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Effect of Rho-kinase pathway on neurite outgrowth of rat hippocampal neurons under atomic force microscopy★

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Abstract

Hippocampal neurons of neonatal rats were cultured in serum-free culture medium for 5 days *in vitro*, and treated with the Rho-kinase inducer lysophosphatidic acid. Atomic force microscopy revealed that the numbers of level-1, -2 and -3 neurites protruding from rat hippocampal neurons was significantly reduced. After treatment with the Rho kinase inhibitor Y27632, a significant increase in the numbers of these neurites was observed. Our experimental findings indicate that the Rho-kinase pathway is closely associated with the neurites of hippocampal neurons.

Key Words: atomic force microscopy; Rho-kinase; nerve cells; neurites; hippocampus; rats; neural regeneration

Abbreviations: LPA, lysophosphatidic acid

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INTRODUCTION

There is strong evidence showing that the RhoGTP enzyme family is involved in the development of nerve cells, including neurite growth, cell differentiation and formation of dendritic spines, and that it plays a crucial role in nerve cell polarization and synapse formation[1-3]. Based on sequence and function, members of the RhoGTP enzyme family can be divided into three subfamilies, namely Rho, Rac and Cdc42 subfamilies. The main signaling molecule downstream signal of Rho is RhoA, while Rho kinase (also known as ROK/ROCK) family members are downstream of RhoA. Rho kinase mediates neurite retraction. Rho kinase can regulate the outgrowth of neurites through its downstream signals, and it participates in transformation of dendrites into axons as well as axonal growth. The Rho kinase pathway is considered a key regulator of many glial-derived growth factors and inducers of neurite outgrowth[4-5]. *Via* the action of Rho kinase, RhoA induces neurite collapse, because the formation of neurites is accompanied by the presence of target neurite growth and non-target neurite collapse and pruning. Therefore, we speculated that the Rho-kinase inducer lysophosphatidic acid (LPA) could activate neurite collapse resulting in a rapid retraction of neurites, and cause cells to become round. The Rho specific inhibitor Y-26732 has been shown to trigger neurite formation[6] and reduce neurite collapse. Atomic force microscopy is an ideal tool to study biological materials, as magnifications can reach up to one-billion times, which enables the nanostructure of these materials to be viewed[7]. In this study, the effects of the LPA and inhibitor (Y-27632) on the regulation of neurite growth in primary cultured hippocampal neurons was observed using atomic force microscopy, in a broader attempt to conduct a micro-level elucidation of the Rho-kinase pathway in rat hippocampal neurites *in vitro*.

RESULTS

Morphology of rat hippocampal neurons under atomic force microscopy

Rat hippocampal neurons were isolated and cultured *in vitro*. The cells were divided into three groups: control (neurobasal-B27 culture medium), LPA (neurobasal-B27 culture medium + LPA), and LPA + Y27632 (neurobasal-B27 culture medium + LPA + Y27632). Cells were cultured in 6-well culture plates, with six wells in each group.

Control group: Scanning images (Figures

1A, C, E; scale bars: 60 μm) showed that the hippocampal nerve cells in the control group were polygonal with round nuclei. There were more than three level-1 neurites protruding from the cell body, and these were of different lengths and sizes. Among them, one process was relatively short, while other neurites were smaller. These neurites extended tiny branches and short protrusions (Figure 1A). Scanning images of the neurites and their branches (scale bar: 30 μm) showed that level-1 neurites extended long level-2 neurites, thin level-3 neurites and short level-4 neurites. The diameters of nerve cell neurites increased with decreasing neurite level, and no level-5 neurites were seen (Figure 1B).

LPA group: Scanning images (scale bar: 60 μm) showed that the morphology of hippocampal nerve cell bodies in the LPA group was similar to that in the control group: several level-1 neurites were still visible, but the number of neurites was lower. Neurite length was no more than the diameter of the cell body, with only the root of the residual neurites being seen. Other neurites maintained normal morphology, but the number of level-2 neurites was significantly reduced and the neurites were short (Figure 1C). Scanning images (scale bar: 30 μm) of the neurites and their branches showed that level-2 neurites were significantly shortened and only short protruding roots were visible. The number of level-3 neurites was significantly reduced and these neurites became short (Figure 1D).

LPA + Y27632 group: Scanning images (scale bar: 60 μm) showed that, compared with the control group, although no significant changes to cell bodies were found in the LPA + Y27632 group, the number of level-1 neurites was significantly increased. A large number of neurites extended radially from the hippocampal cell body, and the majority of these neurites were thin and slender, with abundant level-2 neurites (Figure 1E). Scanning images (scale bar: 30 μm) of the neurites and their branches showed a number of thin level-2 neurites, but they extended for a short distance, with the protruding end appearing like a droplet-like structure. There were many level-3 and level-4 neurites, which were short, and the quantity seemed low (Figure 1F).

Quantity of neurites at all levels in rat hippocampal neurons

The majority of cell neurites in the hippocampus of rats in the control group were level-2 and level-3, and the number of level-1 and level-4 neurites was relatively small. After treatment with LPA, the numbers of level-1, -2, -3 neurites in hippocampal nerve cells were significantly lower than those in the control group ($P < 0.05$); the number of level-4 neurites was not significantly different compared with control group. After treatment with LPA + Y27632, the numbers of level-1, -2 and -3 neurites in hippocampal nerve cells were significantly higher than those in the LPA group ($P < 0.05$), the number of level-1, -2 neurites was significantly higher than that in control group ($P < 0.05$), and the number of level-3 neurites was not significantly different compared with the control group. The numbers of level-4 neurites in the three groups were similar to the numbers of the three other types of neurite, but there was no significant difference between groups (Table 1).

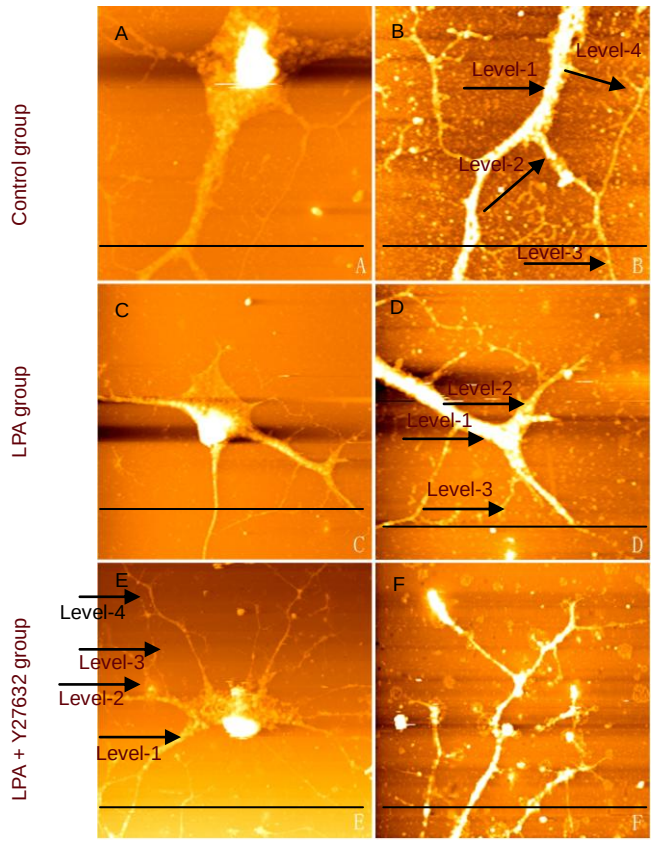

Figure 1 Scanning images of primary cultured rat hippocampal neurons and their neurites after lysophosphatidic acid (LPA) and Y27632 treatment (atomic force microscopy).

Neurites were graded as follows: level-1 neurites protrude directly from the cell body; level-2 neurites extend from level-1 neurites; level-3 neurites extend from level-2 neurites; and level-4 neurites extend from level-3 neurites.

LPA treatment was found to significantly reduce the numbers of level-1, -2 and -3 neurites in rat hippocampal neurons. After treatment with the Rho-kinase inhibitor Y27632, the numbers of these neurites were significantly increased.

Scale bars: 60 μm in A, C, E and 30 μm in B, D, F.

Table 1 Changes in the number of neurites of different levels in hippocampal nerve cells at 1 hour after lysophosphatidic acid (LPA) and Y27632 treatments

Group	Neurite grading (level)			
	1	2	3	4
Control	5.00±1.49	12.50±2.46	13.80±3.85	4.80±2.78
LPA	3.20±0.79[a]	5.90±1.29[a]	7.80±2.44[a]	4.00±2.75
LPA+Y27632	7.40±1.07[ab]	31.20±11.67[ab]	15.00±3.64[b]	5.40±0.84

[a]$P < 0.05$, *vs*. control group; [b]$P < 0.05$, *vs*. LPA group.

The number of neurites (*n*) was calculated as follows: the level-1 neurites were first measured, and a straight line was drawn along the longitudinal axis of the process, starting from the midpoint of the cell membrane between the neurites, and ending at the starting point for the level-2 neurites.

The number of level-2 neurites was calculated using the midpoint of the starting point of level-2 neurites as the starting point. If the neurites were curved or irregular in shape, a number of straight lines was drawn to link the head and the end.

The number of neurites on each line was counted and their sum was taken as the number of level-2 neurites.

Data are expressed as mean±SD of six wells in each group. Data were statistically analyzed using one-way analysis of variance and Dunnett's test.

DISCUSSION

Nerve cells develop from non-polar cells to extend dendrites and axons with stable polarity, and the formation of polarity depends on the neurite extension and their differentiation into axons or dendrites[8]. The cellular neurites occur as a result of the regulation of neuron-specific genes; for example, neural stem cells only transform into various types of nerve cells[9], and the induction of exogenous factors, for example stem cells induced by exogenous factors can develop into a variety of cell types[10-11]. Exogenous factors usually alter the cell form through the activation of the corresponding receptors on the cell membrane and their coupling to GTP-binding proteins. GTP-binding proteins are small G-proteins that contribute to the regulation of cytoskeleton movement, *via* actions on microtubules and actin polymerization and depolymerization[12-14]. The movement of the cytoskeleton is dependent on cellular migration and neurite growth, so small GTPases play a crucial role in actin cytoskeletal regulation[15-17]. Rho kinase is downstream of these small G-proteins, and changes in its activity can affect neurite outgrowth through different signaling molecules regulating the movement of the cytoskeleton[18]. LPA and Y27632 can respectively activate and inhibit the activity of Rho-kinase, and thus, it could be utilized as a tool to study the role of Rho-kinase[19-20]. LPA-induced activation of Rho-kinase can trigger the retraction of nerve cell neurites, and inhibition of Rho-kinase activity by Y27632 can inhibit neurite growth.

Previous studies addressing cell morphology have mainly adopted common optical microscopes or electron microscopy to determine the numbers and lengths of cell neurites, although fluorescent staining is another means of detecting protein expression in the neurites. Optical microscopy cannot be used to observe neurites smaller than micrometer size, while scanning electron microscopy cannot be used to measure the lengths of process and is prone to missing tiny neurites. The emergence of atomic force microscope offers a possibility for quantitative analysis of neurites at the micrometer and nanometer levels.

In this study, cells were directly extracted and not treated prior to observation by atomic force microscopy; therefore, the morphology and characteristics of nerve cells could be preserved well. After Rho-kinase was activated by LPA, the number of level-1 neurites protruding from the nerve cells was reduced or these simply disappeared, leaving the protruding roots only. Level-2 and level-3 neurites were similarly affected; their number was significantly reduced, which suggested that LPA can induce neurite collapse and inhibit neurite growth. We speculate that LPA-mediated induction of neurite collapse and inhibitory neurite growth commences on tiny neurites, gradually leading to the collapse and disappearance of level-1 neurite. Y27632 increased the numbers of level-1 and level-2 neurites, but the number of level-3 neurites was not significantly changed compared with the numbers in the control group, although the number was significantly improved compared with the LPA group. The number of level-4 neurites was not significantly different among groups. The results of this study show that the kinase activity is an important factor regulating the growth of nerve cell neurites. Activation of the Rho-kinase pathway can induced neurite collapse, with a reduction in the numbers of level-1 and level-2 neurites and a shortening of neurites. Therefore, inhibition of the Rho-kinase pathway may increase the numbers of level-1 and level-2 neurites and promote neurite growth; in this way, Y27632 could inhibit LPA-induced neurite collapse and promote neurite growth.

MATERIALS AND METHODS

Design

A parallel controlled, comparative, *in vitro* experiment.

Time and setting

Experiments were performed from January 2009 to July 2010 at the Gastroenterology Institute, Sun Yat-sen University, China.

Materials

A total of 150 healthy, clean, neonatal Sprague-Dawley rats within 24 hours after birth, of specific pathogen free level, either male or female, were purchased from the Experimental Animal Center of Sun Yat-sen University, China. Experimental treatment of animals was in strict accordance with the *Guidance Suggestions for the Care*

and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China[21].

Methods

Isolation and culture of hippocampal neurons

A total of 150 Sprague-Dawley rats were used at 1 day after birth. The bilateral hippocampi were exposed and removed to prepare a single cell suspension. Cells were seeded on poly-L-lysine (1 mg/mL)-coated 6-well culture plates at a density of 1×10^6 cells/mL.

Cell culture and intervention

Control group: Cells were cultured in neurobasal-B27 culture medium (Gibco, New York, NY, USA; Guangzhou Pubo Instruments Co., Ltd., China; serum-free and antibiotics-free) at 37°C in a humidified atmosphere of 5% CO_2 for 12 hours. Then, the culture medium was replenished with serum-free medium, and half of the medium was changed every 3 days. Cells were cultured for 8 days for further use.

LPA group: Cells were pretreated in neurobasal-B27 culture medium supplemented with LPA (Sigma, St. Louis, MO, USA; final concentration 200 ng/mL) for 1 hour, and then seeded onto neurobasal-B27 medium like the control group for further use.

LPA + Y27632 group: Cells were pretreated in neurobasal-B27 culture medium containing LPA for 1 hour, and then with neurobasal-B27 medium containing Y27632 (Sigma; final concentration 200 ng/mL) for 1 hour. Finally, cells were seeded onto neurobasal-B27 medium like the control group for further use.

Atomic force microscopy observations of nerve cell neurite in rat hippocampus

Cells from every group were seeded onto one cell culture plate with six wells, with one culture plate for each of the control group, LPA group and LPA + Y27632 group. Cells were incubated for 8 days, and the coverslips were removed. Cells were rinsed with phosphate-buffered saline, fixed with 4% paraformaldehyde for 1 hour, and then rinsed with 0.01 M phosphate-buffered saline three times, each for 10 minutes. Excessive phosphate-buffered saline on the coverslips was rinsed with double-distilled water, and the excessive liquid was absorbed using filter paper. Coverslips covered with cells were fixed on dedicated slides for atomic force microscopy using double-sided tape, and then fixed on the microscope objected stage.

Cells were observed under an atomic force microscope (Autoprobe CP Research; Thermo Company, Boston, MA, USA) in tapping mode at 25°C in 50% air humidity. Cells were *in situ* scanned using 60-μm and 30-μm scanners (X-axis scan lines 256, cantilever length 85 μm, force constant 2.5 N/m, probe tip radius of curvature 10 nm).

Image acquisition and data collection

Cell neurites and large nerve cells were screened out using a microscope monitor, and the atomic force microscope probe was gradually transferred to the cell surface. Images were captured with constant force and backed up to a computer using Image Pro software for the atomic force microscope (Thermo Company), followed by smoothing processing. Neurites were graded according to the standards of Havton *et al* [22]: neurites directly protruding from the cell body are level-1 neurites; level-1 neurites protrude level-2 neurites; level-2 neurites protrude level-3 neurites; and level-3 neurites protrude level-4 neurites. The lengths of level-1 neurites are twice the diameter of the cell body. Three fields of vision were randomly selected from each coverslip with a total of 10–12 cells analyzed. Typical nerve cells with round bodies and several neurites protruding, without interference or impurities, were fixed at the center of the image for scanning; then, the cell neurites were scanned and measured.

Statistical analysis

Measurement data are expressed as mean ± SD and were statistically analyzed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Results are presented as the numbers of measured neurites. Mean differences among groups were compared using one-way analysis of variance, and pairwise comparisons of multiple mean differences between groups were performed using Dunnett's test. A P level of < 0.05 was considered to represent a statistically significant difference.

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Author contributions: Jing Chen completed the experiments, provided and integrated data. Hu Hao was responsible for data analysis, wrote the manuscript and supervised the study. Guoqing Guo provided research ideas and research design. Sitao Li completed the statistical analysis and provided data. Xin Xiao guided the study.

Conflicts of interest: None declared.

Ethical approval: The study was approved by the Animal Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University, China.

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