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Acetaminophen reduces lipopolysaccharide-induced fever by inhibiting cyclooxygenase-2

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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 E_2 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 E_2.

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 non-steroidal 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 PGE_2 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

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

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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) (Morham et al., 1995) on a B6 background, and *Ptges*$^{+/-}$ mice (Trebino et al., 2003) on a DBA/1lacJ 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 PGE_2

Mice were mounted in a stereotaxic frame under anesthesia with 1% isoflurane in a 30/70% mixture of O_2/N_2, 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 PGE_2 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 CO_2. 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 Biotechnology, Santa Cruz, CA), 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 CO_2 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 h 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% ethylene 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 min, 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% H_2O_2 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-peroxidase complex (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 sulfate, and 0.01% H_2O_2 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 CO_2, 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 ± 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