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Metformin ameliorates high uric acid-induced insulin resistance in skeletal muscle cells

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ABSTRACT

Hyperuricemia occurs together with abnormal glucose metabolism and insulin resistance. Skeletal muscle is an important organ of glucose uptake, disposal, and storage. Metformin activates adenosine monophosphate-activated protein kinase (AMPK) to regulate insulin signaling and promote the translocation of glucose transporter type 4 (GLUT4), thereby stimulating glucose uptake to maintain energy balance. Our previous study showed that high uric acid (HUA) induced insulin resistance in skeletal muscle tissue. However, the mechanism of metformin ameliorating UA-induced insulin resistance in muscle cells is unknown and we aimed to determine it. In this study, differentiated C2C12 cells were exposed to UA (15 mg/dl), then reactive oxygen species (ROS) was detected with DCFH-DA and glucose uptake with 2-NBDG. The levels of phospho-insulin receptor substrate 1 (IRS1; Ser307), phospho-AKT (Ser473) and membrane GLUT4 were examined by western blot analysis. The impact of metformin on UA-induced insulin resistance was monitored by adding Compound C, an AMPK inhibitor, and LY294002, a PI3K/AKT inhibitor. Our data indicate that UA can increase ROS production, inhibit IRS1-AKT signaling and insulin-stimulated glucose uptake, and induce insulin resistance in C2C12 cells. Metformin can reverse this process by increasing intracellular glucose uptake and ameliorating UA-induced insulin resistance.

1. Introduction

Hyperuricemia (serum uric acid [UA] level >420 μmoL/L for men, >360 μmoL/L for women) is a disease of disordered purine metabolism that has become increasingly common in China over the past several decades because of enhanced living standards (Zhu et al., 2014; Li et al., 2015). The condition is related to metabolic syndrome such as hypertension, hyperlipaemia and hyperglycemia. As well, hyperuricemia is connected to gout disease, inflammatory response, and endothelial dysfunction, for example (Zhu et al., 2011; Yang et al., 2012; So and Thorens, 2010). Hyperuricemia occurs together with abnormal glucose metabolism and also insulin resistance; it participates in the pathological process of diabetes and cardiovascular diseases and thereby induces a variety of complications that may affect the quality of life of patients. Nevertheless, the therapeutic scheme of hyperuricemia is limited and diversified treatment is in need of research.

Skeletal muscle is one of the most important organs of glucose uptake, disposal, and storage, accounting for approximately 75% of the entire body's glucose uptake under insulin stimulation (Gogoi et al., 2014). Increasing production of reactive oxygen species (ROS) may account for the inhibition of insulin signaling by oxidative stress in cells (Gogoi et al., 2014; Zhang et al., 2013). Insulin receptor substrate 1 (IRS1), AKT and glucose transporter 4 (GLUT4) are crucial counter-regulating mediators of the insulin

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signaling pathway (Qin et al., 2012; Choi et al., 2014; Kou et al., 2011). Our previous study showed that high UA level may activate IRS1 and inhibit AKT and induce insulin resistance via oxidative stress in hepatocytes and cardiomyocytes *in vitro* (Zhu et al., 2014; Zhi et al., 2016). Furthermore, we found that high UA level induced skeletal muscle insulin resistance in 10-week-old male C57BL/6J mice (Zhu et al., 2014). However, the molecular mechanism of high UA-induced insulin resistance in skeletal muscle cells is still unknown.

Metformin, as the most commonly used biguanide drug, has potent hyperglycemic, antilipidemic and insulin-sensitizing properties (Nepomnyashchikh et al., 2007). Adenosine monophosphate-activated protein kinase (AMPK) is an intracellular energy sensor that plays a key role in regulating cellular metabolism. AMPK activation can increase ATP synthesis, then promote fatty acid oxidation (Popovics et al., 2015). Metformin activates AMPK to regulate insulin signaling and promote the translocation of GLUT4, thereby stimulating glucose uptake to maintain energy balance (Wu et al., 2015; Shi et al., 2015; Bujak et al., 2014; Tzatsos and Tsichlis, 2007). As well, metformin can promote insulin resistance via traditional insulin signaling (Nepomnyashchikh et al., 2007). Nevertheless, how and whether metformin ameliorates UA-induced insulin resistance in skeletal muscle cells is unknown, as is the precise mechanism.

In this study, we examined whether metformin alters UA-induced insulin resistance in skeletal muscle cells by activating AMPK and regulating insulin signaling. We investigated the molecular mechanism of UA-induced insulin resistance in C2C12 skeletal muscle cells and the effect of metformin on AMPK activity, IRS1/AKT insulin signaling, GLUT4 level and glucose uptake. Metformin could ameliorate UA-induced insulin resistance in muscle cells by AMPK-GLUT4 and AKT-GLUT4 signals, which may provide a new theoretical basis for hyperuricemia as a new target of abnormal glucose metabolism and insulin resistance therapy.

2. Materials and methods

2.1. Reagents

2-[N-(7-Nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxy-d-glucose (2-NBDG) was from Invitrogen (Carlsbad, CA, USA). 2,7,-dichlorodihydrofluorescein diacetate (DCFH-DA), LY294002, Compound C, UA and metformin were from Sigma (St. Louis, MO, USA). Anti-phospho-AKT (Ser473) and anti-AKT antibodies were from Bioworld (St. Louis Park, MN, USA). Anti-phospho-IRS1 (Ser307) and anti-IRS1 antibodies were from Millipore (Billerica, MA). Anti-GAPDH antibody was from Abcam. Anti-AMPK (recognizing the N-terminal domain of both 1 and 2) and anti-phospho-AMPK Thr172 antibodies were from CST. N-acetylcysteine (NAC) was from ENZO Life Sciences (Farmingdale, NY, USA). All chemical reagents were of analytical grade. For primary buffer, UA stock solution was prepared at 15 mg/ml in 0.5 M NaOH. NAC was prepared at 1 M in ultrapure water.

2.2. Cell culture and treatment

C2C12 mouse myoblasts were grown in DMEM medium supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS) and 100 units/ml penicillin, 100 μg/ml streptomycin (complete medium) in a humidified atmosphere of 5% CO2/95% air at 37 °C. For all experiments, cells were plated in 6-well plates at 2.0×10^5 cells/ml. Cells were split every 2 days when confluence did not exceed 90%. To stimulate cell differentiation, cells were grown to 100% confluence and then regular growth medium was replaced with differentiating medium containing 2% horse serum instead of FBS. After 7-day stimulation with 2% horse serum, more fusion of cells occurred, and longer and larger myotubes appeared. Cells at this stage were treated with NAC (10 or 20 mM), Compound C (10 μM), LY294002 (50 μM) or metformin (1 mM) for 2 h before UA was added. UA (15 mg/dl) dissolved in ultrapure water was added to the fresh cell-culture medium 24 h before an experiment. Cells were incubated for the indicated times, then harvested for biochemical or molecular assays. All experiments were repeated at least 3 times.

2.3. Measurement of intracellular ROS levels

C2C12 cells were subcultured in 6-well plates (2.0×10^5 cells/well), allowed to attach for 24 h, incubated and differentiated as described (Popovics et al., 2015), exposed to UA at 15 mg/dl for 24 h, and stained with 10 mM DCFH-DA for 30 min at 37 °C as described. ROS generation was evaluated by fluorescence microscopy in cells stained with DCFH-DA and analyzed by flow cytometry at excitation and emission of 480 and 530 nm, respectively.

2.4. Insulin resistance induction and glucose (2-NBDG) uptake in C2C12 cells

Glucose uptake of C2C12 cells was assessed by measuring the fluorescent glucose analog, 2-NBDG. Briefly, differentiated muscle cells on 6-well plates were treated without FBS in low-glucose DMEM supplemented for 24 h. Then we added UA (15 mg/dl for 24 h), metformin (0.25 or 0.5 or 1 mM for 26 h), Compound C (10 μM for 26 h), or LY294002 (50 μM for 26 h) depending on the experiment. Cells were treated, then the medium was replaced with Krebs-Ringer-Bicarbonate buffer containing insulin (100 nM; final concentration) and 2-NBDG (80 μM; final concentration) for 30 min at 37 °C with fluorometry excitation and emission at 485 and 535 nm, respectively. Free 2-NBDG was washed from cultures after treatment and 2-NBDG was measured by flow cytometry (BD Biosciences, San Jose, CA, USA).

2.5. Western blot analysis

C2C12 cells were lysed, sonicated and homogenized in RIPA lysis buffer, supplemented with protease inhibitors (1 mmoL/L phenylmethanesulfonyl fluoride) and phosphatase inhibitors (phosphatase inhibitor mixture I). The supernatant protein concentration was determined by use of the BCA Protein Assay Kit (Pierce, IL, USA), then equal amounts of total protein underwent 10% SDS-PAGE and were transferred to polyvinylidene difluoride membranes (Millipore, Shanghai), which were blocked with 5% non-fat milk and incubated with primary phosphorylated and total antibodies (1:1000 dilution), then horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution). An enhanced chemiluminescence kit (Pierce, IL, USA) was used for signal detection. Images of blots were acquired by using a digital image processing system (Universal HoodII76S/0608, Bio-Rad, Hercules, CA) and quantified by use of Quantity One (Bio-Rad).

2.6. Statistical analysis

Data are described with mean ± SEM from more than three independent experiments and were analyzed by using SPSS 19.0 (SPSS Inc., Chicago, IL) with unpaired Student's *t*-test or one-way ANOVA. Significant differences were determined by Duncan's multiple range test. Results were considered statistically significant at $P < 0.05$.