Molecular and Cellular Endocrinology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Metformin suppresses growth and adrenocorticotrophic hormone secretion in mouse pituitary corticotroph tumor AtT20 cells

Kai Jin, Lunliang Ruan, Jiujun Pu, Ailing Zhong, Fuchao Wang, Song Tan, Hua Huang, Jiamin Mu, Gang Yang*

Department of Neurosurgery, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China

ARTICLE INFO

Keywords:
Pituitary corticotroph tumors
Metformin
Apoptosis
AMP-Activated protein kinase(AMPK)
Insulin-like growth factor-1 receptor(IGF-1R)

ABSTRACT

Pituitary corticotroph tumors lead to excess adrenocorticotrophic hormone (ACTH) secretion, resulting in Cushing's disease (CD), which is associated with significant mortality. Standard treatments include neurosurgery, radiotherapy and medical therapy. Both surgery and radiotherapy have undesirable complications and high recurrence rates. At present, there is only one medical option available that targets pituitary adenoma and ACTH secretion, the drug pasireotide. However, hyperglycemia is common during pasireotide treatment. In addition, some patients have discontinued pasireotide treatment because of hyperglycemia-related adverse events or uncontrolled diabetes. New medical treatments directly targeting the corticotroph cells and suppressing ACTH secretion are urgently required. Metformin is a commonly used antidiabetic drug that has been widely used to control the hyperglycemia that occurs in patients with CD, which is secondary to both cortisol excess and pasireotide treatment. Recent studies suggest that metformin has direct anticancer activities against many tumor cell lines. In the present study, we investigated whether metformin exerts an anti-tumor effect by directly targeting pituitary corticotroph tumors and exploring the underlying mechanisms. Using the mouse corticotroph tumor cells, AtT20 cells, we report that metformin inhibited cell proliferation, promoted cell apoptosis and decreased ACTH secretion but did not block the cell cycle in cells. The apoptosis induced by metformin was accompanied by increased caspase-3 activity. Meanwhile, metformin down-regulated the anti-apoptotic protein B cell lymphoma 2 (Bcl-2) but up-regulated the pro-apoptotic protein Bcl2-associated X (BAX), which suggests the involvement of the mitochondrial-mediated apoptosis pathway. Furthermore, metformin promoted AMP-activated protein kinase (AMPK) phosphorylation but inhibited insulin-like growth factor-1 receptor (IGF-1R) expression, protein kinase B (PKB/AKT) phosphorylation and mammalian target of rapamycin (mTOR) phosphorylation. Finally, the IGF-1R inhibitor picropodophyllin (PPP) significantly inhibited the cell proliferation of AtT20 cells. We conclude that metformin inhibits cell proliferation and induces apoptosis in AtT20 cells by activating AMPK/mTOR and inhibiting IGF-1R/AKT/mTOR signaling pathways. Metformin may have direct antitumor activity against pituitary corticotroph tumors.

1. Introduction

Pituitary corticotroph tumors, composing 10–15% of pituitary adenomas (Gong et al., 2014), result in Cushing's disease (CD) due to the excessive secretion of adrenocorticotrophic hormone (ACTH), which is associated with increased morbidity and mortality (Shimon et al., 2012). CD leads to severe symptoms caused by excessive cortisol secretion including central obesity, muscle weakness and easy bruising and fatigue (Cannavo et al., 2016). CD also often causes hypertension, osteoporosis, hyperglycemia and other complications (Bangaru et al., 2010). Pituitary surgery, especially transsphenoidal microsurgery, is the primary therapy for pituitary corticotroph tumors, but the complication of hypopituitarism is common (reported in up to 80% of patients), and reported recurrences rise to 20–25% of patients (Gong et al., 2014). In addition, some patients have unresectable invasive tumors and may be at high surgical risk. Radiotherapy or medical therapy are considered when surgery is not an option or unsuccessful. Considering that the treatment effects of radiotherapy are often delayed and the adverse effects of brain irradiation such as hypopituitarism are common (Colao et al., 2014; Ceccato et al., 2015), medical therapy may be a better choice. However, many of the available drugs are experimental; only pasireotide has been approved by both the European

* Corresponding author. Department of Neurosurgery, The First Affiliated Hospital of Chongqing Medical University, 1 Youyi Road, Chongqing 400016, China.
E-mail address: yg_angcq@163.com (G. Yang).

https://doi.org/10.1016/j.mce.2018.07.007
Received 20 January 2018; Received in revised form 9 July 2018; Accepted 15 July 2018

Medical Agency and the US Food and Drug Administration (Shimon et al., 2012; Cannavo et al., 2016; Colao et al., 2014). Although pasireotide is the first medicine that directly targets pituitary corticotroph tumors and has achieved beneficial curative effects by reducing tumor volume and ACTH secretion (Colao et al., 2014), the side effects cannot be ignored, such as diarrhea, nausea, abdominal pain, hypotension, asthenia and hyperglycemia (Ceccato et al., 2015). The most serious side effect is hyperglycemia, which is especially common (affecting 73% of the patients involved in phase 3 clinical trials) (Colao et al., 2012, 2014). In addition, 6% of patients stop taking pasireotide due to hyperglycemia-related adverse events or uncontrolled diabetes mellitus (Ceccato et al., 2015; Colao et al., 2012). Besides, it is difficult to normalize hormone levels with a single pasireotide treatment in the majority of patients (Ceccato et al., 2015). Furthermore, pasireotide has not yet been approved for use in humans in most countries. Therefore, a new medical treatment directly targeting corticotroph tumors and ACTH secretion is urgently needed.

Metformin is an oral hypoglycemic drug for the treatment of type 2 diabetes mellitus (T2DM) that also plays a crucial role in the management of blood glucose and weight of patients with CD. How to control the hyperglycemia induced by pasireotide treatment has recently become a research focus in the management of CD (Colao et al., 2014; Ceccato et al., 2015). Recently, European experts in pituitary disease and diabetes mellitus recommend that the medical treatment for managing pasireotide-induced hyperglycemia should begin with metformin (Colao et al., 2014). A study reported that there is a glucose-lowering effect when pasireotide treatment is used together with oral metformin compared with pasireotide alone (Breitschaft et al., 2014). Metformin is also used to control the high risk of developing impaired glucose tolerance (IGT) and diabetes due to increased insulin resistance secondary to hypercortisolism in patients with CD (Colao et al., 2014; Ceccato et al., 2015). In a word, metformin has been widely used to control the hyperglycemia occurring in patients with CD, which is secondary both to cortisol excess and to pasireotide treatment.

Recent studies have reported that metformin can exhibit direct antitumoral effects against many tumor cells (Lei et al., 2017; Karnevi et al., 2013; Han et al., 2015; Sośnicki et al., 2016). The anti-tumor mechanism of metformin is being researched and mainly includes the activation of AMP-activated protein kinase (AMPK), thus inhibiting the mammalian target of rapamycin (mTOR) pathway (Lei et al., 2017; Karnevi et al., 2013; Han et al., 2015). Both the activation of AMPK and the inhibition of mTOR play important roles in tumor growth inhibition for some antineoplastic agents (Lei et al., 2017; Karnevi et al., 2013; Han et al., 2015). Some studies also indicate that metformin has antitumor effects involving the inhibition of insulin like growth factor 1 receptor (IGF-1R) signaling by reducing the expression of IGF-1R and repressing its downstream signaling protein kinase B (AKT) and mTOR (Lei et al., 2017; Karnevi et al., 2013; Zi et al., 2015). The IGF-1R signaling pathway is a pivotal signaling pathway for tumor cell survival and proliferation (Karnevi et al., 2013; Zi et al., 2015). New research about the genome-wide mutational landscape of pituitary adenomas suggests that, as a downstream target of IGF-1R, phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR pathway is preferentially targeted by mutations and considered a potential drug target in ACTH-producing pituitary adenomas (Song et al., 2016).

Metformin has the properties of low cost, easy availability and good security in that it has been clinically used to manage hyperglycemia for patients with CD. Therefore, it is necessary to explore whether metformin is an antitumor agent for pituitary corticotroph tumors simultaneously. However, there is hardly any study that has attempted to demonstrate the antitumor effects of metformin in pituitary corticotroph tumors. In the present study, we evaluated the growth suppressive effect of metformin on AtT20 mouse corticotroph tumor cells. We also report that the antitumor mechanism of metformin involved activation of the AMPK and inhibition of the IGF-1R signaling pathways. In addition, metformin potently suppressed ACTH secretion was also explored.

2. Materials and methods

2.1. Reagents and drugs

Metformin was purchased from Sigma-Aldrich (MO, USA), dissolved in phosphate buffered saline (PBS) and stored at −20 °C. An ELISA Kit for ACTH was purchased from Cloud-Clone Corp (USCN Life Science Inc; Wuhan, China). A cell counting kit-8 (CCK-8) was purchased from Bimake (Shanghai, China). Picropodophyllin (PPP; a selective IGF-1R inhibitor) was purchased from Selleck (Shanghai, China), dissolved in dimethyl sulfoxide (DMSO) and stored at −80 °C. The following primary antibodies (rabbit, anti-mouse) were used: anti-caspase-3 and anti-phospho-mTOR (p-mTOR) (Ser2448) polyclonal antibodies (pAbs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-bcl-2 associated X (Bax) polyclonal antibody (pAb) was purchased from Sangon Biotech (Shanghai, China); anti-bcl-2 and anti-β-actin pAbs were purchased from Proteintech Group (Wuhan, China); anti-cleaved caspase-3, anti-AKT (protein kinase B), anti-ACTH and anti-AMPKα1/2 pAbs were purchased from Wanleibio (Shenyang, China); anti-mTOR and anti-IGF-1R monoclonal antibodies were purchased from Abcam (MA, USA); anti-phospho-AKT (p-AKT) (S473) pAb was purchased from R&D Systems (Minnesota, USA); and anti-phospho-AMPKα (p-AMPKα) (Thr172) pAb was purchased from Cell Signaling Technology (CST; MA, USA). Horseradish peroxidase-labeled goat anti-rabbit IgG (H + L) was purchased from ABclonal (Boston, MA, USA). Annexin V-fluorescein isothiocyanate/propidium iodide (annexin V-FITC/PI) apoptosis detection kit and ribonuclease A (RNase A) were purchased from Solarbio (Beijing, China).

2.2. Cell culture

AtT20 cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). AtT20 cells were cultured in RPMI-1640 medium (HyClone, UT, USA) supplemented with 100 mL/L fetal bovine serum (PAN Biotech GmbH; Aidenbach, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (Beyotime Biotechnology; Shanghai, China) at 37 °C in a humidified incubator with 50 mL/L CO_2.

2.3. Cell viability assay

Cell proliferation was evaluated using the CCK-8 assay. AtT20 cells in log phase were seeded (2×10^4/well) into 96-well plates and incubated with different concentrations of metformin (0, 2.5, 5, 10, 20, 30 and 40 mM) at 37 °C with 5% CO_2. After incubation for 24, 48 and 72 h, 10 μl CCK-8 were added to each well followed by 4 additional hours of incubation. The optical density (OD) at 450 nm was measured using a microplate reader (Thermo, MA, USA). The cell viability was calculated as follows: [(OD of treated cells-OD of blank)/(OD of control-OD of blank)] ×100%. To calculate the IC50 value of metformin, the GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) was used. The experiments were conducted in five replicates and repeated thrice.

To determine the effect of PPP (IGF-1R inhibitor) on the proliferation of AtT20 cells, cells were divided into the following groups: a blank control group, DMSO (concentration used was < 1 ml/L) group and different concentrations of PPP (0.1, 1 and 10 μM) groups. Cells were treated for 48 h. The OD was measured as described above.

2.4. Cell cycle analysis

AtT20 cells were seeded into 6-well plates at a density of 6×10^5/well and incubated with serum-free medium for 12 h. Then, the medium was replaced with fresh complete RPMI medium containing the indicated concentrations of metformin (0, 5, 10 and 20 mM) and