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

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Rheumatoid arthritis (RA) is an autoimmune disease characterised by persistent synovial inflammation, during which fibroblast-like synoviocytes (FLS) are one of the key effector cells that can release inflammatory cytokines and matrix metalloproteases (MMPs). In a study by Garcia-Carbonell et al., it was reported that glycolysis plays a significant role in the pathology of RA. By targeting glucose metabolism, the biological functions of FLS can be affected, leading to a reduction in the production of cytokines and alleviation of arthritis severity (1). Methotrexate (MTX) is the anchor drug used for treating RA and its known treatment mechanisms include inhibiting purine and pyrimidine synthesis, regulating adenosine signalling, generating reactive oxygen species, decreasing adhesion molecules, altering cytokine profiles, and inhibiting polyamine production (2). In this report, we present a new mechanism of action for MTX in RA, which involves the inhibition of glycolysis.

On the basis of different cell growth and proliferation rates in different cell culture media (galactose or glucose), Gohil et al. conducted a screening of FDA approved compounds (n=3695) and identified 338 drugs, including MTX, that could inhibit glycolysis (3). Garcia-Carbonell et al. reported that lipopolysaccharide (LPS) could redirect oxidative metabolism to glycolysis in FLS (1). Therefore, LPS was used to

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*Fig. 1.* Methotrexate inhibits LPS induced glycolysis in FLS isolated from patients with RA.

A: CCK8 assay to detect the proliferation of FLS in presence or absence of LPS (1μg/mL) after treated with MTX for 72 hours. B: Glycolysis rate test to detect the ECAR after treated with MTX (72 hours) in presence of LPS (1μg/mL). C: ELISA to detect the lactate level in the cell culture supernatant after treated with MTX for 24 hours in presence of LPS (1μg/mL) and 2DG, a known glycolysis inhibitor, as the treatment control. D: WB to detect the protein levels of glycolytic related molecules after FLS were treated with MTX for 24 hours in presence of LPS (1μg/mL). E-F: FLS were seeded in 6-well plate and after treated with MTX for 24 hours in presence of LPS (1μg/mL), cells were collected for mRNA extraction. Then qRT-PCR to detect the mRNA expression levels of inflammatory cytokines (E) and MMPs (F). G: DARTs to test the binding between MTX and HDAC1. H: HDAC1 activity. I: WB to test the protein level of HIF1α. FLS were seeded in 6-well plate and after treated with MTX for 24 hours, cells were collected for protein extraction. See the Supplementary material. The experiments were repeated two or three times. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
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treat cells in order to enhance glycolysis. To confirm the inhibitory effect of MTX on LPS-induced glycolysis, we performed cell counting kit-8 (CCK8) assay, glycolysis rate test, and ELISA. The results showed that MTX could inhibit the proliferation of FLS isolated from patients with RA both in absence and presence of LPS. MTX also increased the extra-cellular acidification rate (ECAR) and reduced lactate production in the cell culture media (Fig. 1 A-C). These findings confirmed the inhibitory effect of MTX on glycolysis. To investigate whether the inhibitory effect of MTX on glycolysis was mediated through the expression levels of related glycolytic molecules, we conducted qRT-PCR and WB analysis. The results revealed that MTX could inhibit both the mRNA (data not shown) and protein levels of glucose transporter 1 (GLUT1), creatine kinase 2 (HK2), pyruvate kinase type 2 (PKM2) and pyruvate dehydrogenase kinase 2 (PDK2) stimulated by LPS (Fig. 1D).

In a previously published study (4), it was reported that glycolysis inhibition leads to a reduction in the production of cytokines and MMPs. Therefore, we examined the mRNA expression levels of cytokines and MMPs, using 2-deoxyglucose (2DG), a known glycolysis inhibitor, as a positive control, and found that MTX could reduce the LPS-induced mRNA expression levels of IL-1β, IL-6, IL-8, MMP1, MMP3 and MMP13, similar to the effect of 2DG (Fig. 1E-F). MTX was found to suppress the production of inflammatory cytokines and MMPs by inhibiting glycolysis. To find out how MTX regulated glycolysis, the binding target of MTX was predicted by using bioinformatics analysis on the website (http://targetnet.scbdd.com), which indicated a 0.972 binding affinity of MTX to HDAC1. To confirm this binding, the drug affinity responsive target stability (DARTS) assay was performed. The results showed that at the promiscuous protein ratio of 1:400 and 1:800, the levels of HDAC1 showed that at pronase v.

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Inhibition of histone deacetylase activity by methotrexate in human cancer cells (6). This finding sup.

ports our study results, which show that MTX could inhibit LPS-stimulated glycolysis enzymes (5). Therefore, the expression levels of HIF1α were tested, and the results showed that MTX could decrease both LPS-induced mRNA expression levels (data not shown) and protein levels of HIF1α in FLS (Fig. 1I). Thus, the inhibitory effect of MTX on glycolysis is achieved through the inhibition of HDAC1 activity, resulting in reduced HIF1α expression and subsequently decreased expression of glycolytic molecules.

The previous study carried out by Yang et al. demonstrated that MTX could dock into the active site of crystal structure of a HDAC homologue by molecular modeling, thus inhibiting the activity of HDAC1 in human cancer cells (6). This finding supports our study results, which show that MTX can also inhibit LPS-stimulated glycolysis in FLS isolated from patients with RA. The inhibition of HDAC1 activity by MTX is through their binding and leads to a decrease in the expression of HIF1α, as well as the inhibition of related glycolytic molecules. Consequently, the production of cytokines and MMPs is also inhibited.

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Written informed consent was obtained. The patients were not involved in the design, or conduct, or reporting, or dissemination plans of our research.

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References


