Our novel findings in the current studies demonstrate that 1) Alda-1 alleviates Ca^{2+}-triggered atrial arrhythmogenicity in HHS hearts; 2) Alcohol impairs human RyR2 channel function in a JNK2 dependent manner; 3) Alda-1 suppresses the JNK2 activity, to consequentially mitigate binge alcohol-evoked Ca^{2+}-triggered atrial arrhythmogenesis. Thus, our study here mechanistically presents Alda-1 as a promising preventative agent against binge alcohol-evoked AF risk *via* a previously unrecognized action in JNK2 inhibition.

2. Materials and methods

2.1. Mouse Models

All animal studies followed the Guide for the Care and Use of Laboratory Animals (NIH publication, 8th edition, 2011) and were approved by the Institutional Animal Care and Use Committees of The Ohio State University. Wild-type (WT) C57B/6j male mice (The Jackson Laboratory; Bar Harbor, ME) and $RyR2^{E4872Q+/-}$ transgenic mice [18] harboring a loss-of-function mutation E4872Q of the cardiac RyR2 channels were used (2–3 months old). We employed our previously established binge alcohol HHS model here. [11,15] Briefly, alcohol (2 g/kg body weight, intraperitoneal injection (i.p.) every other day for a total of 4 doses) was administered to mice as previously described. [11,15] To test the role of Alda-1 (Caymen Chemical; Ann Arbor, MI) in HHS-caused AF, Alda-1 was administered to HHS mice (20 mg/kg body weight; i.p. every other day for a total of 4 treatments) *in vivo* starting one day prior to the binge alcohol exposure. To assess the role of binge alcohol or activated JNK2 in RyR2 channel-modulated triggered arrhythmic activities, $RyR2^{E4872Q+/-}$ mice were exposed to either binge alcohol or anisomycin (a JNK activator, 20 mg/kg; i.p. every other day for a total of 4 injections) as previously described. [11,14,17,19,20] Terminal studies were performed within 24 h following the last alcohol dose. The mice were sacrificed under a surgical plane of anesthesia using 4 % isoflurane delivered with 100 % oxygen in the collection of intact hearts for confocal imaging studies or heart tissue for biochemistry assays.

2.2. Human Heart Tissue Samples

Human donor hearts were obtained from Illinois Gift of Hope (GOH) Organ & Tissue Donor Network. The alcohol-exposed hearts were from donors with a history of repeated binge drinking while the control hearts were from donors without a history of alcohol use. All

donors had no history of AF or any major cardiovascular diseases. Supplemental_Table-1 shows de-identified general data (age, sex, race, *etc.*) of the donors obtained from GOH. The studies were approved by the Human Study Committees of Rush University Medical Center and The Ohio State University.

2.3. Intact Atrial Confocal Ca^{2+} Imaging and Atrial Arrhythmia Inducibility Studies

Freshly excised mouse hearts were cannulated and subjected to Langendorff perfusion with oxygenated Ca^{2+}-free Tyrode's solution (composed of 137 mM NaCl, 0.36 mM NaH_2PO_4, 5.56 mM Glucose, 1 mM $MgCl_2$, 10 mM HEPES, 12 mM $NaHCO_3$, pH 7.4) at 4 °C to flush remaining blood out of the coronary system. Subsequently, hearts were loaded with the Ca^{2+} indicator Rhod2-AM (5 μM in 10 % Pluronic acid F-127 (AAT Bioquest; CA, dissolved in DMSO) for 35 min at room temperature. Following the dye loading, the heart was perfused by an oxygenated 1.8 mM Ca^{2+} Tyrode's solution (37 °C) followed by confocal Ca^{2+} imaging (Nikon Eclipse TE2000-U, 40× objective, NA 1.3). Confocal line scan images on the intact atria were acquired under either sinus rhythm or electrical pacing at set frequencies (8 Hz, 10 Hz, 20 Hz) in *ex vivo* Langendorff-perfused hearts. Detected Ca^{2+} transient signals were normalized to the background fluorescence level ($\Delta F/F_0$), wherein F_0 corresponded to the diastolic fluorescence value determined in the same cardiac myocyte. The Ca^{2+} transient decay constant (τ) was calculated as the time from the Ca^{2+} transient peak to the 63.2 % decay value. Aberrant Ca^{2+} release events were analyzed and presented as frequency of the Ca^{2+} waves per millimeter per second. All the data were analyzed using our well-established MATLAB (The MathWorks: Inc., Natick, MA algorithm as previously described. [11,14,20] The inducibility of atrial arrhythmias was assessed in Langendorff-perfused mouse atria using an established electrical burst pacing protocol (10-Hz and 20-Hz with a voltage of 2× and 3× diastolic pacing threshold). *In vivo* AF induction was conducted in sedated mice, and electrogram data were recorded using a 1.1F octapolar catheter inserted into the right atrium as previously described. [17,20] Electrical signal recordings were acquired through a multichannel data acquisition system (AD Instruments; Dunedin, New Zealand). Perfused hearts were observed and left for 50s in order to return to sinus rhythm. Atrial tachycardia (AT) incidence was defined as three or more regular fast atrial beats in a row, at two times or more the normal sinus rhythm. AF was determined to be three or more irregularly fast beats in a row with atrial-ventricular uncoupling. The AT/AF data were normalized and reported as AT/AF incidences/challenges per animal, while the AT/AF duration was recorded and analyzed as the mean value of all incidences in each mouse.

2.4. Cultured Cell Models

We utilized the widely used H9c2 cell line (America Tissue Type Collection, Manassas, VA; Cat # CRL-1446) with 1.0 % FBS plus all-trans-retinoic acid (RA; 1 μM) for 5 days to induce differentiation towards a more cardiac-like myocyte phenotype as previously described. [21,22] Briefly, the cells transformed into low-proliferating multinuclear cells that exhibited phenotypes typically observed in adult myocytes. These cells were cultured in regular maintenance media (DMEM (Gibco) that was supplemented with 10 % fetal bovine serum (FBS; GeminiBio; Sacramento, CA), HEPES, and penicillin/streptomycin) then later differentiated using a medium with 1.0 % FBS and RA once they reached 80 % confluency

in order to prevent loss of differentiation potential. They were treated with RA for four consecutive days followed by treatment with 50 mM alcohol with and without Alda-1 (20 μM, 4 h prior to the alcohol treatment plus simultaneously for an additional 24 h of culture). Cells were harvested 24 h after the treatments, and cell lysates were subsequently used for biochemical and molecular biology studies. To evaluate the effect of ALDH2 on JNK2 phosphorylation and activation, HEK-293 T cells (America Tissue Type Collection, Manassas, VA) were co-transfected with FLAG-tagged ALDH2 and turbo(t)-GFP-tagged active JNK2 (tGFP-JNK2). FLAG-ALDH2 only transfected cells were used as negative controls. All cells were harvested 48 h after the transfection for immunoblotting studies.

2.5. Single Channel RyR2 Recording

To measure single channel RyR2 function, heavy SR vesicles were isolated from human organ donor hearts and then fused into planar lipid bilayers. [17] Planar lipid bilayers were formed using 5:4:1 lipid mixture in *n*-decane (50 mg/ml): bovine brain phosphatidylethanolamine, –serine, and -choline, respectively (Avanti Polar Lipids; Birmingham, AL). The single RyR2 studies were performed at room temperature and their function is assumed to reasonably reflect single RyR2 function at body temperature as previous studies suggested. [23] Single RyR2 recordings were made at +40 mV in cell-like solutions at room temperature. The luminal recording solution contained 200 mM Cs-HEPES, 1 mM Ca^{2+} and pH 7.4. The cytosolic recording solution contained 114 mM Tris, 250 mM HEPES, 0.5 mM EGTA, 5 mM total ATP, 10 μM free Ca^{2+}, 1 mM free Mg^{2+} and pH 7.4. The JNK2-specific inhibitor, JNK2I, was applied to the cytosolic side of the single RyR2 channels after their incorporation into the bilayer. Ag/AgCl electrodes were used to connect the recording solutions to the head stage of an Axopatch 200B amplifier (Molecular Devices, LLC; San Jose, CA). RyR2 unitary currents were digitized at 50 kHz by an Axon Digidata 1544B AD converter and analyzed using pCLAMP software (Molecular Devices) after being filtered at 1 kHz. Opening/closing events (openings or closings) shorter than 0.5 ms were excluded.

2.6. Enzyme Activity and Protein Assays

Quantitative immunoblotting was employed for assessing protein abundance in mouse atrial samples and H9c2 cell lysates using target-specific antibodies. JNK2 and JNK1 enzyme activities were assessed using an ADP-Glo™ Kinase assay kit (Promega) as previously described. [11,17,24] Briefly, JNK2 and JNK1 proteins were immunoprecipitated (IPed) from H9c2 cells samples using either anti-JNK2 and anti-JNK1 specific antibodies, respectively. The IPed JNK2 or JNK1 proteins were incubated with the JNK-specific substrate c-jun protein in ATP-containing kinase activity buffer followed by the measurement of ADP production as previously described. [11,17,24] The effect of Alda-1 on the JNK2 enzyme activity was assessed by incubating 30 ng JNK2 pure protein with JNK-specific substrate c-Jun with or without Alda-1 (20 μM) in ATP-containing kinase activity buffer followed by measuring APD production.

2.7. Statistical Analyses

Analyses and graphs were generated using GraphPad software (San Diego, CA) or OriginPro software version 2023b (OriginLab Corporation, Northampton, MA). All data

are presented as mean ± interquartile range. Statistical analyses were conducted using non-parametric methods independent of a normal distribution. When two groups are compared, Mann-Whitney *U* test was applied. When more than two groups were compared, nonparametric 1-way ANOVA with *post hoc* Dunnett test was applied.

3. Results

3.1. Alda-1 Alleviates Alcohol-evoked Arrhythmia Inducibility in Intact Mouse Atria

We assessed burst pacing-induced AT/AF incidence in Langendorff-perfused intact HHS hearts [11,14,15] with and without Alda-1 treatment. Summarized data in Figs. 1A–1B show that the HHS alcohol challenge (red bar) substantially increased AT/AF incidence in response to electrical pacing, while sham controls had no occurrence of AT/AF events (0.21 *vs* 0 incidences/pacing attempts/animal in sham controls; **1A**). Also, HHS mice had a long AT/AF duration compared to sham controls (0.93 *vs.* 0.00 s in sham controls; **1B**). Strikingly, Alda-1 treatment prevented binge alcohol-evoked AT/AF inducibility, evidenced by the absence of pacing induced AT/AF in Alda-1 treated HHS mice as compared to the high AT/AF incidence in untreated HHS mice (Figs.1A–1B, far right bars). And six out of eight HHS mice showed pacing-induced AT/AF, while none of the sham controls or Alda-1-treated HHS mice showed pacing-induced arrhythmia. Fig. 1C shows representative electrograms of pacing induced AT/AF events following the abrupt cessation of electrical pacing (10 Hz) in an HHS mouse (upper panel) compared to the absence of any AT/AF incidence observed in an Alda-1-treated HHS mouse heart (lower panel). Given that alcohol consumption in humans occurs through the gastrointestinal system, we also assessed atrial arrhythmic susceptibility in Langendorff-perfused hearts from alcohol gavaged HHS mice and found a comparable result of AT/AF inducibility (Supplemental_Fig. S1A) as that seen where interperitoneally injected alcohol was given. Similarly, an increased inducibility of *in vivo* intra-atrial catheter pacing-induced AT/AF was also observed in HHS mice (intraperitoneal injection) as seen in Langendorff-perfused intact HHS hearts (Supplemental_Fig. S1B).

3.2. The Effect of Binge Alcohol Exposure on ALDH2 and Apoptotic Signaling Pathways

Alda-1 is an agonist of aldehyde dehydrogenase 2 (ALDH2), a mitochondrial enzyme responsible for breaking down the toxic alcohol metabolite acetaldehyde to mitigate alcohol-caused cellular response. [13–16] Here, we assessed ALDH2 expression and the apoptotic signaling in alcohol exposed differentiated H9c2 myocytes, which exhibit well-characterized cardiac phenotypes as previously described. [21,22] The use of all-trans-retinoic acid (RA) is a key factor in inducing the differentiation of H9c2 cells into cardiomyocytes, as evidenced by morphological changes, gene expression related to cell contraction, and positive staining for cardiac differentiation marker proteins such as α-actinin and cardiac troponin T (cTnT) as previously described. [21,25–27] As shown in Supplemental_Fig. S2, cardiac Ca^{2+} handling proteins RyR2 and cardiac-specific alpha actin (*ACTC1*) are significantly greater in differentiated cells compared to undifferentiated cells. We found that 50 mM alcohol treatment for 24 h increased the abundance of ALDH2. The presence of Alda-1 did not affect the ALDH2 protein level (compared to controls, no alcohol) as shown in representative immunoblotting images (Fig. 2A) and summarized quantitative

data (Fig. 2B). Moreover, an increased ALDH2 abundance was also observed in binge alcohol-exposed mouse hearts compared to sham controls as shown in Supplemental_Fig. S3A. Similarly, this increased ALDH2 level was also exhibited in human atrial tissue from binge alcohol-exposed organ donors compared to non-alcohol-exposed age-matched controls (see Supplemental_Fig. S3B).

Alda-1 is reported to be cardioprotective through the boosting of ALDH2-alleviated apoptosis. [16] We thus assessed the pro- and anti-apoptotic signaling in alcohol-exposed H9c2 cells using immunoblotting. Both BAX and Bcl2 are two important pro-apoptotic protein markers. However, phosphorylated Bcl2 at Serine70 (Bcl2-P^{70}) has an anti-apoptotic action. [28,29] Summary results and representative blotting images in Figs.2C & 2E (upper panel) show unchanged BAX levels in alcohol-exposed H9c2 cells compared to sham controls. Meanwhile, the ratio of Bcl2-P^{70} to tBcl2 (total Bcl2,) in alcohol-exposed H9c2 cells was unchanged compared to sham controls as shown in Fig. 2D. Likewise, the alcohol challenge in HHS mice had no effect on the expression of pro-apoptotic protein BAD (Fig. 2F) along with an unchanged ratio of Bcl2-P^{70}/tBcl2 (Fig. 2G) compared to controls. Correspondingly, Alda-1 treatment did not significantly alter the balance of anti-apoptotic and pro-apoptotic signaling in binge alcohol exposed mouse hearts. Accordingly, our results show that binge alcohol increases ALDH2 expression, which could contribute to the maintained balance of pro- *vs* anti-apoptotic signaling, which is therefore, unlikely to be a key factor in the binge alcohol-evoked atrial arrhythmogenesis.

3.3. Alda-1 Alleviates Alcohol-evoked JNK2-dependent Ca^{2+} Triggered Activities

Next, we performed intravital confocal Ca^{2+} imaging in HHS-WT intact mouse atria with and without *in vivo* Alda-1 treatment compared to sham controls as shown in representative images (Fig. 3A). We found that alcohol-exposed WT mouse atria exhibited significantly increased pacing induced Ca^{2+} waves (20 Hz) (Fig. 3B) and a markedly prolonged Ca^{2+} transient decay τ compared to sham counterparts (Fig. 3C, red bar; 32.39 ± 0.68 in sham *vs.* 45.33 ± 5.25, $p < 0.05$). Intriguingly, Alda-1 treatment abolished these binge alcohol-evoked triggered Ca^{2+} waves and prolonged τ in HHS atria compared to sham controls (Figs. 3A–3C, purple bars). This is consistent with an Alda-1 action on attenuated AT/AF inducibility shown in Fig. 1. We have recently reported that binge alcohol-exposure activates the stress response kinase JNK2. And JNK2 increases aberrant diastolic SR Ca^{2+} leak *via* RyR2 channels, which enhances AF risk in HHS mice. [11,14,17,30] Here, we further demonstrated that JNK2 inhibition in cardiac-specific JNK2dn mice (with overexpression of inactive dominant negative JNK2 in heart only) abolished the binge alcohol-evoked Ca^{2+} waves and normalized alcohol-prolonged Ca^{2+} transient decay (τ) compared to that of sham controls (Figs. 3B–3C, purple bars). These findings suggest a possible role of Alda-1 in JNK2-driven Ca^{2+}-triggered atrial arrhythmogenicity in the HHS heart.

Ca^{2+} triggered aberrant Ca^{2+} release RyR2 channel function is a key determinant of diastolic SR Ca^{2+} leak, which leads to prolonged intracellular Ca^{2+} removal due to increased diastolic intracellular Ca^{2+} as previously described. [11,14,17,30–33] Recently, we reported that activated JNK2 enhances mouse RyR2 single channel Ca^{2+} sensitivity, which contributes to enhanced diastolic SR Ca^{2+} leak and triggered arrhythmic activities. [31] To further

detail the role of RyR2 channels in alcohol-evoked atrial arrhythmogenicity, we treated $RyR2^{E4872Q+/-}$ mice overexpressing a loss-of-function RyR2 mutation of E4872Q [18] with alcohol using our HHS protocol. [11,14] Summarized data of confocal Ca^{2+} imaging on Langendorff-perfused intact hearts showed increased Ca^{2+} wave frequency in response to a train of burst pacing in HHS WT littermates, while the loss-of-function mutation in $RyR2^{E4872Q+/-}$ mice prevented alcohol-evoked triggered Ca^{2+} waves (Fig. 4A). Anisomycin is a potent JNK activator. [20,31]. The anisomysin action on Ca^{2+} wave frequency in WT and $RyR2^{E4872Q+/-}$ intact hearts is shown in Fig. 4B. Ca^{2+} waves were more frequently observed in the anisomysin-treated WT hearts (compared to $RyR2^{E4872Q+/-}$ hearts) as expected when activated JNK2 enhances RyR2 function. Whether alcohol has an impact on human RyR2 channel function has not previously been investigated, we thus assessed the action of alcohol-activated JNK2 on human RyR2 channel function in isolated SR vesicles from human organ donor's hearts. Example recordings of isolated human RyR2 channel function are shown in Fig. 4C. Alcohol increased the RyR2 channel activity (middle panel) compared to control (no alcohol, upper panel) and subsequent addition of a JNK2 inhibitor (with alcohol still present; bottom panel) reduced alcohol-enhanced channel activity. Summary data of single RyR2 open probability (Po) and mean opening time (MOT) results are presented in Figs. 4D–4E. The alcohol application significantly increased single RyR2 Po and addition of the JNK2 inhibitor significantly reduced the alcohol-increased Po and MOT. Thus, alcohol-associated JNK2 activation increases human RyR2 channel activity that in turn promotes RyR2-associated Ca^{2+} triggered arrhythmic activities.

3.4. Alda-1 Alleviates Binge Alcohol-evoked Activation of JNK2 in an ALDH2 Independent Manner

We next assessed the influence of Alda-1 on binge alcohol activated JNK2 and JNK1 isoform-specific enzyme activities in RA-differentiated H9c2 myocytes. We pulled down JNK2 or JNK1 with anti-JNK2 or anti-JNK1 specific antibodies, respectively, using an immunoprecipitation approach as previously described. [11,14,24] The JNK2 or JNK1 enzymatic activity was determined by the production of ADP from a kinase reaction utilizing c-jun as a substrate as previously described. [11,24] Summary data show that alcohol-exposed H9c2 myocytes have significantly increased JNK2 (Fig. 5A), but not JNK1 (Fig. 5B) enzyme activity, compared to sham controls. Intriguingly, subsequent Alda-1 addition substantially suppressed this binge alcohol-evoked JNK2 activation compared to sham controls (Fig. 5A, far right bar). We also assessed the protein expression of both JNK1 and 2 in binge alcohol exposed H9c2 myocytes using immunoblotting assays. Summarized data and representative images show significantly increased phosphorylated JNK (JNK-p, reflecting the activation status of total JNKs) detected by the anti-phosphor-specific JNK antibody (Figs. 5C & 5F, top panel), while Alda-1 suppressed the JNK-p to a level normally seen in sham controls (Fig. 5C, far right bar). On the other hand, the expression levels of either JNK2 (Figs. 5D & 5F, middle panel) or JNK1 (Figs. 5E & 5F, bottom panel) remained unchanged in alcohol exposed H9c2 myocytes compared to sham control. Similarly, Alda-1 had no effect on the expression levels of JNK1 and JNK2 proteins (Figs. 5D–5E, far right bars). Thus, our results demonstrate a striking inhibitory role of Alda-1 on JNK2 kinase activity.

Given that Alda-1 is an ALDH2 agonist, [34–36] we thought Alda-1 suppression of JNK2 activity might be linked to ALDH2. To this end, we co-transfected HEK293T cells with FLAG-tagged ALDH2 and turbo (t)-GFP-tagged active JNK2 (tGFP-JNK2) vectors followed by immune-blotting assays. Unexpectedly, Fig. 5G shows the unchanged phosphorylation status of JNK2 (detected by anti-JNK-P antibody) and expression verification of tGFP-JNK2 (detected by anti-tGFP antibody) in association with overexpressed ALDH2 (detected by anti-FLAG antibody) in tGFP-JNK2/FLAG-ALDH2 co-transfected HEK293T cells (lanes 3–4) compared to tGFP-JNK2 only cells (lanes 1–2). The bottom panel of Fig. 5G shows strongly expressed FLAG-ALDH2 bands in lane 3–5 as compared to the absence of a FLAG band in JNK2 only transfected cells (lanes 1–2), demonstrating the successful co-expression of ALDH2. Thus, an upregulation of ALDH2 alone would not alter JNK activity.

To further assess the inhibitory effect of Alda-1 on JNK2 kinase activity, we incubated 20 μM Alda-1 with pure active human JNK2 protein (Millipore Sigma) followed by the JNK2 enzyme activity assessment by measuring ADP production using an ADP-Glo™ kit. In this simple two-component only (JNK2 & Alda-1) system, the presence of Alda-1 suppressed JNK2 activity (Fig. 5H). This shows that the Alda-1 inhibitory action on JNK2 activity is ALDH2 independent (as no ALDH2 was present).

4. Discussion

In this study, we report for the first time that Alda-1, a known cardiac protective agent, effectively alleviates binge alcohol-enhanced AF risk. We also revealed a previously unknown action of Alda-1 in the suppression of JNK2 activity that underlies the anti-arrhythmia effect of Alda-1 on binge alcohol-evoked RyR2 channel dysfunction and Ca^{2+}-triggered arrhythmic activities in the HHS heart. Our exciting new findings suggest that Alda-1 could be a promising preventive agent against binge alcohol-evoked AF genesis.

Excessive binge alcohol intake has been widely recognized as a high-risk factor for AF, which is the most common arrhythmia diagnosed in clinical practice. Although to date, significant efforts have been made to reduce binge drinking, repeated binge drinking remains prevalent worldwide and consequently the prevalence of alcohol associated AF is high. Thus, there is an urgent need for effective pharmacological therapies for alcohol-provoked AF.

In the current studies, we demonstrated that Alda-1 prevents binge alcohol-evoked atrial arrhythmogenesis by suppressing the Ca^{2+} triggered arrhythmogenic activities. Alda-1 has gained attention due to its pivotal role in the context of cardiac protection as it is known to activate ALDH2, a main enzyme to catalyze the oxidation of acetaldehyde, a highly toxic intermediate alcohol detoxification product, towards acetate. [13–16,34–36] Mutations of ALDH2, such as ALDH2*2 (commonly observed in those of Asian descent), can lead to a slowed alcohol clearance due to its reduced enzyme activity, which could augment alcohol-evoked cellular injury and thus increase AF risk. In addition, it was reported that Alda-1 was even able to alleviate aldehyde overload and to prevent cell death, fibrosis and AF burden in long-term alcohol-exposed transgenic mice with knock-in of the loss-of-

function ALDH2*2 by restoring the ALDH2*2 activity, indicating an important mechanism of Alda-1 augmented ALDH2 activity against alcohol-induced cellular apoptosis and tissue injury. [34,37] On the other hand, studies suggest that Alda-1 can reduce cardiac apoptosis after myocardial infarction evidenced by reduced caspase-3 activity and cleaved caspase-3 levels. [38] In non-cardiac tissue, Alda-1 is also known to inhibit apoptosis and pyroptosis in intestinal, hepatic, neuronal tissue and bone tissue after I/R injury and/or chronic alcohol consumption. [39–42] In the current studies, we found that ALDH2 expression was increased in response to binge alcohol exposure in both human hearts from organ donors, our HHS mouse model and H9c2 differentiated myocytes, while the balance of pro- and anti-apoptosis signaling pathways including BAX, BAD, ratio of Bcl-P^{70} to tBcl2, and caspase 3 and 9 remained unchanged, suggesting a protective effect of increased ALDH2 on these apoptotic signaling pathways. However, the susceptibility of atrial arrhythmia is significantly increased in the HHS heart. Thus, this binge “alcohol-boosted” increase of ALDH2 expression appears to be anti-apoptotic, but it is insufficient to prevent alcohol-evoked atrial arrhythmogenesis in the HHS hearts.

We recently discovered that the activated stress response kinase JNK2 causatively activates CaMKII, and that this JNK2 regulated kinase-to-kinase regulatory mechanism is ROS-independent. [11,14,17,20]. Also, enhanced CaMKII-dependent phosphorylation of RyR2 leads to sensitized RyR2 channels (increased RyR2 channel open probability P_o) and thus, increased diastolic SR Ca^{2+} leak and triggered arrhythmic Ca^{2+} activities to enhance AF risk. [17,20] A recent study showed dantrolene, one of the RyR2 channel blockers, alleviated AF risk in a HHS rat model, suggesting the important role of RyR2 dysfunction in HHS AF risk. [43] However, HHS myocytes also exhibit an elevated SR load, thus, the effect of dantrolene on increasing SR Ca^{2+} load could become a double-edged sword when aberrant diastolic SR Ca^{2+} leak is accompanied by SR overload. [11,14,43,44] However, we reported that alcohol activated JNK2 is responsible for increased diastolic SR Ca^{2+} leak and elevated SR Ca^{2+} content in freshly isolated myocytes from HHS mice and alcohol-treated (24 h) rabbit myocytes. [11,14]. Additionally, cardiac-specific JNK2 inhibition in alcohol-exposed JNK2dn mice and a well-characterized JNK2-specific inhibitor *in vivo* treatment effectively alleviated both leak and load and consequently reduced the AF risk in HHS hearts. To date, the role of alcohol in human RyR2 channel activities has not been previously investigated. In the current study, we showed, for the first time, that alcohol significantly increased the open probability and prolonged the mean open time of planar lipid bilayer fused single RyR2 channels isolated from human organ donor hearts. Thus, increased duration of channel opening may underlie the alcohol-evoked RyR2 dysfunction. An earlier report showed that alcohol exposure suppressed RyR2 channel P_o. [45] However, the RyR2 single channel recordings conducted in that report used an ATP-free solution, which did not represent a physiological state. In our studies, we used a physiological solution containing 5 mM ATP for single RyR2 channel recording and demonstrated that alcohol exposure increases RyR2 channel P_o and prolonged channel opening time, which is in a JNK2-dependent manner. This was supported by the evidence of a complete elimination of the alcohol-increased RyR2 channel P_o and mean opening time (MOT) by JNK2 specific inhibition using our well-characterized JNK2 inhibitor JNK2I (Figs. 4A–4B). Moreover, the consequence of alcohol-activated JNK2 in RyR2 channel dysfunction in triggered Ca^{2+} activities in intact

atria was further supported by the striking rescue effect in a unique mouse model with the loss-of-function $RyR2^{E4872Q+/-}$ mutation. We employed this unique RyR2 mutant mouse model expressing the $RyR2^{E4872Q+/-}$ mutation that specifically reduces the open duration of the RyR2 channels. Our results showed that the RyR2 open time limiting mutation $RyR2^{E4872Q+/-}$ precluded alcohol-evoked RyR2 dysfunction, Ca^{2+}-triggered arrhythmic activities, and AF inducibility. Moreover, specific inhibition of JNK2 eliminated this alcohol-evoked RyR2 channel dysfunction in isolated human RyR2 single channels. Thus, our results support that alcohol or activated JNK2-caused RyR2 channel dysfunction is responsible for SR Ca^{2+} triggered arrhythmic activities and enhanced AF risk.

A recent study showed that Alda-1 exerts protective effects in reducing chronic alcohol-induced AF in ALDH2*2 knock-in mice through the detoxification of 4-HNE and ROS production. [46] However, AF in HHS patients often occurs 12–36 h post-alcohol exposure with no detectable alcohol in the blood. [11,14] The acute aldehyde/ROS insult from metabolized alcohol is unlikely to be the key arrhythmogenic cause, but the alcohol-activated JNK2 could act as a key stress signaling hub. [11,35] Others have also reported that Alda-1 prevented prolonged intracellular Ca^{2+} decay and cell shortening in a mouse model of binge alcohol exposure, while Alda-1 can abolish abnormal cardiac Ca^{2+} homeostasis due to post-cardiac arrest by inhibiting CaMKII activation. [16,47] Notably, our recent findings showed that JNK2 activates CaMKII, which leads to CaMKII-dependent diastolic SR Ca^{2+} leak and AF pathogenesis. [11,17] Consistent with these previous findings, our results here further demonstrate a previously unknown action of Alda-1 in suppressing JNK2 activity, which underlies the anti-Ca^{2+}-triggered arrhythmic activities and anti-AF action of Alda-1. We and others have shown that the heart expresses both JNK1 and JNK2 isoforms. [17,48,49] In H9c2 differentiated myocytes, alcohol exposure also increased enzyme activity of JNK2, but not JNK1, which aligns with our previous findings of activated JNK2 (not JNK1) in the HHS mouse atria. [11,14] Strikingly, we found that Alda-1 significantly suppresses alcohol-evoked JNK2 enzyme activity, while this action is independent of ALDH2, evidenced from the lack of effect of ALDH2 on the JNK2 activation status in HEK293T cells with co-transfected ALDH2 and active JNK2 (Fig. 5G) as well as the inhibitory effect of Alda-1 on active JNK2 proteins (Fig. 5H). Taken together, our studies provide new evidence suggesting a novel mechanism of anti-arrhythmia effect of Alda-1 on binge alcohol-evoked AF risk by suppressing the JNK2 activity in the heart.

In conclusion, the key findings in the current studies demonstrate the anti-AF action of Alda-1 is through a previously unrecognized mechanism of suppressed JNK2 activity. The results we have reported here are highly novel. The previously reported mechanism of action for Alda-1 was to counter alcohol-related organ injury *via* ALDH2 activation, however, our findings reported here suggest that ALDH2 is unlikely a critical factor in binge alcohol-evoked AF risk. In contrast, our findings demonstrate that Alda-1 suppressed JNK2 enzyme activity underlies the striking anti-arrhythmic effect of Alda-1 on AF risk in HHS hearts. Thus, Alda-1 could be a promising preventive agent in mitigating binge alcohol-evoked AF risk through Alda-1 inhibited JNK2 activity. Our novel findings are of high clinical relevance, especially in the context of binge alcohol consumption as a well-established risk factor for AF. While nearly 1/3 of the new onset cases of AF are related to alcohol consumption, binge alcohol could even have a significant impact on healthy young

individuals who have no pre-existing cardiac conditions. During the COVID-19 pandemic, the social isolation and stress further promoted binge drinking behavior. Considering the high morbidity and mortality AF brings to patients, and the high burden it adds to the health care system, an effective prevention and treatment approach towards AF is a paramount need. The current study sheds new light on Alda-1 as a promising preventative agent for binge alcohol triggered AF risk in HHS patients.

However, we do acknowledge that the findings reported through the use of our HHS mouse model only demonstrated binge alcohol-enhanced atrial susceptibility evidence from enhanced pacing-induced atrial arrhythmias. Further studies are clearly needed to elucidate the role of alcohol activated JNK2 in spontaneous AF onset *in vivo*. In addition, the anti-AF therapeutic potential and possible therapeutic strategy of using Alda-1 in AF management require further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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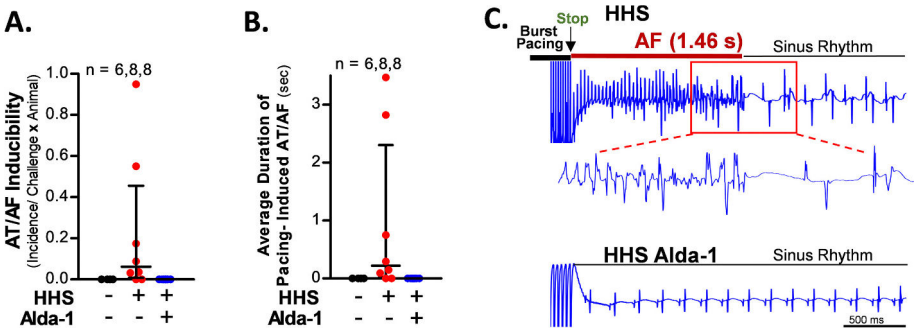

Fig. 1.

Alda-1 eliminated pacing-induced atrial arrhythmias in HHS mice. **A**) Summarized data of pacing induced incidence of atrial tachycardia (AT) and AF (quantified as incidence of AT/AF per burst pacing challenge per animal) in HHS mice and sham controls. Alda-1 treatment in HHS mice completely eliminated pacing-induced AT/AF episodes (far right bar). **B**) Summarized data of average duration of pacing-induced AT/AF in HHS mice and sham controls. **C**)**.** Representative electrogram traces showing burst pacing-induced AF in an HHS heart (upper panel), while Alda-1 treated HHS heart showing self-restored normal sinus rhythm after burst pacing. Individual data points were shown alongside mean ± interquartile range with statistical analysis by Kruskal-Wallis one-way ANOVA and Dunn's test for multiple comparison.

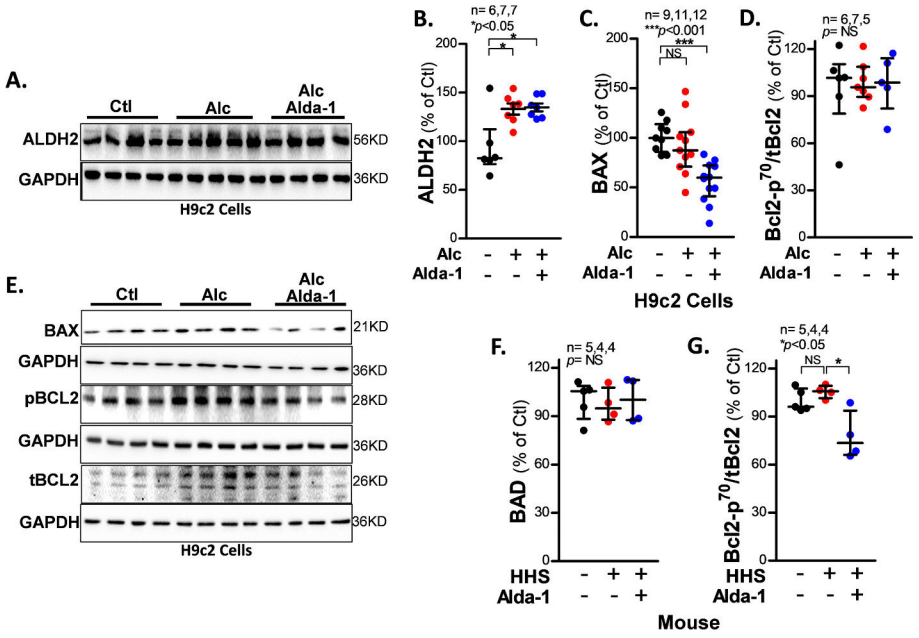

Fig. 2.

Binge alcohol increases ALDH2 abundance but does not affect apoptotic signaling markers. **A-B)** Representative immunoblotting images and summarized quantification data of ALDH2 protein expression in 24 h alcohol treated H9c2 myocytes with and without the presence of Alda-1 compared to sham controls. **C-E**) Pooled immunoblotting data and example images of unchanged apoptosis marker Bax (**C** & **E**, upper panel) and the ratio of pBcl2/tBcl2 (**D** & **E**, middle panels) in alcohol treated H9c2 myocytes with and without the presence of Alda-1 compared to sham controls. **F-G**) summarized immunoblotting data show unaltered levels of the apoptosis marker BAD and unchanged ratio of pBcl2/tBcl2 in HHS mouse hearts compared to sham controls, while Alda-1 treatment reduced the ratio of pBcl2/tBcl2 in HHS hearts compared to controls. Three technical replicates (immunoblotting) were performed. Individual data points were shown alongside mean ± interquartile range with statistical analysis by Kruskal-Wallis one-way ANOVA and Dunn's test for multiple comparison.

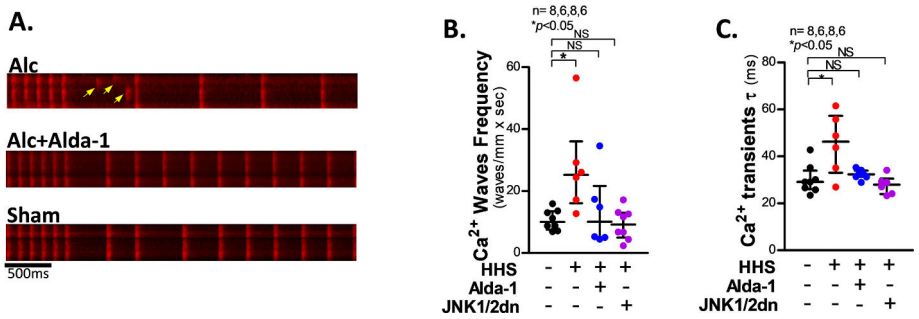

Fig. 3.

Either Alda-1 treatment or JNK2 inhibition eliminates binge alcohol-evoked Ca^{2+} mishandling in intact mouse atria. **A**) Representative confocal images showing increased pacing-induced diastolic Ca^{2+} wave (yellow arrows) frequency in HHS intact atria, while there are no pacing-induced Ca^{2+} waves in sham controls and Alda-1-treated HHS atria. **B**) Summarized data showing increased frequency of Ca^{2+} waves in binge alcohol-exposed mouse atria, while either Alda-1 treatment or JNK2 inhibition in JNK2dn transgenic mice with cardiac-specific overexpression of inactive dominant negative (dn) JNK2 precluded binge alcohol-prompted Ca^{2+} waves. **C**) HHS mouse atria exhibited prolonged Ca^{2+} decay constant, **τ**, compared to sham controls. However, either Alda-1 treatment in HHS-WT mice or cardiac JNK2 inhibition in JNK2dn mice normalized **τ** of Ca^{2+} decay as seen in sham controls. Individual data points were shown alongside mean ± interquartile range with statistical analysis by Kruskal-Wallis one-way ANOVA and Dunn's test for multiple comparison. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

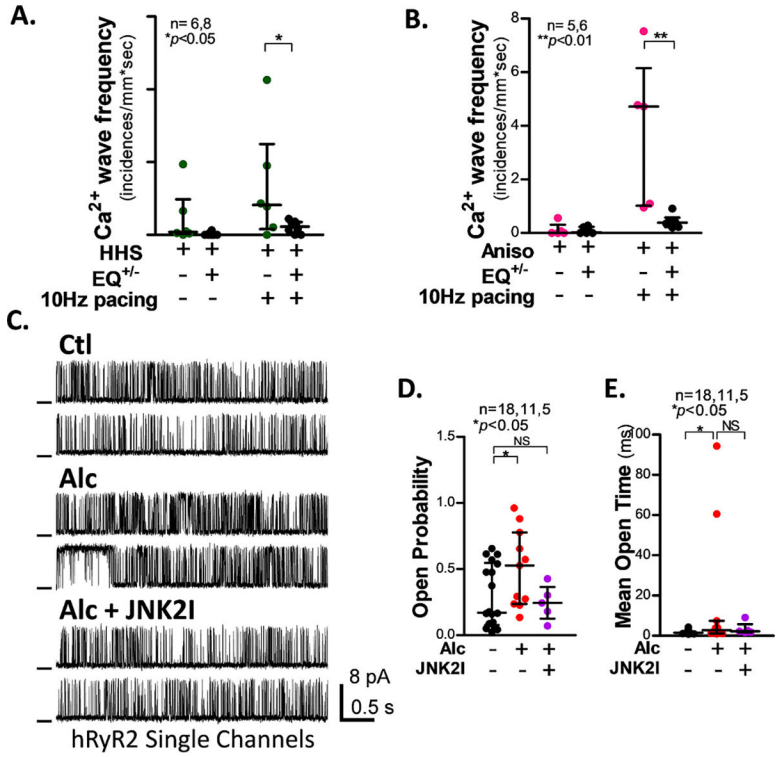

Fig. 4.

Alcohol drives RyR2 channel dysfunction in a JNK2-dependent manner. **A**) Summarized bar graphs showing alleviated binge alcohol-evoked Ca^{2+} waves frequency in HHS $RyR2^{E4872Q+/-}$ ($EQ^{+/-}$) with loss-of-function of RyR2 channels, while HHS WT (littermates, WT-Lit) mouse atria exhibited significantly increased spontaneous Ca^{2+} wave frequency (sinus rhythm (SR) before burst pacing) and 10 Hz pacing-induced Ca^{2+} wave frequency during the recovery period (when burst pacing was stopped) in comparison to that of WT young controls. **B**) Pooled data showing precluded arrhythmic Ca^{2+} waves in JNK activator anisomycin (Aniso)-treated $EQ^{+/-}$ mice. In contrast, Aniso-treated WT littermates showed significantly increased Ca^{2+} waves during sinus rhythm (SR) and in response to burst-pacing in Aniso-treated WT young mouse atria. Single channel recordings were performed on isolated human atrial RyR2 channels (hRyR2). **C**) Sample human single RyR2 channel recordings showing before (control) and after cytosolic addition of 39 mM alcohol (without and with pre-treatment of JNK2I-IX. The zero current levels are indicated by a dash). **D-E**) summarized bar graphs showing the mean single RyR2 Po and mean opening time after alcohol treatment alone or with JNK2 inhibitor JNK2I present. Individual data points were shown alongside mean ± interquartile range with statistical analysis by Kruskal-Wallis one-way ANOVA and Dunn's test for multiple comparison.

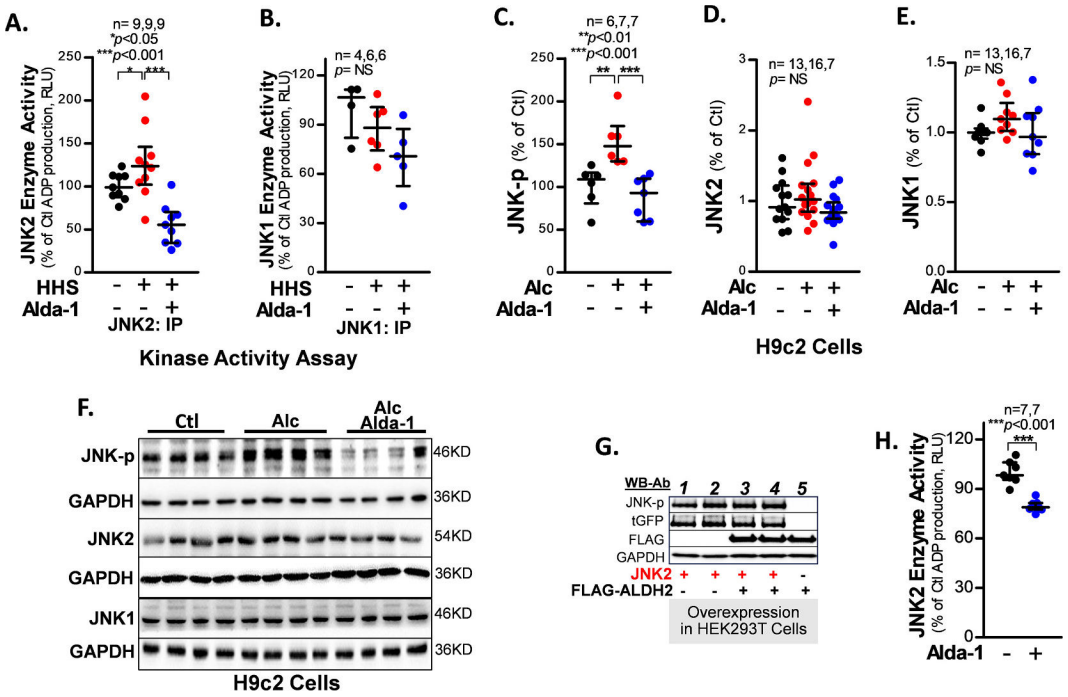

Fig. 5.

Alda-1 suppresses binge alcohol-activated JNK2 activity, but not JNK1. (**A-B**) An *in vitro* kinase activity assay shows that immunoprecipitated JNK2 (IPed: JNK2 with JNK2 specific antibody; **A**) but not JNK1 (IPed: JNK1 using JNK1 specific antibody; **B**) activity is significantly elevated in alcohol-exposed H9c2 myocytes. **C**) Summarized data showing a significantly increased level of JNK-P in HHS mice, while Alda-1 pre-treated HHS mice showed suppressed JNK2 activity. **D**) Pooled immunoblotting data demonstrating unchanged levels of JNK2 (**D**) and JNK1 (**E**) expression in HHS mouse hearts with and without Alda-1 treatment. **F**) Representative immunoblotting images of JNK-P, JNK2, JNK1 bands in alcohol-treated H9c2 myocytes with and without the presence of Alda-1. **G**) Immunoblotting images showing comparable level of phosphorylated JNK (JNK-P) in HEK293T cells co-transfected with FLAG-tagged ALDH2 and turbo(t)-GFP-tagged active JNK2 (tGFP-JNK2) vectors (lanes 3–4) compared to tGFP-JNK2-only transfected cells (lanes 1–2), while a JNK-P band is absent in the FLAG-ALDH2-only transfected cells (lane 5). **H**) A summarized bar graph showing significantly reduced enzyme activity of active JNK2 pure protein in the presence of Alda-1 compared to the baseline JNK2 enzyme activity in the absence of Alda-1. At least three technical replicates (immunoblotting & enzyme activity assay) were performed. Individual data points were shown alongside mean ± interquartile range with statistical analysis by Mann-Whitney test for two-group comparison and Kruskal-Wallis one-way ANOVA and Dunn's test for multiple comparison.