Systems/Circuits

Deletion of Prostaglandin E_2 Synthesizing Enzymes in Brain Endothelial Cells Attenuates Inflammatory Fever

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Fever is a hallmark of inflammatory and infectious diseases. The febrile response is triggered by prostaglandin E_2 synthesis mediated by induced expression of the enzymes cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase 1 (mPGES-1). The cellular source for pyrogenic PGE_2 remains a subject of debate; several hypotheses have been forwarded, including immune cells in the periphery and in the brain, as well as the brain endothelium. Here we generated mice with selective deletion of COX-2 and mPGES1 in brain endothelial cells. These mice displayed strongly attenuated febrile responses to peripheral immune challenge. In contrast, inflammation-induced hypoactivity was unaffected, demonstrating the physiological selectivity of the response to the targeted gene deletions. These findings demonstrate that PGE_2 synthesis in brain endothelial cells is critical for inflammation-induced fever.

Key words: **COX-2; endothelium; fever; mPGES-1; PGE_2; prostaglandin**

Introduction

Inflammatory challenge, typically in the form of an acute infection, elicits a number of distinct autonomic responses, including fever (Dantzer, 2001; Bartfai and Conti, 2010; Furuyashiki and Narumiya, 2011; Saper et al., 2012). Fever, which is a highly conserved trait of acute inflammatory activation (Kluger, 1991), is dependent on the prostaglandin cascade; inhibition of prostaglandin synthesis is the main mechanism of action for common antipyretic drugs, such as aspirin (Flower and Vane, 1972) and acetaminophen (Hinz et al., 2008; Engström Ruud et al., 2013). Furthermore, selective interventions with prostaglandin E_2 (PGE_2) synthesis (Engblom et al., 2003) or its receptor binding (Ushikubi et al., 1998; Lazarus et al., 2007) block fever. Cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1), the enzymes responsible for the generation of pyrogenic PGE_2 (Li et al., 1999; Engblom et al., 2003), are strongly induced in many cell types and tissues upon systemic inflammation (Cao et al., 1995; Breder and Saper, 1996; Ek et al., 2001; Yamagata et al., 2001; Ivanov et al., 2002; Eskilsson et al., 2014). The CNS target region for fever-inducing PGE_2 has been mapped to the anterior preoptic hypothalamus (Scammell et al., 1998; Lazarus et al., 2007). The brain endothelium is a strong candidate for being the critical site of prostaglandin production in this context because immune challenge induces both COX-2 and mPGES-1 in brain endothelial cells (Cao et al., 1995; Laflamme et al., 1999; Ek et al., 2001; Yamagata et al., 2001; Engblom et al., 2003; Engström et al., 2012). This source of PGE_2 is also suggested by studies showing a role for endothelial activation in fever induced by interleukin-1 (IL-1) (Ching et al., 2007; Ridder et al., 2011). However, the view that the cerebrovascular endothelium is the critical site for PGE_2 production in fever is challenged by studies indicating an important role for COX-2 in peripheral macrophages (Steiner et al., 2006) and/or brain perivascular cells (Breder and Saper, 1996; Elmquist et al., 1997; Schiltz and Sawchenko, 2002; Serrats et al., 2010). Thus, the cellular localization of COX-2 and mPGES-1 involved in the febrile response still remains a subject of debate, and direct *in vivo* investigations toward this end are lacking (Saper et al., 2012). Here, we examined the role of brain endothelial PGE_2 synthesis in inflammatory fever using mice with targeted deletions of COX-2 and mPGES-1 in brain endothelial cells.

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Materials and Methods

Animals. To obtain deletions specific to the brain endothelium, we used a mouse line expressing a codon-improved Cre recombinase (iCre) coupled to a mutated ligand binding domain of the human estrogen receptor (ER^{T2}). In the mouse line used, Cre is expressed under control of the *Slco1c1* promoter (*Slco1c1-Cre* ER^{T2}) (Ridder et al., 2011). The *Slco1c1*

gene encodes the solute carrier organic anion transporter 1c1 (also called Oatp 14) that is expressed in brain endothelial cells but not in endothelial cells in other organs (Ridder et al., 2011). The *Slco1c1-Cre ER*^{T2} mouse line, which efficiently recombines in cerebrovascular endothelium (Ridder et al., 2011), was used in the present study to selectively ablate the *Ptgs2* (COX-2) and the *Ptges1* (mPGES-1) genes in brain vasculature. The *Slco1c1-Cre ER*^{T2} mouse line was generated on a mixed Bl6/DBA background (B6D2F1), with all subsequent breeding on a pure C57BL/6 background. It was crossed to mice in which exons 4 and 5 of *Ptgs2* are flanked by loxP sites (Ishikawa and Herschman, 2006). Removal of these exons results in a frame shift mutation and early stop codons. The *Ptgs2*$^{flox/+}$ line was kept on a C57BL/6129SJ background. The *Ptges1* flox line was generated by introducing loxP elements on the both sites of exon 2 in the *Ptges1* gene. Deletion of exon 2 results in a frame shift mutation. The genetic modification was done by targeted mutagenesis in ES cells. The procedures used have been described in detail previously (Lee and Liu, 2009). ES cells were screened by Southern blotting and PCR, and clones with the targeted deletion were used for blastocyst injection (done by KCTT). Mice positive for the genetic modification of *Ptges1* were identified by PCR (forward primer: AGG AAT TCT GGG TAG GAG ATC CTG GCC TTT, reverse primer: GGT AGA AGC CAT TAA GGC CAC TCC TTG AGC) using DNA from ear biopsies as template. Gene deletions were induced by intraperitoneal injection of tamoxifen in adult mice (1 mg tamoxifen diluted in a mixture of 10% ethanol and 90% sunflower seed oil twice a day for 5 consecutive days). All animal experiments were approved by the local Animal Care and Use Committee and followed international guidelines. Mice of both sexes were used in the experiments.

Intraperitoneal injection of LPS and IL-1β. LPS from *Escherichia coli* serotype O111:B4 (Sigma-Aldrich; 100 μg/kg) was diluted in 100 μl 0.9% saline solution and injected intraperitoneally 1.5–3 h after lights on. In fever experiments, following a washout period of 1 week, animals previously injected with saline solution were given LPS and vice versa and injected 3 h after lights on. Recombinant murine IL-1β (PeproTech; 600 ng) was diluted in 100 μl 0.9% saline solution and injected intraperitoneally 1.5–3 h after lights on in fever experiments, whereas injections for locomotor activity studies were performed 1 h before lights off. The control group was injected with 100 μl 0.9% saline solution. After a washout period of 1 week, animals previously injected with IL-1β were given saline solution and vice versa. In all other experiments, animals were killed on the day of injection. The doses of LPS and IL-1β have been used previously by us (Engblom et al., 2003; Nilsberth et al., 2009; Engström et al., 2012) and were chosen because they induce robust fevers with a duration of ~6 h without causing strong hypothermic responses.

Measurements of fever and locomotor activity. Deep body temperature and locomotor activity were monitored using continuous telemetry with an indwelling abdominal transmitter (model TA11TAF10, Data Sciences International). Activity was quantified as midline crossings during the dark period.

qPCR measurements. Mice were injected intraperitoneally with either LPS (120 μg/kg) or saline and killed 3 h later by asphyxiation with CO_2, and perfused with saline to remove blood cells. Hypothalami were dissected accordingly to previously published protocols (Reyes et al., 2003) and placed in RNA later stabilization reagent solution (QIAGEN) and stored at −70°C until further use. RNA was extracted with RNeasy Universal Plus kit (QIAGEN), and reverse transcription was done with High Capacity cDNA Reverse Transcription kit (Applied Biosystems). 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 were for *Ptgs2:* Mm00478374_m1, *Cxcl10*: Mm00445235_m1, *Ccl2*: Mm00441242_m1, *Cebpd*: Mm00786711_s1 and for *GAPDH*: Mm99999915_g1 (used as reference gene). The levels of *Cox-2* mRNA were normalized against the reference gene (ΔCT) both in the stimulated and in the control group as $CT_{target\ gene} - CT_{reference\ gene}$, and the difference between the $\Delta CT_{stimulated} - \Delta CT_{control}$ was expressed as ΔΔCT. The gene expression changes were then analyzed as fold change values: $2^{-\Delta\Delta CT}$.

Immunohistochemistry and microscopy. Animals were killed by CO_2 asphyxiation and perfused transcardially with buffered PFA solution (4%). Coronal 40-μm-thick sections were cut on a freezing microtome (Leica Biosystems). Primary antibodies were as follows: rabbit anti-COX-2 (R1747, 1:1000 for peroxidase-based immunohistochemisty, and 1:500 for fluorescent labeling; Santa Cruz Biotechnology), goat anti-lipocalin 2 (AF-1857, 1:1000 and 1:500, respectively; R&D Systems), and sheep anti von Willebrand factor (1:500, Abcam). Secondary antibodies were as follows: biotinylated goat anti-rabbit IgG (1:1000; Vector Laboratories), AlexaFluor-488 donkey anti-rabbit (A21206, 1:500; Invitrogen), biotinylated horse anti-goat IgG (1:1000; Vector Laboratories), AlexaFluor-568 donkey anti-goat (A11057, 1:500; Invitrogen), and AlexaFluor-568 donkey anti-sheep (A21099, 1:500; Invitrogen). An avidin-biotin-HRP system with 3,3′-diaminobenzidine as chromogen was used for detection in the quantitative analyses, according to standard protocols (Engblom et al., 2002).

Immunohistochemical quantification. Quantification of COX-2 and lipocalin-2 was performed on photomicrographs comprising one full field of vision at 20× magnification, centered around the third ventricle at the level of the anterior hypothalamus. Counting was performed by two blinded investigators who were not aware of genotype or treatment. No significant differences in quantification results were seen between the two investigators.

Measurements of IL-1 in plasma. The concentration of IL-1β in plasma of animals after intraperitoneal administration of LPS was measured by a sandwich ELISA (QuantikineELISA Kit; R&D Systems) according to the manufacturer's instructions. The minimal detectable concentration of mouse IL-1β was 4.8 pg/ml. Optical densities were read at 450 nm with correction at 540 nm. The values were then calculated using a 4-PL curve fit, ranging from 0 to 800 pg/ml.

Surgical procedures. Preoperative and postoperative analgesia was provided with buprenorphine (25 μg/kg; Temgesic, RB Pharmaceuticals). Anesthesia was induced by 4% isoflurane (Abbot) in 100% O_2 in an induction chamber and maintained with 1.5% isloflurane in 100% O_2 administered via a face mask. Telemetry transmitters (Data Sciences International) were implanted via an abdominal midline incision. The peritoneum and skin were closed in layers. Postoperatively, animals were allowed to recover for 7 d. Basal temperature was recorded for 24 h before any experimentation. From the surgery to the end of the experiment, mice were kept in a thermoneutral environment (29°C).

Statistical analysis. Experiments in which four groups were used in 2 × 2 factorial design were analyzed by two-way ANOVA followed by Tukey's multiple-comparisons test. Experiments with two groups were analyzed with Student's *t* test. *p* values <0.05 were considered statistically significant.

Results

We first interfered with prostaglandin synthesis in brain endothelial cells by deletion of COX-2 selectively in these cells. We crossed Slco1c1-Cre ER^{T2} mice, which mediate recombination in brain endothelial cells and show only very limited recombination in other cell types, including peripheral endothelial cells (Ridder et al., 2011), with mice in which critical parts of the gene encoding COX-2 are floxed (Ishikawa and Herschman, 2006) resulting in offspring with deletion of COX-2 in the brain endothelium (*COX-2ΔbEnd*) and mice without any deletion (COX-2$^{fl/fl}$ mice; called WT here). To assess the deletion efficiency of COX-2 in *COX-2ΔbEnd* mice, we first used qPCR. We observed a strong induction of COX-2 mRNA in the hypothalamus of WT mice 3 h after immune challenge with bacterial wall LPS (100 μg/kg i.p.). The induction was markedly blunted in *COX-2ΔbEnd* mice (Fig. 1*a*; genotype $F_{(1,20)} = 6.625$, $p = 0.0139$, treatment $F_{(1,20)} = 33.47$, $p < 0.0001$, interaction $F_{(1,20)} = 6.625$ $p = 0.0181$, LPS:WT vs LPS:*COX-2ΔbEnd*; $p = 0.0046$). Next, we performed immunohistochemical staining of COX-2, 3 h after LPS injection. In WT mice, COX-2 was induced robustly along the vasculature throughout the brain (Fig. 1*b*). In contrast, in brains from *COX-2ΔbEnd* mice, the majority of vessels showed no or only sparse labeling (Fig. 1*c*). To confirm that the difference between genotypes resulted from a loss of COX-2 in endothelial cells in the *COX-2ΔbEnd* mice, we identified endothelial cells by labeling for

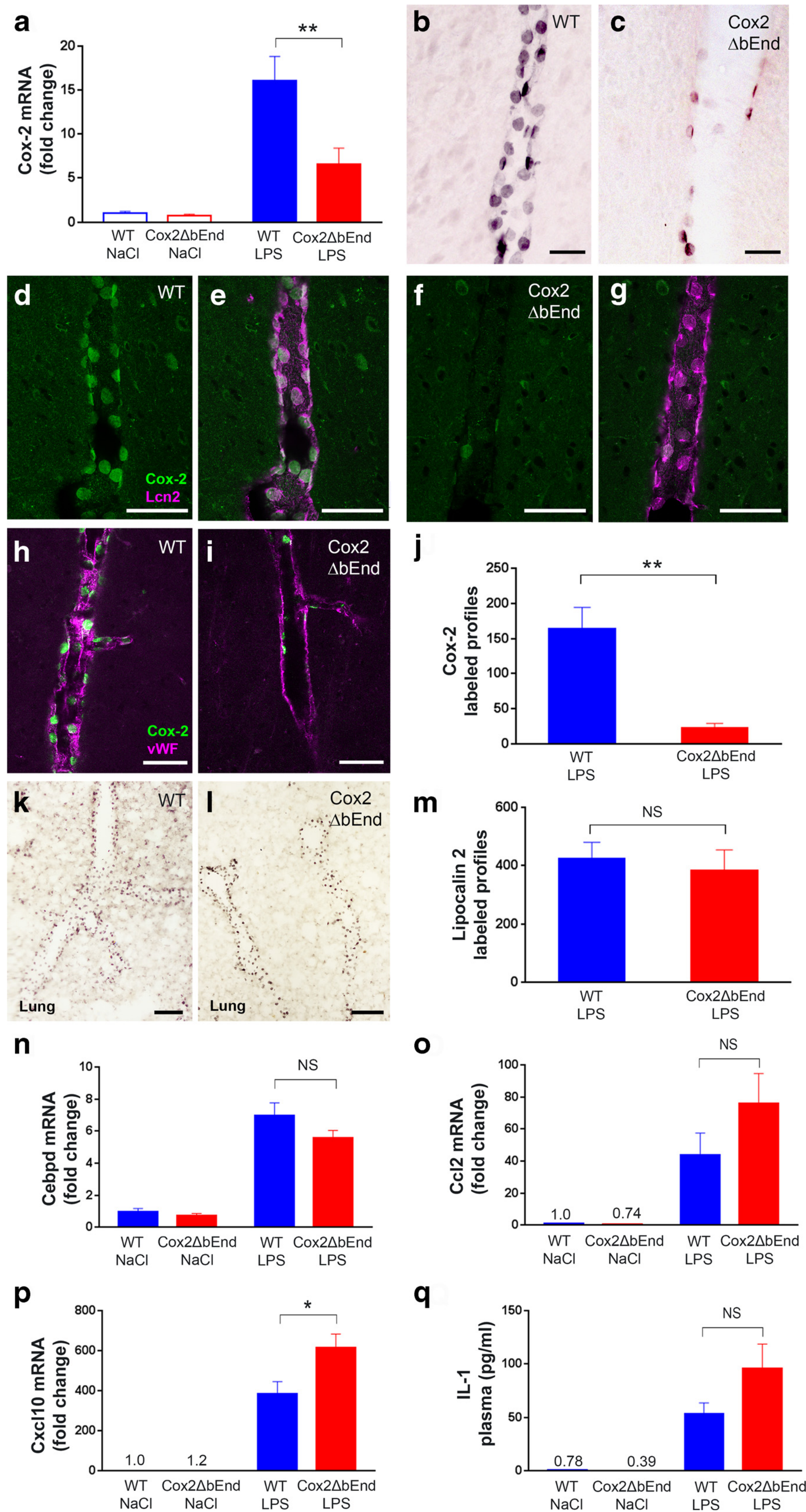

Figure 1. Selective deletion of COX-2 in brain endothelial cells. ***a***, Quantification of COX-2 mRNA levels in hypothalamus 3 h after intraperitoneal vehicle or LPS injection. COX-2 mRNA levels were significantly lower in LPS-treated *COX2ΔbEnd* mice ($n = 6$) than in WT ($n = 6$) mice. ***b***, ***c***, Micrographs showing COX-2 immunoreactivity in brain vasculature in the anterior hypothalamus of WT (***b***) and *COX2ΔbEnd* mice (***c***) 3 h after LPS injection. ***d–g***, Confocal micrographs showing COX-2 expression in hypothalamic blood vessels with lipocalin-2 (Lcn-2) as a marker of endothelial activation. ***h***, ***i***, Confocal micrographs of COX-2 expression in vWF-positive brain endothelial cells 3 h after intraperitoneal vehicle or LPS injection. ***j***, Immunohistochemical quantification of COX-2-positive endothelial cells at the level of the anterior hypothalamus. ***k***, ***l***, Low-power images showing similar COX-2 expression in lung blood vessels in WT (***k***) and *COX2ΔbEnd* animals (***l***). ***m***, Lcn-2-positive endothelial cells at the level of the anterior hypothalamus 3 h after intraperitoneal vehicle or LPS injection. ***n–p***, Quantification of inflammatory gene expression in the hypothalamus 3 h after LPS injection. ***q***, IL-1 levels in the plasma of WT and *COX2ΔbEnd* mice. Scale bars: ***b–i***, 50 μm; ***k***, ***l***, 100 μm. $^*p < 0.05$, $^{**}p < 0.01$. NS, Not significant.

lipocalin-2 (Hamzic et al., 2013), or for the von Willebrand factor, and colabeled these cells for COX-2 (Fig. 1*d–i*). The number of COX-2-positive cells in the hypothalamic area was reduced by ~85% in *COX-2ΔbEnd* animals compared with WT littermates (Fig. 1*j*; $p = 0.006$, $n = 6$, $n = 4$). To investigate whether the deletion also affected endothelial cells in the periphery, we performed immunohistochemical staining of COX-2 in lungs from LPS-treated mice because COX-2 is induced in the lung endothelium upon immune challenge (Engström et al., 2012). In contrast to the case in the brain endothelium, endothelial COX-2 labeling was equivalent in lungs from LPS-treated *COX-2ΔbEnd* and WT mice (Fig. 1*k*, *l*), showing that the COX-2 deletion had the expected cell-type specificity.

To validate that the COX-2 deletion in the brain endothelial cells in the mutant mice did not interfere with endothelial activation in general or with factors upstream of their activation (e.g., circulating cytokine levels), we examined the expression of lipocalin-2, which is strongly induced in endothelial cells upon peripheral immune challenge (Hamzic et al., 2013). *COX-2ΔbEnd* mice and WT littermates exhibited similar degrees of lipocalin-2 expression upon LPS-administration (Fig. 1*m*; $p = 0.669$, $n = 4$, $n = 5$). We also quantified hypothalamic mRNA levels for *Cebpd*, *Ccl2*, and *Cxcl10* in WT and mutant mice because they are induced in brain endothelial cells upon immune challenge (Reyes et al., 2003). All these genes were strongly induced 3 h after LPS administration in both genotypes (Fig. 1*n–p*; *Cebpd*: genotype not significant, treatment $F_{(1,20)} = 83.94$, $p < 0.0001$, interaction not significant. *Ccl2*: genotype not significant, treatment $F_{(1,20)} = 20.32$, $p = 0.0002$, interaction not significant. *Cxcl10*: genotype $F_{(1,20)} = 5.219$, $p = 0.0334$, treatment $F_{(1,20)} = 96.09$, $p < 0.0001$, interaction $F_{(1,20)} = 5.197$, $p = 0.0337$. LPS:WT vs LPS:*COX-2ΔbEnd* $p = 0.0147$). Finally we measured IL-1 levels in plasma 3 h after LPS. Also here, LPS caused a robust elevation in both genotypes (Fig. 1*q*; genotype

not significant, treatment $F_{(1,32)} = 14.78$, $p = 0.0005$, interaction not significant). Collectively, these data show that the deletion of COX-2 did not blunt the inflammatory process or immune-induced endothelial activation in general. If anything, the targeted brain endothelial cell COX-2 mutation slightly enhanced the general inflammatory response (Fig. 1*p*; and trends in Fig. 1*o*,*q*).

To determine the role of brain endothelial COX-2 in the febrile response, we next injected *COX-2ΔbEnd* mice and WT littermates with IL-1β (600 ng) or vehicle intraperitoneally and recorded body temperature using telemetry. In response to saline injection, both genotypes reacted with an identical early peak in temperature related to the stress associated with the injection, but they then both remained afebrile for the rest of the period monitored (Fig. 2*a*). WT mice injected with IL-1β showed an initial hypothermia after the handling-induced temperature spike, followed at ∼4 h after injection by a pronounced rise in body temperature. Mice with COX-2 gene deletion in cerebrovascular endothelial cells displayed significantly lower body temperature in response to IL-1β (Fig. 2*a*,*b*; genotype $F_{(1,31)} = 12.68$, treatment $F_{(1,31)} = 69.30$, $p < 0.0001$, interaction $F_{(1,31)} = 1.910$, IL-1:WT vs IL-1:*COX-2ΔbEnd* $p = 0.0086$), but an intact initial hypothermia. Thus, COX-2 in the brain endothelium is important for fever induced by IL-1β.

To test whether the phenotype seen in *COX-2ΔbEnd* animals may be specific to fever or whether it also may affect other central nervous sickness symptoms, we examined locomotor inhibition after administration of IL-1β (Ridder et al., 2011). IL-1β injection reduced the locomotor activity of both WT and *COX-2ΔbEnd* mice to a similar extent (Fig. 2*c*; genotype not significant, treatment $F_{(1,40)} = 27.00$, $p < 0.0001$, interaction not significant). These data indicate that brain endothelial prostaglandin production is dispensable for the reduction in locomotor activity seen after immune challenge.

To test whether COX-2 in the brain endothelium is involved in the febrile response to a more natural immune challenge, we injected mice of both genotypes with LPS (100 μg/kg) or saline. Again, mice of both genotypes showed an identical stress-induced hyperthermia associated with the injection (Fig. 3*a*). WT littermate mice responded with a robust rise in body temperature in response to LPS. As observed for the response to IL-1β, *COX-2ΔbEnd* mice showed significantly attenuated body temperature elevations (Fig. 3*a*,*b*; genotype $F_{(1,46)} = 9.766$, $p = 0.0031$, treatment $F_{(1,46)} = 60.58$, $p < 0.0001$, interaction $F_{(1,46)} = 5.364$, $p = 0.0251$. LPS:WT vs LPS:*COX-2ΔbEnd* $p = 0.0020$). Together, these data show that COX-2 in brain endothelial cells is important for immune-induced fever, whereas it is dispensable for stress-induced hyperthermia and immune-induced inactivity.

To determine whether the next step in pyrogenic PGE_2 production, the conversion of PGH_2 to PGE_2, is also occurring in the brain endothelium, we deleted mPGES-1 in brain endothelial cells, again using the *Slco1c1-Cre* ER^{T2} line. To this end, we generated a new mouse line in which mPGES-1 is floxed and crossed these mice with Slco1c1-Cre mice to generate mice lacking mPGES-1 in the brain endothelium (*mPGES-1ΔbEnd*). In response to LPS, *mPGES-1ΔbEnd* mice reacted with an initial hypothermia not seen in WT mice. Subsequently, they showed a markedly attenuated LPS-induced elevation of body temperature (Fig. 3*c*,*d*; genotype $F_{(1,40)} = 8.318$, $p = 0.0063$, treatment $F_{(1,40)} = 41.42$, $p < 0.0001$, interaction $F_{(1,40)} = 7.603$, $p = 0.0087$, WT:LPS vs *mPGES-1ΔbEnd*:LPS $p = 0.0015$).

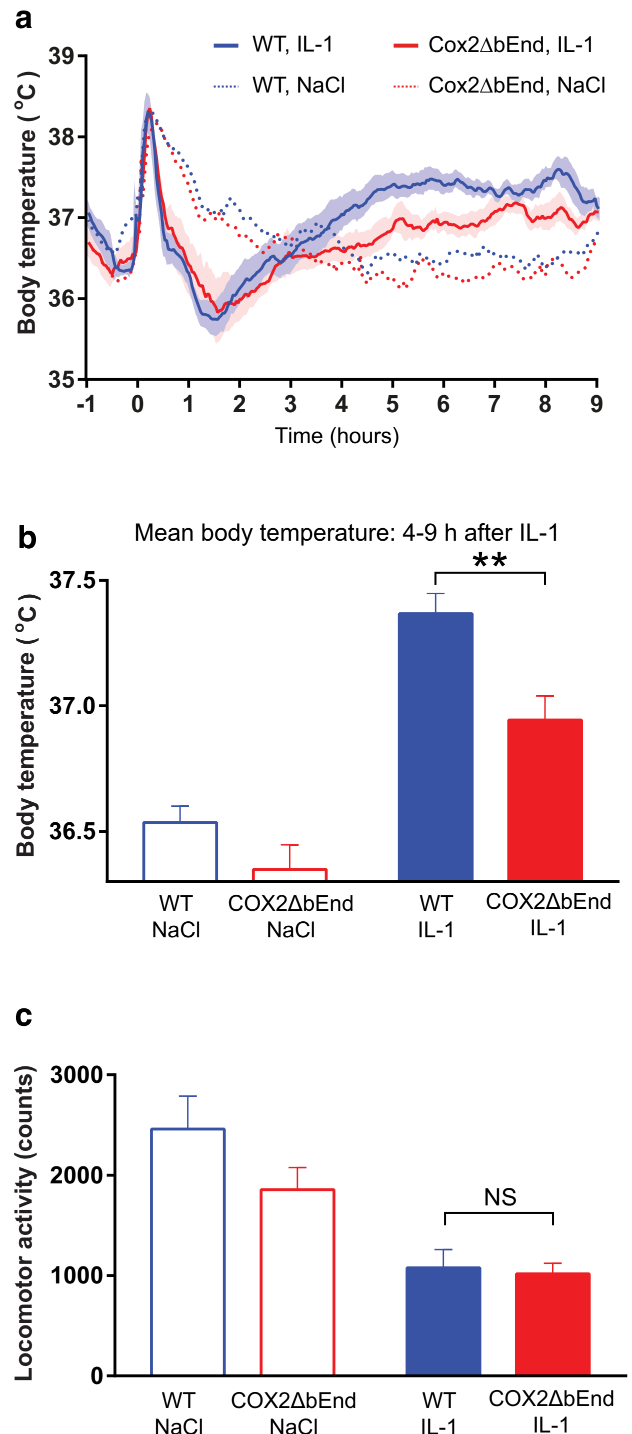

Figure 2. Attenuated febrile response to IL-1β in mice with COX-2 deletion in the brain endothelium. ***a***, Telemetric temperature recordings in freely moving animals showing the core body temperature of WT and *Cox2ΔbEnd* animals after IL-1β (IL-1) or vehicle injected intraperitoneally. ***b***, Mean body temperature is significantly lower in *COX2ΔbEnd* mice than in WT mice for the duration of the febrile response. ***c***, Locomotor activity is significantly reduced in both WT and *COX2ΔbEnd* mice injected with IL-1β. There is no significant difference between genotypes in the degree of locomotor inhibition caused by IL-1β). $^{**}p < 0.01$. NS, Not significant.

Discussion

Prostaglandins are key mediators of the febrile response. However, the cellular source of the prostaglandins involved has not been directly demonstrated. Histological studies have shown that

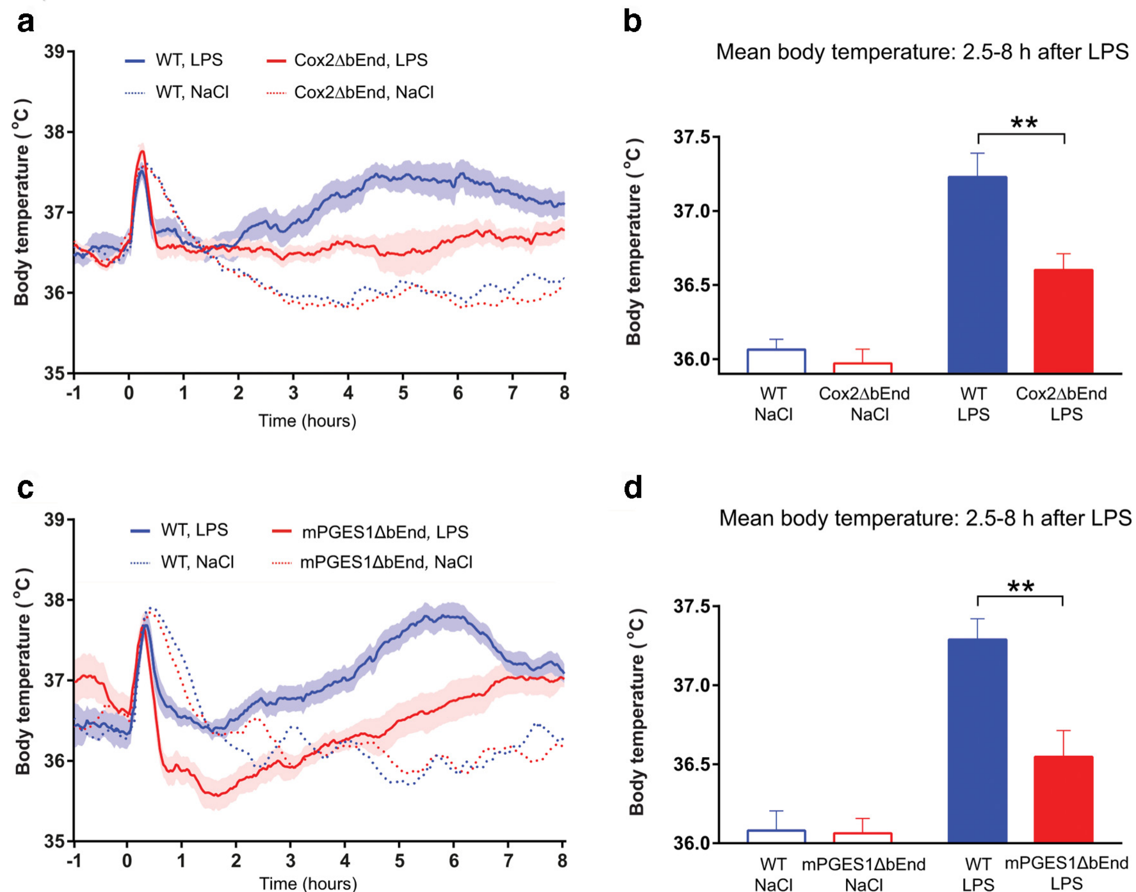

Figure 3. Blunted fever in response to LPS in mice with deletions of COX-2 and mPGES-1 in the brain endothelium. ***a***, Core body temperature in WT and *COX2ΔbEnd* mice after injection of NaCl or LPS. ***b***, Mean body temperature after LPS injection is significantly reduced in *COX2ΔbEnd* animals compared with WT mice. ***c***, Body temperature in WT and *mPGES1ΔbEnd* mice after injection of NaCl or LPS. ***d***, Mean body temperature during the febrile response in WT and *mPGES1ΔbEnd* mice. $^{**}p < 0.01$.

the two enzymes (COX-2 and mPGES-1) that mediate the PGE_2 synthesis important for fever are induced in endothelial cells throughout the brain by inflammatory stimuli (Ek et al., 2001; Yamagata et al., 2001). However, no cell-type-specific inhibition of brain endothelial prostaglandin synthesis has so far been performed, and it has not been known whether blockade of PGE_2 synthesis in the brain endothelium would attenuate the febrile response. Here, to test this hypothesis, we used transgenic mice with targeted, selective deletion of the genes encoding COX-2 and mPGES-1 in blood–brain barrier endothelial cells. These animals exhibited strongly attenuated febrile responses, indicating a pivotal role for brain endothelial cell PGE_2 synthesis in the induction of inflammatory fever.

This is the first study using a brain endothelium-specific intervention with prostaglandin synthesis. Earlier studies showing that deletion of the IL1-receptor or TAK1 (Ching et al., 2007; Ridder et al., 2011) in the brain endothelium attenuates IL1-induced fever could not exclude the possibility that endothelial activation triggers pyrogenic prostaglandin release by an effect on other cells, such as perivascular macrophages, or that endothelial activation is completely unrelated to pyrogenic prostaglandin release. Our demonstration that the induction of other inflammatory genes known to be induced in the brain endothelium upon immune challenge (lipocalin-2 (*Lcn2*), *Cebpd*, *Ccl2*, and *Cxcl10*) was intact in the hypothalamus of the *COX-2ΔbEnd* mice strongly indicates that there was little or no difference in general endothelial activation and that the major, and perhaps only, missing components in the immune-induced machinery were COX-2 and mPGES-1. Consequently, our findings bridge histological data on COX-2 and mPGES-1 expression in the brain (Cao et al., 1995; Ek et al., 2001; Yamagata et al., 2001; Engström et al., 2012; Eskilsson et al., 2014) and studies showing that brain endothelial activation is important for fever (Ching et al., 2007; Ridder et al., 2011), by providing direct evidence for a role of brain endothelial PGE_2 synthesis in inflammation-induced fever.

Fever in response to LPS and IL-1β is nearly completely blocked in mice with global genetic deletion or pharmacological inhibition of COX-2 (Li et al., 1999; Nilsberth et al., 2009), mPGES-1 (Engblom et al., 2003; Engström et al., 2012), or EP3 receptors (Ushikubi et al., 1998). Thus, the residual fever seen in *COX-2ΔbEnd* mice, and to some extent in *mPGES-1ΔbEnd* mice, likely depends on COX-2, mPGES-1, and PGE_2, perhaps resulting from residual COX-2/mPGES-1 activity in some brain endothelial cells. Indeed, the gene deletion in the present study was not complete; *COX-2ΔbEnd* mutant mice still display ~15% of the number of COX-2-positive endothelial cells present in WT mice. However, Ching et al. (2007) reported a residual fever response, in the absence of induced COX-2 expression in the brain endothelium, after intraperitoneal injection of IL-1β in mice lacking IL-1 receptors in the endothelium. Thus, there might be an additional source(s) of PGE_2 that account for the residual fever observed in *COX-2ΔbEnd* and *mPGES-1ΔbEnd* mice. One possibility is peripheral PGE_2 synthesis by macrophages of the liver and lung (Steiner et al., 2006). This alternative PGE_2 source

has been suggested to be important for the first phase of fever (0.5–1.5 h) in response to intravenous LPS administration. Perivascular cells at the blood–brain barrier have been suggested as another potential source for COX-2-dependent PGE_2 production in fever (Breder and Saper, 1996; Schiltz and Sawchenko, 2002; Serrats et al., 2010). In the present study, the first phase of the febrile response is masked by stress-induced hyperthermia related to the injection procedure. As a result, we cannot rule out a contribution of COX-2 in hematopoietic cells at early time-points. Also, the residual fever that we see at later time-points could, in principle, be triggered by COX-2 and mPGES-1 in hematopoietic cells. However, mice lacking mPGES-1 in perivascular cells and macrophages due to irradiation, followed by transplantation of bone marrow cells lacking mPGES-1, mount an intact febrile response to both intraperitoneal and intravenous LPS injection. Moreover, transplantation of mPGES-1-expressing hematopoietic cells to mPGES-1 KO mice does not rescue the missing febrile response (Engström et al., 2012), arguing against a role of hematopoietic cells in fever-generating PGE_2 production. Finally, COX-2 deletion in neurons and glial cells does not affect fever (Vardeh et al., 2009). Accordingly, despite a comprehensive literature, it is not clear which (if any) cell type(s) act in concert with the brain endothelial cells to produce the pyrogenic PGE_2 (Rivest, 1999; Saper et al., 2012). This ambiguity is due to the fact that the conclusions drawn so far in the field are based on correlational expression data and interventions that are either not cell-type-specific or are not specific to the prostaglandin cascade.

In conclusion, we show here the first example of a blunted systemic inflammatory symptom after a cell-type-specific intervention with prostaglandin synthesis. We show that a full inflammation-induced fever requires COX-2 and mPGES-1 in brain endothelial cells and thus directly demonstrate an important role of brain endothelial PGE_2 production in the generation of fever. This resolves, at least in part, a long-standing issue on which cells are the critical interface in transmitting the pyrogenic signal from the periphery to the brain (Elmquist et al., 1997; Rivest, 1999; Furuyashiki and Narumiya, 2011; Saper et al., 2012).

References

Bartfai T, Conti B (2010) Fever. Sci World J 10:490–503. CrossRef Medline

Breder CD, Saper CB (1996) Expression of inducible cyclooxygenase mRNA in the mouse brain after systemic administration of bacterial lipopolysaccharide. Brain Res 713:64–69. CrossRef Medline

Cao C, Matsumura K, Yamagata K, Watanabe Y (1995) Induction by lipopolysaccharide of cyclooxygenase-2 mRNA in rat brain: its possible role in the febrile response. Brain Res 697:187–196. CrossRef Medline

Ching S, Zhang H, Belevych N, He L, Lai W, Pu XA, Jaeger LB, Chen Q, Quan N (2007) Endothelial-specific knockdown of interleukin-1 (IL-1) type 1 receptor differentially alters CNS responses to IL-1 depending on its route of administration. J Neurosci 27:10476–10486. CrossRef Medline

Dantzer R (2001) Cytokine-induced sickness behavior: where do we stand? Brain Behav Immun 15:7–24. CrossRef Medline

Ek M, Engblom D, Saha S, Blomqvist A, Jakobsson PJ, Ericsson-Dahlstrand A (2001) Inflammatory response: pathway across the blood–brain barrier. Nature 410:430–431. CrossRef Medline

Elmquist JK, Scammell TE, Saper CB (1997) Mechanisms of CNS response to systemic immune challenge: the febrile response. Trends Neurosci 20: 565–570. CrossRef Medline

Engblom D, Ek M, Andersson IM, Saha S, Dahlström M, Jakobsson PJ, Ericsson-Dahlstrand A, Blomqvist A (2002) Induction of microsomal prostaglandin E synthase in the rat brain endothelium and parenchyma in adjuvant-induced arthritis. J Comp Neurol 452:205–214. CrossRef Medline

Engblom D, Saha S, Engström L, Westman M, Audoly LP, Jakobsson PJ, Blomqvist A (2003) Microsomal prostaglandin E synthase-1 is the central switch during immune-induced pyresis. Nat Neurosci 6:1137–1138. CrossRef Medline

Engström L, Ruud J, Eskilsson A, Larsson A, Mackerlova L, Kugelberg U, Qian H, Vasilache AM, Larsson P, Engblom D, Sigvardsson M, Jönsson JI, Blomqvist A (2012) Lipopolysaccharide-induced fever depends on prostaglandin E_2 production specifically in brain endothelial cells. Endocrinology 153:4849–4861. CrossRef Medline

Engström Ruud L, Wilhelms DB, Eskilsson A, Vasilache AM, Elander L, Engblom D, Blomqvist A (2013) Acetaminophen reduces lipopolysaccharide-induced fever by inhibiting cyclooxygenase-2. Neuropharmacology 71:124–129. CrossRef Medline

Eskilsson A, Tachikawa M, Hosoya KI, Blomqvist A (2014) Distribution of microsomal prostaglandin E synthase-1 in the mouse brain. J Comp Neurol 522:3229–3244. CrossRef Medline

Flower RJ, Vane JR (1972) Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). Nature 240:410–411. CrossRef Medline

Furuyashiki T, Narumiya S (2011) Stress responses: the contribution of prostaglandin E(2) and its receptors. Nat Rev Endocrinol 7:163–175. CrossRef Medline

Hamzic N, Blomqvist A, Nilsberth C (2013) Immune-induced expression of lipocalin-2 in brain endothelial cells: relationship with interleukin-6, cyclooxygenase-2 and the febrile response. J Neuroendocrinol 25:271–280. CrossRef Medline

Hinz B, Cheremina O, Brune K (2008) Acetaminophen (paracetamol) is a selective cyclooxygenase-2 inhibitor in man. FASEB J 22:383–390. CrossRef Medline

Ishikawa TO, Herschman HR (2006) Conditional knockout mouse for tissue-specific disruption of the cyclooxygenase-2 (Cox-2) gene. Genesis 44:143–149. CrossRef Medline

Ivanov AI, Pero RS, Scheck AC, Romanovsky AA (2002) Prostaglandin E(2)-synthesizing enzymes in fever: differential transcriptional regulation. Am J Physiol Regul Integr Comp Physiol 283:R1104–R1117. CrossRef Medline

Kluger MJ (1991) Fever: role of pyrogens and cryogens. Physiol Rev 71:93–127. Medline

Laflamme N, Lacroix S, Rivest S (1999) An essential role of interleukin-1beta in mediating NF-kappaB activity and COX-2 transcription in cells of the blood–brain barrier in response to a systemic and localized inflammation but not during endotoxemia. J Neurosci 19:10923–10930. Medline

Lazarus M, Yoshida K, Coppari R, Bass CE, Mochizuki T, Lowell BB, Saper CB (2007) EP3 prostaglandin receptors in the median preoptic nucleus are critical for fever responses. Nat Neurosci 10:1131–1133. CrossRef Medline

Lee SC, Liu P (2009) Construction of gene-targeting vectors by recombineering. Cold Spring Harb Protoc 2009:pdb.prot5291. CrossRef Medline

Li S, Wang Y, Matsumura K, Ballou LR, Morham SG, Blatteis CM (1999) The febrile response to lipopolysaccharide is blocked in cyclooxygenase-2(−/−), but not in cyclooxygenase-1(−/−) mice. Brain Res 825:86–94. CrossRef Medline

Nilsberth C, Elander L, Hamzic N, Norell M, Lönn J, Engström L, Blomqvist A (2009) The role of interleukin-6 in lipopolysaccharide-induced fever by mechanisms independent of prostaglandin E_2. Endocrinology 150: 1850–1860. CrossRef Medline

Reyes TM, Walker JR, DeCino C, Hogenesch JB, Sawchenko PE (2003) Categorically distinct acute stressors elicit dissimilar transcriptional profiles in the paraventricular nucleus of the hypothalamus. J Neurosci 23:5607–5616. Medline

Ridder DA, Lang MF, Salinin S, Röderer JP, Struss M, Maser-Gluth C, Schwaninger M (2011) TAK1 in brain endothelial cells mediates fever and lethargy. J Exp Med 208:2615–2623. CrossRef Medline

Rivest S (1999) What is the cellular source of prostaglandins in the brain in response to systemic inflammation? Facts and controversies. Mol Psychiatry 4:500–507. CrossRef Medline

Saper CB, Romanovsky AA, Scammell TE (2012) Neural circuitry engaged by prostaglandins during the sickness syndrome. Nat Neurosci 15:1088–1095. CrossRef Medline

Scammell TE, Griffin JD, Elmquist JK, Saper CB (1998) Microinjection of a

cyclooxygenase inhibitor into the anteroventral preoptic region attenuates LPS fever. Am J Physiol 274:R783–R789. Medline

Schiltz JC, Sawchenko PE (2002) Distinct brain vascular cell types manifest inducible cyclooxygenase expression as a function of the strength and nature of immune insults. J Neurosci 22:5606 –5618. Medline

Serrats J, Schiltz JC, García-Bueno B, van Rooijen N, Reyes TM, Sawchenko PE (2010) Dual roles for perivascular macrophages in immune-to-brain signaling. Neuron 65:94 –106. CrossRef Medline

Steiner AA, Ivanov AI, Serrats J, Hosokawa H, Phayre AN, Robbins JR, Roberts JL, Kobayashi S, Matsumura K, Sawchenko PE, Romanovsky AA (2006) Cellular and molecular bases of the initiation of fever. PLoS Biol 4:e284. CrossRef Medline

Ushikubi F, Segi E, Sugimoto Y, Murata T, Matsuoka T, Kobayashi T, Hizaki H, Tuboi K, Katsuyama M, Ichikawa A, Tanaka T, Yoshida N, Narumiya S (1998) Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3. Nature 395:281–284. CrossRef Medline

Vardeh D, Wang D, Costigan M, Lazarus M, Saper CB, Woolf CJ, Fitzgerald GA, Samad TA (2009) COX2 in CNS neural cells mediates mechanical inflammatory pain hypersensitivity in mice. J Clin Invest 119:287–294. CrossRef Medline

Yamagata K, Matsumura K, Inoue W, Shiraki T, Suzuki K, Yasuda S, Sugiura H, Cao C, Watanabe Y, Kobayashi S (2001) Coexpression of microsomal-type prostaglandin E synthase with cyclooxygenase-2 in brain endothelial cells of rats during endotoxin-induced fever. J Neurosci 21:2669 –2677. Medline