

Original Article

Insulin-like growth factor-1 protects ischemic myocardial cells via PI3K/AKT pathway

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Abstract: Ischemic heart disease (IHD) is the primary cause of death because of aging population in the world, which is characterized by reduced blood or oxygen supply to the heart. This study was aimed to indicate the biological process of Insulin-like growth factor 1 (IGF-1) on the IHD, as well as the functional role. Cell viability was determined by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT). Apoptosis cells were performed by flow cytometry. Reactive oxygen species (ROS) levels were determined with 2,7-dichlorofluorescein diacetate (DCFH-DA) by flow cytometry. Western blot was used to analyze the protein expression of cytochrome C, B-cell lymphoma-2 (Bcl-2), Phosphatidylinositol 3-kinase (PI3K)/AKT signaling after treatment with IGF-1 or IGF-1 receptor (IGF-1R) inhibitor. A model of ischemic and starved model was successfully induced in cardiac myoblast H9c2 cell line. An appropriate concentration of IGF-1 (50 ng/ml) was screened in ischemic H9c2 cells. Administration of IGF-1 statistically increased the cell viability ($P < 0.05$) compared to the control group, but decreased the cell apoptosis and ROS ($P < 0.05$). These effects were reversed by IGF-1R inhibitor. Besides, we demonstrated IGF-1 dramatically decreased cytochrome C release ($P < 0.05$) and up-regulated the Bcl-2 protein expression ($P < 0.05$), with p-AKT 308, p-AKT473, p-ERK, and p-S6 protein expression was increased ($P < 0.05$). Our studies indicate IGF-1 could promote cell viability, and inhibit cell apoptosis and ROS level by regulating PI3K/AKT signaling pathway.

Keywords: Ischemic heart disease (IHD), insulin-like growth factor 1 (IGF-1), apoptosis, reactive oxygen species (ROS), cytochrome C, phosphatidylinositol 3-kinase (PI3K)/AKT

Introduction

Presently, ischemic heart disease (IHD) is the main reason of threatening human health [1]. Myocardial ischemia implicates simultaneous nutrient and oxygen deprivation, which is characterized by reduced blood supply to the heart, and leads to sudden cardiac arrest. In the absence of hypoxia, the cell activates biological processes to help the ischemic cells combine with the oxygen. As a result of the starvation, the biological process of cell apoptosis would be activated to ensure its survival and recover the myocardial ischemia [2]. Therefore, it is essential to explore the mechanisms of preventing the ischemia at the cellular level to supply the target treatments.

Insulin-like growth factor 1 (IGF-1) belongs to the receptor tyrosine kinase (RKT) family, which

is activated by the plasma membrane IGF-1 receptor (IGF-1R). The IGF-1R expression has been detected in human heart, endothelial cells, fibroblasts, and red blood cells [3]. IGF-1 deficiency has been described in patients with liver cirrhosis, intrauterine growth restriction, and age-related cardiovascular disease [4]. The IGF-1 levels are negatively correlated with the risk of developing IHD [5]. A low level of IGF-1 has been used as a affirm indicator for IHD [6]. For the clinical outcomes of ischemic disease, IGF-1 represents a valuable tool as a predictor [7].

However, it is still unclear about the function of IGF-1 at the cellular of IHD [8, 9]. In this paper, a model of myocardium starvation and ischemia in myoblast cell line H9c2 was induced. We analyzed IGF-1 could promote the ischemic cells viability, with inhibiting apoptosis and

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reactive oxygen species (ROS) level, via Phosphatidylinositol 3-kinase (PI3K)/AKT signaling.

Materials and methods

Cell culture

The cardiac myoblast cell line H9c2 (Sigma, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, USA) [10], supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA), 1% Penicillin/Streptomycin (100 U/ml: 0.1 mg/ml, Gibco, Carlsbad, CA) and 1% GlutaMAX (Gibco, Carlsbad, CA), at 37°C under 5% CO₂. The dishes were placed in hypoxic pouches with an Indicator (GasPak™ EZ BDBiosciences, USA), by a buffer exchange to an ischemia-mimetic solution, and metabolic ischemia was induced for 2 h. According to the manufacturer's instruction, an atmosphere containing 10% carbon dioxide and 1% oxygen was produced by the Anaerobe Gas Generating Pouch System. IGF-1 and IGF-1R inhibitor was purchased from Takara (Takara, Dalian, China) and was prepared in 1 mM filtered phosphate buffer.

Cell viability

The cell viability was determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) (MTT) assay [11]. The cells were seeded in 96 well-plates, and were added 100 µl suspension in density of 2×10^5 cells/ml in every well, then placed in incubator to culture for various days (1 day, 2 days, 3 days, 4 days). The colorimetric assay was used with 0.5% MTT for 10 µl/well, and incubated for 1.5 h at 37°C. The formazan was dissolved by dimethylsulfoxide (DMSO), and the absorbance value was detected by microplate reader at 492 nm. Each experiment was performed for three times.

Apoptosis assay

Using Annexin V-FITC/ Propidium Iodide (PI) apoptosis detection kit (Shanghai Beyotime Biotechnology, Shanghai, China), flow cytometry analysis was performed to quantify the apoptotic cells. The H9c2 cells were seeded in 6 well-plates for 100,000 cells/well. With cold phosphate buffered saline (PBS), treated cells were washed twice, and resuspended in buffer. Following to the manufacturer's instruction, the

adherent and floating cells were combined and treated, and measured with flow cytometer (BD, USA) to identify apoptotic cells.

Antioxidant assay

Using 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Nanjing Jiancheng, Nanjing, China) which was dissolved in serum-free culture medium, ROS was measured by flow cytometry [12]. The cells were cultured in a 6-well plate in an incubator, then were washed twice with PBS, and co-incubated with 0.1% DCFH-DA in a condition of 37°C for 20 min in dark. After staining, the cells were washed twice with PBS. With a trypsin digestion, cell samples were collected and centrifuged, and then the supernatants were removed. Finally, the fluorescent intensities were detected by a flow cytometer.

Western blot

RIPA lysis buffer and protease inhibitors were purchased from Takara (Takara, Dalian, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody, rabbit-anti-mouse second antibodies and rabbit-anti-mouse primary antibodies including with p/t-AKT 308, p/t-AKT473, p/t-extracellular signal-regulated kinase (ERK), and p/t-S6 were obtained from Univ-bio (Univ-bio, Shanghai, China). The BCA™ Protein Assay Kit (Sigma, USA) was used to quantify the proteins. Following the manufacturer's instruction, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was established. In 5% blocking buffer, primary antibodies were prepared at a dilution of 1:1,000, and incubated with the membrane at 4°C overnight, followed by wash. The secondary antibody was incubated for 1 h at room temperature. After rinsing, the signals were captured, and the intensities were quantified by using Image Lab™ Software (Thermo, USA).

Statistical analysis

All experiments were performed three times. The values were presented as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) analysis was used to calculate the *P*-values. A *P*-value of < 0.05 was considered as a statistically significant result.

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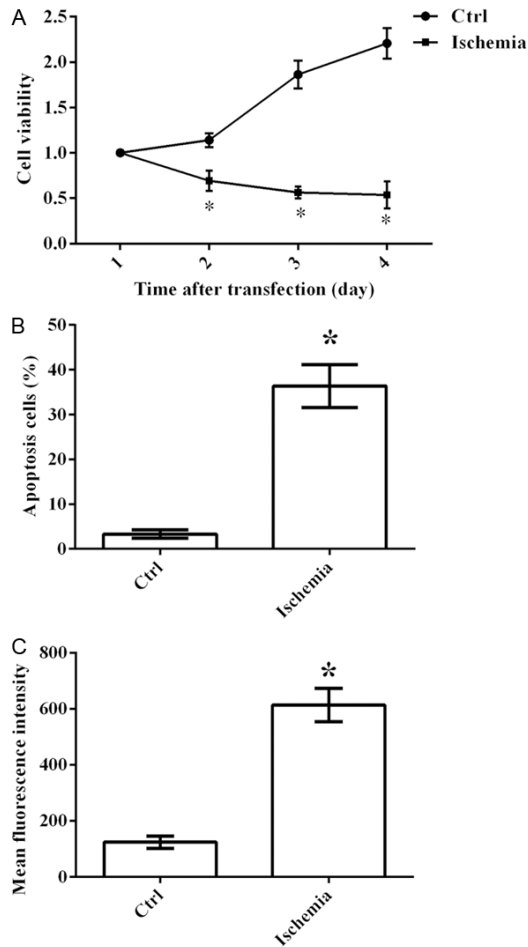


Figure 1. Cells viability, apoptotic cells and ROS assay were analyzed to confirm the H9c2 cell line in a model of myocardium starvation and ischemia. **A.** The cell viability of ischemic cell line was significantly increased compared with the untreated control. **B.** Apoptotic cells were significantly decreased compared with the untreated control. **C.** ROS was analyzed by DCFH-DA. Values are mean \pm SD of three independent experiments. ROS, Reactive oxygen species; DCFH-DA, 2, 7-dichlorofluorescein diacetate; SD, standard deviation. * $P < 0.05$ significant different between control with ischemic cells.

Results

Inducement of myocardium starvation and ischemia model

To confirm whether the model of myocardium starvation and ischemia was successfully induced in the H9c2 cell line, we detected the cell viability, apoptosis, and ROS level. **Figure 1A** had shown that the treated ischemic cell viability was significantly decreased ($P < 0.05$). Furthermore, treated ischemic cell apoptosis was significantly increased compared to the control group (**Figure 1B**, $P < 0.05$), as well as

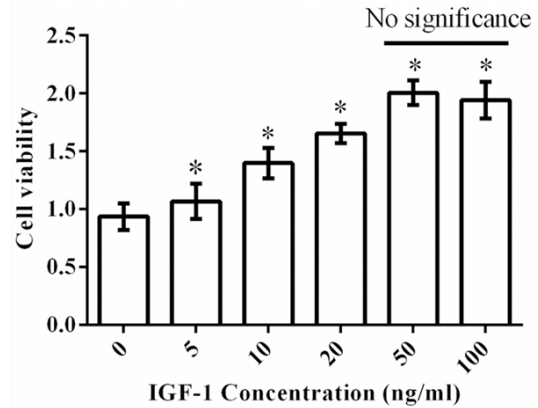


Figure 2. Cell viability was assayed with different IGF-1 concentration (0, 5 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml, and 100 ng/ml), values are the mean \pm SD of three independent experiments. SD, standard deviation; IGF-1, Insulin-like growth factor. * $P < 0.05$ significant different between control and ischemia.

ROS level (**Figure 1C**, $P < 0.05$), so the H9c2 cell line was successfully induced.

Screening of appropriate IGF-1 concentration for treating H9c2

After treatment with different concentrations of IGF-1, the cell viability assay was performed. The results showed that IGF-1 could promote cell viability, which is dependent on the concentration of IGF-1 (**Figure 2**). In 50 ng/ml IGF-1, the cell viability was the highest ($P < 0.05$), so we selected this concentration as the best in subsequent process of experiments.

Effects of IGF-1 and IGF-1R inhibitor on cell viability, apoptosis and ROS

Figure 3A-C had shown that cell viability was increased, accompanying with apoptotic and ROS level decreasing after adding IGF-1. To detect the effects of IGF-1 on apoptosis, the protein expression level of cytochrome C and Bcl-2 were performed. **Figure 3D, 3E** indicated that cytochrome C expression was also decreased ($P < 0.05$), while Bcl-2 expression was significantly increased ($P < 0.05$). However, after adding IGF-1R inhibitor, results showed a significant difference compared with adding IGF-1 ($P < 0.05$). Especially, the protein expression of Bcl-2 had a huge difference ($P < 0.05$).

Effects of IGF-1 and IGF-1R inhibitor on PI3K/AKT signal key factors

The PI3K/AKT pathway is one of the main pathways activated by IGF-1 signal, and is known to

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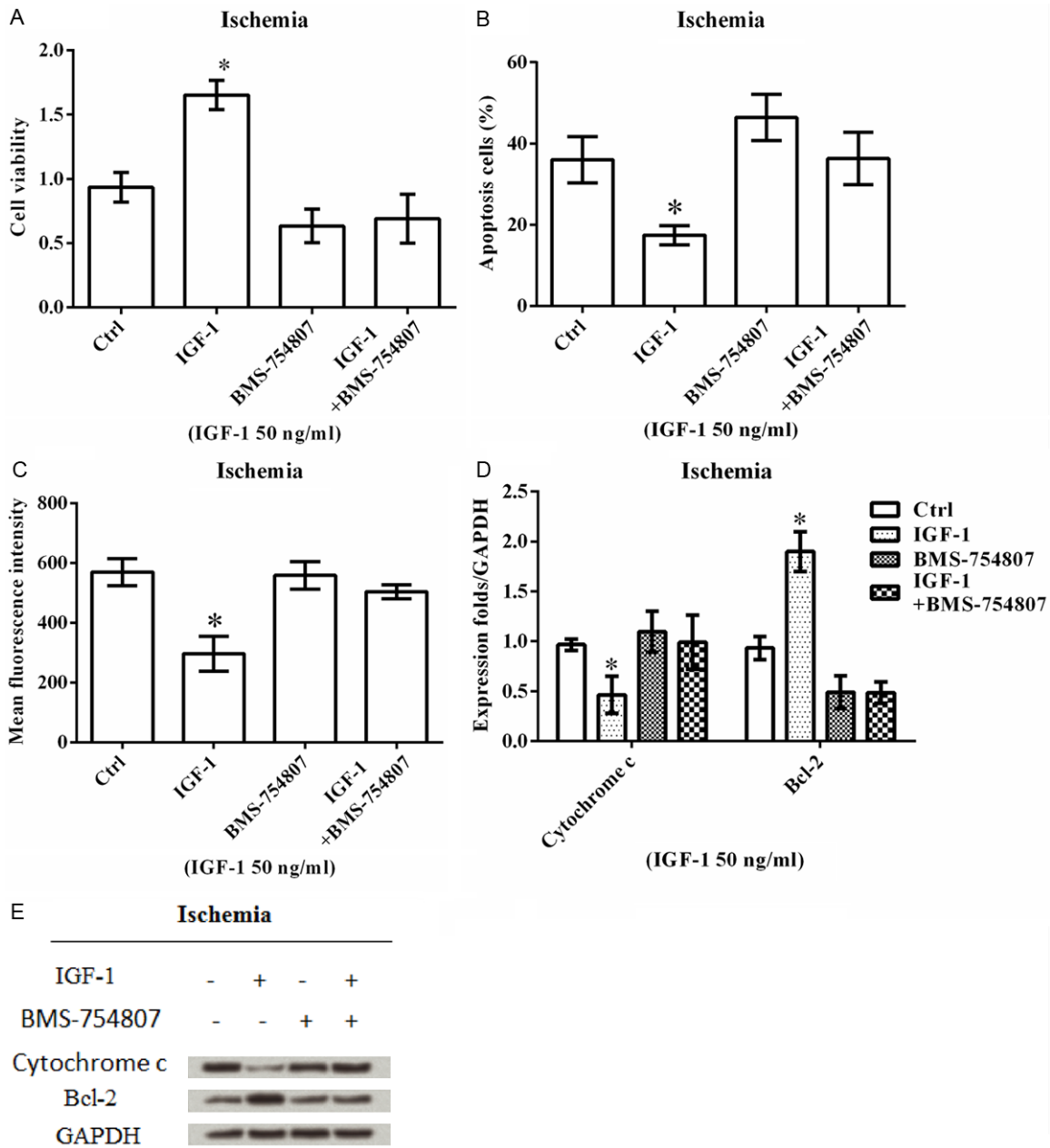
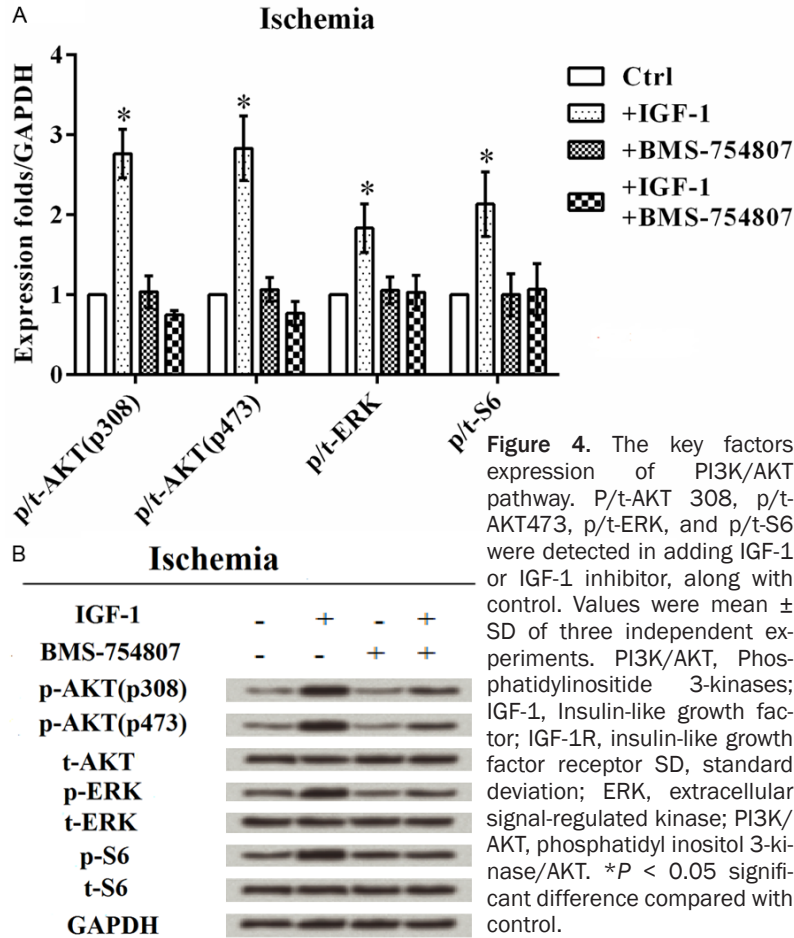


Figure 3. Cell viability, apoptotic cells, ROS level, and the protein expression of apoptosis factor cytochrome C and Bcl-2 were detected after adding IGF-1 and IGF-1R inhibitor to the ischemic cells. A. Cell viability was significantly increased after adding IGF-1, compared with control. B. Apoptotic cells were assayed between adding IGF-1 or IGF-1R inhibitor. C. ROS was analyzed by DCFH-DA after adding IGF-1 or IGF-1R inhibitor. D. The protein expression of cytochrome C and Bcl-2, untreated ischemic cells as control; E. Effects of IGF-1 and IGF-1R inhibitor on expression of apoptosis associated protein. Values are mean \pm SD of three independent experiments. IGF-1, Insulin-like growth factor; IGF-1R, insulin-like growth factor receptor; ROS, reactive oxygen species; SD, standard deviation; DCFH-DA, 2, 7-dichlorofluorescein diacetate. * $P < 0.05$ significant difference compared with control.

play an important role in apoptosis, so the key factors were subjected to western blot analysis, such as p-AKT 308, p-AKT473, p-ERK and p-S6. **Figure 4** showed that the expression of

p-AKT, p-AKT473, p-ERK and p-S6 presented a dramatic increase ($P < 0.05$), while this effect was abolished after adding the IGF-1R inhibitor which blocked AKT phosphorylation.

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Discussion

Here, while added IGF-1 in ischemic H9c2 cells, the cell viability was increased. Increasing of cytochrome C could induce cell apoptosis, and Bcl-2 is an important factor of apoptosis. In this study, we found cytochrome C was decreased and Bcl-2 expression was significantly increased. Respectively, we showed ROS level was statistically decreased in the condition of IGF-1 overexpression. So it is indicated that IGF-1 overexpression could suppress apoptosis and ROS level in ischemic H9c2 cells. Furthermore, the key protein expression of PI3K/AKT signal was increased. These results indicate IGF-1 overexpression could promote cell viability of ischemic H9c2 cells, with suppressing apoptosis and ROS level through PI3K/AKT pathway. Evidence show that IGF-1 could regulate the function of ischemic cells.

IHD is the primary cause of death in the world and epidemiologic studies have projected mor-

tality because of the aging population [13]. Cell death is a main maker of various cardiac diseases which includes myocardial infarction and IHD [14, 15]. A variety of risk factors participates in heart failure including starvation, ischemia, hypoxia, and oxidative stress, all of which are potential to induce the cardiomyocyte cell death [16]. In the condition of oxygen deficit, a series of biological processes were activated to help the ischemic cells combine with the oxygen, while the result of starvation could lead to the biological process of cell apoptosis activated recover the myocardial ischemia [2].

Apoptosis is a programmed cell death, which involves a series of activation, expression, and regulation of genes. In biological mechanisms, it is not a pathological condition, but for better

adapting to the environment and actively fighting for a dying process. Among the several treatments of IHD, the antiapoptotic properties of IGF-1 play an important role. When the cell is not able to adapt to stress and occurs an increase in the production of ROS, the mechanism of cell apoptosis is activated [17]. D'Amario *et al.* have shown that in c-Kit⁺ cells isolated from human hearts was correlated with higher telomerase activity, proliferation, and decreased apoptosis, whereas absence of IGF-1R led to cell senescence and increased apoptosis [18]. In addition, IGF-1 increases oxidative and improves insulin sensitivity [19, 20]. Long-term IGF-1 could decrease the cell viability [21, 22], but short-term protect against starvation and ischemia in the heart [16, 23]. In this study, we found IGF-1 could promote cell viability, and suppress cell apoptosis in ischemic H9c2 cells. After administration of IGF-1R inhibitor, the cell viability was significantly suppressed and cell apoptosis was promoted.

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This activation by IGF-1 contains two canonical pathways, which one is the PI3K/AKT pathway [24]. In several recent studies, the signaling mechanism of IGF-1 in ischemic cells has been described [25-27]. Kim *et al.* searched through overexpressing IGF-1R in mice, IGF-1-dependent ischemia mimics physiological and cardiac growth by promoting protein translation, rather than by activating cardiac gene expression [28-30]. Troncoso *et al.* found that the antiapoptotic effects of IGF-1 could be mediated by the activation of PI3K in the cellular level [23, 31]. Activation of AKT presents as an essential step for the antiapoptotic actions, and Bcl-2 family are the downstream targets of AKT [32, 33]. Mitochondria-bound Bcl-2 could progress the apoptotic function, and promoting cell death [31]. Here, after adding IGF-1 in ischemic H9c2 cells, the cell apoptosis, ROS level, and cytochrome C were suppressed, while the expression of Bcl-2 and the protein of PI3K/AKT signaling were significantly promoted. These results indicated the mechanisms of apoptosis were regulated by PI3K/AKT pathway. Nevertheless, IGF-1 was considered a potential candidate for the treatment of IHD.

In conclusion, our studies demonstrate IGF-1 could promote cell viability, and inhibit ROS level, cytochrome C release, and Bcl-2 expression. IGF-1 could regulate the mechanisms of ischemic H9c2 cell apoptosis by PI3K/AKT signaling.

Disclosure of conflict of interest

None.

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