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Acetaminophen: antipyretic or hypothermic in mice? In either case, PGHS-1b (COX-3) is irrelevant

Shuxin Li¹, Wenkai Dou^{2,*}, Ying Tang¹, Sarita Goorha², Leslie R. Ballou², and Clark M. Blatteis¹

¹Department of Physiology, College of Medicine, University of Tennessee Health Science Center, Memphis, TN 38163, USA

²Department of Medicine and Molecular Sciences, College of Medicine, University of Tennessee Health Science Center, Memphis, TN 38163, USA, and Research Service, Veterans Affairs Medical Center, Memphis, TN 38104, USA

Abstract

Acetaminophen (AC) reduces the core temperatures (T_c) of febrile and non-febrile mice alike. Evidence has been adduced that the selectively AC-sensitive PGHS isoform, PGHS-1b (COX-3), mediates these effects. PGHS-1b, however, has no catalytic potency in mice. To resolve this contradiction, AC was injected intravenously (iv) into conscious *PGHS-1* gene-sufficient (wild-type [WT]) and -deficient (*PGHS-1*^{-/-}) mice 60 min before or after pyrogen-free saline (PFS) or *E. coli* LPS (10 μ g/kg) iv. T_c was monitored continuously; brain and plasma PGE₂ levels were determined hourly. AC at <160 mg/kg did not affect T_c when given before PFS or LPS; at 160 mg/kg, it caused a ~2.5 °C T_c fall in 60 min. LPS given after AC (all doses) induced a ~1 °C fever, not different from that in AC-untreated mice. But this rise was insufficient to overcome the hypothermia of the 160 mg/kg-treated mice; their T_c culminated 1 °C below baseline. LPS given before AC similarly elevated T_c ~1 °C. This rise was reduced to baseline in 30 min by 80 mg AC/kg; T_c rebounded to its febrile level over the next 30 min. At 160 mg/kg, AC reduced T_c to 4 °C below baseline in 60 min, where it remained until the end of the experiment. WT and *PGHS-1*^{-/-} mice responded similarly to all the treatments. The basal brain and plasma PGE₂ levels of PFS mice and the elevated plasma levels of LPS mice were unchanged by AC at 160 mg/kg; but the latter's brain levels were reduced at 1 h, then recovered. Thus, AC could exert an anti-PGHS-2 effect when this enzyme is upregulated in the brain of febrile mice. The hypothermia it induces in non-febrile mice, therefore, is due to another mechanism. PGHS-1b is not involved in either case.

1. Introduction

According to current concepts, prostaglandin (PG)E₂ is believed to be the final fever mediator in the brain, specifically in the preoptic area of the anterior hypothalamus (POA), the fever-mediating locus [1]. It is produced by the conversion of arachidonic acid (AA) to PGE₂ catalyzed by prostaglandin H₂ synthase (PGHS)-2 (COX-2), the isoform of the enzyme specifically induced by propyretic agents. It is controversial, however, whether this PGE₂ is generated within the POA parenchyma or reaches it by passage across the blood-brain barrier

*Deceased (10/22/2006)

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(BBB) from extra-CNS sources [2–6]. The constitutive isoform of this enzyme, PGHS-1 (COX-1), is not believed to have a role in febrigenesis [2–6].

The catalytic mechanism of PGHS involves two steps occurring at distinct active sites within the enzyme, viz., the cyclooxygenase (COX) and the peroxidase (POX) sites. To abate a fever, nonsteroidal anti-inflammatory drugs (NSAIDs), which act by competing with AA non-selectively for the COX active site of both isozymes, are popularly used [7]. Acetaminophen (AC), which inhibits PGHS at its POX active site by reducing the higher oxidative states of the PGHS protein, thereby blocking the further progress of the catalytic cycle [8,9], is also widely used as an antipyretic drug, particularly because it is largely devoid of the untoward gastrotoxic effects of most NSAIDs [7]. Indeed, AC was shown long ago to reduce fever simultaneously with a decrease in the concentration of a PGE-like material in the cerebrospinal fluid of cats [10,11], thus suggesting, in the context of current concepts, the inhibition of PGHS-2 in the CNS. Yet AC shows no systemic anti-inflammatory activity, indicating no inhibition of peripheral PGHS-2, although several recent reports have indicated that it may selectively block PGHS-2 in certain cells under certain conditions [12–14]. It also only weakly inhibits peripheral PGHS-1. This is relevant because Kupffer cell-derived PGHS-1- and PGHS-2-dependent PGE₂ has recently been implicated as the peripheral trigger of the febrile response to bacterial endotoxic lipopolysaccharide (LPS), an exogenous pyrogen [15,16]. *A priori*, the mechanism of the antipyretic action of AC would thus appear to differ from that of the traditional NSAIDs. To account, therefore, for its effect, it was suggested that another fever-mediating PGE₂ catalyst, the recently discovered AC-sensitive PGHS isoform PGHS-1b (COX-3), a splicing variant of PGHS-1 retaining intron-1, could be involved [17–19].

AC has also been reported to induce hypothermia at therapeutic doses in non-febrile humans and mice, in the latter in correspondence with a reduction of brain PGE₂ levels [19]. PGHS-1b has also been invoked to account for this hypothermic effect. Thus, Ayoub et al. [19] suggested that a constant biosynthesis of PGE₂ may underlie the maintenance of T_c of mice kept at ambient temperatures (T_as) below (22 °C in the referenced study) their thermoneutral zone (32–34 °C), inferring that PGHS-1b could have an inherent role in the temperature regulation of this species. There exists, however, no substantive evidence in the literature that central PGE₂ participates as a matter of course in the control of normal T_c in any species [20,21].

To determine, therefore, whether the observed fall in T_c produced by AC in both non-febrile and febrile subjects is indeed mediated by similar or distinct mechanisms and whether PGHS-1b could a role, notwithstanding its probable lack of catalytic activity [22], we compared the effects of AC on the thermal responses and plasma and brain PGE₂ levels of PGHS-1^{+/+} [wild-type, WT] and PGHS-1^{-/-} mice to the intravenous (iv) injection of pyrogen-free saline (PFS) or LPS.

2. Materials and methods

2.1. Animals

Adult, wild-type, PGHS-1-sufficient ([WT] C57BL/6J, PGHS-1^{+/+} [COX-1^{+/+}]) mice and mice with congenital deletions of the *Pgbs-1* gene (PGHS-1^{-/-} [COX-1^{-/-}]) were used in these experiments; all the animals weighed 25–30 g during the study. The WT mice were purchased from Jackson Laboratories, Bar Harbor, ME; the PGHS-1^{-/-} mice were provided by one of us (LRB); the targeted gene deletions of the knockout mice were verified using standard PCR techniques. All the PGHS-1^{-/-} mice appeared to be clinically healthy and thriving under the conditions of our normal laboratory housing.

Following receipt, the animals were quarantined for 3 weeks, four to a cage, before any experimental use. Tap water and food (Agway Prolab® mouse diet) were available ad libitum.

The T_a in the animal room was $23 \pm 1^\circ\text{C}$, in accord with the Institute of Laboratory Animal Resources' "Guide for the Care and Use of Laboratory Animals" [23]; light and darkness alternated, with light on from 0600 to 1800 h. After quarantine, to moderate the psychological stress associated with the experiments, all the mice were habituated to the experimental procedures 4 h daily for at least two weeks by handling, colonic probe insertion, and placement in individual, locally fabricated, 12×3.5 cm semi-cylindrical, wire-mesh confiners. These were configured to simulate mouse holes and designed to prevent their turning around and minimize forward and backward movements, but without causing restraint stress. The front of the confiners was tapered to accommodate the mice's snout; the rear (entry port) was closed by a removable, fitted, plastic cap with a hole in its middle to allow the passage of the tail and a thermocouple wire. To obviate possible effects of circadian variations, all the experiments were begun at the same time of day (0830). All animal protocols were approved by the University of Tennessee Health Science Center Animal Care and Use Committee and fully conform to the standards established by the US Animal Welfare Act and by the documents entitled "Guiding Principles for Research Involving Animals and Human Beings" [24].

2.2. Drugs

LPS was *Escherichia coli*, serotype 0111:B4 (lot #36F4019, prepared by trichloroacetic acid extraction; protein content, 0.5%; Sigma-Aldrich, St. Louis, MO); $10 \mu\text{g}$ of this pyrogen/kg body weight ($\sim 0.25 \mu\text{g}/\text{mouse}$) were administered in all cases. AC (N-acetyl-p-aminophenol) was also purchased from Sigma-Aldrich; different doses of this putative antipyretic were tested. The vehicle for LPS was pyrogen-free saline (PFS) and for AC 3% dimethylsulfoxide (DMSO). All glassware and plasticware used in the preparation of these solutions were sterilized by autoclaving, and electrochemical-grade, high-purity-water (Baxter Healthcare, Muskegan, MI) was used exclusively. Before use, the stock solutions were passed through a sterile $0.22\text{-}\mu\text{m}$ Miller-GS filter unit (Millipore, Bedford, MA) as an added precaution against bacterial contamination. Absence of endotoxic contamination in all fluids not containing LPS by design was verified by the Limulus amoebocyte lysate test (Pyrochrome®; Associates of Cape Cod, Falmouth, MA).

2.3. Drug injections

On the experimental day, the previously trained, fully conscious mice were connected to the relevant measuring devices, placed in their confiners, and allowed to stabilize for at least 3 h until their T_c varied not more than $\pm 0.1^\circ\text{C}$ over five consecutive 2-min periods. Treatment began after thermal stabilization was achieved. With the mice in their confiners and their tails extending outside the confiners, 0.1 ml of the desired drug or its vehicle was injected into a tail vein with a sterile $30\text{G} \times 1/2$ needle and tuberculin syringe.

2.4. Temperature measurements

The animals, fully conscious in their confiners, were placed under a plastic hood (free convection through open ports) to prevent undue disturbances from noise and fluctuations in T_a $23 \pm 1^\circ\text{C}$, their habitual housing T_a . It was chosen in preference to the mice's thermoneutral zone ($26 - 34^\circ\text{C}$) to obviate the several hours-long hyperthermia that acute exposure to this T_a induces in mice not previously acclimatized to it [25]; this T_a was also previously used in different studies by us [26] and others [27]. The T_{cs} of the mice were monitored constantly and recorded at 2-min intervals for the duration of the experiments on a Macintosh Plus 1 Mb computer through an analog-to-digital converter, using precalibrated copper-constantan thermocouples inserted 2 cm into the colon and taped to the tail. The data were displayed on a video monitor, printed digitally on an ImageWriter® printer, and stored on a disk for subsequent statistical analysis. Control measurements were begun when the animals' T_{cs} had become stabilized, as described above. The treatment pertinent to a given experiment was then

administered and T_c recorded for 1 h according to the same routine as before treatment. At that point, the second treatment was administered in the same manner as the first and the measurements continued for another 3 h.

2.5. PGE₂ assay

2.5.1 Extraction of PGE₂ from brain and plasma—The procedure was modified from that of Powell [28]. Briefly, fresh brain tissue pieces were immediately frozen in liquid N₂ and pulverised with a N₂ bomb (Biospec Products, Bartlesville, OK). One ml of 15% (v/v) ethanol in distilled water (pH 3) was added and the homogenates incubated at 4 °C for 10 min, then centrifuged at 375 × g for another 10 min also at 4 °C. Columns (C-18 Sep-Pak cartridges) were conditioned with, successively, 4 ml of 15% ethanol and 4 ml of distilled water at a flow rate of 5–10 ml/min; the supernatant from the homogenates was then applied to these columns at a flow rate of 5 ml/min.

Freshly collected blood was promptly centrifuged and the plasma samples were similarly applied to conditioned columns. The columns were then washed in 4 ml of distilled water again followed by 4 ml of 15% ethanol in distilled water; the samples eluted with 2 ml of ethyl acetate at a flow rate of 5 ml/min. They were freeze-dried overnight, then stored at –80 °C until assayed.

2.5.2. PGE₂ immunoassay—Brain and plasma PGE₂ levels were evaluated using a commercial enzyme immunoassay kit from Amersham Pharmacia, according to the manufacturer's protocol. Briefly, the extracted PGE₂ was incubated on a goat anti-mouse IgG-coated plate along with anti-PGE₂ antibody and horseradish peroxidase-labeled PGE₂. A blue color was developed with 3,3',5,5'-tetramethylbenzidine substrate and the optical density read colometrically at 630 nm. The concentration of PGE₂ in the samples was determined by reference of the optical density of PGE₂ in the samples to that of a standard PGE₂ curve (0.05 – 6.4 ng/ml).

2.6. Experimental design

2.6.1. Experiment 1. Effect of AC on the T_c of WT (PGHS-1^{+/+}) and PGHS-1^{-/-} mice—To verify initially the reported dose-dependent hypothermic effect of AC in mice, four doses of the drug (80, 120, 140, and 160 mg/kg body weight in 0.1 ml of 3% DMSO/mouse) or its vehicle (3% DMSO, 0.1 ml/mouse) were injected into the tail vein of conscious WT [PGHS-1^{+/+}] and PGHS-1^{-/-} [COX-1^{-/-}] mice. PFS (0.1 ml/mouse) was the null solution for DMSO. It was also administered 1 h later, as the control solution for LPS in the following experiment.

2.6.2. Experiment 2. Effect of AC pretreatment on the febrile response to LPS of WT mice—To determine whether prophylactic AC affects the development of fever induced by LPS, four doses of AC (80, 120, 140, and 160 mg/kg body weight) or its vehicle (3% DMSO) were injected into conscious WT mice 1 h before LPS (10 µg/kg [0.25 µg/mouse]) or its vehicle (PFS). In this protocol, PFS was also the control injection for DMSO.

2.6.3. Experiment 3. Effect of AC on the course of the febrile response to LPS of WT mice—To examine the effect of AC on an on-going fever induced by LPS, 80 or 160 mg of AC/kg or DMSO was injected iv into WT mice 1 h after the iv injection of LPS or PFS. In this experiment, PFS was also the null solution for DMSO.

2.6.4. Experiment 4. Effect of AC on the course of the febrile response to LPS of PGHS-1^{-/-} mice—To establish whether PGHS-1b is the target of the putative antipyretic action of AC, 80 mg of AC/kg or DMSO was injected iv into PGHS-1^{-/-} mice 1 h after the iv injection of LPS or PFS.

2.6.5. Experiment 5. Effect of AC on the plasma and brain PGE₂ levels of non-febrile and febrile WT mice—Two protocols were followed, using 3–4 WT mice per time point.

Blood was withdrawn by cardiac puncture and the brains quickly removed, and PGE₂ extracted thereafter. Deep ketamine-xylazine (50–50 mg/kg in 0.3 ml of PFS/mouse, ip) anesthesia was induced immediately before sampling.

2.6.5.1. Protocol A: AC (160 mg/kg) or DMSO was administered iv. Plasma and brain samples were obtained before (0 time) and hourly for 3 h after this treatment.

2.6.5.2. Protocol B: Plasma and brain samples were collected before (0 time) and 1 and 2 h after the injection of PFS or LPS. Immediately following the latter collection, AC (160 mg/kg) or DMSO was injected iv and samples obtained 1 and 2 h after this second treatment.

2.7. Statistical analyses

The results are presented herein as means $\pm$ SE. The values of T_c are reported as the changes from basal values (T_{ci} [initial], the T_c at 2-min intervals averaged over the last 10 min of the preceding 3-h stabilization period). Student's unpaired *t*-test was used to compare the maximal response to two different treatments (e.g., PFS vs LPS, AC vs DMSO) at the same time point. Student's paired *t*-test was used to compare pre- (basal) and post- (the maximal [LPS] or minimal [AC]) treatment data within a treatment. Differences between treatments were evaluated by a two-factors repeated-measures analysis of variance model (Microsoft Excel Analysis ToolPak), where factor 1 was the between-groups factor (the experimental treatment) and factor 2 the within-subject factor (the different sampling periods). Each variable was considered to be independent. The 5% level of probability was accepted as statistically significant.

3. Results

3.1. Experiment 1

The injection of PFS or DMSO promptly induced in all the mice transient, ca. 0.6 °C rises in T_c ; this effect is a characteristic response of rodents to the manipulations associated with invasive techniques (handling stress hyperthermia [29]). All the T_c s returned to their initial levels within 60 min (Fig. 1A and Fig. 2A). Identical hyperthermic responses were produced by the injection of AC at doses <160 mg/kg (Fig. 1B, 1C, and Fig. 2B). The decline of the T_c s after their peak, however, stabilized in all cases ca. 0.5 °C below their original values (Figs. 1B and 1C). The injection of PFS 60 min after AC again produced temporary, small T_c rises associated with the handling procedure. Handling stress hyperthermia, however, did not occur in both WT [PGHS-1^{+/+}] and PGHS-1^{-/-} [COX-1^{-/-}] mice treated with AC at 160 mg/kg (Figs. 1B and 1C). Instead, this dose provoked within 10 min a profound (2–3 °C) fall in the T_c s of both groups of mice (Fig. 1B, 1C, and Fig. 2B); the lowest point was reached in 1 h. It was not affected by the injection of PFS 60 min after AC and persisted essentially unchanged over the following 3 h, when the experiment was ended (Figs. 1B and 1C). An apparently smaller hypothermic response to AC by the PGHS-1^{-/-} than the WT mice (Figs. 1B and 1C) was not statistically significant.

3.2. Experiment 2

LPS administered to WT mice 60 min after PFS or DMSO, i.e., after the animals had recovered from their handling stress hyperthermia, elevated T_c further than the handling stress-associated rise *per se*, to a febrile maximum of ~1 °C; it was reached 30 min after the injection. T_c remained at this level for ca. 2 h, then slowly decreased toward its pre-LPS level (Fig. 2A).

LPS similarly administered 60 min after AC at doses of 80, 120, and 140 mg/kg, i.e., at the nadir of the AC-induced hypothermia, raised T_c by amounts not different than the febrile rises it induced in its AC-untreated counterparts. It also culminated in ~30 min, stabilized at this level for ca. 2 h, then began to decrease toward its pre-LPS value (Fig. 2B). Thus, fevers of the dose-commensurate height (~1 °C) developed and abated normally in all these AC-pretreated mice.

LPS injected 60 min after AC at 160 mg/kg caused a similar ~1 °C rise of T_c above its hypothermic level. It reached its peak 45 min later and remained at this level until the conclusion of the experiment. This rise, however, did not return the T_c s of these more profoundly hypothermic mice to their pre-LPS levels so that they remained hypothermic throughout the duration of this experiment (Fig. 2B, closed circles). In view of the definitive nature of these results, we considered it unnecessary to replicate them in scarce PGHS-1^{-/-} mice.

3.3. Experiment 3

The thermal responses of WT mice to PFS injected at time 0 and to PFS, DMSO or AC injected 60 min later were similar to those described in Experiment 1 (Fig. 1A and B). LPS injected at time 0 induced its prototypic ~1°C fever. As in Experiment 2 (Fig. 2A), it continued at this level for ca. 3 h before breaking, and the injection of PFS or DMSO 60 min later had no effect on this febrile course (Fig. 3B). On the other hand, the administration of 80 mg of AC/kg at this time produced, after a brief delay, a ~2 °C T_c fall, attained 45 min later. T_c recovered at approximately the same rate as that at which it had decreased, returning to the same febrile level as that of its AC-untreated counterparts 90 min after AC (Fig. 3B, closed circles). AC at 160 mg/kg given 60 min after LPS caused immediately a far greater and more rapid T_c fall. It reached its nadir of 5 °C below its febrile level 60 min after AC, then recovered a statistically insignificant 0.8 °C over the remainder of the experimental period (Fig. 3B, closed triangles).

3.4. Experiment 4

The T_c s of PGHS-1^{-/-} mice treated with PFS at time 0 and again 60 min later exhibited the characteristic, transient rises associated with handling stress already described (Experiment 1). Eighty mg/kg of AC given 60 min after PFS caused a ~0.5 °C T_c fall similar to that in WT mice (Fig 3A). LPS given at time 0 also induced a febrile response similar to that in WT mice (Fig. 3B). PFS injected 60 min after LPS further increased the T_c slightly (superimposed handling stress hyperthermia). The fever lasted about 150 min, then gradually abated over the following 90 min. Eighty mg/kg of AC administered 60 min after LPS depressed T_c immediately from its febrile level. It fell to its nadir ~1.5 °C from its peak ~40 min after AC, then recovered at approximately the same rate as that at which it had declined to the febrile level of its PFS-treated counterparts 90 min after AC (Fig 4B). The response patterns of these PGHS-1^{-/-} mice were not significantly different from their WT (PGHS-1^{+/+}) controls (Fig. 3). In view of the definitive nature of these results, we considered it unnecessary to test higher doses of AC.

3.5. Experiment 5

Protocol A—Neither DMSO nor AC at 160 mg/kg significantly affected the brain and plasma levels of PGE₂ of non-febrile WT mice 1, 2, or 3 h after administration (Fig. 5; DMSO data not illustrated). In view of this absence of effects, PGHS-1^{-/-} animals were not tested.

Protocol B—The injection of PFS had no significant effect on the plasma and brain PGE₂ levels of these WT mice (Fig. 6A and B, open circles). The injection of LPS, on the other hand, induced significant rises of both the plasma and brain PGE₂ levels 1 and 2 h after its administration (Fig. 6A and B, closed circles). AC or DMSO given to the febrile mice 2 h after LPS had no demonstrable effect on their elevated plasma PGE₂ level for 1 h; however,

PGE₂ declined toward its basal value during the second hour (Fig. 6A, closed circles; DMSO data not shown). By contrast, AC caused the immediate fall of the elevated brain PGE₂ level of these mice to its basal value within 1 h; PGE₂ then rebounded to its pre-AC high level within the next hour (Fig. 6B, closed circles). Neither DMSO nor AC given 2 h after PFS affected the plasma and brain PGE₂ levels of these mice (Fig. 6A and B, open circles; DMSO not shown). Again, in view of the consistency of these results, we considered it unnecessary to replicate them in scarce PGHS-1^{-/-} animals.

4. Discussion

Although AC has been shown to inhibit the activities of both PGHS-1 and PGHS-2 *in vitro*, it does so only in certain isolated tissues and cells. Its inhibitory effect on PG synthesis, moreover, is more potent in intact than in homogenized cells and under conditions of low rather than high AA availability [8,12]. AC has also been reported to reduce PG production *in vivo*, but only at certain sites [30]. Thus, while its antipyretic potency suggests inhibition of inducible PGHS-2 in the CNS, its low anti-inflammatory activity indicates weak inhibition of peripheral inducible PGHS-2. It is also poorly effective on platelets, indicating weak inhibition of peripheral PGHS-1. It does, however, reduce the urinary levels of 2,3-dinor-6-keto-PGF_{1α}, an *in vivo* metabolite of PGI₂ in humans, suggesting inhibition of PGHS within blood vessels [31]. Although PGHS-2 is usually not expressed in healthy blood vessels [32], the induction of PGHS-2 mRNA under inflammatory conditions has been demonstrated in rodent venular endothelial cells throughout the brain microvasculature [33–35]. In agreement with the previously reported weak peripheral anti-PGHS-2 activity of AC *in vivo* [30], the finding in the present study that the LPS-induced rise of plasma PGE₂ was unaffected by AC treatment indicates that the upregulation of PGHS-2 in peripheral cells implicated as sources of febrigenic PGE₂, specifically Kupffer cells and venular endothelial cells of the cerebral microvasculature [5,15,16], progressed without impediment. Taken together, therefore, these results would infer that neither constitutive PGHS-1 nor inducible PGHS-2 in the periphery could be the targets of AC. By deduction, therefore, the antipyretic target of AC should be PGHS-2 in the POA.

Indeed, in the present study, when given 1 h before LPS, AC reduced the mice's T_c, the extent and duration of the fall depending on the dose; but it did not prevent the subsequent, LPS-induced T_c and associated plasma and brain PGE₂ rises from their then existing levels to their usual, dose-related height, identically in both WT and PGHS-1^{-/-} mice. AC would thus appear to lack the ability to inhibit the rapid LPS-induced, complement C5a-mediated activation of Kupffer cell constitutive PGHS-1 and -2 [2,15,36] and, hence, the ability to act as a prophylactic antipyretic. But when given 1 h after LPS, it again reduced the T_cs of both the WT and PGHS-1^{-/-} mice from their febrile level to the same dose-dependent extent and duration as when given before LPS. However, in this case, it also transiently reduced the fever-associated rise of brain PGE₂, although it did not affect that of plasma. AC thus suppressed brain PGE₂ release at a time point (~2 h, i.e., the onset of the second phase of fever [37,38]) associated with iv LPS-induced PGHS-2 activation in microglia and/or cerebral microvessels [32–35]. Supporting the likelihood that brain PGHS-2 is the target of AC under these conditions are previous findings that it inhibits the activity of PGHS-2 in rat brain microglial cultures [39, 40] and that it and a selective PGHS-2 inhibitor, NS398, are equally effective in inhibiting dose-dependently LPS-induced PGE₂ production in the cerebral microvasculature [41]. Since, by contrast, PGHS-2 presumptively was not upregulated in the present non-febrile mice, AC had no demonstrable effect on these animals' plasma and brain PGE₂ levels. It would thus appear that AC may be acting selectively against PGHS-2 only when the latter is elevated, as in the POA of the present febrile animals, and so for only a relatively short, albeit dose-dependent, time.

Several mechanisms for the anti-PGHS-2 property of AC have been adduced, but none is as yet definitive. Thus, a direct action of AC was inferred from the finding that it is metabolized in the brain to N-arachidonoylphenolamine, which was demonstrated to inhibit PGHS-1 and PGHS-2 [42]; this conversion is minimal in peripheral tissues. A diclofenac-inducible variant form of PGHS-2 sensitive to inhibition by low concentrations of AC has also been described in cultured mouse macrophages [43]. This variant was more sensitive to AC inhibition than LPS-induced PGHS-2 and, in contrast to LPS-induced membrane-bound PGHS-2, this PGHS-2 existed in cytoplasm. The characteristics of this PGHS-2 variant have, however, not been explored further, to our knowledge. It is unclear whether it also exists in brain microglia, the PGHS-2 of which is activated not by LPS, but secondarily by norepinephrine [37]. It has also been suggested that the action of AC could be related to the production of reactive metabolites by the POX function of PGHS-2, which could deplete glutathione, a co-factor of mPGES [44]. Finally, it was proposed most recently that AC inhibits PG synthesis by scavenging the PGHS-activator peroxynitrite [45], consistent with the weak ability of AC to inhibit PGHS in the presence of high peroxide levels, as in inflamed tissue, but its higher ability when the local concentration of hydroperoxides is naturally low, as in brain tissue [8,12,14].

Because AC is an efficacious antipyretic, yet a weak inhibitor of PGHS-2, the PGHS isozyme induced by pyrogens, it was suggested by Ayoub et al. [19] that a fever-mediating, AC selectively sensitive PGHS isoform, PGHS-3 (COX-3), is its alternate therapeutic target. In support, they noted that 1) AC reportedly readily crosses the blood-brain barrier [46–50], rapidly depletes brain PGHS-3 mRNA [51] and reduces CSF and brain PGE₂ levels [19,52, 53], 2) the structurally similar, presumptively selective PGHS-3 inhibitors aminopyrine and antipyrine also cause dose-dependent T_c falls in rats and mice [17,54], and 3) AC injected intracerebroventricularly promptly induces the fall of T_c [55]. It is now clear that PGHS-3 is a splicing variant of PGHS-1 retaining intron-1 (PGHS-1b). The retention of intron 1 in rodent PGHS-1 introduces an insertion at the amino terminus of PGHS-1 mRNA, resulting in a shift in the reading frame that yields a prematurely terminated, truncated protein [56,57] and very likely precludes its having any functional activity. Hence, explaining the antipyretic action of AC on the basis of its inhibition of central PGHS-1b is tenuous. Indeed, the recently cloned rat and mouse PGHS-1b proteins do not possess PGHS activity [22,58]. Similarly, the finding that the present febrile PGHS-1^{+/+} and PGHS-1^{-/-} mice reacted to AC identically corroborates the previously well-established view that PGHS-1 is not involved in febrigenesis [33] and, hence, expands it to include PGHS-1b. For that matter, PGHS-1^{-/-} mice do not express the *Pghs-1b* gene [22].

An entirely different mode of action was proposed by Huang et al. [59], viz., that the antipyretic effect of AC may be achieved by its inhibiting the release of hypothalamic glutamate; the activation by peripheral pyrogens of glutaminergic pathways in the brain has been demonstrated [60–63]. In a series of studies [64–66], these workers showed in rabbits that the consequently glutamate-induced elevated hypothalamic levels of 2,3-dihydroxybenzoic acid, indicative of hydroxyl radical generation, were antagonized by pretreatment with antioxidants or N-methyl-D-aspartate (NMDA, the ionotropic receptor for glutamate) receptor antagonists. They further showed that the increased levels of hypothalamic PGE₂ induced by iv LPS were similarly suppressed by these pretreatments and suggested, therefore, that an NMDA-receptor-dependent pathway in the hypothalamus could mediate LPS fever. Indeed, it had been shown earlier in rats that the blockade of NMDA receptors by its antagonist, dizocilpine, injected ip significantly reduced LPS-induced *c-fos* expression in the paraventricular nucleus, supraoptic nucleus, and the A1/A2 regions of the nucleus tractus solitarius (NTS) [62]. Similarly, the inhibition of LPS-induced central glutamate release by tetrodotoxin microdialyzed into the NTS significantly attenuated all acute-phase responses to LPS [61]. A2 neurons, in particular, are synaptically connected to vagal afferents and project to the POA via the ventral noradrenergic bundle, leading to the induction of fever [2,15,67]. Other related sites containing

glutamate receptors are the dorsomedial hypothalamus, whence autonomic premotors to thermoeffectors originate, and the rostral raphe pallidus, which relays these sympathoexcitatory signals downstream to their thermogenic and vasomotor targets [68]. All these regions also express PGE₂ EP₃ receptors, which are critical for the febrile response [69,70]. It should be noted in this regard that AC was administered iv in the present study and that, in view of its ready penetration into the brain, it is likely that it was distributed quite widely, reaching these other brain regions; its possible action at these sites could therefore also have contributed to its inhibition of the LPS-induced preoptic PGE₂ and T_c rises. It is thus not a given that AC's sole brain site of antipyretic action is the POA.

The AC-induced hypothermia recorded in this study is not a new observation. It was reported previously in mice [19,55,71] and humans [72–75]. As in this study, the extent and duration of the T_c fall in all the previous instances were dose-dependent, i.e., an acute effect that approximately coincided in time with the reported half-life of AC in plasma [76]. It was suggested by Ayoub et al. [19] that the hypothermic action of AC could also be mediated by its selective inhibition of PGHS-1b in the hypothalamus. To explicate the phenomenon, these workers speculated that a constant biosynthesis of PGE₂ underlies the maintenance of T_c of mice kept at T_as below (22 °C in their study) their thermoneutral zone (32–34 °C). There exists, however, no substantive evidence in the literature that PGE₂ participates as a matter of course in the control of normal T_c [20,21]. Indeed, contrary to Ayoub et al.'s study [19], the hypothermia of the non-febrile mice in the present study was not paralleled by a significant, similarly transient decrease in brain or plasma PGE₂. Since, in the present study, AC lowered non-differentially the T_cs of febrile and non-febrile mice to the same extent, it would appear that its hypothermic activity is distinct from its anti-PGHS-2, antipyretic activity.

As mentioned earlier, based on their findings that pretreatment with antioxidants antagonized the elevation of hypothalamic hydroxyl radicals generated by the release of glutamate, Huang et al. [59] suggested that the antipyretic activity of antioxidants could be due to their reduction of the thiol groups attached to NMDA, thereby depressing glutamate-mediated neuronal excitability and, hence, limiting fever. While it is currently controversial whether central NMDA or non-NMDA (metabotropic glutamate receptors) mediate the febrile response to LPS [63,68], the fact that PGE₂ production is inhibited and fever prevented when the production of free radicals is blocked has been demonstrated previously [77–81]. AC, as a phenolic compound with antioxidant potency, is an effective scavenger of free radicals [45,82]. It inhibits, for example, lipid peroxidation and superoxide anion generation in quinolinic acid-stimulated rat hippocampus in vivo [82]. It is therefore interesting to speculate that this mechanism could also account for the T_c fall caused by AC in mice when central PGE₂ is not elevated. i.e., in non-febrile conditions. In support, dithiothreitol (DTT), a sulphydryl-reducing agent that modulates the redox state of NMDA receptors, produces dose-dependent, transient T_c falls in rats maintained at T_a 20–22 °C very similar to those caused by AC in the present study by releasing the full autonomic pattern of heat loss effector responses [79,80,84]. Thiol reductants like DTT have been demonstrated previously to depress NMDA-induced responses [85]. Thus, it may be speculated that the hypothermic effect of AC in non-febrile states could be due to the anti-glutamate and antioxidant activities of this phenolic compound. More studies will be required to determine whether these molecular mechanisms, in fact, account for the T_c falls caused by AC under these conditions.

Another possible mechanism of the hypothermic action of AC could be related to the reported elevation of norepinephrine levels in the forebrain following its systemic administration [86, 87]. This neurotransmitter has been demonstrated to evoke an initial, transitory fall in T_c by its action on α₂-adrenoceptors when microdialyzed into the POA [88], the temporal characteristics of which are similar to the effect of AC observed in the present study. Verification of the role of this potential mechanism also awaits further study.

Because of their large surface area-to-mass ratio, mice maintain a high metabolic (i.e., thermogenic) rate in order to compensate for the continuous, large heat loss from their shell that occurs at prevailing room T_{as} . To help preserve heat balance under these conditions, the skin of the tail (which serves as a variable heat exchanger depending on its blood flow) is ordinarily in a vasoconstrictive state. It is likely, therefore, that the fall in T_c produced by AC in non-febrile and febrile subjects alike in this and previous studies [19,51,71–75] was more the result of an inhibitory action by this drug on thermogenesis than on the tail's cutaneous circulation. Indeed, the absence of any change in plasma PGE_2 in both the non-febrile and febrile mice after AC and the previously reported insensitivity of peripheral PGHS-2 to inhibition by AC [8] would preclude a direct vasodilatory action by PGE_2 (and presumably also by other PGHS-2-derived prostanoids with vascular effects) as accounting for the antipyretic and/or hypothermic response. In rodents, heat production is primarily dependent on brown adipose tissue (BAT) stimulated by norepinephrine released from the sympathetic nervous system [89]. PGE_2 released in the POA acting on EP_3 receptors on warm-sensitive neurons (which drive heat loss) inhibits these, thus eliciting a marked increase in BAT sympathetic nerve activity and BAT thermogenesis [90,91] via the pathways mentioned earlier. Similarly, the blockade by AC of other interconnected regions that comprise the circuitry controlling the sympathetic outflow to BAT could interrupt this thermogenic pathway and, consequently, result in the fall of T_c .

In conclusion, the present results indicate that AC does not prevent the occurrence of the dose-commensurate T_c rise induced by 10 μ g of *E. coli* LPS/kg injected iv into mice. Rather, it provokes a fall of T_c from its pre-existing level that is AC dose-dependent and identical in magnitude and duration in WT (PGHS-1^{+/+}) and PGHS-1^{-/-} mice, regardless of whether it is administered before or after fever has developed; i.e., AC exerts a dose-related hypothermic action that is not different in non-febrile and febrile animals and thus is independent of the initial T_c . Since AC depressed both the elevated T_c and brain PGE_2 levels induced in these mice by LPS, it would appear to exhibit some anti-PGHS-2, i.e., antipyretic activity when PGHS-2 expression is upregulated. Alternative or cooperative mechanisms could involve the reported inhibition by AC of the LPS-induced release of hypothalamic glutamate and/or the excitation of NMDA receptors as well as its own antioxidant activity. Since neither the plasma nor brain PGE_2 levels of the present non-febrile mice were changed by AC, the hypothermic effect it exerts is probably not due to its PGHS-2 inhibiting activity. We speculate that it could be due to the anti-glutamate and antioxidant activities of this phenolic compound. The AC-induced release of norepinephrine in the POA could also be a factor. Finally, since the observed responses were identical in PGHS-1^{+/+} and PGHS-1^{-/-} mice, neither its antipyretic nor its hypothermic activity could be mediated by PGHS-1 or PGHS-1b (COX-3). The present results thus corroborate previous data showing that PGHS-1b does not play a role in the formation of pyrogenic PGE_2 in mice, consistent with the view that this splice variant has little, if any, catalytic activity in this species.

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Fig. 1.

Effects of PFS (0.1 ml/mouse, iv), 3% DMSO (0.1 ml/mouse, iv; panels **A** and **C**), and AC (140 and 160 mg/kg in 0.1 ml/mouse, iv; panels **B** and **C**) on the 4-h course of the T_c s of conscious WT [COX-1^{+/+}] (**A** and **B**) and COX-1^{-/-} (**C**) mice. DMSO is the vehicle of AC and PFS the solvent of DMSO. PFS is also the solvent of LPS; it was administered 1 h after these treatments. These results are thus the control data for the next experiment (Fig. 2). The T_c s are expressed as differences (ΔT_c) relative to their initial levels (T_{ci} : average of the T_c over the last 10 min before the injection of PFS, DMSO, or AC). The values are means $\pm$ S.E.; (n) = number of animals.

Fig. 2.

A. Effect of 3% DMSO (0.1 ml/mouse, iv) or PFS (0.1 ml/mouse, iv) on the 4-h course of the febrile response of conscious WT mice to LPS (10 μg /kg [0.25 μg /mouse] in 0.1 ml of PFS/mouse, iv), administered 1 h after the initial treatment. **B.** Effect of four doses of AC (80, 120, 140, and 160 mg/kg in 0.1 ml of 3% DMSO/mouse, iv) on the 4-h course of the febrile response of conscious WT mice to LPS (0.25 μg /mouse, iv), administered 1 h after AC. Conventions and abbreviations as in Fig. 1.

Fig. 3.

Effect of AC (80 and 160 mg/kg in 0.1 ml of 3% DMSO/kg, iv), its vehicle (3% DMSO, 0.1 ml/mouse, iv), or PFS (the solvent of the latter, 0.1 ml/mouse, iv) on the 4-h course of the thermal response of conscious WT mice to PFS (0.1 ml/mouse, iv; **A**) or LPS (0.25 μg /mouse in 0.1 ml of PFS/mouse, iv; **B**), administered 1 h earlier. Conventions and abbreviations as in Fig. 1.

Fig 4.

A. Effect of AC (80 mg/kg in 0.1 ml of 3% DMSO/mouse, iv) or PFS (the solvent of DMSO, 0.1 ml/mouse, iv) on the 4-h course of the T_c of conscious COX-1^{-/-} mice, administered 1 h after PFS (the solvent of LPS, 0.1 ml/kg, iv). **B.** Effect of AC (80 mg/kg in 0.1 ml of 3% DMSO/mouse, iv) or PFS (the solvent of DMSO, 0.1 ml/mouse, iv) on the 4-h course of the febrile response of conscious COX-1^{-/-} mice to LPS (0.25 µg/mouse in 0.1 ml of PFS/mouse, iv), administered 1 h after LPS. Conventions and abbreviations as in Fig. 1.

Fig 5. Effects of AC (160 mg/kg in 0.1 ml of 3% DMSO/mouse, iv) on the plasma and brain PGE₂ levels of WT mice. The samples were collected before a treatment (0 time) and at hourly intervals for 3 h thereafter.

Fig. 6.

Effect of PFS (0.1 ml/mouse, iv), LPS (0.25 μ g/mouse in 0.1 ml of PFS/mouse, iv), 3% DMSO (0.1 ml/mouse, iv), or AC (160 mg/kg in 0.1 ml of 3% DMSO/mouse, iv) on the plasma (**A**) and brain (**B**) PGE₂ levels of WT mice. The samples were collected before a treatment (0 time) and at hourly intervals for 4 h thereafter. The DMSO and AC were injected 2 h after PFS or LPS. The DMSO results are not illustrated.