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Original Article

Insulin-like growth factor-1 inhibits the expression of autophagic ID1 in cerebrovascular endothelial cells

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Abstract: Insulin-like growth factor (IGF)-1 has the effect of reducing blood glucose. However, it may cause obvious complications, which may be related to the autophagy-related protein ubiquitin binding protein P62, inhibitor of DNA binding/differentiation 1 (ID1), or activated caspase-3. This study intends to discuss the protective role of IGF-1 on endothelial cells injured by autophagy and its impact on P62, ID1, and activated-caspase-3 expressions. IGF-1 was used to treat an endothelial cell autophagy injury model induced by hydrogen peroxide. Cell viability was assessed using an MTT assay. Cell autophagy and ID1 expression were detected by Western blot. IGF-1 is a protective molecule in endothelial cell autophagy injury. IGF-1 suppresses activated-caspase-3 expression and upregulates ID1 and P62 levels. IGF-1 contributes to cerebrovascular disease by inhibiting autophagy and promoting ID1 expression to antagonize endothelial cell autophagic death.

Keywords: IGF-1, cerebrovascular endothelial cell, autophagy, oxidative stress

Introduction

With the acceleration of the aging of the population, stroke incidence is rising at a rate of about 8.7% a year. Arteriosclerosis obliterans is one of the most common symptoms of peripheral vascular disease. Cardio-cerebrovascular disease has become the leading cause of death in China. Arteriosclerosis obliterans-induced ischemic vascular disease seriously affects the quality of life. Cerebrovascular disease causes multiple impacts on patients, their families, and public health. Searching for the risk factors and for effective treatments are of great significance to improve the prognosis and reduce the incidence rate of cerebrovascular disease.

It was found that ischemic injury may cause endothelial cell lesions. Oxidative stress-induced endothelial cell autophagic death is the main outcome of injured blood vessels [1], which may cause arteriosclerosis obliterans [2]. As a member of the HLH transcriptional factor family, inhibitor of DNA binding/differentiation 1 (ID1) is characterized as promoting cell proliferation and regulating the cell cycle [3]. It was thought that the regulation of ID1 on autophagy is an important mechanism of ID1 in repairing endothelial cell injuries. It participates in the repair process of vascular endothelial cell injuries [4]. It was confirmed that ID1 plays a critical role in cerebrovascular atherosclerosis. Insulin-like growth factor (IGF)-1 has the effect of reducing blood glucose. However, it may bring obvious complications at the same time it decreases blood glucose. It especially regulates microvascular lesions, but the specific mechanism has not been clarified [5]. Meanwhile, IGF-1 is an important factor in anti-apoptosis and promoting cell growth, which is closely associated with cerebral arteriosclerosis. However, there is still lack of research about its influence on endothelial cell apoptosis and autophagy [6]. This study intends to explore the impact of IGF-1 on ID1 and P62 expressions and autophagic death in human umbilical vein endothelial cells (HUVECs).

Materials and methods

Main reagents and instruments

Cell culture flasks, microtubes, and culture plates were purchased from Corning (USA). Hank's

balanced salt solution, DMEM medium, penicillin-streptomycin, and trypsin were bought from GIBCO (USA). VEGF was obtained from PROSPEC (Israel). FBS was obtained from Tianjin Haoyang Biological Manufacture Co., Ltd (China). DMSO was provided by Sigma (USA). Skim milk was derived from BD (USA). 30% hydrogen peroxide (v/v) was purchased from Tianjin Fuyu Chemical Co., Ltd (China). Activated-caspase-3 and ID1 antibodies were bought from Bioss (Beijing, China). Super ECL and ubiquitin binding protein P62 antibody were obtained from CST (USA). IGF-1 was derived from Cabiochem. The 0.45 μm PVDF membrane was purchased from Merck Millipore (USA). Agilent Technologies 6890N Network GC System and Agilent 5973 Network Mass selective Detector were provided by Agilent (USA). Trans-Blot transphor was purchased from Bio-Rad (USA). A vertical electrophoresis chamber and a DYY2C electrophoresis apparatus were bought from Liuyi (Beijing, China).

Cell line

HUVECs were purchased from Saliai (Guangzhou, China).

HUVECs cultivation and modeling

HUVECs were seeded in a flask at $1\times10^6/cm^2$ and cultured at 37°C and 5% CO_2. The cells were maintained in a DMEM medium containing 100 mg/L penicillin-streptomycin, 4 ng/mL VEGF, and 10% FBS. The HUVECs in their logarithmic phase were digested by 2.5 g/L trypsin for 5 min for passage. The HUVECs were treated by 0.5 mmol/mL H_2O_2 for 3 h to establish the HUVEC injury model.

MTT assay

HUVECs in the logarithmic phase were seeded in 96-well plate at 3000/well for 24 h. After 4 h incubation, 20 μl MTT at 5 mg/mL was added to the plate for 4 h. Then the plate was treated with 100 μl DMSO for 10 min and tested at 490 nm to measure the absorbance value.

The cells were divided into the model group, the normal control, and the treatment group (F1, 40 μl; F2, 20 μl; F3, 10 μl; F4, 5 μl). The HUVECs in the model group were treated with 0.5 mmol/L H_2O_2 for 3 h and further cultured for 24 h. The HUVECs in the treatment group were treated with different concentrations of IGF-1 before the H_2O_2 intervention.

Western blot

The HUVECs were mixed with RIPA for 15-30 min. Next, the protein was separated by 10% SDS-PAGE and transferred to a PVDF membrane. After being blocked by 5% skim milk for 1 h, the membrane was incubated in β-actin, ID1, P62, and activated-caspase 3 antibodies (1:500) at 4°C overnight. Then the membrane was incubated in a HRP labeled secondary antibody (1:1000) at room temperature for 45 min. Next, the membrane was treated with developer for 1 min and exposed to observe the result. The film was scanned and analyzed by Image J.

Flow cytometry

The cells were resuspended in a 100 μl binding buffer and incubated in 5 μl Annexin V-FITC and 5 μl PI in the dark for 15 min. Next, the cells were tested using BD flow cytometry to evaluate the cell apoptosis.

The cells were incubated in Annexin V-FITC at room temperature and in the dark for 30 min. Then the cells were washed with a buffer and fixed with 1 ml of formaldehyde. Next, PI was added to the cells to test the apoptosis cycle.

Statistical analysis

All data analyses were performed on SPSS 20.0 software. The measurement data were compared using ANOVA and an LSD test. $P < 0.05$ was considered statistically significant.

Results

The impact of IGF-1 on endothelial cell autophagic death model apoptosis

Compared with the normal control, the IGF-1 treatment significantly enhanced cell apoptosis in the endothelial cell autophagic death model (**Figure 1**).

The impact of IGF-1 on endothelial cell autophagic death model cell viability

40 μl IGF-1 exhibited a protective effect on the endothelial cell model ($P < 0.01$). It is considered that 40 μl IGF-1 plays a crucial role in pro-

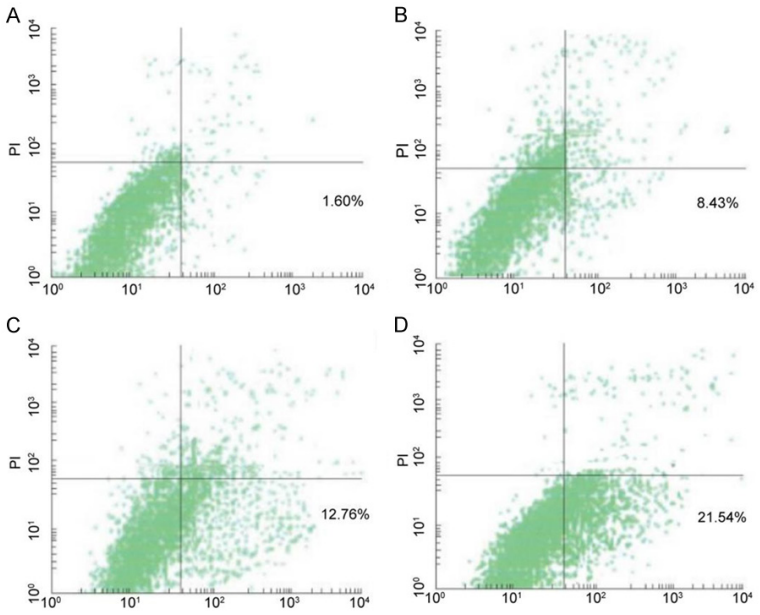

Figure 1. The impact of IGF-1 on endothelial cell autophagic death model apoptosis. A, normal control; B, 20 μl group; C, 5 μl group; D, model group.

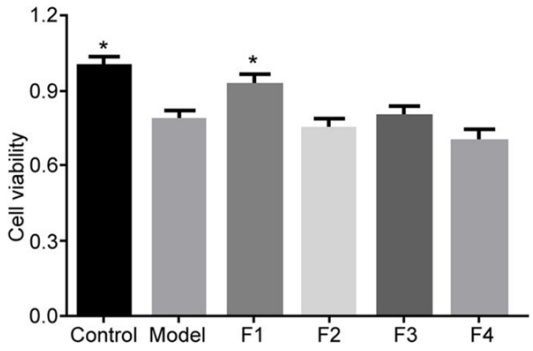

Figure 2. The impact of IGF-1 on HUVECs viability. F1, 40 μl group; F2, 20 μl group; F3, 10 μl group; F4, 5 μl group. *P < 0.05, compared with model group.

tecting endothelial cell autophagic death (**Figure 2**).

IGF-1 influenced activated caspase-3, ID1, and P62 expressions in the endothelial cell injury model

Western blot revealed that IGF-1 treatment clearly downregulated activated-caspase-3 expression and elevated ID1 and P62 levels compared with the model group ($P < 0.05$) (**Figure 3**).

Discussion

Arteriosclerosis obliterans (ASO) involves arterial occlusion or stenosis caused by peripheral artery atherosclerosis. It may lead to ischemic vascular disease, even resulting in disability or death [7]. Surgery is the main treatment for the disease. Although surgical treatment can quickly relieve the patient's clinical symptoms, surgery does not protect the endothelial cells from injury very well or effectively improve the body's internal environment. Therefore, it is difficult to prevent ASO recurrence since surgery cannot pathogenically treat the disease [8]. It was found that autophagic damage is one of the important reasons for this. Affected vascular endothelial cell injury is also an important factor in this disease [9]. Recently, the treatment effect of IGF-1 on ASO has drawn much attention from researchers. IGF-1 is mainly used in the treatment of diabetes, especially with vascular complications. Animal and clinical experiments have shown that IGF-1 can effectively treat ASO mainly by maintaining the normal function of endothelial cells to reduce ASO recurrence [10]. This study investigated the protective role of IGF-1 on endothelial cell autophagic injury and observed its impact on endothelial cell autophagy, aiming to provide a theoretical basis for the application of IGF-1 in endothelial cell protection and ASO treatment.

Since IGF-1 is a focus of diabetes treatment, we discussed it from the perspective of endothelial cell growth and failed to find the treatment mechanism. Clinical studies demonstrated that IGF-1 improves circulation and protects ischemic injury in blood vessels [11]. In addition, IGF-1 was found to confront oxidative stress injury and clear cell oxygen free radicals in vitro [12]. At the same time, it was shown that IGF-1 caused damage to endothelial barrier function recovery and inhibited autophagy [13].

This study exhibited that IGF-1 suppressed activated-caspase-3 expression. As a member of the caspase family, caspase-3 is known as an apoptosis execution factor. Caspase-3 usually exists in the form of pro-caspase-3 with no activity. Apoptosis signal activation or destruc-

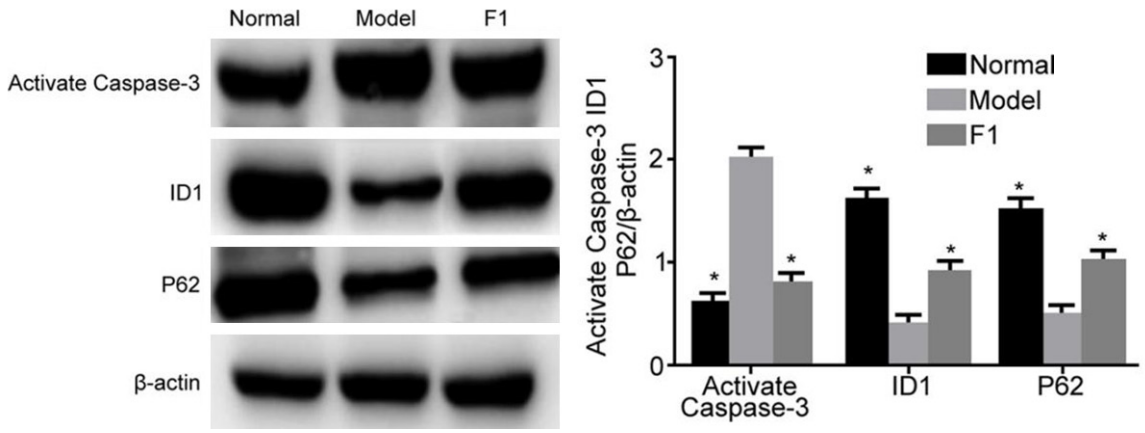

Figure 3. 40 μl IGF-1 influenced activated caspase-3, ID1, and P62 expressions in HUVECs. *P < 0.05, compared with the model group.

tive stimulus activated-caspase-3 works to cleave caspase-3 with activity to regulate cell apoptosis. Inhibition of cleaved caspase-3 activation and expression can alleviate cell apoptosis. Cleaved caspase-3 is treated as a marker to judge the cell apoptosis level [14]. Furthermore, H_2O_2 stimulus can induce endothelial cell apoptosis and elevate cleaved caspase-3 expression. IGF-1 treatment clearly decreases activated-caspase-3 expression, suggesting that IGF-1 can suppress endothelial cell apoptosis induced by H_2O_2 [15, 16].

Autophagy is the process of cytoplasmic material degradation and recycling by lysosomes in eukaryotic cells. It plays an important role in protecting cell environmental change, maintaining cell survival, and stabilizing the internal environment. It provides materials and energy for protein synthesis and degrades macromolecules and organelles in cytoplasms under nutrition deficient conditions [17]. Meanwhile, it degrades harmful substances and prevents their accumulation. Excessive autophagy activation may "digest" cellular organelles, leading to programmed cell death. Although the autophagy-lysosome can degrade harmful substances, it may cause environmental disorders [18, 19]. During autophagy activation, P62 sends the misfolded protein to autophagosomes after ubiquitin. As a kind of ubiquitin binding protein, P62 downregulation can be treated as a marker of autophagy elevation, leading to the degradation of misfolded proteins and ubiquitin binding protein P62. Our results showed that H_2O_2 stimulus elevated the activated-caspase-3 protein, while it reduced the P62 level in HUVECs, indicating that H_2O_2 stimulus induced HUVECs autophagic death. IGF-1 intervention significantly increased P62 expression, revealing that IGF-1 can inhibit HUVEC cell model autophagy to restrain cellular autophagic death.

ID1 is an important regulator of the cell cycle. It was demonstrated that ID1 can facilitate cell proliferation by inhibiting the promoter activity of the cycle related P21 gene. It can suppress P21 protein expression and reduce cell autophagy to inhibit the binding of P21 and Beclin-1. Moreover, ID1 can restrain cellular autophagic death by suppressing the promoter activity of P53 and upregulating Bcl-2 expression [19]. In this study, Western blot showed that ID1 was enhanced in the model group after IGF-1 treatment [20], suggesting that IGF-1 suppressed cellular autophagic injury by regulating the ID1 level. H_2O_2 induced endothelial cell autophagy works mainly by suppressing ID1 activity.

Our study demonstrated that IGF-1 suppresses cell autophagy and upregulates ID1 expression. However, there is still a lack of information about the central role of the ID1 protein in endothelial cell protection. Thus, we intended to treat cells with the ID1 protein inhibitor and IGF-1 together, aiming to provide a theoretical basis.

Disclosure of conflict of interest

None.

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