Aquaporin in the proliferation and apoptosis of diabetic myocardial cells

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ABSTRACT. The aim of this study was to explore the effect of aquaporin on the molecular mechanism of human diabetic myocardial cell apoptosis. The methylthiazolyle tetrazolium assay was used to detect the inhibitory effect of different concentrations of aquaporin on cell growth. The rate of aquaporin-induced myocardial cell apoptosis was detected by flow cytometric analysis of Annexin V-fluorescein isothiocyanate/propidium iodide double-stained cells. We also attempted to quantify the expression of Bcl-2, Bax, caspase-3, and survivin in diabetic myocardial cells by western blot analysis. Aquaporin was found to inhibit the proliferation of diabetic myocardial cells in a concentration-dependent manner; the increase in aquaporin concentration led to an increase in Bax (apoptosis protein) expression, decrease in Bcl-2 expression (anti-apoptosis protein), increase in the Bax/Bcl-2 ratio, and a decrease in caspase-3 and survivin expression (P < 0.05). Therefore, aquaporin significantly inhibits the proliferation of diabetic myocardial cells and cell apoptosis in a dose-dependent manner by upregulating the ratio of Bax/Bcl-2 protein expression, activating the caspase-3 protein cascade, and regulating the expression of survivin.

Key words: Aquaporin; Diabetes; Myocardial cell; Cell proliferation; Apoptosis
INTRODUCTION

Aquaporin is an inner-membrane protein that is responsible for forming "channels" in the cell membrane, which control the movement of water in and out of the cells, similar to a "cell pump" (Sasaki et al., 2003; Ferrari and Palmerini, 2007). Previous research has shown that aquaporins have immunomodulatory, anti-inflammatory, and antioxidant functions, impart cardiovascular protection, inhibit the enzyme activity, and have other biological functions (Horvathova et al., 2005), in addition to exerting an inhibitory effect on different types of tumor cells. So far, studies have confirmed that aquaporins regulate the proliferation and apoptosis of leukemia cells (Li et al., 2001), and prostate cancer (Fang et al., 2007; Zhou et al., 2009), liver cancer (Lee et al., 2005; Yoo et al., 2009), gastric cancer (Wu et al., 2008; Zhang et al., 2009), lung cancer (Kim et al., 2006), and other tumor cells. However, the effects of aquaporins on myocardial cells remain to be comprehensively reported.

In this study, the pharmacologic activity of aquaporins on diabetic myocardial cells was investigated using a diabetic myocardial cell model, by analyzing the inhibition of cell proliferation and the apoptosis induction effect. In addition, we attempted to detect the presence of diabetic myocardial cell apoptosis-related proteins, and elucidate the molecular mechanism of aquaporin-induced apoptosis of myocardial cells.

MATERIAL AND METHODS

Experimental material

Diabetic myocardial cells were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Aquaporin was purchased from the Chinese Pharmaceutical and Biological Products Assay Institute, while Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and dimethyl sulfoxide (DMSO) were obtained from Gibco (Grand Island, NY, USA). Methylthiazole tetrazolium (MTT) was purchased from Axygen (Corning, NY, USA); the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit was obtained from Biovision (San Francisco, CA, USA), and the bicinchoninic acid (BCA) protein concentration assay kit and RIPA lysis buffer were purchased from Jiangsu Haimen Biyuntian Institute of Biotechnology. Anti Bcl-2, Bax polyclonal antibody, and peroxidase-coupled sheep anti-rabbit polyclonal antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while anti-caspase-3 and the survivin mouse monoclonal antibody were purchased from Cell Signaling Technology (Boston, MA, USA).

Instruments

The experiments involved the use of a CO₂ incubator (Thermo Forma, Grand Island, NY, USA), a super-clean workbench (Suzhou Purification Equipment Factory, Suzhou, China), a microplate reader, an inverted microscope (Nikon, Tokyo, Japan), a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), a vertical electrophoresis tank, a protein transfer tank, and an electrophoresis apparatus and power (Bio-Rad, Hercules, CA, USA).

Diabetic myocardial cell culture

Human diabetic myocardial cells were conventionally cultured in DMEM (supplemented
with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin) at 37°C in a 5% CO₂ incubator; the liquid medium was changed (subculture) according to the cell metabolism (after digestion).

**MTT assay to detect the inhibitory effect of aquaporin on human diabetic myocardial cell proliferation**

Diabetic myocardial cells in the logarithmic phase of growth were trypsinized; the cell concentration was adjusted to 1 x 10⁴ cells/mL, and the cells cultured on a 96-well plate (100 µL/well). A control group (blank wells with cells containing <0.1% volume fraction of DMSO) and aquaporin drug groups (0.5, 2.5, and 12.5 µg/mL) were established; each group was assigned to 6 wells. After the cells adhered to the walls of the well, aquaporin was added to the wells to obtain final concentrations of 0.5, 2.5, 12.5 µg/mL aquaporin. The culture was terminated after 48 h, and the cells were incubated for an additional 4 h with 20 µL (5 g/L) MTT solution. The supernatant was subsequently removed and the cells incubated with 150 µL DMSO. The culture plate was gently shaken for 10 min until the blue crystal was completely dissolved. The optical density at 570 nm was measured for each well, and the cell survival rate calculated using the following formula: Cell survival rate (%) = (1 - (control hole absorbance - experimental hole absorbance) / control hole absorbance) x 100%.

**Detection of aquaporin-induced apoptosis of diabetic myocardial cells by Annexin V-FITC/PI double-staining**

Diabetic myocardial cells in the logarithmic phase of growth were inoculated on a 6-well plate at a cell density of 5 x 10⁵ cells/mL (2 mL/well). A control group (blank well containing cells cultured in <0.1% volume fraction DMSO) and three aquaporin drug groups (0.5, 2.5, and 12.5 µg/mL) were established. Aquaporin was added to the adhered cells, in order to ensure the final concentrations (of the drug group) of 0.5, 2.5, and 12.5 µg/mL aquaporin. The cells were incubated for 48 h; subsequently, the cells were collected and the early apoptosis rate was detected by flow cytometry, according to the protocols provided by the manufacturers of the Annexin V-FITC/PI kit.

**Detection of Bax, caspase-3, and Bcl-2 after aquaporin treatment by western blotting**

Diabetic myocardial cells in the logarithmic phase of growth were inoculated on a 6-well plate at a cell density of 5 x 10⁵ cells/mL (2 mL/hole). A control group (blank wells with cells incubated in <0.1% volume fraction DMSO) and three aquaporin drug groups (0.5, 2.5, and 12.5 µg/mL) were established. After ensuring cell adherence, aquaporin was added to obtain final aquaporin concentrations of 0.5, 2.5, and 12.5 µg/mL in the respective wells. The cells were pre-incubated for 12 h and the supernatant was subsequently removed by centrifugation. The cells were then washed thrice with pre-cooled phosphate-buffered saline (PBS) and incubated with 100 µL RIPA lysate (for pyrolysis) on ice for 30 min. Subsequently, the cells were scratched off, centrifuged at 12,000 g for 10 min at 4°C, and the supernatant collected for subsequent protein concentration determination and quantitative western blot analysis. The protein concentration was determined by BCA analysis, according to the standard instructions provided in the kit. Twenty micrograms of protein from each group was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12%); the
separated protein bands were transferred to a polyvinylidene fluoride membrane, and incubated (closed) at room temperature for 1 h or at 4°C overnight. The membrane was then separately incubated overnight with anti-Bcl-2, anti-Bax, anti-caspase-3, and anti-survivin antibodies (1:1000) at 4°C. The primary antibodies were eluted with PBST solution; subsequently, the membrane was incubated with the secondary antibody (1:1000) for 1 h. The membrane was washed with PBS, proteins were visualized using an enhanced chemiluminescence detection system (Millipore, Chicago, IL, USA).

RESULTS

Effect of aquaporin on human diabetic myocardial cell proliferation

Aquaporin inhibits the proliferation of diabetic myocardial cells in a concentration-dependent manner, wherein the 2.5 µg/mL aquaporin drug group exhibited a lower level of inhibition compared to the 12.5 µg/mL aquaporin drug group, but a higher level of inhibition compared to the control group, with statistical significance (P < 0.05). The cell viability in the 0.5, 2.5, and 12.5 µg/mL aquaporin drug groups were 91.33 ± 9.62, 72.03 ± 8.42, and 35.06 ± 4.56%, respectively (Figure 1).

![Figure 1](image-url)

**Figure 1.** MTT assay detection of effect of aquaporin on the proliferation of diabetic myocardial cells. Aquaporin can inhibit the proliferation of diabetic myocardial cells in a concentration-dependent manner. **P < 0.05 compared to control group.

Effect of aquaporin on human diabetic myocardial cell apoptosis

Diabetic myocardial cell apoptosis was analyzed using an Annexin V-FITC/PI double-staining flow cytometric analysis technique following aquaporin treatment for 48 h. In the quadrant diagram, the normal cell group, early apoptotic cells, the autophagic cell death and nonspecific cell death subsets, and the cell subsets undergoing late apoptosis and necrosis were distributed in the lower left, lower right, upper left, and upper right quadrants, respectively. The proportion of
apoptotic cells increased with the increase in drug concentration, with the dosage exhibiting an obvious effect. At aquaporin concentrations of 0, 0.5, 2.5, and 12.5 µg/mL, the percentage of early apoptotic cells were 3.5, 14.4, 29.8, and 58.9%, respectively (Table 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Percentage of early stage apoptosis (%)</th>
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<tbody>
<tr>
<td>A</td>
<td>3.50</td>
</tr>
<tr>
<td>B</td>
<td>14.4</td>
</tr>
<tr>
<td>C</td>
<td>29.8</td>
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<tr>
<td>D</td>
<td>58.9</td>
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Table 1. Effect of aquaporin on apoptosis of human diabetic myocardial cells.

Western blot detection of Bcl-2, Bax, caspase-3, and survivin protein expression following aquaporin treatment

The mitochondrial pathway is an apoptosis pathway; the expression of mitochondrial pathway-related factors Bcl-2, Bax, and caspase-3 were detected by western blot analysis in order to explore the molecular mechanism of aquaporin-induced diabetic myocardial apoptosis. We observed an increase in the expression of the apoptosis protein Bax, decrease in the expression of the anti-apoptosis protein Bcl-2, and an increase in the Bax/Bcl-2 ratio with the increase in aquaporin concentration; on the other hand, while caspase-3, the downstream protein of the mitochondrial pathway of apoptosis was activated, its expression reduced in a concentration-dependent manner (Figure 2). Therefore, aquaporin may induce diabetic myocardial cell apoptosis through the mitochondrial pathway. Survivin, an apoptosis inhibitor, is an important regulatory factor of cell apoptosis. We observed a concentration-dependent decrease in survivin expression with the increase in aquaporin concentration; this suggested that survivin is a water-channel target protein that regulates apoptosis in diabetic myocardial cells.

![Western blot detection of the expression of caspase-3, Bcl-2, Bax, and survivin in diabetic myocardial cells after the aquaporin treatment.](image-url)
DISCUSSION

Recent studies have identified many proteins that inhibit tumor cell proliferation or induce tumor cell apoptosis, thereby causing an anti-tumor effect. Aquaporin is widely distributed in the cell membrane (intrinsic membrane protein); aquaporins form “channels” on the cell membrane, which can control the movement of water in and out of the cells, similar to a “cell pump”. Tumors are mainly inhibited through 1) the inhibition of tumor cell proliferation, 2) accelerated tumor cell apoptosis, 3) change in the cyclic distribution of tumor cells, 4) sensitizers that prepare the tumor cells for apoptosis, 5) inhibition of tumor cell neovascularization, and 6) the antioxidant effect that exercises an apoptotic function. In this study, the MTT method was adopted to detect the diabetic myocardial cell activity; the results revealed that aquaporin significantly inhibits myocardial cell proliferation in a concentration-dependent manner.

Apoptosis is a means by which multicellular organisms regulate and maintain their normal growth and stability; recent findings have suggested that the apoptosis pathway is mainly divided into the endoplasmic reticulum pathway, endogenous mitochondrial pathway, and the exogenous death ligand pathway (Fiandalo and Kyprianou, 2012). Among these, the mitochondrial pathway functions mainly by regulating the expression ratio of the upstream anti-apoptotic protein Bcl-2 (of the Bcl-2 family) and the apoptosis accelerating protein Bax, subsequently triggering the release of cytochrome C and inducing changes in the mitochondrial membrane potential, which in turn activates the downstream caspase-9 and caspase-3 proteins (of the caspase protease family), thereby initiating a cascade reaction (Yang et al., 1997; Ohtsuka et al., 2003; Elmore 2007; Siu et al., 2008). Flow cytometric detection of diabetic myocardial cells after aquaporin treatment identified a significant, concentration-dependent increase in the apoptosis rate of cells in the aquaporin drug group compared to the control group, indicating that aquaporin effectively induces apoptosis in diabetic myocardial cells. The molecular mechanism of diabetic myocardial cell apoptosis induction by apoptosis by aquaporin was studied by western blot analysis, by detecting the expression of Bcl-2, Bax, caspase-3, and surviving proteins. Aquaporin was found to significantly inhibit the expression of the anti-apoptotic protein Bcl-2, and promote the expression of the apoptosis protein Bax, thereby activating the caspase-3 cascade reaction. Survivin is an important member of the apoptosis inhibitor protein family, and plays an important role in the suppression of apoptosis, and promotion of cell proliferation. Therefore, its expression is an independent indicator of osteosarcoma (Suzuki and Shiraki, 2001; Osaka et al., 2007; Altieri, 2008). In this study, the increase in aquaporin concentration resulted in a dose-dependent decrease in the expression of survivin in myocardial diabetic cells. Therefore, we concluded that survivin was also a molecular target for apoptosis-induced myocardial cell apoptosis.

Aquaporin is a newly identified targeting protein, which regulates the mitochondrial apoptosis pathway and inhibits the expression of the apoptosis inhibitor protein surviving. Aquaporin was found to effectively induce diabetic myocardial cell apoptosis; in addition, it could potentially be applied for the treatment of diabetic myocarditis. However, further research must be conducted to determine if it can be combined with the routine treatment strategies for diabetic myocarditis, and to determine the related medication dosage. In addition, the use of aquaporins in the human body and the related pharmacokinetics remains to be further examined via clinical trials.

Conflicts of interest

The authors declare no conflict of interest.
REFERENCES


