

Clusterin protects H9c2 cardiomyocytes from oxidative stress-induced apoptosis *via* Akt/GSK-3 β signaling pathway

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DOI 10.3858/emm.2011.43.1.006

Accepted 13 December 2010

Available Online 22 December 2010

Abbreviations: Apo J, apolipoprotein J; ROS, reactive oxygen species; TRPM-2, testosterone-repressed prostate message-2

Abstract

Clusterin is a secretory glycoprotein, which is highly up-regulated in a variety of normal and injury tissues undergoing apoptosis including infarct region of the myocardium. Here, we report that clusterin protects H9c2 cardiomyocytes from H₂O₂-induced apoptosis by triggering the activation of Akt and GSK-3 β . Treatment with H₂O₂ induces apoptosis of H9c2 cells by promoting caspase cleavage and cytochrome c re-

lease from mitochondria. However, co-treatment with clusterin reverses the induction of apoptotic signaling by H₂O₂, thereby recovers cell viability. The protective effect of clusterin on H₂O₂-induced apoptosis is impaired by PI3K inhibitor LY294002, which effectively suppresses clusterin-induced activation of Akt and GSK-3 β . In addition, the protective effect of clusterin is independent on its receptor megalin, because inhibition of megalin has no effect on clusterin-mediated Akt/GSK-3 β phosphorylation and H9c2 cell viability. Collectively, these results suggest that clusterin has a role protecting cardiomyocytes from oxidative stress and the Akt/GSK-3 β signaling mediates anti-apoptotic effect of clusterin.

Keywords: apoptosis; clusterin; glycogen synthase kinase 3 β ; myocytes, cardiac; oxidative stress; proto-oncogene proteins c-act

Introduction

The reactive oxygen species (ROS) and free radicals are widely believed to be important in pathological events causing apoptotic cell death. In particular, they play central roles in cardiac pathophysiology when the heart is subjected to significant oxidative stress governed by ischemia and reperfusion (MacLellan and Schneider, 1997; Saraste *et al.*, 1997; Lee *et al.*, 2009). Clusterin, also known as apolipoprotein J (apo J) or testosterone-repressed prostate message-2 (TRPM-2), was originally isolated from the rat testis (Blaschuk *et al.*, 1983). Clusterin is a hetero-dimeric glycoprotein secreted by a number of cell types (Jones and Jomary, 2002). Clusterin is an extracellular chaperone protein, which has a cytoprotective effect in response to diverse stresses (Poon *et al.*, 2000; Whang *et al.*, 2005). Previous studies suggest that clusterin plays important roles in cell adhesion, spermatogenesis, tumor metastasis, and lipid transportation (Wilson and Easterbrook-Smith, 2000; Jones and Jomary, 2002; Lau *et al.*, 2006). In addition to chaperone activity, clusterin may have an antiapoptotic function. With regard to the role of clusterin in apoptotic cell death, multiple lines of evidence have demonstrated its cytoprotective effects in several tumors, epithelial cells, endothelial cells and so on (Kim *et al.*, 2007, 2010;

Sallman *et al.*, 2007). Moreover, clusterin protects cells from heat shock and TNF- α , suggesting that clusterin could be a survival factor related to the apoptotic pathway (Sensibar *et al.*, 1995; Miyake *et al.*, 2000; Trougakos and Gonos, 2006). The anti-apoptotic effect of clusterin was also reported in the cardiac system based on its increased expression in the injured heart (Swertfeger *et al.*, 1996; Ishikawa *et al.*, 1998; McLaughlin *et al.*, 2000; Miyata *et al.*, 2001). It is previously reported that clusterin protects cardiomyocytes from ischemia-induced cell death (Krijnen *et al.*, 2005) or ethanol-induced cardiac injury (Li *et al.*, 2007). Despite the potential cardioprotective effect of clusterin, little is known about the action of clusterin against the individual oxidative stress, and the mechanism by which clusterin represents cardioprotection needs to be elucidated in detail.

In the present study, therefore, we investigated the protective effect of clusterin on the survival of H9c2 cardiomyocytes against H₂O₂ stimulus as well as the underlying signaling pathway. We found that clusterin exerts cardioprotection against H₂O₂-induced oxidative injury and the activation of Akt/GSK-3 β signaling pathway plays an important role in mediating the anti-apoptotic effect of clusterin.

Results

Effect of single treatment of H₂O₂ or clusterin on H9c2 cells viability

We first determined the dose at which cytotoxicity develops in a period of 24 h upon H₂O₂ exposure in H9c2 cells through the MTS assay. Cells were treated with increasing doses of H₂O₂ for 24 h. As shown in Figure 1A, H₂O₂ impaired cell viability in a concentration-dependent manner over the tested concentration range (100-500 μ M). A maximum reduction was $56 \pm 2\%$ of the control group and it was observed at 500 μ M of H₂O₂. We chose the level of H₂O₂ 400 μ M for use in our subsequent experiments based on this result. In order to evaluate whether clusterin is cytotoxic to H9c2 cells, we determined the viability of cells treated with clusterin (1-20 μ g) for 24 h using the MTS assay. Cell viability was not significantly affected by treatment with any tested clusterin concentrations (Figure 1B).

Effects of clusterin on H₂O₂-induced apoptosis in H9c2 cells

In order to determine the effects of clusterin on H₂O₂-induced cell death, the H9c2 cells were pre-treated with 10 μ g clusterin for 2 h, after which

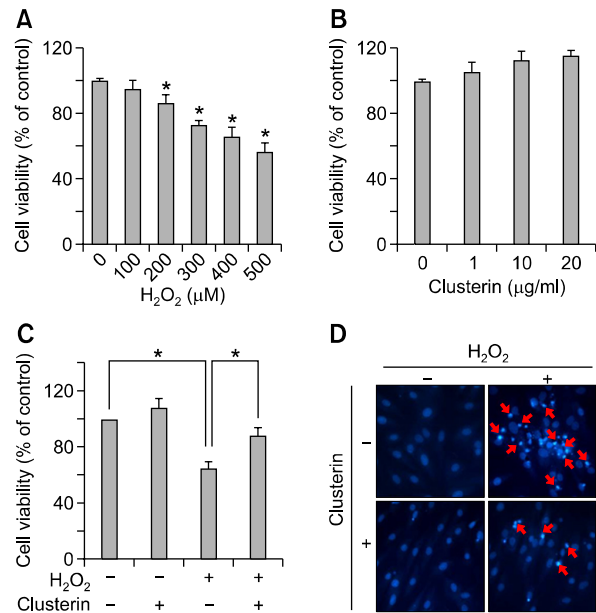


Figure 1. Clusterin attenuates H₂O₂-induced apoptotic cell death. (A) H9c2 cells were incubated with indicated concentrations of H₂O₂ (100-500 μ M) for 24 h, then cell viability was measured by MTS assay. (B) After treatment of clusterin (1-20 μ g/ml) for 24 h, cell viability was determined by MTS assay. (C) H9c2 cells were treated with 10 μ g/ml clusterin in the presence or absence of 400 μ M H₂O₂ for 24 h. Cell viability was measured by MTS assay. (D) Cell apoptosis was evaluated by DAPI staining. Each value represents the mean \pm SD of three independent experiments (* $P < 0.05$).

co-incubated with 400 μ M of H₂O₂ for additional 24 h. As shown in Figure 1C, co-treatment of clusterin relieved reduced viability of H₂O₂-stimulated cells. To clarify whether clusterin protects H9c2 cells from apoptosis, we compared apoptotic nuclei and DNA fragmentation in H₂O₂-treated cells with or without clusterin. As shown in Figure 1D, most nuclei in either control or clusterin alone group displayed uniform blue chromatin with organized structure. In contrast, intense DAPI stained nuclei that indicate cells undergoing apoptosis were frequently observed in H₂O₂-stimulated cells compared to control cells. However, co-treatment with clusterin decreased the rate of apoptotic nuclei induced by H₂O₂. These data propose that clusterin has a protective role in H₂O₂-induced apoptotic cell death of H9c2 cells.

Caspases are key mediators of cell death and caspase-3 is an executioner for the death program in many cells in response to oxidant, such as H₂O₂. Thus we examined the effect of clusterin on H₂O₂-stimulated caspase-3 and caspase-9 activation. As shown in Figure 2, H₂O₂ treatment was associated with significant increase in caspase-9 and caspase-3 cleavage as compared to control. Clusterin pretreatment significantly decreased the

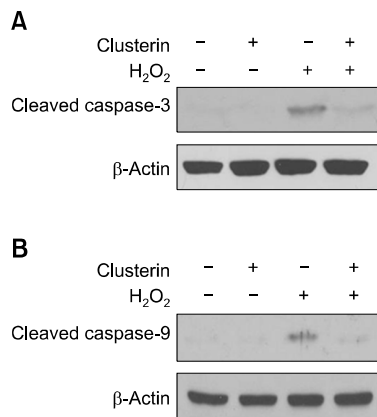


Figure 2. Clusterin inhibits H₂O₂-induced caspase-3 and caspase-9 activation in H9c2 cells. After 2 h pre-incubation with 10 μg/ml clusterin, H9c2 cells were treated with 400 μM H₂O₂ for 24 h. The activity of caspase-9 (A) caspase-3 (B) was assessed by western blot analysis.

level of both cleaved caspase-9 and -3 in H₂O₂-treated H9c2 cells, indicating that clusterin attenuated H₂O₂-induced apoptosis occurring through caspase activation.

Effect of clusterin on mitochondria-mediated apoptosis in H9c2 cells

Changes in the induction of Bcl-2 family proteins are closely related to an imbalance in the mitochondrial homeostasis. In particular, the release of cytochrome c into the cytosol is an important step for the induction of apoptosis in a number of different cell types, which is tightly regulated by the equilibrium between the anti-apoptotic Bcl-2 and pro-apoptotic Bad and Bax. As shown in Figure 3A, cytochrome c mainly located in mitochondria and could not be found in cytosol of myocytes in control group. H₂O₂ treatment reduced the level of cytochrome c in mitochondria but increased it in cytosol, indicating its translocation from mitochondria to cytosol. However, the level of cytosolic cytochrome c was lower in clusterin co-treatment group compared to H₂O₂ single treatment indicating that clusterin might inhibit translocation of cytochrome c. Next, we examined the level of Bcl-2 and Bax protein. As shown in Figure 3B, H₂O₂ treatment resulted in decreased Bcl-2/Bax ratio compared to control, but clusterin treatment recovered the ratio of Bcl-2/Bax proteins.

Effects of clusterin on Akt and GSK-3β phosphorylation

To explore the potential signaling pathways contributing to the anti-apoptotic function of clusterin,

we examined Akt and GSK-3β activation. As shown in Figure 4, H₂O₂ treatment decreased both Akt (Figure 4A) and GSK-3β phosphorylation (Figure 4B) compared to control, but the decreased phosphorylation of Akt and GSK-3β were recovered by clusterin treatment. To determine whether the Akt/GSK-3β signaling pathway is involved in the pro-survival function of clusterin, H9c2 cells were pre-incubated with PI3K inhibitor LY294002 and thereafter treated with H₂O₂ in the absence or presence of clusterin for 24 h. LY294002 reversed the protective effects of clusterin on H₂O₂-induced apoptosis as determined by the caspase-3 activity (Figure 4C). The efficacy of inhibitor was confirmed through the reduction of phosphorylation of Akt (Figure 4D) and GSK-3β (Figure 4E). Therefore, these data imply that the apoptotic effect of clusterin on oxidative stress-induced apoptosis of cardiomyocytes is mediated at least in part through Akt/GSK-3β signaling.

Independency of megalin on the protective effect of clusterin

Megalyn is described as a receptor of clusterin (Zlokovic *et al.*, 1996). Clusterin is bound and taken

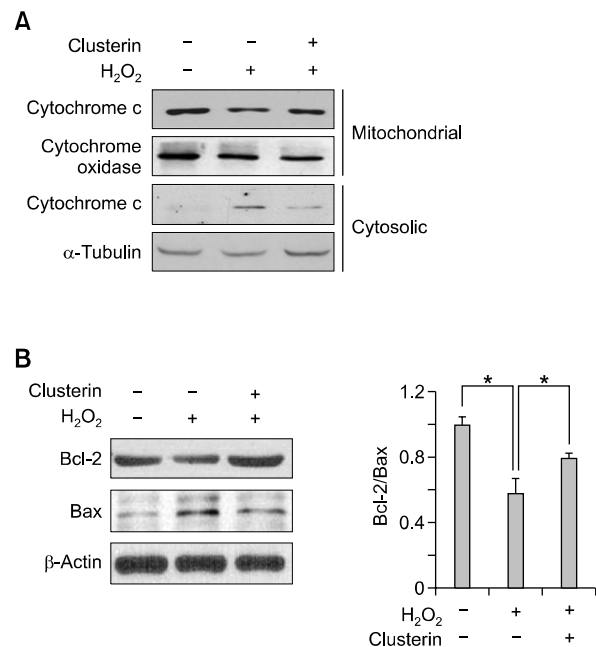


Figure 3. Clusterin reduces cytochrome c release and the ratio of Bcl-2/Bax. After 2 h pre-incubation with 10 μg/ml clusterin, H9c2 cells were incubated in the presence of 400 μM H₂O₂ for 12 h. (A) Cytochrome c release from mitochondria to cytosol was measured by western blot analysis. (B) Bcl-2 and Bax expression were assessed by western blot analysis. Each value represents the mean ± SD of three independent experiments (**P* < 0.05).

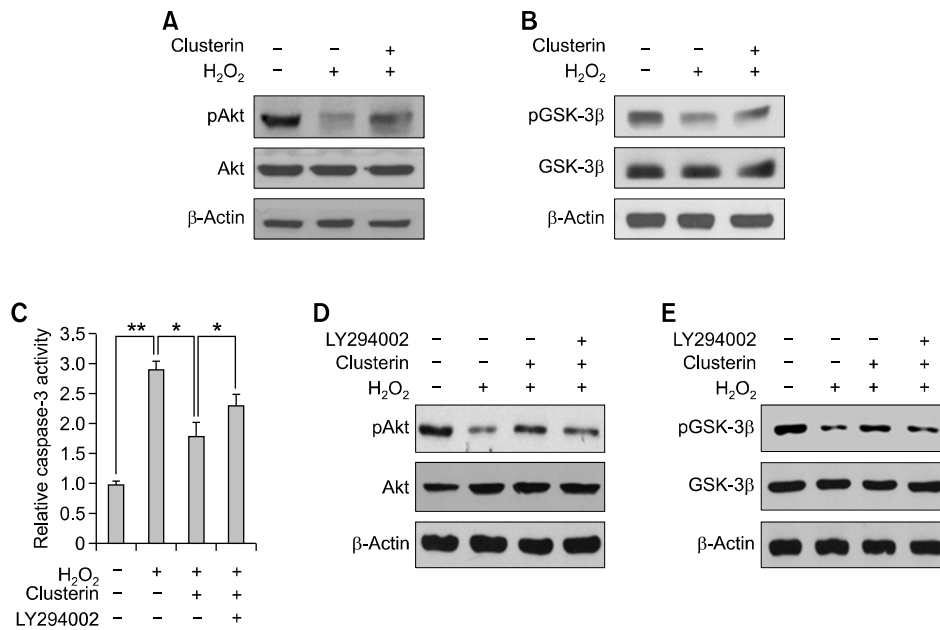


Figure 4. Cardioprotective effect of clusterin is mediated by the activation of Akt/GSK-3β signaling. (A, B) After 2 h pre-incubation with 10 μg/ml clusterin, H9c2 cells were treated with 400 μM H₂O₂ for 12 h. Western blot analysis was performed to examine phosphorylation of Akt and GSK-3β. (C) H9c2 cells were pre-incubated with 10 μM LY294002 and treated with H₂O₂ alone or in combination with clusterin. Then, caspase-3 activity was measured. (D, E) Phosphorylation of Akt and GSK-3β was examined in LY294002 pre-treated cells by western blot analysis. Each value represents the mean ± SD of three independent experiments (**P* < 0.05, ***P* < 0.01).

up by megalin (Kounnas *et al.*, 1995). Even though clusterin certainly has a survival effect in various cell types, there are some controversial results about whether the survival effect of clusterin is dependent or independent on its receptor megalin (Girton *et al.*, 2002; Ammar and Closset, 2008; Van Dijk *et al.*, 2010). We examined whether or not megalin is involved in the survival effect of clusterin in H9c2 cells using either RAP, an inhibitor of megalin (Van Dijk *et al.*, 2010), or megalin siRNA. H9c2 cells were treated with clusterin with and without RAP, and the phosphorylations of Akt and GSK-3β were analyzed. Decreased phosphorylations of Akt and GSK-3β by H₂O₂ were recovered by clusterin treatment but co-treatment of RAP did not alter the effect of clusterin (Figure 5A). In addition, knock-down of megalin using siRNA did not affect Akt and GSK-3β phosphorylation (Figure 5B). Furthermore, there was no change in cell viability after treatment of RAP with clusterin as determined by the MTS assay (Figure 5C). Therefore, these results suggest that clusterin does not exert its protective effect through megalin in H9c2 cells.

Discussion

During ischemia and reperfusion, cell death of cardiomyocytes causes direct loss of heart function. Therefore, massive amount of studies has focused on ways to protect cardiomyocytes from cytotoxic stimuli such as oxidative stress. Due to its importance in heart function, cardiomyocyte seems to have mechanisms protecting itself against cytotoxic stimuli (Lamendola *et al.*, 2009). One of the self-protective mechanisms of cardiomyocyte could be an induction of cytoprotective protein, clusterin. Despite some controversy about the exact location of clusterin up-regulation, it seems to be clear that clusterin expression increases in the myocardium of the infarcted heart (Vakeva *et al.*, 1993; Silkenen *et al.*, 1998). In particular, recent report showed that the factors released from conditioned medium of mouse embryonic stem cell, including clusterin, cystatin-c, osteopontin, and TIMP-1, inhibited H₂O₂-induced apoptosis in H9c2 cells (Singla and McDonald, 2007). Consistently with these results, our study presents new direct evidence showing the protective effect of clusterin on H₂O₂-induced apoptosis in H9c2 cells. Clusterin was previously reported to decrease intracellular ROS level in human corneal endothelial cells and human retinal pigment epithelial cells (Shin *et al.*,

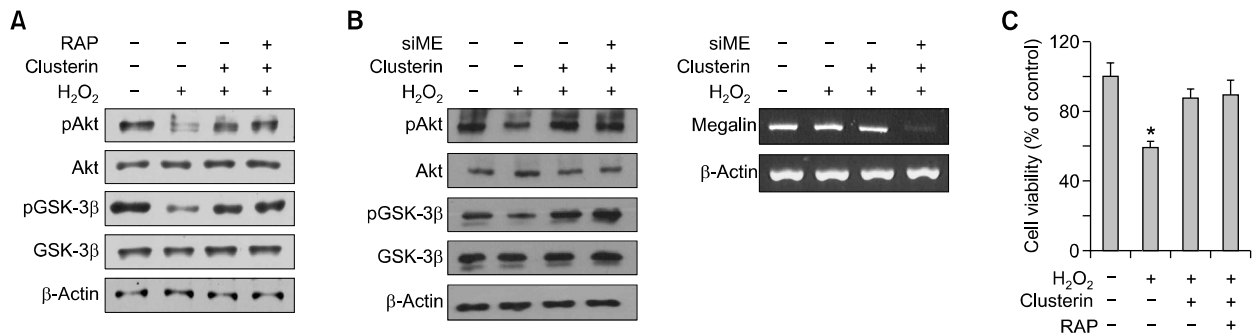


Figure 5. Cardioprotective effect of clusterin is megalin independent. (A) After 2 h pre-incubation with 10 $\mu\text{g/ml}$ clusterin and/or 0.5 μM RAP, H9c2 cells were treated with 400 μM H₂O₂ for 12 h. Western blot analysis was performed to examine phosphorylation of Akt and GSK-3 β . (B) H9c2 cells were transfected with either negative control siRNA (20 nM) or megalin siRNA (20 nM). After 24 h, the transfectants were exposed to 400 μM H₂O₂ for 12 h. RT-PCR analysis was carried out using specific primers for *megalyn* and β -actin to show the efficient knock-down of megalin (right) and western blot analysis was performed to examine phosphorylation of Akt and GSK-3 β (left). (C) After 2h pre-incubation with 10 $\mu\text{g/ml}$ clusterin and/or 0.5 μM RAP, H9c2 cells were treated with 400 μM H₂O₂ for 24 h. Cell viability was measured by MTS assay. Each value represents the mean \pm SD of three independent experiments (* $P < 0.05$).

2009; Kim *et al.*, 2010). Compatible with these previous studies, we also observed that clusterin treatment in H9c2 cells strongly inhibited H₂O₂-enhanced intracellular ROS production (Supplemental Data Figure S1). Similar to the pattern of intracellular ROS production, H₂O₂-induced cell death was also significantly inhibited by clusterin treatment (Figures 1C and D) and accordingly clusterin significantly attenuates H₂O₂-induced cytochrome c release and apoptosis in H9c2 cells. These data imply that clusterin may protect H9c2 cells from oxidative stress through inhibition of ROS production.

However, clusterin represented cytoprotection in H9c2 cells at much higher concentration compared to in retinal pigment epithelium cells against the same H₂O₂ concentration (Kim *et al.*, 2010). In the previous study, we observed anti-apoptotic effect of clusterin in retinal pigment epithelium at 1 $\mu\text{g/ml}$ concentration (Kim *et al.*, 2010) whereas it showed anti-apoptotic effect in H9c2 cells at more than 10 $\mu\text{g/ml}$ concentration (Figure 1). The possible explanation of this difference is that H9c2 cell might express clusterin higher than retinal pigment epithelial cell upon oxidative stress. In accordance with this, Krijnen *et al.* (2005) showed that clusterin expression increased by ischemic challenge in H9c2 cells. Thus, our previous and current data may inform that clusterin administration should be differently adapted in different tissues. In addition to the functional study of clusterin, we also showed the mechanisms whereby it exerts anti-apoptotic effect in H9c2 cells.

We observed that clusterin regulates the expression of Bcl-2 family members such as Bcl-2 and Bax, which is important to control cytochrome c release (Pollack *et al.*, 2002; Susnow *et al.*,

2009). Co-treatment of clusterin with H₂O₂ increased anti-apoptotic protein Bcl-2 and decreased pro-apoptotic protein Bax compared to H₂O₂-treated H9c2 cells. Accordingly, H₂O₂-induced cytochrome c release and caspase activation were also reduced by co-treatment of clusterin.

Besides Bcl-2 family, GSK-3 β is also recognized as a critical factor mediating cytochrome c release from mitochondria to cytosol (Park *et al.*, 2006). Akt directly phosphorylates GSK-3 β at Ser9 which negatively regulates its kinase activity. Thereafter, phosphorylated GSK-3 β inhibits the opening of mitochondrial permeability transition pore (mPTP) (Juhászova *et al.*, 2009). In this study, clusterin recovered the decrease in Akt and GSK-3 β phosphorylation induced by H₂O₂ (Figures 4A and B). Activation of Akt/GSK-3 β seems to be responsible for anti-apoptotic effect of clusterin because LY294002, the inhibitor of PI3K which is an upstream activator of Akt, could abolish cytoprotection by clusterin (Figure 4C). Indeed, accumulating evidence supports that Akt might protect injured heart through normalizing mitochondrial regulation (Miyamoto *et al.*, 2009) and GSK-3 β is recognized as a potential therapeutic target for cardiac protection (Miura and Miki, 2009). In particular, the previous report suggested that phosphorylated GSK-3 β provides cardioprotection against myocardial I/R injury (Gross *et al.*, 2004). In our results, the treatment of LY294002, however, did not completely recovered caspase-3 activity compared to H₂O₂-treated cells (Figure 4C), suggesting that additional pathway(s) could be involved in anti-apoptotic effect of clusterin.

Similar to our results, Ammar and Closset (2008) previously reported that clusterin protects TNF-alpha-induced apoptosis through activation of

PI 3-kinase/Akt pathway in prostatic cells. In this study, they also showed that the activation of PI 3-kinase/Akt pathway by clusterin is dependent on the expression and phosphorylation of megalin, the receptor of clusterin. However, in the present study, clusterin-induced Akt phosphorylation seems to be megalin independent even though we observed similar activation of Akt pathway by clusterin in H9c2 cells. To examine whether or not megalin is involved in the survival effect of clusterin in H9c2 cells, we used either RAP, an inhibitor of megalin, or megalin siRNA. Expression of megalin was not changed by clusterin treatment in H9c2 cells and the inhibition of megalin using RAP and siRNA has no effect on clusterin-induced Akt/GSK-3 β phosphorylation (Figure 5), suggesting that clusterin does not exert its protective effect through megalin in H9c2. These differences could be explained by the different system used. While Ammar and Closset used prostatic cells, we used H9c2 cardiomyocytes. Therefore, we could say that clusterin shows similar cytoprotective effect mediating PI 3-kinase/Akt pathway in both prostatic cells and H9c2 cardiomyocytes but it may utilize different upstream mediator; it involves megalin in prostatic cells but not in H9c2 cardiomyocytes. In accordance with our results, Van Dijk *et al.* (2010) previously reported that endogenous clusterin does not co-localize with megalin in H9c2 cells and its protective effect on cardiomyocytes after acute myocardial infarction *in vivo* and ischemia *in vitro* is independent on its receptor, megalin.

In summary, our study reveals that clusterin protects H9c2 cardiomyocytes against oxidative stress by inhibiting mitochondria-mediated apoptosis and that the activation of the Akt/GSK-3 β signaling pathway is involved in the cardioprotective effect of clusterin. Therefore, clusterin could be applicable to therapeutics for heart injury after ischemia and reperfusion.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Invitrogen (Carlsbad, CA). DAPI (4,6-diamidino-2-phenylindole) was obtained from Sigma (St. Louis, MO). Receptor-associated protein (RAP) was obtained from Progen (Heidelberg, Germany). Rabbit polyclonal anti-Bcl-2, and anti-Bax antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-Akt, anti-p-Akt^{ser473}, anti-caspase-3, anti-caspase-9, anti-GSK-3 β , and anti-p-GSK-3 β ^{ser9} antibodies were purchased from Cell Signaling (Danvers, MA). All other reagents, including H₂O₂ and LY294002, were

purchased from Sigma.

Cell culture

H9c2 cardiomyocytes were obtained from American Type Culture Collection (Rockville, MD) and cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin.

Purification of clusterin

Clusterin was purified from human serum as previously described (Shin *et al.*, 2006). Briefly, human serum in 0.5 mM phenylmethylsulfonyl fluoride (PMSF) was stirred with 12% polyethylene glycol (PEG, Sigma) overnight at 4°C. After centrifugation, the supernatant was reprecipitated with 23% PEG. This precipitate was subjected to DEAE-sepharose and heparin sepharose column chromatography (GE Healthcare Life Science, Buckinghamshire, England). The serum clusterin was finally purified by affinity chromatography using cyanogen bromide-activated sepharose covalently conjugated with monoclonal clusterin antibody. The eluted protein was dialyzed and lyophilized prior to being stored at -70°C.

Cell viability

Cell viability was determined colorimetrically using MTS assay. H9c2 cells were cultured in 48-well plates and treated with H₂O₂ and/or clusterin. After 24 h, MTS was added to each well according to the manufacturer's instruction (Promega, Madison, WI). After 2 h incubation at 37°C, the cell viability was determined by measuring the absorbance at 490 nm using microplate spectrophotometer (Molecular Device, Sunnyvale, CA). Three independent experiments were performed for each experimental condition.

DAPI staining

Apoptosis of H9c2 cardiomyocytes were determined by DAPI staining, which allows determination and quantification of cells with fragmented and condensed chromatin. After washing with phosphate-buffered saline (PBS), H9c2 cells were fixed for 10 min with a 4% paraformaldehyde at room temperature. Then, the cells were washed with PBS and stained with DAPI (5 μ g/ml in PBS) at room temperature for 5 min. Cell nuclei analysis was conducted with the TCS NT fluorescence imaging system comprising of inverted microscope (Leica, Germany).

Caspase-3 activity assay

Caspase-3 activity was measured by a cleavage of fluorogenic substrate according to the manufacturer's recommendation (BD Bioscience, San Jose, CA). H9c2 cells were pre-incubated with LY294002 (10 μ M) for 30 min, then incubated with 400 μ M H₂O₂ or in combination with 10 μ g/ml clusterin for 24 h. Following treatment, cell lysate and caspase-3 substrate (Ac-DEVD-AMC) were added to the reaction buffer and incubated for 1 h at 37°C. Cleaved

substrate by caspase-3 was determined using microplate spectrofluorometer (Molecular Device, Sunnyvale, CA) with a 380 nm excitation filter and a 460 nm emission filter. Assays were performed in three independent experiments.

Western blot analysis

Cells were lysed in a whole cell extract buffer containing 10 mM HEPES (pH 7.9), 1 mM EDTA, 5% Glycerol, 1 mM DTT and 400 mM NaCl. Protein concentration was determined with the use of a BCA protein assay kit following the manufacturer's instruction (Thermo Scientific, Rockford, IL). Protein samples of whole cell lysate were mixed with an equal volume of $5 \times$ SDS sample buffer, boiled for 5 min, and then separated by 8-15% SDS-PAGE gels. After electrophoresis, proteins were transferred to nitrocellulose membrane. The membranes were blocked in 5% non-fat dry milk for 1 h, rinsed, and incubated with specific antibodies against clusterin, caspase-3, caspase-9, Akt and p-Akt in PBS-T (PBS containing 0.1 % Tween-20) overnight at 4°C. Primary antibody was removed by washing the membranes 3 times in PBS-T, and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody. Following 3 times of washing in PBS-T, immuno-positive bands were visualized using chemiluminescent reagent (Amersham, Piscataway, NJ) and exposed to X-ray film (Agfa, Germany).

Cell fractionation

Cultured cells were washed with PBS and then Dounce-homogenized (40 strokes) in homogenization buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol, 250 mM sucrose and 0.1 mM PMSF) and centrifuged twice at 4°C for 5 min at 1,000 *g*. The supernatants were transferred to new tubes and the pellets were discarded to remove nuclei and unbroken cells. The supernatant were re-centrifuged at 4°C for 15 min at 14,000 *g*. The collected pellets was washed twice by homogenization buffer and resuspended with lysis buffer for mitochondria fraction, and the supernatants were used for cytosolic fraction. Protein concentration was determined using the BCA assay. Proteins were analyzed by SDS-PAGE as described above.

siRNA transfection

siRNA targeting rat megalin was chemically synthesized and purified in the 2-deprotected and desalted form (Bioneer, Daejeon, Korea). The sequences of megalin siRNA pair were 5'-CAGUGAUGAGCUUCCUACA-3' and 5'-UGU-AGGAAGCUCAUCACUG-3'. Nonspecific siRNA (Cat. no: SN-1002, Bioneer, Daejeon, Korea) was used as a control for comparison. Transfection of siRNA was performed using lipofectamine and plus (Invitrogen, Rockville, MD), according to the manufacturer's instructions. Briefly, H9c2 cells were seeded at 2×10^5 cells in a 6-well plate. After 24 h, cells were transfected at the final concentration of 20 nM siRNA duplexes. The efficacy of knockdown was assessed RT-PCR using a megalin specific primer.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA from cells was isolated using TRI Reagent[®] (Molecular Research Center, Cincinnati, OH), according to the manufacturer's instructions. First-stranded cDNA was synthesized with 5 µg of total RNA and oligo(dT)₁₈ by M-MLV reverse transcriptase (Invitrogen, Rockville, MD). Equal amounts of cDNA were subsequently amplified by PCR using PCR reaction kit (Bioneer, Daejeon, Korea) and specific primer for megalin (5'-GGTGTGTGACGAGGAT-3' and 5'-AGTTGCAATTGCGCTCATCG-3') and β-actin (5'-ATTGCCGATAGTGATGACCT-3' and 5'-CGTGAAAAGATGACCCAGAT-3'). PCR was performed with an initial denaturation step followed by denaturation, annealing, and extension. PCR products were separated on 1% agarose gels and visualized using SYBR[®] Safe DNA gel stain (Invitrogen, Rockville, MD) under UV transillumination.

Statistical analysis

Data were expressed as mean ± SD. Differences were analyzed for significance by Student's *t*-test. The results were considered significant at $P < 0.05$.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Ministry of Education, Science & Technology (MEST) through the Creative Research Initiative Program (Grant R16-2004-001010010, 2009) and the grant No. R31-2008-000-10103-0 from the WCU project of the MEST and the NRF.

Supplemental data

Supplemental Data include a Figure and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-43-1-07.pdf.

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