Metformin Suppresses Proliferation and Viability of Rat Pheochromocytoma Cells

AB Min Li*
Xiuli Jiang*
Tingwei Su
Lei Jiang
Weiwei Zhou
Weiqing Wang

* Min Li and Xiuli Jiang equally contributed to this work

Corresponding Author: Weiqing Wang, e-mail: wqingw61@163.com

Source of support: This study was supported by the National International Science Cooperation Foundation (2015DFA30560) and the Shanghai Municipal Commission of Health and Family Planning (2013SV016)

Background: Previous studies have clearly demonstrated that metformin inhibits cell proliferation and cell growth in many types of human cancers. Increased survival rates in patients with breast and lung cancer receiving metformin have also been observed. However, the effect of metformin on pheochromocytoma cells remains unexplored.

Material/Methods: Rat pheochromocytoma cells (PC12 cells) were cultured and treated with metformin or vehicle control. Cell proliferation, cell-cycle, apoptosis, genes expression, and the signaling pathways involved were analyzed in PC12 cells.

Results: The metformin treatment reduced cell viability and proliferation in rat pheochromocytoma PC12 cells in a dose- and time-dependent manner. Furthermore, metformin exposure led to an increased apoptosis rate and cell-cycle arrest accompanied with downregulation of \( \text{Ccna2} \) and \( \text{Ccnb2} \). At the molecular level, the AMPK signaling pathway was activated, whereas the mTOR and ERK1/2 signaling pathways were inhibited by metformin.

Conclusions: Our data suggest an antiproliferative role of metformin in pheochromocytoma development, which may provide a novel option for future cancer therapy.

MeSH Keywords: AMP-Activated Protein Kinases • Cell Proliferation • Metformin • Pheochromocytoma • TOR Serine-Threonine Kinases

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/903348
Background

Pheochromocytoma, a neuroendocrine tumor originating in the medulla of the adrenal glands, usually causes paroxysmal hypertension, palpitations, diaphoresis, and weight loss, due to elevated catecholamine levels [1]. Genetic studies have demonstrated that mutations of several genes could increase the risk of pheochromocytoma, such as multiple endocrine neoplasia 2A, 2B (MEN2A and MEN2B), and von Hippel-Lindau (VHL) [2–4]. Although surgical resection of the tumor is the first choice of treatment, such surgery should be performed only at centers experienced in the management of this serious disorder. Therefore, an alternative candidate for its prevention and treatment is urgently required.

Metformin is one of the most widely used antihyperglycemic drugs and insulin sensitizers, which inhibits hepatic glucose production and enhances peripheral glucose uptake [5,6]. Interestingly, recent studies found that metformin has antiproliferative activity in many types of human malignancies, including breast, gastric, colon, and prostate, and renal cancers [7–10]. In addition, several retrospective reports showed that metformin is also associated with a decreased risk of developing cancer and cancer-related mortality [11]. Indeed, in vitro and in vivo analyses showed that metformin modulates many molecular pathways, including the activation of AMPK signaling and suppression of mTOR and PI3K/ATK signaling [12–14]. Furthermore, the expression of cell-cycle regulators, such as Ccnd1, Cdkn1b, and Cdkn1c, was also inhibited by metformin treatment [15–17]. However, whether metformin inhibits the development and progression of pheochromocytoma remains unknown.

In the present study, we aimed to examine whether metformin exerts an antitumor effect in PC12 cells, a rat pheochromocytoma cell line, and to analyze the underlying mechanisms.

Material and Methods

Ethics statement

This study was reviewed and approved by the Research Ethics Committee of Rui-jin Hospital, Shanghai Jiao-Tong University School of Medicine (Shanghai, China).

Cell culture and treatment

Rat pheochromocytoma cells (PC12 cells) were purchased from the Shanghai Institute of Biological Science (SIBS, Shanghai, China). The cells were maintained in RPMI-1640 (GBICO, Rockville, MD, USA) with 10% horse serum and 5% fetal calf serum at 37°C with infusion of 5% CO₂ and humidified air. Metformin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in milliQ water and applied at concentrations from 1 to 50 mmol/L. PC12 cells were cultured for 24 h, followed by treatment with metformin for different times and doses.

Cell viability and proliferation assays

PC12 cells at a density of 3×10⁴ per well were seeded on 96-well plates. PC12 cell proliferation curves were observed following the protocol of CCK-8 (Cell Counting Kit-8, Dojindo Molecular Technologies, Japan) after the application of different doses and times of treatment with metformin. Further, CCK-8 analysis was performed on day 3, when the cells were in the stationary phase of growth, to determine the survival rate of PC12 cells in the treatments with metformin or vehicle. The final absorbance of each well was determined at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Shanghai, China).

Flow cytometry experiments

To analyze cell proliferation, EdU staining was performed using Click-iT EdU Flow Cytometry Assay Kits (Invitrogen, Shanghai, China) according to the manufacturer’s protocols. Dispersed cells were plated in a 6-well dish. Forty-eight hours after the treatment with metformin, the cells were incubated with 10 μM EdU for another 4 h. Then, the cells were fixed and permeabilized, followed by EdU detection and treatment with PI staining before the analysis. To analyze cell-cycle progression, cells were harvested and immobilized in 70% ethanol at 4°C for 12 h, followed by washing with phosphate buffer solution (PBS). Then, the cells were incubated with RNase (10 μg/mL), resuspended in propidium iodide (50 μg/mL), and placed in a dark room. To analyze cell apoptosis, cells were stained with the Annexin V-FITC reaction reagent (5 μL of Annexin V-FITC and 5 μL of propidium iodide) and detected using an Accuri C6 flow cytometer.

RNA extraction and quantitative real-time PCR

A TRIzol Kit (Invitrogen, Shanghai, China) was used to extract total RNA from PC12 cells following the manufacturer’s protocols. cDNA was converted from 1 mg of total RNA by Reverse Transcription System (Promega, Madison, WI, USA). Quantitative real-time PCR analysis was conducted using SYBR Premix Ex Taq reagents (Takara, Shiga, Japan), and calculations were performed using the 2⁻ΔΔCt method. Rn18s were employed to normalize target genes expression. The gene primers were listed as follows: Ccnab2: F: 5’-GATGCCCTGGCTTTTAGTGC-3’; R: 5’-TGCTCGCTGCTCCTC-3’; Ccnb2: F: 5’-GACCGGCTCAAGTGGCTAAG-3’. R: 5’-CATTCACTGGCTT TTCGTCTTC-3’; Ccna2: F: 5’-GATGCCCTGGCTTTTAGTGC-3’; R: 5’-TCAGAG AAAGCTTGGCAGAGG-3’; Ccnb2: F: 5’-CATTCACTGGCTT TTCGTCTTC-3’; R: 5’-CATTCACTGGCTT TTCGTCTTC-3’.
**Figure 1.** Cell growth of PC12 cells. (A) Cell growth of PC12 cells treated with different doses of metformin (1, 2, 5, 10, and 50 mM) or vehicle for 48 h was analyzed by CCK-8 assay. (B–D) Cell growth of PC12 cells treated with different doses of metformin (2, 10, and 50 mM) or vehicle for different time periods (Day 1 to Day 6) analyzed by CCK-8 assay. (E) Cell survival rate of PC12 cells treated with different doses of metformin or vehicle analyzed by CCK-8 assay. The proliferation and survival rates of PC12 cells were decreased by metformin in a dose- and time-dependent manner.

**Statistical analysis**

Results from at least 3 independent experiments were analyzed by GraphPad Prism software (GraphPad Software, version 6.01) and expressed as mean ± standard error of the mean (SEM). The significant differences were determined using the Student’s t-test for two-group comparisons or the one-way ANOVA for multi-group comparisons. Statistical significance was accepted at *P < 0.05, **P < 0.01, or ***P < 0.001.

**Results**

**Metformin suppressed the viability and proliferation of PC12 cells**

To explore whether metformin affects the growth of pheochromocytoma cells, PC12 cells were exposed to different doses of metformin or vehicle. Cell growth was inhibited after 72 h of metformin treatment in a dose-dependent manner (Figure 1A).

PCNA: F: GGCGTGAGGCACTACAGAC AT-3';
R: 5'- CGGTTGAACCTACAGAC AT-3';
Cdkn1b: F: 5'-GTGTCCAGGGATGAGGAG C-3';
R: 5'-TCGGAGCTGTTTACGTCTGG-3';
Cdkn1c: F: 5'-TCGGAGCTGTTTACGTCTGG-3';
R: 5'-CTGAGCAGGTCTCTGAGCA-3';
Ccnd1: F: 5'-CGCACAACGC ACTTTCTTTC-3';
R: 5'-TTGGGATCGATGTTCTGCTG-3';
Ccnd3: F: 5'-AACCAC GCCCCTGACTATTG-3';
R: 5'-GGTCACTGGGCAGAGAGG-3';
Rn18s: F: 5'-ATTCGAACGTCTGCCCTATCA-3';
R: 5'-CGGGAGTGGGTAATTTGCA-3'.

Western blotting

Total proteins were prepared with an ice-cold lysis buffer containing 50 mM of Tris-HCl, 100 mM of 2-Mercaptoethanol, 2% w/v SDS, and 10% glycerol. Then, separation by 6% or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was done, transferred onto polyvinylidene fluoride (PVDF) membranes, followed by blocking by 10% bovine serum albumin (BSA). Next, the proteins were incubated with the following primary and secondary antibodies: ERK1/2, mTOR, AMPK, and GAPDH (Cell Signaling Technology, Beverly, MA, USA).
cell viability was significantly inhibited by the different doses of metformin, as evidenced by CCK-8 assays (Figure 1E). Consistently, the EdU flow cytometry analysis showed that the cell proliferation potential was suppressed by metformin treatment (Figure 2A–2E). These data suggest an inhibitory effect of metformin on the growth of pheochromocytoma cells.

Metformin caused cell-cycle arrest and increased cell apoptosis of PC12 cells

Next, cell-cycle progression and cell apoptosis were analyzed in PC12 cells administered with metformin or vehicle. Flow cytometry analysis also revealed a cell-cycle arrest at the G0/G1 phase of the PC12 cells treated with metformin (Figure 3A–3E). For apoptosis analysis, flow cytometry was performed after staining with Annexin V/PI, showing that the metformin treatment induced a higher apoptosis rate (Figure 4A–4E). Most importantly, the cell-cycle and apoptosis of PC12 cells were inhibited by treatment with metformin.

Modulation of cell-cycle regulators by metformin

Given that metformin inhibits cell-cycle progression in PC12 cells, we speculated that expression of cell-cycle regulators might be modulated by metformin treatment. Therefore, the mRNA levels of genes responsible for cell-cycle progression were determined by quantitative real-time PCR. Among them, Ccna2, Ccnb2, and PCNA were significantly downregulated by metformin treatment (Figure 5A), whereas other cell-cycle regulators, including Cdkn1b, Cdkn1c, Ccnd1, and Ccnd3, remained unaffected (Figure 5B). Therefore, the expression levels of certain cell-cycle regulators may be modulated by metformin treatment of PC12 cells.

Signaling pathways affected by metformin

It has been shown that several pathways are involved in the execution of the suppressive function of metformin on tumor growth, such as the activation of AMPK signaling and suppression of mTOR signaling [12–14]. The Western blot analyses in our study revealed that AMPK phosphorylation was enhanced in PC12 cells treated with metformin (Figure 6A, 6B). Moreover, phosphorylated ACC, a downstream target of AMPK, was also induced by metformin (Figure 6A, 6B). On the other hand, phosphorylated mTOR and ERK1/2 levels were reduced (Figure 6A, 6B), suggesting that these downstream signaling pathways were inhibited in the PC12 cells upon metformin treatment. Therefore, the regulation of AMPK, mTOR, and...
ERK1/2 pathways might be responsible for the antitumor effects of metformin on pheochromocytoma cells.

**Discussion**

Although the biological roles of metformin have been explored in many types of human cancers, its effect on the growth of pheochromocytoma cells remains poorly understood. In the present study, using in vitro models, we showed that metformin inhibited cellular proliferation in rat pheochromocytoma cells. This finding is supported by several lines of evidence. First, cell growth rate and proliferation ability were decreased in a dose- and time-dependent manner after treatment with metformin. Second, the cell-cycle was arrested in the G0/G1 phase, accompanied by changes in the expression of related genes. Third, Annexin V/PI staining analysis confirmed that metformin treatment increased the cell apoptosis rate. Therefore, the remarkable suppressive efficiency of metformin on pheochromocytoma cell proliferation and growth might have important implications for the treatment of this serious disease. However, in vivo studies, such as xenograft models, are needed to further determine the anti-proliferative effect of metformin on pheochromocytoma cells.

At the molecular level, our results indicate that Ccna2 and Ccnb2, which are 2 critical regulators of cell-cycle progression, are downregulated by metformin and may be intracellular targets of its antiproliferative activities. Ccna2 is expressed at the onset of S phase and is involved in the G2/M transition [18], whereas Ccnb2 was shown to control the proper timing of centrosome separation and transforming growth factor beta-mediated cell-cycle transition [19,20]. It has been well-established that the expression levels of these 2 cyclin proteins are significantly upregulated in many types of human cancers, suggesting that their inhibition might be beneficial to the control of cancer cell proliferation. It should be noted that our data suggest that other important cell-cycle regulators, including Ccnd1, Cdkn1b, and Cdkn2c, were not affected by metformin treatment. Previous studies have shown that metformin exerts an antitumor effect in breast and prostate cancer cells through a decrease of cyclin D1 level [21]. Cai et al. reported that metformin induced G0/G1 phase arrest in esophageal squamous cell carcinomas, which was accompanied by the upregulation of p21 and p27 [22]. Moreover, Rodriguez-Lirio et al. demonstrated that metformin blocked leukemia cell proliferation by inducing arrest of S and G2/M phases through downregulation of cyclin A and cyclin B1, but not that of cyclin E [23].
Although the reason for this inconsistency remains unknown, we speculate that the molecular targets of metformin might be cell- or tissue-specific.

Mechanistically, initial studies found that metformin activates the AMPK pathway, which is essential for the antiproliferative effects of metformin [24]. However, subsequent studies showed that genetic inhibition of the AMPK pathway did not inhibit the effect of metformin, suggesting that additional mechanisms are involved [25]. For instance, it has been shown that metformin regulates several intracellular signaling pathways, including mTOR, ERK, and PI3K/AKT. Here, we found that metformin also activates phosphorylated AMPK, whereas it inhibits phosphorylated mTOR and ERK1/2. Therefore, we speculate that the effect of metformin on pheochromocytoma cell growth, at least in part, is dependent on the modulation of these signaling pathways.
Figure 6. Downstream signaling pathways in PC12 cells. (A) Representative protein levels of phosphorylated AMPK, mTOR, and ERK1/2 in PC12 cells treated with metformin or vehicle control, as indicated. Total AMPK, mTOR, ERK1/2, and GAPDH were used as loading controls. (B) Statistical histogram of the relative protein levels of phosphorylated AMPK, mTOR, and ERK1/2. AMPK phosphorylation was activated by metformin in PC12 cells, whereas the activation of mTOR and ERK1/2 was inhibited.

In summary, we evaluated the antiproliferative effects of metformin in pheochromocytoma cells. Given that metformin has a superior safety profile and few adverse effects [26], it will be of crucial importance to determine its effects in patients with pheochromocytoma.

Conclusions

Metformin treatment reduced cell viability and proliferation ability of PC12 cells in a dose- and time-dependent manner. Metformin also increased the apoptosis rate and induced cell-cycle arrest, accompanied by downregulation of Ccna2 and Ccnb2. At the molecular level, AMPK, mTOR, and ERK1/2 signaling pathways were regulated by metformin. Taken together, our data suggest a previously unknown role of metformin in pheochromocytoma development, which might provide a novel option for future cancer therapy.

Conflict of interest

None.
References:

1. Favier J, Amar L, Gimenez-Roqueplo AP: Paraganglioma and phaeochromocytoma: From genetics to personalized medicine. Nat Rev Endocrinol, 2015; 11(2): 101–11

2. Bravo EL, Tagle R: Pheochromocytoma: State-of-the-art and future prospects. Endocr Rev, 2003; 24(4): 539–53

3. Dahia PL: Pheochromocytoma and paraganglioma pathogenesis: Learning from genetic heterogeneity. Nat Rev Cancer, 2014; 14(2): 108–19

4. Burnichon N, Buffet A, Gimenez-Roqueplo AP: Pheochromocytoma and paraganglioma: Molecular testing and personalized medicine. Curr Opin Oncol, 2016; 28(1): 5–10

5. Madiraju AK, Erion DM, Rahimi Y et al: Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. Nature, 2014; 510(7506): 542–46

6. Miller RA, Chu Q, Xie J et al: Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. Nature, 2013; 494(7436): 256–60

7. Chae YK, Arya A, Malecek MK et al: Metformin in rat pheochromocytoma cells. Med Sci Monit, 2017; 23: 29–37

8. Ben Sahra I, Laurent K, Loubat A et al: Metformin inhibits growth inhibition and cell cycle arrest by upregulating MicroRNA34a in renal cancer cells. Med Sci Monit Basic Res, 2016; 22: 27–33

9. Sośnicki S, Kapral M, Węglarz L: Molecular targets of metformin antitumor action. Pharmacol Rep, 2016; 68(5): 918–25

10. Li M. et al.: Metformin in rat pheochromocytoma cells © Med Sci Monit, 2017; 23: 3253-3260

11. Rodríguez-Lirio A, Pérez-Yarza G, Fernández-Suárez MR et al: Metformin induces cell cycle arrest and apoptosis in drug-resistant leukemia cells. Leuk Res Treatment, 2015; 2015: 516460

12. Zakikhani M, Dowling R, Fantus IG et al: Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer Res, 2006; 66(21): 10269–73

13. Steber CJ, Perkins SL, Harris KB: Metformin-Induced fixed-drug eruption confirmed by multiple exposures. Am J Case Rep, 2016; 17: 231–34