

Polyphenol epigallocatechin-3-gallate alleviates high glucose-induced H9C2 cell damage through PI3K/Akt pathway

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Abstract. – OBJECTIVE: The aim of this work was to study the protective effect of polyphenol epigallocatechin-3-gallate (EGCG) on high glucose-induced oxidative damage of H9C2 cells and to investigate the relationship between this effect and phosphatidyl inositol 3 kinase-serine/threonine kinase (PI3K/Akt) signal transduction pathway.

MATERIALS AND METHODS: H9C2 cells were used as objects of study, 350 mM glucose serum-free medium was used as the high glucose molding condition, and LY294002 (10 μ M) was used as the PI3K/Akt inhibitor. 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay was used to detect the cell viability, lactate dehydrogenase (LDH) assay was used to detect the cytotoxicity, flow cytometry was used to detect the proportion of cell apoptosis, and Western blotting was used to detect the expressions of cell-associated proteins.

RESULTS: Cell viability was reduced and cell apoptosis was increased by 350 mM high glucose. The high glucose-induced apoptosis was alleviated and the Akt expression in cells was increased by EGCG. The protective effect of EGCG was reduced after inhibition of PI3K/Akt pathway.

CONCLUSIONS: EGCG protects H9C2 cells from high glucose-induced damage. EGCG plays the protective effect through inducing the PI3K/Akt pathway activation.

Key Words:

EGCG, High glucose, H9C2 cells, PI3K/Akt, Apoptosis.

Introduction

It is well known that diabetes mellitus will increase the risk and mortality of cardiovascular diseases^{1,2}. Compared with non-diabetic patients, diabetic patients are more likely to die from myocardial infarction³ and there is a higher prevalence of heart failure complications in the

traditional treatment for diabetic patients after myocardial infarction⁴. Diabetes will eventually affect the myocardium, leading to diabetic cardiomyopathy (DC)⁵. Sustained high glucose stress can make myocardial cells in excessive oxidative stress state⁶, and the excessive oxidative stress can activate many pro-apoptotic signaling pathways⁷, eventually leading to myocardial cell apoptosis and cardiac function injury⁸. It has been proved that the expression of phosphatidyl inositol 3 kinase-serine/threonine kinase (PI3K/Akt) plays an important regulatory role in myocardial cell injury caused by oxidative stress⁹. The study found that the sustained activation of Akt can activate downstream anti-apoptotic signaling pathways in the cell oxidative damage, thereby preventing the oxidative stress stimulating factor-induced apoptosis¹⁰. It was found in the study on H9C2 cells that the number of apoptotic myocardial cells was increased with the decrease of P-Akt level¹¹. Some studies have also suggested that the decreased P-Akt protein expression may be involved in insulin resistance¹². Also, it was found in the study on myocardial cells in neonatal rats that the high glucose stimulation can reduce Akt activity and lead to more apoptotic cells¹³. High glucose can also promote the myocardial cell apoptosis and insulin resistance of adult rats through reducing the P-Akt activity⁹, reflecting the close relationship between PI3K/Akt pathway and high glucose damage of myocardial cells. Polyphenol epigallocatechin-3-gallate (EGCG) is an active ingredient extracted from green tea, which can increase the insulin activity and glucose tolerance¹⁴. Researches have shown that EGCG can effectively improve the glucose tolerance level in rats with type 2 diabetes¹⁵. In addition, EGCG has a protective effect on the cardiovascular system, and studies have shown that drinking green tea can significantly

reduce the prevalence of cardiovascular events¹⁶. However, there is still no further study on whether EGCG has a protective effect on high glucose-induced myocardial damage. The primary purpose of this study was to investigate the protective effect of polyphenol epigallocatechin-3-gallate (EGCG) on high glucose-induced oxidative damage of H9C2 cells and the relationship between this effect and PI3K/Akt signal transduction pathway.

Materials and Methods

Cells and Experimental Materials

H9C2 cells were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). DMEM (Dulbecco's Modified Eagle Medium), 350 mM glucose medium and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA), PI3K/Akt inhibitor (LY294002, LY) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and antibodies used for experiments were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other reagents belonged to purified and analytical grade.

Cell Culture and Modeling

The cell suspension was transferred to a 10 mL centrifuge tube and 5 mL DMEM containing 10% FBS was added. After the cell suspension was centrifuged at 1000 rpm for 10 min, the supernatant was discarded, and the appropriate amount of DMEM containing 10% FBS was added. The cell density was adjusted to 1×10^5 /mL and cells were inoculated into a 25 mL culture flask and cultured in an incubator containing 5% CO₂ at 37°C, and the medium was replaced after 24 h.

Cells in the logarithmic growth phase were cultured in an incubator containing 5% CO₂ at 37°C using DMEM containing 10% FBS for 24 h. Cells were treated with FBS-free DMEM (containing 350 mM glucose) for 48 h as the high-glucose damage model. In EGCG treatment groups, EGCG with the final concentration of 5 μM or 10 μM was added into the FBS-free DMEM (containing 350 mM glucose). LY294002 with the final concentration of 10 μM was added into the PI3K/Akt inhibition group.

CCK8

Cell Counting Kit 8 (Dojindo, Kumamoto, Japan) was used to detect the cell viability. Cells in the logarithmic growth phase were digested using

0.25% trypsin ethylene diamine tetra acetic acid (EDTA). After centrifugation, the supernatant was discarded, and cells were resuspended in DMEM containing 10% FBS. Cells were counted and diluted to 1.0×10^5 cells/mL, and inoculated onto a 96-well plate. Cells were divided into 5 groups: blank group, control group (10 μM EGCG), high glucose treatment group, high glucose treatment + 5 μM EGCG group and high glucose treatment + 10 μM EGCG group. 3 control wells were set up for each group. After the treatment, 10 μL CCK8 solution was added to each well of a 96-well plate and incubated in an incubator containing 5% CO₂ at 37°C for 4 h. The absorbance at 450 nm was measured using the microplate reader.

LDH Detection

Cytotoxicity was detected via lactate dehydrogenase (LDH) (Beyotime, Shanghai, China). Cells in the logarithmic growth phase were digested using 0.25% trypsin ethylene diamine tetra acetic acid (EDTA) and resuspended in DMEM containing 10% FBS. Cells were counted and diluted to 1.0×10^5 cells/mL, and inoculated onto a 96-well plate (5.0×10^3 cells/mL). Cells were divided into 5 groups: blank group, control group (10 μM EGCG), high glucose treatment group, high glucose treatment + 5 μM EGCG group and high glucose treatment + 10 μM EGCG group. 3 control wells were set up for each group. After the intervention, the supernatant was taken (20 μL/well) and the corresponding reagents were added according to the instructions of kit; the solution was placed at room temperature for 3 min, followed by zero setting via 440 nm double distilled water and 1 cm optical path cuvette. The OD value of each tube was measured via the microplate reader. Data statistics were performed according to the ratio of each group to blank group.

Flow Cytometry Detection

The proportion of apoptotic cells was detected via flow cytometry (Partec AG, Arlesheim, Switzerland). Cells in the logarithmic growth phase were taken for experimental intervention. Then, cells were digested and washed, and prepared into the single-cell suspension. The cell density was adjusted into $(1-5) \times 10^5$ cells/mL; 500 μL Binding Buffer was added to suspend cells and mixed gently. 5 μL Annexin-V-FITC and 5 μL PI were added to incubate cells in a dark place at room temperature for 15 min, followed by machine detection within 1 h; the experimental results were analyzed using BD Cell Quest software.

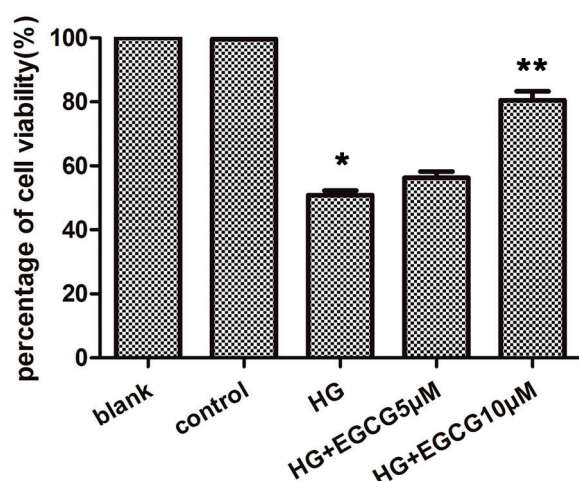


Figure 1. Detection of cell viability via CCK8. The cell viability in blank group, control group (10 μ M EGCG), HG group, HG + 5 μ M EGCG group and HG + 10 μ M EGCG group is detected via CCK8. * Compared with blank group, $p < 0.05$; ** compared with HG group, $p < 0.05$.

Western Blotting

Cells in each group were taken and washed twice with D-Hank's, which was sucked clean using absorbent paper. 150 μ L pre-cooled lysis buffer was added to each group to lyse cells on ice for 30 min. Next, the protein in each group was collected into an Eppendorf (EP) tube (Hamburg, Germany) using cell scraper, and centrifuged at 12000 rpm at 4°C; the supernatant was taken and transferred to a new EP tube. The protein concentration was detected using bicinchoninic acid (BCA) method, 5 $\times$ loading buffer was added and the protein was heated at 100°C for 6 min. 30 μ L protein was added to the loading holes of prepared separation gel and spacer gel, followed by electrophoresis in the electrophoretic buffer under a suitable voltage. After electrophoresis, the gel was adhered to polyvinylidene fluoride (PVDF) membrane, followed by membrane transfer in transfer buffer at 0°C under the constant voltage of 100 V for 60 min. The PVDF membrane was sealed at room temperature in 5% skim milk powder for 1 h; it was cut according to the molecular weight and sealed in the primary antibody at 4°C overnight. The next day, the PVDF membrane was rinsed with tris buffered saline-tween (TBST) and the secondary antibody anti-IgG (1:5000) was added for incubation for 1 h at room temperature. After incubation, the PVDF membrane was rinsed again with TBST, followed by color development using Tannon 5200 fluorescence immunoassay development system and gray scale measurement.

Statistical Analysis

The results were presented as mean $\pm$ variance, and SPSS 16.0 (Version X; IBM, Armonk, NY, USA) software was used for statistical processing and analysis of data. The measurement results received the homogeneity test of variance. Comparison between groups was done using One-way ANOVA test followed by LSD (Least Significant Difference). $p < 0.05$ suggested that the difference was statistically significant.

Results

EGCG Reduced the Effect of high Glucose on H9C2 Cell Viability

In this study, changes in H9C2 cell viability were measured using CCK8 method. Cells were divided into blank group, control group (10 μ M EGCG), high glucose group, high glucose + 5 μ M EGCG group and high glucose + 10 μ M EGCG group. The results showed that there was no significant difference between blank group and control group, and the cell viability in high glucose group was decreased significantly to $53.62 \pm 2.28\%$ compared with that in blank group ($p < 0.05$). In the low-concentration EGCG (5 μ M) group, there was no significant effect on H9C2 cell viability, and EGCG showed a significantly protective effect on cell viability in the high-concentration (10 μ M) group and the cell viability was increased to $82.6 \pm 2.1\%$ compared with that in high glucose group ($p < 0.05$) (Figure 1).

EGCG had a Protective Effect on high Glucose-Induced H9C2 Cell Damage

LDH release was used as an index of measuring cell injury. There was no significant difference in LDH release between blank group and control group. After treatment with high glucose, the LDH release amount was increased significantly to 4.3 ± 0.2 times of that in blank group ($p < 0.05$). After treatment with 10 μ M EGCG, compared with that in high glucose group, LDH release amount was decreased by 1.63 ± 0.4 times ($p < 0.05$). There was no significant abnormality in 5 μ M group compared with H/R group (Figure 2). So EGCG with the concentration of 10 μ M was selected for the following experiments.

EGCG Reduced high Glucose-Induced H9C2 Cell Apoptosis

Flow cytometry was used to detect the proportion of apoptotic cells. Compared with those in

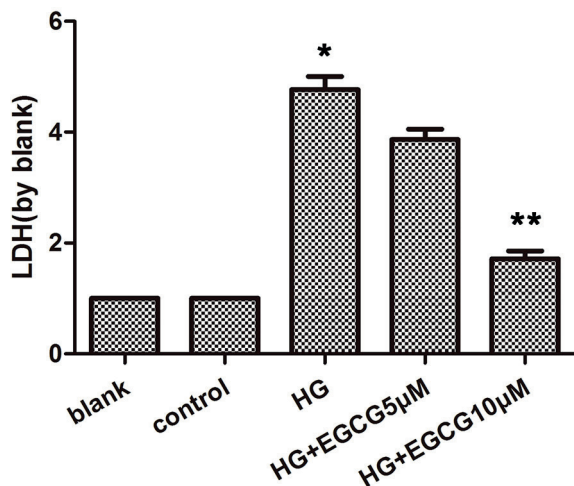


Figure 2. Detection of cytotoxicity via LDH. The LDH release concentration in blank group, control group (10 μ M EGCG), HG group, HG + 5 μ M EGCG group and HG + 10 μ M EGCG group is detected. * Compared with blank group, $p < 0.05$; ** compared with HG group, $p < 0.05$.

control group, the proportions of early apoptotic cells and late apoptotic cells had no significant differences. After high glucose treatment, the proportion of early apoptotic cells was increased to $42.4 \pm 2.2\%$, while that of late apoptotic cells was increased to $5.6 \pm 0.9\%$, and there were statistically significant differences compared with those in blank group ($p < 0.05$). The proportion of apoptotic cells in high glucose + EGCG (10 μ M) group was significantly decreased compared with that in high glucose group ($p < 0.05$); the proportion of early apoptotic cells was decreased to $15.6 \pm 1.7\%$ and that of late apoptotic cells was $3.4 \pm 0.4\%$ (Figure 3). The results of Western blotting showed that the expression of apoptosis-related protein, Cleaved-Caspase 3, in high glucose treatment group was significantly increased compared with that in blank group ($p < 0.05$), while that in high glucose + EGCG group was significantly decreased compared with that in high glucose group ($p < 0.05$) (Figure 4).

PI3K/Akt Inhibitor Inhibited the Effect of EGCG on Reducing Apoptosis

The flow cytometry showed that the proportion of apoptotic cells in high glucose + LY294002 (10 μ M) group was similar to that in high glucose group without significant difference; after LY294002 (10 μ M) was added into high glucose + EGCG group, the proportion of apoptotic cells was significantly increased compared with that

in high glucose + EGCG group ($p < 0.05$); the proportion of early apoptosis was increased to $40.3 \pm 2.5\%$, while that of late apoptosis was increased to $7.1 \pm 1.5\%$ (Figure 3). Western blotting showed that the expression of apoptosis-related protein, Cleaved-Caspase 3, in high glucose + LY294002 group was not significantly different from that in high glucose group; after LY294002 was added into high glucose + EGCG group, the expression of Cleaved-Caspase 3 in cells was significantly increased ($p < 0.05$) (Figure 4).

EGCG Induced Akt activation in H9C2 Cells

The expression of activated Akt (p-Akt) in cells in each group was detected via Western blotting. The results showed that there was no significant difference in the expression of p-Akt between blank group and control group. The expression of p-Akt in high glucose treatment group was increased compared with that in blank group, but there was no statistically significant difference. The expression of p-Akt in high glucose + LY294002 group was increased significantly compared with that in high glucose group ($p < 0.05$); after LY294002 was added, the Akt excitation was inhibited and the expression in high glucose + LY294002 group was decreased; the expression of p-Akt in high glucose + EGCG + LY294002 group was significantly decreased compared with that in high glucose + EGCG group ($p < 0.05$) (Figure 5).

Discussion

This study found that 10 μ M EGCG significantly increased the survival rate of high glucose-induced H9C2 cells and decreased LDH activity, proportion of cell apoptosis and apoptosis-related protein expression, indicating that EGCG has the effect of anti-high glucose-induced H9C2 cell damage and can significantly up-regulate the expression level of p-Akt in H9C2 cells with high glucose damage. PI3K/Akt signaling pathway inhibitor LY294002 could eliminate the cytoprotective effect of EGCG and increase the proportion of H9C2 cell apoptosis and apoptosis-related protein expression, suggesting that EGCG plays its cytoprotective effect through PI3K/Akt signaling pathway.

Diabetes mellitus is a kind of metabolic disease characterized by elevated blood glucose caused by the absolute or relative deficiency of insulin secretion¹⁷. DC is currently one of the

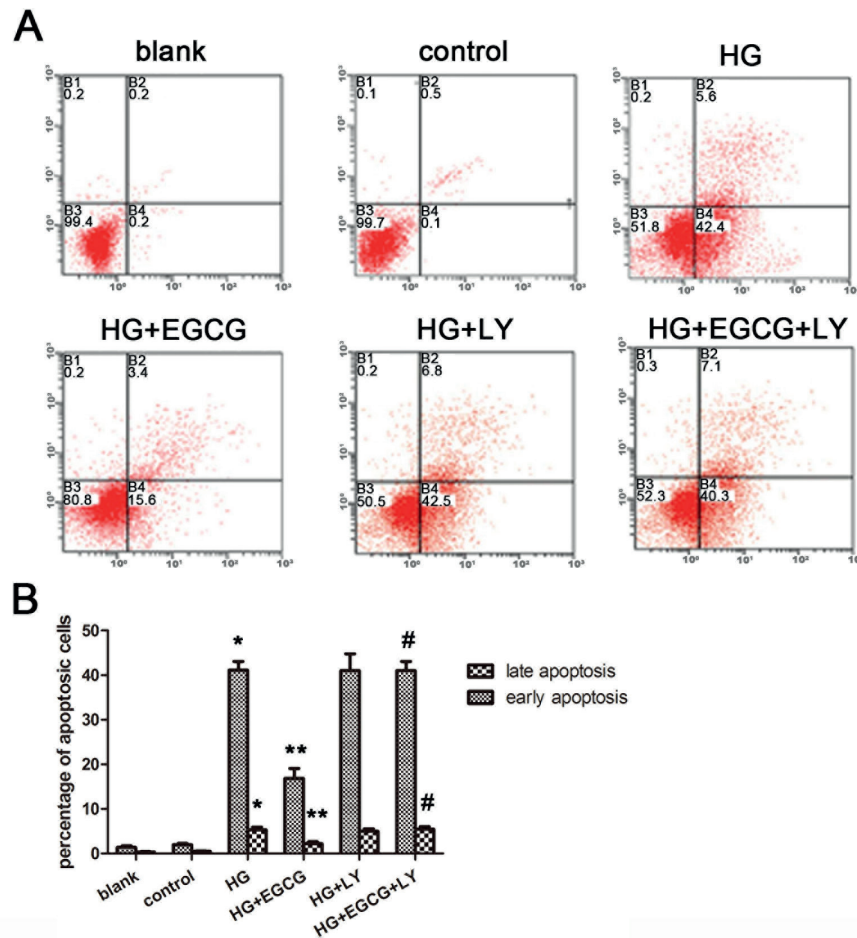


Figure 3. Detection of cell apoptosis via flow cytometry. **(A)** The cell apoptosis in blank group, control group (10 μ M EGCG), HG group, HG + EGCG group, HG + LY294002 (HG+LY) group, and HG + EGCG + LY294002 (HG+EGCG+LY) group is detected via flow cytometry; **(B)** Data statistics of proportion of cell apoptosis. *Compared with blank group, $p < 0.05$; **compared with HG group, $p < 0.05$; # compared with HG+ EGCG group, $p < 0.05$.

important complications of diabetes¹⁸. The study suggests that the sustained high glucose stress in the DM state can lead to the production of a large number of reactive oxygen species, thus inducing oxidative stress¹⁹, causing abnormal gene expression, changing signal transduction and activating programmed cell death pathways²⁰. The resulting myocardial cell loss and cardiac dysfunction play important roles in the development of DC²¹. In this study, high glucose was used to induce the oxidative damage of H9C2 cells to stimulate the process of oxidative damage of myocardial cells caused by sustained high glucose level in DM state, and the model was established successfully.

At present, most studies have suggested that the early DC is mainly caused by the mitochondrial damage, abnormal cell energy metabolism, lipid toxicity, calcium dyshomeostasis and apop-

toxis, etc²². Recently, Lorenzo et al²³ have proposed the “oxidative stress theory” which is of great significance in initiating and promoting the development of DM complications. Studies have suggested that oxidative stress is involved in the development and progression of DC and there is growing evidence that oxidative stress participates in the development and progression of DC²⁴.

EGCG, as a kind of green tea extract, mainly has the effects of reducing the production of reactive oxygen and inhibits the oxidative stress response²⁵. Studies have confirmed that EGCG has a definite effect of reducing blood glucose and improving the symptoms of DM^{15,26}, but there is little research on whether EGCG has the cardio protective effect in DC.

In this study, it was found that EGCG had a definite protective effect on the high glucose-in-

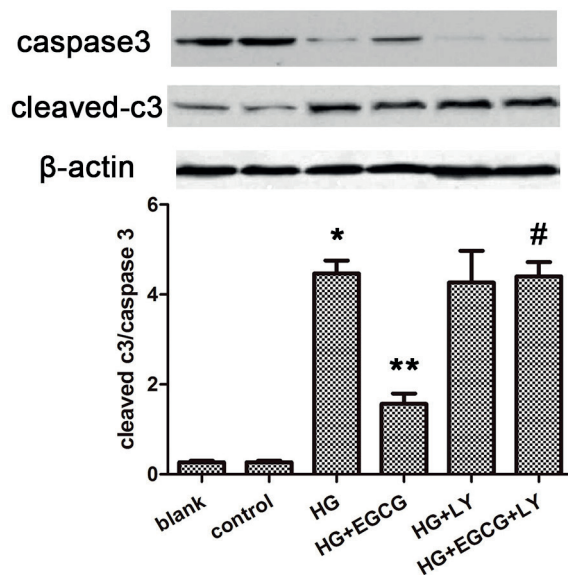


Figure 4. Detection of apoptotic proteins via Western blotting. The expressions of apoptosis-related proteins Caspase 3 and Cleaved-Caspase 3 in blank group, control group (10 μ M EGCG), HG group, HG + EGCG group, HG + LY294002 (HG+LY) group, and HG + EGCG + LY294002 (HG+EGCG+LY) group are detected via Western blotting. *Compared with blank group, $p < 0.05$; **compared with HG group, $p < 0.05$; # compared with HG+ EGCG group, $p < 0.05$.

duced H9C2 cell damage. Flow cytometry showed that EGCG could reduce the proportion of H9C2 cell apoptosis; the detection of apoptosis-related protein Caspase 3 and cleaved-Caspase 3 showed that EGCG reduced the expressions of apoptosis-related proteins. To further study the molecular pathway of anti-apoptotic effect of EGCG, the expression of activated Akt in cells was detected in this work, and it was found that EGCG could effectively enhance the Akt activation in H9C2 cells under high glucose damage. The regulatory effect of EGCG on Akt activation in cells was blocked after the PI3K/Akt pathway inhibitor LY294002 was used, and the proportion of cell apoptosis and the expressions of apoptosis-related proteins were significantly increased. This proves that EGCG protects H9C2 cells from apoptosis under high glucose damage through regulating the Akt activation.

Conclusions

In the H9C2 cell oxidative damage model prepared by 350 mmol/L glucose for 48 h, the cell survival rate was decreased moderately, the reproducibility was good and the model was

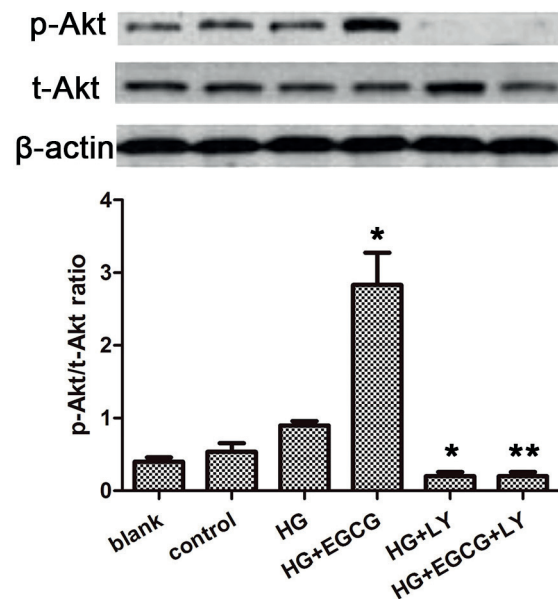


Figure 5. Detection of Akt protein via Western blotting. The Akt protein activation levels in blank group, control group (10 μ M EGCG), HG group, HG + EGCG group, HG + LY294002 (HG+LY) group, and HG + EGCG + LY294002 (HG+EGCG+LY) group are detected via Western blotting. *Compared with blank group, $p < 0.05$; **compared with HG group, $p < 0.05$.

established successfully. 10 μ M EGCG can improve the survival rate of H9C2 cells, reduce the LDH release and decrease the proportion of cell apoptosis, thus playing the effect of anti-high glucose-induced H9C2 cell damage. EGCG can up-regulate the p-Akt protein expression level in H9C2 cells induced by high glucose and the cytoprotective effect of EGCG can be blocked by PI3K/Akt inhibitor LY294002, suggesting that EGCG plays the effect of anti-high glucose-induced H9C2 cell oxidative damage through PI3K/Akt signaling pathway.

Conflict of Interest

The authors declare no conflicts of interest.

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