Inhibitors of alternative pathways of arachidonate metabolism differentially affect fever in mice

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Kozak, Wieslaw, Inez Archuleta, Kimberly P. Mayfield, Anna Kozak, Karin Rudolph, and Matthew J. Kluger. Inhibitors of alternative pathways of arachidonate metabolism differentially affect fever in mice. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R1031–R1040, 1998.—Inhibitors of cyclooxygenases prevent fever. The purpose of this study was to test the hypothesis that selective and dual inhibitors of the other enzyme systems of arachidonic acid oxygenation (i.e., lipoxygenase and epoxyen- nase) affect the time course or magnitude of fever in mice. Swiss Webster mice kept at 30°C ambient temperature were implanted with biotelemeters to monitor body temperature. Fever was induced by intraperitoneal injection of lipopolysaccharide at doses from 10 µg/kg to 2.5 mg/kg. Phenindone (20–30 mg/kg ip), a dual lipoxygenase and cyclooxygenase inhibitor, prevented fever in these mice, but esculetin (1–10 mg/kg ip), a selective inhibitor of lipoxygenases, did not affect fever. Intramuscular injection of nordihydroguaiaretic acid (10–20 mg/kg), a dual lipoxygenase and epoxygenase inhibitor, as well as SKF-525A (5 mg/kg ip) and clotrimazole (20 mg/kg im), inhibitors of the cytochrome P-450/epoxygenase pathway, augmented fever in mice. Indomethacin (5 mg/kg ip), an inhibitor of cyclooxygenase, suppressed the exacerbation of fever due to clotrimazole, suggesting that the epoxygenase inhibitor induced potentiation of fever in mice is a prostaglandin-mediated effect. From this study, we hypothesize that the cytochrome P-450/epoxygenase branch of the arachidonate cascade is involved in antipyresis and in controlling the upper limit of fever.

Fever results from the interaction of the central nervous and immune systems and is a common manifestation of injury, tumor, infection, and inflammation. Specifically, the elevation of body temperature during disorders results from the pyrogen-induced upward resetting of the thermoregulatory set point that is believed to reflect the functioning of thermosensitive neurons located in the anterior hypothalamus (6). The current model of fever involves pyrogenic cytokines, e.g., interleukin (IL)-1 and IL-6 (22), and the subsequent induction of prostaglandins (PGs) by these cytokines, particularly stimulation of PGE$_2$ (4), as a common pathway of induction of the rise of the hypothalamic thermoregulatory set point. The actions of classic antipyretic agents, nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ketoprofen, ibuprofen, indomethacin, and piroxicam, are primarily attributed to the inhibition of cyclooxygenases and the subsequently reduced generation of PGE$_2$. It is important to recognize, however, that the precursor of PGs, arachidonic acid, once liberated from the membrane phospholipids, can be oxygenated by any of three major enzyme systems: cyclooxygenases, lipoxygenases, and cytochrome P-450-dependent monoxygenases (also termed the "epoxygenase" pathway) (9). These three pathways lead to the synthesis of PGs, leukotrienes (LTs), and epoxy and hydroxy tetraenoic acids, respectively. Data indicate that inhibition of one of the enzymes of arachidone metabolism may facilitate the metabolism of arachidonic acid through other pathways (5, 8, 26, 29, 39). We hypothesize that the increased products of the alternate pathways due to application of a given inhibitor may influence the metabolic and behavioral responses during inflammation. For example, we have observed that in mice injected with lipopolysaccharide (LPS; a classic laboratory pyrogen extracted from the cell walls of gram-negative bacteria) at a dose that induces initial hypothermia followed by fever, indomethacin prevents fever and also prolongs the LPS-induced hypothermia (24, 25). This effect of indomethacin is associated with an overexpression of the LPS-induced cytokine tumor necrosis factor (TNF)-α in the circulation (25).

Expression of all three enzyme systems of arachidonate metabolism have been demonstrated in brain structures (7, 20, 44), including hypothalamus (18), and in liver, kidney, and lung (21), among other tissues. Localization of cyclooxygenases, lipoxygenases, and cytochrome P-450-dependent monoxygenases in the key tissues responsible for initiation of the acute-phase response to inflammation (23) is consistent with a possible role for these enzymes in the generation of fever. Both the cyclooxygenase (7) and lipoxygenase (20) can be activated or induced by LPS and pyrogenic cytokines. The effect of pyrogens on the cytochrome P-450 enzyme family is complex and involves a partial and time-dependent inhibition of certain isoenzymes (31). Whereas the role for the cyclooxygenase pathway and prostaglandin formation in fever is well documented (4), involvement of biological products of the other two pathways of arachidonic acid oxygenation in fever remains largely unknown. O'Rourke and Rudy (38) injected LTB$_4$, LTD$_4$, and LTE$_4$ into cerebral ventricles of rats and found that none of them affected normal body temperature. In similar studies, Mash-
burn et al. (28) found that LTB$_4$, LTC$_4$, and LTD$_4$ injected into the preoptic hypothalamus of a guinea pig did not induce a rise in body temperature. Hynes et al. (20) also found no evidence for the involvement of LTs in the central mechanism of fever in the cat. These data suggest that LTs, the products of the 5-lipoxygenase pathway, do not participate in the regulation of normal body temperature. The effect of pharmacological inhibition of lipoxygenases on fever has not been thoroughly studied, and results are conflicting. For example, it was reported that propyl gallate, a 5-lipoxygenase inhibitor, did not alter fever induced by peripheral or central injection of IL-1 (12), whereas another 5-lipoxygenase inhibitor, BW A797C, reduced yeast-induced fever in rats (3). On the other hand, in studies by Muller-Peddinghaus et al. (32), an inhibitor of LTB$_4$ and LTC$_4$ biosynthesis, BAY X1005, did not affect baker's yeast-induced fever in the rat. Whether or not the 12- and 15-lipoxygenases and/or a cytochrome P-450/epoxygenase system have a role in the generation or regulation of fever is unknown.

We report the effects of dual and selective inhibitors of cyclooxygenase, lipoxygenase, and epoxygenase on fever in mice. Specifically, 1) the dual lipoxygenase and cyclooxygenase inhibitor phenidone (Phe) potentiates an early hypothermia due to a high dose of LPS (2.5 mg/kg). Phe prevents a subsequent fever in a similar manner as does a more specific inhibitor of cyclooxygenase, indomethacin, as we reported earlier (24, 25). 2) An inhibitor of 5-, 12-, and 15-lipoxygenases, esculetin (Esc), does not affect fever. 3) Finally, the dual inhibitor of lipoxygenases and cytochrome P-450-dependent monoxygenases (epoxygenases), nordihydroguaiaretic acid (NDGA), as well as more selective inhibitors of cytochrome P-450/epoxygenases, SKF-525A (proadifen) and clotrimazole (an imidazole antimycotic), potentiate fever. Indomethacin abrogated the effect of clotrimazole on LPS-induced fever in mice, suggesting that enhanced generation of PGE$_2$ may be responsible for the potentiation of fever due to application of the inhibitor of cytochrome P-450/epoxygenase. On the basis of these data, we conclude that the cytochrome P-450/epoxygenase branch of the arachidonate cascade is involved in antipyresis and in controlling the upper limit of fever. We also report that SKF-525A prevents the LPS-induced increase of plasma TNF-α and augments the elevation of IL-6. In contrast, inhibition of the cyclooxygenase-dependent branch of arachidonic acid cascade by indomethacin potentiates the LPS-induced elevation of circulating TNF-α and reduces the elevation of IL-6. These data indicate that products of cyclooxygenases and the cytochrome P-450-dependent oxygenation of arachidonic acid play opposing roles in the regulation of the generation of cytokines involved in fever.

MATERIALS AND METHODS

Animals. Specific pathogen-free male Swiss Webster mice [Tac(SW)FBR], age 24–35 days, were obtained from Taconic Laboratories (Germantown, NY). The care and treatment of the mice were approved by the Institutional Animal Care and Use Committee of the Lovelace Respiratory Research Insti-

ute. After being received, the mice were housed in individual cages in a temperature-, humidity-, and light-controlled chamber. The chamber was set at 30 ± 1°C (thermoneutral zone for the mice) and on a 12:12-h light-dark cycle, with lights on at 0600. Food (Teklad Rodent Diet W 8604) and tap water were provided ad libitum throughout all experiments. Once mice reached an average body weight of 25–30 g, they were implanted with Mini-Mitter transmitters (model VMHF; Mini-Mitter, Sunriver, OR) to monitor body temperature. Mice were used only once for each experiment, and all experiments were conducted in climatic chambers set as above.

Body temperature measurement. Deep body temperature ($T_b$) was measured using a battery-operated biotelemetry device (Mini-Mitter) implanted intra-abdominally. Recordings were made at 5-min intervals using a peripheral processor (Dataquest III System) connected to an IBM personal computer (for details see Ref. 24).

LPS. Purified lyophilized extract of Escherichia coli LPS (serotype 0111:B4, Sigma L-2630) was dissolved in pyrogen-free 0.15 M sodium chloride (saline) at a concentration of 2 mg/ml and kept frozen at −20°C as a stock solution. To induce a systemic inflammation and fever, LPS was injected intraperitoneally at doses as shown in Figs. 1–8. Saline was used as a control injection. During injection of LPS, the mice were briefly restrained, but not anesthetized.

Test agents. Phe (1-phenyl-3-pyrazolidinone), Esc (6,7-dihydroxycoumarin), NDGA, and SKF-525A (proadifen; 2-dicyethylaminooethyl-2,2-diphényl-n-pentane dioxyphlorocoll) were purchased from BIOMOL (Plymouth Meeting, PA). Clotrimazole and indomethacin were from Sigma. Phe and Esc were dissolved in DMSO (40 mg/ml) and reconstituted with saline (final concentration of DMSO ~5%). NDGA was dissolved in ethanol and reconstituted with saline (final ethanol concentration ~5%). Clotrimazole was dissolved in ethanol and reconstituted with corn oil. SKF-525A was dissolved in saline. Indomethacin, which was prepared as an aqueous sodium solution in 0.01 M anhydrous sodium carbonate, was injected intraperitoneally at a dose of 5 mg/kg. Other test agents were administered at doses as shown in Figs. 1–8. Phe and Esc were injected intraperitoneally at the time of LPS administration. NDGA and clotrimazole were injected intramuscularly 60 min before LPS. SKF-525A was injected intraperitoneally 30 min before LPS. No injection volume exceeded 0.1 ml·mouse$^{-1}$·injection$^{-1}$. Test agents or vehicle controls were injected between 0830 and 0930 into nonanesthetized animals. Doses of test agents and time of administration selected for this study were based on brief preliminary studies testing a dose-related effect of these agents on fever in mice. These preliminary studies are not shown here in details, and only the most effective dose range is demonstrated.

Blood collection and assays. Mice were anesthetized with halothane, and blood for assays was collected in sterile heparinized syringes by cardiac puncture. The plasma obtained after centrifugation was kept at −20°C until used for measurements of TNF-α and IL-6. TNF-α bioactivity in plasma was determined using lysis of WEHI 164 subclone 13 cell line (gift from Dr. Anders Waage, University of Trondheim) as described elsewhere (see, e.g., Ref. 25). The activity of IL-6 in plasma was measured by the B9 bioassay as described (27). Because of a well-known variability in bioassays, TNF-α and IL-6 measurements for all plasma samples within a given protocol were determined in the same assay.

Data analysis. Values are reported as means ± SE. Data collected for $T_b$ at 5-min intervals were collapsed into hourly averages before statistical analysis and for presentation.
Data were analyzed using Statview SE + Graphics (Abacus Concepts, Berkeley, CA). ANOVA with repeated measures was used to determine differences in time-dependent patterns of temperature among groups. ANOVA followed by Scheffé’s pairwise comparisons was used to test for statistical differences among groups at individual time points. Differences were considered significant at P < 0.05.

RESULTS

A dual cyclooxygenase and lipoxygenase inhibitor prevents fever in mice. Phe is a dual cyclooxygenase (IC\textsubscript{50} \sim 12 \mu M) and lipoxygenase (IC\textsubscript{50} \sim 24 \mu M) inhibitor (2, 15). Figure 1 presents the effect of Phe on changes of Tb over 2 days in mice injected intraperitoneally with LPS at a dose of 2.5 mg/kg. This dose of LPS in mice caused a transient decrease in Tb followed by fever that peaked \sim 24 h after injection (Fig. 1). In a dose-dependent manner, Phe injected intraperitoneally at a dose of 20 mg/kg did not significantly affect circadian variations of the normal Tb of mice. Daytime and nighttime Tb of the control injected mice, i.e., injected with 20 mg/kg Phe + saline (Fig. 1) and 5% DMSO + saline (for clarity of presentation these data are not shown in Fig. 1), did not differ from each other and from that seen for these mice during days before injections.

A dual epoxygenase and lipoxygenase inhibitor potentiates fever in mice. NDGA inhibits lipoxygenases (IC\textsubscript{50} between 0.2 and 30 \mu M for 5-\textsubscript{12}- and 15-lipoxygenase), as well as the microsomal cytochrome P-450-mediated monooxygenase pathway of the arachidonic acid cascade (IC\textsubscript{50} \sim 40 \mu M; see Refs. 1 and 10). Figure 2 shows that neither dose of NDGA (10 or 20 mg/kg in a volume of 0.1 ml of \sim 5% ethanol in saline injected intramuscularly 60 min before administration of LPS 2.5 mg/kg) affects the peak Tb (fever) \sim 24 h post-LPS. This inhibitor does, however, influence an early “hypothermic” phase of the response to a high dose of LPS.

The low NDGA dose prevented the drop of Tb; the high dose facilitated (or accelerated) the onset of fever in mice. That high dose of NDGA, similar to Phe described above, did not significantly affect the normal daytime and nighttime Tb of mice.

In the next series of experiments, we used Esc to assess whether inhibition of lipoxygenases could account for the potentiation of LPS fever in mice treated with NDGA. Esc is a specific inhibitor of LT biosynthesis (IC\textsubscript{50} \sim 4 \mu M) that does not affect other pathways of arachidonic acid oxygenation (36). Graded doses of Esc (from 1 to 10 mg/kg ip) did not alter the generation or time course of LPS (2.5 mg/kg)-induced fever in mice (data not shown; Esc at doses higher than 20 mg/kg itself induced hypothermia and death for normal mice). Since this specific lipoxygenase inhibitor did not affect LPS-induced fever in mice, the effect of Phe, a dual cyclooxygenase and lipoxygenase inhibitor, was presumably due to the inhibition of the former enzyme system. Similarly, the enhancement of fever seen following treatment with NDGA, a dual inhibitor of lipoxygenases and cytochrome P-450 monoxygenase, was presumably due to the inhibition of the latter enzyme.

To further test the hypothesis that inhibition of cytochrome P-450-dependent oxidation of arachidonate is responsible for the potentiation of fever seen following treatment with NDGA, in the next series of experiments we used more selective (over cyclooxygenases and lipoxygenases) inhibitors of cytochrome P-450, SKF-525A (proadifen) and clotrimazole. Using indomethacin, we also tested whether or not this exacerbation of fever could be ascribed to a potentiation of PGE\textsubscript{2} generation.

Inhibitors of cytochrome P-450 augment fever in mice. SKF-525A inhibits a considerable number of various cytochrome P-450 enzymes and is widely used as an inhibitor of oxidative drug metabolism (33). It also inhibits a cytochrome P-450/epoxygenase-dependent arachidonic acid metabolism (IC\textsubscript{50} \sim 20 \mu M) over cyclooxygenases and lipoxygenases (10, 17, 29). In our experiments, SKF-525A dissolved in sterile saline was...
injected intraperitoneally in graded doses (from 2 to 20 mg/kg) into mice 30 min before LPS. At doses lower than 10 mg/kg, SKF-525A did not significantly affect the normal Tb of mice. Figures 3–5 demonstrate effects of SKF-525A at a dose of 5 mg/kg on changes in Tb induced by injection of mice with LPS at doses 2.5 mg/kg (Fig. 3), 100 µg/kg (Fig. 4), and 10 µg/kg (Fig. 5).

As shown in Fig. 3, SKF-525A inhibited the drop of Tb and accelerated the onset of fever in mice injected with the highest dose of LPS (2.5 mg/kg). LPS at a dose of 100 µg/kg produced a moderate fever starting soon after the injection (Fig. 4), whereas a dose of 10 µg/kg did not induce fever (Fig. 5). SKF-525A injected 30 min before LPS significantly augmented fever produced by 100 µg/kg LPS (Fig. 4). Interestingly, treatment with the cytochrome P-450 inhibitor provoked elevation of Tb in mice that were injected with LPS at a dose of 10 µg/kg (Fig. 5), even though this dose of LPS alone did not produce fever in control mice (P < 0.05 for average Tb calculated between 1130 and 1830, between LPS-injected and SKF-525A- or saline-treated groups of mice in each experiment shown in Figs. 4 and 5).

Because SKF-525A has been shown to have a limited selectivity (10) and it inhibits a wide spectrum of cytochrome P-450-based reactions (33), in the next series of experiments we tested whether clotrimazole, an imidazole antimycotic, can also enhance the LPS fever in mice. Clotrimazole has been reported to be a potent inhibitor of a cytochrome P-450/epoxygenase-dependent arachidonic acid metabolism (IC50 0.5 µM, Ref. 10). In our studies, a corn oil solution of clotrimazole administered intramuscularly 60 min before LPS at doses of 10, 20, and 30 mg/kg significantly potentiated LPS-induced fever in mice in a dose-dependent manner. Clotrimazole at a dose of 5 mg/kg also augmented the response of mice to LPS; however, the change was not statistically significant. The augmenting effect of clotrimazole at a dose of 50 mg/kg on fever

Fig. 2. Effect of nordihydroguaiaretic acid (NDGA, 10 and 20 mg/kg) on LPS (2.5 mg/kg)-induced changes in Tb in mice recorded over 2 days. At time −1 h, mice were intramuscularly injected with NDGA (5% ethanol, EtOH, in saline served as a solvent for NDGA). Sixty minutes later, at time 0, mice were injected intraperitoneally with LPS. See legend to Fig. 1 for further explanation.

Fig. 3. Effect of proadifen (SKF-525A, 5 mg/kg) on LPS (2.5 mg/kg)-induced changes in Tb in mice recorded over 2 days. At time 0 mice were injected intraperitoneally with SKF-525A (saline solution) and 30 min later with LPS. See legends to Figs. 1 and 2 for further explanation. During this experiment, body weight of mice was controlled. Mice were weighed at time 0, then at time 24 h (arrowhead, notice sharp peak of Tb due to handling).
in mice, on the other hand, was not significantly different from that of 30 mg/kg (data not shown). We did not test the effects of clotrimazole at doses higher than 50 mg/kg on fever in mice, since that dose itself had a tendency of producing hyperthermia.

The influence of clotrimazole on changes in $T_b$ of mice injected with a high dose of LPS (2.5 mg/kg) was similar to that seen after treatment of mice with NDGA or SKF-525A (described above; see Figs. 2 and 3). Clotrimazole prevented an initial drop of $T_b$ and accelerated fever response, but it did not affect the peak of fever seen 24 h after the injection of a high dose of LPS into mice (data not shown). Figure 6 demonstrates results of the experiment in which mice were pretreated intramuscularly either with clotrimazole at a dose of 20 mg/kg or corn oil as a control, then 60 min later injected intraperitoneally with LPS at a dose of 50 µg/kg or saline. Five

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Fig. 4. Changes of $T_b$ of mice injected with SKF-525A (5 mg/kg) and LPS (100 µg/kg) recorded over 24 h. At 0900 mice were injected intraperitoneally with SKF-525A (saline solution), then 30 min later with LPS (arrowhead). Black horizontal bar indicates dark period in the 12:12-h light-dark cycle. Values are means of hourly averages ± SE.

Fig. 5. Changes of $T_b$ of mice injected with SKF-525A (5 mg/kg) and LPS (10 µg/kg) recorded over 24 h. At 0900 mice were injected intraperitoneally with SKF-525A (saline solution), then 30 min later with LPS (arrowhead). See Fig. 4 for further explanation.

Fig. 6. A: changes of $T_b$ of mice pretreated intramuscularly for 1 h with clotrimazole (20 mg/kg; arrowhead 1) or corn oil, then injected intraperitoneally with LPS (50 µg/kg; arrowhead 2) or saline. After 5 h from LPS (or saline) injections, mice were treated intraperitoneally with indomethacin (5 mg/kg; arrowhead 3) or sodium carbonate (vehicle solution) as shown. *Significant difference ($P < 0.05$) in $T_b$ between 2 selected groups, i.e., clotrimazole/LPS treated with sodium carbonate and clotrimazole/LPS treated with indomethacin. B: average $T_b$ of groups of mice shown in A and calculated for the time between 1030 and 1830. *Significant difference in mean $T_b$ among groups indicated.
hours after the LPS injection, one-half (5 mice) of the clotrimazole/LPS group was injected intraperitoneally with indomethacin (5 mg/kg) and the other one-half with control vehicle. As can be seen from Fig. 6, clotrimazole augmented fever in mice and indomethacin suppressed this effect (note that $T_b$ of clotrimazole/LPS group was calculated as a single group, n = 10, before and at the indomethacin injection). One interpretation of these data is that augmentation of fever following administration of clotrimazole is related to the enhancement of PG generation.

Inhibitors of cyclooxygenase and cytochrome P-450 differentially affect plasma levels of IL-6 and TNF-α in mice injected with LPS. Groups of mice were treated either with SKF-525A (5 mg/kg) or with indomethacin (5 mg/kg) and 30 min later injected intraperitoneally with LPS at a dose of 100 µg/kg. As expected, indomethacin at a dose of 5 mg/kg blocked fever in mice injected with LPS as reported elsewhere (data not shown; see, e.g., Ref. 24). Appropriate control groups of animals were injected intraperitoneally with saline (vehicle solution for LPS and SKF-525A) or with 0.01 M sodium carbonate (vehicle solution for indomethacin). Blood samples for plasma IL-6 and TNF-α bioassays were taken 90 min after the LPS (or control vehicle) injection. The assays to test the effects of these inhibitors of cyclooxygenase and cytochrome P-450 on LPS-induced elevations in plasma IL-6 and TNF-α were run separately for each inhibitor. However, all samples for a given protocol were measured in a single assay. Therefore, the difference in levels of the unit values for the particular cytokine, e.g., for IL-6, between experiments presented in Fig. 7 is due to a well-known interassay variability of the bioassay.

Control injections of vehicle solutions and inhibitors at a dose of 5 mg/kg did not alter the basal levels of IL-6 and TNF-α in mice: bioactivity of the cytokines of the control-injected animals, i.e., those injected with inhibitor + saline and those injected with vehicle + saline, did not differ among groups. Injections of cyclooxygenase and/or cytochrome P-450 inhibitors did, however, influence the LPS-induced plasma elevations of IL-6 and TNF-α. Figure 7 demonstrates the effect of indomethacin (Fig. 7A) and SKF-525A (Fig. 7B) on post-LPS IL-6 level. Injection of indomethacin significantly reduced (P < 0.05), whereas SKF-525A potentiated (P < 0.05), the LPS-induced elevation of IL-6. In contrast, the inhibitor of cyclooxygenase (indomethacin) augmented the LPS-induced increase of TNF-α (Fig. 8A), whereas the inhibitor of cytochrome P-450 (SKF-525A) significantly reduced it (Fig. 8B).

**DISCUSSION**

These studies demonstrate that compounds known to inhibit the three enzyme systems of arachidonic acid oxygenation (cyclooxygenases, lipoxygenases, and epoxygenases) differentially affect the thermal responses of mice to a systemic inflammation induced by intraperitoneal administration of LPS. Phe, a dual inhibitor of cyclooxygenase and lipoxygenase, dosedependently augmented a drop of $T_b$ triggered by a high dose of LPS and prevented fever in a similar manner to that previously reported for indomethacin (24, 25). Esc, a selective inhibitor of lipoxygenases, did not affect the febrile response in mice injected with LPS. NDGA, a dual inhibitor of lipoxygenases and cytochrome P-450 monoxygenase, as well as SKF-525A and clotrimazole, selective inhibitors of the cytochrome P-450-dependent oxygenation of arachidonic acid over cyclo- and lipoxygenases, augmented the LPS-induced fever in mice. The effects of enzyme inhibitors involved in arachidonic acid metabolism on fever of mice during inflammation induced by LPS are summarized in Fig. 9. Our data showing that NDGA, SKF-525A, and clotrimazole potentiated LPS-induced fever in mice are consistent with results of Nakashima et al. (34) demonstrating that inhibitors of cytochrome P-450, clotrimazole, and econa-
Zole augmented fever induced by intrahypothalamic injection of IL-1 in rats. In their report, Nakashima et al. (34) described the effect of one dose of clotrimazole (15 mg/kg) administered intramuscularly in sesame oil. We have observed that a rather narrow range of doses of clotrimazole significantly augmented fever in mice in a dose-dependent manner (doses between 10 and 30 mg/kg), whereas SKF-525 produced a similar effect at lower doses (2–10 mg/kg). Clotrimazole, having a similar molecular weight to SKF-525A (344.8 and 389.9, respectively), is a more potent and specific inhibitor of cytochrome P-450/epoxygenase in vitro than SKF-525A (IC50 0.5 and 20 µM, respectively; see Refs. 10, 17, 29). Therefore, one could expect that lower doses of clotrimazole might be required to produce similar effects in vivo.

We cannot explain this discrepancy other than that SKF-525A as an aqueous solution given intraperitoneally could be more readily absorbed and reach threshold saturation in circulation and/or in certain tissue faster than that of clotrimazole given intramuscularly in an oily solution. Another possibility is that SKF-525A, in addition to the inhibition of the cytochrome P-450/epoxygenase enzyme system, also affects other homeostatic systems that are important for regulating thermal responses during inflammation.

Since the first demonstration that PGE1 and PGE2 are pyrogenic (13, 30), the role of cyclooxygenases in fever has been well documented. As a result, the hypothesis proposed by Vane (42) that inhibition of cyclooxygenases underlies the mechanism of NSAIDs-mediated antipyresis has received considerable experimental support and is now approaching the status of dogma. The fever-reducing effect of Phe in this study is most likely due to this mechanism. However, arachidonic acid liberated by the activation of phospholipases during inflammation can be further metabolized by lipoxygenases and epoxygenases, even though the activity of cytooxygense pathway is inhibited by aspirin-like drugs. In other words, the inhibition of cyclooxygenases does not arrest the biological actions of arachidonic acid because it can still be oxygenated by the other two pathways affording bioactive eicosanoids, LTs and a considerable number of regional isomers of hydroxyeicosatetraenoic, epoxyeicosatrienoic, and dihydroxyeicosatrienoic acids. Results from studies using injection of LTs into rats, guinea pigs, and cats (20, 28, 38), and data using Esc in mice presented in this report do not support a role for lipoxygenase products in the generation of fever. We conclude, therefore, that exacerbation of the drop of Tb and inhibition of fever in mice observed in the present study following administration of Phe, a dual cyclooxygenase and lipoxygenase inhibitor, was due to the inhibition of cyclooxygenes rather than lipoxygenases. The previously shown effects of indomethacin on fever in mice (24, 25), and of Phe, NDGA, proadifen, and clotrimazole found in the present study, suggest that the cytochrome P-450/epoxygenase enzyme family (and perhaps their products) is involved in the mechanism of endogenous antipyresis.

Fig. 8. Effect of indomethacin (A) and SKF-525A (B) on LPS (100 µg/kg)-induced elevation of plasma tumor necrosis factor-α (TNF). Refer to legend to Fig. 7 for further explanation. *Significant difference (P < 0.05) between groups indicated.

Fig. 9. Schematic representing inhibitory effects of agents used in our study on the enzymes involved in arachidonate cascade via cyclooxygenase (inhibited by indomethacin and Phe), lipoxygenase (inhibited by Phe, esculentin, and NDGA), and the cytochrome P-450 pathway (inhibited by NDGA, SKF-525A, and clotrimazole). Sequential use of inhibitors shifted the thermal responses of mice during inflammation (induced by injection of LPS) either to pyretic (left side of the metabolism) or antipyretic (right side of the metabolism).
Drugs that inhibit the activity of cytochrome P-450/epoxygenase could augment LPS-induced fever in mice by several possible mechanisms. It is well established that immunological stimuli, including exogenous pyrogens and cytokines, partially depress the activity of cytochrome P-450 (16, 31, 41). We report that additional inhibition of cytochrome P-450 using SKF-525A and/or clotrimazole potentiates fever in mice, implying that the larger the inhibition of cytochrome P-450, the higher and longer the fever. Altogether, these data are consistent with the hypothesis that fever is associated with the inhibition of cytochrome P-450. We speculate that arachidonic acid may be metabolized either by cyclooxygenase to form the fever-producing metabolites such as PGE\textsubscript{2} or by a cytochrome P-450/epoxygenase-dependent pathway to contribute to endogenous antipyresis. In support of this hypothesis, indomethacin prevented the exacerbation of LPS fever in mice treated with clotrimazole (Fig. 6), suggesting that this enhancement of fever due to inhibition of epoxygenase may be related to a potentiation of the activity of cyclooxygenases and, in turn, increased generation of PGE\textsubscript{2}. Due to a lack of experimental data, it is unclear whether the products of cytochrome P-450-dependent arachidonate metabolism act directly as antipyretics. Another possibility is that the cytochrome P-450-dependent oxygenation of arachidonic acid may constitute a biological "safety valve," which may utilize the excess of free arachidonic acid. The pathway may simply yield products that do not affect a thermoregulatory function while limiting the pool of free arachidonic acid available for the fever-inducing cyclooxygenase pathway. Nevertheless, the relative rates of these two alternate pathways of arachidonate oxygenation may control the onset and the height of fever. Thus suppression of the activity of cytochrome P-450 may divert more arachidonic acid into the profebrile cyclooxygenase pathway, whereas the inhibition of cyclooxygenases may result in diversion of more arachidonic acid into the antipyretic (or thermally inactive) epoxygenase pathway. In support of this model, Boynaems et al. (5) reported that SKF-525A facilitates stimulation of cyclooxygenases and formation of prostacyclin. On the other hand, acetylsalicylic acid, a classic antipyretic and cyclooxygenase inhibitor, stimulates a cytochrome P-450/epoxygenase-dependent \( \omega-1 \) hydroxylation of arachidonic acid (8, 26, 39). On the basis of these data, we conclude that the shift of a pool of liberated arachidonic acid and the activation of a certain enzymatic system of arachidonate oxygenation can constitute a regulatory mechanism. This mechanism may control the timing and the rate of the formation of a certain class of eicosanoids in a course of inflammation and induction of the acute-phase responses, including fever.

Alternative explanations of data presented in our report are that 1) inhibitors of cytochrome P-450 augment fever by inhibiting a cyclooxygenase P-450-dependent clearance of PGs (29) or 2) inhibitors of cytochrome P-450 amplify fever by eliminating an epoxycosatrienoic acid (EET) inhibitory modulator of the cyclooxygenase pathway (14). Both mechanisms may result, in turn, in an enhanced signaling from the increased PGE\textsubscript{2}, possibly in the brain and peripheral tissues. Certain isomers of EET also potently stimulate the release of arginine vasopressin (AVP) from the neural lobe of the rat pituitary (35). AVP has been implicated in the mechanism of endogenous antipyresis (40) and stimulates a microsomal cytochrome P-450-related metabolism of arachidonic acid (43). Thus EETs, one product of cytochrome P-450-dependent arachidonicate oxygenation, may actually contribute to the antipyresis by inhibiting cyclooxygenases and stimulating release of AVP. Hydroxycetomisinic acids, the other products of cytochrome P-450-dependent arachidonate metabolism, on the other hand, may also contribute to antipyresis since they inhibit Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (29), an enzymatic system involved in thermogenesis (11).

Evidence is increasing that eicosanoids, and PGs in particular, are involved in regulation of the synthesis and release of cytokines (19, 25, 37). Therefore, inhibitors of arachidonic acid metabolism may affect fever due to interference with the generation of cytokines, in particular those cytokines that are defined as endogenous pyrogens, such as IL-6 (22). Data presented in Fig. 7A indicate that inhibition of cyclooxygenases by indomethacin is associated with suppression of the LPS-induced IL-6. In contrast, inhibition of cytochrome P-450 using SKF-525A augmented the elevation of plasma IL-6 (Fig. 7B). These alterations to in vivo generation of IL-6 provoked by administration of indomethacin and SKF-525A paralleled the effects of these inhibitors on fever in mice. Although IL-6 is considered a major endogenous pyrogen, TNF-\( \alpha \) has also been proposed to contribute to fever generation (22). However, the actual role of TNF-\( \alpha \) in fever remains unclear because this cytokine may exhibit both pyretic and antipyretic activities (22). In the present study we observed that, in the context of fever versus plasma cytokine level, the effects of cyclooxygenase and cytochrome P-450/epoxygenase inhibitors on LPS-induced plasma TNF-\( \alpha \) (Fig. 8, A and B) were opposite of those found for IL-6. Our data indicate that the augmented LPS fever in mice (provoked by a cotreatment with epoxygenase inhibitor) was associated with a suppressed elevation of TNF-\( \alpha \), whereas inhibition of fever by indomethacin was accompanied by an exacerbation of plasma TNF-\( \alpha \) activity. This observation supports the hypothesis that TNF-\( \alpha \) either is not critical for the generation of fever or is involved in the antipyretic mechanisms. These data also suggest that products of cyclooxygenases and the cytochrome P-450-dependent oxygenation of arachidonic acid play contrasting roles in the regulation of generation of IL-6 and TNF-\( \alpha \).

**Perspectives**

Fever and its pathophysiological correlates, such as pain and body wasting, are common responses to infection and injury and extremely important diagnostic tools. Multiple mechanisms are responsible for fever. Considerable evidence indicates that generation of fever involves synthesis and release of inflammatory mediators of protein origin, such as cytokines, and...
those of lipid origin, including eicosanoids. Among them, PGE$_2$ has been documented to play a critical role in the generation of fever. Besides this mechanism that serves to elevate $T_d$ and an endogenous antipyretic system acts to modulate fever and presumably prevents elevations in $T_d$ from rising to dangerous levels. Thus fever appears to be a carefully orchestrated host defense response. Over the past 20 years, investigators have shown that AVP, $\alpha$-melanocyte-stimulating hormone, glucocorticoids, and, in some cases, TNF may act as endogenous antipyretics. Our studies may support the hypothesis that the activity of epoxygenase (i.e., a cytochrome P-450-dependent metabolization of arachidonic acid) also plays a role in regulating the upper limit of fever. The mechanism(s) by which cytochrome P-450 regulates the ceiling of fever may be complex and may involve the clearance and biodegradation processes, as well as the generation of antipyretic metabolites.

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