Metformin Inhibit Cell Proliferation and Secretion Function in NCI-H295R Cells

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Abstract

Background: The evidence about effects of metformin on adrenocortical carcinoma cell lines is lacking. This study aims to investigate the effects of metformin on proliferation and secretion function of H295R cells.

Methods: H295R cells were treated with different doses of metformin for 3 days or with 20 mmol/L metformin for different times. Cell proliferation was detected by MTS method. Cortisol and aldosterone in culture medium was determined by chemiluminescent method and radioimmunoassay, respectively. H295R cells were treated with 20 mmol/L metformin for 24 hours, and mRNA expressions of 11 beta-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) were detected by real-time quantitative PCR.

Results: The results showed that no differences of cell proliferation and secretion of cortisol and aldosterone were detected between control group and metformin group with doses less than 5 mmol/L. Metformin with large doses (≥ 10 mmol/L) significantly inhibited cell proliferation and secretion of cortisol and aldosterone in a dose-dependent pattern. Metformin (20 mmol/L) inhibited cell proliferation after 12 hours’ incubation. And the proliferation inhibitory effects of metformin were in a time-dependent manner. Compared with control group, metformin decreased secretion of cortisol and aldosterone, and mRNA expression of CYP11B1.

Conclusions: Metformin can inhibit cell proliferation and secretion of cortisol and aldosterone in H295R cells.

Background

Metformin is the first-line treatment for patients with type 2 diabetes. In recent years, studies have found that metformin has anti-tumor effect in colon cancer patients, breast cancer patients, pancreatic cancer patients and lung cancer patients with type 2 diabetes [1, 2]. Adrenocortical carcinoma (ACC) is a rare malignant and aggressive tumor with poor prognosis. The 5-year survival rate is 16–40%. Treatment of ACC patients with distant metastases is very difficult. The tumor is not sensitive to traditional radiotherapy and chemotherapy. Mitotane has proved to be of limited efficacy [3, 4]. New medicine is yet to be explored.

Only one report explored the effect of metformin on adrenocortical carcinoma. The results showed that metformin inhibited the proliferation of adrenocortical carcinoma cells and the growth of metastatic tumors, increased AMPK phosphorylation and inhibited mTOR phosphorylation [5]. However, this study did not pay attention to the effect of metformin on the secretory function of ACC cell line. Therefore, we aim to investigate the effects of metformin on the cell proliferation and the synthesis and secretion of cortisol and aldosterone in human adrenocortical carcinoma H295R cells.

Materials And Methods
Reagents

Metformin (Sigma-Aldrich), DMEM/F-12 medium and Trizol (Invitrogen-Gibco), Nu serum™ and ITS + premix [including insulin (6.25 µg/mL), transferrin (6.25 µg/mL), selenium (6.25 ng/mL), bovine serum albumin (1.25 mg/mL) and linoleic acid (5.35 µg/mL)] (BD Biosciences), reverse transcription kit and MTS (Promega), Power SYBR® Green PCR Master Mix (ABI) USA, cortisol chemiluminescence immunoassay kit (Siemens), aldosterone radioimmunoassay kit (Beijing North Biotechnology Research Institute).

Cell culture

The human adrenocortical carcinoma cell line NCI-H295R cells were provided by the Cell Center of the Institute of Basic Medicine, Peking Union Medical College. NCI-H295R cells were cultured in DMEM/F-12 complete medium (containing 2.5% Nu-serum™, 1% ITS + premix, 50 units/mL penicillin and 50 units/mL streptomycin), at 37°C in an incubator supplied with humidified air. Cells were passaged by digestion with trypsin-EDTA (0.25% trypsin and 0.02% EDTA in PBS without Ca^{2+} and Mg^{2+}).

Cell Proliferation Assay

Cells were plated into 96-well culture plates (6 × 10^4 cells/mL, 200 µL per well) in complete medium. At logarithmic growth phase, cells were incubated in fresh serum-free DMEM/F12. After serum starvation for 6 hours, cells were treated as follows: 1) Cells were treated with different concentrations of metformin (0.01 mmol/L, 0.1 mmol/L, 1 mmol/L, 5 mmol/L, 10 mmol/L, 20 mmol/L, 50 mmol/L) for 3 days. 2) Cells were treated with 20 mmol/L metformin for 12 hours, 1 day, 2 days, 3 days and 5 days respectively. Culture medium was removed and 100 µL DMEM/F12 medium and 20 µL MTS were added to each well. Cells were incubated at 37 °C for 40 min. Finally, the plates were read on a microplate reader (Bio-TEK Instruments, Vermont, USA) at 490 nm with a reference filter at 630 nm. The experiment was repeated three times.

Detection of Hormone Concentration

Cells were cultured in 24-well culture plates (4 × 10^5 cells/mL, 1 mL per well) in complete medium. Then cells were treated as aforementioned method in cell proliferation experiments. The culture medium was collected for measurement of cortisol (chemiluminescence assay) and aldosterone (radioimmunoassay). The experiment was repeated three times.

Real-Time Quantitative PCR
Cells were cultured in 6-well culture plates (5 × 10^5 cells/mL, 2 mL per well) in complete medium. At logarithmic growth phase, cells were incubated in fresh serum-free DMEM/F12 for starvation for 6 hours, and then treated with metformin (20 mmol/L) for 24 hours. Cells were lysed with Trizol reagent, and total RNA isolation and cDNA synthesis were conducted by following the standard protocol provided by the manufacturer. PCR amplification for CYP11B1 and CYP11B2 genes was conducted by using the Power SYBR ® Green PCR Master on an ABI ViiA7 PCR system. The relative gene expression levels were calculated by using the cycle threshold value and were normalized against the expression of β-actin gene. All real-time PCR was performed in triplicate for each sample. The experiment was repeated three times.

The primers used in the experiment were as follows: CYP11B1 primer: forwards 5'-AATGCGGAAGTGCAGGGGAAACACCGTC-3', backwards 5'-TCAGCAAGGGAAACACCGTC-3'; CYP11B2 primer: forwards 5'-ACTCGCTGGGTGCAATG-3', backwards 5'-AGTGTCTCCACCAGGAAGTG-3'; β-actin primer: forward-s 5'-TCCCTGGAGAAGAGCTACG-3', backwards: 5'-GTAGTTTCGTGGATGCCACA-3'.

**Statistical Analysis**

The statistical analyses were performed by using the SPSS version 13.0 software package. Data were presented as means ± standard deviation, and significance of differences was assessed by using independent samples t-test. *P*<0.05 was considered to be statistically significant.

**Results**

**Effect of metformin on proliferation of H295R cells**

When the concentration of metformin was below 5 mmol/L, there was no significant difference in cell proliferation between the treatment group and the control group. While the concentration of metformin above 10 mmol/L, cell proliferation was significantly inhibited as compared to the control group. And this inhibitory effect was dose-dependent (P<0.001) (Fig. 1A).

After 12 hours’ incubation, metformin inhibited cell proliferation, and the inhibitory effect was time-dependent (P<0.001) (Fig. 1B).

**Effect of secretory functions**

Compared with the control group, the concentration of cortisol and aldosterone in the treatment group with metformin concentration below 5 mmol/L did not change significantly. When the concentration of metformin was 10 mmol/L in the treatment group, the secretion of cortisol (P = 0.0988) and aldosterone (P = 0.0197) decreased compared with the control group, and the inhibition was dose-dependent (P<0.001) (Fig. 2A, 2B).
After 12 hours’ incubation, metformin (20 mmol/L) inhibited the secretion of cortisol (P < 0.001) and aldosterone (P < 0.05) in H295R cells. Over time the inhibition rate was significantly enhanced (Fig. 3A, 3B).

Compared with the control group, the expression of CYP11B1 mRNA in the metformin-treated (20 mmol/L) group decreased significantly (P < 0.05) (Fig. 4A). No statistical difference expression of CYP11B2 was found between the two groups although the treatment group had a downward trend (P = 0.1706) (Fig. 4B).

**Discussion**

Several studies have shown that metformin has anti-cancer activity in in vitro and in vivo tumor models by directly inhibiting proliferation, promoting apoptosis and indirectly regulating metabolism of cancer cells [6, 7]. However, the effect of metformin on adrenocortical carcinoma was rarely studied. One research indicated that metformin treatment can significantly reduce cell viability and proliferation with inhibition of ERK1/2 and mTOR phosphorylation as well as stimulation of AMPK activity. Metformin also triggers the apoptotic pathway and interferes with the proliferative autocrine loop of IGF2/IGF-1R, which supports adrenal cancer growth [5]. Our study demonstrate that metformin inhibits the proliferation of adrenocortical carcinoma cells in a concentration- and time-dependent manner, consistent with previous report [5]. This suggests that metformin may have a therapeutic effect on adrenocortical tumors.

Usually in cell experiments, the concentration of metformin that effectively inhibits cell proliferation (usually greater than 5 mM) is much higher than the plasma concentration in patients with metformin treatment (5–50 µM) [8]. A much larger dose of metformin may be required to achieve the same concentrations in tumor tissues as that in the cell experiments, which seems to limit the use of metformin in the treatment of tumors. However, it has been reported in the literature that in some tissues, the cumulative concentration of metformin is several times higher than in blood [9], such as the adrenal gland and liver [10]. The adrenal gland is one of the tissues with high levels of organic cation transporters 1 and 3 [11] which are responsible for cellular uptake of metformin [12]. In addition, metformin can specifically accumulate within mitochondria [13]. Therefore, some scholars speculate that in routinely treated type 2 diabetes patients, although the concentration of metformin in plasm was in micromolar levels, it may reach millimolar levels in the adrenal gland [5]. Some clinical studies have shown that metformin can decline androgen levels in patients with polycystic ovary syndrome [14, 15]. Steroid enzymes that catalyze androgen biosynthesis, including 17α-hydroxylase (CYP17A1) and 3β-hydroxysteroid dehydrogenase (HSD3β2), are overexpressed in ovarian cells of these patients. In H295R cells, metformin inhibits androgen production by decreasing the expression of HSD3β2 and the activity of CYP17A1 and HSD3β2 [8]. In vitro studies have also found that in granulosa cells of bovine ovaries, metformin can reduce the synthesis of progesterone and estradiol [16]. However, the effects of metformin on cortisol and aldosterone secretion in adrenocortical cells have not been reported so far. Our study found that metformin significantly inhibited the secretion of cortisol and aldosterone in H295R cells, but
only slightly inhibited the expression of CYP11B1 and CYP11B2 mRNA, indicating that the main inhibitory effect of metformin on secretion is due to its inhibition of cell proliferation.

Metformin can inhibit the proliferation of adrenocortical carcinoma H295R cells and secretion of cortisol and aldosterone. In view of the possible inhibitory effect on the growth of adrenal cortical tumors, metformin may have a therapeutic effect of reducing hormone secretion and improving clinical symptoms for functional adrenal tumors. Metformin is expected to become a new approach for clinical treatment. The specific mechanism of the effect of metformin on the proliferation and secretion functions of adrenocortical cells is not fully understood and needs further studies.

Conclusions

Metformin can inhibit cell proliferation and secretion of cortisol and aldosterone in H295R cells.

Statement

The abstract of this article had been presented as conference abstract in A5520 Effects of Metformin on cell proliferation and secretion function in adrenocortical carcinoma H295R cells [17].

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request

Competing interests

The authors declare that they have no competing interests involved.

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Authors’ contributions

F.W. drafted manuscript; C.-Y.L performed primary culture experiments; C.-Y.L and F.W. analyzed data; C.-Y.L and F.W. interpreted results of experiments; A.-L.T. design the research; C.-Y.L prepared figures; A.-L.T., Z.-L.Y. and Y.-X.L. edited and revised manuscript; A.-L.T., Z.-L.Y. and Y.-X.L. approved final version of manuscript.

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Figures

![Graph 1](image1)

![Graph 2](image2)

**Figure 1**

Effects of Metformin with different concentrations (A) and at different stimulating times (B) on the proliferation of H295R cells (N=3). *P < 0.001 compared with control
Figure 2

Effects of Metformin with different concentrations on the secretion of cortisol (A) and aldosterone (B) in H295R cells (N=3). *P < 0.05, **P < 0.01 compared with control

Figure 3

Effects of Metformin at different stimulating times on the secretion of cortisol (A) and aldosterone (B) in H295R cells (N=3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with control
Figure 4

Effects of Metformin on the mRNA expression of CYP11B1 (A) and CYP11B2 (B) in H295R cells (N=3). *P < 0.05 compared with control