Methotrexate inhibits LPS induced glycolysis in fibroblast-like synoviocytes isolated from patients with rheumatoid arthritis

X. Cen, Y. Chen, H. Liu, Y. Luo, M. Dong, Q. Xie

Supplementary material

Methods

Reagents

Dulbecco's Modified Eagle's Medium (DMEM, 10-017-CM) was purchased from Corning, Inc. (Corning, NY, USA) and fetal bovine serum (FBS, 10099-141) was purchased from Gibco (Grand Island, NY, USA). Methotrexate (HY-14519), 2-Deoxy-D-glucose (2DG, hy-13966), and LPS (HY-D1056) were purchased from MedChemExpress (Monmouth Junction, NJ, USA). The RNeasy® Mini kit (74104) was purchased from Qiagen (Hilden, Germany). Anti-GAPDH antibody (EPR16891, ab181602) was purchased from Abcam (Cambridge, UK). Antibodies of GLUT1 (AF0173), HK2 (DF6176), PKM2 (AF5234), PDK2 (DF4366), HDAC1 (AF0178), HIFα (AF1009) were bought from Affinity Biosciences (Cincinnati, OH, USA). ChamQ SYBR qPCR Master Mix (Q311-02) and HiScript® III RT SuperMix for qPCR (+gDNA wiper, R323-01) were purchased from Vazyme (Nanjing, China). Pronase from *Streptomyces griseus* (C756W53, 10165921001) was purchased from MilliporeSigma (Temecula, CA, USA). The Immobilon®-P transfer membrane, 0.45 µm (IPVH00010), was purchased from Merck Millipore (Billerica, MA, USA). Cell lysis buffer (P0013) and the BCA protein quantitation assay (P0010) were purchased from Beyotime (Shanghai, China). The protease inhibitor cocktail (GK10014) was purchased from Glpbio (Montclair, CA, USA). PBS (1×, G4202) was purchased from Servicebio (Wuhan, China). Cell counting kit-8 (CCK8, IV08) was bought from Invigentech (CA, USA). Seahorse XF Glycolytic Rate Assay Kit (103344-100) was bought from Agilent Technologies Inc. (CA, USA). Human lactate ELISA kit (MM-12907H1) was bought from Bolai Biotechnology Co., LTD (Jiangsu, China). HDAC1 inhibitor screening assay kit (10011564) was bought from Cayman chemical company (Ann Arbor, Michigan, USA).

Cell culture

FLS were isolated from synovial tissues obtained from patients with RA, following previously reported methods (1). The FLS were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin.

CCK8 assay

The proliferation of FLS in the presence or absence of LPS (1µg/mL) after treatment with MTX for 72 hours was detected using the CCK8 assay. FLS cells were seeded in 96-well plates at a density of 3000 cells per well. After treatment with MTX and LPS, a CCK8 kit was used according to the kit specifications. The cells were then incubated in the dark for 4 hours, and the optical density (OD) values were measured at 450 nm using a plate reader (CLARIOstar, BMG LABTECH, Germany).

Glycolysis rate test

The glycolysis rate test was conducted to measure the ECAR, which indicates the level of glycolysis in the cells. In this experiment, FLS were seeded in the XF24 cell culture plate and treated with MTX for 72 hours in the presence of LPS (1µg/mL). The ECAR was measured using the Seahorse XFe24 analyzer (Wave 2.6.1, Agilent Technologies, USA) according to the kit specifications.

qRT-PCR

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was employed to assess the relative expressions of the target genes. The expressions were normalised against the internal reference GAPDH. In brief, FLS were seeded in a 6-well plate and treated with MTX for 24 hours in the presence of LPS (1µg/mL). Subsequently, cells were collected and whole cell RNA was extracted following the specifications of the RNA extraction kit. The RNA was then reverse transcribed to generate cDNA, which was used for synthesizing the target gene with the specific primers. The levels of gene expression were calculated using the $2^{-\Delta\Delta Cq}$ method. The following primers were used: hGAPDH: F 5′-3′: CAC ATG GCC TCC AAG GAG TAA, R 5′-3′: TGA GGG TCT CTC TCT TCC TCT TGT; hIL-1β: F 5′-3′: TCC AGG AGA ATG ACC TGA GC, R 5′-3′: GTG ATC GTA CAG GTG CAT CG; hIL-6: F 5′-3′: TGA GGA GAC TTG CCT GGT GA, R 5′-3′: TTG GGT CAG GGG TGG TTA TT; hIL-8: F 5′-3′: TGA ATG GGT TTG CTA GAA TGT G, R 5′-3′: TGA GGT AAG ATG GTG GCT AAT AC; hMMP1: F 5′-3′: CAC GCC AGA TTT GCC AAG AG, R 5′-3′: GTC CCG ATG ATC TCC CCT GA; hMMP3: F 5′-3′: CAC TCA CAG ACC TGA CTC GGT T, R 5′-3′: AAG CAG GAT CAC AGT TGG CTG G; hMMP13: F 5′-3′: CCT TCC CAG TGG TGG TGA TG, R 5′-3′: CGG AGC CTC TCA GTC ATG GA.

ELISA

Secretion levels of lactate in the cell culture supernatant were detected using ELISA method. Briefly, FLS were seeded in a 6-well plate and treated with MTX for 24 hours in the presence of LPS (1µg/mL). The cell culture supernatant was collected, and ELISA was performed following the instructions provided with the kit. OD values were measured at 450 nm using a plate reader (CLARIOstar, BMG LABTECH, Germany), and sample concentrations were calculated using the standard curve.

Western blotting

Western blotting was performed to detect protein expression. FLS were seeded in a 6-well plate and treated with MTX for 24 hours in the presence of LPS (1µg/mL). Cells were then collected for protein extraction. After determining protein concentrations, loading buffer was added and the proteins were denatured at 95 °C for 10 minutes. Protein samples of 20 µg were loaded onto a gel and separated via gel electrophoresis. A wet transfer system was used to transfer the proteins onto a nitrocellulose membrane. The membrane was then blocked with 5% (w/v) non-fat milk in TBST for 30 minutes at room temperature. The primary antibody (1:1000) was added to the membrane and incubated overnight at 4 °C. After washing three times with TBST, the secondary antibody (1:50000) was added and incubated for 1 hour at room temperature. Following washing, the bands on the membrane were visualised using a gel scanner (ChemiDoc XRS, BIO-RAD, USA) with an enhanced chemiluminescent substrate.

Binding target prediction

To predict the binding target of MTX, we first searched for the drug's SMILES structure on PubChem via the website:
https://pubchem.ncbi.nlm.nih.gov/
Next, we inputted the SMILES structure into the SwissTargetPrediction site:
http://targetnet.scbdd.com
to predict the binding targets (2).

DARTs assay

The drug affinity responsive target stability (DARTs) assay was performed to validate the binding between MTX and HDAC1, following previously published methods (3, 4). If a drug binds to a predicted protein, it may protect the protein from pronase digestion by altering its spatial configuration or occupying the protein binding site with other molecules. In brief, protein was extracted from FLS and its concentration was determined. Then, 1 µL of DMSO or MTX (10 mM) was added to 99 µL of cell lysis buffer, and the mixture was incubated at room temperature for 30 minutes with shaking. Subsequently, 20 µL of the resulting solution was added to four tubes. Pronase was added to the tubes at ratios of 0, 1:400, 1:800, and 1:1600 based on the total protein in each tube, and the tubes were left at ambient temperature for 5 minutes. Digestion was stopped by adding a protease inhibitor (1 µL, 20-fold), followed by denaturation of the samples at 95 °C for 10 minutes. The expression level of HDAC1

was then detected using western blotting. The binding of MTX to HDAC1 was assumed when darker bands were observed in the MTX lane compared to the control.

HDAC1 activity

The activity of HDAC1 was determined using the HDAC1 inhibitor screening assay kit, and the fluorescent values were measured using a plate reader (CLARIOstar, BMG LABTECH, Germany) with excitation wavelengths of 350±10 nm and emission wavelengths of 450±10 nm.

Statistical analysis

All data were organised and analysed using SPSS v. 22.0 (SPSS, Inc., Chicago, IL, USA) or GraphPad Prism v. 6.0 (GraphPad, Inc., La Jolla, CA, USA). The data were expressed as means with standard errors. The t-test was used to compare two means, while one-way ANOVA was used to compare more than two means. Bonferroni corrections were applied to correct the pairwise comparisons among multiple groups. A p-value less than 0.05 was considered statistically significant, and asterisks indicated results with a p-value less than 0.05 (*<0.05, **<0.01, ***<0.001,****<0.0001).

References

1. ZAFARI P, RAFIEI A, FARAMARZI F, GHAFFARI S, AMIRI AH, TAGHADOSI M: Human fibroblast-like synoviocyte isolation matter: a comparison between cell isolation from synovial tissue and synovial fluid from patients with rheumatoid arthritis. *Rev Assoc Med Bras* (1992) 2021; 67(11): 1654-58.
2. YAO ZJ, DONG J, CHE YJ *et al*.: TargetNet: a web service for predicting potential drug-target interaction profiling via multi-target SAR models. *J Comput Aided Mol Des* 2016; 30(5): 413-24.
3. LOMENICK B, JUNG G, WOHLSCHLEGEL JA, HUANG J: Target identification using drug affinity responsive target stability (DARTS). *Curr Protoc Chem Biol* 2011; 3(4): 163-80.
4. PAI MY, LOMENICK B, HWANG H *et al*.: Drug affinity responsive target stability (DARTS) for small-molecule target identification. *Methods Mol Biol* 2015; 1263: 287-98.