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The Rho-kinase inhibitor fasudil restores normal motor nerve conduction velocity in diabetic rats by assuring the proper localization of adhesion-related molecules in myelinating Schwann cells

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ABSTRACT

The Rho/Rho-kinase signaling pathway has been shown to be involved in the complications of diabetes. In this study, we found that fasudil, a specific Rho-kinase inhibitor, had a beneficial effect on the motor nerve conduction velocity (MNCV), which is delayed in rats with streptozotocin (STZ)-induced diabetes. Cadherin-dependent adherens junctions (AJs) in myelinating Schwann cells, necessary for proper myelin formation and rapid propagation of action potentials, are regulated by Rho/Rho-kinase signaling. These AJ structures are maintained by E-cadherin and catenin complexes such as β-catenin and p120 catenin. To elucidate the mechanism underlying the effect of fasudil on MNCV, we examined alterations in AJ structure in the peripheral nerves of the experimental rats. Our results showed that the activities of Rho and Rho-kinase increased simultaneously in the sciatic nerves of the diabetic rats. Fasudil restored the MNCV by suppressing the up-regulation of the Rho-kinase. In the diabetic state, enhanced Rho and Rho-kinase activity reduced p120 catenin expression and altered the distribution of p120 catenin and E-cadherin, which are normally localized in the paranodal compartment of the nodes of Ranvier and Schmidt–Lanterman incisures where autotypic AJs stabilize myelin structure. Fasudil restored normal p120 catenin expression and the distribution of p120 catenin and E-cadherin in the myelin sheath.
In conclusion, reduced expression and altered distribution of the adhesion molecules in the myelin sheath might contribute to the slowing of the MNCV in the diabetic rats. Fasudil, through its effect on the distribution of the adhesion-related molecules, might prevent slowing of the MNCV.

Introduction

Peripheral diabetic neuropathy, one of the most devastating complications of diabetes mellitus, affects at least half of diabetic patients (Obrosova, 2009). Aberrant cell signaling induced by hyperglycemia plays a critical role in the development of complications in diabetic patients; there is increasing evidence that the Rho/Rho-kinase signaling pathway is involved in the pathogenesis of vascular complications in diabetes (Arita et al., 2009; Gojo et al., 2007; Kolavennu et al., 2008; Peng et al., 2008; Wu et al., 2010; Yokota et al., 2007). We previously found that Rho/Rho-kinase was upregulated in the kidney and retina of diabetic rats, and contributed to the development of diabetic nephropathy and retinopathy; a specific Rho-kinase inhibitor, fasudil, prevented these complications (Gojo et al., 2007; Yokota et al., 2007).

In this study, we show that Rho/Rho-kinase activity is enhanced in the peripheral nerves of rats with streptozotocin (STZ)-induced diabetes and fasudil has a beneficial effect on the delayed motor nerve conduction velocity (MNCV) in the diabetic rats. Rho, a small G protein, and its effector Rho-kinase, a serine/threonine-specific protein kinase, regulate various cell functions by controlling cytoskeletal assembly (Amano et al., 2010; Heasman and Ridley, 2008). Activation of Rho/Rho-kinase participates in a wide range of events, one of which is the formation of cell adhesion complexes (Braga et al., 1997; Fukata and Kaibuchi, 2001; Takaishi et al., 1997). Proper regulation of Rho/Rho-kinase signaling is necessary for physiological cell structure and function. In peripheral nerves, RhoA is mainly located in the paranodal compartment of nodes of Ranvier and Schmidt–Lanterman (S–L) incisures in the myelin sheath (Melendez-Vasquez et al., 2004; Scherer and Gutmann, 1996). These structures contain E-cadherin-positive autotypic adherens junctions (AJs), which are maintained by E-cadherin and catenin complexes such as β-catenin and p120 catenin (p120 ctn), and connect the cytoplasm of the Schwann cell layers to maintain the structure of the myelin sheath

Abbreviations: MNCV, Motor nerve conduction velocity; STZ, Streptozotocin; AJ, Adherens junction; p120 ctn, p120 catenin.

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(Arroyo and Scherer, 2000; Fannon et al., 1995; Poliak et al., 2002; Tricaud et al., 2005). Since the proper formation of myelin structure in Schwann cells is necessary for rapid propagation of action potentials, we hypothesize that enhanced Rho/Rho-kinase activity might alter the conformation of E-cadherin-dependent AJs under diabetic conditions. To elucidate the mechanism underlying the effect of fasudil, we examined alterations in AJ structure in peripheral nerves of the experimental rats.

Materials and methods

Materials and experimental protocol

Male Sprague–Dawley (SD) rats were purchased from Charles River Japan (Yokohama, Japan). The following items were also purchased: streptozotocin (STZ) from Sigma Chemical Co. (St Louis, MO, USA), G-LISA RhoA Activation Assay Biochemical Kit from Cytoskeleton, Inc. (Denver, CO, USA). Fasudil (Rho-kinase inhibitor) was obtained from Asahi Kasei Pharmaceutical Co. (Tokyo, Japan).

Eight-week-old rats weighing 260–300 g were rendered diabetic with a single intravenous (i.v.) injection of STZ (50 mg/kg) dissolved in 20 mM citrate buffer pH 4.5. The animals were examined 48 h later; those with plasma glucose levels ≥300 mg/dl were considered to be diabetic. Control rats were injected i.v. with 1 ml/kg of vehicle. The rats were then divided into the following 4 groups: normal control rats (control group, n = 6), diabetic rats (Diabetes group, n = 6), normal control rats treated with fasudil (control fasudil group, n = 6), and diabetic rats treated with fasudil (Diabetes fasudil group, n = 6). Fasudil was dissolved in physiological saline and administered 10 mg/kg/day by intraperitoneal (i.p.) injection throughout the study. Normal control rats were injected i.p. with the same volume of saline. After 4 weeks of treatment, the rats were anesthetized i.p. with pentobarbital (50 mg/kg), and motor nerve conduction velocities were determined as described below. Serum glucose was measured with the glucose oxidase method. All experimental procedures were approved by the Animal Care and Use Committee of the Jikei University School of Medicine, and performed according to the guidelines for Animal Experiments.

Measurement of sciatic motor nerve conduction velocity (MNCV)

Surgery was performed on the right hind limb of anesthetized rats to expose the sciatic nerve. The skin was incised and detached between the biceps femoris and semitendinous muscles to expose the sciatic nerve, to which an electrode was attached. The sciatic nerve was stimulated at two points, and conduction was detected by a fine needle electrode at the ankle (Moore et al., 1980). The stimulus was digitized by a bio-amplifier, using the Power Lab System (AD Instruments, Hampstead, London, UK), and captured with MacLab Scope version 4 software (AD Instruments, Hampstead, London, UK). The sciatic nerve was stimulated with constant-current (1 mA) square-wave pulses (0.25 ms). The temperature adjacent to the sciatic nerve was monitored by an electronic thermometer and maintained at 37 °C with an incandescent lamp.

Sciatic nerve sample preparation (in vivo cryotechnique)

Isopentane–propane cryogen (−196 °C) was poured on the exposed left sciatic nerve opposite to the side on which the nerve conduction velocity was measured under anesthesia (Ohno et al., 2007). The frozen sciatic nerves were resected and divided for immunohistochemistry and protein extraction.

For immunohistochemistry, the frozen sciatic nerves were put in a freeze-substitution solution (absolute acetone containing 2% paraformaldehyde) and kept at −80 °C for 24 h. The temperature of the sciatic nerves was gradually returned to room temperature. After washing in pure acetone, they were transferred into chloroform and embedded in paraffin wax. To extract protein, the nerve samples were homogenized in ice cold lysis buffer (Lysis Buffer contained in the G-LISA RhoA Activation Assay Biochemical Kit, supplemented with 50 mM NaF, 20 mM Na pyrophosphate, 1 mM p-Nitrophenyl phosphate, 1 μM Microcystin LR, Protease Inhibitor Cocktail) for assays of RhoA, myosin phosphatase target subunit-1 (MYPT-1) and Phosphorylated-MYPT-1 (p-MYPT-1) and RIPA buffer (50 mM Tris HCl pH 7.6, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, Protease inhibitor) for assays of E-cadherin, p120 ctn, β-catenin and GAPDH. After centrifugation, the supernatants were snap-frozen and conserved at −150 °C.

Immunoblotting

Primary antibodies were RhoA polyclonal antibody (ProteinTech, Chicago, IL, USA) (1:1000), MYPT-1 polyclonal antibody (Cell Signaling, Danvers, MA, USA) (1:1000), p-MYPT-1 (Thr850) polyclonal antibody (Upstate, Lake Placid, NY, USA) (1:5000), E-cadherin monoclonal antibody (BD Bioscience, Franklin Lakes, NJ, USA) (1:10,000), p120 ctn monoclonal antibody (Abcam, Cambridge, UK) (1:10,000), β-catenin monoclonal antibody (BD Bioscience, Franklin Lakes, NJ, USA) (1:1000), and GAPDH polyclonal antibody (Abcam, Cambridge, UK) (1:10,000).

Protein concentrations were measured with the Precision Red Advanced Protein Assay Reagent (Cytoskeleton, Inc., Denver, CO, USA). The lysates (40 μg) were separated on 12.5% (for RhoA and GAPDH) and 7.5% (for MYPT-1, p-MYPT-1, E-cadherin, p120 ctn and β-catenin) SDS-polyacrylamide gels. After electroblotting onto nitrocellulose transfer membranes (Whatman, Dassel, Germany), the membranes were incubated for 2 h at room temperature with blocking buffer (10 mM Tris HCl pH 7.5, 150 mM NaCl, 0.1% Gelatin, 0.1% Casein, 0.05% Tween 20), then over night at 4 °C with the primary antibody diluted in Can Get Signal Immunoreaction Enhancer Solution 1 (TOYOBO, Osaka, Japan). After washing three times in wash buffer (TBS, 0.05% Tween 20), the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody for RhoA, MYPT-1, p-MYPT-1, p120 ctn and GAPDH and horseradish peroxidase-conjugated anti-mouse secondary antibody for E-cadherin and β-catenin (VECTOR, Burlingame, CA) (1:5000) in Can Get Signal Immunoreaction Enhancer Solution 2 for 60 min with gentle shaking at room temperature. The signals were detected with ECL Plus Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) and X-ray film (Hyperfilm, Amersham Bioscience, Buckinghamshire, UK). The densitometric analysis was performed simultaneously with Light-Capture II (ATTO, Tokyo, Japan). The expression levels of E-cadherin, p120 ctn, and β-catenin were normalized to GAPDH.

Determination of RhoA and Rho-kinase activity

Rho-kinase activity was expressed as the ratio of p-MYPT-1 to total MYPT-1. RhoA activity was determined by the G-LISA RhoA Activation Assay Biochemical Kit, a modified ELISA system that specifically detects the complex between the active GTP-bound form of RhoA and rhotekin. Active RhoA was evaluated according to the manufacturer's instructions. In brief, the 50 μl of the protein sample, diluted to 1.0 μg/μl, were added to 96-well plates coated with the Rho binding domain of Rho effector proteins and incubated at 4 °C for 30 min with vigorous shaking. The plates were subsequently incubated with anti-RhoA antibody and secondary horseradish peroxidase-conjugated antibody for 45 min at room temperature. Active RhoA levels were determined by measuring absorbance at 490 nm using a microplate spectrophotometer. In addition, total RhoA was evaluated by immunoblotting using an anti-RhoA antibody. RhoA activity was expressed as the ratio of active RhoA to total RhoA.