Induction of COX-2 and down-regulation of COX-1 expression by LPS control prostaglandin E₂ production in astrocytes*

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Background: The relative contribution of COX-2 and COX-1 to prostanoid formation under neuroinflammation is complex.

Results: LPS induced COX-2 and mPGES1 but down-regulated COX-1 and TS in astroglia. These effects accounted for high production of PGE₂.

Conclusion: PGE₂ after LPS results from the coordinated COX-2 up-regulation and COX-1 down-regulation in astrocytes.

Significance: Changes in COX-2 and COX-1 expression mediate astrogial PGE₂ generation in neuroinflammation.

SUMMARY

Brain pathological conditions and pro-inflammatory stimuli induce cyclooxygenase-2 (COX-2), a key enzyme in arachidonic acid metabolism mediating the production of prostanoids that, amongst other actions, have strong vasoactive properties. Although low basal cerebral COX-2 expression has been reported, COX-2 is strongly induced by pro-inflammatory challenges, whereas COX-1 is constitutively expressed. However, the contribution of these enzymes in prostanoid formation varies depending on the stimuli and cell type. Astrocyte feet surround cerebral microvessels and release molecules that can trigger vascular responses. Here we investigate the regulation of COX-2 induction and its role in prostanoid generation after a pro-inflammatory challenge with the bacterial lipopolysaccharide (LPS) in astroglia. Intracerebral administration of LPS in rodents induced strong COX-2 expression mainly in astroglia and microglia, while COX-1 expression was predominant in microglia, and did not increase. In cultured astrocytes, LPS strongly induced COX-2 and microsomal prostaglandin-E₂ (PGE₂) synthase-1, mediated by the MyD88-dependent NFκB pathway and influenced by mitogen-activated protein kinase pathways. Studies in COX-deficient cells and using COX inhibitors demonstrated that COX-2 mediated the high production of PGE₂ and, to a lesser extent, other prostanoids after LPS. In contrast, LPS down-regulated COX-1 in a MyD88 dependent fashion, and COX-1 deficiency increased PGE₂ production after LPS. The results evidence that astrocytes respond to LPS by a COX-2 dependent production of prostanoids, mainly vasoactive PGE₂, and suggest that the coordinated down-regulation of COX-1 facilitates PGE₂ production after TLR-4 activation. These effects might induce cerebral blood flow responses to brain inflammation.

Cyclooxygenases (COX), also known as prostaglandin G/H synthases, play a crucial role in inflammation and are targets of widely used non-steroidal anti-inflammatory drugs. There are two main COX enzymes COX-1 and COX-2 that participate in the metabolism of arachidonic acid generating the unstable product prostaglandin (PG) G₂ that is reduced to PGH₂. PGH₂ is the substrate of prostaglandin isomerases that give rise to a family of vasoactive compounds called prostanoids, including molecules such as prostaglandins, thromboxane and prostacyclin. There is cellular specificity for the production of certain prostanoids and they exert different actions depending on the type of molecule produced and on the specific receptors that become activated (1). While COX-1 is constitutively expressed in most tissues, COX-2 is an inducible enzyme that responds to pro-inflammatory stimuli.
COX-2 is induced in brain cells under pathological conditions, but the role of the COX isoforms in brain diseases is not clearly established. COX-2 deficient mice are protected against brain ischemia (2), and inhibition of COX-2 provides beneficial effects against ischemic damage and neuronal death (3-6), suggesting a detrimental effect of COX-2 in stroke. In contrast, in neurodegenerative diseases, COX-2 inhibitors are not protective in mouse models of Alzheimer’s disease (AD) (7) and did not show benefits in clinical trials in Alzheimer’s disease patients (8) or in patients with mild cognitive impairment (9). Furthermore, COX-2 inhibitors increase the risk of cardiovascular and cerebrovascular pathology (10) and COX-2 deficient mice show exacerbated brain inflammation, leukocyte infiltration and blood-brain barrier (BBB) damage after exposure to the bacterial lipopolysaccharide (LPS) (11-15) suggesting some beneficial action of COX-2 in inflammation. Furthermore, COX-2 might contribute to neurovascular coupling since COX-2 inhibitors abrogate the increases in cerebral blood flow (CBF) induced by neuronal activation in rats (16). Exposure to LPS has been reported to induce vasodilation (17) and increase CBF (18) through a mechanism involving inducible nitric oxide synthase (iNOS) and the nox2 subunit of the superoxide-producing enzyme NADPH oxidase. Since LPS induces strong expression of COX-2 in the brain, it is feasible that vasoactive COX-2 products might also be involved in CBF regulation.

In the present study we examined the effect of intracerebral administration of LPS on the cellular expression of COX-2 and found strong up-regulation in microglia and astrocytes. Since astrocytes are recognised as important players in CBF regulation under physiological and pathological conditions (19) we then investigated the prostanooids induced by LPS and the COX isoforms involved in prostanooid generation in purified astrocyte cultures. The results show that the LPS challenge strongly induced COX-2 in astrocytes through a MyD88/NFκB-dependent mechanism, show the crucial role of COX-2 in prostanooid production after LPS, and evidence that PGE₂ is the major product of arachidonic acid metabolism under these experimental conditions. Furthermore, we found that LPS down-regulates Cox-1 gene expression and that COX-1 deficient cells produce more PGE₂ than the wt, indicating some negative effect of COX-1 on the COX-2-dependent production of PGE₂ in astrocytes after LPS.

**Experimental Procedures**

**Animals**- Animal work was authorized by the Ethical Committee of the University of Barcelona (CEEA), and it was performed in agreement with the local regulations and in compliance with the Directives of the European Community. Four-month old male Sprague-Dawley rats were obtained from Charles-River (Lyon, France). MyD88 knock-out (KO) mice in a C57Bl/6 background were obtained from Oriental Bioservices, Inc. (Kyoto, Japan). MyD88 KO mice (+/-) were crossed with wild-type (wt) (+/+) C57Bl/6 mice (Charles River) and a colony of MyD88 heterozygous mice (+/-) was kept in the animal house of the School of Medicine (University of Barcelona). Each individual animal born from the heterozygous progenitors was genotyped and the MyD88 KO and the MyD88 wt animals were selected for the studies described below. COX-1 and COX-2 heterozygous mice were from Taconics Inc. (Hudson, NY, USA). COX-2 or COX-1 heterozygous females (+/-) were crossed with homozygous (-/-) males; all had a mixed B6;129P2 background. We genotyped each animal of the offspring and the KO animals were selected for the studies, while the corresponding wt animals were used as controls.

**Genotyping protocols**- Genotyping was carried out by doing PCR on DNA extracted from tail biopsies. The Extract-N-Amp Tissue PCR Kit (Sigma) was used for the extraction of DNA and preparation of PCR reactions according to the manufacturer’s instructions. For experiments with COX KO or wt cells, the following sets of primers (20) were used for PCR: Cox-1 KO forward 5’-GCAGCCTCTGTTCACATACATACAC-3’, Cox-1 wt forward 5’-AGGAGATGGCTGTGAGTTGG-3’, Cox-1 reverse (common) 5’-GAATCTGACTTTCTGAGTTGCC-3’. Amplicon sizes were as follows, Cox-1 wt: 601 bp; Cox-1 KO: 646 bp; Cox-2 wt: 725 bp; Cox-2 KO: 905 bp. The cycling parameters were: 94 °C, 5 min
for initial denaturation, followed by 34 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C (Cox-1) or 57°C (Cox-2) for 30 s, extension at 72°C for one min. The last cycle was followed by an additional extension step at 72°C for 5 min. Supporting information depicts an example of a genotyping reaction (Supplementary Fig. 1). For experiments with MyD88 KO mice, the genotyping procedure has been reported (21).

**LPS administration to rodents**- Rats and mice were anesthetized with isoflurane and placed in a stereotaxic apparatus for injection of LPS or the vehicle (phosphate-buffered saline, PBS) in the right striatum. For rats, LPS (5 µl of 1µg/mL) or the same volume of vehicle was injected at the following coordinates according to the atlas of Paxinos and Watson (22) in relation to Bregma: 0.5 mm antero-posterior, 3 mm lateral, and 5 mm ventral. For mice, LPS (0.7 µl of 1µg/mL) or vehicle was injected at the coordinates: 0.5 mm antero-posterior, 2 mm lateral, and 3 mm ventral. After 8h, animals were anesthetized with isoflurane, perfused through the heart with saline to remove blood from the brain vessels, and brain tissue was obtained after dissection of the ipsilateral and contralateral striatum and was immediately frozen and kept at -80 °C until further use. A different set of animals was processed for immunohistochemistry.

**Cell cultures**- Glial cell cultures enriched in astrocytes were prepared from the cerebral cortex of 1- to 2-day-old rats or mice as previously described (21, 23), with minor modifications. In brief, cells were maintained at 37°C in a humidified atmosphere of 5% CO₂-95% air in culture medium: DMEM (Dulbecco’s modified Eagle medium, Gibco-BRL, Invitrogen, Paisley, UK) for rat astrocytes, and DMEM:F-12 nutrient (1:1) (Gibco-BRL) for mouse astrocytes. Media were supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) and 4 mL/L of a mixture of penicillin/streptomycin 10,000U/ 10,000 µg/mL (Gibco-BRL). Cells were subcultured to obtain purified astroglia cultures, as follows: at confluence after 8-10 days in vitro, cells were treated with 4 µM antimitotic cytosine arabinoside (Ara-C, Sigma-Aldrich) for 5 days to eliminate dividing cells, i.e. mostly microglia and progenitors. Flasks were shaken overnight and the remaining astrocyte adherent monolayer was detached with trypsin 0.0125%/EDTA 0.2 mM and seeded at 10x10⁴ cells/mL with incubation medium (as above). Purified astrocytes were treated when cells reached confluence at four days after subculturing. FBS was reduced to 1% 16h prior to treatments.

Rat astrocyte cultures contained only 2.01±1.68% of contaminating microglia cells, as reported (21). Purified mouse astrocyte cultures also contained very little microglia, as estimated by immunofluorescence and by examining the expression of CD11b mRNA (see below for description of these methods). After immunofluorescence with an antibody against a microglial marker (Iba-1) and an antibody against glial fibrillary acidic protein (GFAP) to label astrocytes (Supplementary Fig. 2), we counted (n=24 fields x 2 cultures, using x20 magnification) the percentage of CD11b expression per culture by real time RT-PCR as a marker of microglia, and used purified microglia cultures (obtained as previously reported (24)) as a reference for 100% CD11b expression. According to this procedure, CD11b expression (mean±SD, n=5) in astroglia cultures was 1.41±1.22%, supporting that contaminating microglia cells were very scarce in the purified astroglia cultures.

For experiments with MyD88 KO, Cox-1 KO, and Cox-2 KO cells, individual astrocyte cultures were obtained from each newborn animal and, after genotyping, the -/- (KO) and +/- (wt) cultures were selected for use in further experiments. Experiments in KO and wt cells were carried in parallel.

**Drug treatments**- Cells were exposed to 10 ng/mL LPS (Escherichia coli 055:B5) (Sigma-Aldrich, St. Louis, MO, USA) for time periods ranging from 4h to 24h. Cells were treated with the following mitogen activated protein kinase (MAPK) inhibitors (Calbiochem, San Diego, CA, USA): MAPK kinase (MEK) inhibitors 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) (1-25 µM); and stress-activated SAPK/MAPK inhibitor anthra (1,9-cd)pyrazol-6(2H)-one (SP600125) (1-25 µM); and stress-activated SAPK/MAPK inhibitor anthra (1,9-cd)pyrazol-6(2H)-one (SP600125) (1-25 µM) that were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and were given 30 min prior to LPS. COX-2 inhibitor N-[Cyclohexyloxy
-4-nitrophenyl] methanesulfonamide (NS-398) was purchased from Tocris Bioscience (Ellisville, MO, USA), dissolved in saline pH 13 and used at 3 µM. COX-1 inhibitor 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole (SC-560) was purchased from Calbiochem, dissolved in DMSO, and used at 10 nM. Treatment with COX inhibitors was carried within 30-60 min prior to LPS. The cytosolic phospholipase A2 (cPLA2) inhibitor arachidonyl trifluoromethyl ketone (AACOCF3) (Calbiochem) was dissolved in DMSO and used at 2 µM 30 min before LPS. For all drugs, the corresponding vehicle was used to check for any non-specific effects in all the experiments. The final concentrations of the vehicles, DMSO or saline pH 13, did not exceed 0.25% and 0.3%, respectively. No effects of the vehicles on the parameters studied were detected.

siRNA transfection- Twenty-four hours after subculturing, rat astrocytes were transfected with specific siRNA sequences TARGETplus™ SMARTpool siRNA from Thermo Fisher Scientific Dharmacon Products (Lafayette, CO, USA). TARGETplus™ SMARTpool siRNA directed against NFκB p65 (L-0800033-01, Rat ReLA, NM_199267), NADPH oxidase flavocytochrome b558 gp91(phox) (J-093524), COX-1 (#19224, PTGS1), and against the mouse MAPK: MAPK1 (#26413, Erk2, NM_011949), MAPK10 (#26414, JNK3, NM_009158), and MAPK14 (#24416, p38, NM_011951). ON-TARGETplus non-targeting siRNA (D-001810-01) was used as a negative control (ns). These siRNAs were predesigned by the maker to minimize off-side effects (23), they were used at 100 nM, and were transfected with oligofectamine™ (Invitrogen), as previously described (23). Astrocytes were used four days after siRNA transfection. The silencing effect was verified by RT-PCR and/or Western blotting.

Western blotting- Astrocytes were lysed in buffer containing protease inhibitors. Twenty µg of protein extract were resolved by SDS-PAGE and proteins were transferred to a polyvinylidene difluoride membrane. Rabbit polyclonal antibodies were used against COX-2 (#160126, Cayman Chemical) diluted 1:500; thromboxane synthase, TS (#ab39362, Abcam, Cambridge, UK) diluted 1:1,000; microsomal prostaglandin E synthase-1, PGES-1 (#AS03 031, Agrisera, Vännas, Sweden) diluted 1:1,000; c-Jun terminal kinase, JNK (#J4500, Sigma) and p38 MAPK (#M0800, Sigma) both diluted 1:10,000. A goat polyclonal antibody against COX-1 (Santa Cruz Biotechnology, CA, USA) was used diluted 1:1,000. Mouse monoclonal antibodies were used against ERK1/2 (#610123, BD Biosciences) diluted 1:50,000; and against β-tubulin (T4026, Sigma, St. Louis, MO, USA) diluted 1:50,000, which was used as control for protein gel loading. Antibodies were diluted in tris-buffered saline containing 0.5% Tween-20 and were incubated overnight at 4°C. On the following day, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA, USA) diluted 1:1,000, or goat anti-rabbit IgG (Amersham Biosciences; Piscataway, NJ, USA) 1:2,000, for 2h at room temperature. The blots were developed with the use of a chemiluminescent substrate (ECL Western Blotting Analysis System; Amersham).

Immunocytochemistry- Astrocytes were seeded on poly-lysine-coated coverslips. Cells were washed in phosphate-buffered saline (PBS) and fixed in 4% parafomaldehyde for 30 min. Cells were permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 10 min, blocked with 10% goat or horse serum in PBS for 1h and incubated overnight at 4°C with one of the following primary antibodies: a rabbit polyclonal antibody against Iba-1 (#019-19741, Wako Chemicals GmbH, Neuss, Germany) diluted 1:1,000, or a monoclonal antibody against GFAP (#G3893, Sigma) diluted 1,1000. The next day, cells were washed and incubated with green-fluorescence Alexa Fluor® 488 dye-labelled goat anti–rabbit IgG antibody, and Alexa Fluor® 546 goat anti-mouse IgG diluted 1,1000, or a monoclonal antibody against GFAP (#G3893, Sigma) diluted 1,1000. The next day, cells were stained with Hoechst to visualize the nuclei. The coverslips were mounted onto microscope slides using Mowiol mounting medium (Calbiochem, Merck Chemicals, Darmstadt, Germany). Observations were performed with an Olympus IX70 fluorescence microscope.

For immunohistochemistry in brain tissue, animals were perfused through the heart with saline followed by paraformaldehyde (PFA, 4%) in phosphate buffer (pH 7.4). The brain was removed, post-fixed with PFA overnight and then kept in phosphate buffer before slicing it in a vibratome to obtain 30 µm-thick coronal sections. Brain sections were cryoprotected in a solution containing glycerol and were kept frozen
at -20 °C. Immunohistochemistry was performed free-floating with vibratome sections, as previously reported (25). Endogenous peroxidases were blocked with 3% hydrogen peroxide and 10% methanol in PBS, for 25 min. Sections were incubated for 2h in 3% normal horse or goat serum for mouse monoclonal or rabbit polyclonal antibodies, respectively, to block unspecific binding sites, washed in T-PBS (PBS containing 0.5% Triton X-100), and incubated overnight at 4°C with either mouse monoclonal antibody against COX-1 (#160110, Cayman Chemical) diluted 1:100, or rabbit polyclonal anti-COX-2 antibody (#160126, Cayman Chemical) diluted 1:500. Thereafter, the sections were rinsed in T-PBS and incubated for 1 h with a biotinylated secondary antibody (1:200, Vector Laboratories), followed by incubation with 1% avidin–biotin–peroxidase complex (ABC kit, Vector Laboratories). The reaction was visualized with 0.05% diaminobenzidine in 0.03% hydrogen peroxide in PBS. Double immunohistochemistry was carried out following the first immunoreaction with COX-1 or COX-2. The second primary antibodies used were: a rabbit polyclonal antibody against GFAP (#Z0334, DakoCytomation) diluted 1:500, which labels astroglia, or a rabbit polyclonal antibody against Iba-1 (as above) diluted 1:500 to detect microglia. Sections were then incubated with the avidin-biotin complex, washed with 0.01 M sodium phosphate buffer, pH 6, and pre-incubated for 10 min with 0.01% benzidine dihydrochloride and 0.025% sodium nitroferricyanide in 0.01 M sodium phosphate buffer, pH 6. The reaction was developed with this solution containing 0.005% H₂O₂. Immunoreaction controls included omission of the first or second primary antibodies. The induction of COX-2 in microglia and in astrocytes was assessed by counting the number of GFAP+ and of Iba-1+ cells expressing COX-2 in the ipsilateral striatum. Microscopic photographs (×20 objective) of three areas surrounding the injection site were taken per brain section in three brain sections per animal. The proportion of microglia (Iba-1+) and of astroglia (GFAP+) expressing COX-2 was calculated in each photograph and the average value of all the photographs per animal was calculated. Values are expressed as the mean of four animals treated with LPS and three animals treated with PBS.

Real-time RT-PCR - Total RNA was extracted using Purelink RNA Kit (Invitrogen, Spain). RNA quantity and purity were determined using ND-1000 micro-spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). One-µg total RNA was reverse-transcribed using a mixture of random primers (High Capacity cDNA Reverse Transcription kit, Applied Biosystems). Real-time quantitative RT-PCR analysis was carried out by SYBR green I dye detection (#11761500, Invitrogen) using the iCycler iQTM Multicolor Real-Time Detection System (Bio-Rad). PCR primers were designed with the aid of Primer3 software to bridge the exon–intron boundaries within the gene of interest to exclude amplification of contaminating genomic DNA. Several genes were assayed as loading controls (HPRT1, SDHA, YWHAZ, and RPL14). RPL14 was the control gene showing the best stability after LPS treatment and was chosen for normalization. Primers (see list in Table 1) were purchased from IDT (Conda, Spain). Optimized thermal cycling conditions were: 1 min at 50°C, 8 min and 30 sec at 95°C and 40 cycles of 15 sec at 95°C and 30 sec at 60°C in which an optical acquisition were performed. Data were collected after each cycle and were graphically displayed (iCycler iQTM Real-time Detection System Software, version 3.1, Bio-Rad). Melt curves were performed upon completion of the cycles to ensure absence of nonspecific products. Quantification was performed by normalizing Ct (Cycle threshold) values with the RPL14 control gene Ct, and analysis was carried out with the 2-ΔΔCT method (26).

ELISA immunoassays- The cellular supernatant was used in ELISA assays to measure the concentration of: prostaglandin E2 (PGE₂, #900-001), thromboxane B₂ (TxB₂, #900-002), and 6-keto-Prostaglandin F₁α (PG F₁α, #900-004) (Assay Designs, Ann Arbor, MI, USA).

Statistical analyses- One-way ANOVA was used for comparisons between multiple groups, after testing for normality, followed by the post-hoc Bonferroni test. Comparison between two groups was carried out with the t-test after verifying normal distribution, otherwise the non-parametric Mann Whitney test was used. Linear or non-linear regression analyses were used for curve fits as appropriate using GraphPad software.
RESULTS

Intracerebral administration of LPS induces Cox-2 in microglia and astroglia. Intracerebral administration of LPS to rats induced mRNA expression of TNF-α (Fig. 1A) in the ipsilateral hemisphere at 8h. LPS also increased the expression of Cox-2 mRNA (Fig. 1B) and COX-2 protein (Fig. 1C, D). LPS induces TLR-4 activation and recruitment of the MyD88 adaptor protein that mediates activation of the transcription factor NFκB and induction of target genes, such as the pro-inflammatory cytokine TNF-α (21). In agreement with this, MyD88 KO mice did not show induction of TNF-α (Fig. 1E) or Cox-2 (Fig. 1F) mRNA in the ipsilateral hemisphere after LPS, evidencing that induction of both genes was MyD88-dependent.

LPS up-regulated the expression of Iba-1 mRNA in the ipsilateral hemisphere at 8h suggesting microglial activation, whereas expression of GFAP mRNA was not modified at this time point (Supplementary Fig. 3). However, by immunohistochemistry (Fig. 2), we detected a strong induction of COX-2, not only in microglia (Fig. 2A-D) but also in astrocytes (Fig. 2E-G) of the ipsilateral hemisphere 8h after LPS. Quantification of the immunohistochemistry showed increased number of COX-2 immunoreactive microglia (Iba-1) and astrocytes (GFAP) after LPS (Fig. 2P,Q). COX-1 was expressed under basal conditions preferentially in microglia (Fig. 2H-K) and, with a lower intensity, in astroglia (Fig. 3L-O), and it was not up-regulated by LPS (Fig. 2H-O).

We then undertook an in vitro study in purified cultures of astroglia treated with LPS to unravel the mechanisms underlying COX-2 induction and prostanoid release induced by TLR-4 activation and the effects of deficiency or inhibition of either COX-1 or COX-2.

Regulation of COX-2 expression in astrocytes challenged with LPS. LPS induced TNF-α (Fig. 3A) and COX-2 (Fig. 3B) mRNA and protein expression (Fig. 3C,D) in cultured astrocytes, as it did in vivo (Fig. 1A-D). The transcription factor NFκB was involved in COX-2 induction since silencing the p65 subunit of NFκB with siRNA attenuated Cox-2 mRNA induction (Fig. 3E). However, this effect was not observed by silencing other genes, such as the gp91 subunit of NADPH oxidase complex (Fig. 3E) that was reported to mediate COX-2 induction after LPS in microglia (27). The involvement of NFκB in mediating the induction of COX-2 after LPS was further substantiated by the use of the inhibitor PDTC, which attenuated the effect of LPS (Fig. 3F). We then used astrocytes from mice deficient in MyD88 or corresponding wt mice to explore whether COX-2 induction was dependent on the Myd88 pathway in these cells, as previously observed in vivo (Fig. 1E,F). LPS failed to induce TNF-α mRNA in Myd88-deficient astrocytes (Fig. 3G), which did not express Cox-2 mRNA (Fig. 3H) or protein (Fig. 3I), either. Therefore COX-2 induction after LPS is dependent on Myd88 and Nfkb.

Mitogen-activated protein kinases (MAPK) participate in LPS signaling (28) and can mediate COX-2 induction (29). We used specific inhibitors of MAPK pathways to unravel their contribution in COX-2 up-regulation after LPS in astrocytes. Cox-2 mRNA induction was severely reduced by the p38 inhibitor SB23906 and by the c-Jun NH2-terminal kinase (JNK) inhibitor SP600125, but not by the MEK inhibitor U0126 (Fig. 4A). Likewise, COX-2 protein expression 8h after LPS was very sensitive to SB239063 (from 1 µM) (Fig. 4B), and SP600125 (from 10 µM) (Fig. 4C), while U0126 (1-25 µM) had a negligible effect (Fig. 4D). This finding was validated with another MEK inhibitor, PD98059 (from 10 to 40 µM) (Fig. 4D). The same result was found at 4h (Fig. 4E). In agreement with this, the production of PGE2, as assessed by ELISA 8h after LPS, was reduced by p38 and JNK inhibitors, but not after MEK inhibition (Fig. 4F).

We then silenced the expression of MAPK1 (ERK2), MAPK10 (JNK3) and MAPK14 (p38) using siRNA. Western blotting (Fig. 4G) showed that siRNAs reduced the corresponding protein expression by 65-70%. Silencing p38 and JNK3 attenuated the expression of Cox-2 mRNA and protein, but no significant effects were observed after silencing MAPK1 (Fig. 4H-J). Therefore, we can conclude that induction of COX-2 expression after LPS is strongly dependent on the Myd88 pathway, NFκB, and on p38 and JNK pathways.

Regulation of COX-1 expression after LPS. We also examined whether the expression of constitutive COX-1 was affected by LPS. The expression of Cox-1 mRNA was significantly reduced 8h after LPS in wt astrocytes, and the same effect was observed in COX-2 KO cells.
(Fig. 5A), which we verified that did not express Cox-2 mRNA (Fig. 5B) or protein (Fig. 5C, D). While the reduction of Cox-1 mRNA by LPS was already seen at 4h (Fig. 5G,I), COX-1 protein expression was unaltered at this time point but a slight reduction was seen at 8h and 24h (Fig. 5E,F). The delay in the reduction of the amount of COX-1 protein after the decreased expression of Cox-1 mRNA might be due to the presence of the constitutive protein that needs to follow its turnover before reductions in mRNA can be translated into protein decreases.

LPS-induced reduction of COX-1 was not prevented by MAPK inhibition (Fig. 5G,H) or by silencing MAPK expression with siRNA (Fig. 5I,J). However, LPS-induced down-regulation of Cox-1 mRNA was dependent on the MyD88 pathway since LPS did not reduce it in MyD88 KO mice (Fig. 5K). To better substantiate this finding, we examined whether down-regulation of COX-1 also occurred in vivo in the mouse brain after intracerebral LPS administration. Expression of Cox-1 mRNA was significantly reduced 8h after injection of LPS, but not after injection of PBS (Fig. 5L). This effect was strongly attenuated in MyD88-deficient mice (Fig. 5L), supporting that it was MyD88-dependent. PDTC, attenuated the reduction of Cox-1 mRNA induced by LPS in cultured cells, suggesting that NFκB was involved (Fig. 5M).

Since COX-1 and COX-2 expression responded in an opposite way to the LPS challenge, we examined whether Cox-1 deficient mice (Fig. 5N) showed up-regulation of Cox-2 mRNA (Fig. 5O) and protein (Fig. 4O) after LPS, which they did. These results support that while LPS strongly induces COX-2, it represses the expression of COX-1, and both responses are dependent on the MyD88 pathway, whereas p38 and JNK MAPK are involved in up-regulating COX-2 but not in down-regulating COX-1.

**LPS modifies the expression of prostaglandin isomerasers.** The types of prostanoids that are produced after COX activation depend on the action of specific prostaglandin isomerasers, i.e. the enzymes responsible for the production of prostanoids from COX-derived PGH₂. LPS induced strong mRNA expression of one of the isoforms of PGE₂ synthase, the microsomal PGE synthase-1 (mPGES-1), (Fig. 6A), in wt and COX-1 or COX-2 deficient cells. Like for COX-2, induction of mPGES-1 after LPS was dependent on the MyD88 pathway since MyD88-deficient cells showed no increase of mPGES-1 mRNA expression (Fig. 6B). These findings show that LPS up-regulates the expression of mPGES-1 through the MyD88 pathway, in a manner coordinated with the induction of COX-2, to strongly generate PGE₂. In contrast to the above findings, LPS down-regulated the expression of prostacyclin synthase (PGIS) mRNA (Fig. 6C) and, to a greater extent, thromboxane synthase mRNA (TS) (Fig. 6D). Accordingly, while after LPS the expression of mPGES-1 protein significantly increased (Fig. 6E), the expression of TS protein tended to be progressively lower than in controls (Fig. 6F). The latter effects on TS paralleled the down-regulation of COX-1 expression after LPS (Fig. 5A, E), suggesting common regulatory pathways.

**LPS exposure induces prostanoid production in astrocytes.** COX enzymes metabolize arachidonic acid (AA) to prostaglandins PGG₂ and PGH₂ that are rapidly converted by cell-specific prostaglandin isomerasers into different prostanoids, including PGE₂, PGF₂α, prostacyclin (PGI₂), and thromboxane A₂ (TxA₂), amongst others. We examined the profile of several prostanoids induced by LPS in the culture medium of rat astrocytes by ELISA assays. TxA₂ has a half-life of only a few seconds (30), and its production is typically assessed by measuring TxB₂, which is a stable metabolite. PGI₂ has a half-life of 60 min in plasma but it is stable for only a few minutes in buffer (30) and its production is typically monitored by measurement of 6-keto-prostaglandin F₁α (PGF₁α). LPS caused a very strong accumulation of PGE₂ in the cell culture medium of rat astrocytes from 2 to 24h (Fig. 7A,B) and, to a lesser extent it increased the concentration of TxB₂ (Fig. 7C,D) and of PGF₁α (Fig. 7E,F) at 8h and 24h. The cytosolic phospholipase A₂ (cPLA₂) inhibitor AAOCOF3 fully prevented the production of prostanoids (Fig. 7G-I), suggesting the involvement of cPLA₂ in arachidonic acid mobilization after LPS.

**Prostanoid production after LPS treatment is prevented by COX-2 inhibitors.** COX-2 inhibitor NS-398 strongly blocked LPS-induced PGE₂, TxA₂ and PGI₂ production, whereas COX-1 inhibition with SC-506 only partly attenuated the generation of prostanoids after LPS in rat astrocytes (Fig. 7B, D, F). These results suggested that COX-2 was the main mediator of
prostanoid production after LPS, and pointed to a small contribution of COX-1 due to a weak inhibitory effect of SC-560. In spite that SC-560 is widely used to inhibit COX-1 and that specific inhibition of this enzyme has been shown in cell-free systems, cell studies suggest that this compound may also exert some non-specific inhibitory effects on COX-2 (31). This possible inhibition of COX-2 might explain why we found some partial inhibitory effects of SC-560 on prostanoid production in our system. To further investigate if SC-560 has COX-1-independent effects, we used astrocytes from COX-1 deficient mice. SC-560 significantly (p<0.001) reduced the production of PGE$_2$, PGF1$\alpha$, and TxB$_2$ (Supplementary Fig. 4) induced by LPS in Cox-1 KO cells, thus indicating that this compound may have COX-1 independent effects.

**Prostanoid production after LPS treatment is dependent on COX-2.** We showed above that the induction of COX-2 by LPS was strongly inhibited in MyD88 deficient cells (Fig. 3H, I). For this reason, we then examined whether LPS-induced prostanoid production was abrogated in these cells. Compared to the previous findings of prostanoid release after LPS in rat astrocytes, we noticed that mouse astrocytes produced less thromboxane and more prostaglandin than rat astrocytes, while in both species PGE$_2$ was the prostanoid more abundantly generated in response to LPS. Lack of MyD88 prevented the production of prostanoids after LPS (Fig. 8A-C), thus further supporting that COX-2 was the main mediator of prostanoid production after this challenge. Since COX-2 was not induced in MyD88 KO cells, the slightly higher production of TxB$_2$ after LPS than in control MyD88 KO cells might be attributable to COX-1 activity and related to the finding that the basal COX-1 expression was not down-regulated after LPS in MyD88 KO cells (Fig. 4I).

We then used astrocytes obtained from mice deficient in COX-1 or COX-2, and their corresponding wt controls to validate the above findings, excluding possible interferences due to non-specific effects of the drug inhibitors. Astrocytes lacking COX-2 did not produce PGE$_2$ (Fig. 8D), PGF$_1\alpha$ (Fig. 8E) or TxA$_2$ (Fig. 8F) in response to LPS, thus confirming that COX-2 was the main enzyme involved in the production of prostanoids induced by LPS in astrocytes. Under basal non-stimulated conditions, the concentration of TxB$_2$ was not reduced in cells lacking COX-2 compared to the wt, while they showed very low levels of PGE$_2$ and PGF1$\alpha$, suggesting that COX-1 is involved in the low basal production of TxA$_2$ in astrocytes. This is in agreement with the previous observation in MyD88 deficient cells, where LPS did not induce COX-2 but did not down-regulate COX-1 either. These cells showed an increase in the production of TxB$_2$ after LPS (Fig. 8C) that is attributed to the basal activity of COX-1 metabolizing the AA newly generated after LPS-induced cPLA$_2$ activation.

COX-1 deficient cells produced PGE$_2$ after LPS to a greater extent than the corresponding wt astrocytes (Fig. 8G), evidencing that COX-2 is the enzyme responsible for PGE$_2$ production and suggesting some negative regulatory effect of COX-1 on PGE$_2$ production after LPS. Also, LPS increased the production of PGF$_1\alpha$, as assessed by measuring PGF1$\alpha$ (Fig. 8H), and TxA$_2$, as assessed by measuring TxB$_2$ (Fig. 8I), in COX-1 deficient cells suggesting the involvement of COX-2. To add further support to these findings, we silenced COX-1 with siRNA (Fig. 8J). Under these conditions, a small but significant increase in the production of PGE$_2$ after LPS was observed (Fig. 8K), whereas the production of PGF$_1\alpha$ (Fig. 8L) and TxA$_2$ (Fig. 8M) was not altered. Altogether, these results show that COX-1 activity maintains basal production of prostanoids in cultured astrocytes, but has not a major role in the increased production of prostanoids after LPS.

**DISCUSSION**

The present results show that the production of prostanoids induced by LPS in glial cells is essentially mediated by COX-2. The MyD88-dependent pathway and the transcription factor NFkB were involved in COX-2 gene expression. In addition, p38 and JNK MAPK pathways influenced COX-2 expression, thus revealing a complex regulation of the expression of this gene in response to TLR-4 activation in astroglia. COX-2 induction was accompanied by strong production of PGE$_2$, and, to a lesser extent, other prostanoids. Several lines of evidence suggest that the COX isoforms are coupled to the activity of the various prostaglandin isomerase catalysing the production of certain prostanoids in a cell-type dependent manner (32). The strong production of PGE$_2$ in astrocytes after LPS is in concordance with the enhanced expression of the microsomal isoform of prostaglandin E synthase-
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1 (mPGES-1), in agreement with previous findings (33). In addition, here we report that the expression of the mPGES-1 gene after LPS was up-regulated through the MyD88 pathway, like COX-2. Therefore, LPS induces the coordinated expression of COX-2 and mPGES-1, which appeared to be functionally coupled and account for the high production and release of PGE2 in astrocytes, in a manner similar to the responses described in macrophages (34).

In a previous study, induction of COX-2 and mPGES-1 was mainly found in microglia in the substantia nigra 48h after intracerebral injection of LPS (35). In contrast, we also observed the induction of COX-2 in astrocytes 8h after LPS injection into the striatum. Besides any regional differences in the reaction to LPS, it is likely that the time course of the glial reaction to this challenge accounts for the observed differences. Increased expression of mPGES-1 has been reported under pathological conditions, e.g. in the brain of Alzheimer’s disease patients (36), and the enzyme is up-regulated in astrocytes stimulated with β-amyloid (37). Also, after intracerebral hemorrhage, strong induction of COX-2 and mPGES-1 was found in astrocytes (38). Therefore, the findings reported here in cultured cells might be relevant to certain neuropathological conditions.

In contrast to the increased expression of COX-2 and mPGES-1, the expression of COX-1 was down-regulated in astrocytes after TLR-4 activation. Reduced expression of COX-1, together with increased COX-2 expression, was previously found in the lungs and hearts of LPS-treated rats (39). In addition, we found that LPS down-regulated the expression of the TS gene in astrocytes, suggesting some link in the control of the expression of COX-1 and TS. In spite of down-regulation of PGIS and TS mRNA, LPS enhanced the production of PG and TxA2, but to a lower extent than it increased PGE2. This apparently contradictory effect (i.e. reduction of mRNA but increase in enzymatic products) could be due to the time delay needed for an effective reduction of protein content following decreases of constitutive mRNA expression. It is feasible that down-regulation of these genes limits the production of PG and TxA2 in astrocytes, while favoring the production of PGE2 due to up-regulation of mPGES-1. Under basal non-stimulated conditions, COX-1 deficient astrocytes produced less PG and TxA2 than the wild type cells while COX-2 deficient astrocytes showed unaltered concentrations of these prostanoids, suggesting that COX-1 was involved in the basal production of PG and TxA2. However, after LPS, the production of PG and TxA2 increased in COX-1 deficient cells, but not in COX-2 deficient cells, demonstrating that COX-2 was mainly responsible for their up-regulation after this challenge. Likewise, the production of PGE2 after LPS was fully dependent on COX-2. However, COX-1 deficient cells produced more PGE2 after LPS than the corresponding wt cells, suggesting that COX-1 exerts some negative control on COX-2-dependent PGE2 production after LPS.

Taken altogether, the present results demonstrate the key role of COX-2 in prostanoid production after LPS in astrocytes, and evidence that the production of PGE2 also depends on down-regulation of COX-1 gene expression. Finally, these findings show that astrocytes respond to proinflammatory triggers with a strong generation of vasoactive PGE2 that might exert effects on the adjacent brain microvasculature and contribute to modulate CBF responses to neuroinflammation.

REFERENCES

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FOOTNOTES

MFN had a PhD fellowship of the Spanish Ministry of Science and Innovation (MICINN). Work supported by MICINN (SAF2008-04515) and by the European Community (FP7/2007-2013 project, grant agreement n°201024). We thank Ms. Francisca Ruiz and Ms. Eva Roig for excellent technical assistance.

FIGURE LEGENDS

Fig. 1. LPS administration to the rat brain induced COX-2 in glial cells. Rats received an intrastriatal injection of LPS (5 µl, 1µg/µl) or the vehicle (PBS) and the brain tissue was obtained 8h later to study mRNA and protein expression. A,B) Rats injected with LPS show a very pronounced increase in the expression of TNF-α and Cox-2 mRNA in the ipsilateral hemisphere compared to that in animals receiving the vehicle or in the contralateral hemisphere (n=3-5 rats per group). C) COX-2 protein was detected by Western blotting in the ipsilateral hemisphere 8 h after LPS. D) Semi-quantification of COX-2 band intensity evidenced a significant increase of COX-2 expression after LPS (n=3 rats per group). E, F) Expression of TNF-α and Cox-2 mRNA in the ipsilateral hemisphere of mice 8h after striatal injection of LPS (0.7 µl, 1µg/µl) is powerfully attenuated in MyD88-deficient mice (MyD88 KO) compared to the wt (n=3-4 per group). Control animals received intrastriatal injection of the vehicle (PBS). One symbol: p<0.05; two symbols: p<0.01; three symbols: p<0.001. Symbols indicate comparison versus either (*) control or (&) LPS wt.

Fig. 2. COX-2 is induced in astrocytes and microglia after LPS in the rat brain. Immunohistochemistry against COX-2 (brown in A-G) and COX-1 (brown in H-O) in control rat brain (A,C,E,H,J,L,N) and after LPS (B,D,F,G,I,K,M,O) shows co-localization of COX-2 with markers of microglia (Iba-1) (B,D) and astroglia (GFAP) (arrow in F, G) (dark blue/purple), whereas COX-1 is predominantly expressed in microglia (H-J) and to a lower extent in astroglia (L-N), and it is not upregulated after LPS (I, K, M, O). The areas indicated with rectangles in L, M are magnified in N, O, respectively. Bar scale = 30 µm (A-G, J-K, N,O); 60 µm (L, M); 120 µm (H-I). P,Q) Quantification of the proportion of microglia and astroglia cells expressing COX-2. Values are expressed as % of total Iba-1+ microglia (P) or GFAP+ astroglia (Q). n=4-5 LPS-treated mice and n=3 mice injected with PBS. Controls are taken as the contralateral non-affected hemispheres, n=7. One-way ANOVA, *** indicates p<0.001.

Fig. 3. COX-2 induction after LPS is dependent on NFkB and MyD88 pathways. LPS (10 ng/mL) was added to rat (A,B, E) or mouse (C-D, F-I) astrocyte cultures and mRNA/protein were studied at different time points (A-D), or at 4h. A,B) Time-course expression of TNF-α and Cox-2 mRNA after LPS. C) Time-course of COX-2 protein expression after LPS as assessed by Western blotting. D) Semi-quantification of COX-2 band intensity in (C). Data were fit to the curve with non-linear regression analysis (one-phase exponential association). The goodness of the fit was assessed by r². E) Cells were transfected with oligofectamine carrying small interference RNA (siRNA) against either the p65 subunit of NFkB (si-p65) or the gp91 subunit of NADPH (si-gp91). A non-silencing scramble double-stranded RNA was used as control (ns). In these cells, the induction of Cox-2 mRNA is dependent on NFkB. F) The NFkB inhibitor PDTC also prevents COX-2 mRNA and protein induction after LPS. G-I) Induction of TNF-α (G) and COX-2 (H) mRNA is strongly reduced in astrocytes deficient in MyD88 (MyD88 -/-) versus the wild type (MyD88 +/+). Likewise, COX-2 protein is detected by Western blotting in wt cells but not in MyD88-deficient cells (I). β-Tubulin is shown in the
Western blots as a loading control. Values are expressed as the mean ± SEM of n=3 per condition. One symbol: p<0.05; two symbols: p<0.01; three symbols: p<0.001. Symbols indicate comparison versus either (*) control or (&) LPS (wt or treatment control).

**Fig. 4.** LPS-induced COX-2 is dependent on p38 and JNK MAPK. LPS (10 ng/mL) was added to mouse astrocyte cultures and mRNA/protein were studied at 4h or 8h. A) Astrocytes were treated with LPS in the presence or absence (-) of the indicated doses of MAPK inhibitors (or the vehicle): SB239063 (SB), SP600125 (SP) and U0126 (U), which inhibit the p38, JNK, and MEK pathways, respectively, and Cox-2 mRNA was studied 4h after LPS. The results show the involvement of p38 and JNK in LPS-induced COX-2 mRNA. B-D) COX-2 protein expression 8h after LPS was inhibited by SB239063 (from 1 to 25 µM) (B), and SP600125 (from 10 to 25 µM) (C), but not by MEK inhibitors (up to 25 µM U0126, and up to 40 µM PD98059) (D). E) COX-2 protein was 4h after LPS was also inhibited by p38 and JNK inhibitors by not after MEK inhibition (U0126). F) Accordingly, SB239063 and SP600125, but not U0126, inhibit the production of PGE₂, as assessed by ELISA in the culture medium 8h after LPS. G) Silencing MAPK with siRNA effectively reduces the expression of the corresponding target proteins by 65-70%. H-J) Silencing MAPK10 (JNK3) or MAPK14 (p38), but not MAPK1 (ERK2), reduces Cox-2 mRNA (H), and protein (I, J) versus treatment with non-silencing RNA (ns) 4h after LPS. β-Tubulin is shown in the Western blots as a loading control. Values are expressed as the mean ± SEM of n=3 per condition in at least 3 independent experiments. One symbol: p<0.05; two symbols: p<0.01; three symbols: p<0.001.

**Fig. 5.** LPS downregulates COX-1 expression in astrocytes. Cultured mouse astrocytes were treated with LPS (10 ng/mL) for 4, 8 or 24h. A) Cox-1 mRNA expression is down-regulated 8h after LPS in wt (+/+) and COX-2 deficient cells (-/-). B-D) Lack of expression of Cox-2 mRNA and protein in COX-2 KO cells is shown compared to wt. E, F) COX-1 protein expression decreases from 8h after LPS, but not at 4h. G-J) Down-regulation of COX-1 after LPS is not MAPK-dependent since it is not altered by MAPK inhibitors (G, H) or by silencing the indicated MAPK (I-J) (ns indicates treatment with control non-silencing RNA). K) LPS-induced down-regulation of COX-1 is not observed in MyD88 deficient cells, suggesting that it is MyD88-dependent. (L) Intracerebral administration of LPS to mice also induces a reduction of Cox-1 mRNA expression in the ipsilateral hemisphere 8h after LPS, and this effect is strongly attenuated in MyD88 deficient mice. M) NFκB inhibition with PDTC (10 µM) significantly attenuates the reduction of Cox-1 mRNA induced by LPS at 4h n cultured astrocytes. N) Compared to wt cells, low levels of Cox-1 mRNA are found in COX-1 deficient cells, and COX-1 protein is not detected in these cells by Western blotting (inset). O) COX-1 KO cells produce Cox-2 mRNA and protein 4h and 8h after LPS, respectively. β-Tubulin is shown as a loading control. n=3 per condition in each result. One symbol: p<0.05; two symbols: p<0.01; three symbols: p<0.001. Symbols indicate comparison versus either (*) control or (&) LPS (wt or untreated).

**Fig. 6.** LPS induces expression of mPGES-1 mRNA, but not of the enzymes that synthesize other prostanoids. Astrocytes of COX-1 or COX-2 KO mice (-/-) and their respective wild type (+/+) astrocytes were treated with LPS (10 ng/mL), and mRNA was extracted at 8h. A) LPS strongly induces microsomal PGE₂ synthase-1 (mPGES1) mRNA in the different genotypes. B) The induction of mPGES1 mRNA after LPS is dependent on the MyD88 pathway, as shown by lack of mPGES-1 mRNA up-regulation in MyD88 deficient (MyD88 -/-) cells after LPS. C,D) The expression of prostacyclin synthase (PGIS) mRNA (C) and that of thromboxane synthase (TS) mRNA (D) is reduced after LPS in all genotypes. E,F) Accordingly, in astrocytes from C57 wt mice, LPS significantly up-regulates the expression of mPGES1 protein (E), while TS protein shows a non-significant tendency to progressively decrease with time versus controls (F). n=3 for each genotype. One symbol p<0.05, two symbols P<0.01, three symbols p<0.001. * indicates comparison versus control; & indicates comparison versus LPS in wt.
Fig. 7. LPS induces secretion of prostanoids to the culture medium. Purified cultures of rat astrocytes were exposed to LPS (10 ng/mL) for different time periods and the medium was collected and studied by ELISA. ELISA assays were carried in 5 independent experiments, and curves from representative experiments are shown. A) Time course of PGE\(_2\) concentrations in the culture medium. B) LPS does not induce PGE\(_2\) in the presence of the Cox-2 inhibitor NS-398 (3 \(\mu\)M), and PGE\(_2\) concentration is lower in the presence of the Cox-1 inhibitor SC-560 (10 nM). C) Time course of TxB\(_2\) as an indicator of the generation of TxA\(_2\). D) NS-398 strongly reduces the production of TxB\(_2\) induced by LPS, whereas SC-560 attenuates the effect of LPS. E) Time course of PGF-1\(\alpha\) concentration to indirectly assess the formation of prostacyclin. F) NS-398 strongly reduces the concentration of PGF\(_1\)-\(\alpha\) in the medium, whereas some reduction is observed with the Cox-1 inhibitor SC-560. G-I) The cPLA\(_2\) inhibitor AACOCF3 (2 \(\mu\)M) completely abrogates LPS-induced prostanoid formation as assessed at 8h after LPS exposure. One symbol p<0.05, two symbols P<0.01, three symbols p<0.001. * indicates comparison versus control. & indicates comparison versus LPS alone. Data (mean±SD) were fit to the curve with non-linear regression analysis (one-phase exponential association) in A and C, and with linear regression in E. The goodness of the fit was assessed by \(r^2\).

Fig. 8. LPS-induced prostanoid production is dependent on COX-2 while COX-1 exerts selective regulatory effects. Purified cultures of mouse astrocytes were treated with LPS (10 ng/mL) and the concentration of prostanoids in the culture medium was studied by ELISA. Cells were collected at 4 and 8h (A-C), or 8h (D-I) after LPS. A-C) The production of prostanoids is strongly attenuated in MyD88-deficient cells. D-F) LPS does not induce PGE\(_2\) (G), PGF1\(\alpha\) (H), or TxB\(_2\) (I) in COX-2 KO cells (-/-). G-I) The production of PGE\(_2\) induced by LPS is enhanced in COX-1 deficient astrocytes (-/-) (G). In contrast, the production of PGF1\(\alpha\) (H) and TxB\(_2\) (I), as indirect assessments of PG and TxA\(_2\), respectively, is smaller in Cox-1 KO cells (-/-) compared to the wild-type (+/+), both in the presence or absence of LPS. J-L) Silencing COX-1 expression with siRNA slightly enhances the production of PGE\(_2\) (K) but does not modify the production of PGI\(_2\) (L) or TxA\(_2\) (M). n=3 in at least 2 independent experiments. One symbol p<0.05, two symbols P<0.01, three symbols p<0.001. * indicates comparison versus control. & indicates comparison versus LPS in wt or after treatment with non-silencing RNA (ns). # indicates comparison versus control KO cells.
TABLE 1. List of primer sequences for mouse and rat PCR.

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<th>Accession number</th>
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| TNF-α  | F: GGGGCCACCACGCCTTTCTGTC  
         | R: TGGGCTACGGGCTTTGTCACTCG  | Nm_013693  | 155  | Exon 1 |
| Cox-2  | F: CCACCTCAAGGGAGTCTCGGA  
         | R: AGTCATCTGCTACGGGAGGA  | Nm_011198.3  | 187  | Exon 3 |
| Cox-1  | F: GTGCTGGGGCAGTGCTGGAG  
         | R: TGGGGGCTGAGTAGGCCGCTG  | Nm_008969.3  | 281  | Exon 1 |
| mPGES-1| F: AGGCCAGATGAGGCTCGGGA  
         | R: AGCGAAAGGCGTGGGTTTCAGC  | Nm_002415.2  | 195  | Exon 1 |
| PGIS   | F: GTGGAGGCCCTACAACACGCAC  
         | R: CCCGGGCTGATCTCTCTCTCTCT  | Nm_008968  | 305  | Exon 5 |
| TS     | F: CACACGGGAGGAGCCAGAGAAG  
         | R: GGGCCAGCTTCACAAAGGGCCAG  | Nm_0011539.3  | 194  | Exon 10 |
| CD11b  | F: AAGCGAGCTGAATGGGAGAAC  
         | R: GATAGACCCTCCTCCTGCTCT  | Nm_001082960  | 190  | Exon 7 |
| RPL14  | F: GGCTTTAGTGGATGGACCCT  
         | R: ATTGATATCCCGCCTCTCCCC  | Nm_025974  | 143  | Exon 3 |

<table>
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<th>RAT</th>
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<th>Accession number</th>
<th>Amplicon length (bp)</th>
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| Cox-2  | F: GATTGACAGCCACCCAACAG  
         | R: CGGATAGCCTCCCTGCTCA  | Nm_017232  | 149  | Exon 4 |
| TNF-α  | F: GGGGCCACCGCTCTCCTGTC  
         | R: TGGGCTACGGGCTTTGTCACTCG  | Nm_012657.3  | 155  | Exon 1 |
| RPL14  | F: TCTTTGCTATGCTAGAGGA  
         | R: GATAGGTATCTTATTCGAGTCCC  | Nm_022949  | 144  | Exon 3 |

Cox-2 induction and Cox-1 down-regulation by LPS
Figure 2

Cox-2 induction and Cox-1 down-regulation by LPS

P  Cox-2 / Iba-1

Q  Cox-2 / GFAP

control  PBS  LPS

control  LPS

control  PBS  LPS

control  LPS

control  LPS

control  LPS

control  LPS

control  LPS
Cox-2 induction and Cox-1 down-regulation by LPS
Cox-2 induction and Cox-1 down-regulation by LPS
Cox-2 induction and Cox-1 down-regulation by LPS

Figure 5

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O
Figure 6

A

Cox-2 induction and Cox-1 down-regulation by LPS

B

C

D

E

F
Cox-2 induction and Cox-1 down-regulation by LPS

Figure 8

A

B

C

D

E

F

G

H

I

J

K

L
**Supplementary Material:**

**Supplementary Figure 1**

Legend to Supplementary Figure 1: Genotyping of litters obtained from heterozygous and mutant mice Cox1+/- and Cox1-/-, and Cox2+/- and Cox2-/. Each mouse was genotyped by PCR to differentiate KO (-/-) or heterozygous (+/-). An image of agarose gels showing the result of a representative genotyping is provided for Cox-1 and Cox-2. The asterisk in the Cox-1 panel indicates a band corresponding to a heteroduplex formed by the wt and KO DNA strands amplified in the heterozygote samples.

**Supplementary Figure 2.** Purified cultures of mouse astrocytes contain very low numbers of contaminating microglia. Double immunofluorescence shows astrocytes stained with an antibody against GFAP (red) and microglia with anti-Iba-1 (green). The nuclei are stained with Hoechst (blue). A-D) examples illustrating the low presence of Iba-1 positive cells. Bar scale = 30 μm.
Legend to supplementary Fig. 3. Expression of mRNA for A) microglia (Iba-1) and B) astroglia (GFAP) markers 8h after infracerebral administration of LPS (0.7 µl, 1µg/µl) or the vehicle (PBS) in mice. LPS enhances the expression of Iba-1 mRNA in the ipsilateral hemisphere at 8h, whereas GFAP mRNA expression is not modified at this time point. n=3-4 per group. ‘contra’ and ‘ipsi’ indicate brain hemispheres, either contralateral or ipsilateral to injection, respectively. Primers used were as follows: for Iba1: (+) 5’-GAAGCGAATGCTGGAGAAAC-3’ and (-) 5’-AAGATGGCAGACTCTTGGCC-3; and for GFAP: (+) 5’-AAGGTCCGCTTCCTGGAA-3’, and (-) 5’-GGCTCGAAGCTGGTTCAGTT-3’. ** p<0.01 versus PBS ipsi.

Legend to supplementary Fig. 4. SC-560 exerts effects independently of COX-1. COX-1 deficient astrocytes were treated with LPS (10 ng/mL) for 8h in the presence or absence of the COX-1 inhibitor SC-560 (sc) (10 nM) or the COX-2 inhibitor NS-398 (ns) (3 µM). NS-398 fully prevents PGE2 (A), PGF1α (B), and TxB2 (C) formation after LPS. However, SC-560 also reduces the production of these prostanoids induced by LPS in spite that the cells do not express COX-1. N=6. One symbol p<0.05, two symbols p<0.01, three symbols p<0.001. * indicates comparison versus control. & indicates comparison versus LPS alone.
Induction of Cox-2 and down-regulation of Cox-1 expression by LPS control prostaglandin E2 production in astrocytes
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