Original Research Article

Metformin promotes apoptosis of A549 cells via regulation of p-AMPK protein expression, bax/bcl-2 ratio and ROS levels

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Abstract

**Purpose:** To investigate the influence of metformin on apoptosis of pulmonary carcinoma cells (A549), and the associated mode of action.

**Methods:** Pulmonary carcinoma cells in logarithmic growth phase were treated with graded concentrations of metformin, and the anti-proliferative and apoptotic effects of the drug were measured using MTT assay and flow cytometry, respectively. The levels of reactive oxygen species (ROS) in A549 cell suspension were determined with 2, 7- dihydrodichlorofluorescein diacetate (DCFH-DA) assay. The expression levels of phosphorylated AMP-activated protein kinase (p-AMPK), mammalian target of rapamycin (mTOR), and bax/bcl-2 ratio were measured using Western blotting and real-time fluorescence quantitative polymerase chain reaction (qRT-PCR).

**Results:** Metformin significantly promoted A549 cell apoptosis, but suppressed its proliferative potential in a dose- and time-based fashion. The levels of ROS, superoxide anion and MDA in A549 cells were significantly and dose-dependently increased by metformin (p < 0.05). Moreover, metformin markedly upregulated the mRNA and protein expressions of p-AMPK as well as bax/bcl-2 ratio, but had no impact on the expression level of mTOR (p < 0.05).

**Conclusion:** Metformin promotes apoptosis in A549 cells via regulation of p-AMPK protein expression, bax/bcl-2 ratio, and ROS levels, and hence may play a role in lung cancer therapy.

**Keywords:** Apoptosis, Lung cancer, Metformin, Proliferation, Reactive oxygen species

INTRODUCTION

Pulmonary carcinoma, a frequently diagnosed malignancy in clinical practice, is associated with high morbidity and mortality. According to statistics, there are over 1.2 million new cases of lung cancer annually, with more than 900,000 deaths globally. The disease seriously impacts negatively on the quality of life of patients [1]. At present, surgical resection remains the major strategy for the treatment of lung cancer. However, because the disease has an insidious onset, more than 50% of the patients usually are unable to benefit from treatment at the time of...
diagnosis. Although patients are often treated with a combination of radiotherapy and chemotherapy, the 5-year survival of lung cancer is less than 10 %. The combination therapy produces serious side effects, thereby necessitating the search for novel effective drugs with good prognosis and limited side effects [2].

Metformin is a commonly used hypoglycemic drug. Its hypoglycemic effect is exerted via inhibition of gluconeogenesis and glycogenolysis (glycogen breakdown), as well as stimulation of the sensitivity of peripheral tissues to insulin [3]. It has been reported that metformin significantly reduces the incidence of malignant tumor in type 2 diabetes mellitus (T2DM) patients [4]. It has also been speculated that metformin inhibits the growth of tumor cells via activation of adenosine monophosphate-activated protein kinase (AMPK) signaling pathway, as well as promotion of protein synthesis and cell apoptosis [5]. This research was carried out to determine the influence of metformin on A549 cell apoptosis, as well as the associated mode of action.

EXPERIMENTAL

Materials

Optical microscope (WMJ-9590) was obtained from Shanghai Yuguang Instrument Co. Ltd. Electronic balance (JA2603) was bought from Shanghai Precision Instrument Co. Ltd. Ultra-low temperature refrigerator (DL-86L828) was a product of Beijing Nuohuicheng Technology Co. Ltd. Flow cytometer (B5-R3-V3) was purchased from Beckman Coulter (USA). Cryogenic high-speed centrifuge (TG16G) was obtained from Changzhou Jintan Lepu Instrument Company, while carbon dioxide incubator (ZCP-270WIR) was produced by Shanghai Zhetu Scientific Instrument Co. Ltd. Electric thermostatic water bath (WB-1-15) was obtained from Shanghai Shibei Instrument Equipment Factory. Fetal bovine serum (FBS) was bought from Shanghai Huiying Biotech. Co. Ltd. Metformin was product of Sino-American Shanghai Squibb Pharmaceutical Company, while MTT solution was from Beijing Zhongsheng Ruitai Technology Co. Ltd. Bax antibody was bought from Shanghai Hongyuan Biotechnology Company; bcl-2 antibody was obtained from EmMETt Technologies, while p-AMPK antibody was supplied by Xiamen Huijia Biotech. Co. Ltd.

Cell line and maintenance

The lung cancer cells (A549) were sourced from Shanghai Tongpai Biotech. Co. Ltd. The cells were cultured for 24 h at 37 °C in DMEM containing FBS (10 %) and 1 % penicillin/streptomycin in a 5 % CO₂ incubator. When the cells attained 85 % confluency, the resultant adherent cells were trypsinized with 2 mL of 0.25 % trypsin, centrifuged at 1500 rpm for 10 min, and converted to a suspension of individual cells which were seeded in culture wells (2 x 10^4 cells/well; 100 μL per well). On attainment of 60 - 70 % confluency following incubation, cells at logarithmic growth phase were incubated with varying doses of metformin for 24 h, with metformin-free cell culture as control group.

Cell proliferation assay

The proliferative capacity of A549 cells was determined using MTT assay. The cells were seeded in 96-well plates at a density of 2 x 10^4 cells/well (100 μL per well) and cultured in DMEM for 24 h. Varied concentrations of metformin were incubated with the cells for 72 h, after which the cells were incubated with MTT (0.5 g/100 mL; 20 μL) for additional 4 h. Then, the medium was discarded, and the resultant formazan crystals in each well were solubilized in 150 mL of 0.1 % DMSO (150 mL), followed by absorbance measurement at 540 nm in a microplate instrument. Percentage proliferative potential was measured at 24 h and 48 h.

Measurement of apoptotic potential

Following 24-h culturing, cells plated in 6-well plates (2 x 10^4 cells/well) in 6-well plates were treated with varied concentrations of metformin, followed by further incubation for 2 days. Thereafter, the cells were rinsed in phosphate-buffered saline, and a homogenous cell suspension was produced by addition of 500 μL of binding buffer. This was followed by cell staining (in a dark chamber) with V/FITC (10 μL) and 5 μL of PI. The degree of apoptosis was measured flow cytometrically.

Determination of ROS levels

In this procedure, A549 cells treated with varied concentrations of metformin were PBS-rinsed after they were incubated for 72 h, followed by addition of DCFH-DA (10 μM) to each well. Incubation was carried out again at 37 °C for about 35 min, followed by PBS-rinsing and flow cytometric analysis. The level of MDA in cell suspension was assayed using commercially available kit.

Assay of mRNA expressions using qRT-PCR

Following treatment of A549 cells with graded concentrations of metformin for 48 h, the mRNA
expressions of p-AMPK, mTOR, bax and bcl-2 in the cells were determined with qRT-PCR. Total cellular RNA extraction was done with TRIzol reagent, followed by reverse transcription to cDNA with cDNA synthesis kits as per the manufacturer’s protocol. The mRNA expression levels of the various genes were determined using Light Cycler 1536 RT-PCR equipment, with GAPDH as the house-keeping gene. Relative mRNA expressions were calculated with the 2^-ΔΔCt procedure.

Immunoblot assay

Total protein was extracted from cells in each group using chilled RIPA buffer laced with protease inhibitor. Following centrifugation, the protein content of each lysate was determined using the BCA procedure. Equal amounts of protein samples (40-μg) were subjected to 12 % SDS-PAGE, followed by electro-transfer to PVDF membranes. Thereafter, the membranes were blocked by incubation with 3 % fat-free milk solution, followed by incubation at 4 °C overnight with 1° antibodies for bcl-2, p-AMPK, bax, mTOR and GAPDH (all diluted 1: 800). Then, after rinsing 3 times in TBS-T, the membranes were incubated with HRP-linked 2° antibody at laboratory temperature for 90 min. Blot development was carried out using X-ray film, while ImageJ Launcher software was employed for grayscale analysis of the bands. Relative protein expression levels were calculated with reference to GAPDH which was used standard.

Statistics

Results are presented as mean ± SEM, and they were analyzed with SPSS version 23.0. Two-group comparison was done with t-test. Significant differences were assumed at p < 0.05.

RESULTS

Influence of metformin on cell proliferation

The proliferative capacity of A549 cells was markedly and time- and dose-dependently reduced by metformin (p < 0.05; Table 1).

Effect of metformin on cell apoptosis

As shown in Table 2, exposure of A549 cells to metformin markedly enhanced apoptosis in a concentration-based fashion (p < 0.05).

Impact of metformin on ROS concentrations

The levels of ROS, superoxide anion and MDA in A549 cells were significantly and dose-dependently increased by metformin treatment (p < 0.05; Table 3).

Effect of metformin on expression levels of p-AMPK, mTOR, bax and bcl-2

Metformin markedly and concentration-dependently increased the mRNA and protein expressions of p-AMPK in A549 cells, and also increased bax/bcl-2 ratio (p < 0.05). However, it did not significantly alter the expression level of mTOR (p > 0.05). These results are shown in Table 4.

DISCUSSION

Lung cancer poses serious threat to humans. There has been a global rise in the incidence of lung cancer.
### Table 4: Effect of metformin on mRNA expressions of p-AMPK, mTOR, bax and bcl-2 (mean ± SEM, n = 5)

<table>
<thead>
<tr>
<th>Group</th>
<th>p-AMPK</th>
<th>mTOR</th>
<th>bax/bcl-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.65 ± 0.03</td>
<td>0.47 ± 0.07</td>
<td>6.98 ± 1.03</td>
</tr>
<tr>
<td>Metformin (15 mM)</td>
<td>0.77 ± 0.06*</td>
<td>0.45 ± 0.05</td>
<td>10.14 ± 1.55*</td>
</tr>
<tr>
<td>Metformin (30 mM)</td>
<td>0.92 ± 0.04*</td>
<td>0.43 ± 0.04</td>
<td>14.79 ± 3.11*</td>
</tr>
</tbody>
</table>

F 45.00 0.67 17.62

P-value < 0.001 0.531 < 0.001

*P < 0.05, vs control

In China, lung cancer-related morbidity and mortality rank first among malignant tumors, with lung adenocarcinoma being the most common pathological subtype of non-small cell lung cancer (NSCLC). Lung adenocarcinoma, which occurs mainly around the lungs, is usually seen in patients who are habitual smokers, and it is the predominant cancer type in young women and in Asians. Patients with lung cancer often miss treatment at the time of diagnosis because of the insidious nature of the disease [6].

At present, the main clinical treatment for lung cancer is combination of surgery and chemoradiotherapy. Although the treatment of lung cancer has seen some improvements over the years, the overall prognosis remains poor, thereby necessitating the search for novel and effective drugs with less side effects [2].

Metformin is an antidiabetic drug which has shown great promise in the treatment of malignant tumors. It lowers blood glucose by stimulating the sensitivity of peripheral tissues to insulin [7]. Reports have implicated metformin in the treatment of polycystic ovary syndrome [8]. Studies have shown that T2DM patients treated with metformin medication responded better to chemotherapy than those placed on sulfonylureas [9]. It has been speculated that metformin may significantly reduce the incidence of cancer and cancer-related mortality [9].

An equilibrium between cell proliferation and apoptosis is vital for regulation of cell growth. Apoptosis refers to autonomic programmed cell death informed by gene regulation. Being the main mechanism of anti-tumor drugs, apoptosis is crucial for the maintenance of stable internal microenvironment of normal cells [10]. The gene bcl-2 belongs to the bcl-2 family of proteins that control cell death via inhibition (anti-apoptosis) or induction (pro-apoptosis). It was first isolated from human follicular lymphoma. It participates in tumorigenesis by making the immune system lose its ability to kill abnormal cells [11].

In humans, the bcl-2-associated X protein (bax), also known as bcl-2-like protein 4, is encoded by the bax gene. It antagonizes the inhibitory effect of bcl-2 protein on apoptosis. Studies have shown that the upregulation of bax protein expression markedly stimulates cell apoptosis, while inhibiting cell proliferation. In addition, bax prevents lymphatic metastasis of tumor cells [12]. It has been established that bax/bcl-2 ratio is closely related to apoptosis or survival of cancer cells [13]. The results obtained in the present research demonstrated that metformin markedly promoted A549 cell apoptosis, but inhibited its proliferation.

Under normal physiological conditions, there is a cellular balance between ROS production and their clearance. When the tissues are stimulated, the production of ROS is markedly enhanced. Increases in ROS over and above normal levels damage cell structure. Moreover, ROS act as second messengers in the regulation of cell proliferation and apoptosis [14-16].

Adenosine monophosphate-activated protein kinase (AMPK) is an energy signal-sensing protein ubiquitously expressed in cells of body tissues where it participates in the maintenance of cell homeostasis. On activation by sugar, hypoxemia or oxidative stress, AMPK activates downstream signaling pathways, thereby keeping intracellular energy level constant, while maintaining normal cell division cycle [17-19].

Adenosine monophosphate-activated protein kinase (AMPK) has several receptor-linked signaling pathways such as mTOR signaling pathway, bcl-2/bax ratio, and MAPK, and exerts significant regulatory effects on cell proliferation and apoptosis [20,21]. In this study, treatment of A549 cells with metformin led to marked and concentration-based increases in the mRNA and protein expressions of p-AMPK, and also increased the proportion of bax/bcl-2. However, it did not appreciably alter the expression level of mTOR. These results suggest that the apoptotic effect of metformin may be exerted via regulation of p-AMPK protein expression, bax/bcl-2 ratio and ROS levels.

**CONCLUSION**

Metformin promotes apoptosis in A549 cells via regulation of p-AMPK protein expression, bax/bcl-2 ratio and ROS levels, and there may be beneficial in lung cancer therapy.
DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was performed by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xiaoli Zhang designed the study, supervised the data collection, and analyzed the data. Ziying Yu interpreted the data and prepared the manuscript for publication. Fengtao Liu supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

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