Original Paper

Fasudil Stimulates Neurite Outgrowth and Promotes Differentiation in C17.2 Neural Stem Cells by Modulating Notch Signalling but not Autophagy

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Key Words

Fasudil • Neural stem cell • Neurite outgrowth • Differentiation • Notch signaling • Autophagy

Abstract

Background: Neurite outgrowth is one of the important therapeutic strategies for neuronal plasticity and regeneration in neural disorders. Fasudil is a clinical medication that is used to treat subarachnoid haemorrhage (SAH) and that is beneficial for many animal models of central nervous system (CNS) diseases. In this study, we hypothesised that fasudil administration would promote neurite outgrowth in neural stem cells (NSCs). **Methods:** Changes in cell morphology were imaged under a light microscope, and neurite-bearing cells were counted. Cell viability and the necrosis rate were determined by MTT and LDH assays, respectively. Additionally, western blot and immunofluorescence analyses were performed to detect protein expression levels. **Results:** We found that fasudil promoted neurite outgrowth in C17.2 cells in a time- and dose-dependent manner. The neurite-bearing C17.2 cells were differentiated by detecting the changes in neural and astrocytic markers after fasudil treatment through down-regulating Notch signalling. Previously, fasudil was reported to induce autophagy, which plays an important role in neural differentiation. However, both rapamycin, an autophagy inducer, and 3-methyl-adenine (3-MA), an autophagy inhibitor, had no effects on the fasudil-induced neurite outgrowth, suggesting that autophagy may be not involved in this process. **Conclusion:** In summary, fasudil could stimulate neurite outgrowth and differentiation in C17.2 cells by modulating Notch signalling but not autophagy.

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Introduction

Various cellular functions are induced by Rho kinase (ROCK), which plays a crucial role in cytoskeleton construction. One of its inhibitors, fasudil, is a potent vasodilator that has been applied as a clinical medication for treating SAH. Currently, increasingly new effects of fasudil have been discovered, particularly in the CNS. New evidence has shown that fasudil can suppress the proliferation, migration and invasion abilities of the glioblastoma cell lines T98G and U251 [1]. Moreover, the ROCK and mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK) signalling pathways are involved in the anti-tumour effects caused by fasudil [2]. Subcutaneous injection of hydroxyfasudil improves learning and working memory, which provides new insight into improving the prognosis of Alzheimer's disease (AD) [3]. Additionally, the role of this medication in preventing neurodegeneration may be due to its contribution to promoting NSC proliferation and differentiation [4, 5]. Neuronal differentiation could improve neurological function in stroke models [6] and traumatic brain injury models [7]. Fasudil can maintain and improve neurologic functions during various internal environment disturbances, which may be due to its ability to promote neurite outgrowth [8-10]. Previous reports have shown that autophagy may be involved in neurite outgrowth and cell differentiation [11, 12]. Furthermore, using an automatic analysis of the topology of the drug network, Iorio et al found that one of the unexpected effects of fasudil is autophagy induction [13, 14].

The Notch signalling pathway is essential for maintaining NSCs in the developing brain and plays a crucial role in NSC proliferation and differentiation [15-17]. Neurite development is also influenced by Notch signalling *in vitro* [18]. Hes 1, which belongs to the basic helix-loop-helix family of transcription factors, plays an important role in the Notch signalling pathway [19]. Additionally, Hes 1 regulates its own expression through a feedback loop and oscillates with an approximately 2-hour periodicity [20]. Both Notch 1 and Hes 1 are repressors that influence the NSC fate decision [21]. The activation of these repressors maintains NSCs in a proliferating state, whereas decreasing the expression of these repressors promotes NSC differentiation and depletion [22]. Moreover, the dysfunction of the Notch signalling pathway is associated with neurodegenerative diseases such as AD [23].

In this study, we hypothesised that fasudil would promote neurite outgrowth in C17.2 NSCs and examined whether the Notch signalling pathway and autophagy were involved in the fasudil-induced neurite outgrowth of NSCs. We found that fasudil could stimulate neurite outgrowth and neuronal differentiation in C17.2 cells through modulating Notch signalling but not autophagy.

Materials and Methods

Cell culture and reagents

The C17.2 cell line, which is composed of neural stem cells that were derived from the external germinal layer of mouse cerebellum [24], was a kind gift presented by Dr. Yuming Zhao of Capital Medical University, China. Fasudil (purity>98.0%) was purchased from Melonepharma (Dalian, China), dissolved in PBS and stored at -20°C. Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were obtained from Gibco-BRL (NY, USA). Trypsin, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33258, glutamine, the autophagy inducer rapamycin and the autophagy inhibitor 3-MA were purchased from Sigma-Aldrich (MO, USA).

C17.2 cells were maintained in plastic culture flasks in complete DMEM [25], which is DMEM containing 10% FBS, 5% horse serum, and 2 mM glutamine, in an incubator with a humidified 5% CO₂ 95% air atmosphere at 37°C. The cells were passaged after they reached 80% confluence.

Neurite outgrowth measurement

C17.2 cells were treated with fasudil at various concentrations (0, 25, 50 and 100 µM) for different periods (1, 3, 6, 12, 24 and 48 h) to analyse dose- and time-dependent neurite outgrowth. Cell morphological

changes were observed under a phase contrast microscope at a magnification of 200×. Neurite outgrowth was defined as a process with a length greater than twofold the cell body diameter as described previously [11]. Then, the percentage of cells with neurite outgrowth was quantified for 300 cells per well in randomly chosen fields ($n=3$ /group). Next, the stimuli were removed, and the cells were cultured in complete DMEM for 12 h. Then, percentage of remaining neurite outgrowth cells was calculated again ($n=3$ /group).

Assessment of cell viability by MTT assay

Cell viability was determined by MTT assay. Briefly, cells (1×10^4 cells/well) were seeded in 96-well plates and incubated in complete DMEM for 24 h before being treated with fasudil. MTT (5 g/L, 10 μ L) was added to each well, and then the cells were cultured in the incubator for 2 h, followed by the removal of the culture medium and the addition of 100 μ L of DMSO. The absorbance was measured at 570 nm, with 655 nm as the reference wavelength. All experiments were performed in triplicate.

LDH release assay

C17.2 cells were plated in 96-well plates at a density of 1×10^4 per well. On the following day, the cells were exposed to various concentrations of fasudil for 24 h. The medium was collected and assayed for lactate dehydrogenase (LDH) activity using a Lactate Dehydrogenase Assay Kit (Nanjing, China). Also the intracellular LDH activity was measured as previously described with some modifications [26]. After treatment with or without fasudil at various concentrations for 24 h, the cells were incubated with 0.2 % Triton X-100 at 37°C for 30 min with shaking (800rpm). The cell lysates were collected. Briefly, LDH release is measured by a coupled enzymatic reaction that results in the conversion of a tetrazolium salt into red formazan product. The amount of formazan synthesised correlates with LDH activity. The formazan product was measured using a microplate reader at 450 nm. The results are expressed as the percentage of LDH release. And the absorbance of control cells was set at 100%.

Western blot

The protein levels of Notch 1 (Cell Signaling Technology, 1:1000 dilution), Hes 1 (Cell Signaling Technology, 1:1000 dilution), the NSC marker Nestin (Abcam, 1:1000 dilution), the immature neuronal cell marker doublecortin (DCX; Cell Signaling Technology, 1:1000 dilution), the mature neuronal cell marker microtubule-associated protein (MAP-2; Boster, 1:500 dilution), the astrocytic marker glial fibrillary acidic protein (GFAP; Cell Signaling Technology, 1:1000 dilution), and the autophagy markers P62 (Cell Signaling Technology, 1:1000 dilution) and LC3 (Cell Signaling Technology, 1:1000 dilution) in C17.2 cells were examined by western blot analysis.

Cells were harvested at the indicated time points and then incubated in radio immunoprecipitation assay lysis buffer with a protease inhibitor tablet for 30 min at 4°C. Cell lysates were centrifuged at 20,000 g at 4°C for 15 min, and the supernatant was collected and stored at -20°C for further analysis by western blot. Protein extracts (20 μ g, quantitation performed by the bicinchoninic acid method, Thermo Scientific) were fractionated by electrophoresis on 10% and 15% polyacrylamide gels and transferred to PVDF membranes. After the membranes were blocked in 5% skim milk at room temperature for 1 h, they were incubated with primary antibodies overnight at 4°C. Then, the membranes were incubated with secondary bodies (Thermo Scientific) for 2 h at room temperature. The same membranes were also incubated with anti- α -tubulin or anti- β -actin as loading controls. Proteins were detected using an ECL kit (Thermo Scientific) and a chemiluminescence imaging system (ChemiScope5600, CLINX) in a dark room at 24°C, and signals were quantified using scanning densitometry.

Immunofluorescence

C17.2 cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature after being treated with fasudil (100 μ M) for 24 hs. Then, the cells were incubated with 0.3% Triton X-100 for 15 minutes to permeabilize the cell membranes before the cells were blocked in normal goat serum for 1 h at room temperature. Then, the cells were incubated with primary antibodies (Nestin, 1:500 dilution; DCX, 1:400 dilution; MAP-2, 1:400 dilution; GFAP, 1:400 dilution) at 4°C overnight. Subsequently, the cells were incubated with Alexa Fluor 555-conjugated secondary antibody (1:500) for 1 h at room temperature. The cells were counterstained with Hoechst 33258 to visualise nuclei. Images were acquired using a fluorescence microscope (Olympus 63× oil lens, Japan) equipped with a UV filter in a dark room at 24°C ($n=3$ /group).

Fig. 1. Fasudil promotes the neurite outgrowth of C17.2 cells in a time- and dose-dependent manner. C17.2 cells were stimulated with different concentrations (5 μ M, 25 μ M, 50 μ M and 100 μ M) of fasudil for different periods (1, 3, 6, 12, 24 and 48 h). Cell morphology was observed under a light microscope (200 $\times$), and the neurite-bearing cells with neurite lengths greater than twofold the cell body diameter were counted. * p <0.05 and *** p <0.01 compared with the time-matched control group (CT). Scale bar=50 μ m.

Statistical analysis

The data are presented as the means $\pm$ standard error. Statistical analyses between two groups were performed using unpaired Student's t-test. Differences among groups were tested by one-way analysis of variance (ANOVA). Following ANOVA analyses, Tukey's test was used, and p <0.05 was considered statistically significant.

Results

Fasudil induced neurite outgrowth in C17.2 cells

We examined the effect of fasudil on neurite outgrowth in C17.2 cells. After the cells were treated with fasudil for 1 h, the ratio of neurite-bearing cells obviously and rapidly increased, except for the cells that were treated with 5 μ M of fasudil (Fig. 1), which displayed a slow increase in the ratio of neurite-bearing cells in a time-dependent manner when compared with the untreated control group. However, when compared with dose-matched, untreated cells, the percentage of neurite-bearing cells rapidly increased with 1, 3, 6, 12 and 24 h of persistent treatment but mildly increased from 24 to 48 h in different concentrations of fasudil. In contrast, when compared with time-matched, untreated cells, the percentage of neurite-bearing cells increased 3.4-, 4.3-, 6.7- and 8.8-fold following stimulation with fasudil for 48 h at concentrations of 5, 25, 50 and 100 μ M, respectively. At the end of the treatment periods, the stimuli were removed, and the culture medium was returned to complete DMEM for 12 h. Then, the percentage of remaining neurite-bearing cells was calculated for dose- and time-dependent treatments with fasudil (Fig. 2). Compared with time-matched, untreated cells, the percentage of remaining neurite-bearing cells after treatment with 100 μ M fasudil was significantly greater (p <0.01) at different time points. Based on these data, we chose the concentration of 100 μ M fasudil for the subsequent experiments.

Fasudil was not cytotoxic to C17.2 cells

The C17.2 cells were incubated with various concentrations of fasudil for 24 h, and then cell viability was measured by MTT assay. As shown in Fig. 3B, the net absorbance at 570 nm significantly decreased in cells treated with 50 μ M and 100 μ M fasudil (p <0.05)

Fig. 2. The ratio of remaining neurite-bearing cells. After fasudil treatment, the stimulation was removed, and the medium was changed back to the complete DMEM. The cells were incubated for 12 h, and then the remaining neurite-bearing cells with neurite lengths greater than twofold the cell body diameter were counted and compared with the stimulated time-matched control. Cell morphology changes were observed under a light microscope (200×). * $p < 0.05$ and *** $p < 0.01$ compared with the time-matched control group (CT). Scale bar = 50 μm

Fig. 3. Fasudil was not cytotoxic to C17.2 cells. After the cells were treated with fasudil for 24 h, they were imaged under phase-contrast optics (200×) (A). Cell viability was examined by MTT assay (B); lower concentrations (5 μM and 25 μM) of fasudil had no influence on cell viability. However, cell viability was partially inhibited by middle and higher concentrations (50 μM and 100 μM) of fasudil. No differences were observed in the LDH assay (C/D). * $p < 0.05$. Scale bar = 50 μm. Control group (CT).

compared with untreated cells. When compared with cells treated with 50 μM fasudil, the net absorbance also clearly decreased in cells treated with 100 μM fasudil ($p < 0.05$). However, no differences were found in the LDH assay (Fig. 3C/D), indicating that fasudil was not cytotoxic to the C17.2 cells in our research.

Fasudil promoted C17.2 cell to differentiate into neuronal cells and astrocytes

C17.2 cells differentiated into neuronal cells and astrocytes as detected by western blot and immunofluorescence analyses (Fig. 4). The expression of the NSC marker Nestin

Fig. 4. Fasudil promotes C17.2 cell differentiation after 24 h of treatment. Immunofluorescence and western blot analyses were applied to evaluate the levels of the neural stem cell marker Nestin (A/B), the immature neuronal marker DCX (C/D), the mature neuronal marker MAP-2 (E/F) and the astrocytic marker GFAP (G/H). *** $p < 0.01$ vs. control group (CT). Scale bar = 10 μ m.

(Fig. 4A/B) markedly decreased in the fasudil treatment group compared with that in the control group ($p < 0.01$). In contrast, the expression of DCX (Fig. 4C/D), MAP-2 (Fig. 4E/F) and GFAP (Fig. 4G/H) significantly increased after fasudil treatment ($p < 0.01$). Furthermore, the percentage of Nestin⁺ cells decreased, whereas that of DCX⁺, MAP2⁺ and GFAP⁺ cells increased ($p < 0.01$).

Fasudil modulated the Notch signalling pathway

To determine whether the Notch signalling pathway was involved in fasudil-induced differentiation of NSCs, we examined the expression levels of Notch 1 and Hes 1 in C17.2 cells with or without fasudil treatment. As shown in Fig. 5, fasudil treatment obviously

Fig. 5. Fasudil decreases the levels of Notch 1 and Hes 1 in C17.2 cells. C17.2 cells were treated with fasudil (100 μ M) for 1, 3, 6, 12 and 24 h. Protein extracts were prepared for western blot analysis. * p <0.05 and *** p <0.01 vs. 0 h group.

Fig. 6. Autophagy inhibition and induction did not affect neurite outgrowth in C17.2 cells. C17.2 cell morphology changed after treatment with fasudil (100 μ M, Fas), the autophagy inducer rapamycin (1 μ M, Rap), fasudil+rapamycin (Fas+Rap), the autophagy inhibitor 3-MA (10 mM, 3-MA) and Fasudil+3-MA (Fas+3-MA). Cell morphology changes were observed under a light microscope (200 $\times$) (A), and the percentage of neurite-bearing cells was counted (B). Western blot analysis of the expression levels of the autophagy markers P62 and LC3 in C17.2 cells (C). * p <0.05 and *** p <0.01 vs. control group (CT). Scale bar=50 μ m.

lowered the expression levels of both Notch 1 and Hes 1 in a time-dependent manner. The expression levels of Notch 1 notably decreased by 77%, 60%, 31%, 34% and 32% following fasudil treatment for 1, 3, 6, 12 and 24 hs, respectively, when compared with untreated cells (p <0.01). In addition, the expression levels of Hes 1 also prominently decreased by 81%,

12%, 61%, 49% and increased by 1% following fasudil treatment for 1, 3, 6, 12 and 24 hs, respectively, when compared with untreated cells ($p < 0.01$, $p < 0.05$ or no difference).

3-MA did not affect fasudil-induced neurite outgrowth in NSCs and rapamycin could not induce neurite outgrowth as fasudil did

To determine whether autophagy was involved in the observed neurite outgrowth in C17.2 cells, the autophagy inhibitor 3-MA and the autophagy inducer rapamycin were applied. Both 3-MA and rapamycin had no effect on the observed morphological changes of the cells. The percentage of neurite-bearing cells did not change after rapamycin treatment compared with that of untreated cells (Fig. 6A/B). Additionally, no change in the ratio of neurite-bearing cells was found in the cells treated with fasudil+3-MA compared with the cells treated with fasudil alone (Fig. 6A/B). Western blot analysis (Fig. 6C) was used to detect the protein levels of LC3II and p62, whose levels changed as we expected; fasudil and rapamycin treatment increased the levels of LC3II and p62 expression, and 3-MA decreased these levels after treatment for 12 h.

Discussion

In this study, we examined the neurite outgrowth in C17.2 cells induced by fasudil in a dose- and time-dependent manner. Moreover, fasudil was not cytotoxic to C17.2 cells and caused these cells to differentiate into immature or mature neuronal cells and astrocytes through modulating the Notch signalling pathway. Autophagy was also potentiated in C17.2 cells by fasudil; however, no effect on neurite outgrowth was observed when autophagy was induced or inhibited.

We found that the observed neurite outgrowth could be reversed by washing off fasudil and further incubating the cells for 12 h (Fig. 1 and Fig. 2). The outgrowth of neurite induced by lower concentration of fasudil might be due to its Rho kinase inhibition, which plays a crucial role in cytoskeleton construction [27, 28]. Very recently, two other reports demonstrated that Rho/ROCK pathway is involved with neurite outgrowth in NT2 and PC12 cells [29, 30]. But it is interesting that why the neurite will not retract when cells exposed to high concentration of fasudil (100 μ M). At such concentration, fasudil can also inhibit other kinases, including PKA [31]. Further studies are necessary to uncover the exact mechanism.

The Notch signalling pathway is widely believed to be tightly associated with NSC differentiation and proliferation. The Notch signalling pathway is activated for NSC self-renewal, and NSCs switch from proliferation to differentiation when Notch activity decreases [16]. In our study, the expression levels of Notch 1 and Hes 1, which were examined by Western blot, both significantly decreased. Notably, the expression level of Hes 1 markedly increased after the cells were treated with fasudil for 6 h compared with other treatment groups; this increase may be due to the oscillation of Hes 1 expression [20]. NSC differentiation and proliferation is influenced by many other factors such as cytokine signaling 1 [32], Ketamine [33] and cell volume change [34].

The Notch signalling pathway plays a crucial role in the development of the CNS and in the regulation of NSC proliferation, survival, self-renewal and differentiation, is involved in many neurodegenerative diseases such as AD and Parkinson's disease (PD) [23, 35]. Inhibiting Notch signalling to promote NSC differentiate into dopaminergic neurons may provide a cell replacement therapy for PD [35]. Additionally, down-regulating Notch 1 may reduce tau aggregates in AD to improve the cognitive function of AD patients [23]. Thus, Notch 1 is a potential therapeutic target for neurodegenerative diseases. Additionally, a fancy Notch-ROCK pathway is involved in immature cell self-renewal and differentiation [36]. Our study suggested that fasudil, which inhibits Notch signalling and the ROCK pathway, may be a promising potent strategy for treating neurodegenerative diseases.

The mechanisms involved in neurite outgrowth and NSC differentiation induced by fasudil are complicated and remain unclear. Some evidence has indicated that autophagy

is involved in neurite outgrowth [37] and that fasudil can potentiate autophagy [13]. Thus, we determined whether autophagy is involved in fasudil-induced neurite outgrowth. Although fasudil and rapamycin treatment induced autophagy, only the cells treated with fasudil displayed neurite outgrowth. Furthermore, autophagy was inhibited when cells were treated with both fasudil and 3-MA; however, no effect on neurite outgrowth was observed in C17.2 cells most likely because the concentration of fasudil was so high that more than one signalling pathway was affected simultaneously. In addition, the conventional clinical dosage of fasudil is 30 mg per day, and its peak concentration in human plasma is approximately 190 ng/ml [38] or approximately 58 μ M. Thus, in our study, the cells were treated with a concentration of fasudil that is somewhat higher than that *in vivo*. Moreover, fasudil has a low blood-brain barrier transmittance due to its poor lipid solubility [39]. However, our findings still indicate that fasudil may possess the potential for multi-target treatment in diseases, particularly in CNS disorders.

As a clinical medicine, fasudil is primarily used to dilate arteries in SAH patients. Fasudil also plays multiple roles in disease states, such as suppressing angiogenesis to inhibit tumour growth [2], improving neurological function recovery in neurodegenerative disease [5] and preserving the motor neurons to treat spinal and muscle atrophy [28]. Fasudil may be a multi-target medicine that regulates not only ROCK but also the ERK signal pathway [2], and Akt [40] and Wnt signalling [41]. Thus, with multiple targets, fasudil may be a promising medicine for CNS disorders.

In summary, the present study demonstrated that fasudil stimulates neurite outgrowth in C17.2 cells and promotes their differentiate into neuronal cells through modulating Notch signalling but not autophagy, suggesting that fasudil may be a promising medicine to exert its multi-targeted activity in CNS disorder treatment.

Disclosure Statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of this paper. The authors report no potential conflicts of interest.

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