Rho Signaling Pathway Targeted to Promote Spinal Cord Repair

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The Rho signaling pathway regulates the cytoskeleton and motility and plays an important role in neuronal growth inhibition. Here we demonstrate that inactivation of Rho or its downstream target Rho-associated kinase (ROK) stimulated neurite growth in primary cells of cortical neurons plated on myelin or chondroitin sulfate proteoglycan substrates. Furthermore, treatment either with C3 transferase (C3) to inactivate Rho or with Y27632 to inhibit ROK was sufficient to stimulate axon regeneration and recovery of hindlimb function after spinal cord injury (SCI) in adult mice. Injured mice were treated with a single injection of Rho or Rho-associated kinase inhibitors delivered in a protein adhesive at the lesion site. Treated animals showed long-distance regeneration of anterogradely labeled corticospinal axons and increased levels of GAP-43 mRNA in the motor cortex. Behaviorally, inactivation of Rho pathway induced rapid recovery of locomotion and progressive recuperation of forelimb–hindlimb coordination. These findings provide evidence that the Rho signaling pathway is a potential target for therapeutic interventions after spinal cord injury.

Key words: Rho GTPase; Rho-associated kinase; C3; Y27632; corticospinal tract; regeneration; BBB behavior scale; GAP-43; mouse

Mammalian neurons do not regenerate successfully after lesion. This is explained in part by myelin-derived inhibition (Caroni and Schwab, 1988; McKerracher et al., 1994; Mukhopadhyay et al., 1994; Chen et al., 2000) and the formation of a glial scar expressing inhibitory molecules (Snow et al., 1990; McKeon et al., 1991; Asher et al., 2000). However, numerous studies in animal models using methods designed to overcome the effect of growth inhibitory proteins have confirmed the regenerative potential of the injured spinal cord. These methods include the use of antibodies (Schnell and Schwab, 1990; Huang et al., 1999), peripheral nerve grafts (Cheng et al., 1996), transplantation of cells into the lesion site (Howland et al., 1995; Rapalino et al., 1998; Liu et al., 1999; McDonald et al., 1999; Ramon-Cueto et al., 2000) and limiting the formation of the glial scar (Davies et al., 1999; Moon et al., 2000).

The failure of regeneration in the adult CNS may also be caused by changes occurring in mature neurons (Li et al., 1995, 1996; Shen et al., 1999; Cai et al., 2001). Another approach to stimulate regeneration has been to target neurite growth signaling. For example, different neurotrophin treatments have increased the ability of neurons in adult CNS to regenerate and stimulate both axonal growth and sprouting after injury (Schnell et al., 1994; Sawai et al., 1996; Blesch and Tuszynski, 1997; Weidner et al., 1999; Coumans et al., 2001). Neurotrophins are known to delay apoptosis, prevent atrophy of axotomized neurons, and enhance the expression of growth-associated genes (Fournier et al., 1997; Kobayashi et al., 1997; Bregman et al., 1998; Broude et al., 1999). Recent data suggest that neurotrophins might stimulate regeneration by increasing neuronal cAMP levels to overcome inhibitory signaling (Cai et al., 1999). Therefore, the decreased ability of the mature CNS to regenerate after injury may result from both the intrinsic properties of adult neurons and the extracellular inhibitory environment.

The Rho GTPase is a key intracellular regulator of cytoskeletal dynamics and cell motility (Hall, 1998). Rho is activated when growth cones collapse in response to chemorepulsive factors (Tigyi et al., 1996; Jin and Strittmatter, 1997; Kuhn et al., 1999; Wahl et al., 2000), and inhibiting Rho promotes neurite outgrowth in the presence of myelin (Jin and Strittmatter, 1997; Lehmann et al., 1999). Recently, Rho has been shown to regulate apoptosis (Liu et al., 2001; Trapp et al., 2001). An enzyme from C. botulinum, C3 transferase (C3), blocks Rho function by ADP ribosylation of the effector domain (Dillon and Feig, 1995). Y27632 inhibits Rho-associated kinase (ROK), a serine–threonine protein kinase that is activated by Rho (Dillon and Feig, 1995). Y27632 inhibits Rho-associated kinase (ROK), a serine–threonine protein kinase that is activated by Rho (Ishizaki et al., 1997; Uehata et al., 1997). Inactivation of ROK with Y27632 promotes neurite outgrowth (Katoh et al., 1998; Bitto et al., 2000), but it is not known whether it is sufficient to block growth inhibition as was shown for inactivation of Rho (Lehmann et al., 1999). In the present study, we compare inactivation of Rho or ROK to promote axon growth on inhibitory substrates. We further study in fully adult mice whether inactivation of the Rho signaling pathway promotes axon regeneration and functional recovery after spinal cord injury (SCI). Animals treated to inactivate the Rho signaling pathway show significant improvement in locomotion by open field testing. Thus, inactivation of the Rho signaling pathway is an effective method to improve outcome after SCI.

MATERIALS AND METHODS

Cell culture. Primary cortical neurons were isolated from embryonic day (E) 15–E18 rat fetuses. The cortex was cut into small pieces into Ca²⁺-
zyme was prepared as a glutathione S-transferase (CSPG) substrates were made by incubating 0.5 mg/ml C3 or buffer, or with 31 or 31 μM Y27632. Cells were plated in eight-well chamber slides coated with 25 μg/ml poly-L-lysine or with test substrates. Myelin substrates were made by coating with 8 μg purified bovine brain myelin dried overnight at room temperature. Chondroitin sulfate proteoglycan (CSPG) substrates were made by incubating 0.5 μg/ml mixed CSPG solution (Chemicon, Temecula, CA) overnight in poly-L-lysine-coated chamber slides. Mixed substrate was made by coating and drying 8 μg of myelin in 150 μg of CSPG solution. The plates were fixed with 4% paraformaldehyde and 0.5% gluteraldehyde after 12, 24, or 48 hr, and neurons were identified by immunocytochemistry using a βIII-tubulin antibody (Sigma, Oakville, ON). The longest neurite per neuron was measured on an average of 400 cells per experiment with a minimum of three experiments per condition. Doses for C3 and Y27632 were chosen on the basis of previous experiments (Lehmann et al., 1999; Bito et al., 2000; M. Winton and L. McKerracher, unpublished observations).

Preparation of recombinant C3 and Y27632. Recombinant C3 exoenzyme was purified from expression bacterial cultures using streptomycin-S and stored at −80°C (Lehmann et al., 1999). Y27632 was synthesized from α-methylbenzylamine and exhibited identical 1H and 13C nuclear magnetic resonance spectra as reported in United States Patents 4,997,834 and 5,478,838. Before in vivo use, the activity of C3 and Y27632 was tested in tissue culture with retinal neurons plated on myelin substrates (Lehmann et al., 1999).

Spinal cord injury and delivery of Rho and Rho-kinase inhibitors. BALB-c female mice (n = 70) of ~20 gm were anesthetized with 0.4 ml/kg hypnorm and 5 mg/kg diazepam. A segment of the thoracic spinal cord was exposed using fine rongeurs to remove the bone, and a dorsal over-hemisection was made at T7. Fine scissors were used to cut the dorsal part of the spinal cord, which was cut a second time with a fine knife to create the lesion extension. The central canal was then filled to prevent adhesive delivery system was prepared using a Tisseq VH kit (Immu

NoAG, Vienna, Austria). According to manufacturer’s instructions for slow polymerization, lophylized fibrogen was reconstituted in an aprotinin solution, thrombin was reconstituted in a calcium chloride solution, and both solutions were warmed to 37°C. Fifty microliters of 1 mg/ml C3 or Y27632 were added to 25 μl of the thrombin solution. This was mixed with 25 μl of the fibrinogen solution just before application to the spinal cord to allow infiltration of the mixture into the lesion site before polymerization. In some C3-treated animals and in all Y27632-treated animals, 10 μl of the 1 mg/ml solution was applied to the lesion site immediately after the cord was cut. As controls, a second group of animals received fibrin adhesive with buffer, and a third group was left untreated. C3-containing collagen gels were formed as follows. C3 was lophylized in 1 ml of buffer (0.5 mg/ml) and then reconstituted in 10 μl of 0.7 mg/ml rat tail collagen. NaHCO3, and then 25 μl of 0.7 mg/ml rat tail collagen was added. As with fibrin, 10 μl of C3 was added to the lesion cavity before the C3-containing collagen gel was applied. For retranssections 3 weeks after SCI, the spinal cords were cut at T6 as described above, and the animals were tested using the Bass-o-Beattie-Bresnahan (BBB) locomotor rating scale on days 1, 2, and 6 after the second surgery.

Anterograde labeling. Three weeks to 3 months after injury, corticospinal tract (CST) fibers were labeled by injection of the anterograde tracer wheat germ agglutinin–horseradish peroxidase (WGA-HRP) into the motor cortex as described (Huang et al., 1999). Two days later, the animals were perfused transcardially with saline and then 4% paraformaldehyde, and the spinal cords and brains were removed. Serial longitudinal cryostat sections of the spinal cord were cut at 30 μm, reacted for HRP (Huang et al., 1999), and counterstained with neutral red. Measurement of axon regeneration was assessed independently by two reviewers. Lesion depth was assessed by measuring the depth of damaged tissue in the spinal cord as a percentage of total spinal cord width.

In situ hybridization. GAP-43 mRNA was detected by in situ hybridization on coronal cryostat sections through the motor cortex of mice treated with PBS (n = 2) or C3 (n = 5). In situ hybridization was performed as described previously (Foustoukos et al., 1997). An 18-mer labeled GAP-43 cRNA probe derived from a plasmid provided by Dr. Pate Skene (Duke University Medical Center) (Basi et al., 1987). After the in situ hybridization procedure, sections were Nissl stained and bright-field and dark-field digital micrographs were taken. On the basis of

RESULTS

Inactivation of Rho or ROK promotes growth of primary neurons plated on complex inhibitory substrates

We tested first whether treatment of primary cortical neurons with C3 or with Y27632 was sufficient to stimulate growth on complex inhibitory substrates typical of the glial scar and white matter. Neurons plated on different test substrates were examined into 12, 24, and 48 hr, and similar results were observed at all time points. Neurons plated on CSPG, purified myelin, or a mixture of both did not extend long neurites and had a rounded shape (Fig. 1A,B). After treatment with C3 (Fig. 1C) or Y27632 (Fig. 1D), neurons were able to extend neurites. Measurements at 24 hr showed that treatment with either C3 or Y27632 significantly increased the length of neurites compared with untreated cells plated on myelin, CSPG, or mixed myelin/CSPG substrates (Fig. 1A). Quantitation at 12 hr showed similar results (data not shown), and at 48 hr growth of treated neurons was too extensive to measure neurite length. C3 was significantly better than Y27632 in promoting neurite growth (t test; p < 0.05). These results demonstrate that inactivation of Rho or inhibition of ROK
showed regenerative sprouting into the dorsal white matter and toward the lesion site (data not shown). To assess axons distal to the lesion site, the distance of the longest axon was measured. Axons were found up to 12 mm from the lesion site in C3-treated animals and up to 3 mm from the lesion site in Y27632-treated animals (Fig. 3), whereas buffer-treated animals showed retraction from the lesion site. Therefore, after treatment with C3 or Y27632, axons were found to extend past the lesion into the distal white matter. These axons have a twisted course of growth typical of regenerated axons (Figs. 2D, F). Although neutral red staining showed lesions extended past the central canal (Fig. 2F), these experiments alone cannot rule out the possibility that secondary damage was reduced after injury. This could arise in the damaged CNS because C3 has neuroprotective effects (Liu et al., 2001; Trapp et al., 2001), in addition to promoting growth on inhibitory substrates (Fig. 1).

**Effect of C3 on the expression of GAP-43 mRNA in the motor cortex of spinal cord injured animals**

After thoracic spinal cord lesion, only axons that regenerate long distances show upregulation of GAP-43 mRNA expression (Fe-randes et al., 1999). To confirm the growth response and the long-distance regeneration after Rho inactivation, we examined the pattern of GAP-43 mRNA expression in the motor cortex (Fig. 4A, B) of animals 1 month after CST transection with or without treatment with C3. In situ hybridization using 35S-labeled riboprobes on coronal brain sections revealed high levels of GAP-43 mRNA expression in neurons of the motor cortex of C3-treated animals (Fig. 4D), whereas untreated animals showed GAP-43 signal similar to background (Fig. 4C). Quantitation of grain cluster densities in motor cortex showed significant upregulation of GAP-43 mRNA (Fig. 4E). These results indicate that C3 treatments elicit changes in gene expression consistent with axon regeneration.

**Behavioral testing**

To test functional recovery after SCI and C3 or Y27632 treatment, we measured HL motor function using the BBB locomotor rating scale (Basso et al., 1995) (n = 37 animals). Because a toe clearance phase cannot be evaluated in recuperating mice, we modified the rating to a 17 point scale (see Materials and Methods). Twenty-four hours after surgery, control mice were paralyzed (Fig. 5A) and moved by pulling themselves forward with their forelimbs (Fig. 5B). Mice treated with C3 or Y27632 showed a remarkable recovery within 24 hr (Fig. 5A), already walking with weight support (Fig. 5A, C) (movie 1; available at www.jneurosci.org). Although this early recovery is too rapid to be explained by long-distance regeneration, possible mechanisms include local reorganization of central pattern generator circuitry ( Giménez y Ribotta et al., 2000) that may include sprouting from undamaged ventral fibers or interneurons, pharmacological activation of neurotransmitter receptors (Rossignol et al., 2000), or neuroprotection (C. Dubreuil, M. Winton, F. Yang, P. Morley, L. McKerracher, unpublished observations). Mice that had received C3 or Y27632 treatment continued to recover over the 1 month period of observation and exhibited HL–FL coordination (Fig. 5E, G) (movie 2; available at www.jneurosci.org). By contrast, the average recovery plateau for untreated animals was limited to unstable walking without HL–FL coordination (Fig. 5D, F) (movie 2). Retractection of the spinal cord at 3 weeks (n = 8) eliminated any difference between the C3-treated (n = 5) and control (n = 3) animals (BBB at day 6, 7.6 vs 7.3, respectively).
DISCUSSION

The failure of axon regeneration in the spinal cord is attributable, at least in part, to the growth inhibitory properties of white matter and the lesion site. The Rho signaling pathway is known to be important both for the cell response to growth inhibition (Lehmann et al., 1999) and for neuroprotection (Trapp et al., 2001), and growth-inhibitory substrates activate Rho (M. Winton and L. McKerracher, unpublished observations). We report here that inactivation of either Rho or Rho kinase is sufficient to allow axon growth on inhibitory substrates and leads to improved recovery after SCI. C3 ribosylates asparagine 41 in the effector domain to inactivate Rho (Sekine et al., 1989); Y27632 inhibits the kinase activity of ROK by competing with ATP for binding to the kinase (Ishizaki et al., 1997; Uehata et al., 1997). Our studies target these two different parts of the Rho signaling pathway to demonstrate the importance of Rho signaling for CNS repair. Both C3 and Y27632 promoted axon growth, but C3 was more effective than Y27632. The difference between the effects of C3 and Y27632 suggest the presence of other effectors of Rho that are inactivated by C3 but not by Y27632. Rho signals not only to...

Figure 3. Quantification of regeneration length. Longest regeneration distances after SCI alone or treatment with vehicle, Rho antagonist C3, or Rho kinase inhibitor Y27632. Each point represents one animal. The circles represent animals examined 3 weeks to 1 month after SCI; the triangles represent animals examined at 3 months. Lines indicate averages for each group. Statistical significances were evaluated with the unpaired t test: C3 + collagen versus collagen, p < 0.05; C3 + fibrin versus fibrin, p < 0.001; Y27632 + fibrin versus fibrin, p < 0.05; C3 + collagen versus C3 + fibrin, p < 0.05; C3 + fibrin versus Y27632 + fibrin, p < 0.01.
ROK but also to protein kinase N (Amano et al., 1996), rhoetokin, and other targets (Reid et al., 1996). Therefore, for CNS repair, Rho appears to be a more efficient target than ROK.

The remarkable improvement in function within 1 d of spinal cord lesion and treatment with C3 or Y27632 may be caused by increased neuroprotection in the lesioned spinal cord. Neuroprotection by itself is important for improved functional recovery after SCI (Pencea et al., 1993; Gaviria et al., 2000), and a growing volume of literature suggests that the application of C3 to ischemic CNS tissue has neuroprotective effects (Laufs et al., 2000; Trapp et al., 2001). Moreover, inactivation of Rho in spinal cord reduces the number of apoptotic cells (C. Dubreuil, M. Winton, F. Yang, P. Morley, and L. McKerracher, unpublished observations). It has been demonstrated that the application of C3 after middle cerebral artery occlusion reduces infarct volumes (Trapp et al., 2001). Therefore, Rho signaling pathway is a good target to both prevent cell death and stimulate regeneration. The ability of C3 and Y27632 to block unwanted effects of Rho activation, cell death, and neurite retraction is likely to contribute importantly to improved outcome after SCI. It is also possible that C3 and Y27632 treatments affected other cells, such as leukocytes; immediate improvement in functional recovery after SCI has been observed 24 hr after treatment with gabexate mesilate, a protease inhibitor that inhibits activation of leukocytes (Taoka et al., 1997). Therefore, the short-term effects that we observe after treatment with C3 or Y27632 are likely caused by the ability of these compounds to limit the cell damage that occurs immediately after injury.

It should be kept in mind that mice show important differences from rats in their response to spinal cord injury, most notably the absence of necrotic cavitation (Steward et al., 1999). In our experiments, we used an over-hemisection of the spinal cord to test whether Rho or ROK inactivation was able to promote repair. Many strategies that work well to promote regeneration after hemisection are not effective after complete transection of the spinal cord. It has been shown that sparing of ventrolateral fibers may translate into improved locomotor performance (Brustein and Rossignol, 1998) because these fibers are important in the initiation and control of spinal central pattern generators (for review, see Rossignol et al., 2000). Sprouting of uninjured collaterals (Weidner et al., 2001) or sprouting of fibers that are part of the circuitry of the spinal cord (Giménez y Ribotto, 2000) are likely to contribute importantly to repair. It was demonstrated recently that reorganization of spared pathways also contributes to functional recovery (Rainêteau et al., 2001). Thus, inactivation of Rho may help stimulate and enhance the spontaneous repair process that leads to limited recovery after SCI, in addition to its effects on regeneration.

Figure 4. Expression of GAP-43 mRNA in motor cortex. A, Photomicrograph of a cresyl violet-stained coronal section of mouse brain. Box outlines motor cortex area depicted in B–D. B, Fluorescent micrograph of same section as in A showing neurons retrogradely labeled with Fluorogold applied at the site of a dorsal hemisection at T7. C, D, Dark-field photomicrographs showing sections of motor cortex from an untreated mouse (C) or a C3/fibrin-treated mouse (D) after in situ hybridization for GAP-43. E, Quantitation showing significantly increased grain density in motor cortex after treatment with C3. Differences between C3 and background (Bkgd) were significant (t test; p < 0.05); differences between PBS and background were not significant. Scale bar: A, 1.8 mm; B, 1.2 mm; C, D, 250 μm.
To study the effects of C3 and Y27632 on axons in vivo, we chose to study the CST because it is one of the best characterized tracts for studies of axon regeneration in the spinal cord. Moreover, the CST can be anterogradely labeled, and in mice, most of the fibers within the CST are located just above the central canal in the dorsal spinal cord. The dorsal over-hemisection that we used for our studies would eliminate not only the CST in the dorsal spinal cord. The dorsal over-hemisection that we chose to study the CST because it is one of the best characterized tracts for studies of axon regeneration in the spinal cord. More-

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