Cordycepin modulate body weight by reducing prolactin via an adenosine A₁ receptor

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Abstract

Cordycepin is an extract from the insect fungus *Cordyceps. militaris*, which is a traditional medicine with various biological function. In previous studies, cordycepin had been reported with excellent anti-obesity effect, but the mechanism is unclear. A large quantity of evidences showed that prolactin plays an important part in body weight regulation, hyperprolactinemia can promote appetite and accelerate fat deposition. In this study, we explored the molecular mechanism of the anti-obesity effect of cordycepin by reducing prolactin release via an adenosine A₁ receptor. *In vivo*, obese rats model was induced by high fat diet for 5 weeks, the serum and liver lipids coupling with serum prolactin were reduced by treatment of cordycepin, the results suggested that cordycepin is a potential drug for therapying obesity which could be related with prolactin. *In vitro*, cordycepin could inhibit prolactin secretion in GH₃ cells via upregulating the expression of adenosine A₁ receptor, the inhibition effect could be blocked by an antagonist of adenosine receptor A₁ DPDPX, prolactin induced the upregulation of lipogenesis genes PRLR, and P-JAK2 in 3T3-L1 cells. Intriguingly, cordycepin would down-regulate the expression of prolactin receptor (PRLR). Thus, we concluded that cordycepin modulate body weight by reducing prolactin release via an adenosine A₁ receptor.

Key Works: Cordycepin, Adenosine A₁ receptor, prolactin, anti-obesity

1. Introduction

Obesity is a worldwide chronic metabolic disease caused by excessive accumulation of fat intake due to the imbalance of energy expenditure. Almost 2 billion people worldwide are overweight or obese and the number will reach 20% of the population by 2025 [1]. In China, the prevalence of overweight among men was 33.8% (33.7 - 33.9%) according to Chinese criteria (BMI $\geq$ 24.0 kg/m²), the rates of obesity were 6.3% (6.2 - 6.4%; BMI $\geq$ 28.0 kg/m²) [2]. In USA, data showed that one-third of adults are obese, adolescent obesity reached 17% of the teenagers (defined as a BMI > 95th percentile), meanwhile, 15% of the adolescents in Europe have obesity (defined as a BMI > 97th percentile) which affect not only adolescent but also the society [3]. Since obesity is

multifactorial, improper dietary patterns, endocrine dyscrasia, steroid hormones, genetic obesity, antibiotics abuse may cause obesity [4]. Various diseases such as diabetes, cardiovascular disease (CVD), certain cancers, NAFLD, osteoarthritis, premature death, pregnancy complications and chronic diseases in the offspring were proved to be linked with obesity [5-10]. Considering the public health threat coupled with the overwhelming obesity prevalence rate, the disease is a huge mental and economic burden for not only developed countries but also developing countries[11]. Life-style modifications may not meet disease's progress coupling with severe side-effect of current anti-obesity drugs, there is an urgent need to reveal obesity pathogenesis and complications and devote to develop high efficiency and low toxicity therapeutic drugs.

Prolactin (PRL) is a 23 k Da protein hormone, which is a multifunctional hormone produced in humans by both pituitary and extra-pituitary sites, including adipose tissue [12]. Previous study showed that a disorder in prolactin secretion occurs in obesity individuals [13]. Nira Ben-Jonathan reviewed that PRL affects hypothalamic orexigenic and anorexigenic systems that regulate appetite [14]. Recent work done by Julien Auffret and his colleague elucidated a lack of prolactin receptor (PRLR) may causes resistance to high-fat-diet-induced obesity and may increase metabolic rate [15]. Adenosine had been reported to inhibit prolactin secretion from GH₃ cells via an Adenosine A₁ receptor pathway, then we targeted adenosine to regulate PRL hormone secretion to treat and prevent obesity. Adenosine receptors had been reported to play an essential role from neuromodulation to immune regulation, also vascular function and metabolic control [16]. 4 types of adenosine receptor had been elucidated include A₁, A_{2A}, A_{2B} and A₃, A₁ and A₃ receptors are G I protein-coupled cell membrane receptors, cordycepin exerts inhibitory effect on the proliferation of mouse melanoma and lung carcinoma cells via adenosine A₃ receptors. A_{2A} and A_{2B} receptors are G s protein-coupled receptors, which stimulated adenylated cyclase and cAMP increase [17]. Among all these receptors, Adenosine A₁ receptor is most conserved subtype, and expressed throughout the body with the highest levels found in the brain, especially the secretory system [18]. In our study, we proved that adenosine A₁ receptor could inhibit prolactin release.

Cordycepin (3'-deoxyadenosine), main component of traditional Chinese herb *Cordyceps militaris*, has been shown with many biological activities like selective interruption of nucleolar RNA synthesis, promote cell differentiation, antibacterial, antifungal anti-tumor, anti-inflammation, anti-adipogenesis, anti-apoptosis biological activity via an adenosine transporter pathway [19-23]. Takahashi reported cordycepin can block insulin-PKB/AMPK induced mTORC1-C/EBP β -PPAR γ pathway to suppress adipogenesis and lipid accumulation in mature adipocytes via an adenosine transporter mechanism *in vitro* [24]. As an analogue of adenosine, the anti-obesity function of cordycepin mediated by adenosine receptor has not been revealed.

In this study, we show that cordycepin can reduce body weight via an adenosine receptor pathway to block prolactin to suppress lipid storage and reduce blood lipid accumulation.

2. Results

2.1. Cordycepin reduce body weight and hyperlipidemia *in vivo*

To investigate the anti-obesity effect of cordycepin *in vivo*, 50 rats were randomly divided into 5 groups as NFD groups fed with normal diet, HFD groups fed with high fat diet, cordycepin treatment groups included high concentration with 50 mg/kg/d cordycepin intragastric administration and high fat diet, media concentration groups and low concentration groups treated by the same drug delivery way and high fat diet with 25 mg/kg/d and 12.5 mg/kg/d cordycepin, each rat was weighted every

week for 5 weeks. Blood were taken from caudal vein for lipids detection after rats were killed, white adipose tissue around the epididymis and kidney were taken for fat coefficient calculation, liver tissue and adipose tissue were used for H.E staining (fig 1). Cordycepin treatment can significantly reduce high fat diet induced body weight gain and fat coefficient with the dosage of 50 mg/kg/d and 25 mg/kg/d, but low dosage showed slight effect (fig 1a). High fat diet can induce hyperlipidemia in rats with higher serum total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), and lower high-density lipoprotein (HDL) which were reversed by cordycepin treatment higher than 25 mg/kg/d (fig 1b). Aspartate aminotransferase activity (AST) and alanine aminotransferase activity (ALT) as biomarkers of liver function, maleic dialdehyde (MDA) and superoxide dismutase (SOD) reflect antioxidant capacity of the liver. Cordycepin treatment can significantly reduce obese rat serum AST, ALT, and MDA level and elevate SOD level, which suggested that cordycepin can regulate fat metabolism and improve free radical scavenging and oxidation resistance (fig 1c, d). TC and TG level in liver tissue proved that cordycepin treatment can remove liver fat and cholesterol accumulation in liver tissue (fig 1e). Damage induced by obesity on adipocyte and hepatocyte were observed through H.E staining, cordycepin can maintained the normal size of the adipocyte and reduce the necrosis and inflammatory infiltration of hepatocytes (fig 1f). From the above conclusions, we showed that cordycepin can reduce rat obesity and hyperlipidemia *in vivo*.

2.2. Cordycepin downregulate prolactin secretion via adenosine A₁ pathway in vivo and vitro

Cordycepin could reduce rat obesity and hyperlipidemia *in vivo*, the molecular mechanism is under revealed. In our previous study, an increased prolactin secretion was detected in high fat diet rats, but not cordycepin treated groups (fig 2a). Then we made a hypothesis that cordycepin may work on prolactin as an adenosine agonist to reduce obesity. Previous studies showed adenosine can inhibits prolactin secretion from GH₃ cells via adenosine A₁ receptors. As an analogue of adenosine, we tested whether cordycepin could reduce prolactin secretion by binding to adenosine A₁ receptor. GH₃ cells were cultured and treated with different concentrations of cordycepin suspension to detect prolactin release. CCK8 cytotoxicity tested the minimum safety concentration of cordycepin was 25 µg/ml, the results showed that the survival rate of CH₃ cells were 75% when treated with cordycepin in 100 g/ml and 87% in 50 g/ml (fig 2b). Thus, cordycepin in 0, 6.25, 12.5, 25 µg/ml concentration were used respectively, each can induce adenosine A₁ receptor expression in a dose dependent manner (fig 2c). An adenosine A₁ receptor agonist (R-PIA) can mimic cordycepin to reduce prolactin secretion, the inhibiting effect could be blocked by an antagonist of adenosine A₁ receptor DPCPX, but when DPCPX added into the groups treated with cordycepin in different concentration, the DPCPX didn't work. The cordycepin concentration of 25 µg/ml could significantly reduce prolactin secretion and inhibit DPCPX function (fig 2d).

Prolactin secretion is related with activation of prolactin-R and the associated signal transduction pathways, of which Ras/Raf/MAPK/ERK pathway may play an import role [25]. Activation of Ras/Raf/MAPK/ERK pathway may progress the secretion of prolactin. Down regulate ERK1/2 gene may block prolactin-R signaling to reduce prolactin release. Then we tested P-ERK, P-PI3K, and P-AKT protein expression level using western blot method, the results showed that cordycepin can block ERK1/2 expression through and induce P-PI3K and P-AKT expression in 25 µg/ml concentration (fig 2e). Collectively, our results showed that cordycepin could downregulate prolactin secretion via an adenosine A₁ receptor pathway.

2.3. Cordycepin reduce adipogenesis by reducing prolactin *in vitro*

Previous studies showed prolactin may correlated with obesity. In this study, we cultured 3T3-L1 cell lines treated with prolactin, observation under fluorescent microscope showed that high concentration of prolactin can significantly promote the differentiation of 3T3-L1 cells into mature adipocytes (fig 3a). Then we tested the prolactin secretion associated protein PRLR and JAK2 in adipocytes, the western-blot results showed that both PRLR and JAK2 were significantly induced by prolactin in a dose-dependent manner (fig 3b). Cordycepin treatment may suppress PRLR expression but not JAK2, which explained that cordycepin could reduce lipid accumulation by suppressing a PRLR binding pathway.

3. Discussion

Obesity is a widespread chronic metabolic disease. In our study we found that cordycepin can significantly reduce body weight and hyperlipidemia in high fat diet induced obese rats. Further, we explored the molecular mechanism of cordycepin works in anti-obesity *in vivo* and *in vitro*. We build high fat diet induced obese rats as animal model which were most close to human obesity. Cordycepin, purity is greater than 99%, were prepared as suspension with different concentrations include 12.5 mg/kg/d, 25 mg/kg/d, and 50 mg/kg/d, Serum and liver TC, TG increased as body weight gain caused expansion of adipose tissue and local inflammation which promote insulin resistance [26], resulted with increased free fatty acids into circulation and liver fatty acid in hepatocytes, which is a high risk factor for NAFLD [27], cordycepin treatment can significantly reduce TC and TG level in both serum and liver compared with obese rats. We concluded that cordycepin may promote lipolysis to reduce excess fat from blood and liver. Serum LDL takes cholesterol to arteries which could lead to a buildup of plaque known as atherosclerosis, further may cause stroke and heart attack, HDL helps rid excess cholesterol from liver. Here, we confirmed that cordycepin may down regulate LDL and increase HDL level to avoid hyperlipidemia caused atherosclerosis [28]. Previous studies showed serum (aspartate aminotransferase) AST level, serum ALT (alanine transaminase) level, and their ratio (AST/ALT ratio) are commonly measured clinically as biomarkers for liver health, these enzyme is crucial in both amino acid degradation and biosynthesis [29]. Obesity has been identified as an important factor of elevated AST and ALT [30]. In our study, cordycepin could reduce both AST and ALT level to maintain liver function and prevent lipid accumulation. MDA (Malondialdehyde) is one of several by-products of lipid peroxidation processes which is a biomarker providing an indication of lipid peroxidation level reflected in obesity [31]. SOD (superoxide dismutase) play an important role in effectively suppressing free radical oxidation damage and prevent diseases and balance the metabolism process of oxygen free radical and lipid peroxidation reaction [32]. Cordycepin could suppress MDA level and induce SOD, which indicated a robust antioxidant activity of cordycepin. Adipocyte and hepatocyte may suffer a chronic damage in development of obesity, dysfunction and finally death of adipocyte and excess accumulation of fat in liver caused steatohepatitis were rescued by cordycepin in our H.E staining experiment. Cordycepin could avoid adipocyte from morphological changes and hepatocyte from vacuolar degeneration and inflammatory infiltration. Collectively, cordycepin could effectively reduce body weight and hyperlipidemia.

Obesity has been proved to be associated with increased prolactin release. Prolactin is a pituitary hormone with various function beyond lactotrophic, a lot of studies showed that prolactin may has an important role in determining the metabolism of fat [33, 34]. Roelfsema *et al.* researched about

prolactin secretion in healthy adults in normal condition showed that prolactin release is positive correlated with BMI, and age, but not gender [35]. Jonathan *et al.* review that prolactin as a metabolic hormone mediated whole balance of fat consumption and accumulation [36]. Barrett *et al.* proved that adrenergic hormones and extra-pituitary prolactin (e-PRL) may be the two signals in determining overall lipid turnover/accumulation in adipose [37]. Based on the researches above, we found an obvious rise of prolactin in high fat diet fed rats, which could be blocked by cordycepin. Then we tested the inhibiting ability of cordycepin against prolactin *in vivo*. As an analogue of adenosine, cordycepin may work through an adenosine receptor. Adenosine has long been reported to function in many diseases, which contributing the block for nucleic acids and provide biological energy currency ATP, works through four adenosine receptor signaling pathway. Adenosine receptors had been reported everywhere, A₁ receptor is most conserved and functioning in increasing intracellular calcium, blocking neurotransmitter release, negative chronotropic in heart. A_{2A} receptor modulate inflammation, myocardial oxygen, angiogenesis, the flow of coronary and pathogenesis of cancer [38]. A_{2B} receptors play when adenosine levels elevated by conditions of hypoxia, ischemia or inflammation. A₃ receptors highly expressed in blood when people suffering from rheumatoid arthritis, Crohn's disease and colon cancer, demonstrated an anti-inflammatory, anticancer and cytoprotective effect [39]. Among these adenosine receptors, Delahunty and his colleague found that analogues of adenosine could suppress prolactin release of GH3 cell lines by binding to an A₁ receptor, they found that A₁ receptors could enhance cAMP accumulation by vasoactive intestinal peptide (VIP), which could increase and TRH and stimulate prolactin release [40]. In our study, we found that cordycepin stimulate expression of adenosine A₁ receptor in a dose dependent manner. Blockade of adenosine A₁ receptor by DPCPX (an inhibitor of adenosine A₁ receptor) could promote the release of prolactin. By contrast, stimulated by R-PIA (an agonist of adenosine A₁ receptor) could suppress prolactin release. In all, we concluded cordycepin inhibit prolactin release by binding to an adenosine A₁ receptor.

Previous studies showed that cordycepin could suppress adipogenesis and lipid accumulation by an adenosine transporter pathway but not adenosine receptors, the authors proves that cordycepin could suppress the adipogenesis genes C/EBP β -PPAR- γ by inhibiting insulin/IRS-1-PKB/mTOR path way [41]. Meanwhile, we explored the anti-adipogenesis effect declined prolactin induced by cordycepin. 3T3-L1 pre-adipocytes were treated with prolactin in different concentration, fluorescence staining showed that 50 ng/mL prolactin significantly caused differentiation of pre-adipocytes to lipid-laden adipocytes, prolactin function through a prolactin receptor expression, the related genes PRLR and P-JAK2 expression in 3T3-L1 were upregulated by treating with prolactin, and these expressions would be blocked by cordycepin, which indicated that cordycepin suppress adipogenesis via prolactin inhibition. Coupling with reported studies, we conclude that cordycepin could suppress adipogenesis and lipid accumulation by two distinct pathways as an adenosine analogue (fig 4). The first is adenosine transporter pathway through blocking adipocyte signal transmission and inhibit adipogenesis genes C/EBP β -PPAR γ , the other is adenosine receptor pathway via suppressing prolactin release by binding to an adenosine A₁ receptor. Considering all these studies, obesity is multifactorial, including hormones, genes, drugs, environment, life style and so on, etiology and drug need equal deep research in the future. The relation between adipocytokines such as leptin, adiponectin, and IL6 with prolactin should be further explored in the next step.

As reported everywhere, gut microbiota can be related with obesity, high fat diet may change the

composition of gut microbiota and its metabolites, including short-chain fatty acids, trimethylamine N-oxide, and lipopolysaccharides which could act on downstream of cellular target, thus may involve in adiposity [42]. Transfer of “obese” microbiota can include adiposity suggesting that gut microbiota may affect host molecular pathway that need further explore [43]. Our previous study showed that cordycepin could change the ratio of *Bacteroidetes* and *Firmicutes*, and increase the abundance of both bacteria, but the molecular mechanism need to be further confirmed. To the end, cordycepin may modulate adiposity through intestinal microbiota.

The one thing need to be emphasized, *Cordyceps militaris* has long been used as nourishing food, whose work is slow, as the main component of *Cordyceps militaris*, cordycepin works short according to the pharmacokinetics study, which can be degraded by adenosine deaminase [44]. Our group found that 3'-deoxyhypoxanthine, the main metabolite of cordycepin, has an excellent anti-obesity potential. In addition, adenosine deaminase inhibitor and slow-release agents developing may have good prospects.

4. Materials and Methods

4.1. Ethics statement

SD rats 50 (6 - 8 weeks, 180–200 g), were obtained from the Experimental Animal Centre of Jilin University. The mice were housed in microisolator cages and received food and water. The laboratory temperature was $24 \pm 1^{\circ}\text{C}$, and relative humidity was 40 - 80%. All of the animal studies were conducted according to the experimental practices and standards that were approved by the Animal Welfare and Research Ethics Committee at Jilin University (no. SCXK2015-0001). The protocols were reviewed and approved by the committee. All of the animal studies were performed under isoflurane anesthesia, and every effort was made to minimize suffering.

4.2. Obese rats model induction

Standard feeding for one week after all rats were adapted, then weighed and randomly divided into 5 groups ($n = 10$), blank control group to give normal feed, the model group was given a high-fat diet, cordycepin high, medium and low dose group feeding high fat diet added 12.5 mg/kg and 25 mg/kg, 50 mg/kg of cordycepin each day through Stomach administration. Feeding for 5 weeks, 12 h after fasting after delivery of the end feed, weighing, taking blood from tail vein, blood placed in 4°C for 2 h. the serums were centrifuged at 3000 r/min for 30 min, blood lipids were detected the TC, TG, LDL, HDL kits (JianCheng biology institute, NanJing China). The rats were killed before anesthesia, white fat around abdominal cavity were weighed, fat coefficient was calculated according to the format (Fat coefficient = white fat in the abdominal cavity/whole body weight).

4.3. Liver TG, TC detection

The liver tissue samples preparation: carefully take small pieces of liver tissue, weighted, added to 9 times the volume of slurry medium, mechanical homogenate under ice condition, centrifuged at 2500 r/min for 10 minutes, supernatant was tested using the same ELISA kit as serum TG detection kit. Non-high-fat samples were extracted using normal saline as homogenized medium, and high-fat samples were extracted using anhydrous ethanol.

4.4. Serum AST, ALT, MDA, SOD detection

Serum AST and ALT were detected by an ELISA kit (JianCheng biology institute, NanJing China), serum MDA, SOD detection kit from YouEr biotechnology company, WuHan, China. Serum samples were prepared as serum TG and TC, also the detection principle and detection method.

4.5. Cell lines

GH3 and 3T3-L1 cell lines were purchased from Cell Signaling Technology (Maryland, USA). GH3 cells were cultured in a medium of ham's F12k (containing 2.5 % fetal bovine serum and 15 % horse serum, 100 U/mL penicillin, 100 mg/mL streptomycin), 3T3-L1 cells were cultured using a high sugar medium of DMEM (containing 10 % thermal inactivation bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin), both at 37°C, 5% CO₂ incubator, repeated every two days, transferred when cell density reaches 80-90%. Differentiation of 3T3-L1 cells first cultured in 10 % fetal bovine serum till the cell cohesion reached 70%, then the culture in differentiation medium 1 for 48h and the culture medium 2 was used to culture for 24 h. All cell culture reagents were purchased from Gibco Laboratories (NY, USA)

4.4. CCK8 cytotoxicity detection

GH3 cells under log phase of growth were seeded into 96-well plates at 1×10^4 at 100 μ g per well, after reaching 70-80% confluence, cells were treated with cordycepin in different concentrations of (0, 3.12, 6.25, 12.5, 25, and 50 μ g/mL) for 24 h, then incubated with 10 μ L CCK-8 solution (MedChemExpress) for 4 h. Optical density at 450nm was determined using an ELISA microplate reader (TACAN)

4.5. Western-blot

The cells were treated with cordycepin as described above, and the total protein was collected by centrifugation and was quantified using the BCA reagent (Beyotime, P0012). The images were obtained by a CanoScan LiDE 100 scanner (Canon). Protein blots were measured using Image-J software.

4.6. Prolactin detection

Prolactin detection as previously described, pretreatment with different concentration of cordycepin with R-PIA, and DPCPX (both purchased from Aobersen Beijing China) for 24 h, supernatant of GH3 cells were collected and detected by an ELISA kit (Sigma).

4.7. Fluorescence staining

3T3-L1 cells were fixed with 4% polyformaldehyde for 30min, remove extra polyformaldehyde, gently wash with PBS for twice, each added 20 μ L the fluorescent, which was diluted for 1000 times, dark incubated for 10min, then washed for twice before image acquisition using a fluorescence microscopy (Olympus, Tokyo, Japan)

4.8. Picture constructing

The pictures in this paper were graphed using a Pathway Builder Tool 2.0 software.

4.9. Statistical analysis

All of the results are expressed as the means $\pm$ SD. The group means were compared using a one-way ANOVA, and Student's t-test was used to determine the significance of differences. For p values, $p < 0.05$, $p < 0.01$ compared with the control were considered statistically significant. The data are representative of triplicate experiments and are presented as the mean value $\pm$ the SD.

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FIGURE LEGENDS

Figure 1 Cordycepin reduce body weight and hyperlipidemia *in vivo*. (a) Rats were divided into 5 groups and treated with NFD and HFD with 12.5 mg/kg/d, 25 mg/kg/d, and 50 mg/kg/d cordycepin suspension for 5 weeks, body weight and fat coefficient were calculated weekly; (b) Serum TG, TC, LDL, and HDL level were tested using lipid test kit; (c, d) Serum AST, ALT, MDA, and SOD level were tested using hepatic functional enzyme test kit; (e) Liver tissues were prepared as previous described, tissues were weight and homogenate with 9 times volume under ice condition, centrifuge for 10 minutes, supernatant was tested as lipid test kit for liver TG and TC detection using the same kit. (f) H.E stain for adipocyte and liver tissue.

Figure 2 Cordycepin downregulate prolactin secretion via adenosine A₁ pathway *in vivo* and *vitro*. (a) Serum prolactin level treated with cordycepin; (b) Survival of GH₃ cells detected by CCK8; (c) Cordycepin upregulate the expression of adenosine A₁ (left), the adenosine A₁ inhibitor DPCPX could block the suppression of prolactin treated by cordycepin(right); (d) Cordycepin can block ERK1/2 expression through and induce P-PI3K and P-AKT expression which were related with prolactin release.

Figure 3 Cordycepin reduce adipogenesis by reducing prolactin release *in vitro*. (a) Prolactin promotes differentiation of 3T3-L1 cells into mature adipocytes observed by fluorescent microscope; (b) Prolactin induce expression of PRLP, and P-JAK2 in 3T3-L1 cells. (b) Cordycepin block PRLP expression but not P-JAK2.

Figure 4 Cordycepin modulate body weight. (a) Cordycepin modulate body weight by reducing prolactin production, then prolactin can inhibit adipocyte genesis, which works through an adenosine A₁ receptor pathway. (b) Cordycepin can directly reduce adipocyte genesis by an adenosine transporter pathway. (c) Cordycepin can regulate microbiota, leading a balance of bacteria metabolism, thus help losing weight. (d) Above all, cordycepin can be developed as new targets for anti-obesity drugs.