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Time-dependent changes in the brain arachidonic acid cascade during cuprizone-induced demyelination and remyelination

S. Palumbo¹, C.D. Toscano¹, L. Parente², R. Weigert², and F. Bosetti¹

¹National Institute on Aging, National Institutes of Health, Bethesda, 20892 MD, USA

²National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, 20892 MD, USA

Abstract

Phospholipases A₂ (PLA₂) are the enzymatic keys for the activation of the arachidonic acid (AA) cascade and the subsequent synthesis of proinflammatory prostanoids (prostaglandins and thromboxanes). Prostanoids play critical roles in the initiation and modulation of inflammation and their levels have been reported increased in several neurological and neurodegenerative disorders, including multiple sclerosis (MS).

Here, we aimed to determine whether brain expression PLA₂ enzymes and the terminal prostaglandin levels are changed during cuprizone-induced demyelination and in the subsequent remyelination phase.

Mice were given the neurotoxicant cuprizone through the diet for six weeks to induce brain demyelination. Then, cuprizone was withdrawn and mice were returned to a normal diet for six weeks to allow spontaneous remyelination.

We found that after 4–6 weeks of cuprizone, sPLA₂(V) and cPLA₂, but not iPLA₂(VI), gene expression was upregulated in the cortex, concomitant with an increase in the expression of astrocyte and microglia markers. Cyclooxygenase (COX)-2 gene expression was consistently upregulated during all the demyelination period, whereas COX-1 sporadically increased only at week 5 of cuprizone exposure. However, we found that at the protein level only sPLA₂(V) and COX-1 were elevated during demyelination, with COX-1 selectively expressed by activated and infiltrated microglia/macrophages and astrocytes. Levels of PGE₂, PGD₂, PGI₂ and TXB₂ were also increased during demyelination. During remyelination, none of the PLA₂ isoforms was significantly changed, whereas COX-1 and -2 were sporadically upregulated only at the gene expression level. PGE₂, PGI₂, and PGD₂ levels returned to normal, whereas TXB₂ was still upregulated after 3 weeks of cuprizone withdrawal.

Our study characterizes for the first time time-dependent changes in the AA metabolic pathway during cuprizone-induced demyelination and the subsequent remyelination and suggests that sPLA₂(V) is the major isoform contributing to AA release.

Corresponding Author: Francesca Bosetti, PhD Brain Physiology and Metabolism Section National Institute on Aging National Institutes of Health 9 Memorial Drive Bldg. 9 Rm. 1S126 Bethesda MD 20892-0947 Phone: (301) 594 -5077 Fax: (301) 402-0074 Mobile: (301) 605-4473 frances@mail.nih.gov.

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Keywords

phospholipases A₂; eicosanoids; demyelination; arachidonic acid; cuprizone

Introduction

Arachidonic acid (AA) is an ω -6 polyunsaturated fatty acid (20:4) which plays a major role in intracellular and extracellular signaling events in the human body. Particularly important is the involvement of AA in physiological and pathological processes that occur in the central nervous system (CNS) [1, 2]. For example, AA and its metabolites are known to play a significant role in regulating neurotransmitter release and cerebral blood flow [1, 2]. However, AA metabolites are also involved in certain neurological and neurodegenerative disorders with a neuroinflammatory component, such as multiple sclerosis (MS), stroke, and Alzheimer's disease [1–5]. The AA metabolites that are involved in these processes are produced by a cascade of enzymes dedicated to releasing AA from cell membrane phospholipids and converting it into different classes of metabolites, such as prostaglandins (PG), thromboxanes (TXB), leukotrienes (LT) and lipoxins (LX) [6].

Release of unesterified AA from membrane phospholipids is accomplished by the phospholipase A₂ (PLA₂) family of proteins, which catalyze the cleavage of phospholipids from the *sn*-2 position. More than 19 different isoforms of PLA₂ have been identified to date and classified in three major groups: the calcium-dependent cytosolic PLA₂ (cPLA₂) and secretory PLA₂ (sPLA₂), and the calcium-independent PLA₂ (iPLA₂). PLA₂ enzymes are not only important for regulating the release of AA, but also for the physiological maintenance of cell membrane phospholipids and the regulation of cell differentiation, proliferation, and apoptosis. Under normal signaling conditions, production of free AA is mainly due to activation of cPLA₂, but also of sPLA₂ [7]. Free AA can be metabolized by cyclooxygenase (COX) to PGH₂, which is then converted to bioactive PGs and TXBs through the activity of terminal PG and TXB synthases.

In MS demyelinating lesions, AA cascade is activated with increased activity of PLA₂ and increased production of the COX-derived PGE₂, PGI₂, PGD₂ and PGF₂ α [4, 5, 8]. Studies in the experimental autoimmune encephalomyelitis (EAE) model, which is T-cell mediated, focused particularly on the cPLA₂ α isoform, which is highly expressed in EAE lesions, and showed that cPLA₂ inhibition leads to a significant reduction in the onset and progression of EAE and the development of Th1 and Th17 responses [9, 10]. Supporting these observations, cPLA₂ α -deficient mice are resistant to EAE [11].

In the cuprizone model of MS, which is characterized by a primary, reversible demyelination due to a direct and immune system-independent injury to oligodendrocytes, the activation of AA cascade has not been investigated. To investigate whether the activation of PLA₂ and AA metabolism is a selective feature of EAE immune activation, or also characterizes primary demyelination, we investigated changes in the expression of different isoforms of PLA₂ and other AA metabolizing enzymes during cuprizone-induced demyelination and subsequent remyelination. In this well characterized model, cuprizone is fed to mice through the diet over the course of six weeks, causing a progressive demyelination of several region of the CNS including cortex, corpus callosum, hippocampus and cerebellum [12, 13]. Demyelination begins to manifest histologically at the third week of treatment and is reversible upon cuprizone withdrawal from the diet [13]. Demyelination is accompanied by a well characterized sequence of events including depletion of mature oligodendrocytes, microglia activation, and astrocyte proliferation [13]. For the first time, we describe here how AA cascade enzymes and terminal prostanoids are changed during

cuprizone-induced demyelination and remyelination. We demonstrate that the AA cascade is activated during cuprizone-induced demyelination, with a substantial involvement of the sPLA₂ isoform.

Materials and Methods

Mice and cuprizone treatment

C57BL6 male mice, 6–8 week-old, were purchased from Taconic (Germantown, NY) and maintained on a 12/12 h light dark cycle. Cuprizone (Bis-cyclohexanoneoxaldihydrazone, Sigma, St. Louis, MO) was mixed into a 0.2% powdered diet prepared by Research Diet Inc (New Brunswick, NJ), as previously described [13]. Mice were fed *ad libitum* with either the cuprizone or control diet. To study remyelination, after 6 weeks of cuprizone exposure mice were returned to a normal diet for six additional weeks [13]. Mice were anesthetized with pentobarbital 50 mg/kg i.p (Nembutal, Ovation Pharmaceuticals Inc., Lake Forest, IL) and euthanized by decapitation for each time point from week 0 (control group) to 6 of cuprizone exposure to study demyelination, and from week 7 to 12, during cuprizone withdrawal to study remyelination. Brain was excised and the cerebral cortex was dissected and frozen at –80 °C for molecular analysis (n= 6). For histology, mice perfused with 4% paraformaldehyde, then brains were extracted, postfixed overnight, cryoprotected in a 30% sucrose solution, and frozen at –80 °C until use (n= 3). All procedures were performed under a NIH approved animal protocol, in accordance with NIH guidelines.

Myelin staining

Histology was performed on 30 µm-coronal brain paraformaldehyde fixed sections. Brains were cut on a cryostat (LI-COR Bioscience, Lincoln, NE) and mounted on gelatin-coated glass slides. Sections were stained with the myelin staining Black-Gold II (Histo-Chem, Jefferson, AR) as previously described [14]. Sections were rinsed in distilled water and incubated in a 0.2% Black-Gold II solution for 12–18 minutes, rinsed in distilled water, transferred in a 2% sodium thiosulfate solution for 3 minutes, rinsed in tap water, dehydrated via gradual alcoholic solutions and air-dried. Sections were cleared in xylene and coverslipped using DPX (Sigma) mounting medium. Sections were photographed using a U-CMAD3 camera (Olympus America Inc., Center Valley, PA) at 4 magnification. Images were opened with Spot Advanced 41 software, which was used to set the background subtraction, and imported into Image J to measure the mean optical density (myelin score) in the cerebral cortex. Myelin density for each mouse was normalized against values of control mice using the following formula: myelin score (%) = (density reading / unchallenged density average) 100, as previously described [15].

Immunofluorescence

Immunohistochemistry was performed on 30 µm-coronal brain paraformaldehyde-fixed sections. Floating sections were incubated for 1 hr in a blocking buffer (PBS 1% BSA, 0.1% Triton-X 100), and incubated overnight with specific primary antibodies at 4°C, followed by a fluorochrome-conjugated secondary antibody incubation in the dark at room temperature for 1 hr. Sections were mounted on gelatin-coated glass slides that were covered with coverslips using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) in the dark. Primary antibodies against GFAP (astrocyte marker, 1:600; Cell Signaling, Danvers, MA), CD11b (microglia/macrophages marker, 1:100; Serotec, Raleigh, NC), COX-1 (1:100 Cayman Chemical, Ann Arbor, MI), and COX-2 (1:100 Cayman Chemical) were used, followed by fluorophore-tagged secondary antibodies Alexa Fluor 594 goat anti-mouse IgG, Alexa Fluor 594 goat anti-rat IgG, Alexa Fluor 488 goat anti-rabbit IgG (5 µg/ml, Invitrogen, Carlsbad, CA). Stained sections were imaged with a Fluoview 1000 confocal microscope (Olympus). All images were acquired using a

UPLSAPO $\times 10$ numerical aperture (NA) 0.4 dry objective and a PLANAPO $\times 60$ NA 1.4 oil immersion objective (Olympus). Images were processed using Imaris 5.7 (Bitplane) and assembled using Adobe Photoshop CS.

Real-Time (RT) Polymerase Chain Reaction (PCR)

RNA was extracted using the Qiagen RNeasy Lipid Tissue Mini kit (Qiagen, Valencia, CA) following the manufacturer's procedure. RNA purity and integrity were verified by examining the 260 nm / 280 nm ratio using a spectrophotometer. Extracted RNA was resuspended in RNase-free molecular grade water and stored at -80°C until usage. For Q-PCR, total RNA (5 μg) was reverse transcribed using a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) using appropriate controls to ensure the absence of genomic DNA contamination. Real Time Polymerase Chain Reaction (RT-PCR) was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). RT-PCR results were normalized to phosphoglycerate kinase 1 (PGK; Mm00435617_m1) expression levels, as previously described [16]. Gene expression was analyzed using the following Assays on Demand: glial fibrillary acidic protein (GFAP; Mm01253033_m1); integrin alpha M (CD11b; Mm00434455_m1), cytosolic phospholipase A₂ (cPLA₂; Mm00447040_m1), secretory phospholipase A₂ (V) (sPLA₂(V); Mm00448161_m1), calcium-independent phospholipase A₂ (VI) (iPLA₂(VI); Mm00479527_m1), cyclooxygenase-1 (COX-1; Mm00477214_m1) and cyclooxygenase-2 (COX-2; Mm00478374_m1). Briefly, Taqman Universal PCR Master Mix, Assay-On-Demand primers and cDNA samples were mixed in RNase-free water and added to an optical 96-well reaction plate (Applied Biosystems). Negative controls containing no cDNA and a standard curve spanning 3 orders of magnitude of dilution were run on each plate in duplicate. RT-PCR conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C . The amount of target gene expression was calculated by using the $\Delta\Delta\text{C}_\text{T}$ method [17]. Relative changes in gene expression were expressed as fold change of expression in untreated mice.

Western Blotting

The cytosolic fraction was prepared from cortical samples as described [16]. Briefly, the cortex was homogenized in a homogenizing buffer containing 20 mM Tris-HCl, 2 mM EDTA, 2 mM EGTA, 2 mM DTT, and Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN) (pH 7.5), using a Polytron® homogenizer. The supernatant was centrifuged at 100,000 g for 60 min at 4°C . The supernatant was collected and used as the cytosolic fraction. Protein concentration was measured using a Dc Protein Assay kit (Bio-Rad, Richmond, CA).

Western blotting was performed as previously described [16]. Briefly, proteins (30–60 μg) were loaded on Criterion gels (Bio-Rad), transferred onto a polyvinylidene difluoride membrane (Bio-Rad) and immunoblotted with antibodies against sPLA₂(V) (1 $\mu\text{g}/\text{ml}$; Cayman Chemical), phospho-cPLA₂ ser505 (1:1000; Cell Signaling, Danvers, MA), COX-1 (1:500; Cayman Chemical), COX-2 (1:500; Cayman Chemical) and β -actin (1:3000; Sigma) as a loading control. An Odyssey Infrared Imaging System (Licor Biosciences, Lincoln, NB) was used to detect and quantify protein levels. Results were expressed as percentage of controls.

Lipids extraction and EIA

Lipid extraction was performed using the 3:2 hexane/2-propanol method [18]. Fresh frozen cerebral cortex was homogenized in 6 ml of solvent using a glass Tenbroeck homogenizer and centrifuged at 1500 rpm for 10 min at room temperature. The pellet was resuspended in 2 ml and centrifuged for two times at 1500 rpm for 10 min as previously described [18, 19].

The supernatant was collected and evaporated to dryness under a gentle stream of nitrogen, resuspended in 500 μ l of solvent and stored at -80°C . Prostaglandin levels were determined by ELISA based assays (Oxford Biomedical Research Kit, Burlington, Ontario, Canada, for PGE_2 , PGI_2 and TXB_2 ; Cayman Chemical for PGD_2) following the manufacturer's instructions ($n = 6-7$ per group).

Statistics

For gene expression, log-transformed $\Delta\Delta\text{Ct}$ values were analyzed using a one-way ANOVA followed by a Bonferroni's multiple comparisons test. For western blotting and ELISA, analysis was done using a one-way ANOVA followed by a Bonferroni's test. Data were expressed as mean $\pm$ SEM, and p values < 0.05 were considered statistically significant.

Results

Cuprizone induces progressive demyelination and astrocytes and microglia activation in the cortex

We show the time course of demyelination and remyelination in the cerebral cortex each week during cuprizone intoxication (week 1 to 6) and after cuprizone withdrawal (week 7 to 12). Supporting previous reports that the maximum demyelination in the cortex is reached after 6 weeks of cuprizone [20, 21], we showed that the peak of demyelination occurs between week 5 and 6 of cuprizone exposure ($p < 0.001$) (Fig. 1B–C). However, demyelination was histologically detectable as early as 3 weeks after cuprizone exposure ($p < 0.01$) (Fig. 1B–C). Gene expression of inflammatory markers of astrocytes (GFAP) and microglia (CD11b) was already upregulated after 1 week of cuprizone (Fig. 2A–B), although the peak in the expression was reached between weeks 4 and 6 ($p < 0.001$) for GFAP, parallel to myelin loss, and after 4 weeks ($p < 0.001$) for CD11b.

During the remyelination phase, cortical myelin score was still significantly lower ($***p < 0.001$; $*p < 0.05$) compared to controls until week 9 (3 weeks after cuprizone withdrawal), and a complete remyelination was observed between week 11 and 12 (5–6 weeks after cuprizone withdrawal) (Fig. 1B–C). Gene expression of GFAP and CD11b was significantly decreased 1 week after cuprizone withdrawal (week 7), but still upregulated compared to control animals during the all remyelination period ($p < 0.001$), indicating that the neuroinflammatory response that is associated with demyelination needs a longer time to be completely cleared (Fig. 2A–B).

Gene and protein levels of $\text{sPLA}_2(\text{V})$ and COX-1 are selectively increased in the cerebral cortex during demyelination

Gene expression of PLA_2 and COX isoforms in the cortex was investigated each week of cuprizone exposure and after cuprizone withdrawal. The mRNA expression of different PLA_2 isoforms was differentially affected during cuprizone-induced demyelination. Although the gene expression of $\text{iPLA}_2(\text{VI})$ was not changed (S.Fig. 2A), the expression of both cPLA_2 ($p < 0.01$ week 4 vs. control, $p < 0.05$ week 5 vs. control; Fig. 3B) and $\text{sPLA}_2(\text{V})$ ($p < 0.01$ weeks 4–6 vs. control; Fig. 3D) was increased at the peak of demyelination. After 4 weeks of cuprizone, COX-1 gene expression was also increased compared to controls ($p < 0.05$; Fig. 4D), but was otherwise unchanged at other time points. COX-2 gene expression was increased after 1 week of cuprizone and, again, after 3, 4 and 6 weeks ($p < 0.05$). In the remyelination phase, there was no change in the expression of any of the PLA_2 isoforms (S.Fig. 1A–B, SM 2B). COX-1 mRNA levels (Fig. 4D) were upregulated at week 11 ($p < 0.05$) and COX-2 (Fig. 4B) at week 9 and 12 ($p < 0.05$).

At the protein level, only sPLA₂(V) and COX-1 expression were significantly increased during demyelination, between week 4 and 6 (sPLA₂(V): 35 and 50% increase, respectively, $p < 0.05$, Fig. 3C; COX-1: 25% and 35% increase, respectively, $p < 0.05$, Fig. 4C). No change was observed in cPLA₂ and COX-2 protein levels (Fig. 3A and Fig. 4A).

COX-1 is specifically expressed by astrocytes and microglia during demyelination

The increase in the expression of COX-1 was concomitant with an influx of macrophages/microglia and proliferation of astrocytes. Specifically, the number of COX-1 positive cells dramatically increased after 5 weeks of cuprizone (Fig. 4E). Using fluorescent confocal imaging, we demonstrated that macrophages/microglia (labeled with CD11b; Fig. 4F) and astrocytes (labeled with GFAP; Fig. 4F), express COX-1 during demyelination (week 5 of cuprizone). COX-2 expression was not found in astrocytes or microglia (data not shown).

COX-derived prostanoids are increased in the cortex after cuprizone exposure

We measured prostanoid levels in the cerebral cortex at week 2, 4, and 6 of cuprizone intoxication and during remyelination, at week 9 and 12 (3 and 6 weeks after cuprizone withdrawal, respectively). During demyelination the increased expression of sPLA₂(V) and COX-1 and -2 resulted in increased levels of AA-derived prostanoids. Specifically, PGD₂ ($p < 0.001$), PGI₂ ($p < 0.05$) and PGE₂ ($p < 0.001$), and TXB₂ ($p < 0.05$) levels were increased after 6 weeks of cuprizone (Fig. 5A–D). During remyelination, only TXB₂ remained increased until week 9 (3 weeks after cuprizone withdrawal ($p < 0.05$; Fig. 5D), whereas PGE₂, PGI₂, and PGD₂ returned to normal levels.

Discussion

In this study we describe for the first time the time course of changes in gene and protein expression of enzymes involved in AA release and metabolism, such as PLA₂ and COX, and in the levels of their prostanoid end-products during cuprizone-induced demyelination as well as in the remyelinating phase. While AA metabolism has been shown to be altered in the EAE model of demyelination, which is dependent on the activation of the immune system [10, 11, 22–25], this is the first demonstration that the AA cascade is altered in a model for primary demyelination, in a time- and cell type-dependent fashion.

Increased production of AA metabolites is known to occur in many pathological processes, including lipopolysaccharide (LPS) or β -amyloid_{1–42}-induced neuroinflammation [26, 27], as well as kainate and other glutamate analogs-induced excitotoxicity, which result in neuronal damage [28, 29]. While the involvement of the AA cascade in these neuroinflammatory and excitotoxic processes is well established, its role in the physiology and pathology of oligodendrocytes and myelin is largely unknown.

We demonstrate that in the cuprizone model, there is a selective increase in the gene and protein expression of the sPLA₂(V) isoform, which is likely responsible for the release of AA from the membranes and the subsequent increase in the terminal pro-inflammatory prostanoid levels. In contrast, in the EAE model, the cPLA₂(IV) α isoform seems to be the isoform mainly involved in the disease process [9, 10]. A possible reason for these differences is that cPLA₂ α was associated to Th1 and Th17 lymphocyte response, which are not activated in the cuprizone model [9, 10]. Moreover, a recent study proposed that cPLA₂ GIVA plays a role only in the onset and iPLA₂ GVIA in both onset and progression of EAE, whereas sPLA₂ might have a potential role for in the later remission phase [30]. Although evidence supports the idea that targeting sPLA₂ can be beneficial in the EAE model [31–33], data on the specific involvement of sPLA₂ remain controversial due to the lack of selective inhibitors for this specific target.

Unesterified AA is further metabolized by COX enzymes. We show that both COX-1 and -2 gene expression was upregulated during cuprizone exposure, however, only COX-1 expression was increased at the protein level, likely because of its expression by astrocytes and activated microglia, that proliferate after 5 weeks of cuprizone exposure. The increased expression of COX-1 resulted in an increase of PGE₂, PGD₂, PGI₂ and TXB₂ levels in the cortex at the peak of demyelination (week 6).

We also report for the first time specific changes in the AA cascade during the remyelination phase. During remyelination, none of the PLA₂ isoforms showed an increased expression, indicating that there is no further stimulation of AA release. The gene expression of both COX-1 and COX-2 was sporadically upregulated, but no change was observed in their protein levels. Finally, PG levels returned to normal values during remyelination, whereas TXB₂ levels remained higher for up to 3 weeks after cuprizone. This could be due to the fact that COX-1-expressing microglia and astrocytes are still moderately upregulated during the remyelination phase, possibly causing an increased production of TXBs, which are COX-1 preferential end-products [34].

In conclusion, we describe specific alterations of the AA cascade in the cortex during cuprizone-induced demyelination and during remyelination. We identify a specific involvement of the sPLA₂(V) isoform, suggesting that sPLA₂(V) inhibition can limit the release of AA and the consequent production of pro-inflammatory PGs (PGE₂, PGD₂, PGI₂) and TXB₂ which are increased during primary demyelination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Progressive cuprizone-induced demyelination and remyelination in the cerebral cortex
 B) Black-Gold II staining in the cortex in control mice and every week of cuprizone exposure from week 1 to week 6, and after cuprizone withdrawal (from week 7 to week 12). 4 magnification objective; scale bar = 0.10 mm. C) Quantification of the Black-Gold II staining optical density (myelin score) in the cerebral cortex. Data are means $\pm$ SEM (**p<0.05, ***p<0.001). N= 3.

Figure 2. Gene expression of astrocytes and microglia markers during cuprizone-induced demyelination and remyelination in the cerebral cortex

Fold changes of mRNA as determined by RT-PCR of A) the astrocytes marker GFAP and of B) the microglia/macrophages CD11b every week of cuprizone intoxication (week 1 to 6) and every week after cuprizone withdrawal (week 7 to 12), compared to normal control (cntrl= 1). Data are means $\pm$ SEM (***) p <0.001). N= 6.

Figure 3. Protein and gene expression of cPLA₂ and sPLA₂ during demyelination in the cerebral cortex

% of protein levels as determined by western blotting of A) p-cPLA₂ and C) sPLA₂ after 4 and 6 weeks of cuprizone exposure, compared to normal control (cntrl= 100%). Fold changes of mRNA as determined by RT-PCR of B) p-cPLA₂ and D) sPLA₂ every week of cuprizone intoxication (week 1 to 6), compared to normal control (cntrl= 1). Data are means $\pm$ SEM (*p<0.05, ***p<0.001). N= 6.

Figure 4. Protein and gene expression of COX-1 and COX-2 during demyelination and remyelination in the cerebral cortex

% of protein level as determined by western blotting of A) COX-2 and B) COX-1 after 4 and 6 weeks of cuprizone intoxication and 3 (week 9) or 6 weeks (week 12) after cuprizone withdrawal, compared to normal control (cntrl= 100%). Fold change of mRNA as determined by RT-PCR of B) COX-2 and D) COX-1 every week of cuprizone intoxication (week 1 to 6), and every week after cuprizone withdrawal (week 7 to 12), compared to normal control (cntrl= 1). Data are means $\pm$ SEM (* p <0.05). N= 6. E) immunofluorescence of COX-1 after 5 weeks of cuprizone and F) double immunofluorescence of COX-1 and CD11b (microglia marker), or GFAP (astrocytes marker) after 5 weeks of cuprizone exposure. Confocal images, 60 magnification objective, scale bars = 0.20 μ m and 0.40 μ m. N= 3.

Figure 5. Prostanoids levels during demyelination and remyelination in the cerebral cortex
 A) PGD₂, B) PGI₂, C) PGE₂ and D) TXB₂ levels (pg/mg) in the cerebral cortex after 2, 4 and 6 weeks of cuprizone and after 3 or 6 weeks of cuprizone withdrawal (weeks 9 and 12) compared to normal control (cntrl). Data are means $\pm$ SEM (* p <0.05, ** p <0.01, *** p <0.001). N= 6–7.