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**Acetaminophen reduces lipopolysaccharide-induced fever
by inhibiting cyclooxygenase-2**

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Abbreviations: Cox, cyclooxygenase; mPGES-1, microsomal prostaglandin E synthase-1;
LPS, lipopolysaccharide

Abstract

Acetaminophen is one of the world's most commonly used drugs to treat fever and pain, yet its mechanism of action has remained unclear. Here we tested the hypothesis that acetaminophen blocks fever through inhibition of cyclooxygenase-2 (Cox-2), by monitoring lipopolysaccharide induced fever in mice with genetic manipulations of enzymes in the prostaglandin cascade. We exploited the fact that lowered levels of a specific enzyme make the system more sensitive to any further inhibition of the same enzyme. Mice were immune challenged by an intraperitoneal injection of bacterial wall lipopolysaccharide and their body temperature recorded by telemetry. We found that mice heterozygous for Cox-2, but not for microsomal prostaglandin E synthase- 1 (mPGES-1), displayed attenuated fever, indicating a rate limiting role of Cox-2. We then titrated a dose of acetaminophen that did not inhibit the lipopolysaccharide-induced fever in wild-type mice. However, when the same dose of acetaminophen was given to Cox-2 heterozygous mice, the febrile response to lipopolysaccharide was strongly attenuated, resulting in an almost normalized temperature curve, whereas no difference was seen between wild-type and heterozygous mPGES-1 mice. Furthermore, the fever to intracerebrally injected prostaglandin E₂ was unaffected by acetaminophen treatment. These findings reveal that acetaminophen, similar to aspirin and other non-steroidal anti-inflammatory drugs, is antipyretic by inhibiting cyclooxygenase-2, and not by inhibiting mPGES-1 or signaling cascades downstream of prostaglandin E₂.

Key words: fever, cyclooxygenase-2, cyclooxygenase- 1, microsomal prostaglandin E synthase 1, gene dosage, hypothalamus

1. Introduction

While acetaminophen (paracetamol), because of its analgesic and antipyretic properties, is one of the world's most commonly used drugs, its mode of action has remained unclear. Unlike acetyl salicylic acid and other nonsteroidal anti-inflammatory drugs (NSAIDs), known to inhibit cyclooxygenase (Cox)-dependent prostaglandin production (Vane, 1971), acetaminophen largely lacks peripheral anti-inflammatory properties, suggesting that its action is within the central nervous system. Indeed, acetaminophen, which readily passes the blood-brain barrier (Courade et al., 2001; Kumpulainen et al., 2007), inhibits prostaglandin synthesis in the brain (Flower and Vane, 1972), but not in peripheral tissues, which also explains its favorable lack of several adverse effects associated with NSAIDs, such as stomach ulcers and impaired hemostasis.

The demonstrations that the febrile response is critically dependent on increased synthesis of PGE₂ through the inducible enzymes Cox-2 and microsomal prostaglandin E synthase-1 (mPGES-1) (Engblom et al., 2003; Li et al., 1999; Nilsberth et al., 2009b; Saha et al., 2005), which are expressed by brain endothelial cells (Ek et al., 2001; Engström et al., 2012; Yamagata et al., 2001), seem to imply that acetaminophen exerts its effect by inhibiting this pathway, but in a brain-specific manner. Yet, *in vitro* studies have demonstrated only weak inhibition of Cox-2 and mPGES-1 by acetaminophen (Mitchell et al., 1993; Thoren and Jakobsson, 2000). Based on this and other observations it has been proposed that acetaminophen targets a brain-specific isoform of Cox, Cox-3, which is a splice variant of the constitutive enzyme Cox-1 (Chandrasekharan et al., 2002), but this idea has been refuted (Kis et al., 2005; Li et al., 2008). It has also been suggested that acetaminophen blocks Cox activity not by binding to its active site but by reducing the active oxidized form of Cox to an inactive form (Ouellet and Percival, 2001), a process that only takes place under conditions with low peroxide activity, such as in the brain (Boutaud et al., 2002). In the same vein,

acetaminophen has been suggested to reduce the oxidative stress (Maharaj et al., 2006; Tripathy and Grammas, 2009) that has been implicated in prostaglandin release and fever (Hou et al., 2011; Riedel et al., 2003). Finally, there is evidence that the analgesic effect of acetaminophen may be ascribed to its metabolites, through the activation of endogenous receptor systems, such as the 5-hydroxytryptamine and endocannabinoid systems (Andersson et al., 2011; Bonnefont et al., 2007).

Here, we examined the sensitivity of Cox-2 and mPGES-1 to acetaminophen *in vivo*. We exploited the fact that lowered levels of a specific enzyme make the system more sensitive to any further inhibition of the same enzyme. This approach has been employed to identify drug targets on genome wide level, for example in yeast (Giaever et al., 1999). Our findings demonstrate that acetaminophen exerts its antipyretic effect by inhibiting Cox-2, and that it does not target mPGES-1, or signaling upstream or downstream of induced prostaglandin synthesis.

2. Materials and Methods

2.1. Animals

Ptgs2^{+/-} mice (Cox-2) mice (Morham et al., 1995) on a B6 background, and *Ptges*^{+/-} mice (Trebino et al., 2003) on a DBA/11acJ background were used. Both strains were mated to produce wild type and heterozygous littermates. The animals, which were of both sexes and in about equal proportions, were housed one to five per cage on a 12-h light/dark cycle (lights on at 08.00 h). All experimental procedures were approved by the Animal Care and Use Committee at Linköping University. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

2.2. Telemetric temperature recordings

The mice were briefly anesthetized with 1% isoflurane (Abbot Scandinavia, Solna, Sweden) and implanted intraperitoneally (ip) with a transmitter that records core body temperature (Data Science International, St. Paul, MN). 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). The animals were allowed to recover for at least 1 week before any recordings were made. Prior to immune challenge, the basal temperature of each mouse was recorded for 72 h to assure that they displayed normal body temperature with normal circadian variation.

2.3. Intraperitoneal injections

Lipopolysaccharide (LPS) from *Escherichia coli* (Sigma 0111:B4, 2 µg dissolved in 100 µl saline) or vehicle was injected ip at around 10.00 h. Propacetamol (Pro-Dafalgan®, Bristol-Myers Squibb, New York, NY; dissolved in 100 µl 0.9% NaCl) or vehicle was given ip 1 h prior to the administration of LPS, to assure high concentration of the drug in the brain at the time the immune challenge (Courade et al., 2001). Temperature data were sampled during 10 s every 2 min throughout the entire observation period.

2.4. Intracerebroventricular injection of PGE₂

Mice were mounted in a stereotaxic frame under anesthesia with 1% isoflurane in a 30/70% mixture of O₂/N₂, and kept at 37°C through a feedback controlled heating pad. A drill hole was made in the skull through which a 29 gauge (o.d.) needle connected by a silicon tube to a Hamilton syringe was inserted into the lateral ventricle (0.5 mm posterior to Bregma, 1 mm lateral to midline, and 2.5 mm vertical to the skull surface), and 4 nmol PGE₂ in 2 µl artificial cerebrospinal fluid was injected during 1 min. This dose was chosen since it produces a robust

and pronounced febrile response, as demonstrated previously (Engblom et al., 2003; Nilsberth et al., 2009a). Two minutes after the end of the injection, the needle was removed, the skin sutured, and the gas anesthesia turned off. All animals were awake within 5 min after injection and were immediately returned to their home cage for resumed body temperature recordings.

2.5. Western blot

Mice were injected ip with LPS (2 µg), and killed 5 h later [the time point of the peak expression of Cox-2 in the brain after peripheral immune challenge (Inoue et al., 2002)] by asphyxiation with CO₂. Hypothalami and forebrains (to permit additional analyses) were immediately collected and kept at -70°C until analysis. The tissue was homogenized in lysis buffer (0.1 M phosphate-buffered saline, 1% nonidet P40, 0.5% deoxycholate and 0.1% sodium dodecyl sulfate) containing protease inhibitors (Complete Mini Protease Inhibitor Cocktail Tablets; Roche Diagnostics Scandinavia AB, Bromma, Sweden). The homogenates were then centrifuged and the supernatants collected and analyzed for total protein concentration using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). For detection of Cox-2, 20 mg of protein from each sample was fractionated on a 12% Mini PROTEAN TGX gel (Bio-Rad, Hercules, CA) and transferred to a Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Amersham, UK). Non-specific binding was blocked by immersing the membrane in 5% non-fat dry milk in Tris-buffered saline (pH 7.6) with 0.1% Tween-20, for 1 h. All steps from here were at room temperature if not otherwise stated. The membrane was incubated for 2 h with goat anti-Cox-2 (1:1000; sc-1747 M-19; Santa Cruz), followed by chicken anti-goat IgG-HRP (1:20,000; sc-2961, Santa Cruz) for 1 h. The antibodies were removed through immersion in stripping buffer (100 mM 2-Mercaptoethanol, and 2% sodium dodecyl sulfate in 62.5 mM Tris-HCl) for 30 min at 50°C, blocked and incubated for 2 h with rabbit anti-GAPDH (1:10

000; sc-25778, Santa Cruz) as loading control, followed by donkey anti-rabbit IgG-HRP (1:50,000; sc-2313, Santa Cruz) for 1 h. The bound antibodies were detected using the Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare, UK). The membranes were examined with a Fujifilm LAS-1000 camera connected to a Fujifilm Intelligent Dark Box (Fujifilm, Tokyo, Japan) and data analyzed with Image Reader LAS-1000 Pro version 2.6 (Fujifilm). Each sample was run on three separate gels, the obtained values were normalized against GAPDH, and the mean of the normalized values were calculated and presented with SEM.

Detection of Cox-1 was done in a similar way but with a blocking solution containing Tris-buffered saline (pH 7.6), 0.1% Tween-20, and 2% enhanced chemiluminescence advance blocking agent; GE Healthcare) overnight at 4°C, and using a goat anti-Cox-1 antibody (1:4000; sc-1754 M-20; Santa Cruz) followed by rabbit anti-goat IgG-HRP (1:50,000; sc-2768, Santa Cruz). Each sample was run on three separate gels and the mean of the normalized values were calculated and presented with SEM.

2.6. Immunohistochemistry

Mice with heterozygous deletion of Cox-2 were divided into three groups. One group received an injection of propacetamol (100 mg/kg; corresponding to 50 mg/kg acetaminophen) followed by LPS (2 µg/mouse ip) 1 h later. The other groups received first saline, followed by either saline or LPS. Three hours after the second injection, the mice were killed with CO₂ and perfused with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer (0.1M, pH 7.4). The brains were removed and stored in fixative for 3 hours and subsequently kept in a cryoprotective PBS solution containing 30% sucrose at 4°C overnight. Coronal sections of the rostral hypothalamus were cut at 40 µm on a freezing

microtome and collected in cold cryoprotectant (5 mM PBS, 30% ethylen glycol and 20% glycerol) and stored at -20°C.

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 1% bovine serum albumin (Sigma-Aldrich, St. Louis, USA) and 0.3% Triton X (Roche)] for 45 minutes, followed by incubation overnight at 4°C with goat anti-Cox-2 (1:1000; sc-1747 M-17; Santa Cruz Biotechnologies), rinsed in 0.3% H₂O₂ for 1 h, and then incubated with biotinylated rabbit anti-goat antibody (1:1000; Vector laboratories Burlingame, CA, USA) for 2 h at room temperature. Bound antibody complexes were visualized by incubation in avidin-biotin (Vectastain ABC kit, Vector Laboratories) for 2 h at room temperature, followed by 0.02% 3,3'-diaminobenzidine (DAB, Sigma-Aldrich), 2.5% ammonium nickel sulphate, and 0.01% H₂O₂ in sodium acetate buffer for 8 min. Sections were mounted on slides, air-dried overnight, dehydrated in 100% ethanol, defatted in xylene, and subsequently coverslipped with DPX (VWR International, Stockholm, Sweden). Microphotographs were taken on a SPOT-2 digital camera (Diagnostics Instruments, Sterling Heights, MI, USA).

2.7. *qPCR*

Mice were injected ip with either LPS (2 µg) or saline and killed 3 h later by asphyxiation with CO₂, and perfused with saline to remove blood cells. This time point was chosen because it is when both Cox-2 mRNA and mPGES-1 mRNA are strongly elevated after peripheral LPS-injection (Ivanov et al., 2002). Hypothalami and forebrains were frozen on dry ice. RNA was extracted with RNeasy Universal Plus kit (Qiagen, Hilden, Germany) 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) with samples run in duplicates for each gene, on a 96-well plate (7900HT Fast RT-PCR system; Applied Biosystems). Assays used were for *Ptges* (mPGES-1): Mm00452105_m1; *Ptgs1* (Cox-1): Mm00477214_m1; *Ptgs2* (Cox-2): Mm00478374_m1; and *GAPDH*: Mm99999915_g1.

2.8. Statistics

Data are given as mean $\pm$ SEM. Differences between temperature curves were analyzed with a two-way ANOVA, followed, when appropriate, by Tukey's post-hoc test. For post-hoc analysis of temperature differences along the time course of the temperature recordings, Fisher's LSD test was used. Peak fever was calculated for the LPS-injected animals by subtracting the highest measured temperature for each animal with the average temperature at the corresponding time point for saline-injected animals of same genotype, and the difference between genotypes analyzed with an unpaired *t*-test. Western blot data were analyzed with an unpaired *t*-test. For qPCR data, each gene was normalized against the reference gene (Δ CT) both in the stimulated and in the control group as $CT_{\text{target gene}} - CT_{\text{reference gene}}$, and the difference between the Δ CT_{stimulated} - Δ CT_{control} was expressed as $\Delta\Delta$ CT. The gene expression changes were then analyzed as fold change values: $2^{-\Delta\Delta\text{CT}}$. The SEM for fold differences was obtained by first calculating the SD for each of the two groups that were compared, and then applying these values in the following formula for the confidence interval for difference between means (Altman, 1991, page 192): $((sp^2 * (1/n1 + 1/n2))^{0.5}$, in which $sp^2 = (s1^2(n1 - 1) + s2^2(n2 - 1))/(n1 + n2 - 2)$. Statistical significance was then determined by using the *t*-distribution. $P < 0.05$ was considered statistically significant.

3. Results

We first examined the febrile response to intraperitoneal injection of LPS in wild type mice and mice heterozygous for Cox-2 or mPGES-1, respectively. As shown in Fig. 1A, Cox-2 heterozygous mice showed an attenuated febrile response compared with wild type mice, whereas no difference in the febrile response was seen between mice heterozygous for mPGES-1 and their wild type counterparts (Fig. 1B). Hence, these data indicate that Cox-2 is the rate limiting enzyme in the PGE₂ synthesizing pathway (Li et al., 1999).

To further verify that the loss of an allele influenced the expression of the enzyme, we next determined the amount of Cox-1, Cox-2 and mPGES-1 expressed by the different genotypes. We found that Cox-2 heterozygous mice challenged with LPS expressed reduced amounts of Cox-2 mRNA and protein as compared to wild type mice, whereas the expression of Cox-1 mRNA and protein and mPGES-1 mRNA (protein was not determined) was the same in both genotypes (Fig. 2A, B; Supplemental Fig. 1A, C). Similarly, heterozygous mPGES-1 mice challenged with LPS expressed about half the amount of mPGES-1 mRNA as that expressed by wild type mice, also without any compensatory up regulation of the mRNA for the Cox enzymes (Fig. 2C; Supplemental Fig. 1B).

Having established the differences in the expression of Cox-2 and mPGES-1 between the heterozygous and wild type mice we now pre-treated the mice with acetaminophen (administered as the soluble pro-drug propacetamol that is hydrolyzed to acetaminophen), given intraperitoneally one hour prior to the administration of LPS. The dose of acetaminophen (50 mg/kg) was titrated so that it had no, or only very mild, effects on the febrile response in wild type mice (Fig. 3A-C, Supplemental Fig. 2). We then gave this dose of acetaminophen to mice heterozygous for Cox-2. In these mice, this treatment strongly attenuated the febrile response to LPS, resulting in an almost normalized temperature curve

(Fig. 3A-C). This shows that mice with lowered amounts of Cox-2 are hypersensitive to the antipyretic action of acetaminophen.

We next tested if the observed hypersensitivity to the antipyretic effect of acetaminophen was specific to mice with low levels of Cox-2 or if other interventions in the PGE₂ generating enzyme cascade would lead to a similar results. Thus, we gave the same dose of acetaminophen that was given to the Cox-2 mice to wild type and mPGES-1 heterozygous mice. However, in these mice the treatment did not result in any difference between the two genotypes in the febrile response to LPS (Fig. 3D). We then pretreated mice with a high dose of acetaminophen (200 mg/kg) that completely extinguished the LPS-induced fever (Supplementary Fig. 2), before injecting them intracerebroventricularly with PGE₂. This resulted in a rapid and pronounced febrile response, similar to that seen in mice not pretreated with acetaminophen (Fig. 3E).

Finally, we tested if the effect of acetaminophen could be explained by an interference with Cox-2 transcription, rather than by affecting its enzymatic activity. For this purpose we determined Cox-2 mRNA levels in LPS challenged WT mice that were pretreated either with acetaminophen (100 mg/kg) or given vehicle only. No difference was seen in Cox-2 mRNA levels between treatments (Supplemental Fig. 3E). Furthermore, in line with an absence of effect of acetaminophen on Cox-2 mRNA transcription, staining for Cox-2 immunoreactivity showed that LPS-induced Cox-2 protein expression in brain vascular cells displayed the same pattern and extent in mice pretreated with acetaminophen as in those pretreated with vehicle (Supplemental Fig. 3A-D).

4. Discussion

The present data are consistent with an inhibition by acetaminophen of Cox-2, but not of mPGES-1, as shown by the different responses in Cox-2 heterozygous mice compared to mPGES-1 heterozygous mice. The obtained data cannot be explained by an action of acetaminophen up-stream of Cox-2, because if the substrate for Cox-2 (arachidonic acid) had been reduced, this would primarily have affected the response of wild type mice and not of Cox-2 heterozygous mice. And similarly, provided that heterozygosity resulted in lower protein levels [which could not be determined because of the technical difficulties in the detection of mPGES-1 protein in mice (Engström et al., 2012)], acetaminophen cannot exert its action on mPGES-1, because if so, it would have affected the febrile response of mPGES-1 heterozygous mice more than that of wild type mice. Furthermore, acetaminophen does not exert its antipyretic effect on the PGE₂-receptors or on some signaling system downstream of these receptors, because acetaminophen-treated mice displayed a normal febrile response to PGE₂ given intracerebroventricularly, implying that the antipyretic action was due to reduced PGE₂ synthesis, in turn accomplished by Cox-2 inhibition. Hence, these data show that during systemic inflammation in an intact organism, acetaminophen is antipyretic through inhibition of Cox-2.

Is the antipyretic effect of acetaminophen exerted on Cox-2 transcription or on its enzymatic activity? *In vitro* studies on rat primary hippocampal neurons and a human astrocytic cell line have reported that acetaminophen reduces the nuclear translocation of NFκB (Bisaglia et al., 2002; Mancini et al., 2003), which is known to be the most important transcription factor for Cox-2 induction in the brain (Nadjar et al., 2005). These data hence would suggest that acetaminophen reduces Cox-2 activity by inhibiting Cox-2 transcription. To address this question, we examined if LPS-induced Cox-2 mRNA expression was affected by acetaminophen. However, there was no difference in Cox-2 mRNA levels after LPS-challenge

between mice that were given acetaminophen and those given vehicle only, and, being consistent with this observation, there was neither any obvious effect on induced Cox-2 protein expression in the brain, as determined by immunohistochemical staining. These data hence do not lend any support to the idea that acetaminophen inhibits Cox-2 on the transcriptional/translational level *in vivo*, but that it exerts its effect by inhibiting its enzymatic activity.

A Cox-2 inhibitory effect of acetaminophen has been suggested in some previous studies. Thus, Hinz and collaborators (Hinz et al., 2008) showed an *ex vivo* inhibition of Cox-2 mediated PGE₂ synthesis in peripheral blood from human volunteers that had been given paracetamol orally, and Li and collaborators, examining Cox-1 knock-out mice, showed that while immune challenge with LPS resulted in fever, this response was extinguished by acetaminophen. Because acetaminophen was unable to reduce the LPS-induced PGE₂-increase in plasma [being contradictory to the observations by Hinz et al. (2008)], while reducing it in the brain, the authors deduced that the antipyretic effect of acetaminophen was due to inhibition of Cox-2 in the brain.

In addition to reducing fever, acetaminophen also possesses analgesic properties. The results of some previous work indicate that the mechanisms behind its pain killing effect may be distinct from the antipyretic mechanism described in the present study. In a model of tonic pain in rats, Bonnefont and collaborators (2007) showed an involvement of 5-HT_{1A} receptor-dependent mechanisms in acetaminophen-produced antinociception, and quite recently, Andersson et al. (2011) demonstrated that acetaminophen, via its metabolites *p*-benzoquinone and N-acetyl-*p*-benzoquinoneimine, produces antinociception through the activation of the TRPA1 receptor on the central nerve endings of spinal nociceptive fibers.

In summary, the present findings demonstrate that acetaminophen, similar to aspirin and other non-steroidal anti-inflammatory drugs, is antipyretic by inhibiting Cox-2, and most likely acting on the Cox-2 dependent induced PGE₂ production that occurs in brain endothelial cells upon inflammatory challenge (Ek et al., 2001; Engström et al., 2012; Yamagata et al., 2001). However, the antipyretic action of acetaminophen may be distinct from its antinociceptive effect that may be exerted by different modes of action.

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References

- Altman, D.G., 1991. Practical statistics for medical research Chapman and Hall, London.
- Andersson, D.A., Gentry, C., Alenmyr, L., Killander, D., Lewis, S.E., Andersson, A., Bucher, B., Galzi, J.-L., Sterner, O., Bevan, S., Högestätt, E.D., Zygmunt, P.M., 2011. TRPA1 mediates spinal antinociception induced by acetaminophen and the cannabinoid Δ9-tetrahydrocannabinol. *Nat. Commun.* 2, 551.
- Bisaglia, M., Venezia, V., Piccioli, P., Stanzione, S., Porcile, C., Russo, C., Mancini, F., Milanese, C., Schettini, G., 2002. Acetaminophen protects hippocampal neurons and PC12

cultures from amyloid β -peptides induced oxidative stress and reduces NF- κ B activation.

Neurochem. Int. 41, 43-54.

Bonnefont, J., Daulhac, L., Etienne, M., Chapuy, E., Mallet, C., Ouchchane, L., Deval, C., Courade, J.-P., Ferrara, M., Eschalier, A., Clottes, E., 2007. Acetaminophen Recruits Spinal p42/p44 MAPKs and GH/IGF-1 Receptors to Produce Analgesia via the Serotonergic System. Mol. Pharmacol. 71, 407-415.

Bosetti, F., Langenbach, R., Weerasinghe, G.R., 2004. Prostaglandin E2 and microsomal prostaglandin E synthase-2 expression are decreased in the cyclooxygenase-2-deficient mouse brain despite compensatory induction of cyclooxygenase-1 and Ca²⁺-dependent phospholipase A2. J. Neurochem. 91, 1389-1397.

Boutaud, O., Aronoff, D.M., Richardson, J.H., Marnett, L.J., Oates, J.A., 2002. Determinants of the cellular specificity of acetaminophen as an inhibitor of prostaglandin H(2) synthases. Proc. Natl. Acad. Sci. U S A 99, 7130-7135.

Chandrasekharan, N.V., Dai, H., Roos, K.L.T., Evanson, N.K., Tomsik, J., Elton, T.S., Simmons, D.L., 2002. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: Cloning, structure, and expression. Proc. Natl. Acad. Sci. U S A 99, 13926-13931.

Courade, J.P., Besse, D., Delchambre, C., Hanoun, N., Hamon, M., Eschalier, A., Caussade, F., Cloarec, A., 2001. Acetaminophen distribution in the rat central nervous system. Life Sci. 69, 1455-1464.

Ek, M., Engblom, D., Saha, S., Blomqvist, A., Jakobsson, P.J., Ericsson-Dahlstrand, A., 2001. Inflammatory response: pathway across the blood-brain barrier. Nature 410, 430-431.

Engblom, D., Saha, S., Engstrom, L., Westman, M., Audoly, L.P., Jakobsson, P.J., Blomqvist, A., 2003. Microsomal prostaglandin E synthase-1 is the central switch during immune-induced pyresis. *Nat. Neurosci.* 6, 1137-1138.

Engström, L., Ruud, J., Eskilsson, A., Larsson, A., Mackerlova, L., Kugelberg, U., Qian, H., Vasilache, A.M., Larsson, P., Engblom, D., Sigvardsson, M., Jönsson, J.-I., Blomqvist, A., 2012. Lipopolysaccharide-induced fever depends on prostaglandin E2 production specifically in brain endothelial cells. *Endocrinology* 153, 4849-4861.

Flower, R.J., Vane, J.R., 1972. Inhibition of prostaglandin synthetase in brain explains the anti- pyretic activity of paracetamol (4-acetamidophenol). *Nature* 240, 410-411.

Giaever, G., Shoemaker, D.D., Jones, T.W., Liang, H., Winzeler, E.A., Astromoff, A., Davis, R.W., 1999. Genomic profiling of drug sensitivities via induced haploinsufficiency. *Nat. Genet.* 21, 278-283.

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

Hou, C.-C., Lin, H., Chang, C.-P., Huang, W.-T., Lin, M.-T., 2011. Oxidative stress and pyrogenic fever pathogenesis. *Eur. J. Pharmacol.* 667, 6-12.

Inoue, W., Matsumura, K., Yamagata, K., Takemiya, T., Shiraki, T., Kobayashi, S., 2002. Brain-specific endothelial induction of prostaglandin E(2) synthesis enzymes and its temporal relation to fever. *Neurosci Res* 44, 51-61.

Ivanov, A.I., Pero, R.S., Scheck, A.C., Romanovsky, A.A., 2002. Prostaglandin E(2)-synthesizing enzymes in fever: differential transcriptional regulation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 283, R1104-1117.

Kis, B., Snipes, J.A., Busija, D.W., 2005. Acetaminophen and the Cyclooxygenase-3 Puzzle: Sorting out Facts, Fictions, and Uncertainties. *J. Pharmacol. Exp. Ther.* 315, 1-7.

Kumpulainen, E., Kokki, H., Halonen, T., Heikkinen, M., Savolainen, J., Laisalmi, M., 2007. Paracetamol (Acetaminophen) Penetrates Readily Into the Cerebrospinal Fluid of Children After Intravenous Administration. *Pediatrics* 119, 766-771.

Li, S., Dou, W., Tang, Y., Goorha, S., Ballou, L.R., Blatteis, C.M., 2008. Acetaminophen: antipyretic or hypothermic in mice? In either case, PGHS-1b (COX-3) is irrelevant. *Prostag. Oth. Lipid M.* 85, 89-99.

Li, S., Wang, Y., Matsumura, K., Ballou, L.R., Morham, S.G., Blatteis, C.M., 1999. The febrile response to lipopolysaccharide is blocked in cyclooxygenase-2(-/-), but not in cyclooxygenase-1(-/-) mice. *Brain Res.* 825, 86-94.

Maharaj, H., Maharaj, D.S., Daya, S., 2006. Acetylsalicylic acid and acetaminophen protect against oxidative neurotoxicity. *Metab. Brain Dis.* 21, 180-190.

Mancini, F., Landolfi, C., Muzio, M., Aquilini, L., Soldo, L., Coletta, I., Guglielmotti, A., Mantovani, A., Pinza, M., Milanese, C., 2003. Acetaminophen down-regulates interleukin-1 β -induced nuclear factor- κ B nuclear translocation in a human astrocytic cell line. *Neurosci. Lett.* 353, 79-82.

Mitchell, J.A., Akarasereenont, P., Thiemermann, C., Flower, R.J., Vane, J.R., 1993. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc. Natl. Acad. Sci. U S A* 90, 11693-11697.

- Morham, S.G., Langenbach, R., Loftin, C.D., Tiano, H.F., Vouloumanos, N., Jennette, J.C., Mahler, J.F., Kluckman, K.D., Ledford, A., Lee, C.A., Smithies, O., 1995. Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell* 83, 473-482.
- Muller-Decker, K., 1999. Cyclooxygenases. In: Marks, F., Furstenberger, G., (Eds), Prostaglandins, Leukotrienes, and Other Eicosanoids. Wiley-VCH, Weinheim, pp. 65-88.
- Nadjar, A., Tridon, V., May, M.J., Ghosh, S., Dantzer, R., Amedee, T., Parnet, P., 2005. NF[κ]B activates in vivo the synthesis of inducible Cox-2 in the brain. *J. Cereb. Blood Flow Metab.* 25, 1047-1059.
- Nilsberth, C., Elander, L., Hamzic, N., Norell, M., Lonn, J., Engstrom, L., Blomqvist, A., 2009a. The role of interleukin-6 in lipopolysaccharide-induced fever by mechanisms independent of prostaglandin E2. *Endocrinology* 150, 1850-1860.
- Nilsberth, C., Hamzic, N., Norell, M., Blomqvist, A., 2009b. Peripheral lipopolysaccharide administration induces cytokine mRNA expression in the viscera and brain of fever-refractory mice lacking microsomal prostaglandin E synthase-1. *J. Neuroendocrinol.* 21, 715-721.
- Ouellet, M., Percival, M.D., 2001. Mechanism of Acetaminophen Inhibition of Cyclooxygenase Isoforms. *Arch. Biochem. Biophys.* 387, 273-280.
- Riedel, W., Lang, U., Oetjen, U., Schlapp, U., Shibata, M., 2003. Inhibition of oxygen radical formation by methylene blue, aspirin, or alpha-lipoic acid, prevents bacterial-lipopolysaccharide-induced fever. *Mol. Cell. Biochem.* 247, 83-94.
- Romanovsky, A.A., Kulchitsky, V.A., Simons, C.T., Sugimoto, N., 1998. Methodology of fever research: why are polyphasic fevers often thought to be biphasic? *Am. J. Physiol.* 275, R332-338.

Rudaya, A.Y., Steiner, A.A., Robbins, J.R., Dragic, A.S., Romanovsky, A.A., 2005.

Thermoregulatory responses to lipopolysaccharide in the mouse: dependence on the dose and ambient temperature. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 289, R1244-1252.

Saha, S., Engstrom, L., Mackerlova, L., Jakobsson, P.J., Blomqvist, A., 2005. Impaired febrile responses to immune challenge in mice deficient in microsomal prostaglandin E synthase-1. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 288, R1100-1107.

Thoren, S., Jakobsson, P.J., 2000. Coordinate up- and down-regulation of glutathione-dependent prostaglandin E synthase and cyclooxygenase-2 in A549 cells. Inhibition by NS-398 and leukotriene C4. *Eur. J. Biochem.* 267, 6428-6434.

Trebino, C.E., Stock, J.L., Gibbons, C.P., Naiman, B.M., Wachtmann, T.S., Umland, J.P., Pandher, K., Lapointe, J.M., Saha, S., Roach, M.L., Carter, D., Thomas, N.A., Durtschi, B.A., McNeish, J.D., Hambor, J.E., Jakobsson, P.J., Carty, T.J., Perez, J.R., Audoly, L.P., 2003. Impaired inflammatory and pain responses in mice lacking an inducible prostaglandin E synthase. *Proc. Natl. Acad. Sci. U S A* 100, 9044-9049.

Tripathy, D., Grammas, P., 2009. Acetaminophen protects brain endothelial cells against oxidative stress. *Microvasc. Res.* 77, 289-296.

Vane, J.R., 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat. New Biol.* 231, 232-235.

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.

Figure Legends

Figure 1

Figure 1. Febrile response to 2 μ g LPS ip (injected at time 0) in Cox-2 (A) and mPGES-1 (B) WT and Hz mice. *, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively, between LPS-injected WT and Hz mice. Temperature data for every 2 min are shown. Intraperitoneal injection is associated with a transient stress-induced hyperthermia (Romanovsky et al., 1998); the part of the diagram representing this phase is dampened. In (B) $P < 0.001$ between treatments for WT mice, and $P < 0.01$ between treatments for mPGES-1 Hz mice. $n = 8-9$ in (A), and $n = 7$ in the LPS-treated groups and $n = 4-5$ in the NaCl-treated groups in (B). n.s., not significant.

Figure 2

Figure 2. Gene expression in Cox-2 and mPGES-1 WT and Hz mice. A, Cox-1 protein and Cox-2 protein and mRNA expression in LPS-challenged Cox-2 WT (filled and unfilled blue bars) and Hz (filled and unfilled red bars) mice. B, Western blot showing expression of Cox-2 in WT and Cox-2 Hz mice. A Cox-2 knockout (KO) mouse was included as specificity control. Arrow points at two bands at approximately 72-74 kD (Muller-Decker, 1999). Note the reduced Cox-2 protein levels in Cox-2 Hz mice, and the absence of labeling in the Cox-2 KO mouse. C, Cox-2 and mPGES-1 mRNA levels in LPS-challenged mPGES-1 WT and Hz mice. *, **, *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. $n = 5-6$ in A, and 4-6 in C.

Figure 3

Figure 3. Acetaminophen treatment. A-B, Febrile response in Cox-2 WT and Hz mice given 50 mg/kg acetaminophen (Acet), followed 1 h later by 2 μ g LPS (A) or saline (B). * indicates $P < 0.05$ for the acetaminophen effect on LPS-injected Hz mice. n.s., not significant ($P = 0.6$) for the acetaminophen effect on LPS-injected WT mice. C, Differences in LPS-elicited peak fever between Cox-2 WT and HZ mice treated with/without acetaminophen. *** indicates $P < 0.001$ between treatments. D, Febrile response in mPGES-1 WT and Hz mice pre-treated with 50 mg/kg acetaminophen, followed 1 h later by 2 μ g LPS ip. E, Febrile response in WT mice pre-treated with 200 mg/kg acetaminophen or saline, followed 1 h later by 4 nmol PGE₂ intracerebroventricularly. $n = 7-9$ in A-C, 5-6 in D, and 3-4 in E.

Legend to Supplemental Figures

Supplemental Figure 1

A, B, mRNA expression of Cox-1, Cox-2, and mPGES-1 in the hypothalamus of wild-type (WT) and heterozygous (Hz) Cox-2 and mPGES-1 mice, 3 h after intraperitoneal injection of LPS (2 µg) or saline. Error bars show SEM. $n = 6$ in each group. ** indicates $P < 0.01$. C, Western blot showing Cox-1 expression in wild-type (WT) and Cox-2 heterozygous (Hz) mice 5 h after intraperitoneal injection of LPS or saline. No difference between genotypes was seen.

Supplemental Figure 2

Fever-reducing effect of different doses of acetaminophen. Wild-type mice were injected intraperitoneally with acetaminophen (acet), and 1 – 1.5 h later given LPS (2 µg), also intraperitoneally, at time 0 (the immediately following temperature peaks are elicited by the handling stress). Note that while 200 mg/kg of acetaminophen almost completely prevented the LPS-induced fever, 50 mg/kg had little effect, at least during the first hours after the LPS injection.

Supplemental Figure 3 Treatment with acetaminophen does not affect LPS-induced Cox-2 mRNA or protein expression in the brain. A, No induced Cox-2 immunoreactivity in blood vessel in control mice injected with a saline solution (NaCl). B, Cox-2 expression in cells (arrow) of the blood vessel 3 h after intraperitoneal injection of LPS (2 µg). C, Similar Cox-2 expression as in (B) in mice pretreated with 50 mg/kg acetaminophen (Acet) 1 h prior to the

LPS injection. Scale bar = 20 μm . D, Cell counts of the number of Cox-2 immunoreactive endothelial cells in a 5.3 mm^2 large microscopic field in the region of the hypothalamus 3 h after LPS injection (120 $\mu\text{g/kg}$ ip) in mice pretreated with 100 mg/kg acetaminophen or saline 1 h prior to the LPS injection. $n = 3$ in both groups. Error bar = SEM. E, Cox-2 mRNA levels in the hypothalamus 3 h after LPS injection (120 $\mu\text{g/kg}$ body weight) in mice pretreated with 100 mg/kg acetaminophen or saline 1 h prior to the LPS injection. $n = 6$ in each group. Error bar = SEM.

Supplemental Figure 1

Supplemental Figure 2

Supplemental Figure 3