Rho GTPase activating protein 15 (arhGAP15) siRNA effect apoptosis-induced by ethanol in bovine fibroblast cells

Ikram Ullah[1], Hae Young Lee[1], Min Jung Kim[1], Shahid Ali Shah[1], Haroon Badshah[1], Tae Hyun Kim[1], Hak-Jae Chung[2], Byoung Chul Yang[2] and Myeong Ok Kim[1*]

[1]Division of Life Science, College of Natural Sciences and Applied Life Science, Gyeongsang National University, Jinju, Republic of Korea

[2]National Institute of Animal Sciences, RDA Suwan, Republic of Korea

Abstract: The Rho GTPases are the sub-group of Ras super family and identified in all eukaryotes. The Rho GTPases effect different cellular signaling pathways involved in a number of diseases such as cancer, neurological and cardiovascular disorders. Members of Rho GTPases including RhoA, RhoC and Rac1 play a major role in regulation of apoptosis in different kind of stress conditions. Here we investigated the Rho GTPase activating protein 15 (ArhGAP15) gene knock-down effect on apoptosis induced by ethanol in bovine fibroblast cells. The bovine Fibroblast cells were treated and transfected with two different concentrations (50 and 100 nM) of ArhGAP15 siRNA for 48 h respectively. Both concentrations of siRNA were effective and the results of RT-PCR revealed an efficient knock-down of ArhGAP15 mRNA in fibroblast cells. Further, the normal cells exposed to a 100 mM ethanol concentration showed a reduction in cell viability and induced the ratio of apoptosis related Bax/Bcl-2 proteins compared with ArhGAP15 siRNA transfected ethanol treated cells. Ethanol also increased caspase-3 expression in normal fibroblast cells compared with transfected cells. The ArhGAP15 knock-down cells treated with ethanol decreased Bax/Bcl-2 ratio and lower caspase-3 protein levels in ArhGAP15 knocked-down cells. Our results suggest that apoptosis induced by ethanol involves the activation of Rho GTPase activating protein 15 and silencing of the said gene protects apoptosis.

Keywords: Ethanol, siRNA**,** apoptosis

INTRODUCTION

The Rho GTPases are the sub-members of Ras family consists of 20 known different kind of cellular proteins, called small GTPases named for their small 20 kD size (Lu *et al.,* 2009). Based on their functions and primary sequence five groups of Rho proteins are categorized includes Rho, Rac, Cdc42, Rnd and RhoBTB like proteins (Burridge and Wennerberg, 2004). The Rho GTPases play a major role in transcription of genes, cellular proliferation and migration. These further effects the cellular transduction pathways involved in gene expression, cell cycle progression and cellular proliferation (Bustelo *et al.,* 2007; Hall, 1998). The Rho families of proteins are molecular switches, cycling between active GTP bound state and in active GDP-bound state (Jaffe and Hall, 2005). A number of regulatory proteins like the GTPase activating proteins (GAPs) regulate the activity of Rho GTPase a mechanism involved the spatial activation/ inactivation of Rho proteins in different kind of cells (Ellenbroek and Collard, 2007).The Rho GTPases are negatively regulated by GAPs through enhancing the Rho proteins intrinsic GTPase activity (Luo, 2000; Moon and Zhang, 2003).

Generally apoptosis involves the activation of proteases which damage the cells associated with DNA fragmentation, biochemical and morphological changes related to cells (Cohen, 1997). Evidence shows that the key player mitochondrion is important during cell death through apoptosis involved the release of cytochrome c from mitochondria to cytosol in all type of cells (Green & Reed, 1998; Kluck *et al.,* 1997). The Bcl-2 families of proteins also have a major role in apoptosis. Particularly the anti apoptotic Bcl-2 and pro-apoptotic Bax are involved in apoptosis and mitochondrial related changes (Zimmermann *et al*., 2001).

Ethanol is used commonly in different concentrations for drug preparations, cosmetics and disinfectants used in a numbered industry as a common source (Cross and Roberts, 2000; Rotter *et al.,* 1998). Many of these cosmetics and disinfectants are in common use for face and neck area. By the use of these products it can activate apoptosis and inflammation in skin cells but the mechanism is not fully defined. The Rho GTPases are known to regulate apoptosis in different type of cells. There is a concept that the inactivation of Rho causes apoptosis in epithelial and fibroblast cells (Fiorentini *et al.,* 1998a; Bobak *et al.,* 1997). While in other type of cells such as PC-12 and endogenous cells of spinal cord the inactivation of Rho protects apoptosis favouring the dual function of Rho GTPases (Mills *et al.,* 1998; Dubreuil *et al.,* 2003).

Here we report that fibroblast cells experienced and suffered from apoptosis when treated with ethanol and

**Corresponding author:* e-mail: mokim@gsnu.ac.kr

silencing of the ArhGAP15 gene protects cell death induced by ethanol. Our current findings indicate the mechanism is that may be ethanol activates the Rho family members proteins particularly Rac-1 which positively induce ROS and arhgap15 silencing block this Rac-1 activity through a negative feed back mechanism.

METHODOLOGY

Cell culture

Bovine fibroblast cells were grown in NUNC T75 flasks in Dulbecco's modified medium (DMEM, Thermo, Hycolne), supplemented with 10 % fetal bovine serum (Hyclone, USA), 1% antibiotic containing 50 U/ml penicillin, and 50 mg/L streptomycin (GIBCO) at 37 ^{o}C in a humidified atmosphere containing 5% CO_2.The medium was changed once every 2 days and cells were passage once a week.

RNA interference (RNAi) and transfection

The silencing of ArhGAP15 gene was carried out via using siRNA targeting ArhGAP15 gene and was delivered by a lipid-based method. The ArhGAP15 gene siRNA was purchased from a commercial source (QIAGEN) target sequence 5`-AAAGATGTCATTCCACCACTA-3`, sense strand 5`-AGAUGUCAUUCCACUATT-3, strand 5-UAGUGGUGGAAUGACUCUTT-3 using a final concentration of 50 and 100 nM. One day prior of transfection, the medium was changed to DMEM with out serum and antibiotics. DMEM medium containing lipofectamine (DMEM containing Lipofectamine2000 ^{TM}, Invitrogen) was incubated for 5 min at room temperature was mix with (DMEM containing shortcut siRNA) and the mixture was incubated for short 20 min duration and again mixed before addition. Then the combine solution containing siRNA and lipofectamine was added to the desired culture plates for 48 hours. Afterwards, the medium was aspirated and complete medium was added back to both siRNA transfected and normal treated cells.

Experimental groups

When proper transfection was achieved in the desired 48 hours , after that the cells were divided in to four groups (1) Control: only containing DMEM medium (2) siRNA transfected group (transfection for 48 hours) (3) Ethanol 100 mM for 30 hours (4) siRNA plus Ethanol treated group. The cells were incubated with ethanol for 30 h and then the cells were harvested for RT-PCR and protein analysis using western blot.

Extraction of total cellular RNA and Reverse transcriptase- polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from transfected and non-transfected cells using a modified manual Trizol Reagent method (Life Technologies, Rockville, MD) and was performed as we previously described (Naseer *et al.,* 2010). RT-PCR was carried out using cDNA from the bovine fibroblast cells transfected and non-transfected cells. The amount (4 µl) of cDNA was used from total for the amplification of desired gene in presence of 1µl Taq DNA polymerase enzyme. RT-PCR was performed at the following optimized condition using 94 °C for (5min), 36 cycles at 94 °C (1min) 68 °C for (1 min), and 72 °C for (1 min) followed by 72 °C (5 min) is a final extension. PCR product were run on a 1.2 % agarose gel containing ethidium bromide and viewed under UV light. The primers used were the following: ArhGAP15 forward primer 5`GAAGATGTTTTTCCGGGAGC 3`;ArhGAP15 reverse primer 5 GAGATTCTGGGATTTGG 3 and for GAPDH as a control these sequences were used GAPDH forward: 5` GCCATCAATGACCCCTTCATT 3` GAPDH reverse: 5` CGCCTGCTTCACCACCTTCTT3`.

Cell viability assay

Cell viability assay was carried out using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) , is a calorimetric method based on the conversion of yellow tetrazolium bromide MTT to the purple formazan derivative in leaving cells. The fibroblast cells were cultured in 96 well plates for 2 days, and after that the cells were treated as mentioned in experimental groups. After drug treatment MTT with 5 mg/ml in PBS along with media was added and further incubated at 37 ^{o}C for 4 h. DMSO 100 ul was added to each well and maintain on shaking to dissolve the formazan crystals for 1 hr and optical density was measured at 570 or 540 nm using (Biorad 680 microplate reader). Plates were placed on a shaker and agitated for 10 to 20 min. The final optical density (OD) obtained was used to calculate the percentage of cell survival, which was expressed as absorbance treated wells/absorbance control wells x 100%. The experiment was repeated at least three times.

Western blotting

Western blot analysis and protein extraction was carried out as we previously described with some modifications (Naseer *et al.,* 2009). Fibroblast cells were cultured and then sonicated using cell lysis buffer from (Cell signaling). After ultracentrifugation (12,000rpm, 4^{o}C, 15min), the supernatant was taken and measured by using the bio-Rad (Hercules, CA) protein assay kit, a total 50 µg g protein was applied per lane. The soluble fraction was separated on duplicate 10% SDS-polyacrylamide gels (30% acrylamide, 1% Bis, 1 M Tris, 10% APS, TEMED). And transferred on to PVDF membrane by electroblotting (Fast Semi-Dry Transfer Buffer, 25V, 400mA, 25min, Thermo Scientific) After transferred, nitrocellulose membranes were blocked with blocking solutions (Tris buffer saline (TBS) 0.1% Tween 20, 5% non-fat dry milk) to reduce nonspecific binding. The membrane were incubated with primary antibody anti-Bax (1:500, Santa Cruz), anti-Bcl-2 (1:500, Santa Cruz), anti-caspase-3 (1:500 cell signaling) for 24 hr at 4^{o}C, then washed in TBS, TTBS and incubated with secondary antibody

(HRP-conjugated goat anti- rabbit IgG, anti-mouse IgG, anti-goat IgG for 2 hour at room temperature. For equal loading control Immunoreactions were carried out using β-actin antibody (Cell Signaling). The desired proteins were detected through a chemiluminescence based method using ECL-detecting reagent (Amersham Pharmacia Biotech, western blotting detection reagents) according to the company protocol. After the ECL the blots were exposed to X-ray film. The final developed X-ray film was scanned, and optical densities were analyzed by densitometry using the computer-based Sigma Gel, version 1.0(Jandel Scientific, San Rafeal, Chicago, USA).

STATISTICS AND DATA ANALYSIS

The object band from Western blot were scanned and analyzed by densitometry using a computer based program Sigma Gel System (SPSS Inc., Chicago, IL). Density values were expressed as mean ± SEM. Compari sons between treated groups and controls were done analysis of variance (ANOVA) followed by Student's *t*-test and to determine the significance of differences between relevant treatment groups. In each case, the acceptance level for statistical significance was $P<0.05$.

RESULTS

Confirmation of transfection and effect on cell morphology

After transfection with siRNA of ArhGAP15 gene RT-PCR was performed to confirm the transfection efficacy. The RT-PCR results showed that after 48 h there was almost complete silencing of the gene with in the desired concentration of siRNA. Total RNA was extracted and RT-PCR was performed using GAPDH for equal loading control. The results showed that an efficient know-down was observed in the transfected cells compared with non-transfected group (fig. 1A). Further we carried out to check the morphology of fibroblast cells in control and transfected cells. There was no significant difference observed among the transfected cells and non-transfected cells whiles observed at a light microscope (fig. 1B).

Cell viability

Fibroblast cells were treated with ethanol (100 mM) with or with out sirna transfection for 30 h, and cell MTT assay was performed using 3-[4,5-dimethylthiazol-2]-2,5 diphenyltetrazolium bromide (MTT). Fibroblast cells treated with ethanol showed a decrease in cell viability after 30 h incubation compared with non-treated group.

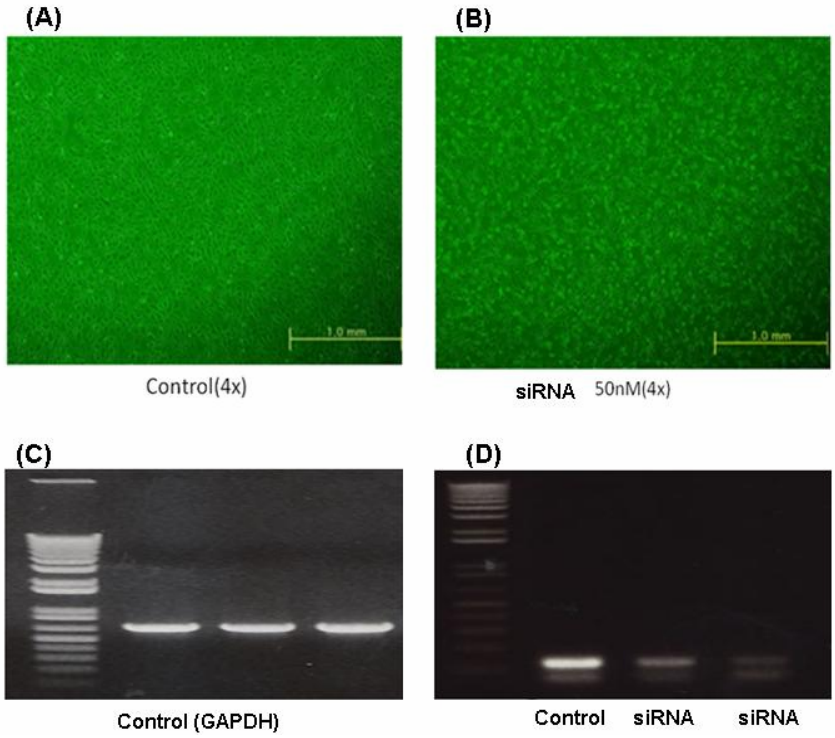

Fig. 1 (A-B): Effect of ArhGAP15 siRNA on fibroblast cells morphology observed by a light microscope. The Panel A fibroblast cells at (4X magnification) with normal media and panel B shows fibroblast cells transfected with 50 nM siRNA for 48 hours. Panels A-B cells observed by light microscope at low magnifications with a 4× objective field, Scale bar=10. **(C-D)** RT-PCR results analysis and transfection efficiency after 48 hours of transfection with 50 and 100 nM ArhGAP15 siRNA. Transfection of siRNA complementary to endogenous ArhGAP15 mRNA led to a significant decrease of ArhGAP15 mRNA as shown by RT-PCR in the PANEL D and no change in the non-transfected group, whereas GAPDH mRNA levels did not show changes.

While the transfected group alone and along with ethanol reversed cell loss compared to the ethanol treated group (fig. 2). The MTT assay showed that ethanol significantly increased cell toxicity in fibroblast cells having the arhgap15 gene while in the transfected group toxicity was reduced.

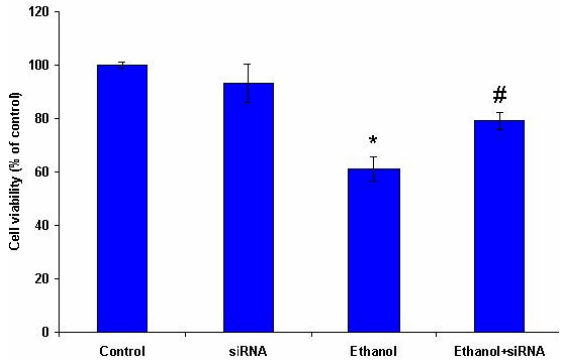

Fig. 2: MTT assay of cell viability for fibroblast cells transfected with siRNA followed by exposure to 100 mM ethanol for 30 h. Cell viability is shown in all groups compared with the O.D of control group. Presented data are the mean±SEM of three independent experiments (n=3), with 3 plates in each experiment. Symbols: $^{\#}P<$ 0.05 significantly different from ethanol; $^{*}P<0.05$ significantly different from control and siRNA transfected group.

Ethanol-induced Bax/Bcl-2 ratio in fibroblast cells

To assess a functional role of ArhGAP15 in fibroblast cells apoptosis mediated by ethanol we concentrated on the expression of apoptosis related proteins Bax and Bcl-2 in the transfected and non-transfected cells. Ethanol increased the expression of pro-apoptotic Bax protein in fibroblast cells while decreased the anti-apoptotic Bcl-2 compared with control and siRNA transfected group. The cell death induced mostly depends on the ratio of Bax/Bcl-2 that's why we focused and determined the ratio between these proteins in our experimental settings. The transfected group using ArhGAP15 siRNA showed a decreased in Bax/Bcl-2 ratio favored the role of this gene in apoptosis (fig. 3).

Ethanol exposure increased the expression of Caspase-3 in fibroblast cells

Caspase-3 is one of the major inducer of apoptosis and has a central role in the initiation of apoptotic cell death (Le *et al.*, 2002; Carloni *et al.*, 2004). In the present study we observed that treatment with ethanol significantly increased the expression of caspase-3 in fibroblast cells. Further we determined the effect of ArhGAP15 siRNA on caspase-3 expression which showed that silencing of ArhGAP15 gene decreased the expression of caspase-3 along with ethanol treated group compared with ethanol treatment alone. Western blot results showed that the cells treated with ethanol have significantly increased the expression of caspase-3 compared with control and ArhGAP15 siRNA transfected group (fig. 4).

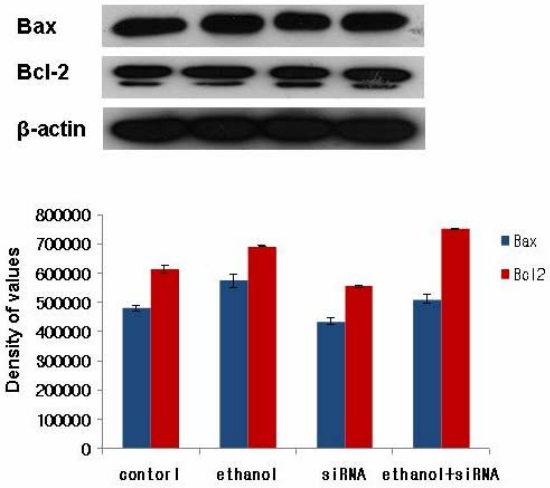

Fig. 3: Western blot analysis of Bax and Bcl-2 proteins ratio in bovine fibroblast cells. Cells were treated for 30 h with normal media as control, siRNA transfected group, Ethanol treatment (100 mM for 30 hours) and siRNA plus ethanol (Transfection for 48 hours followed by ethanol treatment) respectively. β-actin represents as internal equal loading control. Immunoblots are also shown with their respective histograms. Density values were expressed as mean±SEM (n=4) of the corresponding proteins and expressed as arbitrary units. Detail procedures are mentioned in materials and methods section.

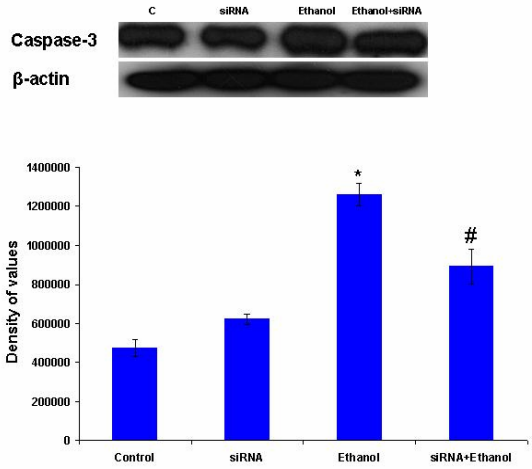

Fig. 4: Western blot analysis of Caspase-3 protein in bovine fibroblast cells. Cells were treated for 30 h with normal media as control, siRNA transfected group, Ethanol treatment (100 mM for 30 hours) and siRNA plus ethanol (Transfection for 48 hours followed by ethanol treatment) respectively. β-actin is the loading control. Immunoblots are also shown with their respective histograms. Density values were expressed as mean ± SEM (n=3) of the corresponding proteins and expressed as arbitrary units. Detail procedures are mentioned in materials and methods section. $^{\#}P<$ 0.05 significantly different from ethanol; $^{*}P<$ 0.05 different from control.

DISCUSSION

In this study we hypothesized that a higher concentration of ethanol (100 mM) cause cytotoxicity and apoptosis in bovine fibroblast cells exposed for 30 h. We observed, that ethanol induced cell death in fibroblast cells through mitochondrial mediated cell death pathway. Here we explored the role of Rho GTPase activating protein 15 (ArhGAP15) in an ethanol exposed *in vitro* model. Additionally we explored the role of ArhGAP15 gene in a system using RNA interference (RNAi) for the mentioned gene. Ethanol induces cell death was compared in both transfected and non-transfected bovine fibroblast cells.

Fibroblast cells are one of the major cell types found in connective tissue and have a major role in maintenance and repair of the injuries in those tissues. We have reported here the apoptogenic effect of ethanol in fibroblast cells by reduction of cell viability and activation of Bax/Bcl-2 and caspase-3 in both transfected and non-transfected cells. It is reported that ethanol triggers apoptosis in different type of cells like heaptocytes, astrocytes, macrophages and mouse fibroblasts (Neuman MG *et al.,* 1998; Holownia *et al,* 1997; Gauthier *et al.,* 2005; Pani *et al.,* 2004). Apoptosis induced in corneal fibroblast cells has been observed that ethanol treatment significantly decreased the cell viability and increased the expression of caspases (Chen *et al.,* 2011). Consistent with these results, we also report here that ethanol treatment reduce cell viability and increased apoptosis in bovine fibroblasts. Our observation that that moderate ethanol concentration induced apoptosis in fibroblast cells via activation of ArhGAP15 which further activate the Rac 1 which is a positive inducer of ROS generation. ROS further activate and up regulate the apoptosis mediator Bax and down regulate the anti-apoptotic Bcl-2 protein and leading to cell death.

The Rho proteins are the members of the Ras superfamily of GTPases including Rho A, Rho C and Rac1 involved in signaling pathways control cell proliferation and apoptosis (Embade *et al.,* 2000). Generally RhoGAP proteins are involved in down regulation of specific GTPase signals. The ArhGAP15 is a good and potential regulator of Rac1 and a good *in vitro* substrate for a number of Rac1 effectors kinases including αPAK and MRCKα (Seoh *et al.,* 2003). Our studies are also in agreement that ethanol may be activate the ArhGAP15 which then further effect through the activation of Rac1 which is involved in apoptosis.

ROS production induced by small GTPase Rac1 regulates apoptosis, differentiation and gene expression. Currently it was investigated that Rac1 mediates TNF-α/CHX-induced apoptosis via JNK1/2 activation. Rac1 increase ROS production through a DPI-sensitive oxidase system followed by mitochondrial dysfunction and activation of caspase-3 (Jin *et al.,* 2008). Additionally, we observed a reduction in apoptosis index in ethanol treated group transfected with ArhGAP15 siRNA compared with ethanol treated group with out transfection. So that proves a role of ArhGAP15 in ethanol induced apoptosis may be involved the activation of Rac1. The data presented here suggest that Rac1 have a dual function depend on the stimulus and type of cells used.

ArhGAP15 have critical role in ethanol-induced apoptosis in fibroblast cells via activation of Rac1. On the other hand silencing ArhGAP15 reduced the induction of Bax and activation of caspase-3.The current finding represents an important progress in our knowledge that how Rho proteins participate in the regulation of apoptosis. Further studies will need to explore the complete mechanism involved in ethanol-induced cytotoxicity and the role of Rho GTPase activating proteins.

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