Experimental Neurology 231 (2011) 247–260

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

RhoA-inhibiting NSAIDs promote axonal myelination after spinal cord injury

Bin Xing, Hui Li, Hongyu Wang, Dhriti Mukhopadhyay, Daniel Fisher, Christopher J. Gilpin, Shuxin Li *

Department of Neurology and Neuroscience Graduate Program, University of Texas Southwestern Medical Center, Dallas, TX, 75390-8813, USA

ARTICLE INFO

Article history:
Received 28 January 2011
Revised 7 June 2011
Accepted 30 June 2011
Available online 14 July 2011

Keywords:
Spinal cord injury
Axon injury
Ibuprofen
NSAIDs
RhoA
Apoptosis
Myelination

ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) are extensively used to relieve pain and inflammation in humans via cyclooxygenase inhibition. Our recent research suggests that certain NSAIDs including ibuprofen suppress intracellular RhoA signal and improve significant axonal growth and functional recovery following axonal injury in the CNS. Several NSAIDs have been shown to reduce generation of amyloid-beta42 peptide via inactivation of RhoA signal, supporting potent RhoA-repressing function of selected NSAIDs. In this report, we demonstrate that RhoA-inhibiting NSAIDs ibuprofen and indomethacin dramatically reduce cell death of oligodendrocytes in cultures or along the white matter tracts in rats with a spinal cord injury. More importantly, we demonstrate that treatments with the RhoA-inhibiting NSAIDs significantly increase axonal myelination along the white matter tracts following a traumatic contusion spinal cord injury. In contrast, non-RhoA-inhibiting NSAID naproxen does not have such an effect. Thus, our results suggest that RhoA inactivation with certain NSAIDs benefits recovery of injured CNS axons not only by promoting axonal elongation, but by enhancing glial survival and axonal myelination along the disrupted axonal tracts. This study, together with previous reports, supports that RhoA signal is an important therapeutic target for promoting recovery of injured CNS and that RhoA-inhibiting NSAIDs provide great therapeutic potential for CNS axonal injuries in adult mammals.

Introduction

Axon disconnection in the central nervous system (CNS) usually results in signal conduction failure and persistent functional deficits in many patients, such as white matter stroke, progressive multiple sclerosis, traumatic brain injury as well as spinal cord injury (SCI). Medical treatments for recovering signal conduction along the injured axons are currently not available due to complexity of injury mechanisms and neuronal growth failure. Although a number of compounds are in clinical trials, the only approved drug for SCI is methylprednisolone, an acute neuroprotective therapy administered only shortly after trauma (Bracken et al., 1997). Following CNS injuries, activation of RhoA is a convergent intracellular pathway for numerous extracellular molecules that restrict axonal growth, including CNS myelin, glial scar source of chondroitin sulfate proteoglycans and possibly a few axonal guidance cues during development (Huber et al., 2003; McGee and Strittmatter, 2003). RhoA signal activation leads to growth cone collapse and neurite growth inhibition in cultured primary neurons (Kozma et al., 1997; Kranenburg et al., 1999). Inactivation of Rho with C3 transferase or its downstream with Y-27632 (Fournier et al., 2003) promotes neurite outgrowth on myelin or glial scar inhibitory substrates. Accordingly, Rho signal suppression has been shown to be a feasible approach to promote axonal regeneration in rodents with transection and contusion SCI or optic nerve crush injury (Bertrand et al., 2005; Dergham et al., 2002; Dubreuil et al., 2003; Fischer et al., 2004; Fournier et al., 2003; Hara et al., 2000; Nishio et al., 2006; Sung et al., 2003). Thus, Rho signaling pathway is an important therapeutic target for promoting functional recovery in adult mammals with CNS axonal injuries (Dergham et al., 2002; Mueller et al., 2005).

Recently, we have reported that NSAID ibuprofen and indomethacin overcome neuronal growth suppression from various axonal growth inhibitors via potently repressing activity of intracellular RhoA signal (Fu et al., 2007). Especially, we have demonstrated that RhoA silence with ibuprofen is able to stimulate significant axonal regrowth of descending fiber tracts in the distal spinal cord following a dorsal transection or contusion trauma (Fu et al., 2007). In addition, ibuprofen promotes remarkable locomotor functional recovery in SCI rodents, even when applied 1 week after injury. Our findings have been independently replicated in other spinal cord injury models (Wang et al., 2009b). Given the axon growth-promoting effect of ibuprofen and frequent use of this drug in humans, it may become an effective treatment for clinical conditions characterized by white matter damages. Most

Abbreviations: DS, dorsal; EthD1, ethidium homodimer-1; GM, gray matter; Ibu, ibuprofen; Ind, indomethacin; LFB, Luxol fast blue; LT, lateral; MBP, myelin basic protein; O-2A, oligodendrocyte-type 2-astrocyte; PPARγ, peroxisome proliferator-activated receptor γ; Nap, naproxen; NSAIDs, nonsteroidal anti-inflammatory drugs; SCI, spinal cord injury; TNF-α, tumor necrosis factor α; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling; WM, white matter; VT, ventral.

* Corresponding author at: Department of Neurology and Neuroscience Program, UT Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd, Dallas, TX, 75390-8813, USA. Fax: +1 214 645 6238.
E-mail address: shuxin.li@utsouthwestern.edu (S. Li).

0014-4886/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2011.06.018

recently, our further research work has illustrated that the transcription factor peroxisome proliferator-activated receptor γ (PPARγ) is essential for coupling ibuprofen to RhoA inhibition and subsequent promotion of neurite growth in neurons (Dill et al., 2010), suggesting that PPARγ may be an additional therapeutic target for the disorders characterized by RhoA activation.

On the other hand, a number of glial cells including oligodendrocytes (OLGs) along the white matter tracts undergo apoptosis, and OLG loss appears to be responsible for demyelination and axonal dysfunction following CNS axonal injuries (Jensen et al., 1999; Tamura et al., 2005; Warden et al., 2001). Several extracellular molecules contribute to the post-injury apoptosis (Beattie et al., 2002; Demjen et al., 2004; Kim et al., 2001; Sanchez-Gomez et al., 2003) and intracellular activation of RhoA appears to be important for apoptotic glial cell death following SCI because RhoA inactivation with membrane-permeable C3 transferase reverses apoptotic cell loss (Dubreuil et al., 2003). Thus, it is very likely that the RhoA-inhibiting NSAIDs improve functional recovery following CNS injuries not only by stimulating axonal regrowth, but also by preventing glial cell loss, consequently reducing demyelination and improving remyelination of spared and/or regenerating CNS axons. In this study, we report that ibuprofen and indomethacin applied at the doses to inhibit RhoA activity dramatically reduced OLG cell death in cultures or along the white matter tracts in SCI rodents. Furthermore, we demonstrate that treatments with RhoA-inhibiting NSAIDs significantly increase myelination of CNS axons following a contusion SCI in rats. Together with previous reports, the present study supports that RhoA signal is an important therapeutic target for promoting functional recovery in the injured CNS and that RhoA-inhibiting NSAIDs provide great therapeutic potential for axonal injuries in adult mammals.

Materials and methods

CG4 cell cultures and treatments

CG4 cell line was cultured on plastic coverslips coated with poly-L-lysine in 24-well plates in DMEM containing 10% fetal bovine serum and N2 supplements. After CG4 cells became confluent (usually 2 days after growth), they were cultured in serum-free medium containing N1 (including insulin) and N2 supplements for 3 days to stimulate cell maturation. CG4 cells develop many processes during maturation 2 days after growth in the second culture medium and the majority of CG4 cells are positive for galactocerebroside C (GalC) staining (Nicholas et al., 2002). Three days after differentiation in the second medium, the differentiated cells were incubated with vehicle (Veh) saline, naproxen (Nap, 400 μM), ibuprofen (Ibu, 400 μM), indomethacin (Ind, 20 μM) or Rho inhibitor C3 transferase (4 μg/ml) for 1 h. Then, cells were stimulated with tumor necrosis factor α (TNF-α, 1.2 μg/ml) for 8 h in serum-free medium to induce cell death in the presence of above drugs. Eight hours following TNF-α incubation, the survival and dead cells on coverslips were stained with fluorescent dyes calcein and ethidium homodimer-1 (EthD1), respectively, via incubation for 30 min in the medium. Cells were fixed with 4% formalin before quantification. The ratio of the dead cells to the total cells was quantified from multiple coverslips in each group. A total of 7268–10,782 cells were counted in each group. In selected experiments, differentiated CG4 cells were treated with drugs for 9 h in the absence of TNF-α.

Animals and contusion spinal cord lesion

In SCI model, female Sprague–Dawley rats (weight 180–250 g) were deeply anesthetized with ketamine (70 mg/kg) and xylazine (8 mg/kg). For the contusion SCI, a laminectomy was performed at T7-8 and a moderate lesion was made at T8 with the NYU Impactor as we reported previously (Dill et al., 2008; Fu et al., 2007). We performed 4 batches of rat experiments with moderate contusion SCI. 1) A total of 34 rats were used for the apoptotic cell analysis in the SCI rats 5 days following the lesion (Figs. 2 and 3), 8 in each of the vehicle, Nap and Ibu groups, and 10 in the Ind group. The drug delivery was started 1 h after the injury until 5 days post-trauma. 2) To confirm the role of Ibu in reducing apoptosis, we performed the second set of SCI rats and analyzed cell apoptosis 7 days after a contusion SCI: 11 and 9 rats in the vehicle and Ibu groups, respectively (Fig. 4). In these 20 rats, vehicle or drugs were initiated 1 h after the injury and terminated 7 days after trauma. 3) For myelination studies at light microscopic level (Figs. 5–6), a total of 34 rats were employed following a moderate contusion injury, 8 in each of the vehicle and Ind groups, 9 in each of the Nap and Ibu groups. These rats started receiving vehicle or drug treatments 1 h after injury, but the delivery persisted for 28 days post-trauma. These SCI rats were perfused for myelination histology 6 weeks after SCI. 4) Twenty SCI rats were used for myelin ultrastructural and Western blotting assays (Figs. 7 and 8), 5 in each of vehicle, Nap, Ibu and Ind groups. These SCI rats received the same drug treatments as in batch 3 and were perfused for histological and biochemical assays 4 weeks after injury. In all those rat experiments, drugs were delivered via daily subcutaneous injections with syringes at the following doses: Nap: 40 mg/kg/day; Ibu: 60 mg/kg/day; Ind: 4 mg/kg/day. The vehicle-treated animals received the same volume of saline. After SCI, a few rats died during the recovery due to general poor condition, and were consequently excluded from the study. In addition, a few rats without SCI were used as uninjured controls for spinal cord histology or Western blot.

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL) staining and immunohistochemistry for OLGs in the spinal cord

Spinal cord samples at the indicated locations rostral to and caudal to the lesion were transversely cut (40 μm) for apoptotic cell staining via TUNEL method. Following dehydration and rehydration in a gradient of ethanol and xylene, TUNEL labeling was performed to detect DNA fragmentation in the apoptotic cells via a diaminobenzidine-based color reaction or a fluorescent dye using ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore, S7101). The sections were co-stained with Hoechst 33342 to label cell nuclei (Sigma-Aldrich). For the quantification of apoptotic cell number, TUNEL-positive cells were counted from multiple sections at a given level in each animal in a blind manner. To co-localize apoptotic cells to OLG cells, we performed double staining for active caspase 3 and OLG marker CC1 (adenomatous polyposis coli 7, Calbiochem, La Jolla, CA) in selected transverse or parasagittal sections of the spinal cord.

To verify that RhoA inhibition with certain NSAIDs increases numbers of OLGs around the lesioned spinal cord, in some transverse sections rostral to or caudal to the lesion, we immunostained OLGs with an antibody against CC1. The CC1 staining signals were visualized via an Alexa488 anti-mouse secondary antibody. The CC1-labeled individual OLGs in the dorsal, lateral and ventral white matter areas of the spinal cord were counted manually from five random transverse sections at a given level in each rat.

Histology for myelination in the spinal cord

Following animal perfusion with 4% paraformaldehyde, the spinal cords containing the lesion area were dissected out. The spinal cord blocks were transversely cut (10 μm) at the indicated levels rostral to or caudal to the lesion. Myelination of the spinal cord was evaluated via Luxol fast blue (LFB) staining and immunostaining for myelin basic protein. For LFB staining, spinal cord sections were dehydrated in a gradient of ethanol and stained in 0.1% solvent blue 38 (Sigma) in acidified 95% ethanol overnight at 60 °C. After rinsing with 95% ethanol and distilled water, sections were then differentiated with 0.05% Li_2CO_3 and 35% ethanol several times until the contrast between