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Hydroxyl fasudil, an inhibitor of Rho signaling, improves erectile function in diabetic rats: a role for neuronal ROCK

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Abstract

Introduction—The pathogenesis of diabetic erectile dysfunction (ED) includes neuropathy, but the molecular basis for neurogenic ED is incompletely understood. The RhoA/ROCK pathway has been implicated in diabetic neuropathy and in ED, but its role in diabetic neurogenic ED is not known.

Aims—The aim of this study was to determine whether hydroxyl fasudil, an ROCK inhibitor, affects diabetic neuropathy-related ED.

Methods—Type 1 diabetes mellitus was induced in male rats by streptozotocin (75 mg/kg, intraperitoneally). After 8 weeks, diabetic rats were administered hydroxyl fasudil, a selective ROCK inhibitor (10 mg/kg/day, intraperitoneally) or vehicle, for 4 weeks. Age-matched control, nondiabetic, rats were treated intraperitoneally for 4 weeks with saline. At week 12, after a 2 day wash-out, neuro-stimulated erectile function was evaluated. Major pelvic ganglia (MPG) were collected for western blot analysis of RhoA, ROCK-1, ROCK-2, phospho (P)-AKT (Ser⁴⁷³), and P-phosphatase and tensin homolog (P-PTEN) (Ser³⁸⁰/Thr^{382/383}).

Main outcome measures—Effect of ROCK inhibitor hydroxyl fasudil on erectile function and ROCK/P-AKT/P-PTEN pathway in the MPG of diabetic rats.

Results—Erectile response was significantly ($P<0.05$) reduced in diabetic compared with nondiabetic rats, and was preserved ($P<0.05$) in diabetic rats treated with hydroxyl fasudil. In diabetic rats, RhoA and ROCK-2 protein expressions in MPG were increased ($P<0.05$) and remained increased in hydroxyl fasudil-treated rats. P-AKT (Ser⁴⁷³) expression was decreased ($P<0.05$), while P-PTEN (Ser³⁸⁰/Thr^{382/383}) expression was increased ($P<0.05$) in MPG of diabetic compared to nondiabetic rats, and both were reversed ($P<0.05$) in diabetic rats treated with hydroxyl fasudil.

Conclusion—Improved erectile function and restored P-AKT and P-PTEN in the MPG with hydroxyl fasudil treatment suggest the role of Rho signaling via PTEN/AKT pathway in neurogenic diabetic ED.

Keywords

P-AKT; P-PTEN; apoptosis; major pelvic ganglia; diabetic neuropathy

Introduction

Diabetes mellitus is one of the major risk factors for erectile dysfunction (ED).¹ Multiple mechanisms involving vasculogenic and neurogenic factors are involved in ED associated with diabetes. Autonomic neuropathy has been attributed to progressive deterioration of nitrergic neurons due to apoptosis and decreased neuronal nitric oxide (NO) synthase (nNOS) content.²⁻⁷ In type 1 diabetic rats reversible decrease in nNOS content in the axons of nitrergic nerves, possibly due to a defect in axonal transport, is followed in more advanced diabetes by apoptosis of nitrergic nerve cell bodies in the major pelvic ganglia (MPG). This leads to irreversible loss of nNOS content and nitrergic function and a decrease in nerve fibers.⁸ Apoptotic cell death has been attributed to increased oxidative stress due to the synergistic action of endogenous neuronal NO and extracellular advanced glycation end products.⁸⁻¹⁰ MPG is the source of nNOS-containing nerve fibers terminating in the penis; nNOS-generated NO both initiates penile erection and participates in the maintenance of penile erection required for normal sexual performance.^{11, 12} Thus, a deficiency in nNOS-derived NO leads to neurogenic ED. However, the molecular mechanisms of nitrergic penile nerve degeneration/ apoptosis in diabetes that lead to functional loss and ED are not well understood.

Recent studies have demonstrated that RhoA, a small GTPase, and its main downstream effector Rho kinase (ROCK) play a pivotal role in erectile function by maintaining the penis in a flaccid state.¹³⁻¹⁵ Elevated RhoA/ROCK activity in the penis contributes to the pathogenesis of vasculogenic ED in conditions such as diabetes, hypertension, and aging.¹⁶⁻¹⁸ In addition, the RhoA/ROCK signaling pathway plays a significant role in the pathogenesis of nerve injury due to its effect on neuronal growth inhibition.¹⁹ Recently, selective ROCK inhibitors such as Y-27632, fasudil and its potent metabolite hydroxyl fasudil, have been introduced with profound neuroprotective, neuroregenerative, and functional recovery effects in experimental animal models of stroke, spinal cord injury, and inflammatory and demyelinating diseases, thus providing a potential new therapeutic for these conditions.²⁰ However, the function of the Rho signaling pathway in normal and diseased penile neuronal mechanisms (MPG neuronal survival and cavernous nerve axonal transport) and its role in the development of diabetic ED are largely unknown.

The aim of this study was to test the hypothesis that hydroxyl fasudil, a selective inhibitor of RhoA/ROCK signaling, inhibits molecular pathways involved in neuronal apoptosis in the MPG, and recovers erectile function associated with type 1 diabetes mellitus.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (375-400 grams, Charles River Laboratories Inc., Wilmington, MA) were used in this study. All animal procedures were conducted in accordance with the ethical standards of the Johns Hopkins University School of Medicine Guidelines for the Care and Use of Laboratory Animals.

Induction of Diabetes and Treatment Groups

Male rats were injected intraperitoneally with streptozotocin (75 mg/kg, Sigma-Aldrich, St. Louis, MO, n=15) or vehicle (0.1 M citrate buffer, pH 4.5, n=7).^{21, 22} The onset of diabetes was confirmed after 3 days by urine glucose levels of >500 mg/dl, by using chemstrips (Roche Diagnostics). Survival rate in diabetic rats was 94%. At 8 weeks after diabetes induction, diabetic rats were treated intraperitoneally with the ROCK inhibitor hydroxyl fasudil (10 mg/kg, Sigma Aldrich, n=8) or vehicle (saline, n=6) for another 4 weeks. This dose of hydroxyl fasudil is within the range used in previous studies.^{23, 24} Age-matched nondiabetic rats were treated intraperitoneally for 4 weeks with saline (n=7). Experiments were performed at week 12 following 2 days of washout of hydroxyl fasudil.

In Vivo Erection Studies

Intracavernosal pressure (ICP) and mean arterial pressure (MAP) were measured in ketamine/xylazine anesthetized rats, as described previously.²⁵ To monitor ICP, the shaft of the penis was denuded of skin and fascia, and a 27-gauge needle attached to polyethylene-50 tubing was inserted into the right crus and connected to a pressure transducer. The prostate was exposed via a midline abdominal incision, and cavernous nerves and MPGs were located bilaterally posterolateral to the prostate. Electrical stimulation of the cavernous nerve was performed by placing a bipolar platinum electrode attached to an S48 stimulator (Grass Instruments, Quincy, MA) around the cavernous nerve. Stimulation parameters were 1 volt at a frequency of 16 Hz, with square-wave duration of 5 milliseconds for 1 minute. ICP was continuously recorded (DI-190; Dataq Instruments, Akron, OH) from the start of stimulation to an interval of 1 minute after stimulation ended. The submaximal stimulation parameter (1 V) was chosen based on our previous studies showing that maximal voltage may obscure pharmacological effects on physiological and molecular parameters in penes.²⁶ Voltage-response was not performed in order to keep the exact timing of MPG collection after initial electrical stimulation of the cavernous nerve consistent among animals for standardizing measurements of P-AKT.¹¹ To continuously monitor MAP, the right carotid artery was cannulated with polyethylene-50 tubing containing heparinized saline (20 U/mL). Maximal ICP (ICP at the plateau) and total ICP (ICP area under the curve) were analyzed using the MATLAB program (Mathworks, Natick, MA) and were expressed per MAP as a difference from baseline. At the conclusion of experiments, animals were sacrificed with a lethal intracardiac injection of saturated potassium chloride. Both MPGs were then excised, rinsed in saline, snap-frozen, and processed for molecular analysis.

Western Blot Analysis

MPG were homogenized in RIPA buffer and centrifuged at $10,000 \times g$ for 30 minutes at 4°C , as described previously.²⁵ Supernatants were loaded on 4-20% Tris HCl gels (Bio-Rad Laboratories, Hercules, CA), transferred to a polyvinylidene fluoride membrane, and incubated with the following primary antibodies overnight at 4°C : anti-phospho (P)-AKT (Ser⁴⁷³) and anti-P-phosphatase and tensin homolog (P-PTEN [Ser³⁸⁰/Thr^{382/383}], Cell Signaling Technology, Beverly, MA) at 1:1,000 dilutions, anti-RhoA (Santa Cruz Biotechnology Inc, Santa Cruz, CA) at 1:1,000 dilution, and anti-ROCK-1 and ROCK-2 (BD Transduction Laboratories, San Diego, CA) at 1:1,000 and 1:2,000 dilution, respectively. Membranes were then stripped and probed with anti-AKT, anti-PTEN (Cell Signaling Technology), or anti- β -tubulin (Abcam, Cambridge, MA) antibodies at 1:1,000, 1:1,000, and 1:7,000 dilutions, respectively. Bands were detected by horseradish peroxidase conjugated anti-mouse or anti-rabbit antibodies (GE Healthcare, Piscataway, NJ, USA), and quantified using NIH Image 1.45. All results were expressed relative to nondiabetic rats treated with vehicle.

Statistical Analysis

The data were expressed as the mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using one-way analysis of variances (ANOVA; SigmaStat Windows Version 3.00), followed by Holm-Sidak multiple comparison tests or by Student's *t* tests when appropriate. A value of $P < 0.05$ was considered to be statistically significant.

Results

Changes in Body Weight in Diabetic Rats

Twelve weeks after the start of the experiment, nondiabetic rats treated with vehicle (603 ± 22 g) significantly ($P < 0.05$) gained weight compared with their initial weight on week 0 (391 ± 16 g). Diabetic rats treated with vehicle (400 ± 25 g) or hydroxyl fasudil (413 ± 22 g) did not gain weight compared with their initial weight on week 0 (391 ± 12 g and 406 ± 6 g; respectively). At week 12, all of the diabetic rats (treated and not treated) had significantly ($P < 0.05$) lower body weight in comparison to nondiabetic control rats.

Hydroxyl Fasudil Improves Erectile Function in Diabetic Rats

Erectile response to electrical stimulation of the cavernous nerve (measured as maximal ICP/MAP and total ICP/MAP) was significantly ($P < 0.05$) decreased in diabetic compared with nondiabetic rats at week 12 after diabetes induction (Figure 1). Baseline ICP was comparable between diabetic (11.6 ± 1.8 mmHg) and nondiabetic (13.2 ± 1.6 mmHg) rats ($P > 0.05$). Hydroxyl fasudil significantly ($P < 0.05$) improved erectile function in diabetic rats. These results indicate that a selective inhibitor of ROCK, given chronically to diabetic animals, preserves erectile function.

Hydroxyl Fasudil Does Not Affect Elevated Protein Expression of Rho Signaling in the MPG of Diabetic Rats

RhoA protein expression was significantly ($P<0.05$) increased in the MPG of diabetic compared to nondiabetic rats, and was not affected by hydroxyl fasudil (Figure 2A). Similarly, ROCK-2 protein expression was significantly ($P<0.05$) increased in the MPG of diabetic compared to nondiabetic rats, and was not altered by hydroxyl fasudil (Figure 2B). Protein expression of ROCK-1 was not affected by diabetes or hydroxyl fasudil treatment (Figure 2C). These results suggest that RhoA/ROCK-2 signaling was disrupted in diabetic MPG and not affected at the protein level with hydroxyl fasudil treatment.

Hydroxyl Fasudil Preserves Antiapoptotic P-AKT (Ser⁴⁷³) Expression in the MPG of Diabetic Rats

Protein expression of P-AKT (Ser⁴⁷³), an antiapoptotic factor, was significantly ($P<0.05$) decreased in the MPG of diabetic compared with nondiabetic rats (Figure 3). Hydroxyl fasudil increased ($P<0.05$) P-AKT (Ser⁴⁷³) expression. These results suggest that a selective inhibitor of ROCK impacts mechanisms associated with apoptosis in the MPG of diabetic rats.

Hydroxyl Fasudil Preserves P-PTEN (Ser³⁸⁰/Thr^{382/383}) Expression in the MPG of Diabetic Rats

Protein expression of P-PTEN (Ser³⁸⁰/Thr^{382/383}), a downstream target of ROCK and a major negative regulator of the PI3K/AKT signaling pathway,²⁷ was significantly ($P<0.05$) increased in the MPG of diabetic compared with nondiabetic rats (Figure 4). Hydroxyl fasudil decreased ($P<0.05$) P-PTEN (Ser³⁸⁰/Thr^{382/383}) expression. These results suggest that a selective inhibitor of ROCK affects PTEN signaling in MPG of diabetic animals.

Discussion

In the present study we demonstrated that an inhibitor of the RhoA/ROCK signaling pathway regulates mechanisms of neuronal apoptosis in penile innervation in a rat model of diabetic ED. ROCK/PTEN/AKT dysregulation in the diabetic MPG is manifested as upregulation of RhoA and ROCK-2 protein expressions, upregulation of the activated form of phosphatase PTEN (an upstream regulator of AKT), and downregulation of the activated form of AKT (an anti-apoptotic factor). This study further established that *in vivo* treatment with the ROCK inhibitor hydroxyl fasudil normalizes P-PTEN and P-AKT protein expressions in the MPG and restores erectile function in diabetic rats. These results suggest that hydroxyl fasudil preserves erectile function in diabetes at least in part through PTEN/AKT pathway in penile innervation.

ROCK, a serine/threonine kinase, has two highly homologous isoforms: ROCK-1 (ROCK β), which is abundantly expressed in the heart, lung and non-neuronal tissue, and ROCK-2 (ROCK α), which is preferentially found in the brain, spinal cord, and muscle.²⁸ ROCK regulates a wide variety of cellular processes including smooth muscle contraction, neurite outgrowth, cell adhesion and motility, and apoptosis. RhoA, a small GTP-binding protein, and ROCK, are important regulators of penile erection by keeping the cavernous smooth

muscle in a contracted state.¹³ Increased RhoA/ROCK activity and upregulated RhoA and ROCK-2 protein expressions in the penis of aged,¹⁶ spontaneously hypertensive,¹⁸ diabetic,^{17, 29} atherosclerotic,³⁰ and cavernous nerve injury-induced ED rat models,^{31, 32} suggest the contribution of this system to the pathogenesis of ED. Activation of the Rho/ROCK pathway also inhibits axonal regeneration and promotes apoptosis in vitro and in vivo.²⁰ Accordingly, inhibition of the RhoA/ROCK pathway promotes axonal outgrowth and prevents neuronal apoptosis, providing neuroprotection in several animal models of nerve injury.^{28, 33} Similar to changes in diabetic corpora cavernosa,²⁹ we found that protein expressions of RhoA and ROCK-2 (but not ROCK-1) were increased and remained increased in the MPG of diabetic rats treated with hydroxyl fasudil, implicating the role of RhoA/ROCK signaling in neuropathy associated with diabetic ED. While unchanged levels of RhoA and ROCK-2 protein expressions in response to hydroxyl fasudil are unexpected, it is plausible that their activity levels are downregulated by the inhibitor.

Prototypical pharmacological inhibitors of ROCK include fasudil and its derivatives, and Y-27632, which inhibit binding of ATP to the catalytic domain of the kinase in a competitive manner.²⁸ Hydroxyl fasudil is the active metabolite of fasudil, and it inhibits ROCK with IC₅₀ value of 0.9-18 μ M. While both compounds have high selectivity for ROCK, their inhibitory effects are nonisoform specific.²⁸ ROCK inhibitors Y-27632¹⁷ and SAR407899, a recently developed novel ROCK inhibitor,³⁴ relax the cavernosal tissue of diabetic animals in a dose-dependent manner. The study by Li et al provided the first long-term in vivo evidence that oral administration of fasudil for 4 weeks, starting at 12 weeks after induction of type 1 diabetes, restored erectile function in rats.³⁵ Similarly, we found in our study that 4 weeks of treatment with hydroxyl fasudil, starting 8 weeks after type 1 diabetes induction, improved erectile function. The recovery of erectile function in hydroxyl fasudil-treated animals was not related to the acute effect of the inhibitor since a 2 day washout (half life of hydroxyl fasudil is about 8 hours)³⁶ was applied. Thus the observed effect conceivably correlates with long-term molecular changes induced by a ROCK inhibitor. Unexpectedly, diabetic animals treated with hydroxyl fasudil had better (although not significantly) erectile responses than non-diabetic control animals. Possible explanations for this phenomenon (long term inhibition of other kinases, long term regulation of smooth muscle activity) should be investigated in future studies.

PI3K/AKT is a central component of cell survival pathways and one of the major intracellular signaling pathways for neuronal survival. Decreased P-AKT expression has been demonstrated in autonomic neurons and ganglia including vagal neurons, myenteric neurons of the enteric nervous system, and penile nerve fibers within corporal tissue in type 1 diabetes,^{35, 37} and in animal models of myocardial infarction³⁸ and cerebral ischemia.³⁹ Inhibition of ROCK increased P-AKT and reduced cellular apoptosis in these models. These studies indicated that AKT activation may protect neurons from the damaging insult.^{38, 39} Our results extend these molecular findings to penile innervation, as decreased P-AKT expression in diabetic MPG was reversed with hydroxyl fasudil treatment in parallel with the improvement of penile erection.

A major negative regulator of the PI3K/AKT signaling pathway is phosphatase PTEN.²⁷ PTEN activity is elevated after stroke and ischemic damage, whereas downregulation of

PTEN activity protects against ischemic damage.³⁹ PTEN itself is activated by ROCK via phosphorylation.⁴⁰ In cardiomyocytes the pro-apoptotic effect of the Rho pathway has been attributed to activation of PTEN by phosphorylation and subsequent inhibition of PI3K/AKT activity.⁴¹ In diabetic rats elevated RhoA/ROCK pathway in the penis increases penile apoptosis via the PTEN/AKT pathway resulting in vasculogenic ED, which was normalized by treatment with the ROCK inhibitor fasudil.³⁵ We now show that ROCK pathway and PTEN/AKT pathway in penile innervation contribute to neurogenic ED associated with diabetes. Beneficial effects of hydroxyl fasudil on AKT activity in the MPG and erectile function in diabetes implies that its mechanism involves, at least in part, decreased activity of PTEN in the MPG.

There are several limitations in our study. Apoptosis, ROCK activity, and membrane-bound RhoA were not measured in the present study due to the limited amount of samples retrieved from MPG, and this limits our interpretation of the results. We attempted to measure phosphorylation of myosin phosphatase targeting protein-1 (MYPT-1), a major downstream ROCK target and a marker of its activity. However, following numerous attempts, we were not able to detect total or phosphorylated MYPT-1 protein expression in the MPG using Western blot. Future studies are needed to delineate whether the activity levels of RhoA/ROCK proteins in penile innervation are affected differentially compared to their protein expressions in response to hydroxyl fasudil treatment. Furthermore, functional and molecular alterations with diabetes and hydroxyl fasudil treatment could be supported by analysis of nNOS content and NOS activity in the MPG and penis, and neuronal survival, such as nerve fiber content, neuronal markers, or retrograde labeling. Further studies are warranted to confirm apoptosis and evaluate neuronal survival in the MPG and nNOS changes.

Conclusions

Diabetic ED is due to vascular and neuronal dysfunction in penile erectile tissue. In this study, using a rat model of streptozotocin-induced diabetes, we have demonstrated that RhoA/ROCK signaling and its downstream targets, PTEN and AKT, are dysregulated in the MPG, which can be associated with decreased erectile function. Reversal of functional changes and PTEN and AKT activities following treatment with a ROCK inhibitor provides further understanding regarding neuropathic mechanisms of diabetic ED and conceivably offers new clinical treatment options.

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Figure 1.

Effect of diabetes and hydroxyl fasudil on penile erection. Erectile response to electrical stimulation of the cavernous nerve is indicated by maximal ICP/MAP corrected for baseline (A) and total ICP/MAP corrected for baseline (B) in nondiabetic rats, diabetic rats, and diabetic rats treated with hydroxyl fasudil (10 mg/kg/day). Each bar represents the mean $\pm$ SEM of 6-8 rats. * $P < 0.05$ vs nondiabetic, # $P < 0.05$ vs diabetic. NonDiab = nondiabetic; Diab = diabetic; Diab + HF = diabetic treated with hydroxyl fasudil; AU = arbitrary units

Figure 2.

Effect of diabetes and hydroxyl fasudil on RhoA (A), ROCK-2 (B), and ROCK-1 (C) protein expressions in the MPG. Upper panels are representative Western immunoblots of RhoA, ROCK-2, ROCK-1, and β -tubulin in nondiabetic rats, diabetic rats, and diabetic rats treated with hydroxyl fasudil (10 mg/kg/day). Lower panel represents quantitative analysis of the proteins in the same treatment groups. Each bar represents the mean $\pm$ SEM of 6-8 rats. * $P < 0.05$ vs nondiabetic. NonDiab = nondiabetic; Diab = diabetic; Diab + HF = diabetic treated with hydroxyl fasudil.

Figure 3.

Effect of diabetes and hydroxyl fasudil on P-AKT (Ser⁴⁷³)/AKT protein expression in the MPG. Upper panels are representative Western immunoblots of P-AKT (Ser⁴⁷³) and AKT in nondiabetic rats, diabetic rats, and diabetic rats treated with hydroxyl fasudil (10 mg/kg/day). Lower panel represents quantitative analysis of P-AKT/AKT in the same treatment groups. Each bar represents the mean $\pm$ SEM of 6-8 rats. * $P < 0.05$ vs nondiabetic, # $P < 0.05$ vs diabetic. NonDiab = nondiabetic; Diab = diabetic; Diab + HF = diabetic treated with hydroxyl fasudil.

Figure 4.

Effect of diabetes and hydroxyl fasudil on P-PTEN (Ser³⁸⁰/Thr^{382/383})/PTEN protein expression in the MPG. Upper panels are representative Western immunoblots of P-PTEN (Ser³⁸⁰/Thr^{382/383}) and PTEN in nondiabetic rats, diabetic rats, and diabetic rats treated with hydroxyl fasudil (10 mg/kg/day). Lower panel represents quantitative analysis of P-PTEN/PTEN in the same treatment groups. Each bar represents the mean $\pm$ SEM of 6-8 rats.

* $P < 0.05$ vs nondiabetic, # $P < 0.05$ vs diabetic. NonDiab = nondiabetic; Diab = diabetic; Diab + HF = diabetic treated with hydroxyl fasudil.