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Effect of ganoderic acid D on colon cancer Warburg effect: Role of SIRT3/cyclophilin D

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ABSTRACT

Ganoderic acid D (GAD) is a highly oxygenated tetracyclic triterpenoid. This study aims to assess the effects of GAD on the energy metabolism of colon cancer through the regulation of SIRT3 expression and whether this effect is related to acetylated cyclophilin D. The results demonstrated that GAD inhibits the energy reprogramming of colon cancer cells including glucose uptake, lactate production, pyruvate and acetyl-coenzyme production in colon cancer cells. Meanwhile, GAD upregulated the protein expression of SIRT3. Furthermore, the interruption of SIRT3 expression significantly reversed all the effects of SIRT3 on the energy reprogramming of colon cancer. In addition, GAD induced the deacetylated cyclophilin D (CypD) by SIRT3, whereas SIRT3-shRNA inhibited its combining effect on CypD. The energy reprogramming effects of GAD on colon cancer seem to be mediated by SIRT3 upregulation via acetylated CypD inhibition.

1. Introduction

Ganoderic acid D (GAD), a highly oxygenated tetracyclic triterpenoid, is the major active component of *Ganoderma lucidum*, which is a medicinal mushroom used in traditional Chinese medicine for 1000 years. *G. lucidum* shows anticancer effects including inhibiting tumour growth, anti-angiogenesis, anti-metastasis, immuno-enhancement, etc. (Yue et al., 2010); however, GAD has not been studied to assess itsrole and mechanism in inhibiting colon cancer.

Warburg effect, the reprogramming energy metabolism, has been an emerging hallmark of cancer and is the best characterized metabolic phenotype in cancer cells (Warburg et al., 1927). Even in the presence of oxygen, colon cancer cells can reprogram their glucose metabolism, and thus, the ATP generation shifts from oxidative phosphorylation to glycolysis, leading to a state termed 'aerobic glycolysis' (Qin et al., 2016; Zhou et al., 2012). In addition, researchers have confirmed that the anti-Warburg effect reduces ATP synthesis to promote apoptosis in colon cancer models (Bianchi et al., 2015). Interestingly, studies have found that metformin prevents colorectal cancer in diabetic rats by reversing the Warburg effect (Jia et al., 2015). Another study confirms that resveratrol induces mitochondrial respiration and apoptosis in colon cancer cells (Blanquer-Rossello et al., 2017). Therefore, we speculated that GAD exhibits an anti-colon cancer effect via inhibiting the Warburg effect.

Mitochondrial aerobic respiration is an efficient method of generating energy in biological systems. SIRT3 is a mitochondrial deacetylase that acts on numerous substrates to activate fat oxidation, amino acid metabolism and electron transport (Huang et al., 2010). Several manuscripts published in the last year provide convincing evidence that SIRT3, the primary mitochondrial deacetylase, is a bona fide TS (Bell et al., 2011; Finley et al., 2011; Kim et al., 2010). Finley et al. (2011) have recently linked aberrant ROS regulation by SIRT3 to the Warburg effect. The study stated that the increased glucose consumption observed in cells lacking *SIRT3* promoted a tumour-permissive phenotype both in vitro and in vivo (Finley et al., 2011).

The mitochondrial matrix-specific protein cyclophilin D (CypD) is an essential regulatory component of mitochondrial permeability transition pore (MPTP), which consists of a voltage-dependent ion channel in the outer membrane of the mitochondria (Baines et al., 2005; Woodfield et al., 1998). Massive prolonged opening of the MPTP in noncancerous cells causes cessation of oxidative phosphorylation, ATP depletion and cell demise due to bioenergetic collapse, which demonstrates that MPTP is needed to shut down mitochondrial function for bioenergetic reprogramming and establishing the Warburg effect in cancer. CypD overexpression found in different tumours should sensitize mitochondria to the MPTP; however, no signs of increased apoptosis or necrosis were observed in these tumours (Schubert and Grimm, 2004). Furthermore, CypD is identified as one of the target proteins of SIRT3, which can be deacetylated bylysine 166 (Hafner et al., 2010). All these evidences support the speculation that CypD mediates the

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function of SIRT3 in the energy metabolism of colon cancer.

Accordingly, in this study, we speculated that GAD can regulate the energy metabolism of colon cancer via the SIRT3/CypD signal pathway.

2. Materials and methods

2.1. Cell culture

The CRC cell lines include HT29 and SW620 were both ordered from American Type Culture Collection. The cells were all cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum at 37 °C in a humidified atmosphere with 5% (v/v) CO_2.

2.2. Measurement of glucose consumption and lactate production

After the cells were treated by GAD, the cell culture media were collected after transfection for 48 h. Glucose uptake and lactate production were measured using the Amplex® Red Glucose/Glucose Oxidase Assay Kit (Cat. #A22189; Invitrogen) and lactate assay kit (Cat. #MAK064; Sigma-Aldrich), respectively. The results were normalized on the basis of the total number of protein molecules present in a cell.

2.3. Pyruvate assay

The concentration of pyruvate in SW620 cells treated by GAD was measured using the pyruvate assay kit (Cat. #K609-100; BioVision). After the cells were treated differently, they were collected and dissolved with 0.5 mL of pyruvate assay buffer. Furthermore, a 50-μL sample was added with 50 μL of reaction mixture and incubated at room temperature for 30 min. A standard curve covering a range of 10–0.1 nmol per well was used as a control. Absorbance was measured at 570 nm. The pyruvate concentration, which was normalized on the basis of the amount of cellular protein, was calculated relative to the standard curve.

2.4. Acetyl-coenzyme assay

To analyse the production of acetyl coenzyme A (acetyl-CoA), cell extracts were prepared using the perchloric acid approach as described previously, with minor modifications (Sze and Li, 2011). CRC cells, treated by ganoderic acid A or D, were harvested and washed with phosphate-buffered saline. Cells were dissolved in 1 mL of washing buffer (10 mM sodium phosphate [pH 7.5], 10 mM $MgCl_2$, 1 mM EDTA), treated with 200 μL of 3 M ice-cold $HClO_4$ and incubated on ice for 30 min. The mixture was centrifuged for 5 min at 10,000 × *g* at 4 °C. The supernatant was neutralized with saturated $KHCO_3$ and centrifuged as described above. The level of acetyl-CoA in the cell extraction was quantified using the acetyl-CoA assay kit (Cat. # K317-100; BioVision). Fluorescence was measured (Ex/Em = 535/589 nm), and the acetyl-CoA concentration was calculated based on the standard curve. The production of acetyl-CoA was normalized based on the total of cellular proteins.

2.5. Western blotting

The expression of several proteins was detected using western blot analysis (Yuan et al., 2014a, 2014b). Proteins were separated on a 10% SDS-PAGE gel and transferred onto a PVDF membrane at 100 V for 1 h. Subsequently, the membrane was incubated in TBS/T buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20) with 5% non-fat milk at room temperature for 2 h. The specific primary antibodies, including rabbit-anti-hexokinase II (HKII, diluted 1:1000, Cell Signalling Technology, Massachusetts, USA), rabbit-anti-glucose transporter 1 (Glut1, diluted 1:1000), rabbit-anti-SIRT3 (diluted 1:500, Santa Cruz, California, USA), acetylated CypD (diluted 1:500) and mouse-anti-GAPDH (diluted 1:2000) were diluted in TBST (50 mM Tris-HCl, with 150 mM NaCl, 0.1% Tween-20, pH 7.4) buffer to incubate the PVDF membrane at 4 °C overnight. The corresponding secondary antibodies, which were conjugated to horseradish peroxidase, were subsequently incubated with the PVDF membrane for 60 min at room temperature. Signal detection was performed with an ECL reagent (Amersham Biosciences, Piscataway, NJ, USA).

2.6. Plasmid transfection

Recombinant PGC-shNegative (PGC-NEG), PGC-shSIRT3 plasmids were used in our study. The plasmids were separately transfected into HT29 and SW620 cells using the FuGENE HD reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. The cells were used for research 48 h after transfection.

2.7. Co-immunoprecipitation

Cells were washed twice with PBS (pH 7.4) and lysed in NP40 buffer (50-mM Tris–Cl [pH 8.0], 150-mM NaCl, 1% NP40). The protein lysates were precleared by the addition of 30 μL of agarose beads for 30 min. Each immunoprecipitation (IP) reaction was initiated with 600 μg of total protein and 4 μg of SIRT3 antibody. The mixture was rotated overnight (4 °C), 30 μg of beads were added to each IP, and the mixture was rotated for another 2 h. After centrifugation (1100*g* for 3 min), the supernatant was removed, and the pellet was washed out 2 times in NP40 buffer. The complexes were eluted in SDS lysis buffer.

2.8. Statistical analysis

Data are expressed as mean ± standard error of the mean (S.E.M.). One-way analysis of variance (ANOVA) followed by the Tukey test was used for multiple comparisons. A P-value that is < 0.05 was considered statistically significant when using two-sided tests. All analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of GAD on colon cancer cells energy metabolism

To evaluate the function of GAD on the level of energy metabolism, glucose uptake, lactate production, pyruvate production and acetyl-CoA were detected. In this research, GAD inhibited all of these levels gradually in a dose-dependent manner in both of HT29 and SW620 cell lines for 24 h (Fig. 1). Treatment of HT29 and SW620 with GAD for 24 h significantly inhibited the level of glycolysis and energy production including glucose uptake (Fig. 1A), lactate production (Fig. 1B), pyruvate production (Fig. 1C) and acetyl-CoA (Fig. 1D) in a dose-dependent manner respectively. Meanwhile, GAD (200 μmol/L) explored the most beneficial on the energy metabolism. These results confirmed that GAD inhibited Warburg effect of colon cancers.

3.2. Effect of GAD on the protein expression related with the energy metabolism

To explore the relative mechanism of GAD on Warburg effect of colon cancer, we detected the proteins relative with the energy metabolism of HT29 and SW620 cells including Glut1, HK1 and SIRT3 located in mitochondria. As the data provided in Fig. 2 indicates, compared with the control group, the regulatory effect of GAD on the expression of Glut1 and HK1 protein was negative (Fig. 2). However, GAD played a significant role in SIRT3 protein upexpression in a dose-dependent manner (Fig. 2). GAD (200 μmol/L, 24 h) upregulated SIRT3 protein expression 2.5-fold of the control group. These results identified that maybe it was SIRT3 was involved the regulatory effect of GAD on the colon cancer Warburg effect.