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Immune-induced fever is dependent on local but not generalized prostaglandin E$_2$ synthesis in the brain

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Abstract

Fever occurs upon binding of prostaglandin E$_2$ (PGE$_2$) to EP$_3$ receptors in the median preoptic nucleus of the hypothalamus, but the origin of the pyrogenic PGE$_2$ has not been clearly determined. Here, using mice of both sexes, we examined the role of local vs generalized PGE$_2$ production in the brain for the febrile response. In wild-type mice and in mice with genetic deletion of the prostaglandin synthesizing enzyme cyclooxygenase-2 in the brain endothelium, generated with an inducible CreER$^{T2}$ under the Slco1c1 promoter, PGE$_2$ levels in the cerebrospinal fluid were only weakly related to the magnitude of the febrile response, whereas the PGE$_2$ synthesizing capacity in the hypothalamus, as reflected in the levels of cyclooxygenase-2 mRNA, showed strong correlation with the immune-induced fever. Histological analysis showed that deletion of cyclooxygenase-2 in brain endothelial cells occurred preferentially in small and medium-sized vessels deep in the brain parenchyma, such as in the hypothalamus, whereas larger vessels, and particularly those close to the neocortical surface and in the meninges, were left unaffected, hence leaving PGE$_2$ synthesis largely intact in major parts of the brain, while significantly reducing it in the region critical for the febrile response. Furthermore, injection of a virus vector expressing microsomal prostaglandin E synthase-1 (mPGES-1) into the median preoptic nucleus of fever-refractive mPGES-1 knock-out mice, resulted in a temperature elevation in response to LPS. We conclude that the febrile response is dependent on local release of PGE$_2$ onto its target neurons and not on the overall PGE$_2$ production in the brain.
Significance statement

By using mice with selective deletion of prostaglandin synthesis in brain endothelial cells, we demonstrate that local prostaglandin E$_2$ (PGE$_2$) production in deep brain areas, such as the hypothalamus, which is the site of thermoregulatory neurons, is critical for the febrile response to peripheral inflammation. In contrast, PGE$_2$ production in other brain areas and the overall PGE$_2$ level in the brain do not influence the febrile response. Furthermore, partly restoring the PGE$_2$ synthesizing capacity in the anterior hypothalamus of mice lacking such capacity with a lentiviral vector resulted in a temperature elevation in response to LPS. These data imply that the febrile response is dependent on the local release of PGE$_2$ onto its target neurons, possibly by a paracrine mechanism.
Introduction

It is now well established that prostaglandin E$_2$ (PGE$_2$) is the final mediator of inflammation-induced fever (Li et al., 1999; Engblom et al., 2003). Fever has long been associated with elevated brain levels of PGE$_2$ (Splawinski, 1977); in response to a peripheral immune stimulus, PGE$_2$ levels in the cerebrospinal fluid increase concomitant with the febrile response (Inoue et al., 2002), and injection of PGE$_2$ into the cerebrospinal fluid elicits fever in a dose-dependent way (Nilsberth et al., 2009b). PGE$_2$ is synthesized by brain endothelial cells through the concerted action of the inducible enzymes cyclooxygenase (Cox)-2 and microsomal prostaglandin E synthase-1 (mPGES-1) (Ek et al., 2001; Yamagata et al., 2001). These correlational studies have later been complemented by functional genetic studies, which have shown that genetic deletion of Cox-2 or mPGES-1 in the brain endothelium strongly attenuates the febrile response (Wilhelms et al., 2014), hence demonstrating that the brain endothelium plays a critical role for the generation of the PGE$_2$ that is seen in the brain during fever.

Because a peripheral immune stimulus elicits the induction of prostaglandin synthesizing enzymes throughout the brain vasculature (Ek et al., 2001; Yamagata et al., 2001), suggesting a generalized PGE$_2$ release, the specificity of the PGE$_2$ elicited responses comes about through the distinct distribution of its receptor subtypes (Zhang and Rivest, 1999; Ek et al., 2000; Oka et al., 2000), as demonstrated by the attenuation of fever through deletion of EP$_3$ receptors in the median preoptic nucleus, but not by deletion of these receptors at other sites (Lazarus et al., 2007). However, it is less clear whether the responses evoked by PGE$_2$ binding to its receptors is the result of PGE$_2$ release locally, in a paracrine fashion, or if PGE$_2$ released at other sites also can elicit fever under physiological conditions (Matsumura et al., 1997). Whereas studies using injections of PGE$_2$ or cyclooxygenase inhibitors support the importance of local synthesis (Scammell et al., 1996; Scammell et al.,
1998), the critical site of prostaglandin synthesis has not been investigated using modern functional genetic techniques. Here we addressed this question by examining in normal mice and in mice with deletion of Cox-2 in the brain endothelium the relationship between the magnitude of the febrile response to a peripheral immune stimulus and the level of induced PGE$_2$ in the cerebrospinal fluid, as seen in individual animals. We also examined the relationship between the magnitude of the febrile response and the level of Cox-2 mRNA in the hypothalamus, as well as the PGE$_2$ levels in plasma, and we determined by using immunohistochemistry how Cox-2 expression in different types of vessels was related to the febrile response. Finally, we examined if local endogenous immune-induced production of PGE$_2$ in the anterior hypothalamus resulted in a temperature response. Our data show that local PGE$_2$ release onto brain PGE$_2$ receptors is the mechanism governing the febrile response to peripheral immune challenge.

**Materials and Methods**

**Animals**

Mice with specific deletion of Cox-2 in brain endothelial cells were generated by crossing animals in which exons 4-5 of the Cox-2 gene ($Ptgs2$) are flanked by loxP-sites (Ishikawa and Herschman, 2006) with animals expressing a tamoxifen inducible CreER$^{T2}$ under the $Slco1c1$ promoter [expressed in the cerebrovascular endothelium (Ridder et al., 2011)]. The tamoxifen (Sigma-Aldrich, St. Louis, MO; 1 mg diluted in a mixture of 10 % ethanol and 90 % sunflower seed oil) was injected intraperitoneally (ip; 0.1 ml) twice a day for five days, followed by a five week recovery period before any further experiments were done. The inducible Cre-line was also crossed with a Cre reporter line, which expresses a $Gt(ROSA)26Sor$ locus with a loxP-flanked STOP cassette preventing transcription of a CAG promoter-driven red fluorescent protein variant (tdTomato) (Jackson Laboratory, Bar Harbor, ME; RRID: IMSR_JAX:007914), and the offspring was treated with tamoxifen as above.
Mice with deletion of the Ptges gene (Trebin et al., 2003), encoding mPGES-1, were from our own breeding and on a C57B/6 background. All animal experiments were approved by the local Animal Care and Use Committee and followed international guidelines.

**Telemetric temperature recordings**

The mice were briefly anesthetized with isoflurane (Abbot Scandinavia, Solna, Sweden) and implanted ip with a transponder that records core body temperature (Mini Mitter, Bend, OR). Immediately after surgery, the mice were transferred to a room in which the ambient temperature was set to 29°C, providing near-thermoneutral conditions (Rudaya et al., 2005).

**Injection of lentiviral vectors**

Mice were anesthetized with isoflurane and mounted onto a stereotaxic frame. The scalp was exposed and two small drill holes were made at the level of the Bregma, on each side of the midline. A Hamilton syringe was lowered to a position that in relation to Bregma was 0.0 mm anteroposteriorly, 0.3 mm mediolaterally, and 5.5 mm dorsoventrally. A lentiviral vector was then injected at a rate of 180 nl/min during 3 min. The syringe remained in place for at least 3 min after the infusion and was then slowly removed, after which the skin was closed. Mice were injected either with a lentiviral vector, in Dulbecco’s phosphate-buffered saline containing MgCl$_2$ and CaCl$_2$ (Sigma-Aldrich; catalogue # D8662), expressing GFP [lenti-5-KP-pgk-GFP; 5 x 10$^8$ – 1 x 10$^9$ TU/ml, produced as previously described (Zufferey et al., 1997; Georgievskia et al., 2004); a gift from Johan Jakobsson, Lund, Sweden], or a lentiviral vector expressing human mPGES-1 (suCMV promoter - human Ptges (NM_004878) - Rsv promoter – puromycin resistance; 10$^8$ IFU/ml; AMS Biotechnology, Abingdon, UK), to which was added 10 % of the lenti-GFP virus to permit subsequent immunofluorescent identification of the injection site (we found no available antibody that could detect mPGES-1). During the same surgical session, a temperature transponder was implanted in the abdominal cavity, as described above.
**Immune stimulation**

Mice were injected ip with bacterial wall lipopolysaccharide (LPS) from *Escherichia coli* (Sigma-Aldrich; O111:B4; 120 µg/kg body weight, diluted in 100 µl), 1 week or 3 weeks (virus injected mice) following implantation of the temperature transponder.

**Tissue collection**

Mice injected with LPS only were killed 5 h after injection [This time point was selected since it corresponds to the time of peak fever in this experimental paradigm (e.g., Hamzic et al., 2013)]. Blood was drawn from the right atrium, transferred to EDTA-coated tubes (Sarstedt, Landskrona, Sweden) to which were added indomethacin (10 µM; Sigma-Aldrich), and centrifuged at 7000 × g for 7 min at 4°C. The plasma was immediately frozen on dry ice and kept at −70°C. The animals were then placed in a stereotaxic frame, the atlanto-occipital membrane was exposed, and cerebrospinal fluid (CSF) withdrawn from the cisterna magna using a Hamilton syringe mounted on a micromanipulator and immediately frozen. Samples that contained traces of blood were discarded. The whole procedure from when the animals were killed until CSF was withdrawn took less than 10 min. A hypothalamic block was then dissected and placed in RNAlater stabilization reagent (Qiagen, Hilden, Germany) and stored at −70°C until analysis. This block was first isolated by two coronal cuts, one placed 0.5 mm rostral to the apex of the optic chiasm and the other at the caudal margin of the mammillary bodies. The resulting slab was then trimmed by sagittal cuts on each side through the sulcus between the hypothalamus and the temporal lobe. Finally, a horizontal cut was placed slightly above the anterior commissure. Mice injected with viral vectors were killed the day after the immune challenge with LPS. After asphyxiation with CO₂ one group of mice were fixed by transcardial perfusion with a phosphate-buffered (0.1 M) paraformaldehyde solution (4 %). The brains were removed and post-fixed for 3 h in the same fixative and then cryoprotected with 25 % sucrose in phosphate-buffered saline (PBS). In another groups of mice the brain
was immediately removed. The hypothalamus was dissected and stored in RNAlater (Qiagen) at −70 °C.

**Immunohistochemistry**

Brains were cut in the frontal plane at 30 µm on a freezing microtome. The immunohistochemical procedures were carried out according to standardized protocols (Engström et al., 2012). In brief, sections were incubated in a blocking solution [PBS containing 3 % normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA), 1 % bovine serum albumin (Sigma–Aldrich), and 0.3 % Triton X (Merck, Darmstadt, Germany)] for 45 min, followed by incubation overnight at room temperature with rabbit anti-Cox-2 antibody (1:500; sc-1747 M-19; Santa Cruz Biotechnologies; RRID: AB_2084976) and goat anti-lipocalin-2 antibody (1:500; AF1857; R&D Systems, Minneapolis, MN; RRID: AB_355022), rinsed in PBS and then incubated with Alexa Fluor 555 donkey anti-rabbit antibody and Alexa Fluor 488 donkey anti-goat antibody (both 1:500; Life Technologies, Carlsbad, CA). Sections from lenti-vector injected brains were incubated with chicken anti-GFP antibody (1:10,000; ab13970, Abcam, Cambridge, UK; RRID: AB_300798), followed by AlexaFluor 488 goat anti-chicken IgG (H+L) antibody (Life Technologies). The sections were finally mounted on SuperFrost Plus glasses (Thermo Fischer Scientific, Waltham, MA) with Prolong gold anti-fade reagent (Life Technologies).

**Assays for PGE2 levels in CSF and plasma**

The concentration of PGE2 in CSF (diluted 1:100) was determined using a High Sensitivity Prostaglandin E2 Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI). The values were calculated using a standard curve ranging from 7.81–1000 pg/ml ($R^2 = 1$). The kit antiserum shows the following cross-reactivity, according to the manufacturer: PGE2 100 %, PGE1 70 %, PGE3 16.3 %, PGF1α 1.4 %, PGF2α 0.7 %, 6-keto-PGF1α 0.6 %, PGA2 0.1 %, PGB1 0.1 %, and < 0.1 % for 13,14-dihydro-15-keto-PGF2α, 6, 15-keto, 13, 4-dihydro-
PGF1α, thromboxane B2, 2 arachidonoylglycerol, anandamide, PGD2 and arachidonic acid. The concentration of PGE2 metabolites in plasma was determined with a Prostaglandin E Metabolite EIA Kit (Cayman Chemical, Ann Arbor, MI). The values were calculated using a standard curve ranging from 0.2–50 pg/ml ($R^2 = 0.999$). The kit antiserum recognizes derivatized 13, 14-dihydro-15-ketoPGE1 and 13,14-dihydro-15-ketoPGE2, and bicycloPGE1, but has less than 0.01 % cross-reactivity with arachidonic acid, leukotriene B4, tetranor-PGEM, tetranor-PGFM, PGD2, PGE1, 6-keto PGE1, PGE2, PGF1α, 6-keto PGF1α, PGF2α and thromboxane B2.

**Real time quantitative PCR analyses (qPCR)**

RNA was extracted with RNeasy Universal Plus kit or RNeasy Micro Kit (Qiagen) and reverse transcription was done with High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Foster City, CA). qPCR was then performed using Gene Expression Master Mix (Applied Biosystems) on a 96-well plate (7900HT Fast RT-PCR system; Applied Biosystems). Assays used (from Applied Biosystems) were: Ptgs2: Mm00478374_m1; human Ptges (Hs01115r610_m1), and Gapdh (Mm99999915_g1).

**Experimental design and statistical analysis**

For all comparisons between genotypes, littermates were used. Mice were of both sexes, and experimental groups balanced with respect to sex and age. Sample size is reported in the figure legends. All statistical analyses were done in Graph Pad Prism (GraphPad Software, La Jolla, CA). Analysis of differences in body temperature at 5 h between LPS-treated WT mice and mice with endothelial specific deletion of Cox-2 was done with a 1-way ANOVA followed by Sidak’s multiple comparisons test. The same analysis was used for differences between groups with respect to PGE2 in CSF, and qPCR data. For PGE2 metabolites in plasma non-parametric statistics were used (Kruskal-Wallis test followed by Dunn’s multiple comparisons test). Temperature responses after injections of viral vectors were analyzed with
a 2-way repeated measures ANOVA followed by Tukey’s *post hoc* test. Regression analysis was done with *F*-statistics. Qualitative data were analyzed with Fisher’s exact test. Results were considered significant when *P* < 0.05.

**Results**

*Deletion of Cox-2 in brain endothelial cells results in attenuated fever response to LPS*

As reported previously (Wilhelms et al., 2014), mice with deletion of Cox-2 in brain endothelial cells (Cox-2ΔSlo1c1) show attenuated fever following ip injection of LPS (Figure 1a). At 5 h post injection, when the animals were killed and tissue collected for analysis, LPS treated Cox-2ΔSlo1c1 mice displayed significantly lower body temperature than their wild type (WT) Cox-2fl/fl littermates (*F*3,37 = 6.283, *P* = 0.0015; LPS Cox-2ΔSlo1c1 vs LPS WT: *P* = 0.0219; Figure 1b).

*No difference in PGE2 levels in CSF between Cox-2ΔSlo1c1 mice and WT mice*

We next examined if the difference in the febrile response to LPS between Cox-2ΔSlo1c1 mice and WT mice was associated with a difference in CSF levels of PGE2. LPS-treatment resulted in elevated levels of PGE2 in CSF at 5 h post-injection (*F*3,37 = 8.003, *P* < 0.003; LPS WT vs NaCl WT: *P* = 0.0005; LPS Cox-2ΔSlo1c1 vs NaCl Cox-2ΔSlo1c1: *P* = 0.0198), but there was no difference between genotypes (Figure 2a).

*Lower levels of induced Cox-2 mRNA in the hypothalamus of Cox-2ΔSlo1c1 mice*

qPCR analysis of the levels of Cox-2 mRNA in the hypothalamus of Cox-2ΔSlo1c1 mice and WT mice showed significantly lower induction following LPS in the gene deleted mice than in the WT mice (*F*3,39 = 21.78, *P* < 0.0001; LPS WT vs LPS Cox-2ΔSlo1c1: *P* = 0.0021; Figure 2b). As expected, there was also a small (but statistically not significant) reduction of Cox-2 mRNA in the NaCl treated Cox-2ΔSlo1c1 mice compared to the WT mice (Figure 2b).
No difference in PGE₂ metabolites levels in plasma between Cox-2ΔSlco1c1 mice and WT mice

To examine if the gene deletion in the Cox-2ΔSlco1c1 mice, which should occur only in brain endothelial cells (Ridder et al., 2011), had influenced prostaglandin synthesis peripherally, the levels of PGE₂ metabolites in plasma were analyzed. This assay was chosen instead of direct measurement of PGE₂ because PGE₂ in plasma is difficult to measure reliably (Samuelsson et al., 1975), since it is rapidly converted \textit{in vivo} to its 13,14-dihydro-15-keto metabolite, with more than 90% of circulating PGE₂ being cleared by a single passage through the lungs (Hamberg and Samuelsson, 1971). The levels of PGE₂ metabolites in plasma were elevated following immune challenge with LPS (Kruskal-Wallis statistic = 19.84, \( P = 0.0002 \); LPS WT vs NaCl WT: \( P = 0.0022 \); LPS Cox-2ΔSlco1c1 vs NaCl Cox-2ΔSlco1c1: \( P = 0.0327 \)), but there was no difference between the genotypes (Figure 2c).

Body temperature following immune stimulation correlates with Cox-2 mRNA levels in the hypothalamus but not with PGE₂ levels in CSF

To further examine the relationship between body temperature and central prostaglandin synthesis, we next performed a regression analysis of the individual temperatures and the levels of PGE₂ in cerebrospinal fluid in Cox-2ΔSlco1c1 mice and WT mice 5 h post LPS injection. As shown (Figure 3a), there was only weak relationship between these two parameters (\( R^2 = 0.157, F_{1,23} = 4.292, P = 0.0497 \); Figure 3a). In contrast, there was a strong correlation between temperature and Cox-2 mRNA in the hypothalamus (\( R^2 = 0.593, F_{1,22} = 32.01, P < 0.0001 \); Figure 3b). There was also moderately strong relationship between body temperature and PGE₂ metabolites in plasma (\( R^2 = 0.4310, F_{1,23} = 17.42, P = 0.0004 \); Figure 3c).
The levels of PGE₂ in CSF correlate only weakly with Cox-2 mRNA expression in the hypothalamus and not with levels of PGE₂ metabolites in plasma

To examine what affects the PGE₂ levels in CSF, regression analysis was performed for the relationship between PGE₂ in CSF and Cox-2 mRNA expression in the hypothalamus of LPS treated mice (Figure 4a) and PGE₂ metabolites in plasma in these mice (Figure 4b), respectively. PGE₂ levels in CSF showed only a weak relationship with the Cox-2 mRNA expression in the hypothalamus ($R^2 = 0.1783$, $F_{1,25} = 5.424$, $P = 0.0282$; Figure 4a). In the same vein, there was no significant relationship between PGE₂ levels in CSF and the levels of PGE₂ metabolites in plasma ($R^2 = 0.1321$, $F_{1,24} = 3.654$, $P = 0.0679$; Figure 4b), indicating that they have different sources of origin.

The gene deletion in Cox-2ΔSlco1c1 mice mainly occurs in smaller vessels in the brain parenchyma but not in large vessels

Since the above data indicated that the gene deletion in Cox-2ΔSlco1c1 mice preferentially affected induced prostaglandin synthesis in the hypothalamus, but not generally in the brain, we examined histologically where the gene deletion took place. We crossed the mice with the tamoxifen inducible CreERT² under the Slco1c1 promoter with a Cre reporter line expressing a Gt(ROSA)26Sor locus with a loxP-flanked STOP cassette, which prevents transcription of a CAG promoter-driven red fluorescent protein variant (tdTomato). Following tamoxifen treatment of the offspring we subjected it to immune challenge with LPS. We found that Cre-induced recombination occurred preferentially in small and medium-sized blood vessels; in the latter tdTomato staining was found to be co-localized with induced Cox-2 immunoreactivity in endothelial cells (Figures 5a, b). In contrast, Cox-2 positive cells in larger blood vessels rarely expressed tdTomato staining (Figure 5b, c). We also examined brains from Cox-2ΔSlco1c1 mice and WT mice for the co-expression of Cox-2 and lipocalin-2 immunoreactivity, in order to evaluate if the endothelial cells had responded to LPS, since
lipocalin-2 is expressed in endothelial cells in response to LPS together with, but independently of Cox-2 (Hamzic et al., 2013; Vasilache et al., 2015). We found that lipocalin-2 expressing cells in larger vessels, and in particular in vessels close to the surface of the neocortex, co-expressed Cox-2 extensively in both genotypes (Figures 6a, b). Strong Cox-2 labeling was also seen among lipocalin-2 expressing cells in meningeal vessel in both genotypes (Figures 6c, d). However, smaller vessels in Cox-2^ΔSlco1c1^ mice, preferentially in the more medial parts of the brain such as in the hypothalamic region, more rarely expressed Cox-2, while displaying extensive labeling for lipocalin-2 (Figure 6f). This was in stark contrast to what was seen in WT mice, in which there was extensive co-expression of both proteins also in small vessels (Figure 6e). Although there was some variation across animals, when a blinded investigator qualitatively determined the genotype of the animals by examining the degree of co-localization of Cox-2 and lipocalin-2 in differently sized vessels, the correct genotype was determined in 83 % of the animals (P = 0.0073; n = 13 for Cox-2^ΔSlco1c1^ mice, and n = 9 for WT mice).

Restoration of PGE2 synthesis in the preoptic hypothalamus of mPGES-1 KO mice results in a temperature response to LPS

To determine if local PGE2 production in the hypothalamus results in a temperature response to LPS, we used intracerebral injection of a viral vector to restore the PGE2 synthesizing capacity in the median preoptic regions of mice with global deletion of mPGES-1, the inducible terminal PGE2 synthesizing enzyme (Jakobsson et al., 1999). We chose to use mPGES-1 KO mice, which previously have been shown to be unable to mount a temperature rise upon peripheral immune challenge (Engblom et al., 2003; Nilsberth et al., 2009a) instead of Cox-2 KO mice, because the latter are difficult to breed and suffer from various health problems, including chronic inflammations (Langenbach et al., 1999). mPGES-1 KO mice with injections of viral vectors expressing GFP aimed at the region of the median preoptic
nucleus, the structure critical for the fever response to PGE₂ (Scammell et al., 1996; Lazarus et al., 2007), displayed the same hypothermic response to immune challenge with LPS as shown previously for these mice (Engblom et al., 2003; Nilsberth et al., 2009a; Engström et al., 2012). In contrast, mPGES-1 KO mice injected with a vector expressing mPGES-1 showed no sustained hypothermia after the initial temperature drop following the handling stress-induced temperature peak, but displayed a body temperature elevation compared to mice injected with a vector expressing GFP (Fig. 7a). Mean body temperature during the period of 60 – 480 min after LPS injection (i.e. after the handling stress induced temperature peak) was significantly different between treatments ($F_{2,29} = 11.17, P = 0.0003$; Lenti mPGES-1 vs Lenti GFP: $P = 0.0042$). While injection sites were determined by immunofluorescence to GFP (GFP lentiviral vector was added at 10 % to the mPGES-1 vector) (Fig. 7b), transcription of mPGES-1 was assured by qPCR analysis. In a total of 8 mice injected with the viral vector expressing mPGES-1, all displayed mPGES-1 mRNA, whereas none of 4 mice injected with the GFP-vector did so, as expected.

**Discussion**

*The febrile response is dependent on PGE₂ synthesis by small to medium sized vessel in the hypothalamus, but independent of global PGE₂ synthesis in brain*

This study shows that the magnitude of the febrile response was strongly correlated with the PGE₂ synthesizing capacity in the hypothalamus, as reflected in the levels of Cox-2 mRNA, but only weakly related to the PGE₂ levels in cerebrospinal fluid. These findings were corroborated by the histological demonstration that genetic deletion of Cox-2 in brain endothelial cells using a tamoxifen inducible CreERT² under the *Slco1c1* promoter (Ridder et al., 2011) occurred preferentially in small to medium-sized vessels deep in the brain parenchyma, such as in the hypothalamus, whereas larger vessels, particularly those close to the neocortical surface, and vessels in the meninges were left unaffected. Accordingly,
whereas the gene deletion attenuated the PGE$_2$ synthesizing capacity in the hypothalamus, in which the EP$_3$ receptor expressing neurons that are critical for the febrile response are located (Lazarus et al., 2007), it left the PGE$_2$ synthesis intact in large parts of the brain. Taken together, these data imply that the febrile response is dependent on the local, possibly paracrine release of PGE$_2$ onto the preoptic EP$_3$ receptor, whereas the overall PGE$_2$ level in the brain, as reflected in the levels measured in the CSF, is not involved. This conclusion was further supported by the finding that local restoration of induced PGE$_2$ synthesis in febrile resistant mPGES-1 KO mice at loci involving the median preoptic nucleus, the critical site for immune induced fever, resulted in a temperature elevation in response to ip LPS.

Previous findings that injection of PGE$_2$ into the cerebral ventricles causes fever in a dose dependent manner (Engblom et al., 2003; Lazarus et al., 2007; Nilsberth et al., 2009b) could seem to indicate that PGE$_2$ synthesized at some other site(s) in the brain than in the immediate vicinity of the preoptic EP$_3$ receptor expressing neurons that are critical for the febrile response also could influence the firing properties of these neurons and hence elicit fever. However, as noted previously (Nilsberth et al., 2009b), the concentration in the CSF of exogenously administered PGE$_2$ that is required for eliciting fever is in the order of 1000-fold higher than that seen in CSF during immune-induced fever. This observation suggests that the concentration of PGE$_2$ at its target neurons that is needed for eliciting fever in response to a peripheral immune stimulus is much higher than that measured in the CSF. It seems likely that such high concentrations of PGE$_2$ during physiological conditions only could be achieved by paracrine release. This idea is supported by the demonstration that intracerebral injection of a threshold dose of PGE$_2$ causes fever when localized to or in the immediate vicinity of the median preoptic nucleus, but not when localized to more distant sites (Scammell et al., 1996). Similarly, microinjections of a cyclooxygenase inhibitor into the
same area attenuated LPS-induced fever, whereas microinjections at other sites did not (Scammell et al., 1998).

Relationship between fever and hypothermia

While injection of a viral vector encoding mPGES-1 resulted in a significantly higher body temperature after peripheral immune challenge than that displayed by mice subjected to control virus injections, the body temperature did not reach levels that could be classified as fever. The temperature response to immune challenge with LPS is likely the central compilation of pyrogenic and hypothermic signaling, the neuronal substrate being reciprocally interconnected cell groups that generate temperature elevating and temperature lowering signals, respectively (Zhao et al., 2017). In the absence of induced PGE₂ synthesis, animals immune challenged with LPS display hypothermia (Fig. 7; see also Engström et al., 2012), and such hypothermia occurs also in mice lacking EP₃ receptors (Oishi et al., 2015). Hypothermia, which is elicited by a yet unidentified cryogen (Almeida et al., 2006), is thus the response to LPS when no pyrogenic PGE₂-EP₃ signaling is present. While mPGES-1 KO mice, similar to WT mice, are fully responsive to intracerebroventricularly injected PGE₂, hence demonstrating intact EP₃ signaling (Engblom et al., 2003), it should be noted that the response to the intracerebroventricularly injected PGE₂ is graded. Low doses give rise to only a slight temperature elevation whereas high doses elicit a body temperature in the order of 40°C (Nilsberth et al., 2009). The inability to fully restore the febrile response by virus vector injection in the present study is therefore likely explained by only partly restored PGE₂ synthesis, which in turn may be due to incomplete mPGES-1 expression and/or deficient mPGES-1 protein coupling to Cox-2. The latter is induced in brain endothelial cells (Engström et al., 2012), however structures that had incorporated the virus (and hence were shown to express GFP) were preferentially of neuron/glial cell type and were only rarely suggestive of endothelial cells (not shown). While speculative, mPGES-1 expressed by other
cells than endothelial cells could perhaps couple with Cox-1 that is more ubiquitously
expressed, hence resulting in PGE2 synthesis, although at levels that did not permit a full-
fledged restoration of the febrile response. Never-the-less, the finding of a temperature
elevation to LPS, although modest, in animals in which mPGES-1 was re-expressed in the
preoptic region demonstrates the critical role for localized PGE2 synthesis for heat production
in response to peripheral immune challenge.

Role of peripherally produced PGE2 for the febrile response

The present data confirm that prostaglandin production in brain endothelial cells is important
for the febrile response (Engström et al., 2012; Wilhelms et al., 2014; Eskilsson et al., 2014a). However, it has been suggested that peripherally produced, circulating PGE2 also is involved
(Steiner et al., 2006), but data from mice chimeric for mPGES-1 imply that the role of PGE2
produced by hematopoietically derived cells is, at most, very small (Engström et al., 2012). Here we found no significant relationship between the levels of PGE2 metabolites in plasma
and PGE2 levels in the cerebrospinal fluid, suggesting that peripherally and centrally
produced PGE2 are of distinct sources of origin, at least at the time point examined.
Accordingly, although there was a moderately strong relationship in the present study
between the levels of PGE2 metabolites in plasma and the magnitude of fever, there is most
likely no causality between these events; in the case of a strong peripheral immune response
there is also a strong central immune response, and, conversely, a weak peripheral immune
response is associated with a weak central immune response. This conclusion is supported by
observations in the mice chimeric for mPGES-1. Thus, whereas WT mice carrying mPGES-1
KO hematopoietic cells displayed normal LPS induced fever, mPGES-1 KO mice carrying
WT hematopoietic cells did not mount a febrile response, despite showing strong induction of
PGE2 metabolites in plasma (Engström et al., 2012). Furthermore, in those mice, central
PGE₂ levels were not significantly increased, providing additional support for the distinct origin of centrally and peripherally produced PGE₂.

**Heterogeneous distribution of transporter proteins among brain endothelial cells**

We observed in the genetically modified mice that Cre recombinase expressed under the control of the *Slco1c1* promoter produced recombination (and hence gene deletion) in brain endothelial cells in small and medium sized vessel but not in larger vessels. The *Slco1c1* gene encodes the organic anion transporter 14 (Oatp14), which has been shown to be expressed selectively in endothelial cells of the brain (Ridder et al., 2011). The organic anion transporter family transports hormones and other organic molecules to and from the brain (Westholm et al., 2008), and it has been demonstrated that other members of the Oatp family also show heterogeneous distributions among the vessels in the brain similar to Oatp14, with the main expression being in smaller vessels (Daneman et al., 2010). Furthermore, it has been shown that transporter proteins overall have a more prominent expression in capillaries than in venules (Macdonald et al., 2010), which is consistent with the idea that the exchange of molecules and nutrients between blood and tissue occurs preferentially in the capillaries. However, induced prostaglandin synthesis, as reflected by induced expression of Cox-2, seems to occur both in large, medium-sized and small vessels but not in capillaries (Fig. 5) (Eskilsson, 2014b), and it is not yet known which transporter is responsible for the transfer of PGE₂ into the brain. As shown here, PGE₂ synthesis in small and medium-sized vessels is critical for the febrile response. The functional role of the PGE₂ that is synthesized in the larger vessels, including vessels in meninges, and which seems to account for most of the PGE₂ that is seen in the CSF, remains to be clarified. It has been reported recently that excitation of pyramidal cells in the cerebral cortex results in increased local cerebral blood flow via neuronal release of PGE₂ and its binding to vasodilatory EP₂ and EP₄ receptors on vascular smooth muscle cells or pericytes (Lacroix et al., 2015). Whether PGE₂ produced by
endothelial cells in response to peripheral immune challenge also takes part in vasodilation of cerebral vessels is not known, and neither what functional role such vasodilation, if present, would subserve.

References


Eskilsson A, Mirrakhimian E, Dufour S, Schwaninger M, Engblom D, Blomqvist A (2014a) Immune-induced fever is mediated by IL-6 receptors on brain endothelial cells


**Figure legends**

**Figure 1.** Attenuated fever in mice with gene deletion of Cox-2 in brain endothelial cells. *a.* Temperature recordings from wild type (WT) and Cox-2ΔSlco1c1 mice immune challenged by intraperitoneal injection of LPS (120 µg/kg). The initial temperature peak is due to the handling stress in conjunction with the injection procedure. It is prostaglandin independent (Saha et al., 2005) and does not differ between genotypes. *b.* Bar graph showing mean fever 5 h after LPS injection in WT type and Cox-2ΔSlco1c1 mice. * indicates $P < 0.05$. In (*a*) and (*b*), *n* = 15 for WT LPS, *n* = 11 for Cox-2ΔSlco1c1 LPS, *n* = 9 for WT NaCl, and *n* = 6 for Cox-2ΔSlco1c1 NaCl.

**Figure 2.** PGE2 levels in brain and plasma and Cox-2 mRNA expression in the hypothalamus in WT mice and in mice with gene deletion of Cox-2 in brain endothelial cells. *a.* Immune stimulation with LPS (120 µg/kg ip) increases the PGE2 concentration in the cerebrospinal fluid to similar levels in both WT and Cox-2ΔSlco1c1 mice. *b.* The immune induced Cox-2 mRNA induction in the hypothalamus is significantly attenuated in Cox-2ΔSlco1c1 mice. ** indicates $P < 0.01$. *c.* The immune-induced levels of PGE2 metabolites in plasma do not differ between WT and Cox-2ΔSlco1c1 mice. In all graphs, *n* = 15 for WT LPS, *n* = 10-11 for Cox-2ΔSlco1c1 LPS, *n* = 9-10 for WT NaCl, and *n* = 5-8 for Cox-2ΔSlco1c1 NaCl.

**Figure 3.** Relationship between body temperature and PGE2 in the cerebrospinal fluid (CSF), Cox-2 mRNA in the hypothalamus, and PGE2 metabolites in plasma, respectively, following immune stimulation with LPS (120 µg/kg ip). *a.* Weak relationship between body temperature and PGE2 in the cerebrospinal fluid. *b.* Strong relationship between body temperature and Cox-2 mRNA in the hypothalamus. *c.* Moderately strong relationship between body temperature and PGE2 metabolites in plasma.
**Figure 4.** Relationship between PGE$_2$ levels in the cerebrospinal fluid (CSF) and Cox-2 mRNA and PGE$_2$ metabolites in plasma, respectively. *a.* Weak relationship between PGE$_2$ in the cerebrospinal fluid and Cox-2 mRNA in the hypothalamus. *b.* No significant relationship between PGE$_2$ in the cerebrospinal fluid and PGE$_2$ metabolites in plasma.

**Figure 5.** Gene deletion with Slco1c1-Cre targets mainly endothelial cells in small and medium-sized vessels in the brain. *a.* Dual labeling (arrowheads) of the Cre reporter protein tdTomato and Cox-2 in a small vessel in the brain. *b.* Large vessel with smaller-sized branch. Most of the Cox-2 immunoreactive cells (green) in the large vessel do not express tdTomato (arrows), whereas those in the smaller-sized branch do so (arrowheads). *c.* Numerous Cox-2 expressing cells (arrows) in a large vessel, but few of those cells also express tdTomato (arrowheads). All micrographs are from immune-challenged mice. Note that endothelial cells in capillaries express tdTomato (small arrows) but not Cox-2. Scale bar = 20 µm.

**Figure 6.** Cox-2 and lipocalin-2 expression following immune challenge. *a, b.* Abundant expression of Cox-2 among lipocalin-2 (LCN2) positive cells in a large vessel in both WT (*a*) and Cox-2$^{\Delta_{Slco1c1}}$ mice (*b*). *c, d.* No difference in Cox-2 expression in the leptomeninges between WT (*c*) and Cox-2$^{\Delta_{Slco1c1}}$ mice (*d*). *e, f.* Reduced Cox-2 expression in small lipocalin-2 stained vessel in a Cox-2$^{\Delta_{Slco1c1}}$ mouse (*f*) and abundant expression in a WT mouse (*e*). Arrowheads point at dual labeled cells and arrows point at single labeled cells. Scale bar = 20 µm.

**Figure 7.** Mice injected into the preoptic hypothalamus with lentiviral vector encoding the terminal prostaglandin E$_2$ synthesizing enzyme mPGES-1 display higher body temperature in response to LPS than mice injected with control vector. *a.* Temperature recordings of immune challenged mPGES-1 knockout mice injected with a viral vector encoding mPGES-1 (magenta trace) or given control injection of lentiviral vector encoding GFP (green trace). *n* =
12 for Lenti mPGES-1 LPS, n = 10 for Lenti GFP LPS, and n = 10 for NaCl (mixed group of Lenti mPGES-1 and Lenti GFP injected mice).  

Micrographs showing immunofluorescent staining for GFP in the preoptic hypothalamus after injection with viral vector. (b) and (c) are from different animals; the plane of the chosen sections corresponds approximately to Bregma +0.14/+0.145 mm (b), and Bregma +0.26/+0.245 mm (c), in the atlas of Paxinos and Franklin (2001) and the Allen Reference Atlas (Dong, 2008), respectively.  

3v, 3rd ventricle; ac, anterior commissure; AVPV, anteroventral periventricular nucleus; f, fornix; lv, lateral ventricle; MnPO, median preoptic nucleus; MPN, medial preoptic nucleus; PV, periventricular nucleus; VLPO, ventrolateral preoptic nucleus. Scale bar = 500 µm.
Figure 1
Figure 2

(a) PGE$_2$ (pg/ml) in CSF

(b) Cox-2 mRNA fold change in hypothalamus

(c) PGE$_2$ metabolites (pg/ml) in plasma

WT, NaCl
Cox-2ΔSlco1c1, NaCl
WT, LPS
Cox-2ΔSlco1c1, LPS
Figure 3

(a) PGE$_2$ (pg/ml) in CSF

(b) Cox-2 mRNA fold change in hypothalamus

(c) PGE$_2$ metabolites (pg/ml) in plasma

R$^2$ values for each graph:

- (a) R$^2 = 0.157^*$
- (b) R$^2 = 0.593^{***}$
- (c) R$^2 = 0.431^{***}$
Figure 4

(a) Cox-2 mRNA fold change in hypothalamus vs. PGE$_2$ (pg/ml) in CSF.

(b) Fold change in hypothalamus vs. PGE$_2$ metabolites (pg/ml) in plasma.
Figure 5
Figure 7

(a) Graph showing body temperature (°C) over time (min) for different groups: Lenti mPGES-1, LPS (magenta), Lenti GFP, LPS (green), and NaCl (blue). The temperature changes are indicated by lines with markers representing each group.

(b) Image showing a section of the brain with labeled structures: f, ac, MnPO, PV, 3v, MPN.

(c) Image showing another section of the brain with labeled structures: lv, ac, MnPO, AVPV, VLPO, 3v.