Role of cytochrome P-450 in endogenous antipyresis

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Kozak, Wieslaw, Matthew J. Kluger, Anna Kozak, Maciej Wachulec, and Karol Dokladny. Role of cytochrome P-450 in endogenous antipyresis. Am J Physiol Regulatory Integrative Comp Physiol 279: R455–R460, 2000.—In previous reports, we (15, 18) and others (29) demonstrated data showing that various inhibitors of cytochrome P-450/epoxygenase augment fever in rats and mice, indicating that the enzyme may be involved in endogenous antipyresis. The aim of this study was to further test the hypothesis that the P-450-dependent epoxygenase pathway of arachidonic acid is part of the homeostatic system to control the height of fever. Sprague-Dawley rats were implanted with biotelemeters to monitor body temperature. Fever was induced by intraperitoneal injection of lipopolysaccharide (LPS; 80 μg/kg). We demonstrate that intraperitoneal administration of P-450 inducers (bezafibrate and dehydroepiandrosterone, 10 and 100 mg/kg) before LPS reduced fever in rats in a dose-dependent manner. In complementary experiments, rats were implanted with brain cannulas in addition to the biotelemeters. Various isomers of epoxyeicosanoids were administered into the lateral ventricle at doses of 0.01 to 10 μg/rat to test their influence on LPS-induced fever in rats. Four of five isomers were antipyretic in a dose-dependent manner. The most potent antipyretic isomers were 11,12-epoxyeicosatrienoic acid (EET) followed by 14,15-EET, 8,9-EET, and 12(R)-hydroxyeicosatetraenoic acid. These data support the hypothesis that the cytochrome P-450/epoxygenase pathway of arachidonate metabolism is part of the endogenous antipyretic system.

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FEVER IS AN UPWARD RESETTING of the set point of thermoregulation, presumably in the preoptic-anterior hypothalamus. Investigators and clinicians have long recognized that fever has an upper limit, indicating that resetting the thermoregulatory set point upward during disease is controlled, and hence the increase of body temperature is carefully guarded from reaching a dangerously high level (5). This resulted in the hypothesis of endogenous antipyresis (23). After administration of lipopolysaccharide (LPS; a component of the endotoxin extracted from the cell walls of gram-negative bacteria and extensively used as an exogenous pyrogen in laboratory settings), fever is triggered by the release of endogenous pyrogens from a large number of cells, including macrophage-like cells (13). Endogenous mediators of fever include cytokines, among which interleukin (IL)-1 and IL-6 are considered most important (4, 14). Research on the mechanisms underlying endogenous antipyresis revealed that certain hormones act as endogenous antipyretics opposing the effects of pyrogens. Among them are arginine vasopressin (34), α-melanocyte-stimulating hormone (28), and glucocorticoids (27). This led to the notion that fever is the result of a balance between pyrogenic cytokines and antipyretic hormones (13). Components of endogenous antipyresis can also be demonstrated within the cytokine network. Studies from our laboratory and from others with the use of various methods of neutralization of cytokines (antibodies, inactivating binding proteins, and receptors) as well as gene deletion techniques (cytokine gene-knockout mice) revealed that there are also antipyretic cytokines. They include tumor necrosis factor-α and IL-10 (12, 16, 20–22, 31).

The current molecular model of fever assumes that the endogenous pyrogens induce liberation of arachidonic acid from structural glycerophospholipids in cell membranes in a variety of tissues. Free arachidonic acid, in turn, is converted into prostaglandin E2 (PGE2). PGE2 is considered to be a key mediator of fever, acting downstream to the endogenous pyrogens, and is ultimately responsible for the upward resetting of the set point of thermoregulation (reviewed in Ref. 2). One may reason therefore that the regulation of the rate of production and/or metabolization of PGE2 might constitute an additional physiological system to complement cytokine:hormone and cytokine:cytokine interactions in endogenous antipyresis and the regulation of fever.

Free arachidonic acid can be metabolized by cyclooxygenase and lipoxygenase enzyme systems to generate proinflammatory eicosanoids such as prostaglandins, thromboxanes, and leukotrienes. A third pathway of arachidonate metabolism exists that produces epoxyeicosanoids, including epoxyeicosatrienoic acids (EETs) and monoxygenase products of arachidonic acid metabolism are part of the endogenous antipyretic system.

bietelemetry: body temperature; lipopolysaccharide; brain stereotaxic surgery; cerebral lateral ventricle; fever mechanism; epoxygenase; bezafibrate; dehydroepiandrosterone; epoxyeicosanoids; epoxyeicosatrienoic acids; hydroxyeicosatetraenoic acids
This pathway is carried out by cytochrome P-450 (CYP) monoxygenases and is referred to as the epoxyenase pathway (P-450). Specific CYP isoforms catalyze monoxygenation of arachidonic acid leading to 1) epoxidation giving rise to four regioisomers 5,6-, 8,9-, 11,12-, and 14,15-EETs, which, in turn, are converted by epoxide hydrolases to corresponding dihydroeicosatrienoic acids (DHETs), 2) allylic oxidation to produce six regioisomers 5-, 8-, 9-, 11-, 12-, and 15-HETEs, and 3) ω-1 hydroxylation to result in 19- and 20-HETEs (9, 25).

P-450 epoxyenase activity has been detected in many tissues, including the hypothalamus (33). Rat astrocytes (1) and isolated brain slices (6) have been shown to make EET regioisomers from arachidonic acid. Generation of HETE regioisomers in cerebral tissues was also documented (11). P-450 arachidonic acid metabolites are currently implicated in a variety of biologic functions, including stimulation of glucagon and insulin release from the pancreatic islets; stimulation of somatostatin release from the hypothalamic median eminence; stimulation of vasopressin, oxytocin and luteinizing hormone release from the anterior pituitary; inhibition of arachidonic acid-induced platelet aggregation; inhibition of the activity of Na⁺-K⁺-AT-Pase in the nephron and corneal epithelium; inhibition of vasopressin-induced water transport; vasodilatation of local microcirculation in the kidney, intestine, brain, and heart; angiogenesis; vasodilatation of arteries; and regulation of blood pressure (6, 9, 11, 24, 25).

The formation of biologically active metabolites of arachidonic acid via P-450s suggests that modulation of these enzymes may also have consequences for the course of fever and other pathophysiological correlates of inflammation. In previous reports, we (15, 17, 18) and others (29) demonstrated that various inhibitors of P-450 exacerbate LPS- and IL-1-induced fever in rats and mice. These data support the hypothesis that CYP is involved in endogenous antipyresis. Accordingly, it has also been suggested that epoxyenase metabolites are antipyretic and that the exacerbation of fever after administration of P-450 inhibitors can result from the suppression of the generation of epoxyeicosanoids (15). In the present studies, with the use of P-450 inducers as well as P-450 arachidonic acid metabolites, we provide additional data supporting this hypothesis.

The rat P-450 monoxygenase isoforms known to catalyze the epoxidation of arachidonic acid are CYP 1A1, CYP 1A2, CYP 4A1, CYP 2B1, CYP 2B4, CYP 2C9, CYP 2C11, CYP 2C23, CYP 2E1, and CYP 2G1 (3, 35). All members of this gene family are markedly inducible as a result of exposure to xenobiotics. To test the effect of P-450 inducers on LPS-induced fever in rats, we used bezafibrate and dehydroepiandrosterone (DHEA). The fibrates (oxysterolburates) are the largest structurally related group of P-450 inducers investigated, and detailed induction protocols have been described for clofibrate, ciprofibrate, clofibrate, and bezafibrate (10). DHEA, a naturally occurring C₁₉-steroid found in mammals, has also been shown to be effective in induction of the P-450 epoxyenases (10). We demonstrate that, in contrast to the effect of P-450 inhibitors on fever reported previously, inducers of P-450 reduce fever in rats.

In complementary experiments, we used various regioisomers of epoxyeicosanoids administered into the rat brain (lateral ventricle) to estimate their effect on fever. This route of administration was used because 1) the preoptic-anterior hypothalamus is considered both the center of thermoregulation as well as regulation of fever (13, 23); 2) expression of P-450 has been demonstrated in the hypothalamus (33); and 3) infusion of proadifen (SKF-525A), an inhibitor of P-450, into the lateral ventricle exacerbated the LPS-induced fever in rats (18). Epoxyeicosanoids significantly attenuated fever in rats. Altogether these data are consistent with the hypothesis that the epoxyenase pathway is a part of an endogenous antipyretic system.

MATERIALS AND METHODS

Animals. Specific pathogen-free, young adult male Sprague-Dawley rats (Charles River, Portage, MI) weighing 250–300 g were housed individually in plastic cages in a temperature-controlled room at 25 ± 1°C with a 12:12-h light-dark cycle (lights on at 0600). Teklad Rodent Diet W8604 and drinking water were provided ad libitum.

Surgery. For measurements of body temperature (Tb), rats under halothane anesthesia were surgically implanted with a battery-operated, temperature-sensitive telemetry transmitter (model VMFH MiniMitter, Sunriver, OR) in the abdominal cavity as described elsewhere (12, 19). For the intracerebroventricular injections, rats were implanted stereotaxically with a 5-mm long, 22-gauge stainless steel, thin-walled cannula (Plastic Products, Roanoke, VA) into the lateral ventricle according to the atlas developed by Paxinos and Watson (32). The cannulas were directed at the coordinates 1.0 mm posterior to bregma and 1.5 mm lateral to the midline. Brain implantations were done as rats were under general anesthesia (ketamine 87 mg/kg and xylazine 13 mg/kg injected intramuscularly). After the surgery, rats were allowed to recover for at least 7 days before experiments were started. After the completion of experiments, the animals were killed. Dye was infused through the cannulas to mark the ventricular space and to verify that the cannula tip was located in the lateral cerebral ventricle.

Measurements of Tₚ, Tᵥ of each rat was monitored with implanted temperature-sensitive telemetry transmitters. Recordings were made at 5-min intervals with use of a peripheral processor (Dataquest III System, MiniMitter) connected to an IBM personal computer (for details, see Ref. 19).

Materials and treatments. LPS (Escherichia coli endotoxin 0111:B4, L2630, Sigma, St. Louis, MO) was dissolved in sterile 0.15 M NaCl (saline) and injected intraperitoneally at a dose of 80 μg/kg in a volume of ~0.25 ml/injection. Saline was used as a control injection. For the induction of CYP, bezafibrate (Sigma) and DHEA (Sigma) were used as described elsewhere (10). Bezafibrate and DHEA were dissolved in warm (~40°C) sterile corn oil (Sigma) at a stock concentration of 80 mg/ml. Stock solutions were rewarmed, sonicated, and diluted with warm corn oil to the desired concentration. Drugs were injected intraperitoneally at doses of 10 and 100 mg/kg in a volume that did not exceed 0.4 ml of rat⁻¹. injection⁻¹ three times at 24-h intervals before the injection of LPS to initiate fever. Sterile corn oil (0.4 ml rat⁻¹. injection⁻¹) was used as a control injection. Peroxide-free ethanol solutions of the arachidonic acid epoxyenase...
metabolites 12(R)HETE, 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET were purchased from Cayman Chemical (Ann Arbor, MI). Before intracerebroventricular infusion into the lateral ventricle, ethanol was evaporated under nitrogen at 10°C, and the specimens were reconstituted with an artificial cerebrospinal fluid ([in mM] 145 NaCl, 3.3 KCl, 1.3 CaCl₂, 1.0 MgCl₂, 3.5 glucose) to a desired concentration. Volume for injections into the lateral ventricle did not exceed 5 μl/rat at a rate of ~1 μl/min.

Data analysis. To unify the influence of the circadian rhythm during the course of experiments and to minimize disturbances due to stress, each experiment was started with injections made at 0900, and the animals were not removed from their isolated climatic chambers. Data are expressed as means ± SE. To assess a difference in the magnitude of fever between groups, a 7-h fever index (FI₇, expressed as °C × 7 h) for each animal was calculated for the statistical analysis. FI₇ was computed as a mean hourly Σ of 5-min ΔT (5-min change in Tb from the baseline, converted into average ΔT/h) for the period of 1050 to 1750 post-LPS and/or postsaline multiplied by 7. Average Tb for an hour preceding each experiment (between 0750 and 0850) was calculated as a baseline for ΔT (during that time before injections, the animals remained undisturbed). Groups were compared with the use of ANOVA followed by Fisher’s protected least-significant differences test. P < 0.05 was considered to be significant.

RESULTS

Agents inducing CYP reduce LPS-induced fever in rats. Bezafibrate and DHEA administered three times before injection of LPS reduced fever in rats in a dose-dependent manner. Figure 1 illustrates changes in fever in rats pretreated three times at 24-h intervals with bezafibrate at doses of 10 and 100 mg/kg and injected 24 h later with LPS at a dose of 80 μg/kg. Regardless of the injecting agents, rats always responded with substantial increase of Tb at the time of injection, i.e., between 0900 and 0930. This transient increase in Tb is regarded as stress induced due to handling and injection. Animals treated with corn oil as a control (vehicle for bezafibrate and/or DHEA) responded to LPS with a biphasic fever starting at 1030. The first phase of fever was completed at 1230. Pretreatment with bezafibrate resulted in reduction of the ovine phase of fever, which is particularly visible for the lower dose of the P-450 inducer. Calculated FI₇ for the control fever (rats pretreated with corn oil and injected with LPS) was 10.4 ± 0.76 (n = 5), whereas for rats pretreated with bezafibrate and injected with LPS, the FI₇ was 7.03 ± 1.10 for 10 mg/kg bezafibrate (P = 0.011 vs. corn oil/LPS group; n = 5/group) and 4.69 ± 0.95 for 100 mg/kg bezafibrate (P = 0.001 vs. corn oil/LPS group; n = 5/group). Bezafibrate did not significantly affect normal Tb, FI₇ for the group of rats pretreated with bezafibrate (100 mg/kg) and then injected with saline (vehicle for LPS). For the group pretreated with corn oil (vehicle for bezafibrate) and injected with saline, Tb/Fl₇ was 2.32 ± 0.5 and 1.78 ± 1.15, respectively (n = 4/group).

DHEA, another inducer of P-450, at a dose of 10 mg/kg (given 3 times before LPS) did not significantly affect fever in rats (data not shown). Computed FI₇ was 11.16 ± 0.66 and 9.9 ± 1.35 for LPS-injected rats pretreated either with corn oil or with DHEA at a dose of 10 mg/kg, respectively. DHEA at a dose of 100 mg/kg, however, significantly reduced fever in rats (Fig. 2B, FI₇ = 5.82 ± 0.84; P = 0.001 vs. corn oil/LPS group; n = 5/group). Similar to the effect of bezafibrate shown in Fig. 1, DHEA (100 mg/kg) affected the second phase of LPS fever in rats. DHEA alone did not influence normal Tb in rats (compare data in Fig. 2, A and B).

Epoxyeicosanoids administered into the lateral ventricle reduce LPS-induced fever in rats. The effect of five regioisomers of epoxyeicosanoids (arachidonic acid epoxygenase metabolites) on fever was examined: 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET, and 12 regioisomer HETE. Fever in rats was induced by an intraperitoneal injection of LPS at a dose of 80 μg/kg. Results of experiments with the most potent regioisomer, the 11,12-EET, are presented in Fig. 3. There is a dose-dependent reduction of fever in rats infused intracerebroventricularly with 11,12-EET at doses of 10 and 100 ng/rat. Computed FI₇ in this experiment was 14.10 ± 1.6 for artificial cerebrospinal fluid (aCSF)/LPS group, 8.46 ± 1.21 for 10 ng/rat 11,12-EET/LPS, and 3.01 ± 0.79 for 100 ng/rat 11,12-EET/LPS group (P < 0.05 among all LPS-treated groups; n = 4/group). The 11,12-EET alone (100 ng/rat) did not significantly affect normal Tb of rats (FI₇ = 1.50 ± 0.61; n = 4).

Figure 4 illustrates combined data of the study on the effect of various regioisomers on fever in rats, demonstrating the antipyretic potency of the examined agents. Average Fl₇ calculated for the control aCSF/
saline groups from all experiments was $1.42 \pm 0.74$ ($n = 11$) and that for the aCSF/LPS groups was $12.79 \pm 0.49$ ($n = 22$). For clarity of presentation, data from the particular control isomer/saline groups are not shown. For each isomer studied, the control average $FI_7$ was not significantly different from that of control aCSF/saline group. Four of five examined arachidonic acid epoxygenase metabolites reduced fever when infused into the lateral ventricle 10 min before the intraperitoneal administration of LPS. The effect was dose dependent at different ranges for each isomer. The most potent antipyretic isomers in our experiments were $11,12$-EET followed by $14,15$-EET, $8,9$-EET, and $12(R)$ HETE. Isomer 5,6-EET at the doses examined (1 and 10 $\mu g$/rat) did not affect fever.

**DISCUSSION**

These studies further support the hypothesis that the P-450/epoxygenase pathway of arachidonate metabolism is part of the endogenous antipyretic system (15, 18, 29).

To date there have been numerous drugs reported to inhibit the P-450 pathway that have been tested for their effects on fever in rodents. These include econazole, clotrimazole, miconazole, SKF-525A, 1-amino benzotriazole, and 17-octadecynoic acid in rats (17, 18, 29) and nordihydroguaiaretic acid, SKF-525A, and clotrimazole in mice (15). Although all of these drugs
undoubtedly have pleiotropic effects in addition to inhibiting the P-450 pathway, they all led to augmentation of fever.

We showed in an earlier study that injection of SKF-525A, an inhibitor of P-450, led to an increase in fever and to increases in brain and circulating concentrations of PGE₂ (18). These data support the hypothesis that blockage of this portion of arachidonate metabolism results in increased catabolism of arachidonic acid via cyclooxygenases. Hence, more PGE₂ can be produced per unit time, which translates into higher fever.

Another possibility supported by other studies (25) is that P-450 itself can be engaged in the inactivation of PGE₂ and that inhibition of P-450 results in a reduction of the rate of PGE₂ catabolism.

Another way to test the role of the P-450 pathway in fever is to inject rodents with inducers of this pathway. Again, although the drugs used (bezafibrate and DHEA) undoubtedly have effects other than simply the induction of this pathway, both drugs led to potent antipyresis. Because the two drugs used in this study induce many P-450 isoforms (10), we do not know which of the isoforms are involved in endogenous antipyresis.

If induction of the P-450 pathway is part of the endogenous antipyretic pathway, then we speculated that products of the arachidonic acid metabolism via epoxygenases should produce antipyresis. The data presented in Fig. 4 indicate that EET isomers 8,9-, 11,12-, and 14,15-, as well as 12(R)HETE have antipyretic properties. There are several possible explanations why the intracerebroventricular treatment with epoxyeicosanoids resulted in antipyresis. One may postulate that products of epoxygenase interfere with the action of PGE₂, e.g., binding of the prostaglandin to PGE₂ EP receptors and/or triggering intracellular signaling processes. Another possibility is that some epoxygenase metabolites act as endogenous suppressors of cyclooxygenases. For example, Node et al. (30) reported that epoxygenase-derived eicosanoids reduced expression of the adhesion proteins and suppressed inflammatory changes in endothelial cells cocultured with LPS and cytokines. These interesting findings may support either hypothesis. In favor of the latter hypothesis, however, Fang et al. (7) and Fitzpatrick et al. (8) demonstrated that certain EET regioisomers are potent inhibitors of prostaglandin synthesis. The potency of some EETs examined, i.e., 8,9-EET and 14,15-EET, surpassed that of aspirin, a well-known anti-inflammatory agent, in the inhibition of the activity of isolated cyclooxygenase enzyme preparation (8). We speculate that induction of the P-450 pathway via the production of epoxyeicosanoids and in turn suppression of cyclooxygenases is responsible for a portion of the antipyretic action of the P-450s. Interestingly, the isomer 5,6-EET did not affect fever in our study. Similarly, 5,6-EET, in contrast to other isomers, was ineffective in the inhibition of cyclooxygenase in the study by Fang et al. (7). Although in studies by Fitzpatrick et al. (8) the isomer 14,15-EET appeared to inhibit cyclooxygenases, in studies by Node et al. (30), unlike the other isomers studied, isomer 14,15-EET did not exert anti-inflammatory effects. These data indicate differential biologic activities of the specific EET regioisomers, depending on the tissue and pathophysiological response investigated.

Besides a possible involvement of the P-450 pathway in regulation within the arachidonic acid cascade, we hypothesize that the antipyretic effect of epoxyeicosanoids is also linked to a negative regulation of the synthesis of IL-6. This cytokine is considered one of the most important endogenous pyrogens, presumably induced and acting as the mediator of fever at the level of the central nervous system (14). We have reported that SKF-525A, an inhibitor of P-450, significantly augmented the level of LPS-induced IL-6 in mice, whereas indomethacin, an inhibitor of cyclooxygenase, suppressed the increase of IL-6 (15). These data indicate that prostanoids and epoxyeicosanoids play contrasting roles in the regulation of IL-6 production.

Perspectives

Fever is the result of the interaction between endogenous pyrogens and endogenous antipyretics. In 1996, Nakashima et al. (29) showed that blockage of the P-450 pathway caused higher fever in rats injected intracerebroventricularly with IL-1β. We recently confirmed and expanded those studies (15, 17, 18), showing that inhibition of the P-450 pathway was associated with increase in levels of brain and circulating PGE₂ and circulating IL-6.

The data presented in our present study show that inducers of the P-450 pathway (bezafibrate and DHEA) lead to antipyresis. Furthermore, microinjection of low concentrations of isomers of epoxyeicosanoids into the lateral ventricles of the brain led to dose-dependent antipyresis. These results clearly support the hypothesis that metabolism of the arachidonic acid via P-450 can give rise to the antipyretic eicosanoids. One might refer these data to the antipyretic mechanism of nonsteroidal anti-inflammatory agents, e.g., aspirin. It is believed that aspirin inhibits fever and attenuates inflammation by blocking cyclooxygenase activity and, in consequence, the generation of PGE₂ (2). It has recently been found, however, that aspirin is a potent inducer of P-450, and administration of a single dose of aspirin produces a significant increase in the activity of CYP 2E1 and CYP 4A1, the CYP isoforms involved in the metabolism of arachidonic acid (see Ref. 17 and references therein). Therefore, one may speculate that aspirin attenuates inflammation and inhibits fever by another mechanism as well, i.e., by inducing CYP, which metabolizes arachidonic acid.

Involvement of the CYP-dependent epoxygenase pathway of arachidonic acid in inflammation and fever has not been thoroughly investigated. It is known, however, that infectious and inflammatory stimuli induce changes in the activities and expression of various forms of CYP in humans and experimental animals (26), suggesting that the generation of epoxyeicosanoids can be a part of the homeostatic mechanisms...
associated with inflammation or infection. We speculate that the induction of the P-450 pathway has much broader implications than simply the production of anti-inflammatory properties. In accord with these data, Fitzpatrick et al. (8) previously and Fang et al. (7) recently have shown that epoxyeicosanoids are potent inhibitors of cyclooxygenases. Taken together, these findings support the hypothesis that modulation of the P-450 pathway can influence inflammation. We speculate that selective epoxyeicosanoids may have therapeutic anti-inflammatory potential.

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