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Authors

López-Vales, Rubèn
Ghasemlou, Nader
Redensek, Adriana
et al.

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Phospholipase A_2 superfamily members play divergent roles after spinal cord injury

Rubèn López-Vales, Nader Ghasemlou, [...], and Samuel David

Abstract

Spinal cord injury (SCI) results in permanent loss of motor functions. A significant aspect of the tissue damage and functional loss may be preventable as it occurs, secondary to the trauma. We show that the phospholipase A_2 (PLA_2) superfamily plays important roles in SCI. PLA_2 enzymes hydrolyze membrane glycerophospholipids to yield a free fatty acid and lysophospholipid. Some free fatty acids (arachidonic acid) give rise to eicosanoids that promote inflammation, while some lysophospholipids (lysophosphatidylcholine) cause demyelination. We show in a mouse model of SCI that two cytosolic forms [calcium-dependent PLA_2 group IVA ($cPLA_2$ GIVA) and calcium-independent PLA_2 group VIA ($iPLA_2$ GVIA)], and a secreted form [secreted PLA_2 group IIA ($sPLA_2$ GIIA)] are up-regulated. Using selective inhibitors and null mice, we show that these PLA_2s play differing roles. $cPLA_2$ GIVA mediates protection, whereas $sPLA_2$ GIIA and, to a lesser extent, $iPLA_2$ GVIA are detrimental. Furthermore, completely blocking all three PLA_2s worsens outcome, while the most beneficial effects are seen by partial inhibition of all three. The partial inhibitor enhances expression of $cPLA_2$ and mediates its beneficial effects *via* the prostaglandin EP1 receptor. These findings indicate that drugs that inhibit detrimental forms of PLA_2 ($sPLA_2$ and iPLA2) and up-regulate the protective form (cPLA2) may be useful for the treatment of SCI.—López-Vales, R., Ghasemlou, N., Redensek, A., Kerr, B. J., Barbayianni, E., Antonopoulou, G., Baskakis, C., Rathore, K. I., Constantinou-Kokotou, V., Stephens, D., Shimizu, T., Dennis, E. A., Kokotos, G., David, S. Phospholipase A_2 superfamily members play divergent roles after spinal cord injury.

Keywords: CNS injury, secondary damage, lipid metabolism, prostaglandin receptors

Spinal cord injury (SCI) causes permanent functional deficits due to disruption of spinal pathways and death of neurons and glial cells. The trauma itself causes initial damage to neural tissue, including glia and neurons at the site of injury. This primary damage, however, spreads to regions rostral and caudal to the injury epicenter during the days and weeks after injury. The inflammatory response that occurs after SCI strongly contributes to secondary damage. The cytotoxic effects of immune cells are likely to be mediated *via* the production of cytokines, chemokines, eicosanoids, proteases, and free radicals, among other factors (1, 2). Reducing inflammation after SCI can therefore be expected to reduce secondary tissue damage and limit functional deficits.

Phospholipase A_2 (PLA_2) enzymes catalyze the hydrolysis of fatty acids at the sn-2 position in phospholipids and thus give rise to the release of fatty acids, such as arachidonic acid, and the production of lysophospholipids, such as lysophosphatidylcholine (LPC) (3). Several types of PLA_2s include both secreted ($sPLA_2$) and intracellular forms, which includes calcium-dependent ($cPLA_2$) and calcium-independent ($iPLA_2$) enzymes (4). Phospholipase A_2s are important enzymes involved in membrane turnover. Recent studies, however, have revealed an important multifaceted role for these enzymes in various aspects of inflammation, including in the nervous system, such as in experimental autoimmune encephalomyelitis (EAE; refs. 5–7), brain ischemia (8, 9) and Wallerian degeneration after sciatic nerve injury (10, 11). One way PLA_2 can play a role in inflammation is through the arachidonic acid pathway, which is the precursor of proinflammatory eicosanoids, such as prostaglandins, thromboxanes, and leukotrienes. Another way PLA_2 can stimulate immune responses is through LPC, which is chemoattractant for monocytes and T cells, activates macrophages, and induces the expression of proinflammatory chemokines and cytokines, and cell adhesion molecules (12–15). Blocking PLA_2 might, therefore, be a good therapeutic target to reduce inflammation and prevent tissue loss and demyelination after SCI.

Little is known about the role of PLA_2 superfamily members in SCI. Recent studies have reported that $cPLA_2$ GIVA and $sPLA_2$ GIIA are up-regulated after SCI in rats (16, 17). Thus far, the role of $sPLA_2$ was assessed indirectly by intraspinal injection of $sPLA_2$ GIII (from bee venom), into the uninjured, normal spinal cord (16), and in a study that assessed the effects of a nonselective PLA_2 inhibitor in SCI over a period of 7 days postinjury (dpi) (18), which blocked both $cPLA_2$ and $iPLA_2$ (19). It is, therefore, not known whether both intracellular forms of PLA_2 ($cPLA_2$ and $iPLA_2$) are involved in contributing to SCI pathology and to what extent. In addition, the role of $sPLA_2$ in the injured spinal cord has not been directly examined.

We now provide direct evidence that of the large number of PLA_2s comprising the PLA_2 superfamily found in mice, the expression of only $cPLA_2$ GIVA, $iPLA_2$ GVIA, and $sPLA_2$ GIIA are increased after spinal cord contusion injury. We also dissected out the contribution of these PLA_2 forms in SCI using selective inhibitors against the three different forms of PLA_2, as well as two pan-PLA_2 inhibitors and the $cPLA_2$-null mouse. We show that $cPLA_2$ GIVA mediates tissue protection after SCI, while $sPLA_2$ GIIA, and to a lesser extent $iPLA_2$ GVIA, contribute to secondary damage and functional loss. These data provide the first clear evidence that different members of the PLA_2 superfamily play divergent roles in SCI. We also show that completely blocking all three PLA2s is detrimental to recovery after SCI, while an inhibitor with partial blocking activity is most beneficial.

MATERIALS AND METHODS

Spinal cord contusion and drug administration

Adult (8–10 wk old) female BALB/c (Charles River, Saint-Constant, QC, Canada), *cPLA$_2$ GIVA*$^{-/-}$ mice, and wild-type littermates were anesthetized with ketamine:xylazine:acepromazine (50:5:1 mg/kg). After performing a laminectomy at the 11th thoracic vertebrae, the exposed spinal cord was contused using the Infinite Horizons Impactor device (Precision Scientific Instrumentation, Lexington, KY, USA). Moderate injuries were made using a force of 50 kdyn, and only animals that had tissue displacements ranging between 400–600 μm were used (20). All surgical procedures were approved by the McGill University Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care.

PLA$_2$ inhibitors

Three types of PLA$_2$ inhibitors were used: 2-oxoamides (AX059 and AX115), fluoroketones (FKGK11 and FKGK2), and an amide (GK115). The 2-oxoamide inhibitors have been extensively characterized (21–23). AX059 is a selective and potent inhibitor of cPLA$_2$ GIVA (**Fig. 1**) and has been used effectively *in vivo* (5, 21–25). AX059 exhibits >95% inhibition of cPLA$_2$ at 0.091 mol fraction, while showing 0% inhibition of iPLA$_2$ and sPLA$_2$ (Fig. 1). Its X_I(50) value, which is the mole fraction of the inhibitor in the total substrate interface required to inhibit the enzyme by 50%, is 0.008 ± 0.002, indicating high potency. FKGK11 is highly selective for iPLA$_2$ GVIA, showing >95% inhibition of iPLA$_2$ at 0.091 mol fraction, as compared to inhibiting only 17% of cPLA$_2$ and 29% of sPLA$_2$. At the high concentration of substrate used for these assays, values ≤25% are not considered significant. Its X_I(50) value (0.0073±0.0007) also indicates that it is a potent inhibitor of iPLA$_2$. FKGK11 has been used effectively *in vivo* (5, 11). The synthesis and details of the inhibition by this and other fluoroketones used in this work have been published previously (26). The novel amide GK115, which was recently characterized (27), is a highly selective inhibitor of sPLA$_2$ (>95% inhibition at 0.091 mol fraction), while showing no inhibition of cPLA$_2$ and iPLA$_2$ (ref. 27 and Fig. 1). It also has a X_I(50) value of 0.003 ± 0.0004, showing high potency (27). In addition, two pan-PLA$_2$ inhibitors were used: one strongly inhibits all three PLA$_2$s (the fluoroketone, FKGK2) while the other partially inhibits all three PLA$_2$s (the 2-oxoamide, AX115). Both these inhibitors (FKGK2 and AX115) have been used previously *in vivo* experiments (5).

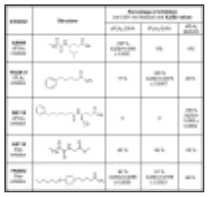

Figure 1.
Structure, selectivity and potency of the PLA, inhibitors used.

PLA$_2$ inhibitor treatment

Mice were given daily intraperitoneal injections of 2-oxoamide (AX059 and AX115), fluoroketone (FKGK11), or amide (GK115), at a dose of 2 mM in 200 μl, starting 1 h after contusion and for 14 d. Control mice that also had SCI were treated daily with vehicle. These doses have been shown to be effective in our previous studies on peripheral nerve injury (11) and experimental autoimmune encephalomyelitis (5).

Western blotting

Protein was extracted from a 5-mm length of spinal cord tissue containing the lesion site harvested at the same time points that were used for the mRNA work. Protein samples (20 μg) were separated on a 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated with antibodies against cPLA$_2$ GIVA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), iPLA$_2$ VIA (Cayman Chemical, Ann Arbor, MI, USA), sPLA$_2$ GIIA (Cayman Chemical), COX-2 (BD Transduction, San Jose, CA, USA), 5-LOX, HPGDS (Cayman Chemical), mPGEs-1 (Cayman Chemical), EP1 (Cayman Chemical), EP2 (Cayman Chemical), and EP4 (Cayman Chemical). Bands were detected using chemiluminescence (Western Lightning Chemiluminescence Reagent Plus; PerkinElmer, Wellesley, MA, USA). β-Actin (Sigma-Aldrich, St. Louis, MO, USA) was used to ensure equal loading of samples. Three samples were used for each time point.

Locomotor assessment

Locomotor recovery was evaluated in an open-field test using the Basso mouse scale (BMS; ref. 28), which was developed specifically for locomotor testing after contusion injuries in mice. The BMS analysis of hind-limb movements and coordination was carried out by 2 independent assessors who were masked to the experimental and control groups, and their consensus score was taken. These individuals were trained in Michele Basso's laboratory (Ohio State University, Columbus, OH, USA). The final scores are presented as means ± SE. The BMS is a compressed scale with a maximum score of 9 as compared to the 21-point BBB scale for rats. Therefore, small differences in the BMS can account for larger functional differences.

Histology

Mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 1, 3, 7, 14, and 28 dpi. A 5-mm length of the spinal cord containing the lesion site was removed, cryoprotected with 30% sucrose in 0.1 M PB, and cut in serial sections (16 μm thick). For double immunofluorescence, sections were incubated with antibodies against cPLA$_2$ GIVA (Santa Cruz Biotechnology), iPLA$_2$ GVIA (Cayman Chemical), or sPLA$_2$ GIIA (Cayman Chemical) and combined with antibodies against Mac-1 (for macrophages/microglia; Serotec, Kidlington, UK), GFAP (for astrocytes; Zymed Labs, Burlingame, CA, USA), CC1 (for oligodendrocytes: Calbiochem, San Diego, CA, USA) and NeuN (for neuron; Chemicon, Temecula, CA, USA). Immunofluorescence labeling for serotonin (5-HT; Sigma-Aldrich) was also performed to assess innervation of serotonergic axons caudal to the lesion. In addition, one series of serial sections of the spinal cord was stained with Luxol fast blue (LFB) histochemistry, which stains myelin, and another series was stained with cresyl violet histochemistry to quantify neuronal loss.

Quantification of histological results

Histological quantification was performed from spinal cord sections harvested at 28 dpi. Tissue sections were viewed with an Axioskop 2 Plus microscope (Zeiss, Oberkochen, Germany), images were captured using a QImaging Retiga 1300 camera (QImaging, Surrey, BC, Canada), and quantification was done using BioQuant Nova Prime image analysis system (BioQuant Image Analysis Corp., Nashville, TN, USA). Tissue sparing was calculated by delineating the GFAP-stained sections. Assessment of myelin sparing was performed by calculating the area occupied by myelin in the lateral white matter. Neuronal survival was assessed by counting the neuron profiles in the ventral horn below the level of the central canal of the spinal cord in tissue sections stained with cresyl violet. Assessment of serotonergic innervation was performed by calculating the area occupied by serotonergic axons in the lateral funiculi and ventral horns of spinal cord sections taken at a distance of 1000 μm caudal to the lesion site.

Statistical analyses

Data are shown as means ± SE. Western blot analyses were done using 1-way ANOVA with *post hoc* Dunnett's test. Statistical analyses of the functional and histological assessments were performed by using 2-way repeated measures ANOVA with *post hoc* Tukey's test for multiple comparisons. Differences were considered significant at values of $P < 0.05$.

RESULTS

Screening of the mammalian PLA$_2$s in SCI

Although a total of 27 mammalian PLA$_2$ have been identified in humans, mice, rats, and bovines, we focused our work on the 14 PLA$_2$s that are found in mice. We first assessed the mRNA expression of the 4 intracellular mammalian PLA$_2$s (cPLA$_2$ GIVA, cPLA$_2$ GIVB, iPLA$_2$ GVIA, iPLA$_2$ GVIB) and 10 secreted PLA$_2$s (sPLA$_2$ GIIA, GIIC, IID, GIIE, GIIF, GV, GVII, GX, GXIIA, GIIB) in the uninjured spinal cord, and at 1, 3, 7, 14, 21 and 28 d after contusion injury. Of these 14 mammalian PLA$_2$s examined, the expressions of cPLA$_2$ GIVB, iPLA$_2$ GVIB, sPLA$_2$ GIIC, IID, GIIE, GIIF, GX, and GXIIA were unchanged, while GV, GVII, and GXIIB were undetectable after SCI (data not shown). The mRNA expressions of cPLA$_2$ GIVA, iPLA$_2$ GVIA, and sPLA$_2$ GIIA, which increased after SCI, were further characterized by Western blotting and immunofluorescence, and their functional roles were assessed.

Expression and role of cPLA$_2$ GIVA in SCI

The activation of cPLA$_2$ protein is correlated with phosphorylation of Ser-505 by MAPKs (4). The higher-molecular-mass band on Western blots that corresponds to phospho-cPLA$_2$ is increased from 3 to 28 dpi (**Fig. 2*A*, *B***). At 3 dpi, cPLA$_2$ is expressed in neurons, particularly the large neurons in the ventral horn, and in some oligodendrocytes located in areas adjacent to the lesion epicenter (Fig. 2*C*) but not in astrocytes or microglia/macrophages.

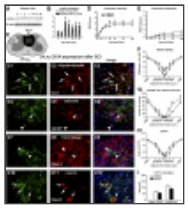

Figure 2.
A) Western blot showing cPLA$_2$ GIVA protein expression after SCI. Samples from uninjured and injured mice show 2 bands corresponding to the phosphorylated (top) and nonphosphorylated (bottom) cPLA$_2$ GIVA forms. *B*) Quantification of protein levels of cPLA ...

The functional role of cPLA$_2$ in SCI was assessed using AX059, a 2-oxoamide compound, which is a highly potent and selective inhibitor for cPLA$_2$ (Fig. 1 and refs. 5, 21–25). Locomotor recovery was assessed using the 9-point BMS, in which a score of 0 indicates complete paralysis, and a score of 9 represents normal locomotion. To our surprise, locomotor recovery was worse in mice treated with AX059 than in vehicle-treated controls (Fig. 2*D*, *E*). Histological analyses revealed that AX059 treatment significantly worsened tissue sparing, neuronal survival, and myelin sparing in areas adjacent to the lesion epicenter (Fig. 2*F–H*). Similar results were also obtained after inducing SCI in cPLA$_2$ GIVA$^{-/-}$ mice (**Fig. 3**). Thus, contrary to other

models of CNS inflammation, such as brain ischemia or EAE, in which $cPLA_2$ has been shown to cause tissue damage (5, 6, 8, 9, 29), our present data indicate a protective role for $cPLA_2$ after SCI.

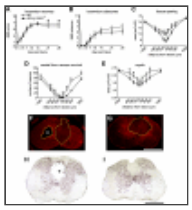

Figure 3.

A, *B*) Time course of locomotor recovery, evaluated using the BMS (*A*) and locomotor BMS subscores (*B*) in $cPLA_2$-null mice. Note that animals lacking $cPLA_2$ GIVA (n=7) show significantly worse motor skills at 21 and 28 d after SCI in the BMS but not in the ...

Expression and role of $iPLA_2$ GVIA in SCI

Quantification of $iPLA_2$ GVIA protein expression by Western blotting showed ~3-fold increase at 14 dpi. $iPLA_2$ has ankyrin-like repeats that negatively control the activity of the enzyme. Removal of these ankyrin-like repeats from the full-length 85-kDa protein results in a smaller 52-kDa form that has enhanced activity (30). We detected the 52-kDa form of $iPLA_2$ after SCI, which increased significantly from 7 to 28 dpi with a peak at 14 dpi (**Fig. 4*A*, *B***). At 14 d, when its expression peaked after SCI, $iPLA_2$ was expressed mainly in oligodendrocytes but also in some astrocytes, microglia/macrophages, and neurons in the injured cord (Fig. 4*C*).

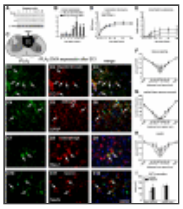

Figure 4.

A) Western blot showing $iPLA_2$ GVIA protein expression after SCI. The activated form (52 kDa) is detected from 7 to 28 dpi. *B*) Quantification of $iPLA_2$ GVIA protein levels from 1 to 28 d after SCI by Western blotting (n=3/time point). The 84-kDa form is ...

We next assessed the role of $iPLA_2$ after SCI using FKGK11, a fluoroketone compound, which is a highly selective and potent $iPLA_2$ inhibitor (Fig. 1 and refs. 5, 11, 26). Although no differences were seen in the BMS scores after FKGK11 administration (Fig. 4*D*), the BMS subscores, which rate finer aspects of locomotor control, revealed a significant improvement at d 28 after SCI (Fig. 4*E*). Inhibiting $iPLA_2$ also resulted in significantly greater tissue and myelin sparing and ~40% increase in serotonergic (5-HT) fibers in the lateral white matter (Fig. 4*F–I*). FKGK11, however, had no effect on neuronal survival (Fig. 4*G*). These data suggest that $iPLA_2$ is likely to have only a minimal detrimental effect after SCI.

Expression and role of $sPLA_2$ GIIA in SCI

$sPLA_2$ GIIA protein was detected at very low levels in the uninjured cord and increased rapidly from 1 to 28 dpi after SCI. Protein levels for $sPLA_2$ were up-regulated from d 1 to 7, and peaked at d 3 (**Fig. 5*A*, *B***). At 3 and 7 dpi, $sPLA_2$ is seen mainly in astrocytes and oligodendrocytes but also in some neurons and microglia/macrophages (Fig. 5*C*).

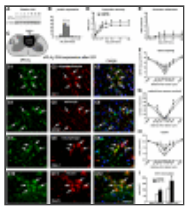

Figure 5.

A) Western blot showing $sPLA_2$ GIIA protein expression after SCI. *B*) Quantification of $sPLA_2$ group GIIA protein levels from 1 to 28 d after SCI, assessed by Western blotting (n=3/time point) shows that expression is significantly increased at 3 and 7 d ...

We next assessed the role of $sPLA_2$ after SCI using the novel amide inhibitor GK115, which is highly selective for $sPLA_2$ (Fig. 1 and ref. 27). Daily administration of GK115, starting 1 h after injury, significantly improved locomotor function as evaluated by the BMS score, beginning at 5 dpi until d 28, the longest time period examined (Fig. 5*D*). In addition, BMS subscores also improved significantly with the GK115 treatment (Fig. 5*E*). In contrast to these experiments done on BALB/c mice, the sPLA2 inhibitor GK115 did not have any effect on locomotor recovery after SCI in C57BL/6 mice (Supplemental Fig. S1), which have a naturally occurring null mutation of $sPLA_2$GIIA (31). The latter evidence provides additional support that $sPLA_2$ GIIA has detrimental effects after SCI. Histologically, treatment with GK115 resulted in significant tissue protection (Fig. 5*F*) and myelin sparing (Fig. 5*H*) at the lesion epicenter and in adjacent areas. GK115 treatment also led to significantly greater neuronal survival in the ventral horn 500 μm rostral and caudal to the lesion epicenter (Fig. 5*G*), and a 50% increase in serotonergic fibers in the lateral white matter and ventral horns (Fig. 5*I*).

We also tested the effects of a 2-oxoamide compound, AX115 (Fig. 1), which is a partial pan-inhibitor of $cPLA_2$, $iPLA_2$, and $sPLA_2$ (5, 27). We found that treatment with AX115 after SCI markedly enhanced locomotor recovery and the various histopathological outcome measures examined, including tissue, myelin, and neuronal sparing, and greater serotonergic innervation (**Fig. 6**). The effects of AX115 are greater than those seen with GK115 ($sPLA_2$ inhibitor) or FKGK11 ($iPLA_2$ inhibitor). This finding suggests that AX115 may not be mediating its effects entirely by partially blocking $sPLA_2$ or $iPLA_2$ but also *via* some other effects (see below). Interestingly, treatment of SCI with FKGK2, a potent fluoroketone pan-PLA_2 inhibitor that almost completely blocks all three PLA_2s (Fig. 1 and refs. 5, 26), had detrimental effects in SCI, causing worsening of locomotor recovery, increased tissue damage, and worsening of other histological parameters (**Fig. 7**).

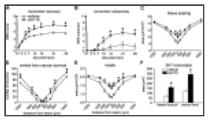

Figure 6.
A) Treatment with AX115 (*n*=9) shows marked improvement in the BMS scores starting from 5 d after SCI as compared to vehicle-treated mice (*n*=9). *B*) AX115-treated mice also display a marked improvement in the finer aspects of locomotor control, showing ...

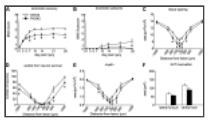

Figure 7.
Effects of treatment with the strong pan-PLA_2 inhibitor (FKGK2) on locomotor and histological changes after SCI. *A*) Locomotor recovery evaluated using the BMS analysis shows that FKGK2-treated mice have markedly poor locomotor recovery starting as early ...

Potential crosstalk between PLA_2s mediates protection after SCI *via* the prostaglandin EP1 receptor

As AX115 was most effective in promoting locomotor recovery and neuroprotection, we sought to assess potential mechanisms by which it could mediate these effects. Because of evidence of crosstalk between different forms of PLA_2 (4, 32), we first assessed whether AX115 modulates the protein levels of $cPLA_2$, $iPLA_2$, and $sPLA_2$. After contusion injury, mice were treated with AX115, and the spinal cords were taken at 3 and 7 dpi for Western blot analysis for PLA_2s and their downstream enzymes. Interestingly, the protein level of $cPLA_2$ but not $iPLA_2$ or $sPLA_2$ is increased in the injured spinal cord of mice treated with AX115 at 3 dpi (**Fig. 8*A***, ***B***). We next examined the expression of two enzymes downstream of $cPLA_2$, namely cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX). The expression of COX-2, but not 5-LOX, was increased in AX115-treated mice at 3 dpi (Fig. 8*A*, *B*), suggesting higher prostaglandin (PG) production in the spinal cord of mice treated with AX115. We therefore assessed the expression of the synthases that regulate prostaglandin E_2 and D_2 expression, namely microsomal-PGE_2 (mPGEs-1) and hematopeitic-PGD_2 synthase (HPGDS). We found that mPGEs-1, but not HPGDS, is increased in the lesioned spinal cord of AX115-treated mice at 3 dpi (Fig. 8*A*, *B*), which suggests higher PGE_2 in the spinal cord of mice treated with AX115. As PGE_2 acts on 4 G-protein receptors (EP1–4), we assessed whether any changes occurred in the expression at the protein level of the three EP receptors, which showed increases in mRNA after SCI (EP1, EP2, and EP4; data not shown). The injured spinal cord showed very high levels of expression of EP1 but very low levels for EP2 and EP4. Furthermore, treatment with AX115 resulted in increased protein levels for EP1 at 7dpi (Fig. 8*A*, *B*), while levels for EP2 and EP4 did not change (data not shown). These data suggest that the protective effects of AX115 in SCI may be mediated, at least in part, *via* PGE_2 and EP1. To confirm these observations, we next assessed the effects of AX115 in combination with an EP1 antagonist (SC51089) on locomotor recovery after SCI. The beneficial effect of AX115 on locomotor control was blocked when EP1 was inhibited (Fig. 8*C*, *D*). EP1 antagonist treatment alone did not cause any change in locomotor recovery (data not shown). As the free acid metabolite of AX115 inhibits $sPLA_2$, these results suggest that $sPLA_2$ may negatively regulate $cPLA_2$ expression and modulate further downstream pathway (COX-2, mPGEs-1 and EP1) that mediate protection after SCI.

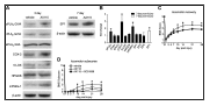

Figure 8.
A, *B*) Effect of AX115 on the pathways downstream of PLA_2 after SCI. *A*) Western blots of spinal cord tissue taken 3 and 7 d after SCI. *B*) Western blot analysis of spinal cord tissue taken 3 d after SCI (*n*=3/time point) shows that in mice treated with AX115, ...

DISCUSSION

In this study, we have characterized the expression and role of different members of the PLA_2 superfamily in SCI. We show that of the 14 PLA_2 forms expressed in mice, only $cPLA_2$ GIVA, $iPLA_2$ GVIA, and $sPLA_2$ GIIA are up-regulated after SCI. We also provide evidence that these three PLA_2 forms have differing roles in SCI.

Phospholipase A_2s play important roles in several inflammatory conditions in the central and peripheral nervous system (5, 6, 8, 10, 11). One way this enzyme can play a role in inflammation is through the release of fatty acids from phospholipids, such as the release of arachidonic acid, a precursor of proinflammatory eicosanoids. The principal pathways of arachidonic acid metabolism are the lipoxygenase pathway, which produces a collection of leukotrienes, and the cyclooxygenase pathway that produces prostaglandins and thromboxanes (33). Several lines of evidence suggest an involvement

of arachidonic acid and its metabolites in the pathophysiology of SCI. Intravenous administration of arachidonic acid increased functional deficits after SCI (34). In addition, lipoxygenase gene deletion or administration of selective COX-2 inhibitors resulted in enhanced locomotor recovery and tissue sparing after SCI (35–38). However, these interventions block only some parts of the pathway downstream of arachidonic acid. In addition, one of the metabolites of PLA_2 action is the production of lysophophatidylcholine a potent demyelinating agent and one capable of inducing proinflammatory chemokine and cytokine expression (12, 13). Therefore, blocking the appropriate PLA_2s rather than one of the downstream enzymes or metabolites may be a more effective therapeutic target for SCI.

Two groups have attempted previously to assess the role of PLA_2 in SCI. One reported that when a nonmammalian form of PLA_2 found in bee venom ($sPLA_2$ GIII) was injected directly into the normal uninjured rat spinal cord, it caused demyelination, tissue damage, and functional impairment (16, 39). The researchers also showed similar effects with intraspinal injection of melittin into the normal uninjured spinal cord. Melittin is the principal active component of bee venom and is a stimulator of secreted phospholipase A_2. In other work, they also showed in cell culture that recombinant human $sPLA_2$ GIIA induced a dose-dependent cytotoxicity of oligodendrocyte precursors (17). Another group assessed the effects of arachidonyl trifluoromethyl ketone ($AACOCF_3$), a nonselective PLA_2 inhibitor, for 1 wk after SCI in rats and reported a modest effect on locomotor recovery and reduction of neuronal and oligodendrocyte loss (18). As mentioned previously, $AACOCF_3$ is a nonselective inhibitor that blocks both $cPLA_2$ and $iPLA_2$ (19). Our work now shows that $cPLA_2$ and $iPLA_2$ play divergent roles in SCI.

Among the PLA_2 superfamily members, $cPLA_2$ shows the strongest preference for arachidonic acid at the sn-2 position in phospholipids. $cPLA_2$-null mice fail to generate arachidonic acid metabolites after brain injury (8, 9). We also showed previously that the $cPLA_2$ inhibitor, AX059, blocked the hydrolysis of arachidonic acid from phospholipids in EAE (5). Several studies have reported a deleterious role for $cPLA_2$ in CNS ischemia (8, 9), EAE (5–7), and Alzheimer disease (40). In contrast to the detrimental effect of $cPLA_2$ in these neuroinflammatory conditions, in the present study we show the opposite in SCI, namely, that $cPLA_2$ plays a beneficial role. Our data revealed that mice treated with AX059, a selective and potent inhibitor for $cPLA_2$, as well as $cPLA_2$-null mice, showed greater locomotor deficits and tissue loss after SCI. The $cPLA_2$ inhibitor and $cPLA_2$-null mice line used here were also used in the earlier studies on EAE (5) and brain ischemia (9), respectively, pointing to the striking difference in the role of $cPLA_2$ in cerebral ischemia, EAE and SCI. We found that $cPLA_2$ was expressed in neurons and oligodendrocytes in the spinal cord after injury, which is in agreement with an earlier report showing $cPLA_2$ expression in neurons and oligodendrocytes in rats after SCI (16). We found that $cPLA_2$-null mice and wild-type mice treated with AX059 displayed greater neuronal and myelin loss after injury, suggesting that inhibition or deletion of $cPLA_2$ make these cells more vulnerable. Thus, contrary to other models of CNS disorders in which $cPLA_2$ contributes to tissue damage (6, 8, 9, 29), our present data indicate a protective role for $cPLA_2$ after SCI. Interestingly, $cPLA_2$ has previously been shown to be required for the survival of cortical and hippocampal neurons *in vitro* (41) and to exert a protective role in autoimmune diabetes in mice (42) and in human embryonic kidney cells after calcium overexposure (43).

Another novel finding of this study is that $iPLA_2$ GVIA is up-regulated after SCI. We recently reported that $iPLA_2$ is involved in the onset and progression of EAE and that treatment with FKGK11, a potent and highly selective $iPLA_2$ inhibitor, ameliorated demyelination and progression of the disease (5). We show here using the same $iPLA_2$ inhibitor that $iPLA_2$ appears to play a minor detrimental role in SCI. Although mice treated with FKGK11 showed only an effect in the locomotor BMS subscore, the histological outcomes showed clear improvements as compared to controls. Our data also revealed that $iPLA_2$ is expressed mainly in oligodendrocytes after lesion and that treatment with FKGK11 reduced myelin loss after SCI. The molecular mechanisms involved in $iPLA_2$ cytotoxicity are not known at present. However, Lauber *et al.* (30) showed that LPC generated by the actions of the lower-molecular-mass forms of $iPLA_2$ (52 kDa), but *not* via $cPLA_2$, acts as an “eat me” signal for macrophages. In the present study, we observed the expression of the 52-kDa form of $iPLA_2$ starting at 7 dpi, which may suggest that LPC might also be generated by $iPLA_2$ after SCI. Evidence indicates that LPC, which acts as a potent demyelinating agent (12, 13, 44), also induces the expression of chemokines, cytokines, and cell adhesion molecules that are important for the recruitment and activation of immune cells into the CNS (12–15). Our recent study on the role of intracellular PLA_2s in myelin breakdown and Wallerian degeneration after sciatic nerve injury revealed that blocking $iPLA_2$ with the FKGK11 inhibitor reduces macrophage recruitment and myelin clearance, and lowers the expression of proinflammatory cytokines (11). In addition, LPC has high affinity to C-reactive protein and IgM antibodies leading to the activation of the complement pathway (10), which is involved in secondary damage after SCI (45). Although further studies are needed to fully elucidate the detrimental effects of $iPLA_2$, these data suggest that LPC generated by the lower-molecular-mass forms of $iPLA_2$ might be responsible for demyelination after SCI. Therefore, $iPLA_2$, in contrast to $cPLA_2$, is likely to contribute to some of the secondary damage in SCI. This finding may also explain the modest effect reported previously after treatment with AACOCF3 in SCI in rats (18). AACOCF3 is a nonselective inhibitor that blocks both cPLA2 and $iPLA_2$ (19). Our results suggest that $AACOCF_3$ would block not only the detrimental effects of iPLA2 but also the beneficial effects of $cPLA_2$.

We found that the expression of $sPLA_2$ GIIA is increased at the protein level by Western blot analysis between d 1 and 7 after lesion. Our data now provide the first direct evidence that $sPLA_2$ GIIA that is up-regulated in the injured mouse spinal cord contributes to secondary damage and functional loss after SCI. This was shown with the treatments with the $sPLA_2$ selective inhibitor GK115 on BALB/c mice. In addition, this inhibitor did not have any effect on locomotor recovery after SCI in the C57BL/6 mouse strain, which has a naturally occurring null mutation of sPLA2 GIIA (31).

An important finding is that the inhibitor that partially blocks all three PLA2s at about the 50% level (AX115) yielded the most beneficial effects in enhancing tissue protection and neuronal, myelin, and axonal sparing, as well as locomotor recovery after SCI. Interestingly, although AX115 partially blocks $sPLA_2$ and $iPLA_2$ activity, it also appeared to enhance the expression of $cPLA_2$, which we have shown has protective or beneficial effects in SCI. However, complete blocking of all three PLA_2s by FKGK2 was detrimental. Our studies revealed that AX115 led to an up-regulation of $cPLA_2$, which is beneficial in SCI. Previous studies in rat ovalbumin-induced bronchoconstriction have shown that $sPLA_2$ can negatively regulate the expression of $cPLA_2$ (32). Our results with AX115 may, therefore, be due at least in part to its ability to block $sPLA_2$, which negatively regulates $cPLA_2$ expression. We also found the $cPLA_2$ downstream pathway involved in AX115-mediated protection was dependent on COX-2, mPGES-1, and EP1 receptor signaling, since the beneficial effects of AX115 were blocked with an EP1 antagonist. In agreement with these results, a previous work also showed that the protective effects of $cPLA_2$ observed in autoimmune diabetes were also dependent on PGE_2 (42). Moreover, the protective effects of $cPLA_2$ in human embryonic kidney cells to calcium overexposure were also dependent on prostaglandins (43). In contrast, in a model of cerebral ischemia, $cPLA_2$ and PGE_2 *via* EP1 receptor signaling exacerbates tissue damage (46, 47), pointing to the diversity of biological effects of the $cPLA_2$-PGE_2 pathway in different CNS injuries.

In summary, our results show that 3 members of the PLA_2 superfamily ($cPLA_2$ GIVA, $iPLA_2$ GVIA, and $sPLA_2$ GIIA) play divergent roles in SCI. Our data reveal that the different PLA_2s have distinct and even opposite effects in SCI. Our work also suggests that drugs such as AX115 that inhibit detrimental forms of PLA_2 ($sPLA_2$ and $iPLA_2$) and up-regulate protective ones ($cPLA_2$) may be useful candidates for the treatment of acute SCI.

Supplementary Material

Supplemental Data:
Click here to view.

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Footnotes

This article includes supplemental data. Please visit http://www.fasebj.org to obtain this information.

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Rubèn López-Vales,[*†] Nader Ghasemlou,[*] Adriana Redensek,[*] Bradley J. Kerr,[*] Efrosini Barbayianni,[‡] Georgia Antonopoulou,[‡] Constantinos Baskakis,[‡] Khizr I. Rathore,[*] Violetta Constantinou-Kokotou,[§] Daren Stephens,[#] Takao Shimizu,[**] Edward A. Dennis,[#] George Kokotos,[‡] and Samuel David[*,1]

[*]Center for Research in Neuroscience, Research Institute of the McGill University Health Center, Montreal, Quebec, Canada;
[†]Departament de Biologia Cel.lular, Fisiologia i Immunologia, Institut de Neurociències, Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Universitat Autònoma de Barcelona, Bellaterra, Spain;
[‡]Department of Chemistry, University of Athens, Panepistimiopolis, Athens, Greece;
[§]Chemical Laboratories, Agricultural University of Athens, Athens, Greece;
[#]Department of Chemistry and Biochemistry, School of Medicine, University of California–San Diego, La Jolla, CA, USA; and
[**]Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Tokyo, Tokyo, Japan
[1] Correspondence: Center for Research in Neuroscience, McGill University Health Center Research Institute, Livingston Hall, Rm. L7-210, 1650 Cedar Ave., Montreal, Québec, Canada, H3G 1A4. E-mail: sam.david@mcgill.ca

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