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ORIGINAL ARTICLE

Fibroblast growth factor 2 upregulates ecto-5′-nucleotidase and adenosine deaminase via MAPK pathways in cultured rat spinal cord astrocytes

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Abstract

Adenosine triphosphate (ATP) and adenosine are neurotransmitters and neuromodulators in the central nervous system. Astrocytes regulate extracellular concentration of purines via ATP release and its metabolisms via ecto-enzymes. The expression and activity of purine metabolic enzymes in astrocytes are increased under pathological conditions. We previously showed that fibroblast growth factor 2 (FGF2) upregulates the expression and activity of the enzymes ecto-5′-nucleotidase (CD73) and adenosine deaminase (ADA). Here, we further demonstrate that this occurs in concentration- and time-dependent manners in cultured rat spinal cord astrocytes and is suppressed by inhibitors of the FGF receptor as well as the mitogen-activated protein kinases (MAPKs). We also found that FGF2 increased the phosphorylation of MAPKs, including extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38 MAPK, leading to the increased expression and activity of CD73 and ADA. Our findings reveal the involvement of FGF2/MAPK pathways in the regulation of purine metabolic enzymes in astrocytes. These pathways may contribute to the control of extracellular purine concentrations under physiological and pathological conditions.

Keywords Ecto-5′-nucleotidase · Adenosine deaminase · ATP · Adenosine · FGF2 · Astrocytes

Introduction

Adenosine triphosphate (ATP) and adenosine are neurotransmitters and neuromodulators in the central nervous system (CNS) that are important for modulating memory, sleep, mood and motivation, and locomotor activity [1] but are also involved in trauma, ischemia, neurodegenerative diseases, and psychiatric diseases [2, 3]. The concentrations of extracellular purines are regulated via their release, uptake, and metabolism [4]. ATP released into the extracellular space is metabolized to adenosine by ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) and ecto-5′-nucleotidase (CD73). Cells uptake adenosine via nucleoside transporters, where it is converted by adenosine kinase to adenosine monophosphate (AMP) or metabolized by adenosine deaminase (ADA) to inosine, which also has various effects in the CNS [5].

Astrocytes, the most abundant glial cell type in the CNS, maintain ion homeostasis, provide energy to neurons, support the structure of the CNS, and modulate neuronal activity by releasing and taking up transmitters [6]. Astrocytes release ATP as a gliotransmitter and metabolize it to adenosine via ecto-enzymes on the cell membrane [7]. The main NTPDase expressed by astrocytes from rat brain is NTPDase2 (CD39L1) [8, 9]. Our previous study also showed that NTPDase2 is mainly responsible for ATP metabolism in rat spinal cord astrocytes [10]. Under pathological conditions, the morphology and functions of astrocytes are altered, and these reactive astrocytes can be both protective and harmful [11, 12]. ATP and adenosine released and metabolized by astrocytes are involved in pathologies such as trauma [13], ischemia [14], and depressive disorder [15]. Furthermore, the expression and activities of NTPDases and CD73 in astrocytes are increased during trauma [16] and ischemia [17].

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The expression of CD39 and CD73 in mouse Th17 cells is regulated by interleukin-6 and transforming growth factor-β [18], and CD73 transcription in human cancer cells is regulated by microRNAs [19]. By contrast, less is known about the factors that regulate the expression of ADA. Furthermore, there are little studies that show the regulatory factors of purine metabolic enzymes in astrocytes. We previously reported that fibroblast growth factor 2 (FGF2), a member of the FGF family that is mainly produced in astrocytes in the CNS [20], enhances the activity and expression of CD73 and ADA but not of NTPDases in cultured rat spinal cord astrocytes [21]. The binding of FGF2 to FGF receptors (FGFRs) activates tyrosine kinase signaling, which then activates downstream signaling pathways, including the mitogen-activated protein kinase (MAPK) pathways [22]. The MAPKs extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK regulate cell proliferation, gene transcription, inflammation, and apoptosis in mammalian cells [23]. However, the precise intracellular signaling pathways that contribute to FGF2-induced CD73 and ADA upregulation have not been determined.

Here, we investigated the signaling pathways downstream of FGF2 that regulate the expression and activity of CD73 and ADA in cultured rat spinal cord astrocytes. We found that FGF2 induced MAPK phosphorylation via FGFR, resulting in upregulated transcription, protein expression, and activity of CD73 and ADA. Furthermore, this largely involved activation of the ERK pathway. Our data elucidate that FGFR/MAPK pathways contribute to the regulation of purine metabolic enzymes in astrocytes.

Materials and methods

Materials

Antibodies against CD73 (#13160, 1:1000), p44/42 MAPK (#4695, 1:4000), phospho-p44/42 MAPK (#9101, 1:4000), SAPK/JNK (#9252, 1:4000), phospho-SAPK/JNK (#9251, 1:4000), p38 MAPK (#9212, 1:4000), and phospho-p38 MAPK (#9211, 1:1000) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against ADA (#ab175310, 1:4000) were purchased from Abcam (Cambridge, UK). Peroxidase-conjugated antibodies against mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (#G9295, 1:50,000), as well as adenosine, inosine, AMP sodium salt, ATP disodium salt, α,β-methylene ADP sodium salt, SU5402, and FGF2, were purchased from Sigma-Aldrich (St. Louis, MO). ADP disodium salt, U0126, SP600125, and SB202190 were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan).

Animals

All animal care and experimental protocols were approved by the Committee on Animal Experimentation, Faculty of Veterinary Medicine, Hokkaido University (no. 19-0009). Wistar rats were obtained from Clea Japan (Tokyo, Japan). The animals had ad libitum access to food and tap water and were maintained in a temperature-controlled environment on a 12:12 h light/dark cycle. Male and female pups aged 0–3 days were used for experiments.

Culture of spinal cord astrocytes

Primary cultures of spinal cord astrocytes were obtained as previously described [10]. In brief, spinal cords were isolated from 0 to 3-day-old rat pups, minced, and incubated with papain (10 U/ml) and DNase (0.1 mg/ml). Dissociated cells were suspended in Dulbecco's Modified Eagle's Medium/Ham's F-12 containing 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The cell suspension was seeded on a poly-L-lysine-coated T75 flask. After 7–8 days, the flask was shaken at 250 rpm at 37 °C for at least 12 h to remove all nonadherent cells. Adherent cells (astrocytes) were detached with trypsin and reseeded on poly-L-lysine-coated 6- or 12-well plates at a density of 1.2×10^4 cells/cm^2. After 3 days, the medium was changed to medium without fetal bovine serum. Inhibitors were added to cells at the same time as medium change, and cells were treated with FGF2 (1–20 ng/ml) 30 min after medium change. Cells were then cultured for another 2 days prior to use in experiments. Almost all cells were positive for the astrocyte marker glial fibrillary acidic protein [21].

Enzymatic activity assay and measurement of extracellular purines

Astrocytes in 12-well plates were preincubated with 400 μl artificial cerebrospinal fluid (ACSF; 138 mM NaCl, 3.5 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM HEPES, and 10 mM glucose (pH 7.3)) for 1 h. Thereafter, the medium was replaced by ACSF containing etheno-derivatized AMP (prepared by etheno-derivatization of 100 μM AMP according to a previously described method [10]) or adenosine (10 μM), and cells were incubated for 60 min. After collecting the external solution for extracellular nucleotides/nucleoside level estimation, cells were suspended in 0.1 N NaOH and sonicated. The protein concentration of the cell lysate was measured using the Quick Start protein assay (Bio-Rad, Hercules, CA).

The levels of extracellular purines were measured by reverse-phase high-performance liquid chromatography [24] with an Accucore aQ column (Thermo Fisher Scientific, Waltham, MA) at 45 °C and a fluorescence detector (FP-2020; Nihon Bunko, Tokyo, Japan). The mobile phase buffer

consisted of 100 mM KH_2PO_4, 5 mM tetrabutylammonium bromide, and 2.0% CH_3CN (pH 3.2). The flow rate was 0.8 ml/min. The concentration of inosine was determined using a UV detector (UV-2070; Nihon Bunko) and mobile phase buffer lacking tetrabutylammonium bromide. The detection limit was approximately 5 nM for adenosine and AMP and 20 nM for inosine. To minimize the effects of differences in cell proliferation on the purine concentrations, the extracellular levels of purines were normalized against the protein concentration of the cell lysate.

Western blotting

Astrocytes in 6-well plates were lysed in RIPA buffer containing a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Burlington, MA). The membranes were blocked with 5% skimmed milk and then incubated overnight at 4 °C with primary antibodies. Thereafter, the membranes were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody, and the proteins were visualized by ECL Prime (GE Healthcare, Little Chalfont, UK). Band intensities were measured using ImageJ software (National Institutes of Health, Bethesda, MD).

Real-time PCR

Total RNA was extracted from astrocytes in 6-well plates using RNAiso Plus (Takara Bio, Tokyo, Japan). To remove genomic DNA and synthesize cDNA, the RNA samples were incubated with qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Real-time PCR was performed using Thunderbird SYBR qPCR Mix (TOYOBO), each primer, and the cDNA reaction solution. The primer sequences and product size are listed in Table S1. Thermal cycles were performed using an Eco Real-Time PCR System (Illumina, San Diego, CA). Cycling conditions were 95 °C for 1 min (for initial denaturation), followed by 40 cycles of denaturation (95 °C, 15 s) and annealing and extension (61 °C, 45 s). The melt curve analysis confirmed that the obtained amplicon was the only one amplified in each reaction. The expression levels of the CD73 and ADA mRNAs relative to that of GAPDH were calculated by the relative quantification of cycle thresholds according to Pfaffl [25].

Data analysis

All experiments were performed five times using cell cultures obtained from different animals. Each individual experiment was carried out in duplicates. Data are presented as medians (interquartile ranges (IQRs)). Two groups were statistically compared using the Mann–Whitney *U* test. Multiple comparisons were performed using the Kruskal–Wallis test, followed by the Steel test. A P value of < 0.05 was considered statistically significant. All statistical analysis was performed with Ekuseru-Toukei 2008 (Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results

FGF2 upregulates CD73 and ADA in concentration- and time-dependent manners in cultured rat spinal cord astrocytes

The activities of CD73 and ADA in astrocytes treated for 48 h with FGF2 (1–20 ng/ml) were measured by incubating the cells in ACSF containing etheno-AMP and adenosine, respectively, and measuring the metabolites by high-performance liquid chromatography. FGF2 increased etheno-adenosine production (CD73 activity; Fig. 1a) and inosine production (ADA activity; Fig. 1b) in a concentration-dependent manner. Specifically, FGF2 at > 10 ng/ml significantly increased the CD73 activity, and FGF2 at 20 ng/ml significantly increased the ADA activity. Western blot analysis also revealed that FGF2 increased the protein levels of CD73 and ADA in a concentration-dependent manner (Fig. 1c). FGF2 at 20 ng/ml significantly increased the CD73 activity, and FGF2 at > 10 ng/ml significantly increased the ADA activity. Moreover, the expression levels of CD73 and ADA in response to 20 ng/ml FGF2 increased in a time-dependent manner for up to 48 h (Fig. 1d). FGF2 treatment for 48 h significantly increased the expression levels of these enzymes. The protein level of CD73 in control cells slightly increased with time but that of ADA did not (Fig. S1). This increase may have resulted from the change to serum-free medium. On the basis of these results, astrocytes were treated for 48 h with 20 ng/ml FGF2 in subsequent experiments.

FGF2 upregulates CD73 and ADA via FGFR

Cultured rat spinal cord astrocytes expressed all FGFR mRNAs (Fig. S2). Treatment of astrocytes with SU5402 (5 μM), a potent and selective inhibitor of FGFR1 [26], significantly decreased the activity of CD73 and ADA in cells incubated with FGF2 but not in control astrocytes (Fig. 2a, b). SU5402 also suppressed the FGF2-induced increases in CD73 and ADA protein levels but did not affect expression in control astrocytes (Fig. 2c). These results indicate that FGF2 upregulates CD73 and ADA via FGFR1 in cultured rat spinal cord astrocytes.

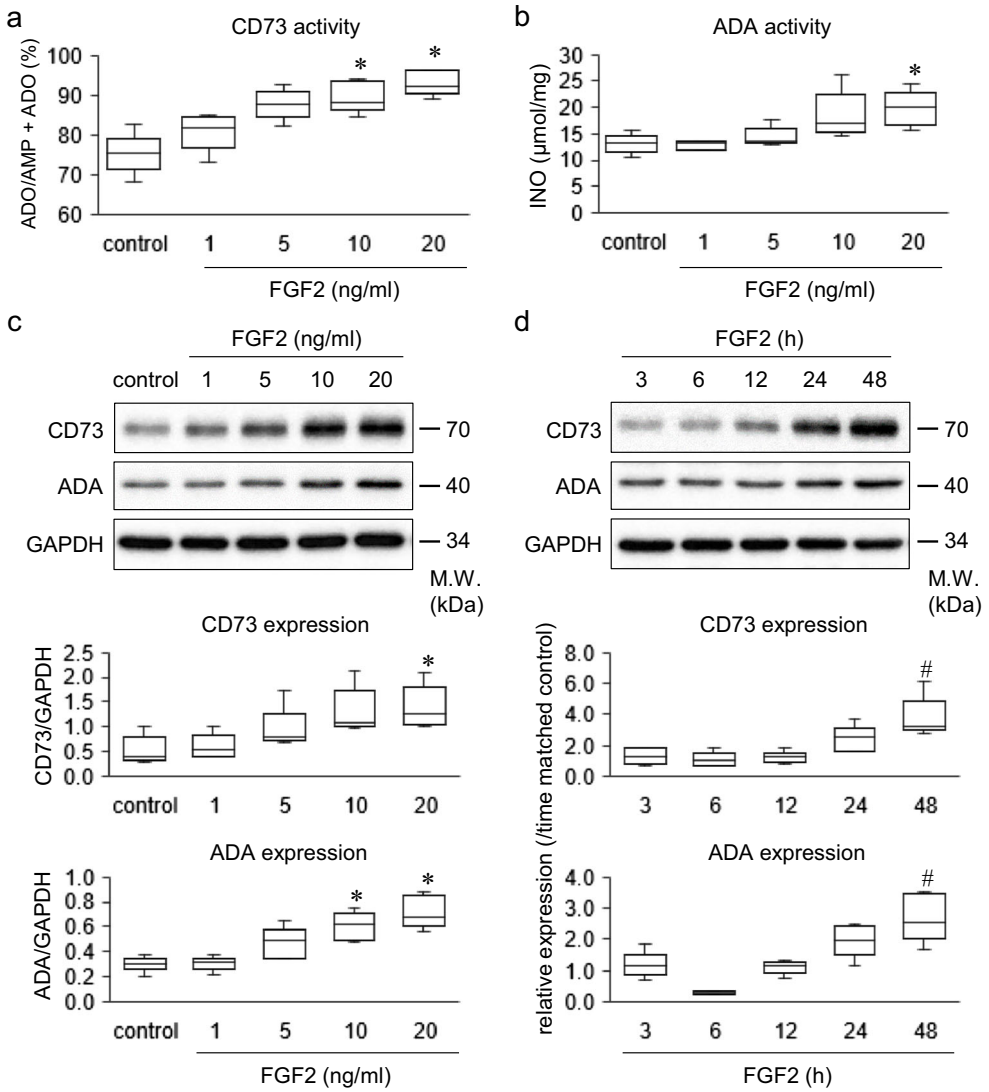

Fig. 1 FGF2 upregulates the activity and protein expression of CD73 and ADA in concentration- and time-dependent manners. Astrocytes were treated with FGF2 (1–20 ng/ml) for 48 h to test upregulation in concentration dependent manner and FGF2 (20 ng/ml) for 3–48 h to test upregulation in time dependent manner. **a** Control and FGF2-treated astrocytes were incubated in ACSF containing etheno-AMP for 60 min. Data show the percentage of adenosine relative to the level of AMP and adenosine. $*P < 0.05$ versus control (Steel test), $n = 5$. **b** Control and FGF2-treated astrocytes were incubated in ACSF containing adenosine (10 μM) for 60 min. Data show the amount of inosine produced in 60 min. $*P < 0.05$ versus control (Steel test), $n = 5$. **c**, **d** Expression levels of CD73 and ADA protein were analyzed by western blotting. Representative western blots of CD73 (70 kDa), ADA (41 kDa) and GAPDH (37 kDa) (top) and summarized data (middle and bottom) are shown. Data for time dependency are expressed relative to the time-matched control. $*P < 0.05$ versus control (Steel test) and $\#P < 0.05$ versus time-matched control (Mann–Whitney U test), $n = 5$. Data are presented as medians (IQRs). ADO, adenosine; INO, inosine

FGF2 activates MAPK pathways to increase CD73 and ADA

Astrocytes treated with FGF2 had markedly increased phosphorylation levels of ERK and JNK, detected within 10 min and lasting for at least 6 h (Fig. 3a). Notably, the magnitude of the increase in p38 phosphorylation was smaller than that of ERK and JNK, and levels returned to that of controls at 30 min, suggesting that ERK and JNK, rather than p38, are the primary contributors to the FGF2-induced upregulation of CD73 and ADA. FGF2-induced phosphorylation of ERK was suppressed by SU5402 and U0126 (10 μM), a MAPK/ERK kinase (MEK) inhibitor (Fig. 3b). The phosphorylation of JNK was suppressed by SU5402 and SP600125 (10 μM), a JNK inhibitor. The phosphorylation of ERK and JNK was also significantly decreased by SP600125 and U0126, respectively. ERK phosphorylation in control astrocytes was significantly reduced by SP600125 and U0126 but only slightly by SU5402 (Fig. S3). The phosphorylation of JNK in control astrocytes was not detected.

To determine if MAPK signaling contributed to the FGF2-induced activation of CD73 and ADA, the corresponding

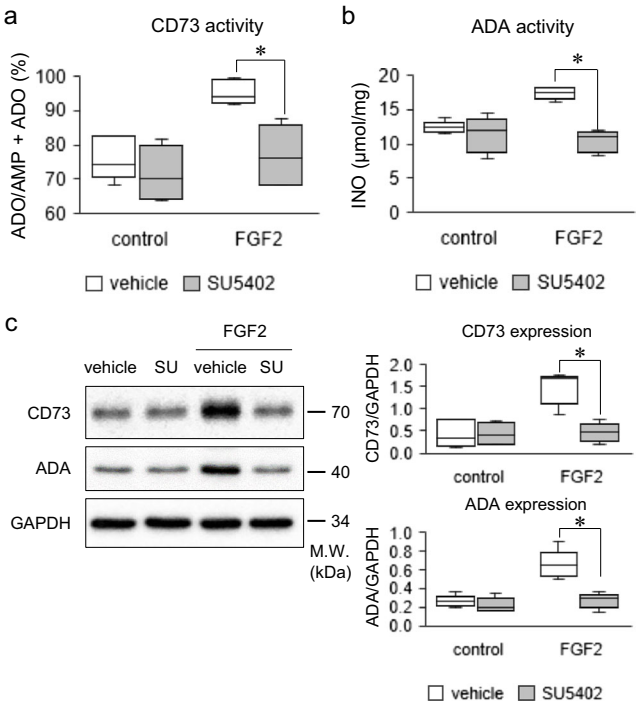

Fig. 2 FGF2 upregulates CD73 and ADA via FGFR1. Astrocytes were treated with FGF2 (20 ng/ml) for 48 h. SU5402 (5 μM) was added to medium 30 min before FGF2 treatment. **a** Control and FGF2-treated astrocytes were incubated in ACSF containing etheno-AMP for 60 min. Data show the percentage of adenosine relative to the level of AMP and adenosine. $*P < 0.05$ (Mann–Whitney U test), $n = 5$. **b** Control and FGF2-treated astrocytes were incubated in ACSF containing adenosine (10 μM) for 60 min. Data show the amount of inosine produced in 60 min. $*P < 0.05$ (Mann–Whitney U test), $n = 5$. **c** Expression levels of CD73 and ADA protein were analyzed by western blotting. Representative western blots of CD73 (70 kDa), ADA (41 kDa), and GAPDH (37 kDa) (left) and summarized data (right) are shown. $*P < 0.05$ (Mann–Whitney U test), $n = 5$. Data are presented as medians (IQRs). ADO, adenosine; INO, inosine

metabolite levels were measured in astrocytes treated with U0126 and SP600125. Although neither inhibitor affected CD73 activity in control astrocytes, they both suppressed activity in astrocytes cultured with FGF2 (Fig. 4a). By contrast, only U0126 suppressed ADA activity in FGF2-treated astrocytes, but it did so also in control astrocytes (Fig. 4b). Western blot analysis demonstrated that U0126 significantly decreased protein levels of CD73 and ADA in FGF2-treated astrocytes, whereas the decreases observed with SP600125 were not significant (Fig. 4c). Neither inhibitor affected CD73 nor ADA protein levels in control astrocytes. These results suggest that MAPKs, mainly the ERK pathway, mediate the upregulation of CD73 and ADA by FGF2.

FGF2 promotes CD73 and ADA gene transcription

As MAPK signaling pathways activate many transcription factors and regulate gene transcription, we investigated the changes in transcription of CD73 and ADA mRNA by FGF2 treatment. FGF2 significantly increased CD73 and ADA mRNA levels beginning 6 h after treatment (Fig. 5a). In control cells, the mRNA levels of CD73 slightly but significantly increased from 6 h after medium change, but those of ADA were not different (Fig. S4). The FGF2-induced increases in mRNA levels of CD73 and ADA were suppressed by SU5402 and U0126, but not by SP600125, at 24 h after treatment (Fig. 5b). In control cells, the mRNA levels of CD73 and ADA were significantly decreased by U0126 but not by SU5402 and SP600125.

We also examined the effects of FGFR and MAPK inhibitors on cell proliferation, as we previously reported that FGF2 enhances cell proliferation [21]. SU5402 and SP600125 decreased the proliferation of FGF2-treated astrocytes but U0126 did not (Fig. S5). SP600125 also decreased the proliferation of control astrocytes.

Discussion

Astrocytes express FGFR1–3 [20], and we detected weak expression of FGFR4 at the mRNA level. Notably, the upregulation of CD73 and ADA induced by FGF2 was abolished by SU5402, a selective FGFR1 inhibitor [26], indicating that FGFR1 mediates the effects of FGF2 in cultured spinal cord astrocytes. There are several signaling pathways downstream of FGF/FGFR, but the FGF2-induced upregulation of CD73 and ADA was completely abolished by the MEK inhibitor U0126. Conversely, the FGFR1 inhibitor blocked the FGF2-induced increase in MAPK phosphorylation. U0126 also decreased the activity of ADA, the mRNA levels of CD73 and ADA, and the phosphorylation of ERK in control astrocytes. These results suggest that MEK/ERK pathway contributes to the regulation of the activity and expression of CD73 and ADA under resting condition. The FGF2-induced upregulation of CD73 and ADA was partially suppressed by the JNK inhibitor SP600125, indicating that the JNK pathway also contributes to the upregulation. The decrease in ERK and JNK phosphorylation by SP600125 and U0126, respectively, demonstrates the cross-talk among MAPK pathways reported previously [27–29]. In addition, SB202190 (10 μM), a p38 MAPK inhibitor, failed to suppress the FGF2-induced phosphorylation of p38 MAPK (data not shown). Although, we could not exclude the possibility of p38 MAPK involvement in the observed effects of FGF2, it seems to be small in proportion to the extent of p38 phosphorylation. The suppression of FGF2-mediated astrocyte proliferation by SU5402 and SP600125, but not by U0126, suggests that the JNK pathway plays a larger regulatory role in proliferation, whereas the MEK/ERK pathway primarily regulates purine metabolism via ADA and CD73.

Extracellular ATP activates ERK pathway via P2Y receptor [30] and can synergistically enhances FGF2-induced

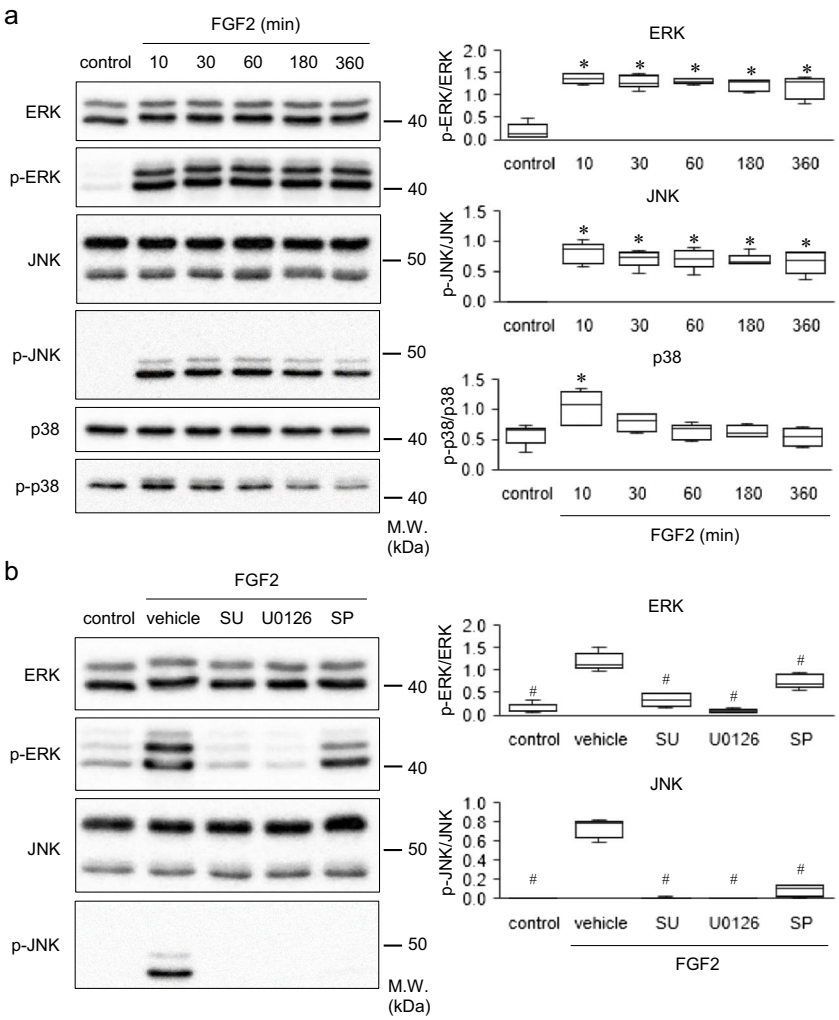

Fig. 3 FGF2 increases MAPK phosphorylation. Astrocytes were treated with FGF2 (20 ng/ml). SU5402 (SU; 5 μM), U0126 (10 μM), or SP600125 (SP; 10 μM) was added to medium 30 min before FGF2 treatment. The phosphorylation levels of MAPKs were analyzed by western blotting for 10–360 min (**a**) or 10 min (**b**). Representative western blots of ERK/p-ERK (42, 44 kDa), JNK/p-JNK (46, 54 kDa), and p38/p-p38 (43 kDa) (left) and summarized data (right) are shown. $*P < 0.05$ versus control and $\#P < 0.05$ versus vehicle (Steel test), $n = 5$. Data are presented as medians (IQRs)

proliferation in astrocytes [31]. Furthermore, P1 receptor activation also stimulates ERK activity [32]. Therefore, it is possible that extracellular purines are involved in the regulation of purine metabolic enzymes via MAPK in astrocytes. In this study, however, the upregulation of CD73, ADA, and ERK phosphorylation was completely suppressed by SU5402, a FGFR1 inhibitor, suggesting that these changes are almost completely mediated via FGF2/FGFR1 pathway. The upregulated purine metabolisms induced by FGF2 may affect to ERK activation via P1 and P2 receptors by changing extracellular balance of purines, resulting in the changes of proliferation and transcription in astrocytes.

MAPK pathways can be activated by a variety of factors, including growth factors [33], cytokines [34], and stress [35], which could therefore also regulate the activity and expression of purine metabolic enzymes. Moreover, CD73 expression is regulated by several transcription factors [36]. Specificity protein 1 (SP1) contributes to CD73 upregulation in rat hepatocytes, and there are two SP1 response elements (− 140 to − 137 and − 114 to −111) in rat CD73 promoter region [37]. Murine ADA promoter region also has SP1 binding site (−211 to −185) [38]. The signal transducer and activator of transcription 3 and growth factor independent 1, which regulate CD73 in mouse Th17 cells, have putative binding site (− 3225 and − 2059 bp upstream, respectively) in CD73 gene [18]. These transcription factors are activated by ERKs [39–41] and thus also may contribute to the upregulation of CD73 and ADA in astrocytes. Further investigation is needed to reveal the transcriptional regulation of these enzymes.

As FGF2 is involved in CNS development [20, 22], its regulation of purine metabolism represents an important contribution to this process. However, FGF2 is also increased during ischemia [42] and trauma [43] and may therefore regulate purine metabolism under these pathological conditions as well as others, such as depressive disorders [44, 45], in which FGF2 is decreased and for which some symptoms are improved by FGF2 treatment [46, 47]. The benefit of FGF2 may be linked to its regulation of purine metabolic enzymes, as ATP and adenosine are implicated in depressive disorders [48, 49].

The present study shows that FGF2 upregulates the activity and expression of CD73 and ADA through

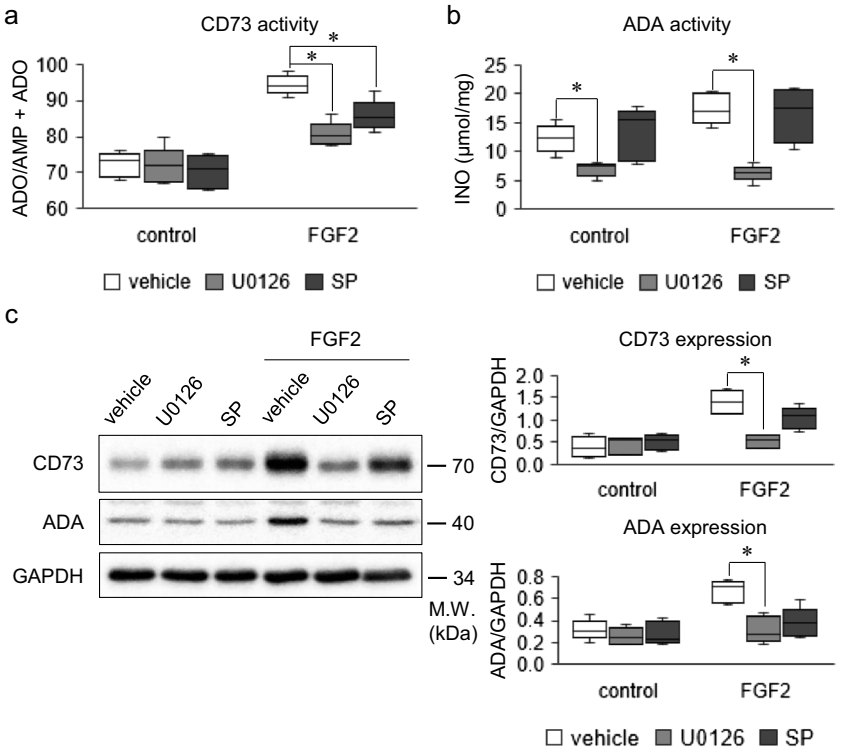

Fig. 4 FGF2 upregulates CD73 and ADA via MAPKs. Astrocytes were treated with FGF2 (20 ng/ml) for 48 h. U0126 (10 μM) or SP600125 (SP; 10 μM) was added to medium 30 min before FGF2 treatment. **a** Control and FGF2-treated astrocytes were incubated in ACSF containing etheno-AMP for 60 min. Data show the percentage of adenosine relative to the level of AMP and adenosine. *$P < 0.05$ (Steel test), $n = 5$. **b** Control and FGF2-treated astrocytes were incubated in ACSF containing adenosine (10 μM) for 60 min. Data show the amount of inosine produced in 60 min. *$P < 0.05$ (Steel test), $n = 5$. **c** Expression levels of CD73 and ADA protein were analyzed by western blotting. Representative western blots of CD73 (70 kDa), ADA (41 kDa), and GAPDH (37 kDa) (left) and summarized data (right) are shown. *$P < 0.05$ (Steel test), $n = 5$. Data are presented as medians (IQRs). ADO, adenosine; INO, inosine

FGFR and MAPK pathways. MAPKs activated via FGFR1 induced the increase in transcription and protein expression of CD73 and ADA. In conclusion, these findings reveal the novel relationship between FGF2/MAPK pathways and the regulation of purine metabolic enzymes. These findings will help to elucidate the precise regulation of purine metabolism under physiological and pathological conditions.

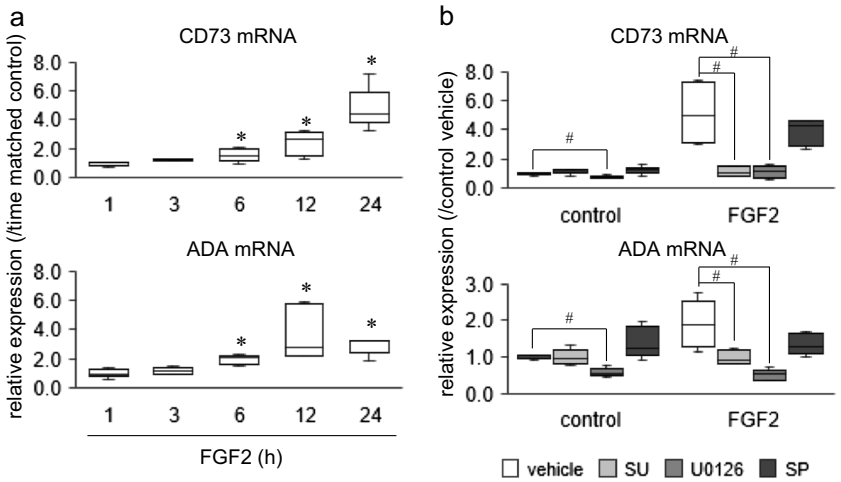

Fig. 5 FGF2 increases the transcription of CD73 and ADA via FGFR1 and MAPKs. Astrocytes were treated with FGF2 (20 ng/ml) for 1–24 h. SU5402 (SU; 5 μM), U0126 (10 μM), or SP600125 (SP; 10 μM) was added to medium 30 min before FGF2 treatment. **a**, **b** Expression levels of CD73 and ADA mRNA were analyzed by real-time PCR. Data are expressed relative to the time-matched control (**a**) or vehicle in control astrocytes (**b**). *$P < 0.05$ versus time-matched control (Mann–Whitney U test) and #$P < 0.05$ versus vehicle (Steel test), $n = 5$. Data are presented as medians (IQRs)

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Data availability The datasets generated and/or analyzed during the current study available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval All animal care and experimental protocols were approved by the Committee on Animal Experimentation, Faculty of Veterinary Medicine, Hokkaido University (no. 19-0009).

Consent to participate Not applicable.

Consent for publication Not applicable.

Code availability Not applicable.

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