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High-dose alcohol induces reactive oxygen species-mediated apoptosis via PKC-β/p66Shc in mouse primary cardiomyocytes

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ABSTRACT

Cardiac dysfunction caused by excessive alcohol consumption is a specific disease, alcoholic cardiomyopathy (ACM). High-dose alcohol has been found to induce oxidation stress and apoptosis in cardiomyocytes, but the signaling link between alcohol-induced oxidation stress and apoptosis in cardiomyocytes remains to be elucidated. To address the issue, we exposed primary cardiomyocytes from neonatal mouse hearts to high doses of alcohol (50 mM, 100 mM, and 200 mM). We found that alcohol induced dose-dependent phosphorylation of p66shc, and reactive oxygen species (ROS) production increased in parallel with phosphorylation levels of p66shc. Exposure to alcohol also led to loss of mitochondrial membrane potential and cytochrome c release. Depletion of p66Shc and inhibition of protein kinase C-β (PKC-β) successfully reversed all the effects and suppressed alcohol-induced apoptosis in cardiomyocytes. Collectively, our study provides a molecular basis for signaling transduction of alcohol-induced oxidation stress and apoptosis of cardiomyocytes, which may facilitate the prevention and treatment of ACM.

1. Introduction

Long-term heavy alcohol consumption, regardless of beverage type, long has been implicated as a risk factor of alcoholic cardiomyopathy (ACM), and may lead to heart failure [1]. ACM is a specific heart muscle disease characterized by left ventricular dilation, increased left ventricular mass, and reduced or normal left ventricular wall thickness [2,3]. The pathophysiology of ACM involves cardiomyocyte apoptosis and changes in many aspects of myocyte function, such as depressed myocardial contractility [2,4].

Apoptotic cardiomyocytes have been found in the heart tissue of high-dose alcohol consumers [5,6], adding to growing evidence that heavy drinking is strongly associated with cardiomyocyte apoptosis. Studies in animal models also showed that excessive alcohol consumption was associated with left ventricular myocyte apoptosis [7,8]. Several markers of apoptosis also were detected in primary rat cardiomyocytes after 24 h of treatment with 0.5% alcohol, including Bax up-regulation, Caspase 3 activation, and DNA fragmentation [9,10]. A recent report found that alcohol (50, 100, 200 mM dosages) induced cardiomyocyte mitochondrial apoptosis and stimulated oxidative stress [10], suggesting that alcohol-induced cardiomyocyte apoptosis may be mediated by reactive oxygen species (ROS) signaling.

ROS refers to free radicals containing oxygen molecules, such as superoxide anions, hydroxyl radicals, peroxyl, alkoxyl, and O_2-derived non-radical species [11]. ROS metabolism is key to disease-resistance and cell-mediated immunity under physiologic and pathologic conditions [12]. However, uncontrolled ROS accumulation may cause oxidation of lipids, proteins and DNA, and often results in cell apoptosis [13,14]. Many signaling molecules take part in ROS production, such as p66Shc (the p66 isoform of the adaptor protein SHC (SRC homology 2 containing)), protein kinase C-β (PKC-β), and prolyl isomerase Pin1 [15]. The role of p66Shc in ROS formation has been widely studied. First, p66Shc promotes Ras-related C3 botulinum toxin substrate 1 (RAC1)-driven ROS production at the plasma membrane. Second, after phosphorylation by PKC-β and prolyl-isomerization by Pin-1, p66Shc translocates to mitochondrial intermembrane space, and induces H_2O_2 production. Third, p66Shc inhibits expression of ROS scavenging enzymes via interaction with forkhead box O transcription factors [16]. Although much has been learned, the underlying mechanism by which alcohol regulates ROS production and finally results in apoptosis is still unclear.

In the present study, we investigated the effect of alcohol on ROS production and cardiomyocyte apoptosis. We found that high-dose alcohol induced oxidative stress in cardiomyocytes via a PKC-β/p66shc signaling cascade. siRNA-mediated depletion of

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p66Shc and inhibition of PKC-β then significantly suppressed alcohol-induced cardiomyocyte apoptosis. Our results reveal the signaling transduction of alcohol-induced cardiomyocyte apoptosis, and suggest a novel mechanism of ACM development.

2. Materials and methods

2.1. Cell culture

Primary cardiomyocytes from neonatal mouse hearts were isolated as described previously [17]. All animal procedures were conducted in accordance with the Guideline for the Care and Use of Laboratory Animals of Harbin Medical University and approved by the Chancellor's Animal Research Committee. Briefly, heart tissue was minced and digested with a collagenase/dispase mixture (Roche, Indianapolis, IN). Tissue fragments were allowed to sediment, and the supernatant-containing suspended cells were preplated for 2 h to remove fibroblasts and endothelial cells. Then enriched cardiomyocytes were cultured in collagen-coated dishes at approximately 1.5×10^5 cells per cm^2.

2.2. Reagents

N-acetylcysteine (NAC), Mito-TEMPO, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and Ly333531 were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Measurement of ROS levels

The overall oxidative status of the cell was analyzed using DCFH-DA. Cells were harvested and incubated with DCFH-DA (10 μM) for 30 min at 37 °C in the dark. Then, the dichlorofluorescin fluorescence was measured by flow cytometry (BD Biosciences, San Jose, CA).

Mitochondrial ROS levels were analyzed using the Elite™ Mitochondrial ROS Activity assay kit (eEnzyme, Gaithersburg, MD). Cells were harvested and incubated with Elite ROS Deep Red stain solution for 60 min at 37 °C. The fluorescence intensity was measured at EX/EM = 650/675 nm.

2.4. Apoptosis assay

Cell apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit with propidium iodide (PI) (Biolegend, San Diego, CA) according to the manufacturer's protocol. Cells were collected and re-suspended in 1× binding buffer at a concentration of 1×10^6 cells per mL. Then cells (1×10^5) were incubated with 5 μL of Annexin V-FITC and 5 μL of PI for 15 min at room temperature in the dark, followed by addition of another 400 μL of 1× binding buffer. Samples were analyzed by flow cytometry within 1 h. All the cells stained positively for Annexin V-FITC were considered apoptotic cells.

2.5. Small interfering RNA (siRNA) and transfection

Scrambled siRNA and p66Shc siRNA (5′-UACAAGGCUUUCUCC UCCUUGUCGC-3′) were purchased from Invitrogen (Carlsbad, CA), and transfected with Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instructions. Cardiomyocytes (5×10^4 cells/well) were seeded in 24-well plates and grew overnight to approximately 80% confluence. The cells were transfected with 30 pmol siRNA and incubated for 48 h, and subsequent experiments were preformed after transfection efficiency analyzed by Western blot.

2.6. RNA isolation and reverse transcription-PCR (RT-PCR)

Total mRNA was extracted using the TRIzol reagent (Invitrogen), and complementary DNA was then synthesized from 1 μg of total RNA, using SuperScript III First-Strand Synthesis System (Life Technologies, Gaithersburg, MD). The expression levels of p66Shc were measured by real-time PCR. The relative expression level of p66Shc mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method versus glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers of oligonucleotides were as follows: 5′-TCCGGAATGAGT CTCTGTCA -3′ (forward) and 5′-GAAGGAGCACAGGGTAGTGG -3′ (reverse) for p66Shc; 5′-TGGACTCCACGACGTACTCAG-3′ (forward) and 5′-CGGGAAGCTTGTCATCAATGGAA-3′ (reverse) for GAPDH.

2.7. Western blot

Cells were harvested and lysed on ice for 30 min in lysis buffer (120 mM NaCl, 40 mM Tris [pH 8.0], and 0.1% NP 40) with proteinase/phosphatase inhibitor (Pierce, Rockford, IL), and centrifuged at 18,000*g* for 15 min at 4 °C. The supernatants were collected and protein concentrations were determined by BCA method. Aliquots of the lysates were electrophoresed on a 10% sodium dodecyl sulfate–polyacrylamide gel. The resolved proteins were then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), which were subsequently incubated with primary antibodies followed by a horseradish peroxidase-conjugated secondary antibody (Boster, Wuhan, China). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Pierce), followed by exposure of the membranes to X-ray film. Primary antibodies for p66Shc, p-p66Shc, Pin1, Cytochrome C, β-actin, and Hsp70 were purchased from Santa Cruz (Santa Cruz, CA).

2.8. Immunoprecipitation (IP)

Cells were lysed in IP lysis buffer (Pierce) supplemented with proteinase/phosphatase inhibitor. Protein concentration was adjusted to 1 mg/ml. Samples were incubated with anti-Pin1 antibody or rabbit IgG for 12 h at 4 °C with gentle orbital rotation. Then 50 μL of protein A-Sepharose beads were added and the incubation was extended for another 12 h. The beads were washed and subjected to 10% SDS–PAGE followed by Western blotting procedures.

2.9. Detection of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi m$) was assessed using a TMRE Mitochondrial Membrane Potential Assay Kit (Abcam, Cambridge, England). Cells were incubated with 100 nM TMRE in the absence or presence of CCCP at 20 μM at 37 °C for 15 min, and washed with 0.2% BSA in PBS. The cell pellet was collected by centrifugation at 1500*g* for 3 min, and resuspended in 1 ml of PBS. Fluorescence was measured by fluorescence plate reader (BioTek, Burlington, VT, USA).

2.10. Statistical analysis

All experiments were performed independently in triplicate. Data shown are representative of at least three experiments and are represented as mean ± SD. One-way ANOVA was used to assess statistical significance of differences among pairs of data sets. Statistical analyses were performed using SPSS software (version 19) with $p < 0.05$ considered statistically significant.

3. Results

3.1. Alcohol induces ROS-mediated apoptosis in cardiomyocytes

We first examined the role of ROS in mediating the alcohol-induced apoptosis of cardiomyocytes by exposing the cells to different levels of alcohol (50 mM, 100 mM, 200 mM). Alcohol increased ROS levels in a dose-dependent manner after 24-h treatment in cardiomyocytes (Fig. 1A). Percentages of Annexin-V labeled apoptotic cells also were dose-dependently up-regulated by treatment with high alcohol concentration (Fig. 1B). To determine if blockage of ROS production could inhibit alcohol-induced apoptosis in cardiomyocytes, the cells were treated with intracellular ROS scavenger NAC and mitochondria-targeted ROS scavenger Mito-TEMPO, respectively. As shown in Fig. 1C, NAC and Mito-TEMPO inhibited alcohol-induced ROS accumulation. Blockage of ROS production then significantly repressed cardiomyocyte apoptosis. These results indicate that alcohol-induced apoptosis of cardiomyocytes is mediated by ROS signaling.

3.2. p66Shc regulates alcohol-induced ROS generation and apoptosis in cardiomyocytes

P66Shc is a redox enzyme mediating ROS generation and apoptosis in response to stress signals [18]. We exposed cardiomyocytes to different concentrations of alcohol and assessed p66Shc mRNA and protein expression levels. As shown in Fig. 2A, alcohol significantly elevated the levels of p66Shc mRNA in a dose-dependent manner. Similarly, protein expression of total p66Shc, as well as phosphorylated p66Shc, increased as alcohol concentration increased (Fig. 2B). Also, protein expression levels of total p66Shc and phosphorylated p66Shc were significantly up-regulated in H_2O_2 –treated cells (Fig. 2C).

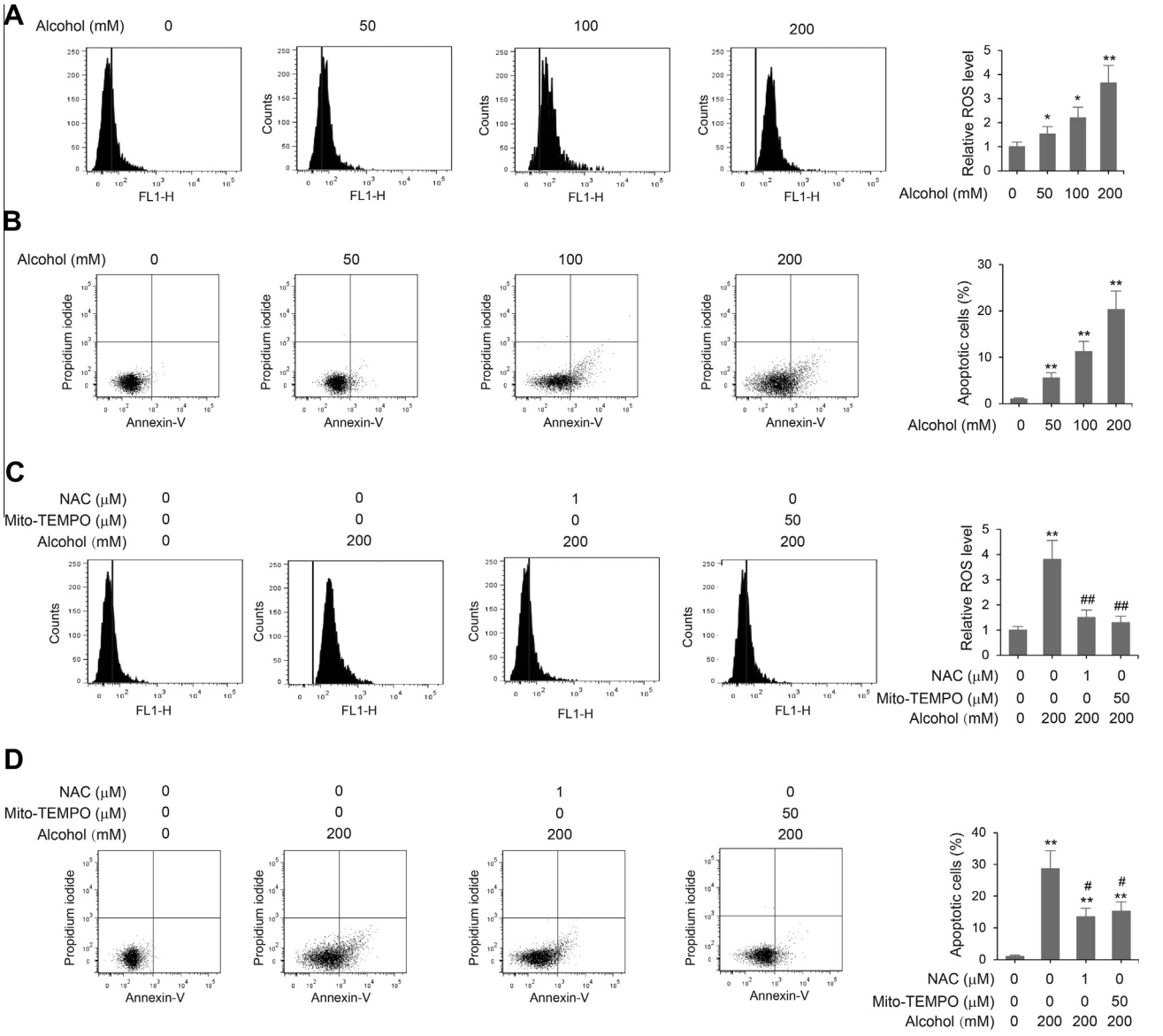

Fig. 1. Alcohol increases ROS-mediated cardiomyocyte apoptosis. (A) ROS levels measured by flow cytometry with DCF-DA in alcohol-treated mouse cardiomyocytes. Cells were treated with alcohol (0, 50, 100 and 200 mM) for 24 h, $n = 4$. (B) Cell apoptosis accessed by flow cytometry using Annexin V-FITC Apoptosis Detection Kit with PI. Cells were treated as indicated, $n = 4$. (C and D) ROS level (C) and cell apoptosis (D) in cardiomyocytes treated with alcohol (200 mM) and NAC (1 μM) or Mito-TEMPO (50 μM). $n = 4$. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to cells without alcohol treatment. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to cells treated with alcohol only.

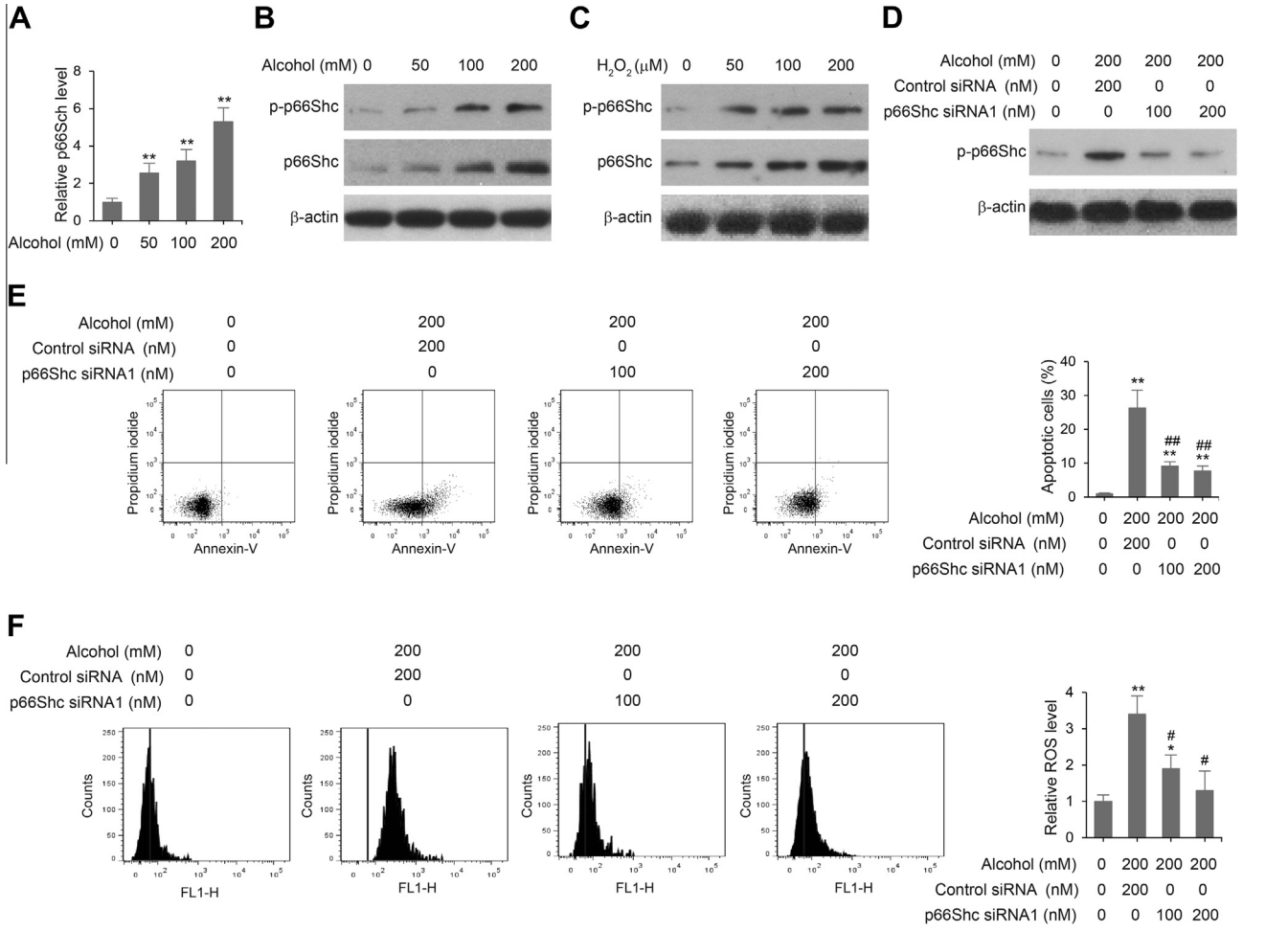

Fig. 2. p66Shc regulates alcohol-induced ROS generation and apoptosis in cardiomyocytes. (A) *p66Shc* mRNA level measured by RT-PCR. Cardiomyocytes were treated with different concentrations of alcohol for 24 h. (B) Expression of p66Shc and p-p66Shc analyzed by Western blot in cardiomyocytes treated with alcohol for 24 h. β-actin served as loading control. (C) Expression of p66Shc and p-p66Shc in cardiomyocytes treated with H_2O_2 treatment for 12 h. β-actin served as loading control. (D) Expression of p-p66Shc in cardiomyocytes transfected with control or p66Shc siRNA in the presence of alcohol. β-actin served as loading control. (E) Cell apoptosis in cardiomyocytes transfected with control or p66Shc siRNA in the presence of alcohol, $n = 4$. (F) ROS level in cardiomyocytes transfected with control or p66Shc siRNA in the presence of alcohol, $n = 4$. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to cells without alcohol treatment. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to control siRNA-transfected cells treated with alcohol.

We further assessed the role of p66Shc in alcohol-induced ROS production and cell apoptosis. Cardiomyocytes were transfected with control or p66Shc-siRNA before exposure to alcohol (200 mM). The knockdown efficiency was confirmed by Western blotting in the presence of alcohol (Fig. 2D). As expected, alcohol-induced ROS production and apoptosis were downregulated by depletion of p66Shc (Fig. 2E and F). These results indicate that p66Shc plays is important to alcohol-induced ROS generation and apoptosis in cardiomyocytes.

3.3. PKC-β modulates alcohol-induced phosphorylation of p66Shc and its binding to Pin1

A recent report found that oxidative stress-activated that PKC-β induced phosphorylation of p66Shc and triggered mitochondrial accumulation of p66Shc after its binding to Pin1 [15]. We then wondered if PKC-β and Pin1 are required for alcohol-induced p66Shc activation. As shown in Fig. 3A, increased p66Shc phosphorylation induced by alcohol (200 mM) was suppressed by Ly333531, a specific PKC-β inhibitor. To investigate if alcohol affects the binding between phosphorylated p66Shc and Pin1, immunoprecipitation was performed after treatment with different alcohol concentrations. Alcohol was found to promote the binding between Pin1 and p-p66-Shc (Fig. 3B), while Ly333531 ameliorated this effect (Fig. 3C), indicating that alcohol enhances the phosphorylation of p66Shc and its interactions with Pin1, which is mediated by PKC-β.

3.4. PKC-β/p66Shc pathway modulates the mitochondrial events of alcohol-induced apoptosis in cardiomyocytes

The mitochondrial electron transport chain is the main source of intercellular ROS production. Recent studies demonstrated that, in response to apoptosis stimuli, p-p66Shc could translocate into the mitochondrial intermembrane space to oxidized cytochrome C, which resulted in excessive ROS generation and mitochondrial depolarization [18]. As shown in Fig. 4A, the cytochrome C level was decreased in mitochondrial extracts of alcohol-treated cardiomyocytes in a dose-dependent manner. Similarly, mitochondrial ROS and mitochondrial potentials were found to be regulated by alcohol treatment (Fig. 4B and C). As expected, this effect was abolished by depletion of p66Shc and inhibition of PKC-β (Fig. 4D–F). Taken together, these results suggest that in alcohol-treated cardiomyocytes, PKC-β/p66Shc signaling induces mitochondrial

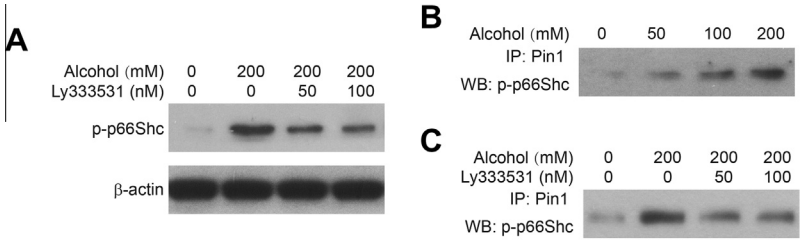

Fig. 3. PKC-β modulates alcohol-induced phosphorylation of p66Shc and its binding to Pin1. (A) Expression of p-p66Shc in cardiomyocytes treated with alcohol (200 mM) in the presence of different concentrations of Ly333531 for 24 h. β-actin served as loading control. (B) Immunoprecipitation analysis between p-p66Shc and Pin1 in cardiomyocytes treated with different concentrations of alcohol. (C) Immunoprecipitation analysis between p-p66Shc and Pin1 in cardiomyocytes treated with alcohol (200 mM) in the presence of different concentrations of Ly333531.

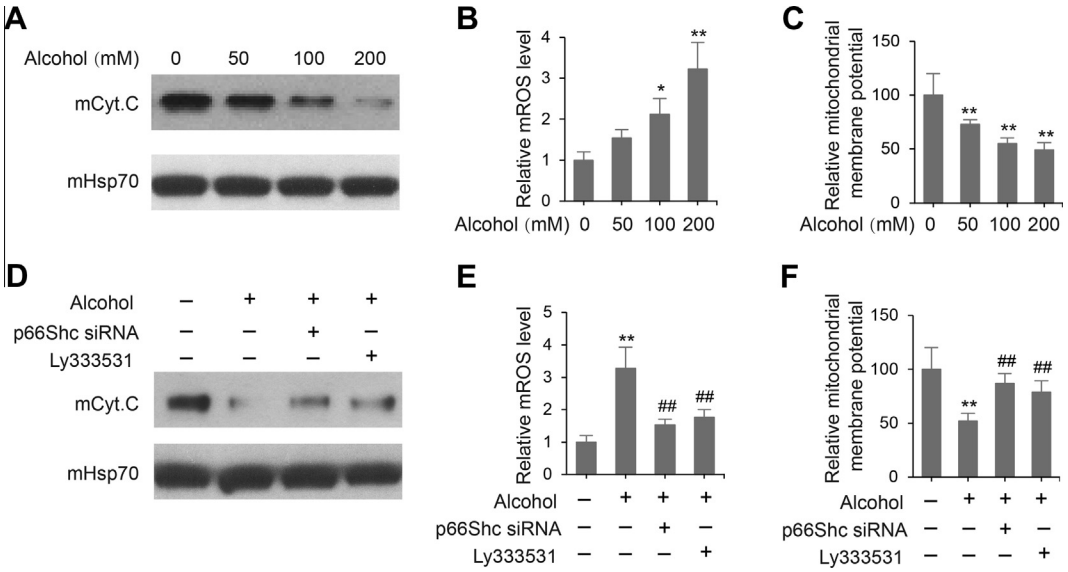

Fig. 4. PKC-β/p66Shc pathway modulates the mitochondrial events related to alcohol induced apoptosis in cardiomyocytes. (A) Mitochondrial cytochrome C levels in cardiomyocytes treated with different concentrations of alcohol for 24 h. Hsp70 served as mitochondrial loading control. (B and C) Mitochondrial ROS levels analyzed by Elite™ Fluorimetric ROS Assay Kit (B) and mitochondrial membrane potential analyzed by TMRE Mitochondrial Membrane Potential Assay Kit (C) in cardiomyocytes treated with different concentration of alcohol for 24 h, $n = 4$. (D–F) Mitochondrial cytochrome C levels (D), mitochondrial ROS levels (E) and mitochondrial potential (F) analyzed in cardiomyocytes subjected to p66Shc siRNA or Ly333531 in presence of alcohol. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to cells without alcohol treatment. $^{\#\#}p < 0.01$ compared to cells treated with alcohol only.

ROS production, mitochondrial potential decrease, and cytochrome C release, which ultimately leads to apoptosis.

4. Discussion

Excessive alcohol consumption is one of the main causes of left ventricular dysfunction. Specifically, ACM is the leading cause of non-ischemic dilated cardiomyopathy in the United States [19,20]. Several potential mechanisms have been proposed to explain the role of alcohol during the development of ACM, such as inhibition of protein synthesis, induction of inflammation and cardiomyocyte apoptosis [2,5,21]. In this study, we found alcohol dose-dependently induced apoptosis in mouse primary cardiomyocytes, which was mediated by ROS production. p66Shc, PKC-β, and Pin1 played key roles in ROS signaling transduction, while inhibition of PKC-β and knockdown of p66Shc rescued alcohol-induced apoptosis of cardiomyocytes.

ROS can be beneficial or harmful to cells and tissues, depending on its concentrations. At physiological levels, ROS function as "redox messengers" in intracellular signaling and regulation, while excessive ROS production inhibits protein function and promotes cell death [22]. We demonstrated that high-dose alcohol induces ROS generation and apoptosis in a dose-dependent manner, while ROS scavengers, such as NAC and Mito-TEMPO, significantly inhibit cardiomyocyte apoptosis, indicating that alcohol-induced cardiomyocyte apoptosis is mediated by ROS signaling. We further observed that phosphorylation of p66shc, a master regulator of mitochondrial ROS production, was upregulated by alcohol exposure. Using a specific inhibitor, we found PKC-β is required for phosphorylation of p66shc. Alcohol exposure also enhanced the interaction between p66shc and Pin1, determined by immunoprecipitation assay.

Mitochondria are sources and targets of ROS [13,14]. Mitochondria generate ROS that are thought to increase intracellular oxidative stress and excessive ROS production can lead to oxidative damage to mitochondrial proteins, membranes and DNA, and impair the ability of ATP generation [23]. In our study, we found exposure to alcohol reduced the mitochondrial membrane potential and led to cytochrome c release in cardiomyocytes. However, siRNA-mediated depletion of p66shc and inhibition of PKC-β could rescue the loss of mitochondrial membrane potential and cytochrome c release, which suggests PKC-β and p66shc are crucial for ROS signaling transduction. Mitochondria also have numerous ROS defense systems [13,24], which are less studied. Since ROS scavengers can inhibit alcohol-induced apoptosis in cardiomyocytes, it would be helpful in the future to test if alcohol affects

the ROS defense systems in mitochondria. ACM also is type of chronic long-term dilated cardiomyopathy. Oxidative stress is considered a major contributor to etiology of senescence [25,26]. Binge drinking may induce senescence of existing cardiomyocytes, and inhibit the formation of new cardiomyocytes.

In summary, we have identified that alcohol-induced cardiomyocyte apoptosis is mediated by ROS signaling, in which PKC-β and Pin1 are crucial to promoting translocation of p66shc into mitochondria. Upregulation of mitochondrial ROS production by alcohol exposure resulted in loss of mitochondrial membrane potential and cytochrome c release, subsequently led to apoptosis. Importantly, prevention of oxidation stress build-up by ROS scavengers or depletion of p66shc can significantly suppress the alcohol-induced apoptosis of cardiomyocytes. This regulatory mechanism of alcohol-induced ROS production could be a potential therapeutic target to treat ACM.

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