IGF-1 Protects Myocardial Cells from ROS Stress-Induced Apoptosis via Up-Regulating ARC

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Abstract: Insulin-like Growth Factor-1 (IGF-1) and Apoptosis Repressor with Caspase recruitment domain (ARC) play an important role in regulating apoptosis. Although, the precise mechanisms of IGF-1 and ARC in this process have not been defined, they have similar anti-apoptotic effects in myocardial cells, suggesting that these effects are related. Researchers found that H₂O₂ can induce ARC reduction in H9C2 cells but IGF-1 can change this trend. To clarify this trend using immunofluorescence and immunoblot analysis, researchers found that LY294002 (PI3K inhibitor) blocked IGF-1 up-regulation of ARC protein and blocked the protective effect of IGF-1 on myocardial cell apoptosis induced by oxidative stress. These results indicate that IGF-1 up-regulates ARC protein expression via the PI3K pathway which protects against myocardial cell apoptosis induced by oxidative stress.

Key words: H9C2 cells, hydrogen peroxide, IGF-1, ARC, apoptosis, China

INTRODUCTION

Insulin-like Growth Factor I (IGF-I), a 70-amino acid polypeptide of 7.5 kDa is a major determinant of proliferation and apoptosis and is a key component in blood along with high-affinity binding proteins (Baserga, 2004). Previous reports have demonstrated a defensive action of IGF-1 against ROS stress-induced apoptosis in myocardial cells (Buerke *et al.*, 1995). Increased expression of the anti-apoptosis proteins, Bcl-2 and Bcl-xL, two members of the Bcl family of proteins has been postulated to be the reason for IGF-1 inhibition of apoptosis (Kim *et al.*, 2002). Although, Bcl-2 and Bcl-xL promote cell survival they are expressed at low or undetectable levels in heart muscle (Miyatake *et al.*, 1998).

The protein apoptosis repressor with caspase recruitment domain (ARC) contains a CARD domain which functions as an inhibitor of apoptosis and has been widely investigated (Koseki *et al.*, 1998; Ekhterae *et al.*, 1999). ARC is highly and predominantly expressed in long-lived tissues such as the heart, skeletal muscle and brain (Koseki *et al.*, 1998). Indeed, ARC is the first anti-apoptotic protein identified so far to be highly expressed in cardiac and skeletal muscle tissues (Mercier *et al.*, 2008). ARC selectively interacts with the initiators caspases-2 and caspases-8, thereby attenuating death receptor-induced (CD95, TNFR1 and DR3) or adaptor-induced (FADD, TRADD and CLARP) apoptosis

(Koseki *et al.*, 1998; Ekhterae *et al.*, 1999). Because of the wide involvement of IGF-1 and the central role of ARC in regulating the apoptotic program in cardiomyocytes, it is reasonable to speculate that there is a correlation between IGF-1 and ARC in their role as apoptosis inhibitors. The goal of this study is to investigate the relationship between IGF-1 and ARC in H9C2 cells under the oxidative stress environment.

MATERIALS AND METHODS

Cell treatment and viability assay: H9C2 cells (ATCC, CRL-1446™) were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) at 37°C with 5% CO₂.

Hydrogen peroxide (H₂O₂) treatment was performed as described previously (Abmayr *et al.*, 2004). In brief, the cultured cells were incubated at 37°C in treatment media (complete media excluding H₂O₂, IGF-1 or LY294002) for 24 h prior to the addition of various reagents. The reaction was stopped by removing the media containing these reagents. Cell death was determined by trypan blue exclusion and the number of trypan blue-positive and negative cells was counted on a hemocytometer.

Immunofluorescence: H9C2 cells cultured on glass coverslips were fixed with 1% paraformaldehyde overnight at 4°C and permeabilized in 0.05% Triton X-100

for 5 min at room temperature. After three washes with PBS-Tween 20, cells were blocked with 1% BSA for 2 h followed by incubation with primary antibody against ARC (abcam Co., Ltd. San Francisco USA) overnight at 4°C. Cells were then washed with PBS followed by incubation with Alexa Fluor 488 anti-rabbit antibody (Invitrogen Co., Ltd. New York USA) for 1 h at room temperature. Then, the nucleus were stained with Hoechst 33342 (1 ug mL⁻¹) (Beyotime Co., Ltd. Jiangsu China) for 10 min.

Immunoblot analysis: Cells were lysed for 1 h at 4°C in a lysis buffer (20 mM Tris pH 7.5, 2 mM EDTA, 3 mM EGTA, 2 mM Dithiothreitol (DTT), 250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride and 1% Triton X-100) containing a protease inhibitor cocktail (Sigma Co., Ltd. Missouri, USA). Samples were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electro-phoresis and transferred to nitrocellulose membranes.

Equal protein loading was controlled by Ponceau Red staining of membranes. Blots were probed using primary antibodies. The anti-ARC antibody (1:1,000) was from Abcam Inc (San Francisco USA). The rabbit immunoglobulin G control (1:200) was from Antigenix America Inc (New York, USA). After four washes with PBS-Tween 20, horseradish peroxidase-conjugated secondary antibodies (1:2,000) were added (Boster Co., Ltd. Wuhan, China). Antigen-antibody complexes were visualized by enhanced chemiluminescence (Thermo Co., Ltd. Illinois USA).

Design and transfection of siRNA: ARC siRNA oligos were synthesized by Qiagen Inc (Dusseldorf Germany). The coding sequence of siRNA oligos was ARC 5'-CCCAGTACCGCTGGAAGTGAA-3' and siRNA oligos were transfected into cells using Lipofectamine™ 2000 (Invitrogen Co., Ltd. New York, USA) following the manufacturer's instructions. Briefly, cells were plated in medium without antibiotics the day before transfection so, that a 50-70% confluence was achieved at the time of transfection. The siRNA oligos (100 nM) were used for each transfection.

Statistical analysis: Data are expressed as the mean±SEM. The significance of differences between samples was analyzed by one-way ANOVA. For each time point or experiment, triplicate or quadruplicate samples were analyzed. A $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

ARC down-regulation occurs upon stimulation with hydrogen peroxide:

The expression levels of ARC were analyzed to assess its role in regulating cell death induced by hydrogen peroxide. As shown by immunofluorescence in Fig. 1, after treatment with hydrogen peroxide, ARC protein expression was very low compared with that in the control group. Moreover, researches also, observed that the IGF-1 inhibited the down-regulation of ARC induced by hydrogen peroxide. To confirm this finding, Western blotting was employed to detect ARC expression. Researchers found that ARC protein levels were down-regulated after hydrogen peroxide treatment (Fig. 1g and h). Increased concentrations of hydrogen peroxide led to decreased ARC expression. We also wished to determine whether ARC is able to affect cell fate upon stimulation with hydrogen peroxide. Researchers observed that H₂O₂ significantly induced the H9C2 cells death (Fig. 1i). Knocking down ARC expression led to a significant increase in cell death compared with that in cells treated with H₂O₂ alone. Corresponding with the cell death, ARC expression level also decreased.

IGF-1 protects H9C2 cells from H₂O₂-induced apoptosis via Up-regulating ARC:

To demonstrate the relationship between IGF-1 and ARC expression, researchers investigated ARC expression from H9C2 cells treated with IGF-1 at different concentrations and time points (Fig. 2a and b). We observed that IGF-1 significantly up-regulated ARC expression in a dose-dependent and time-dependent manner. IGF-1 significantly decreased cell death induced by H₂O₂ (Fig. 2d). IGF-1 also inhibited the decrease in ARC protein levels induced by H₂O₂ (Fig. 2c). These data indicate that ARC plays an important role in IGF-1 protection of apoptosis induced by H₂O₂ in H9C2 cells. To further confirm this finding, siRNA was used to knockdown ARC expression. We found that siRNA (ARC) attenuated cell death induced by H₂O₂ even in the presence of IGF-1 (Fig. 2f). We also found that ARC protein levels were significantly down-regulated by siRNA (Fig. 2e).

LY294002 inhibits ARC expression induced by IGF-1:

PI3K is an important signaling pathway for IGF-1 protection of cell survival. To confirm whether IGF-1 up-regulates the ARC protein expression through the PI3K pathway, we employed LY294002 to inhibit the PI3K pathway. Researchers found that LY294002 increased cell death induced by the H₂O₂ even in the presence of IGF-1

Fig. 1: ARC down-regulation occurs upon stimulation with hydrogen peroxide. Cells were either untreated or were treated with H_2O_2 (1 mM mL^{-1}) or IGF-1 (100 ng mL^{-1}). a, c, e) Expression of ARC protein in H9C2 cells by IFA. b, d, f) The nucleus was stained by Hoechst 33342 (1 $\mu g mL^{-1}$). g) Cells were either untreated or treated for 8 h with 200, 400 or 800 $\mu M mL^{-1}$ H_2O_2 and ARC was assessed by western blotting. h) Cells were transduced with ARC siRNA (100 nM mL^{-1}) and transduced cells were treated with H_2O_2 (1 mM mL^{-1}). Total protein was prepared and total ARC levels were determined by western blotting using anti-ARC antibodies. i) Cell viability was determined as described in the materials and methods

(Fig. 3b). Additionally when detecting ARC protein levels, we observed that the LY294002 inhibitor knocked down ARC protein expression induced by IGF-1 (Fig. 3a). These data strongly indicate that IGF-1-induced ARC protein expression is through the PI3K pathway.

Myocardial cells are highly differentiated cells that typically do not replicate after birth and loss of myocardial cells results in the loss of myocardial function (Kawano *et al.*, 1994; Wakeno *et al.*, 2006; Labovsky *et al.*, 2010) which contributes to the development of heart failure (Ripa *et al.*, 2006; Buerke *et al.*, 1995), first found that IGF-1 is an important anti-apoptotic factor in a murine model of myocardial ischemic reperfusion by finding that IGF-1 could decrease myocardial apoptosis (Ibe *et al.*, 2007). Other research groups have also demonstrated that overexpression of IGF-1 decreases myocardial cell death in a coronary

artery ligation model (Leri *et al.*, 1999; Yamashita *et al.*, 2001). On the other hand, ARC is an apoptosis repressor (Hunter *et al.*, 2007; Foo *et al.*, 2007). It is highly expressed in cardiac tissues and cancer tissue (Li *et al.*, 2008; Zhang and Herman, 2008).

Several lines of evidence suggest a protective role of ARC in cardiomyocyte apoptosis induced by ischemia and reperfusion (Zhang and Herman, 2008). In this study, we found that H_2O_2 down-regulated the ARC protein levels and that IGF-1 inhibited ARC expression by decreasing in response to stimulation with oxidative stress. Previous results have also shown that oxidative stress plays a major role in inhibition of ARC protein expression (Foo *et al.*, 2007; Li *et al.*, 2008). This research has clearly indicated a synergetic effect of IGF-1 on up-regulation of ARC expression.

Fig. 2: IGF-1 protects H9C2 cells from H₂O₂ induced apoptosis via up-regulating ARC; a) Cells were treated with different concentrations (0, 1, 10, or 100 ng mL⁻¹) of IGF-1; b) Cells were treated by the same concentration of IGF-1 (100 ng mL⁻¹) for different times (0, 4, 8 or 12 h); c) Cells were untreated for 30 min with IGF-1 (100 ng mL⁻¹) and then exposed to H₂O₂ (1 mM mL⁻¹) for 6 h; e) Cells were transfected with ARC siRNA (100 nM mL⁻¹), treated with IGF-1 (100 ng mL⁻¹) and then exposed to H₂O₂ (1 mM mL⁻¹) for 6 h. d, f) Cell viability was determined as described in the materials and methods. Cultures were lysed in lysis buffer and the isolated protein was determined using Western blotting analysis. Blots were stripped and analyzed for β -actin to control for protein loading

ARC can block apoptotic cascades in multiple ways (Di Giovanni *et al.*, 2004). ARC not only directs caspase-inhibiting functions but it can also interact with FADD or Bax (Zhang and Herman, 2008). In addition, it also inhibits cytochrome c release and maintains membrane potentials (An *et al.*, 2009). Furthermore, ARC appears to be a calcium-binding protein and can suppress an intracellular Ca²⁺ increase, thereby blocking Ca²⁺-mediated apoptosis (Li *et al.*, 2009) which can stimulate myocardial cell death via down-regulation of ARC protein expression (Abmayr *et al.*, 2004). The present study results are consistent with these previous results. The presence of IGF-1 plays a dual role by inhibiting cell death as well as up-regulating the ARC protein expression under an oxidative stress environment. We also found that ARC

knock-down induced a higher rate of cell death induced by oxidative stress even when IGF-1 was present. From these results we speculate that ARC plays an important role in IGF-1 protection against oxidative stress-induced myocardial cell apoptosis.

It is well documented that IGF-1 promotes cell survival and proliferation via a number of signaling mechanisms such as phosphatidylinositol 3-kinase (PI3-K) or Mitogen-Activated Protein Kinase (MAPK) (Stitt *et al.*, 2004). In fact inhibition of PI3-K can directly result in apoptosis in some cell types (Subramaniam *et al.*, 2005). Activated IGF-1R phosphorylates the signals via MAPK and PI3K-Akt pathways (Laurino *et al.*, 2005).

These signals play important roles in cell proliferation and inhibition of apoptosis. In this study, researchers

Fig. 3: LY294002 inhibits ARC expression induced by IGF-1. Cells were preincubated for 30 min with ly294002 (50 $\mu\text{M mL}^{-1}$, PI-3K inhibitor) or IGF-1 (100 ng mL^{-1}) and then treated with H_2O_2 (1 mM mL^{-1}) for 6 h; a) Total protein extracts were prepared and ARC levels were detected by immunoblot and b) Cell viability was determined as described in the materials and methods

found that PI3K inhibitor blocked IGF-1 up-regulation of ARC protein and it also blocked the IGF-1 protective action against apoptosis induced by oxidative stress in myocardial cells.

CONCLUSION

Researchers successfully demonstrated a correlation between IGF-1 and ACR protein expression under an oxidative stress environment. The results indicate that IGF-1 can up-regulate ARC protein expression via the PI3K pathway to protect against myocardial cell apoptosis induced by oxidative stress.

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