Research article

Gene expression profiling of *liver X receptor α* and *Bcl-2-associated X protein* in experimental transection spinal cord-injured rats

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Background: Study of molecular responses to central nervous system injury would be helpful for controlling the harmful pathways post-injury and triggering the useful pathways required for the treatment of injury.
Objective: To investigate the expression level of *liver X receptor α* (*LXRα*) which has anti-inflammatory effects and pro-apoptotic *Bcl-2-associated X protein* (*Bax*) upon spinal cord injury (SCI).
Design: To induce SCI, transection was carried out at T9 level of male Wister rats. Approximately 8 mm of rostral, caudal, and epicenter tissues of injured sites in treated rats were chosen for quantitative real-time polymerase chain reaction at the 6, 24, and 72 hours, and 7 and 10 days post-surgery.
Results: Our results showed a complicated temporal and spatial pattern of alteration in *LXRα* and *Bax* mRNA expression levels after SCI. *LXRα* expression level followed a homologues pattern (additive and subtractive wave) with a difference in time at three areas of studied. Rostral, caudal, and epicenter expression patterns of *Bax* were dissimilar in these areas. Gradual increase in the expression of *Bax* without any decrease at the rostral area was observed, presumably indicating the active transcription process of this gene, regardless of its protein situation.
Conclusion: A time lapse significant change in *Bax* expression level was observed only in the epicenter of injury, emphasizing that apoptotic responses are limited to this area. Furthermore, an increase in *LXRα* transcription level was observed first in rostral area and then extended to epicentral and caudal areas, implying that inflammation responses extended from rostral to caudal areas.

Keywords: Bcl-2-associated X protein, Liver X receptor α, Quantitative real-time PCR, Spinal cord injuries, Apoptosis, Transcription factors, Paraplegia, Tetraplegia

Introduction

The response to spinal cord injury (SCI) is known to occur in two phases, a primary phase in which mechanical trauma is sustained, and a latter phase that is characterized by tissue damage.[1] Traumatic injury to the spinal cord leads to a rapid destruction of cells at the site of injury consisting an intense inflammatory response, secondary necrotic and apoptotic cell death, and reparative responses. These responses to injury are mediated by changes in mRNA levels. It remains unclear as to whether these changes are due to regulated gene expression or to altered cellular populations.[2] However, it has been demonstrated that SCI leads to induction and/or suppression of several genes.[3]

Up-regulation of several families of inflammatory molecules, including cytokines and chemokines, happens predominantly after SCI. The sources of these molecules are microglia and leukocytes. The potential consequences of sudden presence of these molecules can lead to detrimental events such as breakdown of the blood–spinal code barrier (BSB), demyelination, and axonal injury.[4] A principal player in the regulation of inflammatory gene expression is the nuclear factor-κB (NF-κB) family of transcription factors, and may be important determinants for cell death and disease of the CNS.[5] Meanwhile, nuclear hormone receptors including liver X receptors (LXR) have been described as potential immune-modulatory drug targets for neurodegenerative disorders and SCI.[6] These receptors have

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the ability to inhibit inflammatory gene expression through the mechanism of 'trans-repression' of transcription factors such as NF-κB.[7] Activation of these transcription factors modulates the inflammatory response of glial cells.[8–10] BSB disruption is one of the vascular events that happen in secondary injury after initial contusion and other forms of SCI such as transection. Barrier breakdown can alter the inflammatory responses throughout invasion of neutrophils and macrophages at the site of injury.[11–14] Damage to the spinal cord results in extensive cell proliferation in and around the epicenter, where microglia and macrophages are mostly located.[15] Microglia and oligodendrocytes have been implicated in apoptosis. However, the mechanisms that may underlie the involvement remain poorly understood. The association between microglia and dying oligodendrocytes suggests that microglial activation may be implicated in apoptosis after SCI.[16] Shuman *et al.*[17] indicated maximal oligodendrocyte death 8 days after injury. They assumed that two mechanisms may be implicated: (1) loss of trophic support and (2) microglial activation. In a contusion model of SCI, it was observed that phagocytic microglia and oligodendrocytes undergo apoptosis. Bcl-2-associated X protein (Bax) is a proapoptotic member of Bcl2 family. Bcl-2 family members share one or more Bcl-2 homology domains and are divided into two main groups according to whether they promote or inhibit apoptosis.[18] Stimulation of apoptotic signals leads to Bax interaction with mitochondrial membrane proteins to form trans-membrane permeable channels, that stimulates the release of cytochrome *c* and activates the caspase 3 cascade.[19] Cellular signals consequential from DNA damage, hypoxia, or other types of cell stress can result in triggering of intrinsic pathway by stimulating Bax in the mitochondrial outer membrane.[20] Most studies on Bax have been focused on the presence and activity of Bax protein and its transport from the cytoplasm to the mitochondrial membrane; however, the level of its gene expression after SCI has been little understood. Therefore, this study was designed to define the temporal and spatial distribution of *Bax* and *LXRα* mRNA changes following transection model of SCI in rats.

Materials and methods

Animal experiments

All the ethical aspects and experimental protocols were approved by graduate studies committee of Biology Department of the University of Isfahan. Adult rats weighing 180–240 g were used in this study. Fifty-four (54) rats were randomly divided into six groups of nine rats ($n = 9$). Rats in the CG group (control group) did not undergo surgery or medical intervention, except for anesthesia prior to being sacrificed. Experimental rats were subjected to a laminectomy and a T9 transection and were classified as 6, 24, 72 hours and 10 days according to post-injury periods. There was no intervention at any post-injury period in all groups. Rats were housed three per cage in the animal laboratory with free access to food and water *ad libitum* and were maintained in a 12-hour light/dark cycle at room temperature (22–25°C). Before surgery, all rats were injected with 15 000 IU/kg of *Penicillin–Streptomycin* as prophylaxis against infection after surgery. The animals were anesthetized with halothane. Their dorsal regions were shaved and cleaned with povidone-iodine. Under sterile surgical conditions, and following a midline skin incision at the site of injury and paravertebral muscle dissection, the dura was left intact. T9 laminectomy was used for all experimental groups. After the bisection transection, the muscles and incision were sutured and animals returned to clean cages to recover. Bladders were emptied manually twice a day. At 6th, 24th, 72th hour, 7th and 10th day after injury, rats were anesthetized with halothane. Spinal cords were excised for a length of 2.5 cm; 8 mm rostrally and caudally to the injury site and 8 mm of covering the epicenter of injury was removed and used immediately for total RNA extraction.

RNA isolation and reverse transcription

RNA was isolated from tissue (70–80 mg), using RNX™ plus solution (CinnaGen, Tehran, Iran) according to the protocol described by the manufacturer. The purity of the isolated total RNA was analyzed by absorption at 260 and 280 nm with Eppendorf spectrophotometer (Eppendorf, Hamburg, Germany). The integrity of the total RNA was assessed with TAE agarose gel electrophoresis. Samples were treated with the *DNAase*I, RNase-free kit (Fermentas, Vilnius, Lithuania). First-strand complementary DNA (cDNA) was synthesized from 1 μg total RNA using the RevertAid™ First strand cDNA synthesis (Fermentas) using random hexamer primer.

Real-time polymerase chain reaction

Specific primers were designed according to the the sequences obtained from GeneBank using Beacon Designer 7.5 and oligo6 software to amplify a 147-bp amplicon for *LXRα*, 249 bp for *Bax* and 134 bp for *Tubβ5* (Table 1). Real-time polymerase chain reaction (PCR) was carried out in Chromo4 Detection System (BioRad, Hercules, CA, USA), using the fluorescent

Table 1 Primer list in this study

Gene		Forward (F) and reverse (R) primers (5′–3′)	Primer length	Product length
Tubβ5	F	1494 AAGAGGATTTCGGAGAGG 1511	18	134
	R	1627 GAACAAAAACAGGACAGAGG 1608	20	
LXRα	F	45 CCTGATGTTTCTCCTGACTC 64	20	147
	R	191 TGACTCCAACCCTATCCTTA 172	20	
Bax	F	331 GTTGCCCTCTTCTACTTTGC 350	20	249
	R	579 TCAGCCCATCTTCTTCCAG 561	19	

dye SYBR premix Ex Taq™ II (TaKaRa, Tokyo, Japan). A control cDNA dilution series was created for each gene to establish a standard curve. Each reaction was subjected to melting point analysis to confirm single amplified products. Briefly, 10 ng of cDNA and gene-specific primers were added to SYBR Green PCR MasterMix (TaKaRa, Tokyo, Japan). All samples were run in duplicate on 96-well optical PCR plates in a final reaction volume of 25 µl. The PCR parameters were 1 cycle at 95°C for 30 seconds, 45 cycles at 94°C for 5 seconds, 57°C 20 seconds, and 72°C for 15 seconds. The gene encoding the rattus *Tubβ5* housekeeping gene was used as an internal control to normalize target genes expression. The specificity of PCR products of *LXRα*, *Bax* and *Tubβ5* was confirmed by the single-peak dissociation curves and by agarose gel electrophoresis showing that amplicons had the predicted size.

Statistical analysis

The PCR results were analyzed based on the ΔCT, which is the primary source of data variability. Relative gene expression was calculated by using the $2^{-\Delta\Delta CT}$ method, where CT is the threshold cycle. One-way analysis of variance (ANOVA) was used to analyze expression levels of transcripts of genes. Statistical significance ($P < 0.05$ and $P < 0.001$) was determined with Tukey's *post hoc* test of least significant differences between specific time points in GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

The resulting melting curves showed proper replication of *LXRα, Bax*, and the *Tubβ5* (housekeeping gene) with the absence of primer dimer (data not shown). Therefore, the next steps of analyses carried out and the following results were obtained:

Temporal and spatial alterations in LXRα expression after SCI

LXRα expression was altered after SCI (Fig. 1A–C) which was estimated by one-way ANOVA to be significant. In rostral tissue of the injury site, *LXRα* mRNA was significantly ($P < 0.001$) increased by 134.983700 ± 24.530860 fold at 24 hours post-injury, then returned to control level after 3 days (0.983665 ± 0.220822) (Fig. 1A). However, in the epicenter of injury, *LXRα* mRNA was significantly ($P < 0.001$) increased to 303.415500 ± 63.251800 fold 7 days after injury ($P < 0.001$), and reduced to 163.262600 ± 39.189480 fold at 10 days after injury ($P < 0.05$) (Fig. 1B). In caudal tissue, *LXRα* expression was significantly increased on day 7 after injury (169.469200 ± 18.976610, $P < 0.001$) (Fig. 1C). Thus, the pattern of *LXRα* mRNA expression level showed an additive and subtractive wave with a difference in time at three studied locations (Fig. 2A).

Temporal and spatial alterations in Bax expression after SCI

A different pattern was seen for the expression of *Bax* after SCI (Fig. 1D–F). In rostral of the injury site, *Bax* mRNA was gradually increased. However, significant change was seen after 10 days post-injury (Fig. 1D). Change in the *Bax* expression at epicenter site was more extensive than at the rostral site of injury. In this site, *Bax* mRNAs were significantly increased by 66.134580 ± 12.626100 and 79.953770 ± 16.983280 folds 72 hours and 7 days after injury, respectively ($P < 0.001$) (Fig. 1E). Meanwhile, *Bax* mRNA changes in the caudal site of incision were not significant (Fig. 1F). Thus, the pattern of *Bax* mRNA expression level was different due to three studied locations (Fig. 2B).

Discussion

Pervasive synchronized changes in genes expression occur as a consequence of SCI. Such changes are associated with hemorrhage, metabolic failure, inflammatory/immune activation, loss of ionic homeostasis, lipid deprivation, production of free radicals, and neurotransmitter/neuromodulator imbalances. Taken together, these pathological events lead to cell damage.[13] Further damages after initial trauma take place by prolonged or chronic release of pro-inflammatory cytokines through microglia/macrophages at the site of injury. In

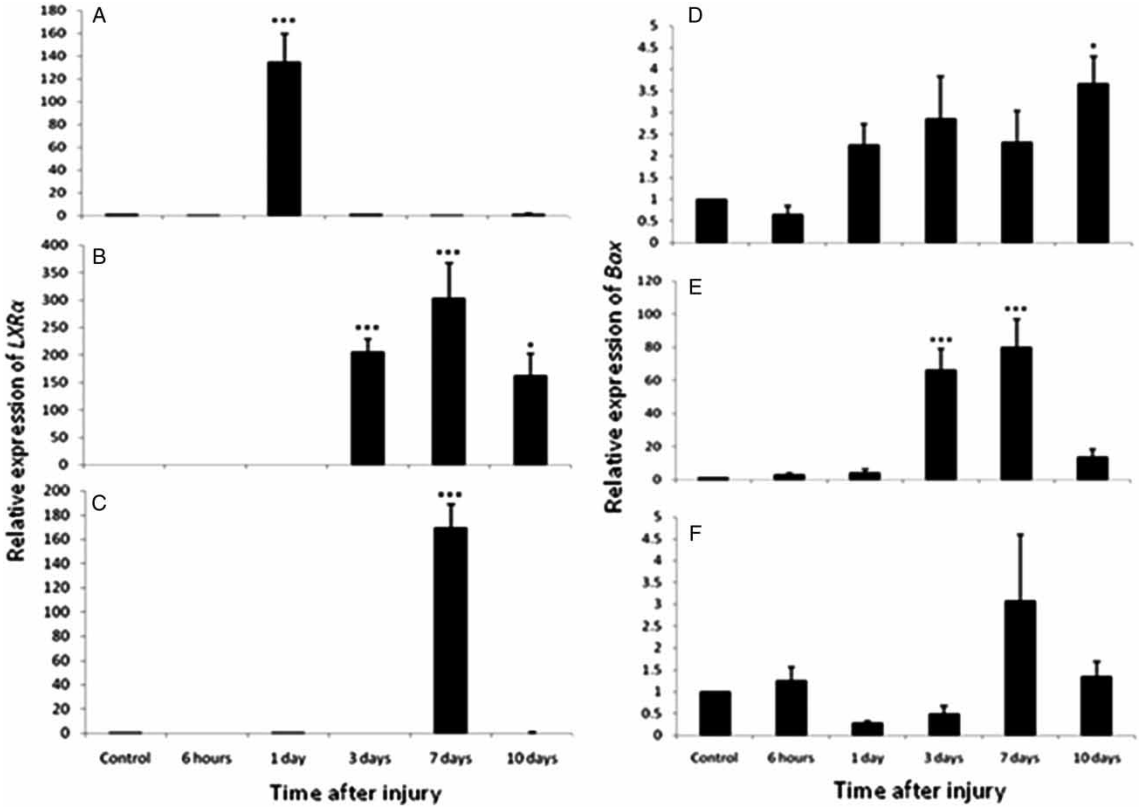

Figure 1 Real-time quantitative PCR analysis of *LXRα* (A–C) and *Bax* (D–F). Spatial–temporal mRNA expression of *LXRα* and *Bax* in injured tissue (rostral tissue, A and D; epicenter, B and E; and caudal tissue C and F) after transection of the spinal cord in adult rats. SEM of three similar experiments in each group ($n = 9$) is shown by error bars. Asterisks indicate significant difference between treated samples and related control at ***$P < 0.001$ and *$P < 0.05$.

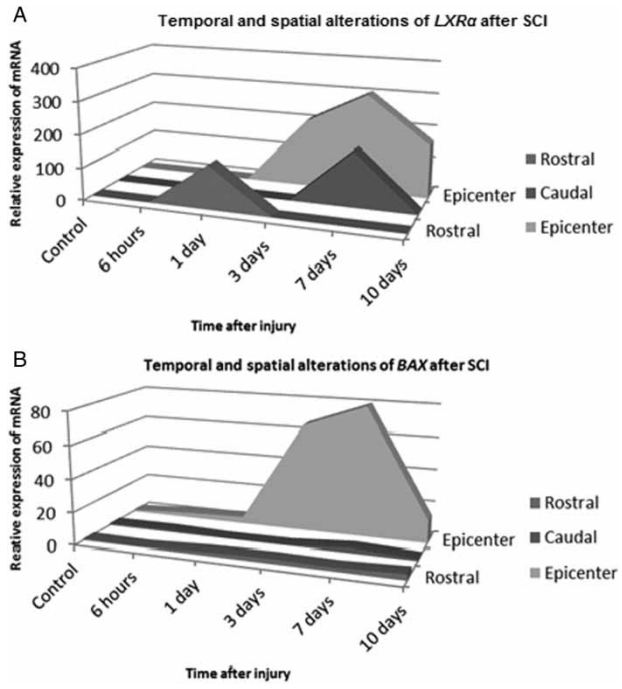

Figure 2 Illustrated schematic diagram of differential mRNA changes of *LXRα* and *Bax* in the epicenter, caudal and rostral tissues post-injury, respectively.

contrast, activated microglia and astrocytes have a role in the production of growth factors, essential for neuronal survival and tissue repair.[11] Support for the involvement of activation of microglia and astrocytes in production of neurotrophic factors comes from studies that transplantation of peripherally activated macrophages may exert beneficial effects on efficient regeneration of spinal cord.[11] Activation of inflammatory factors may also be involved in regeneration of the spinal cord.

Activation of microglia and infiltration of neutrophils are two characterizations of inflammatory response post-SCI. The first step for regulating the inflammatory response after trauma occurs at the transcriptional level.[21] Thus, the mRNA expression level of the involved genes in of inflammatory response seems to be important.Negative regulators in the expression of genes involved in inflammation as NF-κB, are LXRs.[22] LXRs inhibit inflammatory gene expression through the mechanism of trans-repression which is an event that depends on the direct binding of LXR to retinoid X-receptor for occupying promoters of target genes.[7] For instance, LXR activation in activated

microglia and astrocytes inhibits the production of pro-inflammatory factors as NF-κB.[22] Joseph *et al.*[23] showed that a synthetic LXR agonist inhibits transcription of NF-κB.

One of the early features of injury in the CNS is disruption of the BSB. It has been reported that 24 hours after injury, disruption of the BSB is maximal and then gradually declines. BSB breakdown is not limited to the injured site and extends through the cord axis.[11] Thus, activated LXR has an intense effect on the vascular system through controlling molecular mechanisms and gene expression in endothelial cells for instance up-regulation of ATP-binding cassette sub-family B member 1 (ABCB1) and ATP-binding cassette, sub-family C (CFTR/MRP) member 1(ABCC1) transporters, which support endothelial integrity.[14,24]

Our data are consistent with this event that there is a relevant increase in expression level of LXRα at caudal site of SCI. Hausmann has indicated that 24 hours after trauma, microglial activation can be observed which increases by 7 days after injury. Activated microglia, secrete various cytokines including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and reactive free radicals, and nitric oxide.[11] One hour after generation of trauma, apoptotic cells were predominant in the gray matter. In addition, after 8 hours there was an increase in these cells both in the gray and white matters. However, 24–72 hours after injury, the amount of apoptotic cells diminished in the gray matter, but enhanced in the white matter. The focus of apoptotic cells is closest to the epicenter of injury.[11] DNA damage can cause the transcriptional activation of *Bax*. Previous studies have indicated that *Bax* transcripts are up-regulated following SCI. Aimone *et al.*[15] showed an up-regulation of *Bax* 35 day after SCI at epicenter of the contused rat spinal cord. Crisafulli *et al.*[25] provided evidence that T0901317 (synthetic ligand of LXRα) modulates the content of TNF-α and IL-β; and NF-κB; MAPK phosphorylation (ERK, JNK, and p38); and the content of Bax. The LXR pathway antagonize NF-κB signaling pathway and inhibit expression of NF-κB downstream inflammatory genes. Separately, Paterniti and colleagues identified Bax increased protein level 24 hours after SCI. Their data indicated that LXR activation by specific agonist, TO901317, prevented SCI-induced Bax expression. Moreover, they demonstrated that treatment of mice with aforementioned agonist significantly diminished the SCI-reduced expression of the anti-apoptotic protein, Bcl-2.[26] It was already supposed that *Bax* expression plays an important role in the development of apoptosis.[27] Furthermore, Paterniti *et al.*[26] indicated that ligand induced activation of LXRα lead to a substantial reduction of Fas ligand signaling, which plays a central role in SCI.

Thus, an increase in *LXRα* expression may produce a protection from apoptosis, which may be postulated as a prerequisite factor for regenerative approaches to SCI. In the present study, we examined multiple sites of injury at various time points. Our data indicated an increase in expression of *LXRα* after injury, presumably due to inflammatory reactions. It was already implicated the highly expression of *LXR*α in macrophages.[28] Thus, we could suppose that increased influx of macrophages to the site of injury along with increased microglia activation, is the reason for increased expression of *LXRα*. Moreover, we speculate that a decrease in *LXRα* which could be related to a decrease in pro-inflammatory factors. Disruption of microcirculatory can be caused by late expression of this receptor in caudal and epicenter of injury.

Conclusion

Similar increasing pattern in both of *Bax* and *LXRα* transcription levels were observed in the epicenter site of injury after SCI. This event can be explained by possible association between inflammation and apoptosis. On the other hand, a reduction in epicentral *Bax* expression level was observed 10 days post-injury possibly due to the activation of cellular survival pathways or increase of apoptotic cells. Moreover, there was no significant change in *Bax* expression level at both caudal and rostral area emphasizing that apoptotic responses are limited the epicenter of injury. An increase in *LXRα* transcription level was observed first in rostral area and then in epicentral and caudal areas, implying that time-lapse inflammation responses could be extended from rostral to caudal areas.

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