The dopamine D3 receptor regulates dopamine-induced activation and glycolytic metabolism of synovial fibroblasts in rheumatoid arthritis

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Abstract

Introduction: Increased glycolytic metabolism in synovial fibroblasts contributes to their activated phenotype in rheumatoid arthritis (RA). Our previous results revealed that the activation of the dopamine D3 receptor (D3R) in mast cells reduced inflammation in a mouse model of RA. In this study, we explored the role of D3R in regulating dopamine-induced activation and glycolysis in synovial fibroblasts from patients with RA (RASFs). **Method:** RASFs were cultured in the presence of dopamine. Pharmacological modulation of D3R by D3R agonist (7-OH-DPAT) and antagonist (NGB2904) was used to investigate the regulatory role of D3R in dopamine-induced activation and glycolysis in RASFs. **Results:** Dopamine stimulation induced a dose-dependent increase in cell viability and α-SMA expression in RASFs. Dopamine also caused significant and dose-dependent upregulation of glycolysis-related enzymes in RASFs. Treatment with 7-OH-DPAT inhibited dopamine-induced increases inα-SMA expression and inflammatory response in RASFs, whereas NGB2904 treatment resulted in the enhanced effects stimulated by dopamine. NGB2904 treatment upregulated glycolysis and the expression of glycolytic enzymes induced by dopamine, whereas 7-OH-DPAT treatment downregulated glycolysis and glycolytic enzymes in RASFs. NGB2904 attenuated the ability of 7-OH-DPAT to inhibit the dopamine-induced elevation in cAMP levels of RASFs. Involvements of the cAMP pathway was confirmed by findings that H89 (a PKA inhibitor) abrogated the upregulation of activation, glycolysis, and expression of glycolytic enzymes mediated by the D3R antagonist, NGB2904, in RASFs. **Conclusion:** D3R downregulates dopamine induced activation and glycolysis of RASFs by suppressing PKA activity. Therefore, inhibition of glycolysis by manipulating the D3R pathway may provide a novel therapeutic strategy to reduce the activation of RASFs.

Keywords: Dopamine D3 receptor, Synovial fibroblasts, Activation and glycolytic metabolism, Rheumatoid arthritis.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by synovitis and pannus formation, which lead to the progressive destruction of cartilage and bone [1]. RA has a substantial impact on public health owing to its effect on mobility and quality of life caused by joint damage and comorbidities, including pleurisy, pericarditis, and vasculitis [2, 3].

An important advance in our understanding of RA is the realisation that synovial fibroblasts play a major role in the pathogenesis of the disease [4] and are important contributors to the switch from acute to chronic inflammation [4]. Synovial fibroblasts are a key cell type within the hyperplastic pannus and major contributors to joint destruction in RA. The inflammatory milieu in the synovium in RA induces synovial fibroblasts to acquire an aggressive phenotype with aberrant proliferation and enhanced activation properties [5]. Synovial fibroblasts from patients with RA exhibit the increased expression of α-smooth muscle actin (α-SMA), which is associated with synovial fibroblast activation. Moreover, activated synovial fibroblasts secrete inflammatory mediators, such as cytokines, chemokines, and tissue-degrading enzymes to facilitate the recruitment of immune cells to the synovium, resulting in synovial hyperplasia and joint destruction [6, 7].

Mediators of glucose metabolism are likely to become therapeutic targets in RA [8, 9]. Metabolic conversion from oxidative phosphorylation to glycolysis is a typical feature of inflamed joints in patients with RA [10, 11]. An increase in glucose uptake and glycolytic gene expression has also been observed in the stromal compartment of the joints of mice with experimental arthritis. Importantly, the inhibition of glycolysis significantly decreased the severity of arthritis in the same model [7]. Moreover, metabolic changes from using aerobic oxidative phosphorylation to anaerobic glycolysis have been observed in synovial fibroblasts of patients with RA in response to a hypoxic microenvironment to maintain energy homeostasis under low oxygen conditions [12, 13]. There is a notable association

between increased aerobic glycolysis and inflammatory properties of synovial fibroblasts in patients with RA [8, 9]. Consequently, these studies have demonstrated that mediators related to glucose metabolism are probable targets for the treatment of RA.

Based on these findings, there is considerable interest in the identification of endogenous metabolic regulators, and recent findings have highlighted the importance of neurotransmitters, such as dopamine, as potential regulators of glucose metabolism [14, 15]. As a crucial catecholamine neurotransmitter, dopamine plays a vital role in many physiological processes, including cognition, emotion, and memory [16]. Dopamine acts by binding to dopamine receptors (DRs), which are members of the G protein-coupled receptor superfamily and separated into two distinct classes: D1-like (D1R and D5R) and D2-like (D2R, D3R, and D4R). D1-like and D2-like receptors activate or inhibit adenylate cyclase, leading to the elevation or suppression of intracellular cyclic adenosine monophosphate (cAMP), respectively [17]. The D2-like antagonist, ONC206, suppresses the proliferation of uterine serous cancer cells by altering the expression of proteins related to glycolysis [18]. Furthermore, the role of dopamine in the pathogenesis of RA is exemplified by its ability to induce the migration of synovial fibroblasts *in vitro* [19]. Moreover, the density of D3R was significantly higher in synovial fibroblasts from patients with RA compared with that in those with osteoarthritis [20]. Our previous results demonstrated that the activation of D3R in mast cells inhibited inflammation in a mouse model of RA [21]. However, the role of D3R in the regulation of the activation and metabolic activity of synovial fibroblasts from patients with RA (RASFs) remains unknown. In this study, we aimed to elucidate the role of D3R in the activation and glycolysis of RASFs and determine the mechanisms involved in this process.

Materials and methods

Cell culture and reagents

Primary synovial fibroblasts were obtained from the synovial tissues of patients

with RA who underwent joint replacement surgery. All patients were diagnosed with RA based on the American College of Rheumatology/European League Against Rheumatism 2010 classification criteria [22]. Demographic and clinical characteristics of the enrolled patients are summarized in Table 1. Synovial fibroblasts were isolated and cultured as previously described [23]. Synovial tissue was diced and then treated with collagenase A for 2 h at 37°C. Synovial fibroblasts were cultured in DMEM medium (Gibco, Thermo Fisher Scientific) containing 10% foetal bovine serum (FBS; Gibco, Thermo Fisher Scientific) and 1% penicillin/streptomycin (Invitrogen) at 37°C in a humidified incubator in 5% CO_2. RASFs were expanded four to five passages. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University (NO. 2020–872). All the participants provided written informed consent.

Dopamine hydrochloride, (±)-7-hydroxy-2-(di-n-propylamino) tetralin hydrobromide (7-OH-DPAT) and NGB2904 hydrochloride (NGB2904), oligomycin A and 2-deoxy-D-glucose were purchased from Sigma-Aldrich.7-OH-DPAT and NGB2904 was diluted with phosphate buffered saline and 50% polyethylene glycol 400, respectively. NGB2904 (100 nM) and 7-OH-DPAT (100 nM) were used to block or stimulate D3R expression in RASFs. The protein kinase A (PKA) inhibitor, H89, was obtained from Selleck Chemicals.

Cell viability assay

Cell viability was measured using the PrestoBlue™ Cell Viability Reagent (Invitrogen). RASFs were seeded in 96-well plates at a density of 2×10^4 cells per well containing 100 µL of cell culture medium. After 24 and 48 h, cell viability was tested by adding 10 µL of PrestoBlue solution into each well. After incubation for 30 min, cell viability was measured by determining the absorbance value (570 nm) using a BIO-TEK Microplate Reader (Bio-Tek).

Intracellular cAMP concentration assessment

After being washed with PBS, RASFs cultured in a 24-well plate were lysed with 100 µL of lysis buffer. The resulting lysates were transferred to a pre-coated assay

plate, and cAMP levels were quantified using the cAMP-Screen® System (Applied Biosystems) in accordance with the manufacturer's instructions. Absorbance was measured at 450 nm, and concentrations were determined using the standard curve provided with the assay kit.

Measurement of extracellular acidification rate

Assays to detect extracellular acidification rate were conducted using the Seahorse XFp platform (Agilent Technologies). RASFs were seeded in wells at a density of 30,000 cells/well in 80 µl of culture medium and then cultured for 24 h. Subsequently, cells were cultured in fresh culture medium supplemented with 0.1% FBS for a further 24 h prior to treatment with or without 100 nM of 7-OH-DPAT or NGB2904, and with or without 80 µM dopamine for 24 h. To detect glycolytic function, culture medium in the wells was substituted with 175 µl of assay medium (XF DMEM medium, pH 7.4, containing 1 mM sodium l-glutamine). After 1 h of humidified incubation at 37°C, glycolysis was examined using the Seahorse platform. Each treatment was repeated three times. Glycolysis, glycolytic capacity, and glycolytic reserve were determined by sequentially adding glucose, oligomycin A and 2-deoxyglucose (2-DG) into corresponding wells. The final concentrations of glucose, oligomycin A, and 2-deoxyglucose were 10, 2, and 50 mM, respectively.

Measurement of glucose consumption and lactate production

Glucose levels and lactate production were measured using commercial kits in accordance with the manufacturer's instructions (Boxbio, CN). Glucose consumption was interpreted as the difference in the glucose concentration in the medium before and after cell incubation. Finally, glucose consumption and lactate production rates were normalised to the cell number.

Cytokine detection in cell culture supernatants

The culture supernatants of synovial fibroblasts from patients with RA were collected and centrifuged at 800× g for 12 min. The concentrations of interleukin 6 (IL-6), interleukin 8 (IL-8), and matrix metalloproteinase-3 (MMP-3) were detected using enzyme-linked immunosorbent assay, following the manufacturer's

specifications (R&D Systems).

Fluorescence microscopy

The RASFs were left to adhere to glass coverslips, fixed with paraformaldehyde, and permeabilised with 0.25% Triton X-100. Fluorescent antibody staining was performed using the primary antibody against α-SMA (1: 500, Agilent Dako, M085101-2) incubation in blocking buffer overnight at 4℃ and FITC-labelled anti-mouse-IgG. Cell nuclei were stained with 4′, 6-dia-midino-2-phenylindole (DAPI). The analyses were performed using a NIKON Ni-E microscope. The results were analyzed with Image J 2.1.4.7.

Western blotting

Total cell extracts from RASFs were prepared using RIPA buffer (Thermo Fisher Scientific) containing 1% protease/phosphatase inhibitor cocktail (complete Mini, Roche). Protein concentration was determined using a BCA Protein Assay Kit (Pierce Biotechnology). After being diluted in loading buffer and denatured at 75℃for 10 min, protein samples were separated using 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes. The membranes were blocked with casein blocking buffer (Sigma-Aldrich) containing 0.15% Triton X-100 for 1 h followed by incubation with the primary antibodies diluted in blocking buffer overnight at 4℃. The primary antibodies used were anti-HK1 (CST, #2024), anti-HK2 (CST, #2106), anti-PFKL (CST, #89495), anti-PFKM (Proteintech, #55028-1-AP), anti-PFKP (CST, #8164), anti-PFKFB3 (, CST, #13123), anti-p-PKM2 (CST, #3827), anti-LDHA (CST, #2012), anti-p-LDHA (CST, #8176), anti-LDHB (CST, #56298), anti-α-SMA (Agilent Dako, M085101-2), and anti-β-Tubulin (Abcam, ab6046). The membranes were washed thrice with PBST for 10 min each and then incubated with an HRP-conjugated secondary antibody for 1 h at room temperature (25℃). After washing, the membranes were incubated with ECL Plus reagent (Amersham) and the labelled proteins were visualised using a gel scanner (Bio-Rad, USA). Quantification was conducted using densitometric analysis with the Image J software.

Statistical analysis

Data are presented as mean ± standard deviation (SD). The SPSS V.16.0 for Windows software (IBM) was used for the statistical analysis. Comparisons among multiple groups were performed using one-way analysis of variance, followed by Tukey's post hoc test. Statistical significance was set at $P < 0.05$.

Results

The effect of dopamine on the activation and inflammatory response of RASFs

To assess the effect of dopamine on the activation of RASFs, RASFs were treated with dopamine at increasing concentrations (10, 20, 60, 80, and 100 μM) for 24 and 48 h. Cell viability was then measured using PrestoBlue. As shown in Figure 1A, dopamine stimulation increased cell viability in a dose-dependent manner. Compared with the control group, groups treated with 80 or 100 μM dopamine showed significantly higher cell viability at 24 and 48 h. However, no significant differences in cell viability were observed between the control group and the other two groups (10, 20 and 60 μM dopamine treatment) at 24 and 48 h. We also measured expression of the activation marker, α-SMA, in RASFs using western blotting. Consistent with the PrestoBlue cell viability results, A significant increase in α-SMA expression was observed in the dopamine treatment groups (80 and 100 μM) compared with that in the control group (Figure 1B). We also determined the effects of dopamine on the inflammatory response in RASFs. As shown in Figure 1C, the IL-6, IL-8, and MMP-3 production was increased following treatment with dopamine (80 and 100 μM) in RASFs. These results provided evidence that dopamine promotes the activation and inflammatory response of RASFs.

Dopamine induces expression of glycolytic enzymes in RASFs

Accelerated glycolysis has been described as an important characteristic of activated RASFs [9], therefore, the effect of dopamine on the expression of glycolysis-related enzymes in RASFs was investigated. As shown in Figure 1D, stimulation with dopamine induced significant and dose-dependent upregulation of enzymes

associated with glycolysis, including hexokinases (HK1 and HK2), phosphofructose kinases (PFKL, PFKM, PFKP, and PFKFB3), phosphorylated pyruvate kinase M2 (PKM2), and phosphorylated lactate dehydrogenase A (p-LDHA) in RASFs. However, no significant changes were observed in the protein expression of enzymes associated with lactate dehydrogenase A (LDHA) and lactate dehydrogenase B (LDHB). These results revealed a direct stimulatory effect of dopamine on the expression of glycolysis-related enzymes in RASFs.

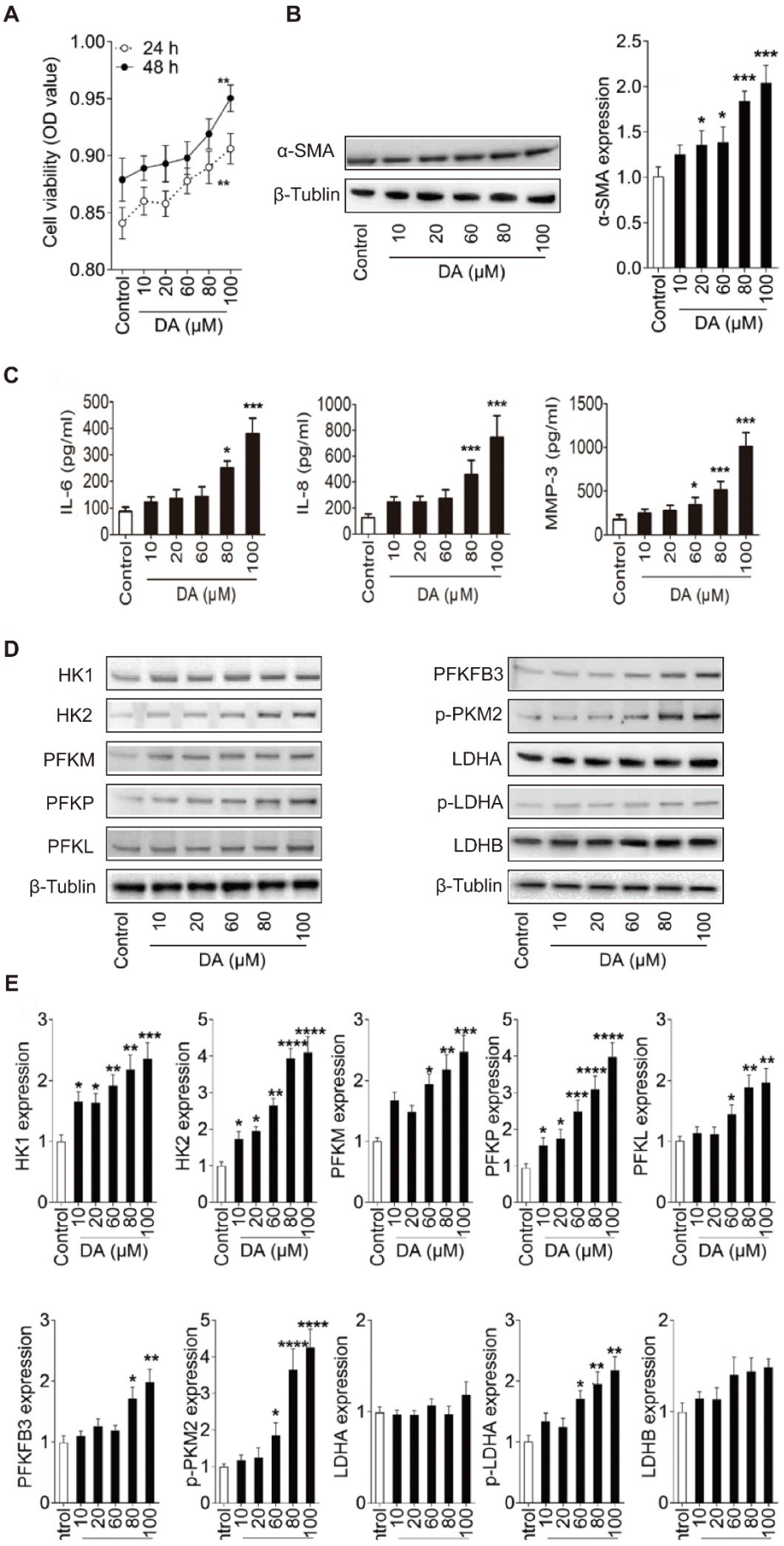
A
Cell viability (OD value)
24 h
48 h
Control
DA (μM)
B
α-SMA
β-Tublin
α-SMA expression
C
IL-6 (pg/ml)
IL-8 (pg/ml)
MMP-3 (pg/ml)
D
HK1
HK2
PFKM
PFKP
PFKL
β-Tublin
PFKFB3
p-PKM2
LDHA
p-LDHA
LDHB
E
HK1 expression
HK2 expression
PFKM expression
PFKP expression
PFKL expression
PFKFB3 expression
p-PKM2 expression
LDHA expression
p-LDHA expression
LDHB expression

Figure 1. Dopamine induces RASF activation and promotes the expression of glycolysis-related enzymes in RASFs. (A) Assessment for cell viability of RASFs using the PrestoBlue assay. Cells were treated with a vehicle control or different concentrations of dopamine (DA; 10, 20, 60, 80, or 100 μM) for 24 or 48 h (n=6). (B and D) Western blotting of expression of activation marker (α-SMA) (B) and glycolytic enzymes (D), including hexokinases (HK1 and HK2), phosphofructokinase (PFKL, PFKM, PFKP, and PFKFB3), pyruvate kinases (p-PKM2), and dehydrogenase (LDHA, p-LDHA, and LDHB) in RASFs. Cells were treated with a vehicle control or different concentrations of dopamine (10, 20, 60, 80, or 100 μM) for 24 h. Densitometric analysis of western blotting results of glycolytic enzymes (n=6). (C) Cytokine detection of RASFs using the ELISA assay. Cells were treated with a vehicle control or different concentrations of dopamine (DA; 10, 20, 60, 80, or 100 μM) for 24 or 48 h (n=12). All data are representative of the means ± SEM.* P <0.05 vs control, ** P<0.01 vs control, *** P<0.001 vs control, **** P<0.0001 vs control. RASF, synovial fibroblast from patients with rheumatoid arthritis.

D3R signalling antagonizes dopamine-induced activation and inflammatory response of RASFs

Dopamine signalling through the D1- and D2-like family receptors has opposing effects on PKA. D1-like receptors (D1R and D5R) enhance intracellular levels of cAMP, leading to the activation of adenylate cyclase and PKA, whereas D2-like receptors (D2R, D3R, and D4R) decrease the levels of cAMP and inhibit the activation of PKA [17]. We previously showed that genetic deletion of D3R results in the exacerbation of experimental arthritis with increased mast cell activity via a TLR-4 dependent mechanism [21]. We investigated whether D3R signalling affects dopamine-mediated activation and inflammatory responses in RASFs. As shown in Figure 2A-2B, pretreatment with the D3R agonist, 7-OH DPAT, significantly suppressed dopamine-induced increase in α-SMA expression in RASFs, whereas pretreatment with the D3R antagonist, NGB2904, restored the ability of dopamine to increase α-SMA expression. In order to further analyze the D3R involved in the dopamine-induced effect, RASFs were pre-incubation with 7-OH DPAT and NGB together followed by dopamine. As shown in Figure 2C, incubation with the selective dopamine D3R receptor antagonist NGB2904 attenuated the ability of 7-OH-DPAT to inhibit the dopamine-induced elevation in cAMP levels. Additionally, consistent with the activation of RASFs, D3R agonist 7-OH DPAT pretreatment led to decreased cytokine production of IL-6, IL-8, and MMP-3 induced by dopamine. In contrast, the D3R

antagonist, NGB2904, caused an elevation in the production of these cytokines compared with the effects induced by dopamine in RASFs (Figure 2D).

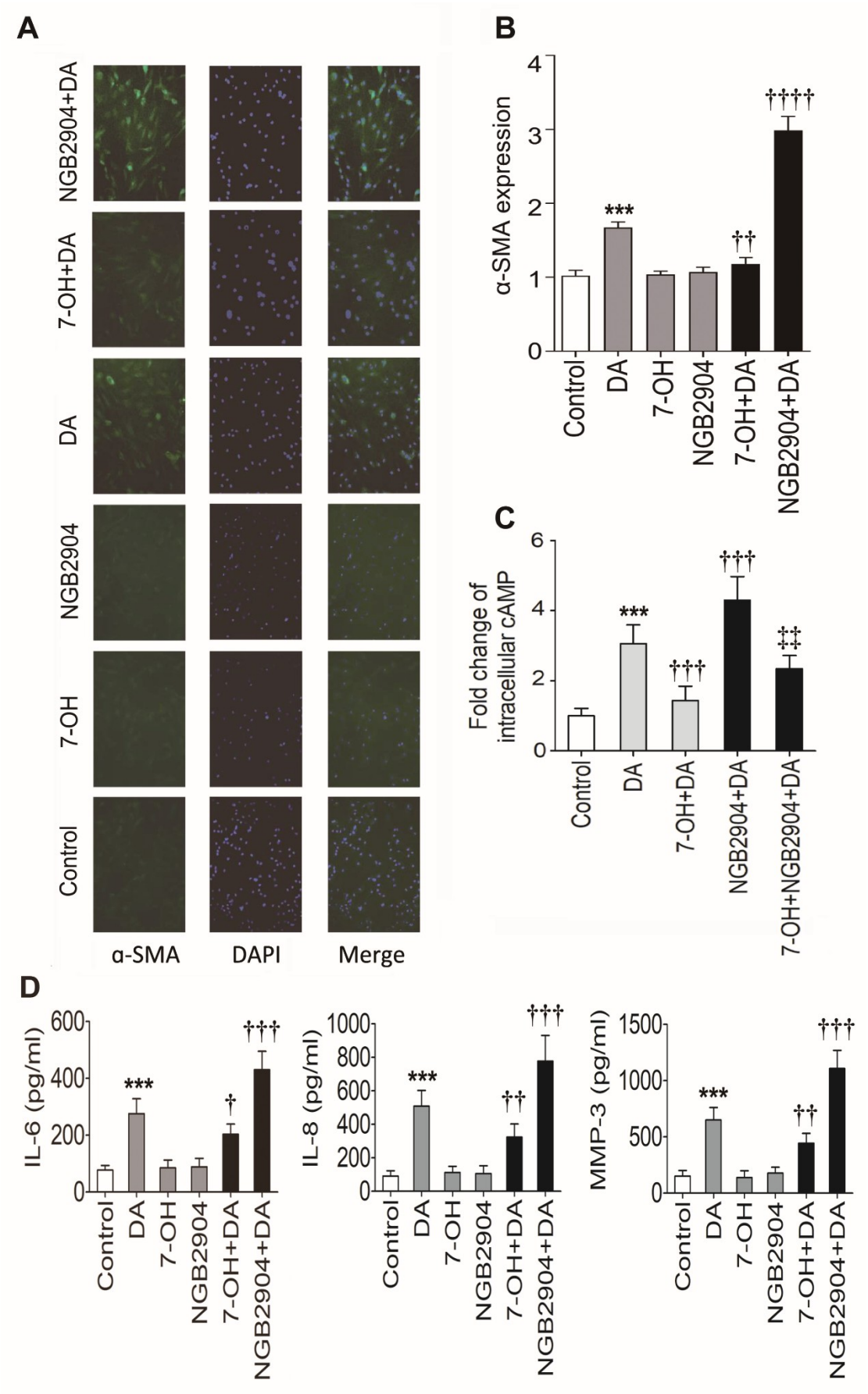
A
NGB2904+DA
7-OH+DA
DA
NGB2904
7-OH
Control
α-SMA
DAPI
Merge
B
α-SMA expression
Control
DA
7-OH
NGB2904
7-OH+DA
NGB2904+DA
C
Fold change of intracellular cAMP
7-OH+NGB2904+DA
D
IL-6 (pg/ml)
IL-8 (pg/ml)
MMP-3 (pg/ml)

Figure 2. D3R suppresses dopamine-mediated activation of RASFs. (A) Fluorescence micrographs with staining for α-SMA (green) and nuclei (blue) after RASFs were pretreated with 7-OH DPAT (100 nM) or NGB2904 (100 nM) for 1 h followed by treatment with or without dopamine (80 μM) for 24 h. (B) Statistical analysis of fluorescence staining results of α-SMA (n=6). (C) CAMP detection of RASFs using the ELISA assay. RASFs were pretreated with 7-OH DPAT (100 nM) and/or NGB2904 (100 nM) for 1 h prior to stimulation with or without dopamine (80 μM) for 24 h (n=12). (D) Cytokine detection of RASFs using the ELISA assay. RASFs were pretreated with 7-OH DPAT (100 nM) or NGB2904 (100 nM) for 1 h followed by treatment with or without dopamine (80 μM) for 24 h (n=12). All data are representative of the means ± SEM. *** P<0.001 vs control, † P <0.05 vs DA, †† P <0.01 vs DA, ††† P<0.001 vs DA, †††† P<0.0001 vs DA, ‡‡ P<0.01 vs 7-OH+DA. DA, dopamine; RASF, synovial fibroblast from patients with rheumatoid arthritis.

D3R signalling abrogates dopamine-induced glycolysis in RASFs

To determine whether D3R participates in the regulation of dopamine-induced glycolysis, we examined the effects of pretreatment with a D3R agonist or antagonist on glycolytic metabolism. The Seahorse XF96 flux analyser showed that stimulation by dopamine induced a significant upregulation of glycolysis and glycolytic capacity in RASFs. The D3R antagonist, NGB2904, enhanced glycolysis and glycolytic capacity in response to dopamine stimulation, whereas pretreatment with the D3R agonist, 7-OH DPAT, significantly inhibited dopamine-induced increase in glycolysis and glycolytic capacity in RASFs (Figure 3A and 3B). This was consistent with the finding that pretreatment with the D3R agonist, 7-OH DPAT, led to the downregulation of key glycolytic enzymes such as HK1, HK2PFKL, PFKP, PFKFB3, p-PKM2 and p-LDHA induced by dopamine in RASFs, whereas pretreatment with the D3R antagonist, NGB2904, increased expression of PFKM, PFKP and p-PKM2 (Figure 4). Moreover, D3R antagonist, NGB2904, led to enhanced glycolytic capacity, lactate production and glucose consumption induced by dopamine in RASFs, while D3R agonist, 7-OH DPAT, led to a significant decrease in glycolytic capacity, lactate production and glucose consumption induced by dopamine (Figure 3).

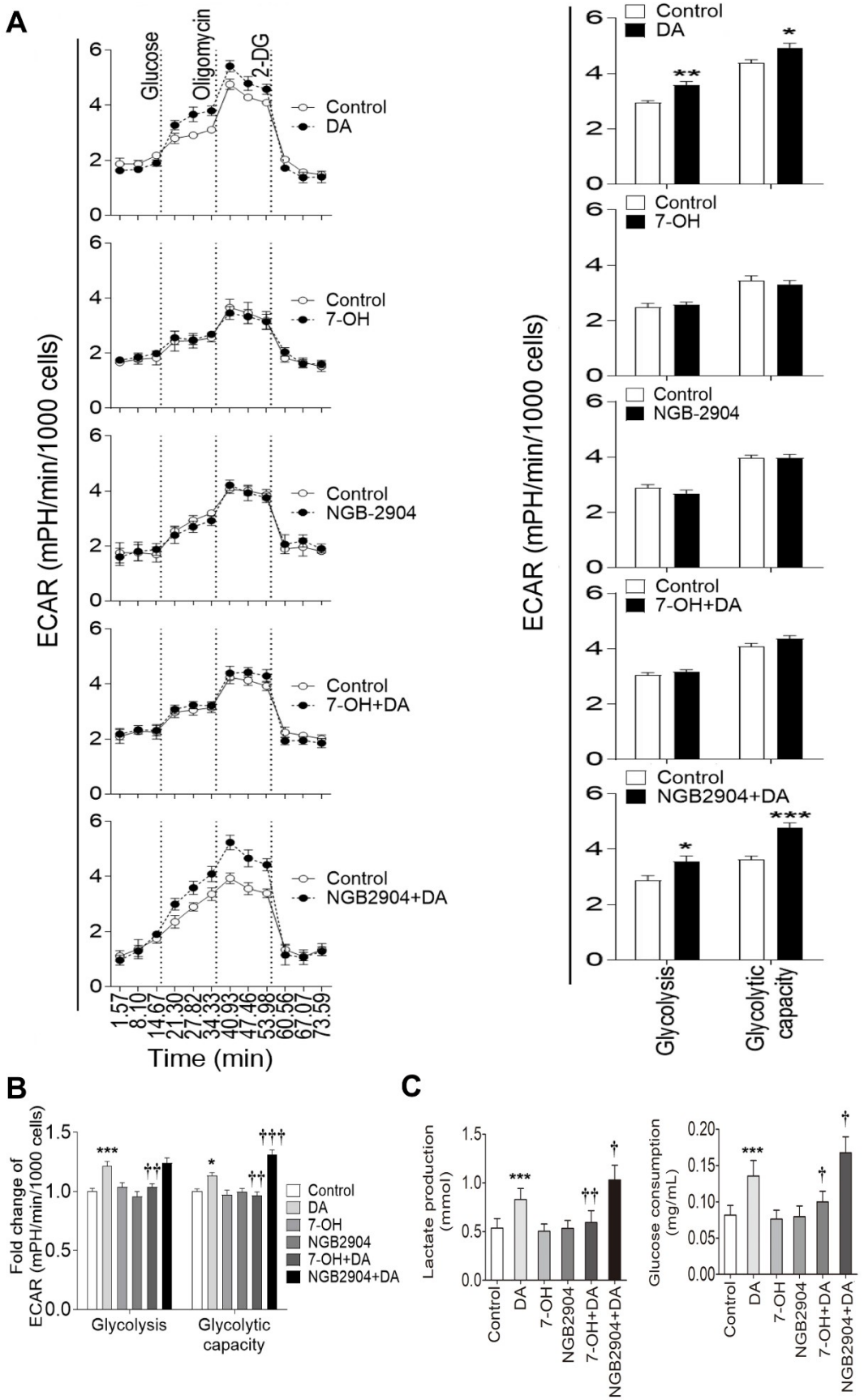
A
ECAR (mPH/min/1000 cells)
Glucose
Oligomycin
2-DG
Control
DA
Control
7-OH
Control
NGB-2904
Control
7-OH+DA
Control
NGB2904+DA
Time (min)
1.57
8.10
14.67
21.30
27.82
34.33
40.93
47.46
53.98
60.56
67.07
73.59
Glycolysis
Glycolytic capacity
B
Fold change of ECAR (mPH/min/1000 cells)
Control
DA
7-OH
NGB2904
7-OH+DA
NGB2904+DA
C
Lactate production (mmol)
Glucose consumption (mg/mL)

Figure 3. D3R suppresses the dopamine-mediated increase of glycolysis in RASFs. (A) Assays for detecting extracellular acidification rate of RASFs using the Seahorse XFp platform after cells were pretreated with 7-OH DPAT (100 nM) or NGB2904 (100 nM) for 1 h and then treated with or without dopamine (80 μM) for 24 h. (B) Analysis of fold change in glycolysis and glycolytic capacity by comparison among multiple groups (n=6). (C) Glucose consumption and lactate production in culture supernatants of RASFs. Cells were pretreated with 7-OH DPAT (100 nM) or NGB2904 (100 nM) for 1 h followed by treatment with or without dopamine (80 μM) for 24 h (n = 6). All data are representative of the means ± SEM. * P<0.05 vs control, ** P<0.01 vs control, *** P<0.001 vs control, † P<0.05 vs DA, †† P<0.01 vs DA, ††† P<0.001 vs DA. DA, dopamine; RASF, synovial fibroblast from patients with rheumatoid arthritis.

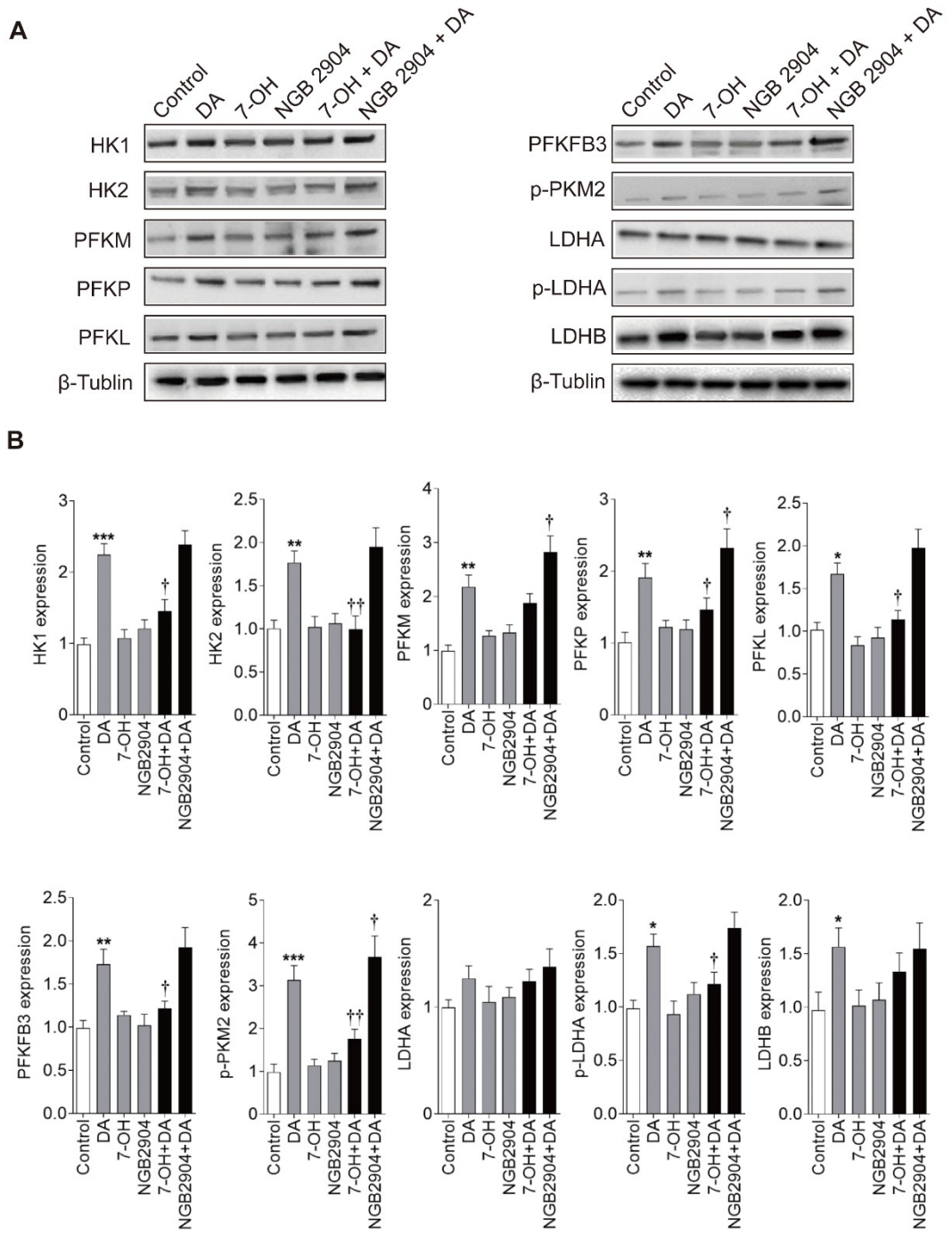

Figure 4. D3R downregulates the dopamine-mediated expression of glycolytic enzymes in RASFs. (A) Effect of the D3R agonist, 7-OH DPAT, and D3R antagonist, NGB2904, on the expression of glycolytic enzymes in RASFs. Western blotting analysis for the expression of glycolysis related enzymes, including hexokinases (HK1 and HK2), phosphofructokinase (PFKL, PFKM, PFKP, and PFKFB3), pyruvate kinases (p-PKM2) and dehydrogenase (LDHA, p-LDHA, and LDHB) after RASFs were pretreated with 7-OH DPAT (100 nM) or NGB2904 (100 nM) for 1 h and then treated with or without dopamine (80 μM) for 24 h. (B) Densitometric analysis of the western blotting results (n=6). All data are representative of the means ± SEM. * P<0.05 vs control, ** P<0.01 vs control, *** P<0.001 vs control, † P<0.05 vs DA, †† P<0.01 vs DA. DA, dopamine; RASF, synovial fibroblast

from patients with rheumatoid arthritis.

Role of PKA in dopamine-mediated activation in RASFs

D3R coupled to the inhibitory G protein $G_{i/o}$ and inhibits cAMP production, resulting in decreased PKA activity [17, 24]. PKA plays a vital role in regulating glucose metabolism related to glycolysis [18]. We hypothesised that a PKA inhibitor would mimic the effect of DR3 stimulation on the dopamine-induced activation of RASFs. As shown in Figure 5, pretreatment with the selective PKA inhibitor, H89, significantly suppressed the stimulatory effect of dopamine on RASF activation. Furthermore, treatment with H89 and the D3R agonist, 7-OH DPAT, further inhibited the dopamine-induced activation of RASFs. These findings confirm the role of PKA in dopamine-induced activation of RASFs.

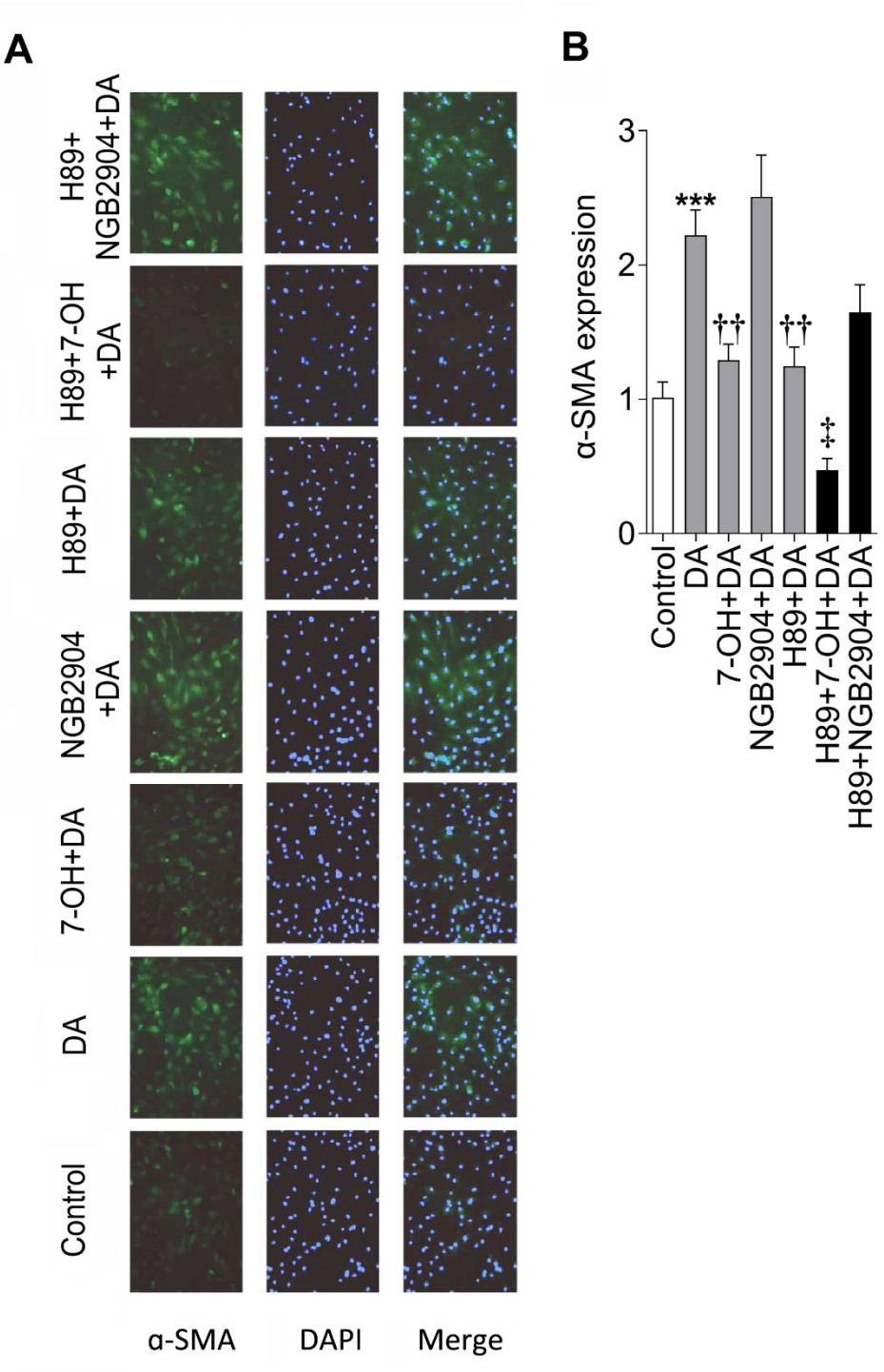

Figure 5. D3R regulates the dopamine-induced activation of RASFs via PKA. (A) Fluorescence micrographs with staining for α-SMA (green) and nuclei (blue) after RASFs were pretreated with or without PKA inhibitor H89 (10 μM) combined with 7-OH DPAT (100 nM) or NGB2904 (100 nM) for 1 h, and then treated with dopamine (80 μM) for 24 h. (B) Statistical analysis of fluorescence staining results of α-SMA (n=6). All data are representative of the means ± SEM. *** P<0.001 vs control, †† P<0.01 vs DA, ‡ P<0.05 vs H89+DA. DA, dopamine; RASF, synovial fibroblast from patients with rheumatoid arthritis.

Role of PKA in dopamine-mediated upregulation of glycolysis in RASFs

We next explored the role of PKA signalling in the dopamine-induced upregulation of glycolysis-related enzymes in RASFs. Treatment with H89 significantly inhibited the effects of dopamine on the expression of glycolytic enzymes, including HK1, HK2, PFKM, PFKP, PFKFB3, p-PKM2 and p-LDHA. An additive inhibitory effect was observed between H89 and the D3R agonist, 7-OH DPAT, on the expression of HK1, PFKM, PFKP (Figure 6A and 6B). Additionally, H89 pretreatment of RASFs significantly diminished the effects of dopamine on lactate production and glucose consumption. Lactate production and glucose consumption were lower in RASFs treated with H89, 7-OH DPAT and dopamine than in those treated with H89 and dopamine. (Figure 6C). These results confirm that PKA signalling contributes to dopamine-induced and D3R antagonist-mediated upregulation of glycolysis in RASFs.

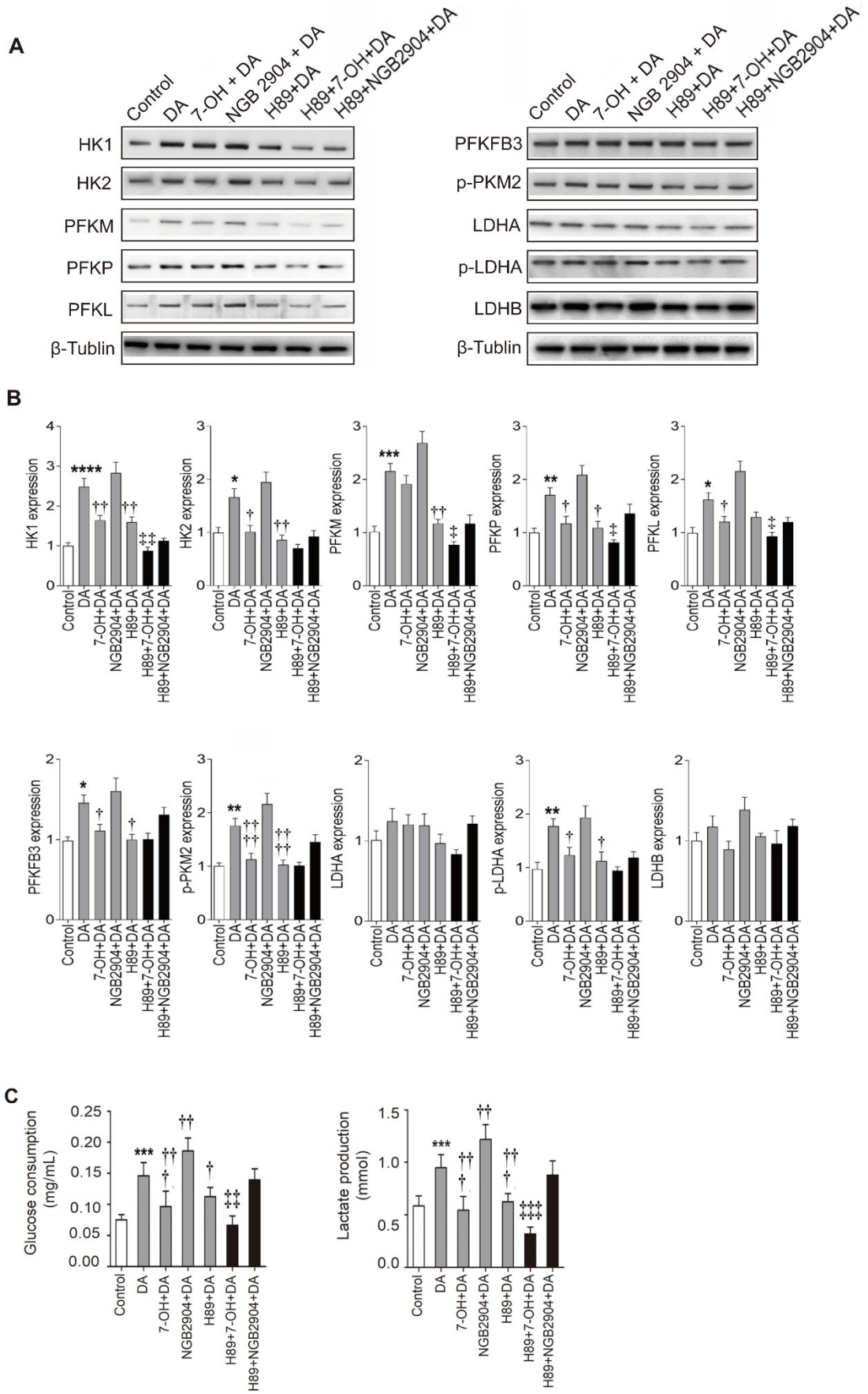
A
Control
DA
7-OH + DA
NGB 2904 + DA
H89+DA
H89+7-OH+DA
H89+NGB2904+DA
HK1
HK2
PFKM
PFKP
PFKL
β-Tublin
PFKFB3
p-PKM2
LDHA
p-LDHA
LDHB
β-Tublin
B
HK1 expression
HK2 expression
PFKM expression
PFKP expression
PFKL expression
PFKFB3 expression
p-PKM2 expression
LDHA expression
p-LDHA expression
LDHB expression
C
Glucose consumption (mg/mL)
Lactate production (mmol)

Figure 6. D3R regulates the dopamine-induced expression of glycolytic enzymes in RASFs via PKA. (A) Western blotting analysis for the expression of glycolysis related enzymes including hexokinases (HK1 and HK2), phosphofructokinase (PFKL, PFKM, PFKP, and PFKFB3), pyruvate kinases (p-PKM2), and dehydrogenase (LDHA, p-LDHA, and LDHB) after RASFs were pretreated with or without the PKA inhibitor, H89 (10 μM), combined with 7-OH DPAT (100 nM) or NGB2904 (100 nM) for 1 h, followed by treatment with dopamine (80 μM) for 24 h. (B) Densitometric analysis of the western blotting results (n=6). (C) Glucose consumption and lactate production in culture supernatants of RASFs. Cells were pretreated with or without the PKA inhibitor, H89 (10 μM), combined with 7-OH DPAT (100 nM) or NGB2904 (100 nM) for 1 h, followed by treatment with dopamine (80 μM) for 24 h (n = 6). All data are representative of the means ± SEM. * P <0.05 vs control, ** P<0.01 vs control, *** P<0.001 vs control, **** P<0.0001 vs control, † P<0.05 vs DA, †† P<0.01 vs DA, ††† P<0.001 vs DA, †††† P<0.0001 vs DA, ‡ P<0.05 vs H89+DA, ‡‡ P<0.01 vs H89+DA, ‡‡‡ P<0.001 vs H89+DA. DA, dopamine; RASF, synovial fibroblast from patients with rheumatoid arthritis.

Discussion

In this study, we demonstrated that dopamine induces the activation and regulation of glycolysis in RASFs, and that this effect was abrogated by the activation of D3R or inhibition of PKA. This strongly suggests that dopamine activates D1-like receptor signalling, leading to PKA activation. Therefore, D3R is an important and targetable G protein-coupled receptor for regulating activation and glycolysis of synovial fibroblasts.

Local dopamine synthesis occurs within the RA synovium, particularly in fibroblasts, macrophages, and B cells [25]. This suggests that dopaminergic pathways in synovial fibroblasts are regulated through autocrine and paracrine mechanisms. Supporting this functional relevance, dopamine D3Rs are strongly overexpressed specifically in RA synovial fibroblasts, implicating dopamine in modulating fibroblast activity within RA pathology [20]. Notably, while D3R expression is elevated in OA synovial tissue adjacent to cartilage [19], the RA-specific dysregulation of the synovial dopaminergic pathway highlights it as a potential therapeutic target for mitigating progressive joint damage in RA patients. We confirmed that inhibition of D3R by treatment of synovial fibroblasts with the D3R antagonist, NGB2904, enhanced the effect of dopamine on activation and glycolysis. However, the D3R agonist, 7-OH

DAPT, impaired the effects of dopamine on activation and glycolysis by downregulating the expression of enzymes that promote glycolysis in RASFs. Moreover, elevated activation and glycolysis after treatment with dopamine alone or NGB2904 plus dopamine was reversed by pretreatment of synovial fibroblasts with the PKA inhibitor, H89. Collectively, these findings demonstrate an important role of D3R in regulating dopamine-induced activation and glycolysis in RASFs. We propose that dopamine predominantly signals through DR1-like receptors, which activate PKA. Hence, the stimulatory effects of dopamine can be abrogated either by a PKA inhibitor or signalling via D3R, a D2-like receptor that results in the recruitment of $G\alpha_{i/o}$ and leads to decreased levels of cAMP synthesis [17].

We focused on the role of D3R in synovial fibroblasts as they are the major effector cells of synovial inflammation and cartilage damage in the joints of patients with RA with RA. Synovial fibroblasts derived from the RA synovium show unique activation profile properties owing to the existence of a hypoxic environment in the RA synovium [26]. Hypoxic conditions have been reported to promote the preponderance of glycolysis in the inflamed joints to promote cell proliferation, inflammation, and angiogenesis in RA [27-29]. Recent studies have documented metabolic changes in the inflamed joints of patients with RA and demonstrated that glucose metabolism is enhanced [30, 31]. Elevated glucose metabolism is a hallmark of proliferative and activated synovial fibroblasts in patients with RA [32]. Glycolysis is enhanced in the synovial fibroblasts of patients with RA under proinflammatory conditions and blocking glycolysis might be beneficial in inflammatory arthritis [9]. Recent evidence suggests that the metabolic response of synovial fibroblasts primarily depends on activating stimuli. Quiescent synovial fibroblasts in RA have a significantly greater glycolytic activity and lactate production compared with those in OA [8, 9]. Stimulation with platelet-derived growth factor led to a substantially higher glycolytic response in synovial fibroblasts from patients with RA than in those from patients with OA, while stimulation with lipopolysaccharide was demonstrated to elicit a metabolic conversion towards aerobic glycolysis and increased lactate

production in synovial fibroblasts of patients with RA and OA [9]. It was also indicated that treatment of activated T helper cells with cell culture supernatants significantly increased the activity of aerobic glycolysis and HK2 expression in synovial fibroblasts of patients with RA and OA compared with those in resting conditions [8]. In this study, we showed that stimulation with dopamine significantly induces the activation of RASFs and altered the metabolic profile of these cells by increasing the expression of glycolytic rate-limiting enzymes, including hexokinases (HK1 and HK2), phosphofructokinases (PFKL, PFKM, PFKP, and PFKFB3), and pyruvate kinases (p-PKM2). Our findings provide key evidence for the ability of dopamine to increase the activation and glycolysis of RASFs. We found that D3R activation reduced the dopamine-mediated activation of synovial fibroblasts from patients with RA. In our further study, we will investigate whether the activation and glycolytic metabolism of synovial fibroblasts to dopamine/D3R modulation may differ according to the disease activity of RA patients.

Our data also suggested that D3R may diminish dopamine-induced glycolysis by inhibiting the overexpression of key glycolysis-related enzymes, such as hexokinases (HK1 and HK2), phosphofructokinases (PFKL, PFKM, PFKP, and PFKFB3), and pyruvate kinases (p-PKM2). D2R-mediated signalling was involved in the regulation of pancreatic inflammation in acute pancreatitis [33]. This is consistent with recent research implicating a protective role for D3R in an acute renal injury model [34]. cAMP is an important secondary messenger involved in multiple biological processes, such as cell proliferation and differentiation [24]. PKA is a key intracellular mediator of cAMP-mediated signalling [24]. As a cAMP-binding regulatory subunit of PKA, PRKAR2B has been reported to exert a tumour-promoting effect by enhancing glycolysis in prostate cancer [18]. Consistent with this finding, the inhibition of PKA activity by H89 suppressed α-SMA protein expression and glycolytic metabolism in RASFs. Further animal studies are required to elucidate whether D3R is involved in the pathogenesis of RA synovitis by regulating the glycolysis of synovial fibroblasts.

In this study, it was shown that dopamine promotes activation, inflammatory response and the expression of glycolysis-related enzymes in RASFs. Dopamine acts by binding to dopamine receptors (DRs), which are members of the G protein-coupled receptor superfamily and separated into two distinct classes: D1-like (D1R and D5R) and D2-like (D2R, D3R, and D4R). D1-like and D2-like receptors activate or inhibit adenylate cyclase, leading to the elevation or suppression of intracellular cyclic adenosine monophosphate (cAMP), respectively [17]. We previously showed that genetic deletion of dopamine D3R results in the exacerbation of experimental arthritis [21]. In this study, based on the previous study and the finding that dopamine promotes activation, inflammatory response and glycolysis, we therefore investigated whether D3R signalling affects dopamine-mediated responses in RASFs. We examined the effects of pretreatment with the D3R preferring agonist or antagonist on the activation and glycolytic metabolism of RASFs to determine whether D3R participates in the regulation of dopamine-induced responses.

In summary, our findings identified the D3R/PKA axis as a key pathway for suppressing dopamine-induced glycolysis and the subsequent activation of RASFs. Notably, treatment with a D3R antagonist leads to an increase in dopamine-induced glycolysis and activation of RASFs, which can be reversed by PKA inhibition. Therefore, our study places PKA as a key downstream effector of dopamine/D3R in glycolysis and RASF activation. The D3R/PKA signalling pathway may play a critical role in modulating the activation of RASFs, and targeting the D3R pathway to inhibit glycolysis may represent a novel therapeutic strategy for reducing the activation of RASFs..

Acknowledgements

We would like to thank Dr. Jan-Willem Taanman in University College London for providing with guidance and assistance in the study.

Funding

This study was supported by the National Natural Science Foundation of China (No.

81802129), Science and Technology Planning Project of Shaanxi Province of China (No. 2023-YBSF-384 and No. 2025SF-YBXM-378), and Basic and Clinical Integration Innovation Project of Xi'an Jiaotong University (No. YXJLRH2022065).

Data availability

No datasets were generated or analysed during the current study.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University. Informed written consent was secured from all participating individuals.

Consent for publication

Not applicable.

Competing interests

Authors declare that they have no competing interests.

Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Xue had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Xue, Abraham and Geng participated in the study conception and design. Xue, Xu, Li, Wang and Kang, participated in the acquisition of data. Xue, Xu, Li, Wang, Zhu, Kang, Felix I. L. Clanchy, Richard O. Williams, Abraham and Geng, participated in the analysis and interpretation of data.

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