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Metabolic changes in synovial cells in early inflammation: Involvement of CREB phosphorylation in the anti-inflammatory effect of 2-deoxyglucose

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ABSTRACT

The involvement of metabolic reprogramming has been suggested to contribute to the pathophysiology of rheumatoid arthritis (RA). Glycolysis is enhanced in synovial cell metabolism in RA patients. Inhibitors of glycolysis are known to have anti-inflammatory effects. But, changes in the metabolism of normal synovial membranes or synovial cells during the early stages of inflammation remains unknown. Moreover, there are still many aspects of inflammatory signaling pathways altered by glycolysis inhibitors, that remain unclear. In this study we found that, in normal, non-pathological bovine synovial cells, most of ATP synthesis was generated by mitochondrial respiration. However, during the early of stages inflammation, initiated by lipopolysaccharide (LPS) exposure, synovial cells shifted to glycolysis for ATP production. The glycolysis inhibitor 2-deoxyglucose (2DG) reversed LPS induced increases in glycolysis for ATP production and suppressed the expression of inflammatory cytokines and proteolytic enzymes. 2DG suppressed the phosphorylation of the transcription factor cAMP response element binding protein (CREB) enhanced by LPS. Treatment with a CREB inhibitor reversed the expression of LPS-stimulated inflammatory cytokines and proteolytic enzymes. This study showed that changes in metabolism occur during the early stages of inflammation of synovial cells and can be reversed by 2DG and signaling pathways associated with CREB phosphorylation.

1. Introduction

Rheumatoid arthritis (RA) is characterized by the inflammation and proliferation of synovium, leading to the destruction of articular cartilage and bone [1]. Recently treatment of RA has improved dramatically, and many patients reach a state of remission with few symptoms. On the other hand, the etiology of RA has not yet been clarified [2], and no treatment methods has been established that can completely cure the disease. In addition, it has been reported that about 5–20% of RA patients remain are difficult to control with the current treatment [3]. Furthermore, it is difficult to discontinue the anti-rheumatic drug even after RA is in remission [4]. Other major problems include the increased risk of complications due to use of anti-rheumatic drugs [5,6] and the long-term financial burden of expensive drugs [7]. Thus, it is desired to develop treatment methods that target a mechanism different from that of current treatment.

In recent years, the involvement of metabolic reprogramming has been suggested to play an important role in the pathophysiology of RA and thus, metabolic pathways have been reported to be a new therapeutic target for RA [8–10]. Synovial proliferation is observed in RA joints, and inflamed synovial cells undergo metabolic changes similar to those observed in tumor cells (Warburg effect), both resulting in cell proliferation [9,11]. Cells in the body utilize nutrients such as glucose and amino acids to produce the energy storing molecule adenosine triphosphate (ATP). When breaking down glucose to produce energy, aerobic glycolysis pathways are typically used -pathways that consume oxygen and produce ATP by oxidative phosphorylation of mitochondria [12]. On the other hand, in tumor cells and RA synovial cells, anaerobic glycolysis pathway come into play, producing ATP without consuming oxygen [13]. It has been reported that glycolysis is enhanced in synovial cells collected from RA patients, and that glycolysis inhibitors have anti-inflammatory effects [8,14].

Previous studies concerning synovial cell metabolism have primarily examined human synovial cells collected during artificial joint replacement [8]; that is, synovial cells associated with late stage RA for experiments. There are no reports on changes in the metabolism of

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normal synovial membranes or synovial cells in the early stages of inflammation. In addition, there are still many unclear points about the signaling pathways in synovial cells [9] that are affected by the anti-inflammatory effects of glycolysis inhibitors.

The purpose of this study is to investigate the intracellular metabolism of the synovial cells in the normal or early stage of inflammation. In addition, we also investigated the signaling pathways of anti-inflammatory effect of glycolysis inhibitors on synovial cells.

2. Methods

Materials. Dulbecco's modified Eagle's medium (DMEM) were obtained from Thermo Fischer Scientific. Trypsin ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma-Aldrich. Fetal bovine serum (FBS) was purchased from PAA Laboratories GmbH (Pasching, Austria). Collagenase P (Cat. No.:11249002001; Roche Applied Science) were used in the dissociation of tissues. Lipopolysaccharide (Cat. No.:437628) was obtained from Sigma-Aldrich. 2-deoxyglucose (Cat. No.:24894205) was obtained from Sigma-Aldrich. Cell lysis buffer was obtained from Cell Signaling Technologies. CREB inhibitor, 666-15-Calbiochem (Cat. No.:538341) was obtained from Sigma-Aldrich.

Cell culture. Cells were obtained from young adult steers (aged 18–24 months), which were provided by Nagoya City Central Wholesale Market. For the method of collecting and isolating bovine synovial cells, we referred to past reports on bovine synovial cells [15–17]. Briefly, primary bovine articular synovial cells were isolated from the articular synovium of metacarpophalangeal joints. Synovial tissue was collected from loose glossy area around the joint. These synovial tissues were digested in 0.025% (0.00625g) collagenase P overnight at 37 °C. After removing tissue debris by filtering through a 70-μm cell strainer (BD Biosciences, Franklin Lakes, NJ, USA), cells were centrifuged for 5 min at 1,500 rpm. Cells were cultured in DMEM, 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin at 37 °C in a 5% CO_2 environment. At confluence, cells were passaged 1:3 by treatment with EDTA. The experiment used cells that had been passaged once, because in previous reports the expression of inflammatory cytokines on bovine synovial cells decreased when passage was performed more than once [16]. Before experiments, bovine synovial cells were seeded into six-well culture plates (TPP, Switzerland) at a density of 4.0×10^5 cells/well. After static incubation for 48 h in 10% FBS, cells were cultured in serum-free medium for 12 h.

qRT-PCR. Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Germany). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Real time RT-PCR was carried out using a Light cycler System with FastStart Master SYBR Green PLUS (Roche, USA). Primers for 18SrRNA, GLUT1, HK2, IL1β, IL6, MMP1 and MMP3 were synthesized by Sigma-Aldrich. The bovine-specific primer sequences are as follows: 18SrRNA, forward (5′-GTA ACC CGT TGA ACC CCA TT-3′) and reverse (5′-CCA TCC AAT CGG TAG TAG CG-3′); GLUT1, forward (5′-GAC ACT TGC CTT CTT TGC CA-3′) and reverse (5′-AAC CTA ATG GAG CCT GAC CC -3′); HK2, forward (5′-TCC AGA GGA GAG GGG ACTT T -3′) and reverse (5′-GGT CCC AAC GGT ATC ATT CA-3′); IL1β, forward (5′-ACG AGT TTC TGT GTG ACG CA-3′) and reverse (5′-AAT CTT GTT GTC TCT TTC CTC TCC TT-3′); IL6, forward (5′-TGA TGA CTT CTG CTT TCC CTA CC -3′) and reverse (5′-TCT GCC AGT GTC CCT TGC T-3′); MMP1, forward (5′-GCC ATC TAC GGA CCT TCC CAA A -3′) and reverse (5′-AGT TAG CTT GCT ATC ACA CAC TTC-3′); MMP3 forward (5′-ATA CTG GAG ATT TGA CGA GAA GAG-3′) and reverse (5′-AAC TGC GAA GAT CCA CTG AAG AA-3′). The-fold increase in copy numbers of mRNA was calculated as a relative ratio of a target gene to 18SrRNA.

Metabolomic studies using Seahorse flux analyzer. The Agilent Seahorse XF Real-Time ATP Rate Assay Kit (Agilent, Cat. No.:103591–100) was used to detect the ATP production rates of mitochondrial oxidative phosphorylation and glycolysis, respectively. Before metabolism measurement, the probe plate was hydrated with HPLC grade water in a CO2-free incubator. The assay phenol red-free solution containing 10 mM glucose, 2 mM glutamine, 1 mM pyruvate and 5 mM HEPES was kept in a 37 °C CO2-free incubator to maintain the pH value. Then the HPLC grade water in the hydration plate was replaced with calibration solution and kept in a 37 °C CO2-free incubator. Bovine synovial cells were seeded into XFp cell culture microplates (Seahorse Bioscience) at the density of 3.0×10^4 cells/well, and allowed to adhere to plate 24h. Then the cell culture medium was replaced with phenol red-free assay solution and placed in a 37 °C CO2-free incubator for 1h. For the ATP Rate standard assay, the cells were incubated with LPS (1 μg/ml) 4h before the cell culture medium was replaced. For the ATP Rate induced assay, LPS (1 μg/ml) was added to the cell before oligomycin and a mix of rotenone and antimycin A was added.

Finally, ATP production rates of mitochondrial oxidative phosphorylation and glycolysis were determined and analyzed on the Agilent's Seahorse Bioscience XFp Extracellular Flux Analyzer (Agilent Technologies) according to the manufacturer's instructions and protocols (Seahorse Bioscience, North Billerica, MA, USA).

For the determination of ATP production rates of mitochondrial oxidative phosphorylation and glycolysis, oligomycin and a mix of rotenone and antimycin A were added according to the manufacturer's instructions and protocols (Seahorse Bioscience, North Billerica, MA, USA).

Live/Dead Cell Viability Assay. Live/Dead Cell Viability Assay Kit (Bio Vision, Cat. No.: K502-100) was used to measure cell viability and cytotoxicity of the reagents in bovine synovial cells. Grow cells in 37 °C incubator containing 5% CO2 in medium containing the reagents with varying concentration. Mix 2 μl of Live Cell Staining Dye and 1 μl of Dead Cell Staining Dye in 1 ml of Assay Buffer. Observe cells immediately under a light and fluorescence microscope (detects green and red wavelength [Ex/Em = 485-495/530-635 nm]). Live Cell Staining Dye stains healthy cells green. Dead Cell Staining Dye stains dead cell red. Acquire several images per well for analysis.

Lactate Measurement Assay. Lactate production were measured by Lactate Assay Kit-WST (Dojindo Molecular Technologies, USA), according to the manufacturer's instructions. The respective reaction buffers were incubated with cell cultured media for 30 min at 37°C, and then the absorbance was measured at 450 nm by a micro-plate reader.

Capillary Western Blot analyses. Capillary Western Blot analyses were performed on a Western blot system (Protein Simple) according to the manufacturer's instruction. Total protein was extracted from cell pellets with Cell Lysis Buffer (Cell Signaling, USA) containing protease and phosphatase inhibitor cocktail. The diluted samples were diluted to 0.5 μg/μL in sample buffer. The diluted samples were combined with fluorescent master mix and heated for 5 min at 95 °C. The prepared samples, blocking reagent, primary antibodies (1:20 dilution for IL1β, 1:40 dilution for MMP1, 1:80 dilution for beta-actin, 1:70 dilution for pNF-κB, 1:70 dilution for total NF-κB, 1:70 dilution for pAMPKα, 1:70 dilution for total AMPKα, 1:20 dilution for pCREB, and 1:10 dilution for total CREB), secondary antibodies, and chemiluminescent substrate were pipetted into designated well in the assay plate. Primary antibodies against, IL1β (#12242), beta-actin (#4970), pNF-κB(#3033), total NF-κB(#8242), pAMPKα(#2535), total AMPKα(#5831), pCREB (#9198) and total CREB (#9104) were purchased from Cell Signaling Technology (USA) and MMP1(ab137332) were purchased from Abcam(UK). The electrophoresis and immunodetection steps were carried out in the fully automated capillary system. Data were analyzed using Compass software (Protein Simple).

Analysis glycolysis metabolites of a mass spectrometry. The sample solution was filtered through a 0.22 μm polyethersulfone (PES) membrane filter capsule (Merck KGaA, Darmstadt, Germany) prior to analysis. Analytical conditions LC/MS/MS (liquid chromatography-tandem mass spectrometry) was performed with a QTRAP6500 mass spectrometer (AB Sciex, Framingham, MA, USA) coupled to a Shimadzu Prominence LC system (Shimadzu Co., Kyoto, Japan) with electrospray

ionization in the negative ion mode. The sample solution (5 μL) was injected into an Inert Sustain C18 column (150 mm × 2.1 mm i.d.; 3 μm particles; GL Sciences Inc., Tokyo, Japan). Analytes were chromatographically separated by gradient elution with mobile phases A (10 mM tributylamine, 15 mM acetic acid) and mobile phases B (methanol) at a flow rate of 0.2 mL/min and the gradient program is shown in Supplementary Table 1. The column oven was maintained at 40 °C. LC/MS/MS was performed in the MRM mode. The MRM transitions and other MS parameters are shown in Supplementary table 2. Calculation of peak areas of the analytes was carried out using Multi Quant ver. 3.0.2 software (AB Sciex).

Statistical analysis. All data, except as noted, were obtained from at least three independent experiments performed in duplicate or triplicate. In some experiments, a two-tailed unpaired Student's t-test was used for direct comparison of the treatment group with control. For multiple comparisons of groups, analyses of variance (one-way ANOVA)

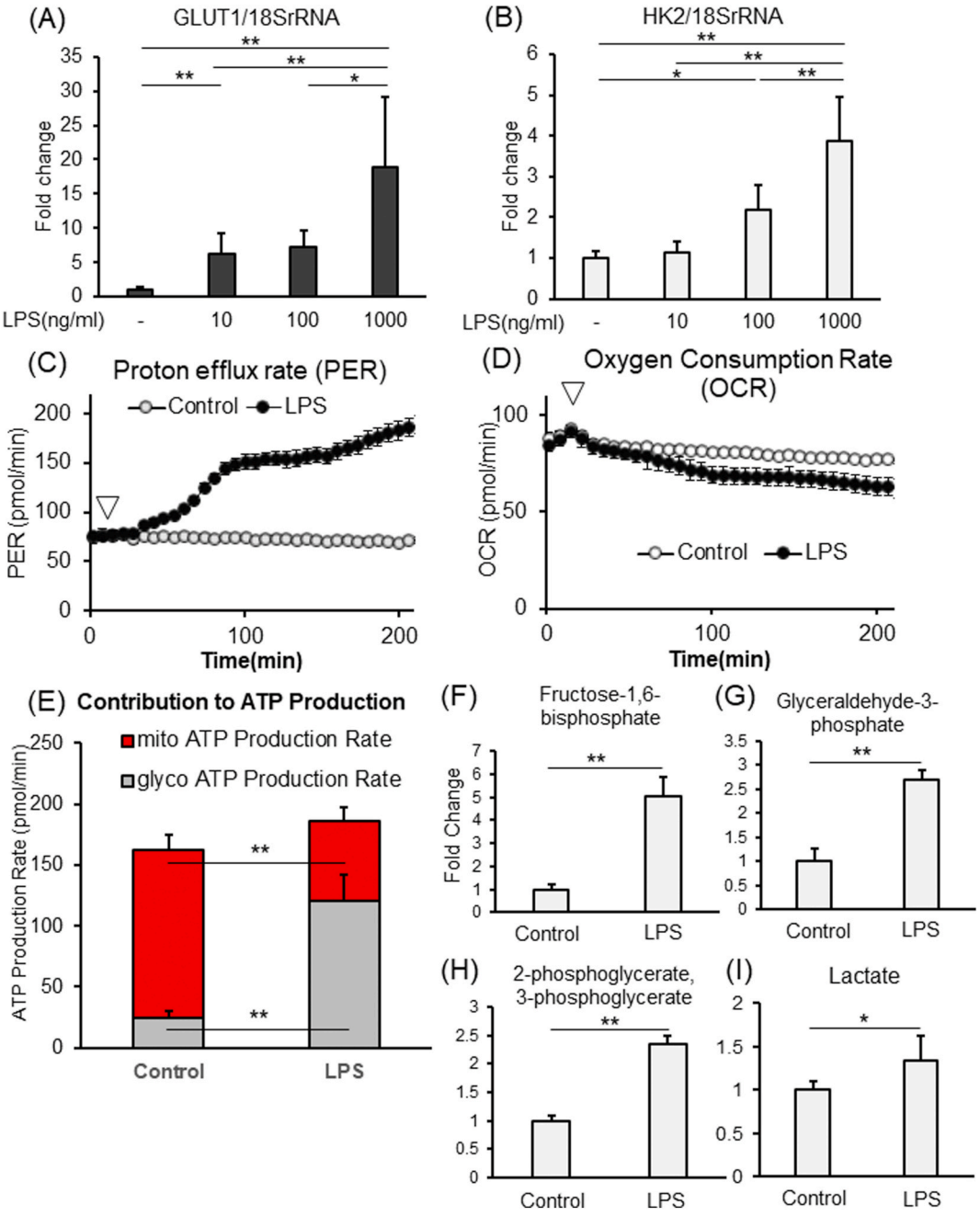

Fig. 1. Lipopolysaccharide promoted glycolysis in synovial cells while downregulating oxidative phosphorylation.
(A, B) Primary bovine synovial cell cultures were treated for 12h without or with LPS at 10, 100, 1000 ng/ml. Total RNA lysates were used for quantitative RT-PCR analyses of relative-fold changes in GLUT1 and HK2 mRNA [mean ± S.D. (error bars), n = 6]. **(C, D)** Representative plot proton efflux rate (PER, pmol/min) and Oxygen Consumption Rate (OCR, pmol/min) levels were versus time in synovial cells stimulated with LPS at 1 μg/ml (▽) by the Seahorse ATP rate induced assay [mean ± S.D. (error bars), n = 3]. **(E)** Representative ATP rate assay wherein the contribution of glycolysis (*gray bars*) and mitochondrial respiration (*red bars*) to ATP production at 4h following stimulation with or without 1 μg/ml LPS by the Seahorse ATP rate standard assay [mean ± S.D. (error bars), n = 3]. **(F–I)** Glycolysis metabolites at 4h following treatment with or without 1 μg/ml LPS by a mass spectrometry relative to control [mean ± S.D. (error bars), n = 3]. *, $p < 0.05$; **, $p < 0.01$ using one-way ANOVA followed by Tukey post hoc tests (A and B) and a two-tailed unpaired Student's t-test(E-I). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were performed, followed by Tukey post hoc tests as indicated in the figure legends. A p-value of <0.05 was considered significant. *, $p < 0.05$; **, $p < 0.01$. Those statistics were calculated by EZR, which is computer software developed by Y Kanda [18].

3. Results

Changes of intracellular metabolism in synovial cell during early inflammation. Changes to metabolism during early inflammation

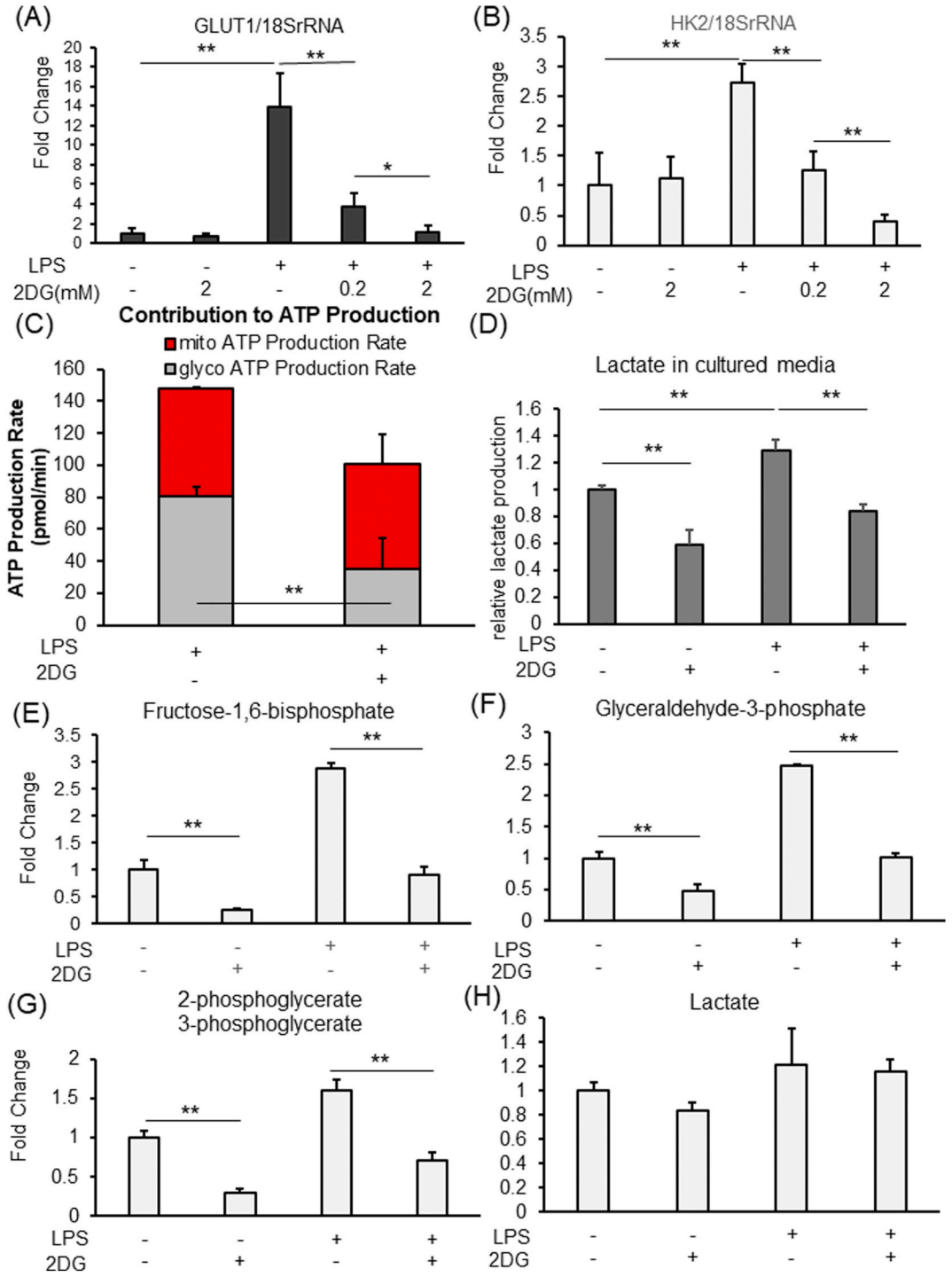

Fig. 2. 2DG suppressed glycolysis promoted with LPS in synovial cells.
(A, B) Bovine synovial cells were treated for 12h without or with 2DG at 0.2, 2 mM in the absence or presence of 1 μg/ml LPS. Total RNA lysates were used for quantitative RT-PCR analyses of relative-fold changes in GLUT1 and HK2 mRNA [mean ± S.D. (error bars), n = 6]. **(C)** Representative ATP rate assay wherein the contribution of glycolysis (*gray bars*) and mitochondrial respiration (*red bars*) to ATP production at 4h following treatment of 1 μg/ml LPS without or with 2DG at 2 mM by the Seahorse ATP rate standard assay [mean ± S.D. (error bars), n = 3]. **(D)** Lactate production in cultured media at 4h following treatment of 1 μg/ml LPS without or with 2DG at 2 mM by lactate assay [mean ± S.D. (error bars), n = 6]. **(E–H)** Glycolysis metabolites at 4h following treatment of 1 μg/ml LPS without or with 2 mM 2DG by a mass spectrometry relative to control [mean ± S.D. (error bars), n = 3]. *, $p < 0.05$; **, $p < 0.01$ using one-way ANOVA followed by Tukey post hoc tests (A, B, D, E-H) and a two-tailed unpaired Student's t-test (C). . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were first explored by monitoring changes in gene expression of two key proteins, glucose transporter-1 (GLUT1) and hexokinase-2 (HK2). Primary bovine synovial cell cultures were treated with varying concentrations of LPS for 12 h and the expression of GLUT1 and HK2 mRNA quantified by qRT-PCR. GLUT1 mRNA expression was increased at LPS concentrations as low as10 ng/ml, with maximum enhancement at 1 μg/ml (Fig. 1A). Enhancement of HK2 mRNA expression was observed at 100 ng/ml and increased in a dose-dependent manner, with maximum enhancement at 1 μg/ml (Fig. 1B).

Next, metabolic changes in bovine synovial cells were examined in real time using a Seahorse flux analyzer. This instrument measures time-dependent changes in the accumulation of H^+ protons in the culture medium (indicative of lactate accumulation) and simultaneously, oxygen consumption (indicative of mitochondrial respiration). In Seahorse ATP rate assay experiments, metabolic pathway inhibitors are administered during the measurement period to provide for true definition of proton efflux rate (PER), oxygen consumption rate (OCR) and more importantly, the relative contribution of glycolysis or mitochondrial respiration pathways to the production of ATP, in living cells, in real time. As shown in Fig. 1C, PER increased in LPS-activated synovial cells after LPS stimulation at 1 μg/ml (▽) and continued to increase gradually over time. On the other hand, OCR decreased after LPS stimulation and continued to decrease gradually over time (Fig. 1D). Increased PER and decreased OCR changes were observed at the same time immediately following LPS administration. When these data are calculated to define the relative contribution to ATP production (Fig. 1E), viewed at 4h after stimulation with or without 1 μg/ml LPS, control synovial cells utilized the mitochondrial TCA cycle to generate the majority of cellular ATP produced (red bars), with approximately one-fifth of their ATP generated from anerobic glycolysis (gray bars). However, in LPS-activated synovial cells a significant increase in dependence on glycolysis for ATP production and decreased dependence on mitochondrial respiration was observed. Hence, the use of glycolysis contributed to the majority of ATP production in LPS-activated synovial cells.

A third method was used to determine changes in metabolic pathways during early inflammation. Changes in glycolysis metabolites in synovial cells (performed 4h after treatment with or without 1 μg/ml LPS) were quantified using mass spectrometry. The intermediate metabolites of glycolysis namely, fructose-1,6-bisphosphate, glyceraldehyde-3-phosphate, 2-phosphoglycerate and 3-phosphoglycerate were all statistically significantly increased in LPS-activated synovial cells as compared to the control groups (Fig. 1F–H). In addition, the late-stage metabolite of glycolysis, lactate, was also increased in LPS-activated synovial cells compared to the control synovial cell cultures (Fig. 1I).

The effect of 2-deoxyglucose on synovial cell metabolism during early inflammation. To determine whether blocking glycolysis could reverse metabolic changes associated with early inflammation events, synovial cells were co-incubated with or without a validated inhibitor of glycolysis (a glucose hexokinase inhibitor) 2-deoxyglucose (2DG) in combination with exposure with or without LPS. First, a cell viability assay was performed to determine the potential for cytotoxicity of 2DG on bovine synovial cells. No major cytotoxicity was observed with 5.0 mM 2DG at relative to control, untreated cells (Supplementary Fig 1). As seen in Fig. 2A–B, 2DG reversed LPS-induced increases GLUT1 and HK2 mRNA expression at 0.2 or 2.0 mM as measured by qRT-PCR. Fig. 2C shows that 2DG treatment (2.0 mM) reduced synovial cell dependence on glycolysis for ATP production (following treatment with 1 μg/ml LPS) and enhanced the contribution of mitochondrial respiration to ATP production. Interestingly, overall ATP production was also reduced. Fig. 2D shows lactate production in cultured media, in which synovial cells were co-incubated 4h after treatment with or without 2DG (2.0 mM) following treatment with 1 μg/ml LPS. Lactate in cultured media treated with LPS was increased. The LPS-enhanced lactate production in cultured media was suppressed by 2DG treatment. Lactate production in 2DG treated synovial cell without LPS treatment were also reduced. Finally, co-incubation of synovial cells with 2DG significantly suppressed the LPS-enhanced production of the key metabolites of the glycolysis pathway to levels of metabolites observed in untreated control cell cultures (Fig. 2E–G). Levels of metabolites in 2DG treated synovial cell cultures without LPS exposure were also reduced suppressive effects of 2DG on lactate accumulation were suggestive but not significant at this time point (Fig. 2H).

Effect of 2DG on proteolytic enzymes and cytokines produced by LPS-activated synovial cells. In our previous studies, we demonstrated that 2DG reduced the expression of matrix metalloproteinase 13 (MMP13) in activated primary bovine chondrocytes [19]. In addition, Ricard et al. reported that 2DG reduced the expression of interleukin-6 (IL6) and matrix metalloproteinase 3 (MMP3) in RA Fibroblast-like synoviocytes (FLS) [8]. To investigate anti-inflammatory effects of glycolysis inhibitors like 2DG, bovine synovial cells were co-incubated with or without 1 μg/ml LPS in the presence or absence of 2DG. As shown in Fig. 3A and B, addition of 2DG at concentrations of 0.2 or 2.0 mM, reversed the LPS-enhanced mRNA expression of two key inflammatory cytokines often associated with RA namely; interleukin-1β (IL1β) and interleukin-6 (IL6), in a concentration-dependent manner. Co-incubation of 2DG with LPS also reversed the enhanced mRNA expression of matrix metalloproteinase-1 (MMP1) and matrix metalloproteinase-3 (MMP3). In addition, 2DG at 2.0 mM decreased the LPS-enhanced protein expression of IL1β (Fig. 3E and F) and MMP1 (Fig. 3G and H) as measured by capillary Western blot analysis.

The effect of 2DG on early signal transduction pathways initiated by LPS stimulation. To determine which signaling pathways were affected by the anti-inflammatory effects of 2DG on LPS-activated bovine synovial cells, key pathways often associated with RA were examined.

First, we evaluated the time course for phosphorylation of nuclear factor-kappa B (NF-κB), which is known as the classical signal pathway activated by LPS. Phosphorylation of NF-κB peaked 1h after the treatment of synovial cell cultures with 1 μg/ml LPS stimulation (Fig. 4A). As shown in Fig. 4C and D, while treatment with LPS alone resulted in a 6-fold increase in phosphorylation of NF-κB, co-incubation of the cells with LPS and 2DG for 1h had little diminishing effect on this pattern of activation.

In our previous study with bovine primary chondrocytes [19], the anti-inflammatory effects of 2DG were found to be associated with reversing phosphorylation of adenosine monophosphate-activated protein kinase (AMPK). While 2DG at 2.0 mM moderately enhanced basal AMPK phosphorylation in normal synovial cell cultures, no reversal of LPS-enhanced levels of pAMPK were observed (Fig. 4E and F).

Next, we evaluated the phosphorylation of cAMP response element binding protein (CREB), a transcription factor involved in metabolism and inflammation. In time course studies, phosphorylation of CREB peaked 1h after LPS stimulation (1 μg/ml), similar to phospho–NF–κB (Fig. 4B). Exposure of synovial cells to 1 μg/ml LPS for 1h resulted in a 7-fold enhancement in phospho-CREB (Fig. 4G and H); a level significantly diminished by co-incubation of LPS with 2DG at 2.0 mM.

To confirm that the anti-inflammatory properties of 2DG were related to its ability to block CREB phosphorylation following exposure of synovial cells to LPS, the cell cultures were co-incubated with LPS in combination with a validated CREB inhibitor, 666-15. First, we investigated potential cytotoxicity effects of 666-15 for bovine synovial cells using a fluorescent live/dead assay. 666-15 was not cytotoxic at 0.5 μM 666-15 (Supplementary Fig 2) but did display an increased proportion of dead cells at 1.0 μM 666-15 significantly inhibited CREB phosphorylation activated with 1 μg/ml LPS for 1h by co-incubation of LPS with 666-15 at 0.5 μM (Supplementary Fig 3). Similar to data shown in Fig. 3, exposure of synovial cells to LPS resulted in a prominent increase in the mRNA expression of inflammatory cytokines, IL1β and IL6 as well as the proteolytic enzymes, MMP1 and MMP3 (Fig. 5A–D). Co-treatment of synovial cells with LPS, in the presence of 0.5 μM 666-15, significantly suppressed the LPS-enhanced mRNA expression of these inflammatory cytokines and proteolytic enzymes with an inhibitory profile like that of

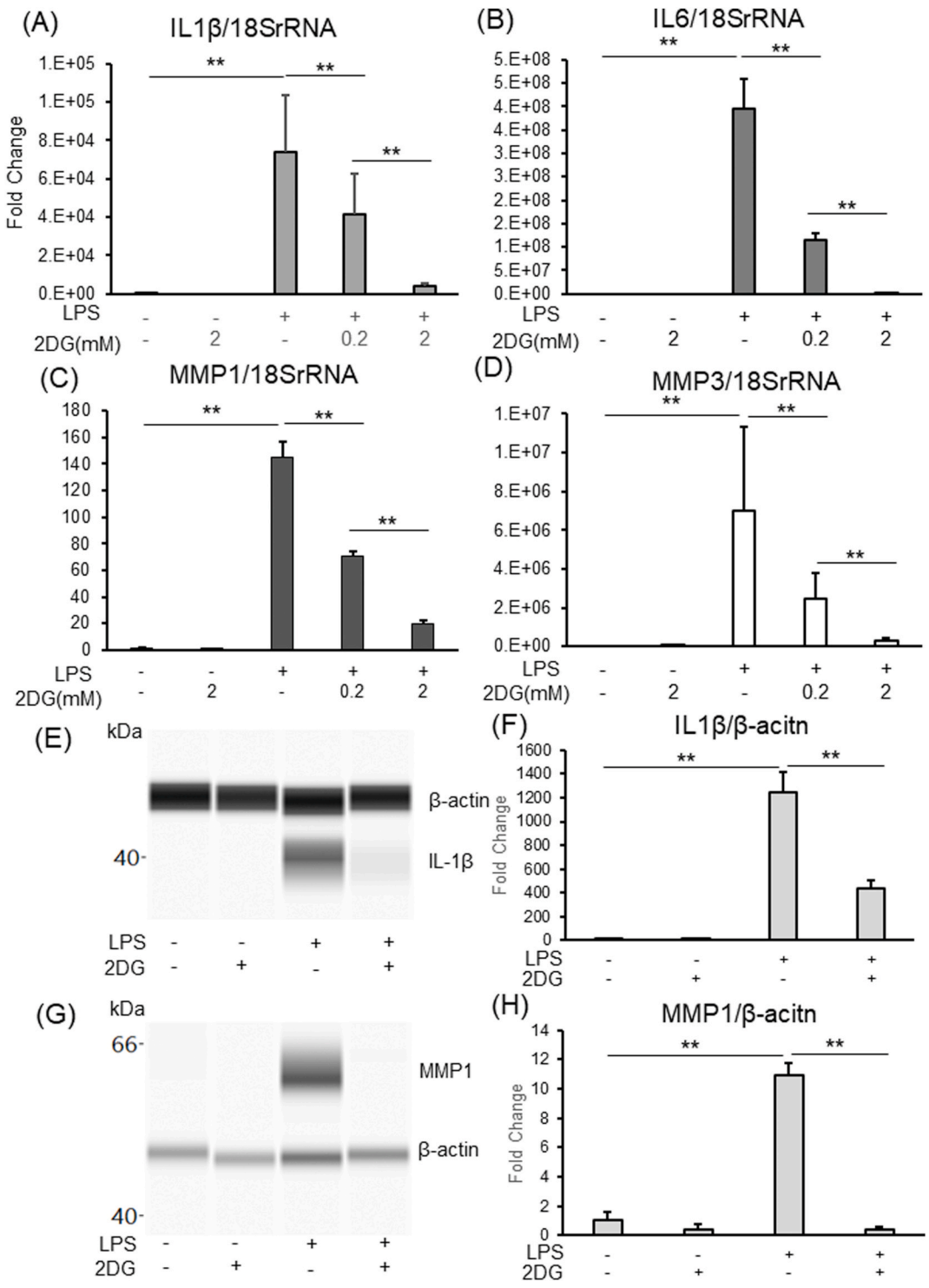

Fig. 3. Anti-inflammatory effect of 2DG in synovial cells activated with LPS
(A-D) Synovial cells were treated for 12h without or with 2DG at 0.2, 2 mM in the absence or presence of 1 μg/ml LPS. Total RNA lysates were used for quantitative RT-PCR analyses of relative-fold changes in IL1β, IL6, MMP1 and MMP3 mRNA [mean ± S.D. (error bars), n = 6]. **(E–H)** Synovial cells were treated for 12h without or with 2 mM 2DG in the absence or presence of 1 μg/ml LPS analyzed by capillary Western Blot analyses for expression of IL1β, MMP1 and β-actin. For quantification, IL1β and MMP1 were normalized to β-actin and presented as density relative to control [mean ± S.D. (error bars), n = 3]. **, $p < 0.01$ using one-way ANOVA followed by Tukey post hoc tests (A-D, F, H).

2DG. In addition, co-treatment with LPS, in the presence of 0.5 μM 666-15, significantly suppressed the LPS-enhanced mRNA expression of GLUT1 and HK2 (Supplementary Fig 4). Co-incubation of synovial cells for 4h with or without 0.5 μM 666-15 significantly suppressed the LPS-enhanced production of fructose-1,6-bisphosphate and lactate but not significant (Supplementary Fig 5).

4. Discussion

In recent years, the involvement of metabolic reprogramming has

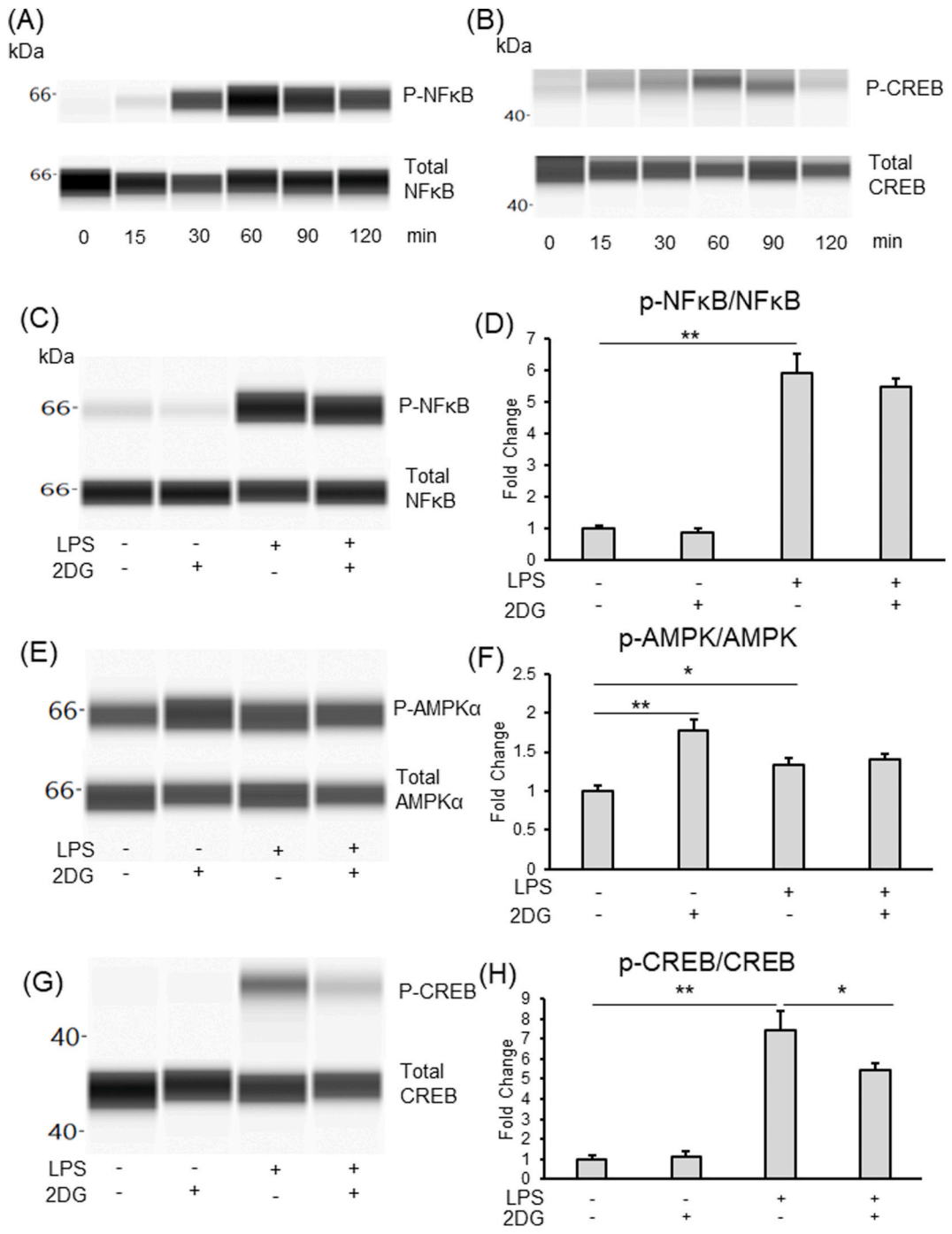

Fig. 4. Effect of 2DG on early signaling events during LPS stimulation of synovial cells.
(A, B) Phosphorylation of NFκB and CREB at 0–120min following the treatment of synovial cell cultures with 1 μg/ml LPS stimulation detected by capillary Western Blot analyses. **(C–H)** Phosphorylation of NFκB, AMPK and CREB at 1h following synovial cells were treated without or with 2 mM 2DG in the absence or presence of 1 μg/ml LPS. For quantification, phospho-NFκB was normalized to total- NFκB and presented as density relative to control as well, AMPK and CREB were normalized. [mean ± S.D. (error bars), n = 3], *, $p < 0.05$; **, $p < 0.01$ using one-way ANOVA followed by Tukey post hoc tests.

been suggested as key to the pathophysiology of RA and thus, pathways related to cellular metabolism become new, potential therapeutic targets for RA [8–10]. In addition, metabolomic studies may provide relevant biomarkers to improve diagnostic accuracy, definition of prognosis and monitoring the efficacy of the treatment in rheumatoid arthritis [20]. Recently several studies reported that glycolysis inhibitors have anti-inflammatory and cellular proliferation inhibition effects in synovial cells, although these studies utilized synovial cells and synovium collected from late stage RA patients or synovial cells isolated from osteoarthritic patients [8,14]. Our present study is the first to show the relationship between the ability of the glycolysis inhibitor 2DG, to reverse changes in intracellular metabolism and, to block anti-inflammatory effects of LPS-activated normal synovial cells, our model for early stage RA. In addition, we have shown that CREB phosphorylation is the key signaling pathway associated with the anti-inflammatory effects of glycolysis inhibitors such as 2DG on synovial cells.

The present study revealed that the balance between glycolysis and

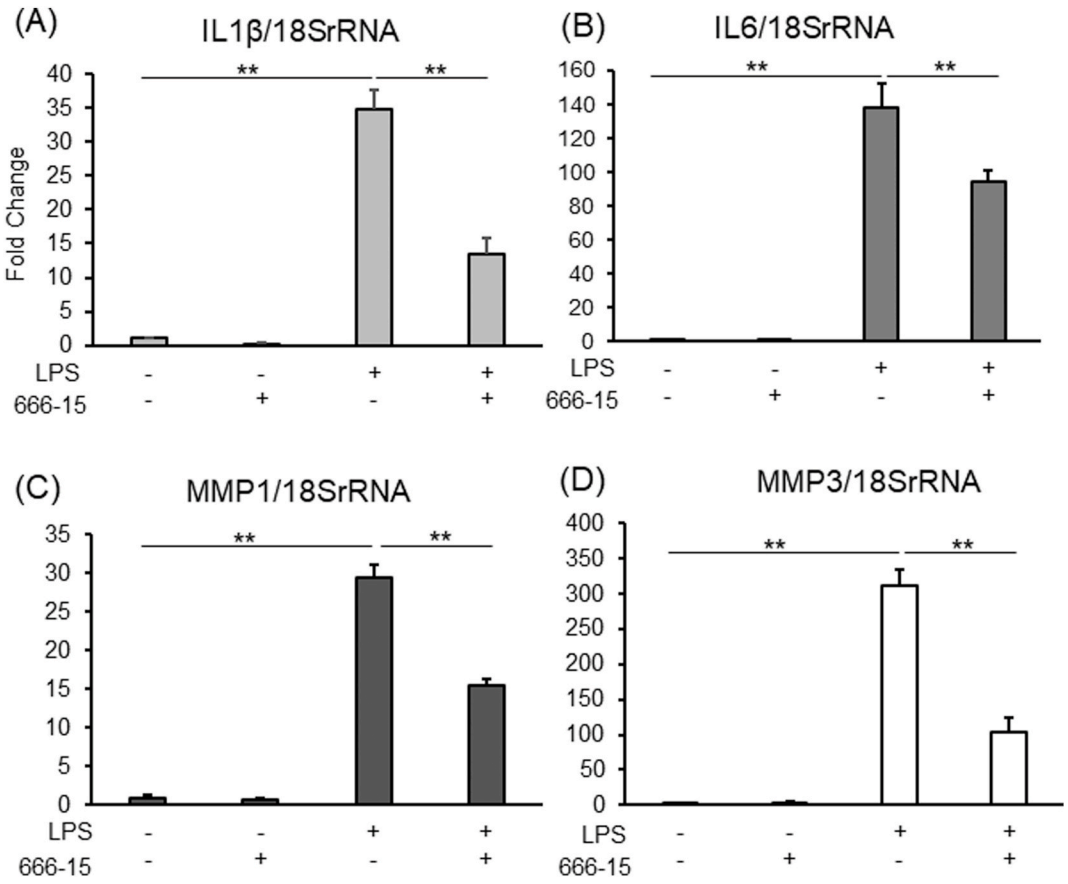

Fig. 5. Effect of CREB inhibitor, 666-15, in LPS -activated synovial cells.
(A–D) Synovial cells were treated for 12h without or with 0.5 mM 666-15 in the absence or presence of 1 μg/ml LPS analyzed by quantitative RT-PCR for expression of IL1β, IL6, MMP1 and MMP3 relative to the housekeeper gene 18SrRNA [mean ± S.D. (error bars), n = 6]. **, $p < 0.01$ using one-way ANOVA followed by Tukey post hoc tests (A–D).

oxidative phosphorylation shifts toward a dependence on glycolysis for ATP production in non-pathological, bovine synovial cells stimulated with LPS. This was confirmed using two complementary approaches; the real time rate measurements using a Seahorse XF flux analyzer and, precise quantification of metabolites by way of mass spectrometry analysis. These results were consistent with that of previous studies performed on human RA FLS compared to OA FLS [8]. As shown in Fig. 1E, a large percentage of ATP synthesis was supplied by mitochondrial respiration in normal synovial cells. On the other hand, LPS stimulation substantially altered this proportion, changing the dependence of glycolysis for ATP production from 15% in control cells to 64% following stimulation with LPS. Interestingly, in our previous study we observed that in normal bovine chondrocytes, a large percentage of ATP synthesis is supplied by the glycolysis pathway even at baseline in control, non-treated chondrocytes [19]. This predominance of glycolysis ATP production in normal quiescent chondrocytes may be due to adaptation by these cells to an avascular environment within the joint. These results suggested that the proportion of metabolism in ATP production varies from cell to cell. Nonetheless, both chondrocytes [19] and synovial cells (used in this study) enhance their dependence of glycolysis for ATP production under experimental conditions used to mimic inflammation. As shown in Fig. 1C and D, immediately after LPS stimulation, a time course study revealed that the proton efflux rate (PER) began increasing coordinate with a decrease in oxygen consumption rate (OCR). This result suggests that intermediary metabolism in synovial cells changes rapidly and likely represents one of the earlies stages of associated with inflammation. Additionally, glycolysis metabolites in synovial cells, quantified using mass spectrometry, also displayed increases in early inflammation (Fig. 1F–I). These results found that dynamic metabolic reprogramming occurred from the early stage of inflammation. These changes, sometimes referred to as "metabolic reprogramming" may due to mitochondrial dysfunction following increased production of reactive oxygen species under inflammation [21]. In past metabolic studies using synovial fluid, synovial fluid in RA patients was found to be low in glucose and high in lactate concentrations as compared to healthy subjects [22]. Thus, these and other metabolites associated with metabolic reprogramming may be provide new and novel biomarkers for acute synovitis.

This study demonstrated that the glycolysis inhibitor, 2DG was effective at both reversing a pro-inflammatory metabolic profile and, reversing the downstream inflammatory phenotype associated with LPS-activated synovial cells namely suppression of synthesis of endogenous inflammatory cytokines and proteolytic enzymes (Fig. 3A–D). 2DG is a glucose analog which blocks the first step of glycolysis. It is phosphorylated by hexokinase and this phosphorylated molecule, 2-deoxy-D-glucose-6-phosphate, cannot be further metabolized [23,24]. As in previous reports, the use of 2DG to alter metabolic profiles also suppressed inflammation in RA FLS [8,14]. In this study, 2DG not only reversed LPS-enhanced usage of the glycolysis pathway but also suppressed the LPS-induced gene expression of proteins that are key to the regulation of glycolysis, GLUT1 and HK2 (Fig. 2A–C). These results demonstrate the close linkage between changes in intermediary metabolism and the development of a pro-inflammatory phenotype that occur during even the earliest stages of inflammation associated with diseases such as RA.

In other systems, the mechanisms responsible for this linkage between metabolism and downstream pro-inflammatory phenotype has been reported to involve changes in AMPK and/or HIF1α [8,9,14].

However, many details of these mechanisms remain unclear as well as whether other factors might be involved. As shown in Fig. 4E and F, 2DG increased AMPK activity and LPS abrogated this AMPK activation in this study. The reason why 2DG increased AMPK activity was considered that 2DG treatment reduced intracellular ATP production by inhibition of glycolysis. As in Fig. 1E, LPS stimulation increased total ATP production by increasing glycolysis. It is considered that the reason why AMPK activation was abrogated was antagonizing the decrease in ATP production by 2DG and the increase in ATP production by LPS.

Interestingly, in this study, changes to CREB phosphorylation were found to be one of the key factors responsive to 2DG suppression of downstream inflammatory cytokines and proteolytic enzymes. CREB is a nucleoprotein transcription factor. CREB functions as a transcription factor in glucose homeostasis as well as cell survival. CREB is phosphorylated in response to various extracellular signals such as changes in cyclic AMP, calcium and various growth factors [25,26]. Once phosphorylated CREB binds to CREB binding protein (CBP) resulting in intrinsic histone acetyltransferase (HAT) activity, which increases the accessibility of the activator to DNA, destabilization of the chromatin structure and enhanced transcription of target genes [25,27]. In previous studies, activated CREB and CBP were associated with changes in synovial cell activity in patients with RA, and suggested as new potential therapeutic targets for RA treatment [28,29]. In addition, enhanced CREB phosphorylation is involved in cell signal pathway activated by LPS, an agonist of Toll-like receptor 4 [30]. Ishizu showed that inhibition of CREB function significantly reduced IL6 production from arthritic synovial cells implicating CREB activation in IL6 production by synovial cells [31]. In this study, CREB phosphorylation was enhanced by LPS stimulation (Fig. 4B) and, the validated CREB inhibitor (666-15) suppressed expression of inflammatory cytokines and proteolytic enzymes (Fig. 5A–D). Riemann showed that acidosis in tumor cells also increased phosphorylation of the transcription factor CREB, which in turn resulted in increased transcriptional activity [32]. Geborek showed that synovial fluid acidosis in RA patients was correlated with joint destruction [33]. Fig. 2D showed that 2DG suppressed this LPS-enhanced production of lactate and, as shown in Fig. 4G and H, 2DG suppressed CREB phosphorylation enhanced by LPS. Thus, the ability of 2DG to reverse LPS-enhanced transcription activity of inflammatory cytokines and proteolytic enzymes (Fig. 6) is likely due to inhibition of CREB phosphorylation and suppression of associated HAT activity. In sum, all of these pathways appear to be closely linked as 2DG reverses enhanced glycolysis, reverses acidosis, suppresses CREB phosphorylation and blocks the enhanced transcription of inflammatory cytokines and proteolytic enzymes. Thus, by suppressing glycolysis and correcting acidosis in joints though the use of glycolysis inhibitors such as 2DG, the progression of joint destruction can be suppressed in RA patients.

5. Conclusions

We confirmed that changes in intermediary metabolism are closely associated with the early stages of inflammation in non-pathological, bovine synovial cells. While most ATP synthesis is supplied by mitochondrial respiration in normal synovial cells, the metabolism shifts to a heavier reliance on the glycolysis pathway for ATP production as one of the earliest inflammation events. Additionally, we showed that the signaling pathways of anti-inflammatory effect of glycolysis inhibitors in synovial cells is associated with CREB phosphorylation. Further studies of synovial cell inflammation and metabolism will lead to the development of new targets for the treatment of RA.

Author contributions

K.T. and N.T. contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting/revising the manuscript critically for important intellectual content, and provided final approval of the version to be submitted. T.K. and S.I. contributed to the conception and design of the study, acquisition of data, revising the manuscript critically for important intellectual content, and provided final approval of the version to be submitted. Y. Y., Y. O., K.H., D.K. and M.M. contributed to analysis and interpretation of data and technical assistance. All authors read and approved the final manuscript.

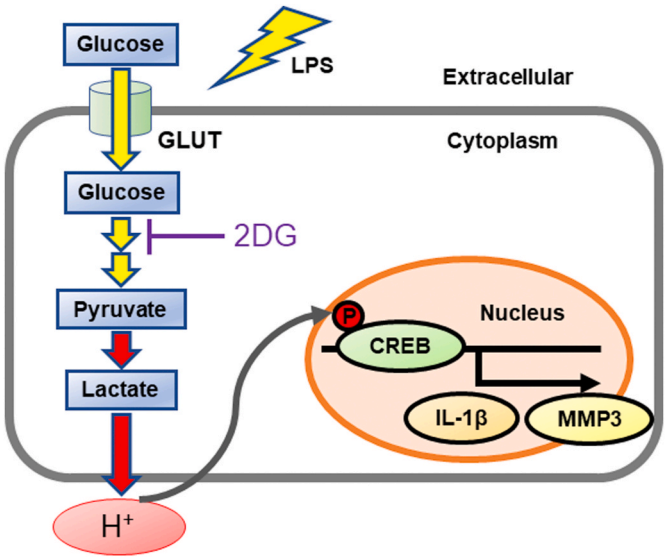

Fig. 6. The anti-inflammatory effect of 2DG is caused by inhibiting glycolysis and suppressing CREB phosphorylation.
Glycolysis is enhanced under inflammation stimulated by LPS. Glycolysis converts glucose into pyruvate and lactate. Lactate is excreted extracellularly, and the intracellular periphery becomes acidosis. Pericellular acidosis enhances CREB phosphorylation, and increases intrinsic HAT activity. 2DG suppresses LPS-enhanced production of lactate, and suppresses CREB phosphorylation, thereby correcting the acidosis around the cells. Inhibition of CREB phosphorylation by 2DG suppresses HAT activity, and reverses enhanced transcription activity of inflammatory cytokines and proteolytic enzymes enhanced by LPS.

Data availability

All data generated or analyzed during this study are includes in this published article.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2021.108962.

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