Restoration of YAP activation rescues HL-1 cardiomyocytes from apoptotic death by ethanol

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ABSTRACT — We reported previously that when mouse atrium-derived HL-1 cardiomyocytes undergo apoptosis upon exposure to 2% ethanol, the cellular cytoskeleton is severely disrupted and the anti-apoptotic transcriptional co-activator Yes-associated protein (YAP) is inactivated. Consistent with our previous observations, the expression of connective tissue growth factor (CTGF), an anti-apoptotic growth factor and a target of YAP, decreases in a time-dependent manner during exposure to 2% ethanol. The restoration of YAP activation rescues the cells from apoptosis: both the retrovirus-mediated expression of constitutively active YAP and the stabilization of the actomyosin cytoskeleton by jasplakinolide prevent cell death. In contrast, YAP inhibitors have no effect on cell death, confirming the inactivation of YAP in ethanol-exposed cells. Thus, a decrease in actin tension and YAP inactivation should be crucially involved in the cytotoxicity of ethanol on HL-1 cardiomyocytes.

Key words: Ethanol, YAP, Cardiomyocytes, Apoptosis

INTRODUCTION

Although the digestive system is the main site of damage to the human body by alcohol consumed by drinking alcoholic beverages, the cardiovascular system is also damaged by alcohol (Laonigro et al., 2009; Fernández-Solà, 2015). Alcoholic cardiomyopathy is a result of alcohol abuse over a long period of time, and can be life-threatening. It has been reported that apoptotic cell death is associated with heart damage in long-term alcohol consumers, suggesting the role of apoptosis in alcoholic cardiomyopathy (Fernandez-Sola et al., 2006). One of the vulnerabilities of the heart is its inability to regenerate, even though heart function and systemic circulation are necessary for life. However, the concept that cardiomyocytes in the adult heart do not proliferate has been challenged by recent studies showing that cardiomyocytes can regenerate through transfer with Yes-associated protein (YAP), a transcriptional co-activator (Xin et al., 2013).

Recent research advances have indicated a central role for YAP in the proliferation, regeneration, and cell-size control of cardiomyocytes (Heallen et al., 2013; Xin et al., 2013; Zhou et al., 2015). In addition to the Hippo kinase pathway that has long been considered the main upstream pathway of YAP, mechanotransduction, which converts mechanical signals from cell-extra cellular matrix (ECM) and cell-cell contact into intracellular signaling pathways via actin tension, has been proposed as another important regulator of YAP (Dupont et al., 2011). Cell-cell as well as cell-ECM interactions play important roles in cell proliferation and the prevention of apoptosis. It has been shown that the forced activation of YAP renders cells able to proliferate even on the 'soft' matrix, suggesting that mechanotransduction of 'rigid' matrix tension to YAP activation gives cells the capability of proliferation (Dupont et al., 2011). Indeed, ethanol, at least in part due to its amphiphilicity, has been reported to cause a decrease in intracellular actomyosin tension (Puszkin and Rubin, 1975; French et al., 1987), a finding that we reproduced in our previous report (Noritake et al., 2015).

To evaluate whether the collapse of the actin cytoskeleton and YAP inactivation, which occur in parallel with cell death during the exposure of HL-1 cells to ethanol (Noritake et al., 2015), are involved in cell death or not, we examined the effect of the forced expression of YAP using a retrovirus as well as the modulation of intracellular actin tension by using actin polymerization/depolymer-
erization inhibitors. The results obtained show the crucial role of these two phenomena in ethanol cytotoxicity on cardiomyocytes.

**MATERIALS AND METHODS**

**Cell culture and materials**

A mouse atrial cardiomyocyte-derived cell line, HL-1, was provided by Dr. William C. Claycomb (Louisiana State University Medical Center, New Orleans, LA, USA) (Claycomb et al., 1998) and maintained according to the instructions of the Dr. Claycomb Lab. Briefly, the cells were cultured on gelatin/fibronectin-coated dishes in a specified medium (Claycomb medium, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.1 mM norepinephrine, and 2 mM L-glutamine. Ethanol (2%) was added to the medium when the cells had grown to confluence and started spontaneous beating. To avoid evaporation, the culture dishes were sealed with film after the addition of ethanol. Antibodies used were anti-YAP (#14074, Cell Signaling, Danvers, MA, USA), anti-CTGF (#sc-101586, Santa Cruz, Santa Cruz, CA, USA or #ab6992, Abcam, Cambridge, UK), anti-cleaved caspase-3 (#9661, Cell Signaling) and anti-hsc70 (#sc-7298, Santa Cruz). Verte- porfin and cytochalasin D were obtained from Sigma-Aldrich. YAP-TEAD inhibitor 1 and jasplakinolide were from Selleckchem (Cedarlane, Canada) and Santa Cruz, respectively.

**Cytochemistry**

The cells were fixed with 4% paraformaldehyde for 15 min., and permeabilized with 0.5% Triton X-100 for 5 min. For immunofluorescence examination of YAP, the cells were incubated with anti-YAP (#14074, Cell Signaling, Danvers, MA, USA), anti-CTGF (#sc-101586, Santa Cruz, Santa Cruz, CA, USA or #ab6992, Abcam, Cambridge, UK), anti-cleaved caspase-3 (#9661, Cell Signaling) and anti-hsc70 (#sc-7298, Santa Cruz). Verteporfin and cytochalasin D were obtained from Sigma-Aldrich. YAP-TEAD inhibitor 1 and jasplakinolide were from Selleckchem (Cedarlane, Canada) and Santa Cruz, respectively.

**Immunoblot analysis**

Immunoblot analysis was performed as described previously (Noritake et al., 2012, 2015). In brief, the cells were scraped from the dish, suspended in STE buffer (0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaF, 2 mM NaVO₄, and protease inhibitor cocktail), and lysed by an ultrasonic disruptor (Sonifier 150, Branson, Danbury, CT, USA). Protein concentrations of cell lysate were measured by the method of Bradford (Bradford, 1976). Proteins were separated by SDS-PAGE by the method of Laemmli (Laemmli, 1970), transferred to a PVDF membrane, and the membrane was blocked in TBS-T (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.05% Tween 20, and 3% skim milk). The membrane was then incubated successively with 1st and 2nd (peroxidase-conjugated anti-IgG) antibodies. A Western Lightning Chemiluminescence Reagent Plus Kit (Perkin Elmer Life Science, Waltham, MA, USA) was used to develop chemiluminescence. Band intensities were quantified using an image analyzer (CS analyzer, Atto, Tokyo, Japan).

**qPCR**

Quantitative real-time PCR (qPCR) was performed using StepOnePlus (Applied Biosystems, Foster City, CA, USA) according to the ∆∆C₅₇ method. The following primers were used for the amplification of templates: 5'-AGGGCCTCTTCTGGATTTTC-3' and 5'-CTTTGGAAGACTGCCTGTACGT-3' for CTGF; 5'-CCCTTAAAGGGATGCTGCCG-3' and 5'-TACGGCCAAATCCGTTCACA-3' for GAPDH. PCR was performed at 95°C for 3 sec and 60°C for 30 sec, for 40 cycles. A GoTaq Green Master Mix for quantitative PCR kit (Promega, Madison, WI, USA), which includes SYBER Green as well as a reference dye, was used for amplification of the templates.

**Retrovirus infection**

Retroviruses expressing wild type YAP (pQCXIH-Myc-YAP) or constitutively active mutant YAP (pQCXIH-Myc-YAP-SSA, mutations: S61A, S109A, S127A, S164A, and S381A) were gifts from Dr. Kun-Liang Guan (Addgene plasmid #33091 and #33093) (Zhao et al., 2007, 2008). Solutions including these viruses were obtained by transfecting 293T cells using a Retrovirus Packaging Kit Eco (Takara Bio, Shiga, Japan) according to the manufacturer’s protocol. HL-1 cells were infected with the diluted virus solution containing polybrene (Sigma Aldrich). After 24 hr of infection, the cells were treated with or without ethanol.

**Statistics**

For multiple comparisons of more than three experimental groups, the Tukey-Kramer and Dunnett’s statistical methods were used as appropriate. P values < 0.05 were considered statistically significant.
RESULTS

Impairment of nuclear translocation of YAP in ethanol-treated cells

As observed in our previous study (Noritake et al., 2015), the structural collapse of the actin cytoskeleton was observed in HL-1 cells treated with 2% ethanol for 24 hr (Fig. 1A). Also, YAP was localized mainly in the cytoplasm in ethanol-treated cells in contrast to its nuclear localization in control cells (Fig. 1B). These results suggest a decrease in actin tension as well as the inactivation of YAP in ethanol-exposed cells.

The levels of both protein and mRNA of CTGF are decreased in ethanol-treated cells

To confirm the inactivation of YAP in ethanol-exposed cells, we next examined the levels of connective tissue growth factor (CTGF) protein and mRNA by immunoblotting and qPCR, respectively. As shown in Fig. 2A, CTGF protein levels showed an obvious time-dependent decrease during exposure to 2% ethanol for 48 hr. CTGF mRNA levels showed the same tendency, but a minimum

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**Fig. 1.** Structural collapse of the actin cytoskeleton and inhibition of YAP nuclear translocation in ethanol-exposed HL-1 cells. Cells were incubated in medium with or without 2% ethanol for 24 hr. (A) Phalloidin-rhodamine staining of the cells to visualize filamentous actin. (B) Immunocytochemical analysis of YAP localization. Nuclei were visualized by DAPI. PH, phase contrast.
level was observed after 24 hr of exposure, after which there seemed to be a recovery during 24-48 hr of exposure (Fig. 2B). These decreases are not due to the duration of culture, since CTGF protein levels did not decrease in the absence of ethanol exposure (Fig. 2C). In conclusion, CTGF levels decreased in response to ethanol exposure, confirming the inactivation of YAP.

**YAP inhibitors do not affect apoptotic cell death by ethanol**

We further examined the effect of YAP inhibitors on cell death. The administration of verteporfin, which inhibits YAP by disrupting the YAP-TEAD transcriptional activator complex (Brodowska et al., 2014), had no effect on caspase-3 activation by ethanol (Fig. 3A), nor did YAP-TEAD inhibitor 1 (peptide 17), another YAP inhibitor (Zhang et al., 2014) (Fig. 3B). These results further support the observation that YAP is localized in the cytoplasm (Fig. 1B), and therefore should be in its inactive state in ethanol-exposed cells.

**Both the retrovirus-mediated gene transfer of constitutively active YAP (SA-YAP or CA-YAP) and the stabilization of filamentous actin suppress apoptosis caused by ethanol**

Finally, we examined whether the artificial amendment of inactive YAP can rescue cells from ethanol cytotoxicity. First, constitutively active (CA) or wild type (WT) YAP (Zhao et al., 2007, 2008) was introduced into the cells by retrovirus-mediated gene transfer. Infections of these viruses by themselves had no cytotoxic effect on the cells (Fig. 4A). However, obviously lower levels of cleaved caspase-3 were detected in CA-YAP-infected cells following exposure to ethanol for 48 hr as compared to WT-YAP-infected cells (Fig. 4A). Second, the effects of substances that stabilize or disrupt actin filaments on cell death were examined. Inhibitors of actin polymerization (cytochalasin D, (Flanagan and Lin, 1980)) or depolymerization (jasplakinolide, (Bubb et al., 1994)) were used for this purpose. As shown in Fig. 4B, neither cytochalasin D nor jasplakinolide had any cytotoxic effect on the cells. However, cytochalasin D showed a tendency to aggravate cell death by ethanol while jasplakinolide significantly ameliorated cell death (Fig. 4B). Thus, the col-
Fig. 3. Effects of YAP inhibitors on ethanol-induced HL-1 cell death. Cells were treated with verteporfin (10 μM) (A) or YAP-TEAD inhibitor 1 (1 μM) (B) and further exposed to ethanol (2%) for 24 hr. Then, caspase-3 activation was assessed by immunoblot analysis. Graphs show means ± S.E. n.s., not significant.

Fig. 4. Effects of retrovirus-mediated YAP gene transduction and cytoskeletal drugs on ethanol-induced HL-1 cell death. (A) Cells were infected with retroviruses expressing WT-YAP or CA-YAP, followed by treatment with 2% ethanol for 48 hr. (B) Cells were treated with cytochalasin D (100 nM) or jasplakinolide (100 nM), followed by further treatment with 2% ethanol for 48 hr. The relative levels of cleaved caspase-3 protein were determined by immunoblot analysis. Hsc70 protein served as a control. Graphs show means ± S.E. *, p < 0.05.
lapse of the actin structure (Fig. 1A) should be one of the causative events in cell death by ethanol.

**DISCUSSION**

In addition to its established deteriorating effect on the contractile ability of the heart and cardiomyocytes (Thomas et al., 1994), ethanol causes the death of cardiomyocytes: it has been reported that moderate but chronic (8 months) ethanol consumption results in the loss of ~14% myocytes in the hearts of rats (Capasso et al., 1992). Coincident with these observations, there are many reports showing that the direct exposure of cardiomyocytes to ethanol results in acute apoptotic cell death (Chen et al., 2000, Wang et al., 2015; Nonitake et al., 2015). An enhanced generation of reactive oxygen species (ROS) has been proposed to be a crucial factor in ethanol cytotoxicity (Walker et al., 2013). On the other hand, structural collapse of the cytoskeleton in ethanol-exposed cells has not attracted much attention, although it was first reported several decades ago (French et al., 1987, Puszkin and Rubin, 1975, Iwata et al., 2011). The relationship between cytoskeletal rupture and cell death has not been pointed out in ethanol cytotoxicity so far, but Loureiro et al. (2011) reported that the ethanol-induced rupture of the cytoskeleton is dependent on ROS formation in glioma cells; actin cytoskeleton collapse can be prevented by antioxidants such as ascorbic acid or the vitamin E analogue Trolox. Thus, ROS generation might be an upstream event leading to the structural collapse of the actin cytoskeleton and the subsequent death of ethanol-exposed cells.

Although the actin cytoskeleton has been reported to activate YAP through the Hippo pathway (Reddy et al., 2013), another report has indicated that actin remodeling can induce cardiomyocyte proliferation even in mice deficient in the Hippo pathway (Morikawa et al., 2015). Thus, the lack of YAP activation observed in ethanol-exposed HL-1 cells might be a result of the collapse of the actin structure, rather than the activation of the Hippo pathway.

It has also been reported that the death of cardiomyocytes by direct exposure to ethanol can be prevented by insulin-like growth factor-I (IGF-I), a prototypic anti-apoptotic growth factor (Chen et al., 2000). IGF-I exerts its anti-apoptotic function mainly by activating the PI3K-AKT pathway. Interestingly, Lin et al., have demonstrated that in HL-1 cells overexpressing YAP, PIK3CB, one of the catalytic subunits of PI3K is upregulated through the direct binding of YAP to its promoter site (Lin et al., 2015; Xiao et al., 2015). Thus, a PI3K-AKT survival pathway might be involved in the rescue of HL-1 cardiomyocytes from ethanol cytotoxicity by YAP, and this possibility should be the subject of a future examination. In conclusion, our current data show that the collapse of the actin structure and YAP inactivation are crucially involved in ethanol cytotoxicity on cardiomyocytes.

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**Conflict of interest**---- The authors declare that there is no conflict of interest.

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YAP rescues apoptosis of cardiomyocytes by ethanol


