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Cytosolic Phospholipase A_2 Plays a Key Role in the Pathogenesis of Multiple Sclerosis-like Disease

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Summary

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that results in motor and sensory deficits. Although MS and its animal model, experimental autoimmune encephalomyelitis (EAE), are thought to be T cell-mediated diseases, the mechanisms underlying the lesions in the CNS are not fully understood. We propose that a strong candidate as a central mediator in evoking the complex pathological changes seen in MS and EAE is the enzyme cytosolic phospholipase A_2 ($cPLA_2$). One of the metabolic products of this enzyme is pro-inflammatory, while the other induces myelin breakdown, demyelination, and chemokine/cytokine expression. We provide evidence that $cPLA_2$ is highly expressed in EAE lesions and show that blocking this enzyme leads to a remarkable reduction in the onset and progression of EAE.

Introduction

Multiple sclerosis is among the most common neurological diseases of young adults (Compston and Coles, 2002). It is thought to involve an autoimmune response whereby myelin-reactive T cells enter the CNS and initiate the disease (Compston and Coles, 2002; Noseworthy et al., 2000; Steinman et al., 2002; Hemmer et al., 2002). The etiology and pathogenesis of this disease are still not fully understood. Although various factors may cause MS, they appear to trigger a common mechanism because the resulting pathology, i.e., immune cell infiltration into the CNS and changes in the CNS leading to demyelination, are similar. Identification of such a common mechanism would help in understanding the pathogenesis of MS and also permit the development of more effective treatment strategies.

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory, demyelinating disease that can be induced by immunizing animals against myelin antigens (Johns et al., 1995; Owens and Sriram, 1995; Slavin et al., 1998; Steinman, 1999). It has been widely used as an animal model for MS as it has similarities based on both the histopathology and the clinical course of the affected animals (Owens and Sriram, 1995; Steinman, 1999). During an attack, there is perivascular infiltration of myelin-reactive T cells into the CNS, which leads to macrophage influx and activation facilitated by an increased permeability of the blood-brain barrier (Compston and Coles, 2002; Noseworthy et al., 2000; Steinman et al., 2002; Hemmer et al., 2002; Owens and Sriram, 1995). A number of pro-inflammatory chemokines and cytokines such as CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CCL7/MCP-3, CXCL9/Mig, CXCL10/IP-10, M-CSF, TNF-α, IL-1α, IL-1β, IL-2, IL-6, IL-12, IFNγ, and GM-CSF are produced by these immune cells and CNS glia and are expressed in the CNS in MS and EAE (Bettelli and Nicholson, 2000; Carmody et al., 2002; Gerard and Rollins, 2001; Glabinski et al., 2003; Godessart and Kunkel, 2001; Godiska et al., 1995; Hulkower et al., 1993; Ibrahim et al., 2001; Lock et al., 2002; Owens and Sriram, 1995; Rajan et al., 2000; Ransohoff, 2002). These factors initiate a pathogenic cascade in the CNS leading to inflammation, demyelination, and axonal damage, which contribute to the functional deficits.

A molecule that could serve as a central mediator of the pathology in MS and EAE is the enzyme phospholipase A_2 (PLA_2). The expression or activation of PLA_2 can be induced by many of the chemokines and cytokines mentioned above such as CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES, CCL7/MCP-3, M-CSF, TNF-α, IL-1α, IL-β, IFNγ, and GM-CSF (Murakami et al., 1997; Locati et al., 1994, 1996). It consists of a family of phospholipid hydrolyzing enzymes whose metabolic products mediate both inflammation and demyelination (Murakami et al., 1997; Dennis, 1994; Ousman and David, 2000). PLA_2 enzymes play a normal physiological role in phospholipid metabolism, host defense, and signal transduction (Brown et al., 2003). They hydrolyze an ester bond at the *sn*-2 position of phospholipids that generates a free fatty acid such as arachidonic acid (AA) and a lysophospholipid such as lysophosphatidylcholine (LPC) (Murakami et al., 1997; Dennis, 1994). Arachidonic acid can give rise to eicosanoids via cyclooxygenase (COX-1 and 2) and 5-lipoxygenase (5-LO) enzymes. Eicosanoids such as prostaglandins, thromboxanes, and leukotrienes are potent mediators of inflammation by increasing vascular permeability and inducing chemotaxis of immune cells (Dennis et al., 1991). In addition, LPC is a myelinolytic agent and can act as a chemoattractant for immune cells (Ousman and David, 2000; Ryborg et al., 1994, 2000). Injection of LPC into the spinal cord causes demyelination as well as inducing the expression of a number of chemokines and cytokines (Ousman and David, 2000, 2001). An abnormally high expression of PLA_2 in the CNS could therefore lead to inflammation and demyelination.

PLA_2 could serve as a convergence point in the induction of MS and EAE pathology because it can be induced by a variety of chemokines and cytokines present in the CNS in the early stages of these diseases and because its metabolic products mediate both inflammation and demyelination. PLA_2 enzymes fall broadly into two main groups: secreted ($sPLA_2$) and cytosolic ($cPLA_2$). The secreted form is a low molecular weight form (14 kDa) that has no preference for the type of fatty acid at the *sn*-2

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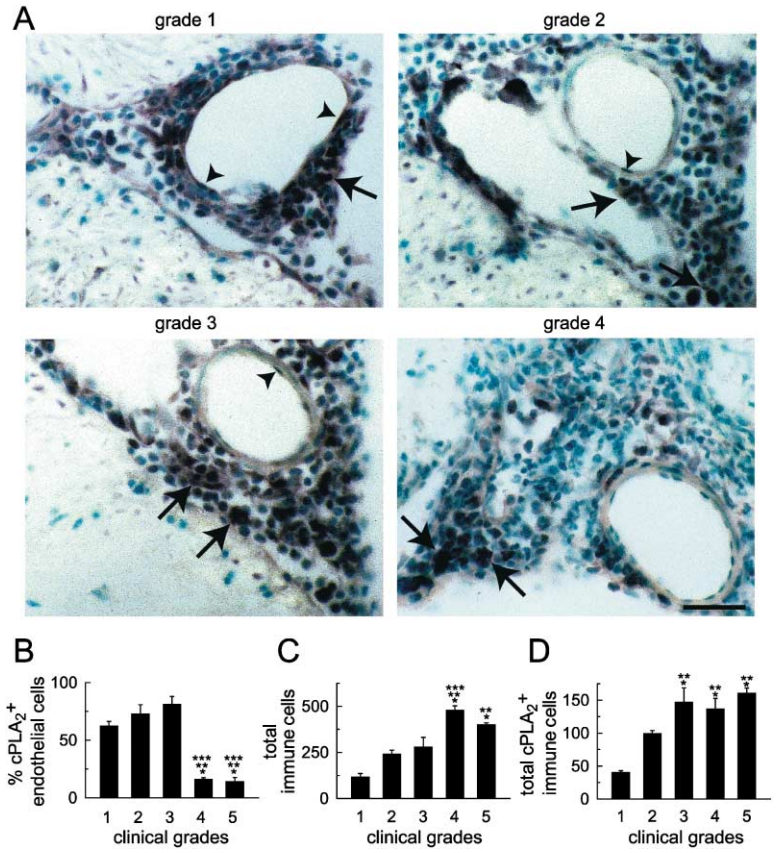

Figure 1. $cPLA_2$ Is Expressed in EAE Lesions

(A) Representative sections of lumbar spinal cord of mice with EAE at clinical grades 1–4 from the first paralytic episode are shown. $cPLA_2^+$ endothelial cells are seen in grades 1–3 indicated by arrowheads. Endothelial cells that do not express $cPLA_2$ are seen in grade 4. $cPLA_2^+$ immune cell clusters in perivascular locations are seen at all clinical grades, indicated by arrows. Scale bars equal 30 μm.

(B) Changes in the percentage of $cPLA_2^+$ endothelial cells in all EAE lesions in cross-sections of the lumbar spinal cord. High percentage of endothelial cells express $cPLA_2$ during the earlier stages of the first attack (grades 1 to 3) and was reduced at grades 4 and 5 (*grade 1 versus 4 and 5, $p < 0.003$; **grade 2 versus 4 and 5, $p < 0.01$; ***grade 3 versus 4 and 5, $p < 0.01$).

(C) Changes in the total number of immune cells in all EAE lesions in cross-sections of the lumbar spinal cord. The number of immune cells in EAE lesions increases at later clinical grades of 4 and 5 (*grade 1 versus 4 and 5, $p < 0.0005$; **grade 2 versus 4 and 5, $p < 0.004$; ***grade 3 versus 4, $p < 0.02$).

(D) Changes in the number of $cPLA_2^+$ immune cells in all EAE lesions in cross-sections of the lumbar spinal cord. The number of $cPLA_2^+$ immune cells in EAE lesions of the first paralytic episode also increases at later clinical grades (*grade 1 versus 3, 4, and 5, $p < 0.04$, 0.02, 0.001, respectively; **grade 2 versus 3, 4, and 5, $p < 0.01$).

position of phospholipids (Murakami et al., 1997; Dennis, 1994). Members of the cytosolic form have a higher molecular mass (85–110 kDa) and selectively hydrolyze phospholipids containing AA (Murakami et al., 1997; Dennis, 1994). Certain mouse strains, such as C57BL/6, 129/Sv, and B10.rIII, have a naturally occurring null mutation of the major form of $sPLA_2$ (Kennedy et al., 1995). We therefore assessed the expression of $cPLA_2$ in EAE lesions and the effects of inhibiting this enzyme on the onset and progression of EAE in C57BL/6 mice.

Results

$cPLA_2$ Is Expressed in EAE Lesions

We first assessed the expression of $cPLA_2$ in the spinal cord of C57BL/6 mice with EAE. If PLA_2 plays a role in MOG-induced EAE in C57BL/6 mice, it has to be mediated mainly by $cPLA_2$, because this strain lacks the major form of $sPLA_2$. Increased expression of $cPLA_2$ was observed by immunohistochemistry at the site of EAE lesions. This increased expression was assessed in cervical, thoracic, and lumbar regions of the spinal cord of mice at clinical grades of 1–5 during their first paralytic attack. The immunohistochemical labeling for $cPLA_2$ occurred in both endothelial cells and immune cells in the CNS inflammatory infiltrates (Figure 1A). A high percentage of $cPLA_2^+$ endothelial cells were seen in the spinal cord early in the course of the disease, ranging from about 60%–85% in grades 1–3 and decreased to about 20% at clinical grades 4 and 5 (Figure 1B). The percentage of $cPLA_2^+$ immune cells in EAE lesions remained at ~30%–50% in various clinical grades at all levels of the spinal cord. However, since the total number of immune cells in the infiltrates increases with increasing clinical grade (Figure 1C), the total number of immune cells expressing $cPLA_2$ also increases with increasing severity of the disease (Figure 1C). The high $cPLA_2$ expression seen in endothelial cells at the early clinical grades (Figure 1C) precedes the largest increase in immune cell entry into the CNS seen at more severe clinical grades (Figure 1C). Furthermore, mice that had reached a grade 3 and remitted to a grade 1 did not have any $cPLA_2^+$ immune cells or endothelial cells in the spinal cord (data not shown).

Double-immunofluorescence labeling indicates that both $CD4^+$ T cells and Mac-1^+ macrophages express $cPLA_2$ in these immune cell infiltrates (Figures 2B and 2C). The number of $CD4^+$ T cells expressing $cPLA_2$ in lesions remains the same throughout the different clinical grades (Figure 2C), whereas the number of macrophages expressing $cPLA_2$ in EAE lesions increases with disease severity (Figure 2). Collectively, these data indicate that $cPLA_2$ is expressed in the CNS very early during the clinical course of EAE, may contribute to the influx of inflammatory cells into the CNS, and may underlie the pathological and clinical changes characteristic of EAE.

Blocking $cPLA_2$ Prevents Onset and Progression of EAE

To assess if $cPLA_2$ is important for the onset of EAE, we blocked it using an AA analog, arachidonyl trifluromethyl ketone ($AACOCF_3$), which is a potent and selective inhib-

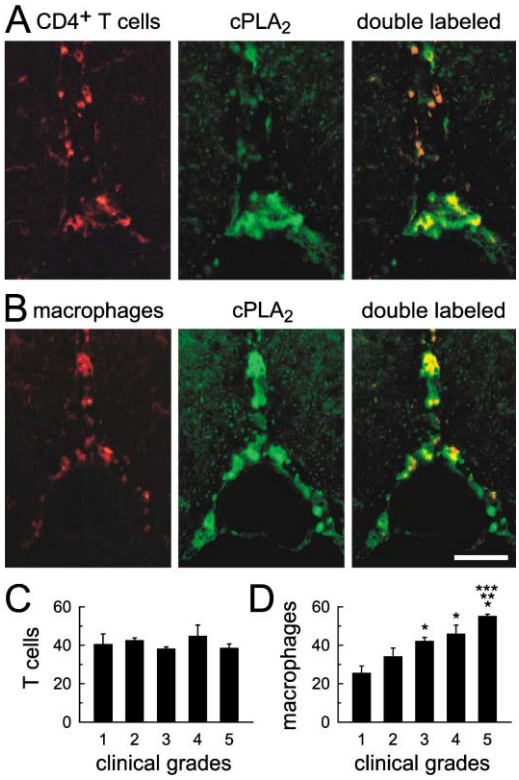

Figure 2. Immune Cell Types Expressing cPLA$_2$

(A) Double-immunofluorescence micrographs showing CD4$^+$ T cells (red, left) expressing cPLA$_2$ (green, middle) and the double labeled cells (yellow, right) in EAE lesions.

(B) Mac-1$^+$ macrophages (red, left) expressing cPLA$_2$ (green, middle) and double labeled cells (yellow, right) in EAE lesions in the lumbar spinal cord. Scale bar equals 100 μm.

(C) Graph showing changes in the mean number of CD4$^+$ T cells in five randomly selected EAE lesions. There were a similar number of CD4$^+$ T cells expressing cPLA$_2$ in the infiltrates at all clinical grades.

(D) Graph showing changes in the number of cPLA$_2^+$ macrophages in five randomly selected EAE lesions. The number of cPLA$_2^+$ macrophages in these lesions increased progressively from grade 1 to grade 5 (*grade 1 versus 3, 4, and 5, $p < 0.03$, 0.03, 0.01, respectively; **grade 2 versus 5, $p < 0.03$; ***grade 3 versus 5, $p < 0.01$).

itor of cPLA$_2$ (Street et al., 1993). This inhibitor shows slow tight binding to cPLA$_2$ in the presence of Ca^{2+} and forms a covalent bond with a serine residue in the active site of the enzyme (Street et al., 1993; Trimble et al., 1993). It is about 500-fold more potent at inhibiting cPLA$_2$ than sPLA$_2$ (Street et al., 1993). It may also be a weak inhibitor of the calcium-independent form of PLA$_2$ (Riendeau et al., 1994; Ackermann et al., 1995; Ghomashchi et al., 1999). Arachidonyl analogs have been shown to be relatively nontoxic to cells (Riendeau et al., 1994). C57BL/6 mice were treated with AACOCF$_3$ on the day of induction of EAE and on day 2 with 50 μl of 2 or 4 mM AACOCF$_3$ intravenously, followed by intraperitoneal injections of the inhibitor (200 μl at 2 or 4 mM) on alternate days until day 24. Treatment with the inhibitor resulted in a remarkable reduction in the onset and progression of EAE. 100% of the control mice got EAE, while 57% of the 2 mM treated and only 28% of the 4 mM treated groups got EAE (Table 1). The progression of the disease was also markedly reduced in the treated mice. Control animals reached an average maximum clinical score of about 3 at day 15, while the 2 mM and 4 mM treated groups reached scores of 1.3 and 0.4, respectively (Figure 3). When only the animals that got EAE symptoms in the 2 mM and 4 mM treated groups were considered, they reached a mean maximal grade of 2.25 and 1.5, respectively, on day 15, compared to a 2.9 seen in the control group (Table 1), indicating that the small percentage of mice treated with 4 mM AACOCF$_3$ that got EAE showed a remarkably milder form of the disease. Unlike the controls, which relapsed into a second paralytic episode between days 25 and 34, mice treated with 4 mM AACOCF$_3$ remained largely unaffected (Figure 3, Table 1). The treatment is well tolerated in that the animals did not show any observable side effects. These data suggest that the inhibitor treatment is well tolerated and can prevent disease when given before symptoms occur.

To examine the effects of the inhibitor treatment at the protein level, we first assessed the expression of cPLA$_2$ and one of the downstream enzymes, COX-2, in the spinal cords of untreated EAE mice that reached a clinical score of grade 3 on day 13; mice treated with AACOCF$_3$ that were also sacrificed on day 13 in which the disease was prevented; and normal healthy control mice. Cytosolic PLA$_2$, seen as an 85 kDa band on Western blots, was elevated in untreated EAE mice as compared to normal controls (Figure 4A). COX-2, a 72 kDa enzyme downstream of cPLA$_2$ that is responsible for the production of prostaglandins and thromboxanes, was also elevated in untreated mice with EAE as compared to normal controls (Figure 4A). In contrast to untreated mice with EAE, both cPLA$_2$ and COX-2 are present at much reduced levels in AACOCF$_3$-treated EAE mice (Figure 4A). These results were consistently seen in three separate experiments.

To further assess the effects of cPLA$_2$ inhibitor treatment on gene expression in the spinal cord of EAE mice, we carried out a gene array analysis of 67 genes for chemokines and their receptors and 96 cytokine genes using arrays from Superarray Bioscience Corp (Frederick, MD). This analysis was carried out on spinal cords of untreated EAE mice with a clinical score of grade 3 on day 13, mice from the inhibitor treated group also sacrificed on day 13 in which the disease was prevented, and normal healthy control mice (Figure 4B). Ten of the cytokine genes and 26 of the chemokines and their receptors were increased >2-fold in mice in the untreated EAE group as compared to normal controls (Tables 2 and 3). In the cPLA$_2$ inhibitor-treated mice, the expression of 80% of these genes returned to normal levels, while 14% were reduced but still remained higher than in normal controls (these include CCR5, CXCR4, CCL19/MIP-3β, Dfy, and angiopoietin 2), and 6% were similar to the EAE levels (CXCL12/SDF-1 and CX$_3$CL1/fractalkine) (Figures 4C and 4D). Many of the chemokine/chemokine receptor and cytokine genes that were elevated in untreated EAE mice are those that have already been implicated in the pathogenesis of EAE (Table 2). Interestingly, several genes not previously implicated in EAE (Table 3) increased in expression substantially in EAE mice. Some of these have or are likely to have biological effects on immune cells and so are possible

Table 1. Clinical Characteristics of Mice Induced with EAE and Treated with the $cPLA_2$ Inhibitor $AACOCF_3$

	n	% Incidence	Mean Day of Disease Onset	Mean Clinical Grade of All Animals at Day 15	Mean Clinical Grade of Only Animals with Disease at Day 15	% Relapse by Day 26	Mean Clinical Grade of All Animals at Day 60	Mean Clinical Grade of Only Animals with Disease at Day 60
Untreated control	9	100% (9/9)	9.6 ± 0.6	2.9 ± 0.2	2.9 ± 0.2	88.9% (8/9)	1.8 ± 0.2	1.8 ± 0.2
Treated: 2 mM $AACOCF_3$	14	57% (7/14)	10.3 ± 0.3	1.3 ± 0.3	2.3 ± 0.4	21.4% (3/14)	1.1 ± 0.2	1.6 ± 0.2
p value		0.01	NS	0.001	NS	0.004	0.03	NS
Treated: 4 mM $AACOCF_3$	14	28% (4/14)	10.7 ± 1.0	0.4 ± 0.30	1.5 ± 0.6	7.1% (1/14)	0.2 ± 0.1	0.8 ± 0.3
p value		0.003	NS	0.0001	0.01	0.001	0.0001	0.005

Animals were given treatment at the time of EAE induction until day 24. Data represent mean ± SEM. p values compared with untreated control. NS, not significant.

new candidates in EAE pathology (Table 3). Others are not known to have their primary effects on the immune system, and their role in EAE remains to be determined. The gene for fibroblast growth factor-12 (FGF-12) was highly expressed in normal controls and was reduced to <0.5-fold in both untreated and treated EAE groups. A few other genes that may have anti-inflammatory roles (CXCR1, IFNα [gene6, gene7, and gene B], erythropoietin, pleiotrophin, TGFβ2, and TGFβ3) (Agnello et al., 2002; Racke et al., 1993; Subramanian et al., 2003; Liu et al., 1998) were also downregulated in the untreated EAE mice and their levels remained lower than normal in the inhibitor-treated mice. Of particular interest is the elevation of LTB_4R2, the receptor for LTB_4, a metabolic product of $cPLA_2$. The differences in expression of a select number of chemokine and cytokine genes (CCL3/MIP-1α, CXCL9/Mig, CXCL10/IP-10, IFN-γ, and M-CSF) that was detected with the gene array screen was further confirmed by RT-PCR (Figure 4E). Taken together, these findings suggest that inhibiting $cPLA_2$ in mice with EAE leads to a decrease in many of the mediators of inflammation such as COX-2, LTB_4R2, and pro-inflammatory chemokines and cytokines, many of which (namely CCL3/MIP-1α, CCL7/MCP-3, M-CSF, IL-1α, IL-1β, and IFN-γ) are known to induce the expression of $cPLA_2$ (Locati et al., 1994, 1996; Murakami et al., 1997) or to be induced by its metabolic product, LPC (Ousman and David, 2001).

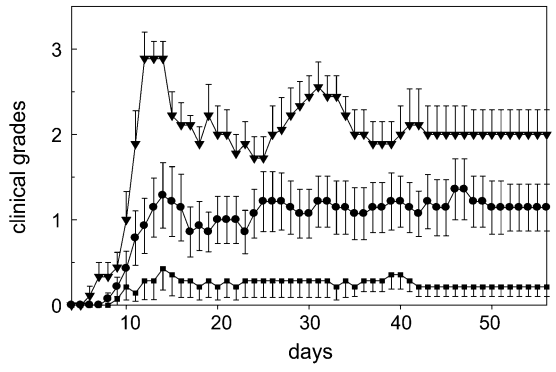

Figure 3. $cPLA_2$ Inhibitor Prevents Onset and Progression of EAE

Graph showing the clinical course of mice induced with EAE that were treated with two doses of $cPLA_2$ inhibitor (closed circle, 2 mM $AACOCF_3$; closed square, 4 mM $AACOCF_3$) from day 0 to 24 compared to an untreated EAE control (closed down triangle). Data represent means ± SEM from two independent experiments, with a total of 14 mice in each of the treated groups and 9 mice for the control group. The difference between 2 mM $AACOCF_3$ and control groups is significant during the first paralytic episode (days 12 to 22; $p < 0.001$), the second paralytic episode (days 29 to 37; $p < 0.01$), and later periods (days 48 to 60; $p < 0.03$). The difference between 4 mM $AACOCF_3$ and control groups is significant from day 9 to 60, $p < 0.0001$.

Therapeutic Effect of Blocking $cPLA_2$ in EAE

To assess if blocking $cPLA_2$ is also effective in improving symptoms of EAE after the disease is well established, C57BL/6 mice that were induced with EAE were given a delayed treatment with 4 mM of $AACOCF_3$. All animals reached a score of grade 3 or above by day 13. Treatments for all mice were begun on day 14, when remissions were first seen. Four 50 μl intravenous injections of the inhibitor were given during a 1 week period. The animals were divided into two groups based on whether they had a clinical score of grade 3 or above or a score of grade of 2 or below on the first day of treatment. Both treated groups showed a similar clinical course as compared to their respective untreated controls during the 1 week treatment period (Figures 5A and 5B). The group that remitted to a clinical score of 2 or below on the first day of treatment showed a remarkable reduction in the progression of the disease (Figure 5A). Although these animals had initially peaked to a mean clinical score of grade 3 prior to treatment, they progressively dropped down to a mean grade of 0.3 and remained virtually symptom-free up to day 60 (Figure 5A, Table 4), the maximum period studied. Their progression into a second relapse was prevented. In contrast, their matching untreated controls, which also remitted to a clinical score of grade 2 or below on day 14 and which had initially peaked to a mean grade of 3.2, progressed into a second paralytic episode (grade 2.9) between days 25 and 37. These mice, which display a relapsing-remitting form of the disease, remained thereafter at a mean clinical score of 1.8 until day 60 (Figure 5A, Table 4). On the other hand, animals that had a clinical score of grade 3 or above on the first day of treatment showed a chronic form of the disease and were unaffected by the treatment regimen, not differing from the control group (Figure 5B). These groups reached a peak mean clinical score of 3.4 (Figure 5B).

Although the mice that displayed a chronic course did not show clinical improvement with the inhibitor treatment, we assessed whether the treatment had any effect on inflammation in the spinal cord. Immune cell

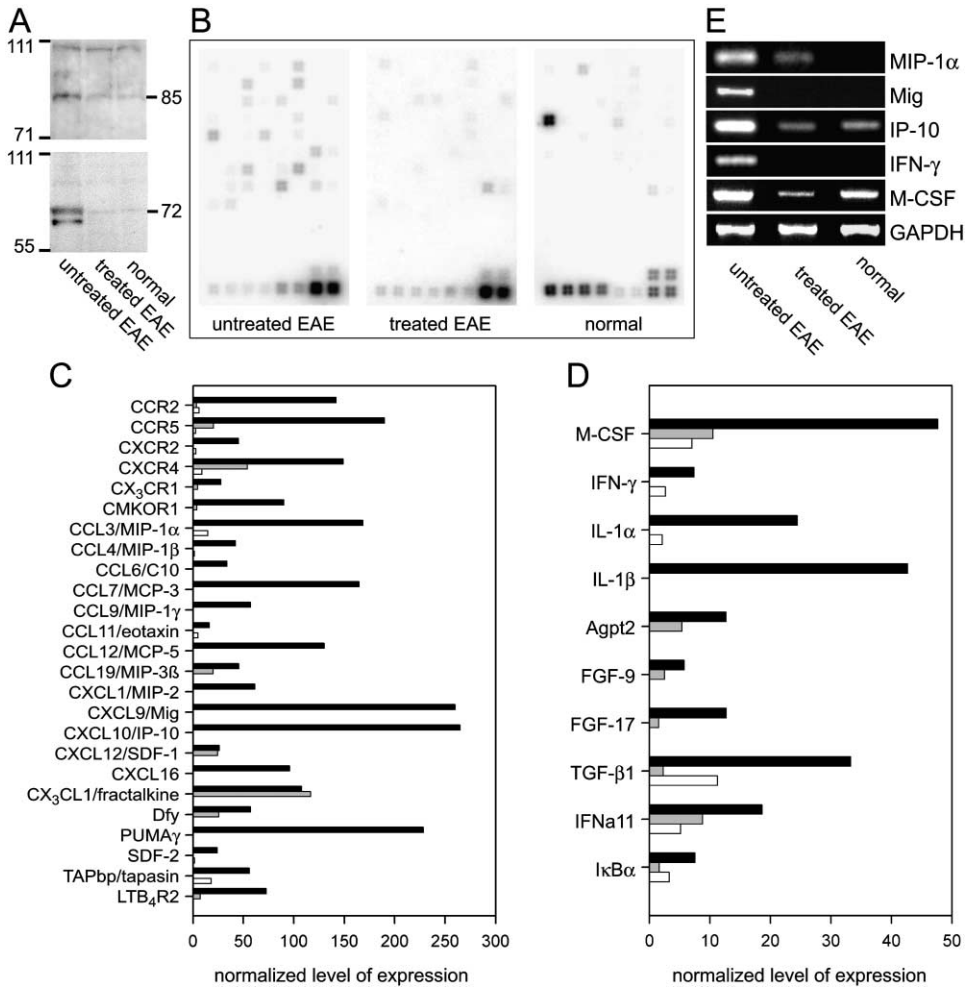

Figure 4. Changes in Gene and Protein Expression in cPLA$_2$ Inhibitor-Treated Mice

(A) Western blot of spinal cord tissue showing expression of cPLA$_2$ in the upper panel as indicated by an 85 kDa band and COX-2 in the lower panel as indicated by a 72 kDa band. There is high expression of both enzymes in the untreated EAE mice. Both enzymes were markedly lower in the AACOCF$_3$-treated mice. The enzymes were present at low levels in the normal control.

(B) Representative chemokine/chemokine receptor gene arrays showing expression in the spinal cords of untreated and treated EAE mice taken at day 13 and a normal control mouse. Expression of many genes are increased in the untreated EAE spinal cord. The number of genes expressed is reduced in the inhibitor-treated EAE mouse. A few genes are expressed at basal levels in the normal animal. The last 10 genes in the bottom two rows are housekeeping genes.

(C and D) Graphs showing the normalized level of expression for the chemokine/chemokine receptor genes and cytokine genes, respectively. The black bars indicate expression in untreated EAE spinal cord, the gray bars indicate expression in inhibitor-treated spinal cord, and the white bars indicate expression in normal control spinal cord.

(E) RT-PCR for several chemokines and cytokines in the same groups of mice as described above. There is high expression of these genes in the untreated EAE spinal cord, which is greatly reduced in spinal cord of AACOCF$_3$-treated mice, and low level or no expression in normal spinal cord.

infiltration into the spinal cord was estimated by cell counts on hematoxylin and eosin (H&E) stained tissue sections of the spinal cord obtained at day 60 ($n = 3$ in each group). The untreated chronic control group (grade 2.9 on day 60) had an average of about 460 immune cells in the lumbar spinal cord, with about six lesions per section, and a lesion area of about 0.39 mm^2 (Figures 6A–6C). In contrast, the treated chronic group that did not show clinical improvement (grade 2.8 on day 60) showed a reduced inflammatory burden. These treated mice had only about 250 immune cells in EAE lesions in the lumbar spinal cord, with an average of four lesions per section, and a lesion area of 0.28 mm^2 (Figures 6A–6C). Similar results were also seen in the cervical and thoracic regions (data not shown). Furthermore, there was a marked reduction in the lesion burden and immune cell infiltration in the other treated group, which had remitted to a grade of 2 or less and which responded well to inhibitor treatment (Figures 6A–6C). These results show that blocking cPLA$_2$ reduces inflammation in mice with EAE.

Although cPLA$_2$ inhibitor treatment was able to partially block inflammation in the chronic group, this reduction in inflammation did not lead to an improvement in the clinical score, suggesting that other factors play a role. We therefore examined Epon embedded sections

Table 2. Genes that Increased in Expression in Untreated EAE Mice and Have Previously Been Implicated in EAE

Gene	Fold Change Treated/Untreated EAE	Effects on	Reference
Chemokine Receptors			
CCR2	0.02	MCP-1/3/5 receptor	Jiang et al., 1998
CCR5	0.11	MIP-1/RANTES receptor	Jiang et al., 1998
CXCR2	−0.15	IL-8 receptor	Glabinski et al., 2000
CXCR4	0.36	SDF-1 receptor	Glabinski et al., 2000
CX_3CR1	0.17	fraktalkine receptor	Jiang et al., 1998
Chemokines			
CCL3/MIP-1α	−0.11	macrophages, granulocytes	Rajan et al., 2000
CCL4/MIP-1β	−0.25	macrophages, granulocytes	Rajan et al., 2000
CCL6/C10	−0.95	recruitment of macrophages	Asensio et al., 1999
CCL7/MCP-3	−0.13	recruitment of monocytes	Godiska et al., 1995
CCL9/MIP-1γ	−0.38	recruitment of $CD4^+$ T cells	Carmody et al., 2002
CCL11/eotaxin	−1.6	recruitment of eosinophil	Rajan et al., 2000
CCL12/MCP-5	−0.18	peripheral blood monocytes	Teuscher et al., 1999
CCL19/MIP-3β	0.4	T cells and B cells	Alt et al., 2002
CXCL1/MIP-2	−0.47	neutrophilic granulocytes	Rajan et al., 2000
CXCL9/Mig	−0.08	recruitment of stimulated T cells	Rajan et al., 2000
CXCL10/IP-10	−0.05	recruitment of activated T cells	Rajan et al., 2000
CXCL12/SDF-1	0.94	lymphocytes and monocytes	Godiska et al., 1995
CX_3CL1/fractalkine	1.08	recruitment of T cells and monocytes	Fischer et al., 2000
Cytokines			
M-CSF	0.2	growth survival/differentiation of monocytes	Hulkower et al., 1993
IFN-γ	−0.6	activates mononuclear phagocytes	Bettelli and Nicholson, 2000
IL-1α	−0.03	both IL-1α and IL-1β enhance expression of adhesion molecules and facilitate $CD4^+$ T cell proliferation	Carmody et al., 2002
IL-1β	−0.04		
TGF-β	0.06	inhibits pro-inflammatory cytokines and proliferation of lymphocytes; role in regulation/entering remission	Bettelli and Nicholson, 2000

Genes presented are those in the untreated EAE mouse spinal cord with values >2-fold as compared to normal control mouse spinal cord. Data represents fold change of treated EAE as compared to untreated EAE spinal cord. A value of 0.5 indicates expression was reduced in half. Those <0.5 indicates a significant reduction of expression in the treated group. A value of 1 indicates the expression is the same in both groups. Abbreviations: MIP, macrophage inflammatory protein; MCP, monocyte chemotactic protein; Mig, monokine induced by gamma interferon; IP, interferon inducible protein; SDF, stromal cell derived factor; M-CSF, macrophage colony stimulating factor; IFN, interferon; TGF, transforming growth factor.

of the spinal cord by electron microscopy to assess whether there was axonal damage. Treated animals that followed a chronic clinical course and their untreated controls both showed a great deal of axonal damage in areas near EAE lesions (Figures 6D–6H). This axonal loss may account for the permanent clinical deficits seen in these groups. In contrast to these chronic groups, the control group with the relapsing-remitting course (mean clinical score of 1.8 on day 60) showed fewer damaged axons, while the companion treated group, which improved with treatment (mean clinical score of 0.3 on day 60), showed virtually no damaged axons (data not shown). These data indicate that the failure of mice with the more severe form of EAE to improve with the inhibitor treatment may be due to permanent axonal damage.

Discussion

Our data indicate an important role for $cPLA_2$ in the pathogenesis of EAE, the animal model of MS. We demonstrate an increased expression of $cPLA_2$ in endothelial cells and immune cells at sites of EAE lesions in the spinal cord. This increased expression in endothelial cells during the early clinical stages of the disease may play an important role in recruiting immune cells into the CNS. The expression of $cPLA_2$ in T cells and macrophages could play a role in recruitment of additional immune cells, leading to further progression of the disease at later stages. We also demonstrate that by blocking the activity of $cPLA_2$ using a chemical inhibitor of this enzyme, the disease incidence can be drastically curtailed and the disease severity markedly reduced when treatment is initiated at the time of induction of EAE. This reduction in disease severity correlates with a decrease in $cPLA_2$ and its downstream mediators such as COX-2, LTB_4R2, and various chemokines and cytokines that contribute to the inflammatory and neural pathology in EAE. Furthermore, we show that inhibiting $cPLA_2$ after the disease is full-blown has a remarkable effect in reducing inflammation, axonal damage, and the clinical severity and preventing further remissions in animals showing a relapsing-remitting form of the disease.

Table 3. Genes that Increased in Expression in Untreated EAE Mice and Have Not Formerly Been Reported in EAE

Gene	Fold Change Treated/Untreated EAE	Effects on	Reference
LTB$_4$R2	0.10	receptor for a downstream metabolite of cPLA$_2$, which is a potent activator of macrophages	Yokomizo et al., 2000
Dfy	0.45	expressed in MS tissue, enhances leukocyte recruitment	Whitney et al., 1999; Lee et al., 2003
TAPbp/tapasin	−0.57	a member of the MHC class I loading complex	Garbi et al., 2003
angiopoietin 2	0.43	blood brain barrier breakdown	Nourhaghighi et al., 2003
IκBα	0.21	inhibitor for NF-κB	Murphy et al., 2002
CCL16	−0.03	chemotactic for CD4$^+$ T-lymphocytes	Nomiyama et al., 2001
PUMA-γ	−0.12	nicotinic acid receptor	Tunaru et al., 2003
FGF-9	0.44	potent mitogen for glial cells	Naruo et al., 1993
FGF-17	0.12	NA	
IFNα gene 11	0.47	NA	
CMKOR1	0.04	NA	
SDF-2	−0.91	NA	

Genes presented are those in the untreated EAE spinal cord with values >2-fold as compared to normal control mouse spinal cord. These genes have not previously been implicated in EAE. Data represents fold change of treated EAE mice as compared to untreated EAE mice. A value <0.5 indicates a reduction of expression in the treated group. Abbreviations: LTB$_4$R2, Leukotrine B$_4$ receptor 2; Dfy, duffy glycoprotein; TAPbp, T cell activating protein binding protein; FGF, fibroblast growth factor; CMKOR, chemokine orphan receptor; PUMA-γ, protein upregulated in macrophages by IFN-γ; NA, not yet associated with immune cells.

One of the major pathologies in MS and EAE is a complex inflammatory cascade in the CNS (Compston and Coles, 2002; Noseworthy et al., 2000; Steinman et al., 2002; Hemmer et al., 2002; Owens and Sriram, 1995). PLA$_2$ may mediate these responses via the release of AA and LPC. One way this enzyme can play a role in inflammation is through the AA pathway, which is the precursor of pro-inflammatory eicosanoids. The principal pathways of AA metabolism are the 5-LO pathway, which produces a collection of leukotrienes (LT), and the COX pathway, which produces prostaglandin H$_2$ (PGH$_2$). PGH$_2$ serves as the substrate for two enzymatic pathways: one leading to the production of several prostaglandins (PG), and the other leading to the production of thromboxanes (TX) (Rocha et al., 2003). These metabolites are collectively referred to as eicosanoids and function by binding to specific cell surface receptors. PGE$_2$, for example, is a potent vasodilator (James et al., 2001; Rocha et al., 2003). TXA$_2$ promotes lymphocyte proliferation and adhesion and enhances macrophage function (James et al., 2001; Rocha et al., 2003). It also facilitates the synthesis of TNFα and IL-1β (Caughey et al., 1997). LTB$_4$ promotes leukocyte adhesion to endothelial cells and extravasation into tissues (Rocha et al., 2003). It also promotes the production of pro-inflammatory cytokines such as IL-1, IL-2, and IFN-γ by monocytes and macrophages (Rocha et al., 2003; Tager and Luster, 2003). Its receptor LTB$_4$R2 is highly expressed on CD4$^+$ and CD8$^+$ T cells (Rocha et al., 2003). These lipid mediators can therefore contribute to inflammation by chemotaxis or activation of immune cells and by increasing vascular permeability. The generation of arachidonic acid by cPLA$_2$ is the rate-limiting step in the production of these eicosanoids. Although AACOCF$_3$ is a potent inhibitor of cPLA$_2$, there is indirect evidence that in platelets it also has COX inhibitor activity under conditions when exogenous arachidonic acid is added to the cells (Riendeau et al., 1994). The positive outcome we have observed with AACOCF$_3$ treatment delivered after onset of EAE as compared to COX inhibitor treatment (Traugott and Raine, 1989; Weber et al., 1991) suggests that the effects of AACOCF$_3$ are likely to reflect its ability to block cPLA$_2$.

Another way cPLA$_2$ can stimulate immune responses is through LPC, which is a chemoattractant for T cells and monocytes and activates macrophages (Ousman and David, 2000, 2001; Ryborg et al., 1994, 2000). LPC also induces expression of the cell adhesion molecules ICAM-1 and VCAM-1 by CNS endothelial cells and rapidly opens the blood-brain barrier (Ousman and David, 2000). Importantly, LPC also induces expression of a number of pro-inflammatory cytokines (Ousman and David, 2001), which can further upregulate cPLA$_2$ expression in a positive feedback loop (Murakami et al., 1997). LPC can be further metabolized into platelet-activating factor (PAF), which is also a strong mediator of inflammatory processes (Murakami et al., 1997; Dennis et al., 1991). Therefore, cPLA$_2$ can set off a robust inflammatory response in the CNS in EAE via multiple pathways.

There is evidence that the inflammatory response in EAE can be reduced by blocking a number of chemokines and cytokines (i.e., CCL3/MIP-1α, CCL2/MCP-1, TNF-α, M-CSF, IL-1, IFNγ, and GM-CSF) (Karpus et al., 1995; Ruddle et al., 1990; Selmaj et al., 1998; McQualter et al., 2001) that are known to induce expression of PLA$_2$. The inflammatory response in EAE can also be reduced by blocking cell adhesion molecules, such as ICAM-1, which is induced by LPC (Kawai et al., 1996), or by directly blocking prostaglandins, a metabolic product of the AA pathway (Reder et al., 1994). Depleting macrophages, which are known to be chemoattracted and activated by LTB$_4$ and LPC, also reduce these inflammatory responses (Tran et al., 1998). These studies provide strong support for the possibility that increased PLA$_2$ expression may trigger the onset and progression of this inflammatory and demyelinating disease.

The second major pathological feature of MS is demyelination and axonal damage (Bjartmar and Trapp, 2001;

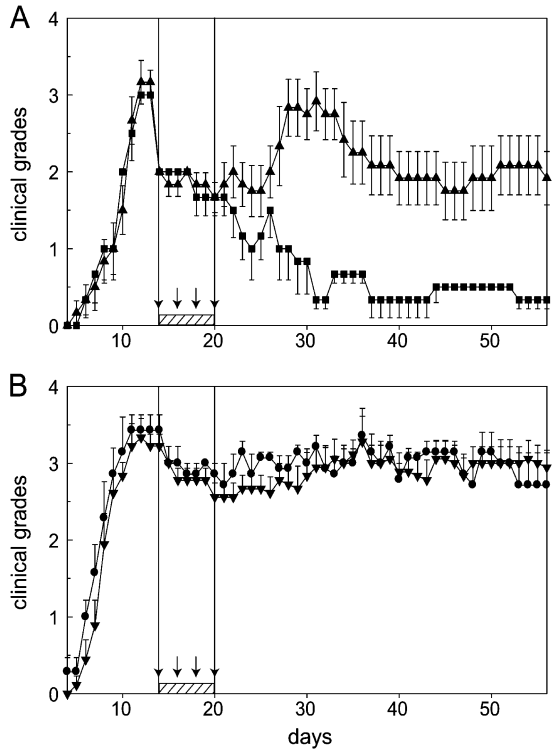

Figure 5. Therapeutic Effect of $cPLA_2$ Inhibitor in EAE

(A) Graph showing the clinical course of mice induced with EAE that were treated with 4 injections of 4 mM $AACOCF_3$ (closed square; n = 6) between days 14 and 20 (indicated by arrows) and the untreated control groups (closed triangle; n = 9). Treated mice that recovered to a grade 2 or below on day 14, the first day of treatment, show a remarkable recovery and remain almost symptom free until day 60. In contrast, untreated control mice that also recovered to a grade 2 or below on day 14 show a relapsing-remitting clinical course. Differences between these groups are statistically significant from day 28 to 60 ($p < 0.005$).

(B) Graph showing the clinical course of mice induced with EAE that remained at a grade 3 or above on day 14, the first day of treatment (closed circle; n = 7), as well as the untreated control mice (closed down triangle; n = 8) that also remained at a grade 3 or above, show a chronic clinical course and no improvement with inhibitor treatment. Data represent means ± SEM from two independent experiments.

Trapp et al., 1998; Wujek et al., 2002). The exact mechanism for demyelination and axonal injury in MS lesions is not known. One possible mechanism is through the destruction of oligodendrocytes, which can occur through antibody, or cytokine-mediated cytotoxicity, and complement activation (Selmaj and Raine, 1988; Genain et al., 1999; Mead et al., 2002). Once the axons are demyelinated, they can become damaged through the actions of certain cytokines, proteolytic enzymes, and nitric oxide (NO) (Bjartmar and Trapp, 2001; Smith et al., 1999). $cPLA_2$ can also contribute to this pathology via its metabolic product LPC. Not only does LPC act as a myelinolytic agent itself, but in late stages of demyelination it may also damage axonal membranes. PLA_2 can also recruit macrophages (Murakami et al., 1997), which produce NO, as well as cytokines that could result in free radical and cytokine-mediated cytotoxicity (Noseworthy et al., 2000; Bjartmar and Trapp, 2001). PLA_2 may also be involved at least in part in glutamate-induced toxicity that leads to axonal and oligodendroglial damage as glutamate release has been shown to be reduced by PLA_2 inhibitors (Sundstrom and Mo, 2002). Additionally, two groups have generated mice lacking $cPLA_2$ (Bonventre et al., 1997; Uozumi et al., 1997), one of which showed that $cPLA_2$ mediates neural damage in ischemia-reperfusion injury as indicated by the reduced infarct size following transient middle cerebral artery occlusion (Bonventre et al., 1997). Induction of EAE in $cPLA_2$ null mutants on an EAE-susceptible background strain would help to further assess the role of $cPLA_2$. Additional confirmation of the importance of $cPLA_2$ in macrophage recruitment, myelin breakdown, and phagocytosis comes from our recent studies on Wallerian degeneration in peripheral nerves (De et al., 2003).

A number of approaches have been tested to ameliorate the clinical course of EAE through a variety of immunomodulatory approaches and methods to achieve protection from oligodendrocyte and axonal damage (Karpus et al., 1995; Ruddle et al., 1990; Selmaj et al., 1998; McQualter et al., 2001; Kawai et al., 1996; Reder et al., 1994; Tran et al., 1998; Lock et al., 2002; Chabas et al., 2001; Fife et al., 2001; Leger et al., 1997; Pitt et al., 2000; Butzkueven et al., 2002; Popovic et al., 2002; Brundula et al., 2002). Of these efforts to develop new treatments for MS, only a few have been approved for human use, with limited success (Polman and Uitdehaag, 2000; Rolak, 2001). Therapies currently being used or in various stages of clinical testing consist of immunomodulatory drugs (Steinman, 1999; Polman and Uitdehaag, 2000; Rolak, 2001). An optimal treatment would aim at preventing both inflammation and axonal damage including demyelination. This study shows that $cPLA_2$ may play a role in contributing to both the inflammatory and axonal pathologies seen in EAE. An elevated level of PLA_2 was detected in MS tissue in one study

Table 4. Clinical Characteristics of Mice that Remitted to a Grade 2 or below on Day 14 Given a Delayed Treatment with the $cPLA_2$ Inhibitor $AACOCF_3$

	n	Mean Day of Disease Onset	Mean Clinical Grade of Animals at 1st Paralytic Attack (Day 13)	% Relapse by Day 25	Mean Clinical Grade of Animals at 2nd Paralytic Attack (Day 31)	Mean Clinical Grade of Animals at Day 60
Untreated RR control	6	7.8 ± 0.6	3.2 ± 0.2	100% (6/6)	2.9 ± 0.4	1.8 ± 0.3
Treated: 4 mM $AACOCF_3$	6	7.7 ± 0.6	3.0 ± 0.2	16.7% (1/6)	0.3 ± 0.2	0.3 ± 0.2
p value		NS	NS	0.05	0.0001	0.005

Animals had a score of 2 or below on the first day of treatment. Treatment given between days 14 and 20. Data represent mean ± SEM. p values compared with untreated, relapsing-remitting (RR) control. NS, not significant.

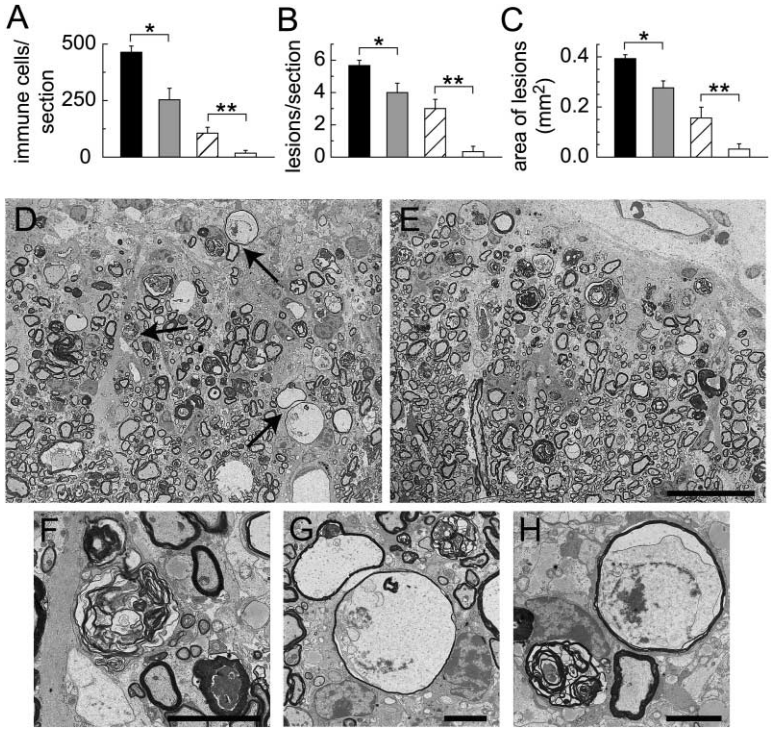

Figure 6. Inflammation and Axonal Damage

(A) Quantitative analysis of H&E stained sections of the lumbar spinal cord revealed a significant reduction in the number of immune cells entering the spinal cord in both groups of treated mice compared to their untreated controls, *$p < 0.02$; **$p < 0.03$. Black bars indicate untreated control mice with chronic clinical course that remained at a score of 3 or above on day 14; gray bars indicate inhibitor-treated mice that showed a chronic course that also remained at a score of 3 or above on day 14; hatched bars indicate untreated control mice that show a relapsing-remitting clinical course that had remitted to a score of 2 or below on day 14; and white bars indicate inhibitor-treated mice that recovered to a grade 2 or below on day 14.

(B) There was also a reduction in the number of EAE lesions in the lumbar spinal cord in both treated groups compared to their untreated controls, *$p < 0.04$; **$p < 0.01$.

(C) A decrease in the area of the EAE inflammatory lesions was also seen in both treated groups versus their controls, *$p < 0.01$; **$p < 0.04$.

(D–H) Electron micrographs of lumbar spinal cord tissue near the site of EAE lesions in the treated chronic group (D, F–H) and their untreated control chronic group (E). Note the large number of degenerating axons in (D) and (E). Scale bar equals 30 μm. The micrographs in (F), (G), and (H) show higher magnifications of degenerating axons indicated by arrows in (D). Scale bars equal 5 μm.

but not in another (Woelk and Peiler-Ichikawa, 1974; Huterer et al., 1995). Further studies are warranted to assess changes in the expression or activation of PLA$_2$ enzymes in MS lesions. However, increases of downstream products of PLA$_2$ such as prostaglandins and leukotrienes have been found in the cerebrospinal fluid of MS patients (Bolton et al., 1984; Dore-Duffy et al., 1986, 1991), and 5-LO, an enzyme downstream of cPLA$_2$ that converts AA to leukotrienes, was recently shown to be increased in MS and EAE lesions (Whitney et al., 2001). As discussed above, the targets of many of the treatments shown to be beneficial in EAE are either chemokines or cytokines that induce expression of PLA$_2$; or enzymes involved in the conversion of AA to eicosanoids, namely, COX and 5-LO; or molecules induced by LPC, such as ICAM-1 and VCAM-1. Taken together, these data suggest that PLA$_2$ could serve as the common link via which various factors could trigger stereotypic cellular changes characteristic of EAE and MS. These studies warrant the designing and testing of more potent and specific inhibitors of cPLA$_2$ and further their development for the treatment of multiple sclerosis and possibly other neuroinflammatory conditions.

Experimental Procedures

Induction of EAE

EAE was induced in female C57BL/6 mice (18–20 g) by subcutaneous injections of 50 μg of myelin oligodendrocyte glycoprotein (MOG$_{35-55}$—MEVGWYRSPFSRVVHLYRNGK) (Sheldon Biotechnology Centre, Montreal, Canada) in Complete Freund's Adjuvant (Incomplete Freund's adjuvant containing 0.5–1 mg/ml of heat-inactivated *Mycobacterium tuberculosis* [Fisher Scientific, Nepean, Canada]). An intravenous injection of 200 ng of pertussis toxin (List Biologicals, Campbell, CA) was also administered on days 0 and 2 of the immunization. All procedures were approved by the McGill University Animal Care committee. The mice were monitored daily for clinical symptoms of EAE using the following 5-point scale: Grade 0, normal (no clinical signs); Grade 1, flaccid tail; Grade 2, mild hindlimb weakness (fast righting reflex); Grade 3, severe hindlimb weakness (slow righting reflex); Grade 4, hindlimb paralysis; Grade 5, hindlimb paralysis and forelimb weakness or moribund.

Immunohistochemistry

Mice at different clinical grades were deeply anesthetized and perfused with 0.1 M phosphate buffer followed by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer. Cryostat sections (12 μm) of the spinal cord in cross-section were processed using standard immunoperoxidase methods (Ousman and David, 2000). The primary antibody used was a polyclonal rabbit anti-cPLA$_2$ (Santa Cruz Biotechnology, Santa Cruz, CA, 1:45). The specificity of this antibody was confirmed by Western blot on extracts of NIH 3T3 cells, which showed a single band at 85 kDa. Immunostaining of tissue sections was lost after pre-adsorption of the polyclonal cPLA$_2$ antibody with rat glomerular epithelial cells transfected with a cPLA$_2$ cDNA (obtained from Dr. A. Cybulsky, McGill University). The secondary was a biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, 1:500). Sections were counterstained with 3% methyl green. For double immunofluorescence, cryostat sections were blocked in 0.1% Triton-X 100 and 2% normal goat serum and incubated overnight with anti-cPLA$_2$ combined with either monoclonal antibodies specific for T cells (rat anti-CD4, BD Biosciences PharMingen, San Diego, CA, 1:100) or macrophages (rat monoclonal antibody Mac-1, hybridoma supernatant), followed by incubation with a biotinylated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:200) combined with a goat anti-rat rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, 1:100). After wash-

ing, the sections were incubated with fluorescein-conjugated streptavidin (Molecular Probes, Eugene, OR, 1:200).

Quantification

Counts were done using an ocular grid. For the immunoperoxidase stained sections, two types of $cPLA_2^+$ cells were counted: endothelial cells and immune cells in EAE lesions. Three levels of the spinal cord (cervical, thoracic, and lumbar) were quantified from 3 animals in each grade (grades 1–5). The mean number of each cell type per cross-section was estimated from three tissue sections per animal, each section being at least 45 μm apart.

For the immunofluorescence stained sections, the number of $cPLA_2^+$ cells, Mac-1^+ cells, and $CD4^+$ T cells were quantified in 5 random lesions in the three levels of the spinal cord from 3 animals in each grade (1–5).

Mice given delayed treatment and their controls were analyzed on day 60. Mice were perfused with 4% paraformaldehyde, and cryostat cross-sections (12 μm) of the spinal cord were stained with hematoxylin and eosin (H&E). Inflammatory lesions were quantified by counting the number of lesions, the number of inflammatory cells, and the area of the infiltrates per section (3 sections per animal and 3 animals per group).

All counts were done blind so that the person doing the quantification was unaware of the clinical grade or experimental groups. Results are presented as the mean number of cells per group $\pm$ standard error of the mean (SEM). The statistical significance ($p < 0.05$) between the various grades was determined by using a two-sample Student's t test.

Treatment of EAE

EAE was induced in C57BL/6 mice as mentioned above. Mice were randomly assigned to each of the treatment and control groups. For the preventative treatment, at day 0 and 2 days after EAE induction, a 50 μl intravenous injection of either 2 mM or 4 mM arachidonyl trifluoromethyl ketone ($AACOCF_3$; Cayman Chemicals, Ann Arbor, MI) diluted in 1% DMSO buffer was administered. This was followed on alternate days by intraperitoneal injections of 200 μl of the same inhibitor at 2 or 4 mM concentrations until day 24. For the delayed treatment, 50 μl intravenous injections of either 4 mM $AACOCF_3$ diluted in 1% DMSO containing buffer or vehicle (1% DMSO containing buffer) was administered on days 14, 16, 18, and 20 after induction of EAE. The mice were scored clinically based on the scoring system described above and were randomly assigned to the control or treatment groups. Monitoring was done blind so that the person doing the scoring was unaware of the experimental groups. The statistical significance ($p < 0.05$) between the various groups was determined by using a Mann-Whitney rank-sum test.

Western Blotting

Spinal cords were removed, homogenized, and centrifuged at 1000 $\times$ g. The supernatant was sonicated and proteins were extracted with RIPA buffer containing 1% NP-40, 1% sodium deoxycholate, 2% SDS, 0.002 M EDTA, and complete protease inhibitor cocktail (Roche Diagnostics, Quebec, Canada). 25 μg of protein was loaded per lane and standard Western blot methods used (Jeong and David, 2003) to detect $cPLA_2$ and COX-2 with anti-$cPLA_2$ (1:500) and anti-COX-2 (1:500, Cayman Chemicals, Ann Arbor, MI) antibodies. Binding of antibodies to the blots was visualized by chemiluminescence (PerkinElmer Life Sciences, Wellesley, MA).

Gene Array Screening

Spinal cords were removed from animals and RNA isolated using the RiboPure kit (Ambion Inc, Austin, TX) and reverse transcribed to cDNA. These cDNA probes were then labeled with [^{32}P]dCTP (Amersham Biosciences, Buckinghamshire, England) and amplified by PCR using the AmpoLabeling-LPR kit (Superarray Bioscience Corp., Frederick, MD). This type of labeling allows detection of low-abundance message such as those of chemokines and cytokines. The labeled cDNA probes were then denatured and hybridized overnight to the GEArray membranes from SuperArray Biosciences. The membrane was then scanned using a Storm PhosphorImager. The data appears as an image of tetra-spots that was quantified using the ImageQuant software. All signals were normalized to a housekeeping gene peptidylprolyl isomerase A, which has been found as a preferred internal control over GAPDH (Feroze-Merzoug et al., 2002). Two types of focused gene expression arrays (GEArray) from SuperArray were used: an array consisting of 67 chemokine and chemokine receptor genes (Cat.# MM-005), and a 97 common cytokine gene array (Cat. #MM-003).

RT-PCR

RNA was purified from spinal cord as mentioned above. RT-PCR was performed using the GeneAmp RNA PCR kit (PerkinElmer Life Sciences). Primers used were as follows: MIP-1α, U-5′-ATGAAGGT CTCCACCACTG-3′, L-5′-GCATTCAGTTCCAGGTCA-3′; IP-10, U-5′-CCCCATCAGCACCATGAAC-3′, L-5′-GCTTCACTCCAGTTAAGGA-3′; Mig, U-5′-TGCCATGAAGTCCGCTGTTC-3′, L-5′-AAAGTAATGGTC TCTTATGTAG-3′; IFN-γ, U-5′-TGAACGCTACACACTGCATCTTGG-3′, L-5′-CGACTCCTTTTCCGCTTCCTGAG-3′; M-CSF, U-5′-AGT GAG GGA TTT TTG ACC CAG GAA GCA AA-3′, L-5′-CTA TAC TGG CAG TTC CAC CTG TCT GT-3′; and GAPDH, U-5′-TGAAGGTCGGTG TGAACGGATTTGGC-3′, L-5′-CATGTAGGCCATGAGGTCCACCAC-3′. PCR was performed as described previously (Jeong and David, 2003) with annealing temperatures of 60°C (MIP-1α, M-CSF, and GAPDH) and 56°C (IP-10, Mig, and IFN-γ).

Electron Microscopy

On day 60, mice were perfused with 0.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer. Tissues were then postfixed in 2% osmium tetroxide and processed and embedded in Epon as described previously (Ousman and David, 2000). Thin sections on Collodion-coated slot grids were stained with lead citrate and examined with a Philips CM10 electron microscope.

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