Insulin in combination with selenium inhibits HG/Pal-induced cardiomyocyte apoptosis by Cbl-b regulating p38MAPK/CBP/Ku70 pathway

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Abstract. – OBJECTIVE: In this study, we investigated whether insulin and selenium in combination (In/Se) suppresses cardiomyocyte apoptosis and whether this protection is mediated by Cbl-b regulating p38MAPK/CBP/Ku70 pathway.

MATERIALS AND METHODS: Firstly, H9c2 cardiomyocytes were treatment with high glucose (25 mmol/L) and palmitate (600 μmol/L) (HG/Pal). Next, H9c2 cardiomyocytes were treatment with HG/Pal+In/Se (10 nmol/L Insulin in combination with 10 nmol/L selenium). Finally, cells were treated with siRNA against Cbl-b, followed by HG/Pal and HG/Pal+In/Se treatment. Then, cell apoptosis was observed by flow cytometry (FCM). The levels of Cbl-b, p-p38MAPK, CBP and Bax were examined by Western blotting. The acetylated Ku70 was detected by immunoprecipitation.

RESULTS: Insulin and selenium in combination reduced cell apoptosis, up-regulated Cbl-b expression, down-regulated p38MAPK, CBP and acetylated Ku70 expressions and prevented Bax translocation, whereas Cbl-b knockdown strongly suppressed In/Se-induced these effects in HG/Pal-treated cardiomyocytes.

CONCLUSIONS: Insulin and selenium synergistically suppressed cardiomyocyte apoptosis by Cbl-b regulating p38MAPK/CBP/Ku70 pathway.

Key Words
Insulin, Selenium, H9c2 cardiomyocytes, Cbl-b, p38MAPK, CBP, Ku70.

Introduction
Diabetic cardiomyopathy (DCM), which is a major cardiac complication in diabetic patients, is one of the leading causes of increased morbidity and mortality in the diabetic population1. It is widely acknowledged that DCM is characterized by a set of structural and functional abnormalities in the heart2. The oxidative stress and apoptosis are regarded as main features of diabetic cardiomyopathy3-5. Therefore, an ideal drug to prevent diabetes-induced cardiomyopathy may need to inhibit oxidative stress and apoptosis simultaneously.

Selenium is an integral part of glutathione peroxidase and protects various cells against oxidative damage. Insulin has a beneficial effect on hyperglycemia in diabetes, but the administration of insulin for controlling hyperglycemia may produce some side effects. Therefore, combined doses of insulin and selenium were superior to administering them alone.

The activation of p38MAPK/CBP/Ku70 pathway initiated mitochondrial apoptotic pathway in a variety of cells6,7. The activation of p38MAPK was negatively regulated by ubiquitin ligase Cbl-b. Whether Cbl-b-mediated p38MAPK/CBP/Ku70 pathway was also involved in insulin in combination with selenium-induced cardiac protection is still unclear.

To study this mechanism, H9c2 cardiomyocytes were treated with HG/Pal and HG/Pal+In/Se, and cells were treated with siRNA against Cbl-b, followed by HG/Pal and HG/Pal+In/Se treatment. We then measured apoptosis, Cbl-b expression and important molecules of p38MAPK/CBP/Ku70 pathway.

Materials and Methods

Cell Culture and Treatments
H9c2 cardiomyocytes were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 ug/ml strepto-
mycin with 5% CO₂ at 37°C. In experiments, cells respectively were treated with normal glucose (control, 5.5 mmol/l glucose), HG/Pal and HG/Pal and In/Se treatment. To inhibit Cbl-b expression, cells were transfected with Cbl-b siRNA using Lipofectamine™ TM 2000 transfection reagent for 24 h. Then cells were cultured by HG/Pal+In/Se for 1 h, followed by HG/Pal 24 h, and cells were cultured by HG/Pal 24 h.

**Material**

Trihydroxymethyl aminomethane (Tris), glycine, sodium dodecyl sulfate (SDS), acrylamide and bis-acrylamide were purchased from Amresco Inc. (Amresco, Solon, OH, USA). Rabbit anti-rat-actin polyclonal antibody, anti-acetyl-lysine antibody and protein A/G bases were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Cbl-b, Anti-p-p38MAPK, Anti-CBP, Anti-Ku70 and Anti-Bax antibody was purchased from Upstate Biotechnology Incorporated (Lake Placid, NY, USA). Rabbit anti-COX4 polyclonal antibody was purchased from Gene Tex (Alton Pkwy Irvine, CA, USA). Lipofectamine 2000 was purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA). RIPA was purchased from Bioteke Corporation (Beijing, PR China). BCA™ protein assay kit, BlueRanger® prestained protein molecular weight marker mix, and Super-Signal® West Pico chemiluminescent substrate were purchased from Pierce Chemical Company (Rockford, IL, USA). Insulin was from Novo Nordisk (Copenhagen, Denmark) and sodium selenite was purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA).

**Cell Apoptosis Analysis**

Cells were plated on 6-well plates. After treatment, cells were harvested and stained with Annexin V-FITC and propidium Iodide (PI) for 10 min at room temperature in the dark. The cell apoptosis was measured by flow cytometry.

**Western Blotting**

The total protein were extracted using RIPA agents. Plasma and Mitochondria proteins were extracted using Cytoplasmic Protein Extraction Kit and Cell Mitochondria Isolation Kit. Protein concentrations of the lysates were determined by the Bradford protein assay system. Equal amounts of protein were separated by SDS-PAGE, transferred onto polyvinylidene fluoride membranes. After nonspecific sites had been blocked with 5% milk-Tris-buffered saline Tween-20 (TBST), membranes were incubated with Anti-Cbl-b, Anti-p-p38MAPK, Anti-CBP and Anti-Bax antibody, anti-β-actin and anti-COX4 antibody overnight at 4°C, respectively. After membranes had been washed in TBST, the membrane was incubated with corresponding secondary antibody conjugated with horseradish peroxidase for 1 h. The blots were developed using an enhanced chemiluminescence Western blotting detection system.

**Immunoprecipitation**

Proteins were extracted from H9c2 cardiomyocytes and, then, the whole lysate was precleared with protein A/G sepharose beads at 4°C for 60 min. The cleared supernatant was incubated overnight at 4°C with the indicated antibody followed by incubation for 2 h at 4°C with protein A/G sepharose beads. Finally, the beads were washed five times, eluted by boiling in sample buffer for SDS-PAGE, and further immunoblotting analysis was conducted with the indicated antibody.

**Statistical Analysis**

Values were calculated as mean ±SD. One-way ANOVA was used to determine general differences, followed by Tukey HSD test for the difference between groups, using SPSS19.0 (SPSS Inc., Chicago, IL, USA). Significance was considered at $p < 0.05$.

**Results**

**Effect of insulin and selenium in combination on myocardial apoptosis**

To investigate effect of insulin and selenium in combination on cell apoptosis. H9c2 cardiomyocytes were treated with HG/Pal, HG/Pal+In/Se. Flow cytometry was performed to measure apoptosis. Our results found that the increased apoptosis was observed in HG/Pal group ($p < 0.01$). However, the increased apoptosis was significantly suppressed by exposure to insulin and selenium in combination ($p < 0.01$) (Figure 1).

To assess the role of Cbl-b in myocardial apoptosis. Cbl-bsiRNA was used to modulate expression of Cbl-b in cardiomyocytes. H9c2 cardiomyocytes were transfected with siRNA-Cbl-b and then harvested by HG/Pal and HG/Pal+In/Se. Cell apoptosis was measured by flow cytometry. Cbl-b gene knockdown led to the increased apoptosis of cells relative to the two in the combination group ($p < 0.01$) (Figure 1).
Effect of insulin and selenium in combination on the levels of Cbl-b, p-p38MAPK and CBP

Our results suggest that insulin and selenium in combination were protective against myocardial apoptosis but how this occurs is unclear. Therefore, we measured Cbl-b, p-p38MAPK and CBP expressions in different groups. The results showed that the level of Cbl-b was significantly lower in HG/Pal group than in the control group, while the levels of p-p38MAPK and CBP were significantly elevated in HG/Pal group than in the control group ($p < 0.01$). However, the decreased Cbl-b expression and the increased p-p38MAPK and CBP expressions were significantly restored by exposure to insulin and selenium in the combination ($p < 0.01$) (Figure 2).
Although we found that insulin and selenium in combination significantly down-regulated the levels of p-p38MAPK and CBP, whether this regulation was mediated by Cbl-b remains unclear. Therefore, Cbl-b siRNA were used to modulate expression of Cbl-b and then harvested by HG/Pal and HG/Pal+In/Se. The relative proteins in H9c2 cardiomyocytes by immunoblotting. As shown in Figure 2, Cbl-b gene knockdown led to the increased expression of p-p38MAPK and CBP relative to the two in the combination group ($p < 0.05$).

**Effect of insulin and selenium in combination on the levels of acetylated Ku70**

To examine the effect of insulin in combination with selenium on Ku70 acetylation in H9c2 cardiomyocytes. Immunoprecipitation assay was used to detect the acetylation status of Ku70. The results showed that acetylated Ku70 was significantly elevated in HG/Pal groups than in the control group ($p < 0.01$). The increased expression of acetylated Ku70 was significantly restored by exposure to insulin and selenium in the combination ($p < 0.01$) (3).

Whether insulin and selenium in combination significantly down-regulated the level of acetylated Ku70 was mediated by Cbl-b. Cbl-b siRNA were used to modulate expression of Cbl-b and then harvested by HG/Pal and HG/Pal+In/Se. As shown in Figure 3, Cbl-b gene knockdown led to the increased expression of acetylated Ku70 relative to the two in combination group ($p < 0.01$).

**Effect of insulin and selenium in combination on the level of Bax in Cytoplasm and mitochondria**

The expression of Bax of cytoplasm and mitochondria in different groups was investigated by immunoblotting. As shown in Figure 4, expression of Bax of cytoplasm was significantly down-regulated and expression of Bax of mitochondria was significantly up-regulated in HG/Pal groups ($p < 0.01$). The treatment with insulin and selenium in combination remarkably prevented Bax translocation ($p < 0.01$).

To illustrate the role of Cbl-b on Bax translocation. Cbl-b siRNA were used to modulate expression of Cbl-b and then harvested by HG/Pal and HG/Pal+In/Se. The relative proteins in H9c2 cardiomyocytes by immunoblotting. As shown in Figure 4, Cbl-b gene knockdown promoted Bax translocation relative to the two in the combination group ($p < 0.05$).

**Discussion**

Diabetic cardiomyopathy, independent of coronary artery disease and hypertension, is defined as left ventricular dysfunction, and it is well

![Figure 3. The protein levels of acetylated Ku70 in different groups. The protein lysates were immunoprecipitated with anti-acetylated lysine antibody. The resulting protein complexes were then immunoblotted for determining Ku70. Data are shown as means ± SD. **p<0.01 versus control group; △△p<0.01 versus HG/Pal group; *** p<0.01 versus HG/Pal+In/Se group.](image-url)
Insulin in combination with selenium inhibits HG/Pal-induced cardiomyocyte apoptosis

established as one of the major causes of heart failure in diabetic patients. The pathogenesis of diabetic cardiomyopathy is complex and is chiefly thought to arise from diabetes-induced apoptosis and oxidative stress. Dead cardiac cells are replaced by an extracellular matrix which impairs myocardial contractility, increases interstitial fibrosis, and leads to cardiac remodeling and dysfunction. Therefore, to prevent diabetic cardiomyopathy, an ideal therapy may simultaneously suppress oxidative stress and apoptosis.

Our previous work suggests that insulin and selenium in combination synergistically decrease blood glucose and improve myocardial collagen remodeling and cardiac function in diabetic rats. However, whether insulin and selenium in combination also inhibit cardiomyocyte apoptosis remains unclear. If so, what is the mechanism behind the two in combination-suppression of cardiomyocyte apoptosis?

To confirm that insulin and selenium in combination are protective against cardiomyocyte apoptosis, we firstly observed the effect of the two in combination on cell apoptosis in HG/Pal-cultured H9c2 cardiomyocytes. The HG/Pal group showed higher apoptosis compared with that in control groups. The two in combination strongly reduced cardiomyocyte apoptosis, whereas Cbl-b knockdown strongly suppressed In/Se-induced anti-apoptotic effect in HG/Pal-treated cardiomyocytes.

p38MAPK is one of the mitogen-activated protein kinase (MAPK) family members that regulates a variety of biological responses, including apoptosis, proliferation, and differentiation. Stimulation of p38MAPK results in activation of transcription factors cAMP-responsive element-binding protein (CREB), which results in the recruitment of ubiquitous co-activators cAMP response element-binding-protein-binding protein (CBP) to promote gene transcription. CBP, a transcriptional co-activator and an acetyltransferase, acetylates Ku70 in various cells. To date, one described function of cytoplasmic Ku70 is to bind Bax, an apoptotic protein, thereby blocking Bax-mediated cell death. The binding between Ku70 and Bax is regulated by Ku70 acetylation. Ku70 acetylation results in Bax release that triggers Bax-dependent cell death. Therefore, suppression of p38MAPK/CBP/Ku70 pathway activity contributes to inhibit apoptosis.

Cbl-b proteins are multifunctional adaptor molecules that modulate cellular activity by targeting the ubiquitylating system, endocytic complexes, and other effectors to a wide variety of regulatory proteins. Cbl-b negatively regulated phosphorylation of p38MAPK in various cells. Next, we mainly focused on evaluating the effect of insulin in combination with selenium on the expression of Cbl-b, p-p38MAPK, CBP, acetylated Ku70 and Bax. The results showed that the level of Cbl-b significantly decreased,
the levels of p-p38MAPK, CBP and acetylated Ku70 significantly increased and Bax translocated from the cytosol into mitochondria in HG/Pal group, suggesting the activation of p38MAPK/CBP/Ku70 pathway in HG/Pal-cultured cardiomyocytes. Insulin in combination with selenium up-regulated Cbl-b expression and down-regulated p38MAPK, CBP and acetylated Ku70 expressions and prevented Bax translocation, whereas Cbl-b knockdown strongly suppressed In/Se-induced these effects in HG/Pal-treated cardiomyocytes. Therefore, we concluded that insulin in combination with selenium prevented cardiomyocyte apoptosis likely via Cbl-b regulating p38MAPK/CBP/Ku70 pathway.

Conclusions

Insulin and selenium in combination reduced cell apoptosis by increasing Cbl-b expression and inhibiting the activation of p38MAPK/CBP/Ku70 pathway, whereas siRNA-mediated silencing of Cbl-b suppressed In/Se-induced these effects in HG/Pal-treated cardiomyocytes. Therefore, Cbl-b is associated with insulin and selenium synergistic anti-cardiomyocyte apoptosis, suggesting that Cbl-b may be a potential target for drug therapy of diabetic cardiomyopathy in future.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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