RhoA/Rho kinase in spinal cord injury

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Abstract
A spinal cord injury refers to an injury to the spinal cord that is caused by a trauma instead of diseases. Spinal cord injury includes a primary mechanical injury and a much more complex secondary injury process involving inflammation, oxidation, excitotoxicity, and cell death. During the secondary injury, many signal pathways are activated and play important roles in mediating the pathogenesis of spinal cord injury. Among them, the RhoA/Rho kinase pathway plays a particular role in mediating spinal degeneration and regeneration. In this review, we will discuss the role and mechanism of RhoA/Rho kinase-mediated spinal cord pathogenesis, as well as the potential of targeting RhoA/Rho kinase as a strategy for promoting both neuroprotection and axonal regeneration.

Key Words: RhoA; Rho kinase; inflammation; cell death; degeneration; regeneration; spinal cord injury

Introduction
It is estimated that 1.5–5.2 million people suffer from spinal cord injury (SCI) and 130,000 new patients are added around the world each year (Schwab et al., 2006). SCI is caused by trauma, a mechanical injury followed by a secondary injury process with much more complex molecular cascade responses (Borgens and Liu-Snyder, 2012). The outcomes of SCI are pain, paralysis, and incontinence. To date, limited effective treatments are available.

RhoA is a small GTPase protein and belongs to the Rho GTPase family which contains seven subfamilies including Rho, Rac, Cdc42, Rnd, RhoD, RhoBTB, and RhoH. Among them, RhoA, Rac1, and Cdc42 are the most studied members. RhoA mediates the formation of focal adhesion and stress fibers, which are contractile acting bundles in non-muscle cells that regulate cell contractility, providing force for cell adhesion, migration, and morphogenesis (Stankiewicz and Linseman, 2014). RhoA and its downstream effector Rho kinase (ROCK) control and regulate cytoskeleton dynamics. Rho kinase has two isoforms, ROCK1 and ROCK2, and belongs to the AGC (PKA/PKG/PKC) family of serine-threonine kinases. ROCK1 and ROCK2 share an overall sequence similarity at the amino-acid level of 65% and in their kinase domains of 92% (Amano et al., 2000). The RhoA/Rho kinase pathway regulates a wide range of fundamental cell functions including contraction, motility, proliferation, gene expression, and apoptosis (Loirand et al., 2006). Studies have shown that the RhoA/Rho kinase signal pathway is involved in many diseases, such as cardiovascular diseases (Loirand et al., 2006), cancer (Sahai and Marshall, 2002), and neurological diseases (Mueller et al., 2005). RhoA/Rho kinase pathway plays a role in stroke, Alzheimer’s disease, neuropathic pain, multiple sclerosis, and SCI (Mueller et al, 2005). In this brief review, we will discuss the expression profile of RhoA/Rho kinase after SCI and the roles of activated RhoA/Rho kinase pathway in mediating inflammation, neuropathic pain and cell death in the acute phase and mediating axon degeneration in the chronic phase. We will also discuss the therapeutic strategies targeting these signal proteins.

RhoA/Rho Kinase Expression Profiles in SCI
Many studies have shown that RhoA/Rho kinase signal is activated after SCI (Dubreuil et al., 2003; Erschbamer et al., 2005; Wei et al., 2014). After spinal transection or contusion injury in rats and mice, active RhoA is dramatically increased (>10 fold). RhoA is active as early as 1.5 hours after injury and sustains at a high level at 1, 3, and 7 days. One week after injury, RhoA mRNA expression level is still 3-fold higher than normal animals and remains 3-fold higher up to 3 months. Active RhoA and its mRNA are detected in neurons, oligodendrocytes, and reactive astrocytes around the lesion area (Dubreuil et al., 2003). Four to fourteen days after contusion SCI, the signal intensity of RhoA mRNA is significantly higher in spinal cord segments below the injury center compared to segments above the injury center (Dubreuil et al., 2003; Erschbamer et al., 2005).

RhoA/Rho Kinase in Injury-induced Inflammation and Neuropathic Pain
As mentioned above, SCI includes a primary injury followed by a secondary injury. The primary injury triggers...
the secondary injury, which involves inflammation, reactive oxidation, and excitotoxicity. The outcomes of SCI are mainly influenced by the secondary injury. Previous studies have shown that the RhoA/Rho kinase pathway regulated inflammatory responses (Bao et al., 2004) and mediated inflammatory cell infiltration and migration, and production of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β), interleukin-2 (IL-2) and CXC chemokines (Angkachatchai and Finkel, 1999; Thorlacius et al., 2006; Impellizzeri et al., 2012). ROCK inhibitors reduced leukocyte infiltration into the injured spinal cord (Hara et al., 2000), decreased cytokine production (Angkachatchai and Finkel, 1999), and impaired lymphocyte (T cell) proliferation (Tharaux et al., 2003).

Rho pathway plays a role in SCI-induced neuropathic pain as well. Inflammatory mediators are potential candidates for the induction of neuropathic pain. Lysoosphatidic acid, present at the lesion sites both in the peripheral nervous system and the central nervous system, has been shown to initiate neuropathic pain (Inoue et al., 2004). The mechanism of lysophosphatidic acid-induced neuropathic pain is through its binding with G-protein-coupled LPA receptors that activate RhoA/Rho kinase signaling. Blocking RhoA (with Clostridium botulinum C3 transferase) or ROCK (with Y27632) prevented the initiation of neuropathic pain after nerve injury or lysophosphatidic acid injection (Inoue et al., 2004). ROCK inhibitors, Y27632 and H-1152, relieved neuropathic pain in mouse dorsal root injury and spinal nerve transection models (Ramer et al., 2004; Tatsumi et al., 2005) (Figure 1). A recent study found that RhoA/Rho kinase pathway mediates p38 MAPK activation and morphological changes by ATP receptors, P2Y12/13, in spinal microglia in neuropathic pain (Tatsumi et al., 2015).

RhoA/Rho Kinase and Cell Death

After SCI, both neurons and glial cells in and around the lesion area undergo apoptosis induced by the secondary injury. The cell death leads to the formation of a lesion cavity (Liu et al., 1997; Shuman et al., 1997). Although mice do not develop cavitation, apoptotic neurons, astrocytes, and oligodendrocytes are still detected (Dubreuil et al., 2003). Many studies show that RhoA/Rho kinase pathway is highly related to cell death. Inhibition of RhoA, both in mice and rats, can significantly reduce the number of apoptotic cell deaths after SCI. The cells that contain RhoA inhibitor are not apoptotic (Dubreuil et al., 2003). This study also shows that activated RhoA promotes the synthesis of proapoptotic protein such as p75NTR, which contributes to the initiation of apoptotic cascades. Reducing p75NTR decreases apoptosis in a contused spinal cord (Brandoli et al., 2001) and protects neurons and glia cells (Dubreuil et al., 2003). Other studies have shown that active RhoA activates p38α and triggers p38α-dependent excitotoxic neuronal death. RhoA is sufficient to induce excitotoxic cell death (Semenova et al., 2007).

Additionally, a number of studies have shown that Rho kinase is very important in the regulation of cell death (Shi and Wei, 2007). Rho kinase regulates myosin light chain phosphorylation and stimulates actomyosin contractility, which induces apoptotic cell membrane blebbing, nuclear disintegration, and cellular fragmentation (Coleman et al., 2001; Croft et al., 2005). ROCK2 can promote apoptosis by increasing erin phosphorylation, which increases Fas, the death receptor, clustering, and expression (Piazzolla et al., 2005). In addition, Rho kinase stimulates phosphatase and tensin homologue (PTEN) and inhibits insulin receptor substrate 1 (IRS1) signaling to inactivate Akt, which plays an important role in cell survival (Begum et al., 2002; Li et al., 2005) (Figure 1). Lastly, Rho kinase mediates inflammation and reactive oxygen species production to induce cell death (Higashi et al., 2003).

RhoA/Rho Kinase and Axon Degeneration

During SCI secondary injury, growth inhibitory proteins such as myelin-associated molecules and glial scar-associated extracellular matrix molecules converge at the RhoA/ROCK pathway to prevent axon regeneration (Forgione and Fehlings, 2014; Fujita and Yamashita, 2014). To date, three myelin-associated growth inhibitors, i.e., Nogo, myelin-associated glycoprotein, and oligodendrocytemyelin glycoprotein (OMgp) (Mckerracher et al., 1994; Chen et al., 2000; Wang et al., 2002), have been reported to block axonal regeneration. For Nogo, there are at least three isoforms: NogoA, NogoB, and NogoC. NogoA is mainly expressed in the nervous system. Two transmembrane domains of Nogo are separated by a 66 amino acid loop, Nogo-66. Nogo-66 is the inhibitory domain that causes growth cone collapse (Fournier et al., 2001). Myelin-associated glycoprotein is required for the formation and maintenance of myelinin normal condition and is identified as a potent inhibitor of neurite outgrowth (Mckerracher et al., 1994). Myelin-associated glycoprotein inhibits axonal growth in older neurons but promotes axonal growth in young neurons depending on the intracellular level of cyclic AMP (Cai et al., 2001). OMgp is a glycosylphophatidylinositol-anchored glycoprotein. It is expressed in both oligodendrocytes and neurons. Notably, all three myelin-associated inhibitory proteins bind to the same receptor, the Nogo receptor. Nogo receptor associates with neurotrophin receptor p75NTR to form a receptor complex. This complex activates RhoA/Rho kinase pathway. Activation of the RhoA/Rho kinase pathway phosphorylates the myosin light chain, LIM kinase, and collapsing response mediator protein-2 to regulate the cytoskeleton dynamics and growth cone collapse, and to inhibit neurite outgrowth (Ohashi et al., 2000; Fukata et al., 2002; Hsieh et al., 2006) (Figure 1).

Additionally, repulsive guidance molecule (RGM) also acts as an inhibitor of axon growth. Three homologs of RGM, i.e., RGMa, RGMb, and RGMc, have been identified. Among these molecules, RGMa plays a role in inhibiting axon regeneration (Mueller et al., 2006) and its expression is enhanced around the lesion site after SCI. Treatment with neutralizing anti-RGMa antibodies after SCI in rats promotes axonal regeneration and functional recovery (Hata et al., 2005).
al., 2006). RGMa binds with receptor neogenin and activates the RhoA/Rho kinase pathway, leading to neurite outgrowth inhibition (Kubo et al., 2008; Hata et al., 2009).

SCI also triggers a cascade of reactive astrogliosis, which leads to the formation of a glial scar. Reactive astrocytes produce inhibitory extracellular matrix molecule proteoglycans. Chondroitin sulfate proteoglycans are the key component of the glial scar and play important roles in inhibiting axonal regeneration (Yiu and He, 2006). Chondroitin sulfate proteoglycans (CSPGs) bind to a transmembrane protein tyrosine phosphatase (PTPσ) (Shen et al., 2009), leukocyte common antigen-related phosphatase (Fisher et al., 2011), and Nogo receptor 1 and 3 (Dickendesher et al., 2012). These complexes activate the RhoA/Rho kinase signal and, through this pathway, inhibit neurite outgrowth (Dergham et al., 2002; Monnier et al., 2003).

Inhibiting RhoA/Rho Kinase Pathway as a Novel Strategy for SCI Repair

Since the RhoA/Rho kinase pathway is involved in multiple pathophysiological processes and is a convergence pathway for many inhibition proteins that prevent axon regeneration after SCI, pharmacologic inhibition of RhoA or Rho kinase could be a promising strategy to prevent cell death and promote axon regeneration.

For RhoA inhibition, Clostridium botulinum C3 exoenzyme is the prototype of bacterial ADP-ribosyltransferases. C3 selectively modifies RhoA by covalent attachment of an ADP-ribose moiety, which results in inactivation of cellular functions of RhoA (Just et al., 2010). In mouse models of SCI, C3 treatment promoted axonal sprouting, locomotor function recovery and prevented p75NTR dependent cell death after hemisection of the thoracic spinal cord (Dergham et al., 2002; Dubreuil et al., 2003). The next generation of C3 is a cell permeable version which was commercially developed into a clinical grade Rho inhibitor known as BA210 (Trademarked as Cethrin). In rat models of SCI, BA-210 has been shown to penetrate the dura of the spinal cord and cell membrane in a nonspecific, receptor independent manner (Lord-Fontaine et al., 2008).

Based on the promised studies in animal models, BA-210 has been evaluated in a phase I/II clinical trial (Fehlings et al., 2011; McKerracher and Guertin, 2013; Nagoshi et al., 2015). In this study, Cethrin was applied to the injury site intraoperatively with a noninvasive, fibrin-mediated delivery system. Forty-eight patients with cervical or thoracic injury were enrolled in this study. Five different doses of Cethrin (0.3–9 mg) were tested. The results showed that the largest neurological recovery occurred in cervical injury patients, whereas patients with thoracic injuries received modest benefits. In the 3-mg dose of Cethrin, 66% of the cervical injured patients changed their ASIA grade from A to C or D (Fehlings et al., 2011).

Another study showed a C3 protein-derived 29 amino-acid (154–182) peptide also significantly improved locomotor functional recovery, enhanced regeneration of corticospinal tract fibers and raphespinal fibers, and improved serotoninergic input to lumbar alpha-motoneurons (Boato et al., 2010). A recent study has shown that RhoA siRNA was delivered through intraspinal and lumbar intrathecal approaches (Otsuka et al., 2011). The intraspinal delivery improved hindlimb walking over 6 weeks. Although the lumbar intrathecal
delivery did not promote locomotion recovery, it decreased tactile hypersensitivity significantly and improved the white matter sparing. The siRNA approach also decreased the accumulation of ED1+ macrophages, increased PKC-γ immunoreactivity in the corticospinal tract rostral to the injury and, increased serotonergic fiber innervation in the caudal site of injury (Otsuka et al., 2011).

Besides RhoA inhibition, Rho kinase inhibition also shows promise in axonal regeneration and functional recovery. Inhibitors of Rho kinase such as Y27632 and Fasudil have been tested on rat or mouse models of SCI. High doses or locally applied Y27632 enhanced the sprouting of corticospinal tract fibers and locomotor function recovery (Fournier et al., 2003; Tanaka et al., 2004; Chan et al., 2005). However, with oral delivery, Y27632 showed no effect (Sung et al., 2003). Immediate treatment with Fasudil resulted in increased sprouting and improved locomotor scores, whereas delayed treatment at 4 weeks post-SCI was not effective (Nishio et al., 2006).

Watzlawick et al. (2014) conducted a systematic review and meta-analysis on RhoA/Rho kinase blocking-related reference to analyze the impact of bias and determine the normalized effect size of functional locomotor recovery after experimental thoracic SCI (Watzlawick et al., 2014). Thirty studies (725 animals) examined the effect of RhoA or Rho kinase inhibition on spinal cord injuries including hemisection, contusion, and transection. Locomotor recovery was measured using the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale or the Basso Mouse Scale. According to the published work, RhoA/Rho kinase inhibition improved locomotor outcome by 21%. In this study, eight different strategies were used to target the RhoA/Rho kinase pathway including RhoA-GTPase inhibitors (BA-210), C3-peptides, C3-ADP-ribosyltransferase, siRNA, ibuprofen, and ROCK inhibitors (fasudil, Y27632 and p21). Additionally, different routes of drug administration were employed such as intra-thecal injection, and topical, intraperitoneal, and oral application. The time of drug administration ranged from 30 minutes before the injury up to 4 weeks after SCI. All these elements may affect the variation of outcome assessments. Finally, different animal species and injury models may also affect the outcomes.

Summary

The RhoA/Rho kinase pathway has been shown to play a unique role in the pathogenesis of SCI. Numerous studies have shown that blocking RhoA/Rho kinase pathway protects cell survival and enhances axonal regeneration leading to functional recovery after SCI. Thus, this pathway is a promising target for SCI treatment in patients. Continued research should be conducted to determine the delivery methods, the dose, and the treatment time window for reaching optimal outcomes.

References


