Published in final edited form as:

J Neuroimmune Pharmacol. 2013 March ; 8(1): 274–286. doi:10.1007/s11481-012-9419-3.

Simvastatin Ameliorates Cauda Equina Compression Injury in a Rat Model of Lumbar Spinal Stenosis

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Abstract

Lumbar spinal stenosis (LSS) is the leading cause of morbidity and mortality worldwide. LSS pathology is associated with secondary injury caused by inflammation, oxidative damage and cell death. Apart from laminectomy, pharmacological therapy targeting secondary injury is limited. Statins are FDA-approved cholesterol-lowering drug. They also show pleiotropic anti-inflammatory, antioxidant and neuroprotective effects. To investigate the therapeutic efficacy of simvastatin in restoring normal locomotor function after cauda equina compression (CEC) in a rat model of LSS, CEC injury was induced in rats by implanting silicone gels into the epidural spaces of L4 and L6. Experimental group was treated with simvastatin (5 mg/kg body weight), while the injured (vehicle) and sham operated (sham) groups received vehicle solution. Locomotor function in terms of latency on rotarod was measured for 49 days and the threshold of pain was determined for 14 days. Rats were sacrificed on day 3 and 14 and the spinal cord and cauda equina fibers were extracted and studied by histology, immunofluorescence, electron microscopy (EM) and TUNEL assay. Simvastatin aided locomotor functional recovery and enhanced the threshold of pain after the CEC. Cellular Infiltration and demyelination decreased in the spinal cord from the simvastatin group. EM revealed enhanced myelination of cauda equina in the simvastatin group. TUNEL assay showed significantly decreased number of apoptotic neurons in spinal cord from the simvastatin group compared to the vehicle group. Simvastatin hastens the locomotor functional recovery and reduces pain after CEC. These outcomes are mediated through the neuroprotective and anti-inflammatory properties of simvastatin. The data indicate that simvastatin may be a promising drug candidate for LSS treatment in humans.

Keywords

Spinal stenosis; Simvastatin; Cauda equina; Demyelination; g-ratio

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Conflict of Interest The authors declare no conflict of interest.

Introduction

Lumbar spinal stenosis (LSS), caused by the narrowing of the spinal canal, is a major clinical problem associated with intermittent claudication or pseudoclaudication. It continues to remain as one of the leading causes of morbidity and mortality worldwide. Five out of 1,000 Americans older than 50 years experience chronic and debilitating LSS (Wong et al. 2012). The available treatment options for LSS include surgical decompression by wide laminectomy. Surgical decompression of lumbar stenosis is the most common surgery for patients older than 65 years of age with an optimum success rate of 66 to 83 % (Gibson and Waddell 2005). Despite the higher rate of success, laminectomy is associated with limitations (Wong et al. 2012). Decompressive laminectomy disrupts the native anatomic support structures like supraspinous ligament, interspinous ligament etc. leading to muscular atrophy (Bresnahan et al. 2010; Yagi et al. 2009). In addition, the mean cost per quality adjusted life year after spinal procedures for degenerative spine conditions is \$ 115,600 (Cahill and Wang 2012). Hence, there exists a compelling need to search for newer therapeutic modalities to treat LSS.

Tissue degeneration after LSS is due to the combined effect of various factors such as physical trauma, ischemia, hemorrhage and pain (Schwab et al. 2006; Schwab and Bartholdi 1996; Koyanagi et al. 1993). LSS involves both primary and secondary injury mechanisms. Secondary de-generation following LSS disrupts the blood spinal cord barrier (BSCB), resulting in an immune cell infiltration and inflammatory insult to the spinal cord. Inflammatory mediators including cytokines, cause neuronal cell death leading to loss of neurological functional (Betha 2000; Hausmann 2003). However, unlike primary injury, secondary injury is preventable (Tator and Fehlings 1991). Therefore, reducing the inflammatory insult by therapeutic drugs is one of the nonsurgical major strategies of neuroprotection and improvement of motor functions after LSS.

Statins are a group of specific inhibitors of HMG-CoA reductase, an enzyme that catalyzes the conversion of HMG-CoA to mevalonate in cholesterol biosynthesis pathway. Statins have established clinical benefits of lowering cholesterol levels (Igel et al. 2002). Hypercholesterolemia and cardiovascular morbidity are routinely treated with statins (Dembowski and Davidson 2009). In addition to cholesterol biosynthesis inhibition, studies attribute immunomodulatory effects to statins (Pahan et al. 1997; Kwak et al. 2000; Weber et al. 2006; Markovic-Plese et al. 2008). In the context of neurodegenerative disorders, the studies with primary cultures of astrocytes, microglia and macrophages from our laboratory demonstrate the anti-inflammatory activity of statins (Pahan et al. 1997). Our studies also show the anti-inflammatory property of statins to ameliorate motor functional loss (Pannu et al. 2005; Pannu et al. 2007) and bladder dysfunction (Shunmugavel et al. 2010) following SCI in rats. In addition, studies with animal models of neuroinflammatory diseases like experimental autoimmune encephalomyelitis (EAE) also support the neuroprotective effect of statins (Paintlia et al. 2009). The findings related with neuroprotective property of statins from our laboratory are well corroborated by the reports from other laboratories (Cimino et al. 2005; Carloni et al. 2009). Statins also improve neurobehavioral functions after traumatic brain injury (TBI) (Qu et al. 2005; Chen et al. 2009). Recently, statins have also been reported to have anti-nociceptive property (Chu et al. 2012; Miranda et al. 2011; Mascitelli et al. 2011). Taken together, these studies justify the use of statins as potential drug to treat LSS related neuropathic pain and locomotor dysfunction.

In this study, we investigated the therapeutic potential of simvastatin in a rat model of LSS. Simvastatin treatment of LSS reduced inflammation and pain and ameliorated the locomotor dysfunction in rats following LSS, through its anti-inflammatory, anti-nociceptive and neuroprotective properties.

Materials and methods

Animals, experimental design and drug

Female Sprague–Dawley rats (weighing 225–250 g), purchased from Harlan laboratories (Durham, NC) and housed in the animal facility at 12 h dark and light cycle, were used. A total of 93 animals were used in the present study. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Medical University of South Carolina. Animals were acclimatized for 1 week before experiments begin. Rats were randomly assigned to three groups; group 1 (Simvastatin) received 5 mg/kg body wt. simvastatin suspended in 0.5 % methyl cellulose solution at 2 h after injury by gavage. The same treatment was continued once/every 24 h until the endpoint. The other two groups (Sham and Vehicle) received the equal volume of vehicle (0.5 % methyl cellulose solution).

Cauda equina compression (CEC) model

CEC rat model of LSS was accomplished as previously described (Shunmugavel et al. 2012b) by following the method of Takenobu et al. (2001). Briefly, animals were anesthetized with ketamine-xylazine cocktail (80 and 10 mg/kg body weight, respectively). After confirming the validity of anesthesia by toe pinching, the animals were depilated on dorsal spine line and the spine was exposed at L3–S2 level. Two appropriate holes were made to expose the dura overlying the spinal cord. Care was taken not to lose cerebrospinal fluid. Two pieces of silicone blocks (4×1×1 mm) (Bentec Medical Inc, Woodland, CA) were placed into the lumbar, L4 and L6 epidural space. The wound was irrigated with phosphate buffered saline (PBS) solution, and the incision was closed in layers, with the skin closed using polysorb 4. Sham operated animals underwent the same procedure except the silicone block implantation. Animals were returned to cages and kept on a 37 °C heating blanket overnight. The injured animals did not show any paralysis and bladder associated problems.

Longitudinal MRI scans

CEC animals from both, the vehicle and the simvastatin groups were scanned post injury using a 7T Bruker Biospec USR system (Bruker Inc, Ettlingen, Germany) equipped with 500 mT/m (rise time 80–120 μ s) gradient set (for performing small animal imaging) and a small bore linear RF coil (ID 119 mm for rat). Inductively-coupled surface coil was used for the localized imaging of the silicone blocks. The rats were anesthetized with 5 % isoflurane in an induction chamber. The animals were kept under 2 % isoflurane delivered in a mixture of 40 % oxygen and 58 % air through a nose mask. The L 4–6 portion of the spinal cord was positioned over the inner loop of the surface coil which was inductively coupled to the outer loop. The coil system mounted with the animal was connected to the scanner's preamplifier and placed into the magnet. Anatomical T2 weighted -2D RARE (Rapid Acquisition with Relaxation Enhancement), TR/TE 4000/65 ms. Geometry: FOV 3.2×3.2 cm and digitized into a matrix of 256×128, slice thickness 2 mm, slice gap 0.2 mm. Vital signs of the anesthetized animals were monitored using a MRI compatible monitoring and gating system (Model 1025, SA Instruments Inc, Stony Bork, NY).

Rotarod test

Latency of rats on rotarod was measured as described previously from our laboratory (Khan et al. 2009). A total of 24 animals under three groups were used for rotarod test. Two groups each consisting 8 animals underwent CEC surgery. After surgery, one group (Vehicle) was fed with 0.5 % methyl cellulose solution and the other with simvastatin (5 mg/kg body wt.) suspended in methyl cellulose solution. Sham group received methyl cellulose solution.

Briefly, animals were pre-trained on an automated 4-lane rotarod unit supplied by Columbus Instruments (Columbus, OH). Pretreatment lasted for 3 days before the actual experimental measurement. All animals were tested during the light cycle at the same time of the day (9:00–12:00 am). Total time in seconds that the animal could stay on the rotarod drum was recorded. Each animal was given three trials and the mean latency of three trials was taken for final calculation.

Mechanical hyperalgesia

The development of neuropathic pain symptoms in the hind paw was evaluated by using analgesy meter (AM) and dynamic plantar aesthesiometer (DPA) as previously described (Shunmugavel et al. 2012b). Analgesy meter (Ugo Basile, Camerio, Italy) was used to measure the development of hyperalgesia after CEC in rats following the procedures previously described (Walker et al. 2003). Experimental animals underwent simulation measurements for 3 days without any actual force applied to acclimatize to the experimental conditions. Hind paw withdrawal threshold to an increasing pressure stimulus was recorded. Briefly, the animal's paw is placed on a small plinth under a cone-shaped pusher with a rounded tip. Using a foot pedal, gradually increasing force was applied to the dorsal side of the paw. When the animal withdraws the paw or struggles, the force application is stopped and the reading on the scale at which the animal felt pain was recorded. Three experimental groups each consisting of six animals were used for AM studies. Measurements were made on the dorsal side of both the hind paws. Three measurements were made on each paw with inter experimental interval of 5 min. Habituation effect was avoided by testing on different spots on the paw. DPA (Ugo Basile, Camerio, Italy) is a modified computer version of the von Frey test used to assess low-threshold mechanical pain threshold prior to CEC and again at different times thereafter. Testing was blinded in regard to experimental groups. The test was performed as previously described (Shunmugavel et al. 2012b). Animals were acclimatized to the DPA for about 15 min before recording. In addition, animals were acclimated to the testing area for 3 days before the actual experiment. Animals were placed individually in the testing area with a wire mesh floor. The instrument raised the filament through the wire mesh to touch the foot and progressively increased the force until it reached a 20 g force. The foot withdrawal/latency was recorded using the software provided by the manufacturer. A total of 18 animals under three experimental groups each consisting of 6 were used for DPA studies. Pain threshold was measured at three different points on each paw with an interval of 5 min between the measurements. Measurement on the same spot was avoided to negate habituation effect.

Histology

Three groups each comprising eight animals were used for histological studies. Two groups after CEC surgery were fed with vehicle and simvastatin respectively. Sham group was also fed with vehicle. Three animals from each group were sacrificed on day 3 and the rest were on day 14 after CEC and drug treatment. Animals were sacrificed at endpoints with an overdose of nembutal (150 mg/kg body weight) and perfused transcardially with saline followed by 4 % paraformaldehyde (PFA) in PBS, pH 7.4. Spinal cord and the cauda equina fibers were extracted as described (Shunmugavel et al. 2012b) and the tissues were post-fixed overnight in PFA. After fixation, the tissues were processed for histological procedures. Tissues were dehydrated in a series of alcohol and infiltrated with paraffin wax (M.P. 60 °C) using Leica TP-1020 automatic tissue processor. Tissue blocks were sectioned (8 µm) with Leica HM-325 rotary microtome. Sections were adhered on to super frost plus gold slides (Fisher Scientific Inc, MA). After suitable drying time, the sections were rehydrated by passing through decreasing grades of ethanol (100, 95, 60, 70 and 30 %) and water. Sections for morphological studies were processed and stained with luxol fast blue (LFB) and hematoxylin and eosin (H&E) as previously described (Lassmann and

Wisniewski 1979; Kiernan 1990). Percentage intensity of LFB staining was measured in sections from five animals from each experimental group using image pro Plus 5.1 software.

Immunohistochemical analysis

Following deparafinization, rehydration and antigen unmasking the tissue sections were blocked in Tris-sodium buffer with 0.5 % blocking reagent (TNB, supplied with TSA-Direct kit; NEN Life Sciences, Boston MA) for 30 min as described previously (Shunmugavel et al. 2012a). Thesections were probed with anti activated microglia (CD68), cytotoxic T cell (CD8: Santa Cruz Biotechnology Inc. CA), glial fibrillary acidic protein (GFAP) and tumor necrosis factor alpha (TNF- α) antibodies. All the primary antibodies were treated at 1:100 dilutions. Detection was performed using Alexafluor conjugated secondary antibodies (Molecular probes, Life Technologies, NY). Secondary antibodies were treated at 1:100 dilutions. Sections were mounted in aqueous based mounting medium (EMS, Fort Washington, PA) and visualized under epi-fluorescence microscope (Olympus, Center valley, PA) using image pro Plus 5.1 software. CD68, CD8 and TNF- α positive cells were counted in at least ten microscopic frames of the dorsal regions of the sections and in equivalent areas of other sections from three animals in an experimental group. GFAP positive staining area was also calculated in the same way using image pro Plus 5.1 software. Independent scoring was also performed by an investigator blinded to experimental groups.

Transmission electron microscopy

Cauda equina from sham, vehicle and simvastatin treated animals were extracted 7 weeks after the induction of CEC. Each group consisted of three animals. Animals were sacrificed with excess nembutal and transcardially perfused with saline followed by 0.1 M sodium cacodylate buffer. Tissues for EM were processed as previously described (Shunmugavel et al. 2012b). Tissues extracted were fixed in 2.5 % glutaraldehyde fixative/0.1 M sodium cacodylate buffer for 1 h and then rinsed in 0.1 M sodium cacodylate buffer three times 10 min each. Tissue blocks were then post fixed in 1 % osmium tetroxide/0.1 M sodium cacodylate buffer for 1 h, thoroughly washed in distilled water three times 5 min each and then dehydrated with acetone. Tissues were embedded in 1:1 acetone: resin for 1h followed by 100 % resin for 1 h and then 100 % resin overnight. The resin was changed 1 h before placing into molds for polymerization in a 60 °C oven overnight. Thin sections of 0.07 μ m were cut and picked up on copper grids for transmission electron microscopy. Specimens were examined under a JEOL 1010 electron microscope. For morphological analysis, acquired images were analyzed for axon inner and outer diameters by measuring the maximum and the minimum diameter and calculating the average as previously described (Brosamle and Schwab 2000). g-ratio of axons was calculated as previously described (Shunmugavel et al. 2012b).

Fluorescent TUNEL assay

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay was performed using ApopTag fluorescein in situ apoptosis detection kit according to the manufacturer (EMD Millipore, Billerica, MA). Basically, the DNA strand breaks are detected by labeling the free terminus with modified nucleotides. Subsequent visualization by light microscopy identifies apoptotic cells stained positive with ApopTag kit. Briefly, the paraffin sections were deparaffinized, rehydrated and treated with proteinase K. After treating with equilibration buffer the sections were incubated with TdT enzyme. After an hour, the reaction was stopped by applying stop buffer followed by the addition of anti-dioxigenine conjugate. The sections were counterstained with DAPI and cover slipped.

For double labeling, spinal cord sections processed for TUNEL staining were further probed with antineuronal nuclei antibody (NeuN; 1:100; Millipore, Billerica, MA). Detection was performed using Alexafluor conjugated secondary antibody (Molecular probes, Life Technologies, NY). Secondary antibody was treated at 1:100 dilution. The sections were visualized by epifluorescence microscopy. TUNEL/NeuN double positive cells were counted in at least five microscopic frames of the sections and in equivalent areas of sections from all the experimental groups.

Statistical evaluation

Statistical analysis was performed using Graph pad prism 3.0 software. Repeated measures of ANOVA were used to compare between groups. Statistical significance was determined using Students' t-test. Data were expressed as mean $\pm$ SD of n determinations. p value less than 0.05 was considered statistically significant.

Results

Silicone-block induced cauda equina compression (CEC)

As described in the materials and methods section, silicone blocks were inserted into the epidural space between L4–L5 and L5–L6 vertebrae. The proper positioning and alignment of the silicone blocks after surgery were determined by MRI and the representative image is given in Fig. 1. This model used in the present study showed consistent locomotor deficit as described by Watanabe et al. (2007). Animals showing deeper or tilted placement of silicone blocks as observed with MR imaging were excluded from the study.

Simvastatin improved locomotor recovery

Motor function after the CEC was measured in sham, vehicle and simvastatin groups in terms of latency on rotarod (Fig. 2). All the animals were pre-trained on the rotarod for 5 days before the experiment and they were able to walk on rotarod for 280 ± 8 s on the day before surgery. Animals in each group were tested on rotarod once in a week until 7 weeks after the surgery. Sham operated animals did not show any change in the locomotor behavior on any day tested (data not shown). Vehicle group showed severe motor deficit following the CEC. On day 2 post injury, the group had a latency of 44.3 ± 8.5 s. With the progression of natural healing, the walking efficiency increased to 120.6 ± 35.3 s on 49th day. However, the latency was significantly ($p < 0.001$) lower than the simvastatin group on all the days tested. The simvastatin group showed significant difference in latency compared to the vehicle group from 7th day to the 49th day of the study. Repeated measures ANOVA revealed an interaction effect of time and treatment ($p = 0.0004$) and treatment and time effects ($p < 0.0001$). Comparisons at each time point revealed simvastatin-treated CEC rats recovered significantly faster than the vehicle group starting on day 7 throughout the rest of the experimental period.

Simvastatin decreased CEC-induced hyperalgesia

The CEC resulted in a significant development of mechanical hyperalgesia indicated by the decrease in hind paw withdrawal threshold after CEC. Mechanical hyperalgesia was determined by AM and DPA. Basal pain threshold measured with AM was about 106 g in the present study. Pain threshold of vehicle group after the CEC on day 2, 4 and 6 were 79.79 ± 2.45 , 81.94 ± 2.50 and 88.06 ± 2.73 g respectively. Simvastatin group had pain threshold of 88.33 ± 1.43 , 89.17 ± 2.17 and 95.28 ± 2.34 g respectively on day 2, 4 and 6 after the CEC (Fig. 3a). In the present study, the rats showed a baseline pain threshold of about 18 s measured with DPA. Vehicle group of CEC rats developed hyperalgesia and had pain threshold of 9.34 ± 1.50 , 9.23 ± 0.95 and 12.08 ± 0.63 s on day 2, 4 and 6 after CEC. The

respective pain threshold of simvastatin group were 12.64 ± 1.41 , 13.99 ± 1.33 and 14.49 ± 2.169 s (Fig. 3b). In both the type of testing, the vehicle group developed significant ($p < 0.001$) hyperalgesia following the CEC. The hypersensitivity to pain stimuli persisted up to 6 days after CEC. We measured the pain sensitivity up to 14 days after the CEC. We did not observe significant difference in pain threshold between the groups after day 6. However, there was a significant ($p < 0.05$) difference in the pain threshold between vehicle and simvastatin groups during the first 6 days after CEC. The data obtained with AM and DPA in the present study demonstrate the antinociceptive property of simvastatin in a rat CEC model.

Simvastatin decreased immune cell infiltration

Representative images of H&E staining of sections of spinal cord of various experimental groups are given in Fig. 4a. Infiltration of cells was predominant in the vehicle group (Fig. 4a arrow). Simvastatin treatment significantly reduced the cellular infiltration. Spinal cord from sham rats showed no cellular infiltration. In order to determine the type of infiltrated immune cell, the spinal cord sections were stained with anti CD68 (activated microglia) and CD 8 (cytotoxic T Cell) antibodies (Fig. 4b). The number of CD68 and CD8 positive cells were counted as mentioned in the material method section and the corresponding histograms are given in Fig. 4c. Spinal cord of sham group did not show CD68 and CD8 positive cells. Vehicle group had significantly ($p < 0.001$) higher number (CD68: 160.44 ± 25.81 ; CD8: 86.64 ± 12.62) of immune cell infiltration compared to both sham and simvastatin groups on day 14 after the CEC. Simvastatin treatment significantly decreased the immune cell infiltration when compared to the vehicle group.

Simvastatin protected myelin in the spinal cord and the cauda equine fibers

The level of myelin in the spinal cord was measured by LFB staining. The representative image of LFB staining and the percentage intensity of LFB measured using image pro plus 5.1 software are given in Fig. 5a&c. Sham group had 57.83 ± 5.325 % of LFB intensity. Vehicle group showed significantly ($p < 0.001$) decreased level (29.88 ± 6.73 %) of LFB. Simvastatin treatment significantly ($p < 0.001$) increased the level of LFB intensity (51.07 ± 6.24 %) when compared to vehicle group, however there was a significant ($p < 0.05$) difference in the level of LFB intensity between the sham and simvastatin groups on day 14 after CEC. The results clearly show that simvastatin treatment protected the spinal cord from the CEC-induced demyelination. The myelin integrity and g-ratio of cauda equina of different experimental groups were also determined in the present study using EM (Fig. 5b&d). Myelin sheath in the vehicle group was less compact and highly degenerated (Fig. 5b arrows) compared to the sham group. Simvastatin group had compact myelin comparable to sham group. The g-ratio of axons of sham, vehicle and simvastatin groups determined in the present study were 0.67 ± 0.02 , 0.70 ± 0.04 and 0.69 ± 0.02 respectively. The corresponding histogram is given in Fig. 5d. Vehicle group had significantly ($p < 0.05$) higher g-ratio when compared to both sham and simvastatin groups. The results show that simvastatin is also neuroprotective and preserves myelin integrity in CEC rats.

Simvastatin decreased the expression of activated astroglia and TNF α

Activated astroglia and cellular expression level of TNF α were determined in the spinal cord of sham, vehicle and simvastatin group, on day 3 and 14 after the CEC. The representative images and corresponding histograms are given in Fig. 6. Increased expression of activated astrocyte and TNF α was seen in the spinal cord of vehicle group on both the day tested showing CEC induced astrogliosis (Fig. 6a & b). The activated astrogliosis was determined based on the area of tissue section positive to GFAP staining. Vehicle group showed significantly ($p < 0.001$) increased level of activated astrocyte (Day 3: 23.24 ± 3.99 ; day 14: 25.16 ± 3.97 %) compared to sham (Day 3: 5.47 ± 2.67 ; day 14: 4.14 ± 2.3 %).

%) and simvastatin groups (Day 3: 13.41 ± 1.87 ; day 14: 7.86 ± 1.41 %) (Fig. 6b). The CEC induced an increase in the number of TNF α positive cells in the spinal cord of rats (Fig. 6a & c). Number of TNF α positive cells were significantly ($p < 0.001$) higher in vehicle group (Day 3: 253.25 ± 44.30 ; day 14: 133.00 ± 22.21) compared to sham and simvastatin (Day 3: 44.50 ± 5.8 ; Day 14: 24.44 ± 9.37) groups (Fig. 6c).

Simvastatin reduced neuronal apoptosis in the spinal cord

Apoptotic cell death (measured by TUNEL assay) in the spinal cord from the vehicle group was increased on day 3 and 14 following the CEC (Fig. 7a&c). Sections stained for TUNEL positive cells were double stained for NeuN positive cells and the cells double positive for TUNEL and NeuN were counted in five frames of 3 sections from each experimental group. Most of the TUNEL positive cells were also found NeuN positive, indicating neurons undergoing apoptosis after the CEC (Fig. 7b). Vehicle group had significantly ($p < 0.01$) increased number of double positive cells both on day 3 (94.33 ± 8.5) and 14 (56.00 ± 4.36) after the CEC. The double positive apoptotic cells were significantly ($p < 0.01$) decreased by the simvastatin treatment on day 3 (37.67 ± 9.07) and 14 (28.67 ± 6.66 , Fig. 7c).

Discussion

In this study, treatment of LSS with simvastatin not only provides neuroprotection but also improves locomotor function in a CEC rat model. These protective effects of simvastatin may be attributed to the down regulation of inflammation (Fig. 4) and demyelination (Fig. 5). Although statins are mainly used to reduce cardiovascular morbidity and mortality through reducing cholesterol and low density lipoproteins levels (Igel et al. 2002), recent studies have documented pleiotropic effects like anti-inflammatory, antiproliferative, antithrombotic and antioxidant properties (Markovic-Plese et al. 2008). Neuroprotective property of statins treatment were reported in animal models of neuroinflammatory SCI (Han et al. 2011; Eroglu et al. 2010; Pannu et al. 2007; Pannu et al. 2005), TBI (Abrahamson et al. 2009; Li et al. 2009; Wu et al. 2010; Chauhan and Gatto 2011) and sciatic nerve injury (Pan et al. 2010). Different doses of statins ranging from 1 mg/kg to 20 mg/kg body weight were tested in previous reports. However, the dose of simvastatin (5 mg/kg body wt.) used in the present study is based on our previous findings that statins are anti-inflammatory and neuroprotective (Pannu et al. 2005; Pannu et al. 2007) and also renoprotective (Shunmugavel et al. 2010) in SCI. The data from this study support the neuroprotective and anti-inflammatory properties of statins in a rat model of LSS in addition to animal models of TBI (Qu et al. 2009), SCI (Pannu et al. 2005), stroke (Cimino et al. 2005), cerebral ischemia (Die et al. 2010) and EAE (Paintlia et al. 2004).

CEC syndrome principally affects locomotor/walking efficiency through radiating low back pain during standing or walking. The voiding function is not usually affected. This may be due to the fact that the sacral nerves are relatively protected from compression owing to their central position within the cauda equina (Siebert et al. 2009). Further, to avoid additional confounding effects due to complications like voiding dysfunction and others along with LSS pathophysiology, MRI was used to ascertain the proper positioning of the silicone gels in the epidural space of CEC rats.

Lack of effective treatment to neuropathic pain developed due to LSS is partially the result of poor understanding of disease pathogenesis. Recent observations on the infiltration of immune cells have documented the possible role of inflammation in the pathobiology of LSS (Seichi et al. 2004; Koike et al. 2003; Kawaguchi et al. 2001; Habtemariam et al. 1998). Inflammatory cells accumulated at the lesion site lead to tissue degeneration (Habtemariam et al. 1998). Therefore, therapeutic approaches designed to attenuate the immune cell

infiltration and reduce the expression of inflammatory mediators are attractive options to treat LSS.

Infiltration of cells into the CNS is controlled by blood/ CNS barrier (blood brain/spinal cord barrier). The permeability changes depend on the microenvironment especially on the expression of extracellular matrix adhesion proteins such as cell adhesion molecules, selectins, cytokines and chemokines. These mediators stimulate the cellular infiltration into the CNS that ultimately results in neuronal loss. Previous studies from our laboratory, showing inhibition of immune cell infiltration in the CNS in a rat model of EAE by lovastatin insinuated the potential of statins in LSS therapy (Stanislaus et al. 2001). In the present study, we have identified simvastatin to decrease the increased influx of CD68 and CD 8 cells in the spinal cord of CEC rats (Fig. 4). In addition, the TNF- α expressing cells were also significantly decreased by simvastatin treatment (Fig. 6).

Compression of lumbar nerve roots or cauda equina is associated with loss of neurons and myelin, resulting in spinal degeneration neurobehavioral deficits (Wang et al. 1999). Spinal degeneration has been the major cause of radicular pain in LSS (Ohtori et al. 2010; Ito et al. 2007; Sekiguchi et al. 2004). In the present study, CEC caused significant demyelination in the spinal cord. The treatment with simvastatin inhibited CEC-induced demyelination. Our observation is supported by earlier reports that statins therapy provides neuroprotection through decreasing demyelination in inflammatory demyelinating EAE disease (Paintlia et al. 2009; Paintlia et al. 2004). Apoptotic cell death of neurons disrupts the axon-myelin structural unit and impairs impulse conduction resulting in functional loss. In the present study, CEC-mediated TUNEL positive cells were also found to be positive to NeuN (Fig. 7b), indicating neuronal apoptosis following CEC. One way to reduce the loss of function of axons is to reduce the extent of cell death. We have seen significantly decreased level of neuronal apoptosis in the spinal cord from simvastatin-treated CEC animals (Fig. 7). The neuroprotective effect of simvastatin described with LSS animal model in the present study is consistent with recent reports witnessing the neuroprotection offered by statins in an experimental model of sciatic nerve crush injury (Pan et al. 2010).

The effect of narrowing of spinal canal can spread from the epicenter to the somatosensory nuclei of brain affecting the locomotor function (Zhou et al. 2002). Hence, LSS patients are characterized by a decrease in walking function (Adamova et al. 2003). Watanabe et al., have reported a decrease in locomotor function with single silicone gel-induced CEC in a rat model of LSS (Watanabe et al. 2007). However, a one level compression has been reported to cause intermittent claudication pain only when the stenosis occurs specifically in the root canal or foramen (Dommissie and Grobler 1976; Kirkaldy-Willis et al. 1982). But two level stenosis has always been shown to end up in intermittent claudication (Porter and Ward 1992). In addition, it is a common observation that most of the LSS patients harbor stenosis at more than one level in their spine (Orendacova et al. 2001). Hence in the present study, we decided to impart stenosis at two levels by implanting of two silicone blocks of same size simulating two level spinal stenosis. As the locomotor functional recovery is a reliable indicator of functional recovery after LSS, we evaluated simvastatin mediated increase in the latency of CEC rats on rotarod as a measure to determine the therapeutic efficacy of the drug. In the present study, the motor function of the simvastatin group started improving from day 7 onwards and stayed significantly higher than that of the vehicle group throughout the experimental period (7 weeks). This observation shows that simvastatin is effective in restoring the motor function following CEC. Furthermore, the effect was not an early or short lived adaptive response but a persistent one (Fig. 2).

Development of neuropathic pain is an intrinsic component of CEC syndrome. Significantly increased hypersensitivity to pain stimulus was seen with rats after CEC in the present study.

Simvastatin treatment, on the other hand significantly decreased the hypersensitivity to mechanical stimuli developed due to the CEC. Our findings are also supported by recent report that simvastatin is effective in ameliorating neuropathic pain in nerve injury mice animal model (Shi et al. 2011). Atorvastatin, another group of statins also protect rats from neuropathic pain inflicted through chronic constriction injury (Chu et al. 2012) through inhibiting NF κ B and iNOS mediated injury pathway. Simvastatin mediated increase in pain threshold may be due to the inhibition of influx of immune cells which release proinflammatory cytokines contributing to hyperexcitability (Reichert et al. 1996).

In the present study, we observed for the first time the reactive astrogliosis in the spinal cord of the CEC rats. Reactive astrogliosis is associated with infection, trauma, ischemia, neurodegeneration and post-surgical healing of CNS. Vehicle group of CEC rats showed significantly elevated levels of active astrocytes in the spinal cord on day 3 following CEC and the astrogliosis was seen to persist for 14 days after CEC. CEC induced significant activation of astrocytes in the spinal cord of rats. Simvastatin treatment on the other hand significantly reduced the activation of astroglia. Reactive astrogliosis is a ubiquitous hallmark of CNS pathology such as trauma and neurodegenerative diseases. Statins were shown to exert their anti-inflammatory activity by inhibiting of various inflammatory molecules like interleukins (IL-1,2,4,5,10, 12), interferon- γ , tumor necrosis factor- α (TNF α), cyclooxygenase-2 (COX-2), thromboxanes, and prostacyclin (Schonbeck and Libby 2004). In the present study we have seen simvastatin mediated decrease in the expression of TNF α and reactive astrogliosis in the spinal cord of the CEC rats. Hence, it can be presumed that the anti-inflammatory property of simvastatin is mediated through the down regulation of TNF α expression in the infiltrated immune cells and thereby decreasing the reactive astrogliosis.

Conclusively, simvastatin ameliorates CEC injury in a rat model of LSS via down regulation of inflammation and apoptosis, leading to an enhanced neuroprotection and motor function recovery. These results indicate that simvastatin therapy has potential to treat LSS patients.

Acknowledgments

This work was supported by grants from The Spinal Research Foundation VA and by grants from Betty L. Beatty and Guy E. Beatty Foundations. The work was also supported in part by grants NS-72511, NS-22576, and NS-37766 and DC00422; 07506 from the NIH, CO6 RR018823 and CO6 RR0015455 from the Extramural Research Facilities Program of the National Center for Research Resources. The authors thank Dr. Hainan Lang, Ph.D., Department of Pathology and Laboratory Medicine for help in histology, Ms. Danielle Clark Lowe for help in proof reading and Ms. Joyce Bryan and Ms. Chara Williams for help in animal and reagents procurement.

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Fig. 1. Representative sagittal MRI showing the orientation of the surgically implanted silicone blocks. Silicone blocks were surgically implanted into the epidural spaces of L4-L5 and L5-L6. The animals were scanned with T Bruker 7T MRI scanner to determine the proper orientation and placement of the silicone blocks. The image shows the silicone blocks are precisely placed in the epidural space under L4-L5 and L5-L6 vertebrae

Fig. 2. Simvastatin reduces locomotor deficits in CEC rats. Simvastatin (5 mg/kg body wt.) improved motor function of CEC rats evaluated by rotarod test. Simvastatin group exhibited significantly higher locomotor recovery compared to the vehicle group from day 7 onward. Results are presented as latency in seconds and data are expressed as mean $\pm$ SD ($n=24$). Sham operated animal had no locomotor deficit on any day tested (data not shown). ** $p<0.05$ and *** $p<0.001$ vs. vehicle

Fig. 3.

Simvastatin enhances the threshold of pain in CEC rats. Rats subjected to CEC surgery and treated either with vehicle or simvastatin after measuring the baseline pain threshold (Day 0) using analgesy meter (a) and dynamic plantar aesthesiometer (b). Pain threshold was measured from post CEC day 2 to 14. Vehicle group had significant hypersensitivity for 4 days measured with AM and 6 days measured with DPA when compared with the baseline values. After that, the animals spontaneously recovered normal sensitivity to pain threshold. Pain threshold of simvastatin group resembled to that of the baseline value on all the days tested. The results demonstrate the antinociceptive property of simvastatin. Data are represented as mean $\pm$ SD. Each group consisted of 6 animals. ** $p < 0.05$, *** $p < 0.001$ vs. baseline and simvastatin group

Fig. 4.

Simvastatin inhibits cellular infiltration in the spinal cord of rats on day 14 after CEC. Spinal cord from each group was extracted and analyzed by H&E staining (a). Spinal cord of sham animals showed normal tissue architecture without any cellular infiltration. However, spinal cord from vehicle group showed increased infiltration of cells and the cellular architecture was also altered with large vacuoles. Simvastatin group had normal cellular architecture in addition to reduced cellular infiltration. Images are representative of sections from 5 animals in each group (magnification 600X). The infiltrated cells were stained for activated microglia (CD68) and cytotoxic T cells (b). Vehicle group showed increased infiltration of CD 68+ and CD 8+ cells in the spinal cord. Simvastatin treatment significantly decreased in infiltration of activated microglia and cytotoxic T cells in the spinal cord of CEC rats. Immunoreactive cells were counted in 4 regions of single section ($n=6$). Summary of the quantification of immunolabeling of CD 68 and CD 8 is given (C, D). Data are given as mean $\pm$ SD. ** $p < 0.05$ vs. sham and simvastatin

Fig. 5.

Simvastatin inhibits CEC-induced demyelination. Simvastatin treatment preserved myelin levels in the spinal cord from CEC rats as determined by LFB staining (**a**). EM was also employed to determine the myelin degeneration (*arrows* **b**) and g-ratio of axons (**d**). Spinal cord of sham animals showed normal levels of myelin. While the vehicle group showed significant loss of myelin, measured in terms of LFB intensity, simvastatin group had myelin level comparable to that of sham group (**a** & **c**). Images are representative of sections from five animals from each group (magnification 600X). g-ratio, a measure of myelin level also showed simvastatin mediated myelin protection in the CEC rats (**d**). Images are representative of sections from three animals from each group (magnification 20000X).

Fig. 6.

Simvastatin reduces inflammation mediated astroglial activation following spinal stenosis. Simvastatin treatment significantly decreased the activation of astroglial cells on day 3 and 14 after CEC measured in terms of GFAP positive area of the spinal tissue section examined (**a: upper panel**). In addition, simvastatin also decreased the number of TNF- α positive cells in the spinal cord of CEC rats on day 3 and 14 post spinal stenosis (**a lower panel**). Photomicrographs are representative of 3 animals in each group (magnification 400X). The respective histogram of GFAP positive area and number of TNF- α positive cell of spinal sections of different experimental groups are given in B and C. GFAP positive area and number of TNF- α positive cell were counted in 4 different locations on each spinal tissue section. Data are presented as mean $\pm$ SD of $n=3$ in each group

Fig. 7.

Simvastatin reduces neuronal apoptosis in spinal cord from the CEC rats. Treatment with simvastatin significantly decreased the cellular apoptosis determined by TUNEL assay (**a**). Colocalization study of TUNEL and NeuN showed that most of the apoptotic cells are neurons (**b**). The graph (**c**) shows the number of cells double positive to TUNEL and NeuN observed in sham, vehicle and simvastatin groups on post CEC day 3 and 14. Photomicrographs are representative of 3 animals in each group. Fig C represents the number of cells counted over 5 different locations on each section in each group. ## $p < 0.001$ vs. sham, ** $p < 0.001$ vs. vehicle