Evidence supporting involvement of leukotrienes in LPS-induced hypothermia in mice

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Evidence supporting involvement of leukotrienes in LPS-induced hypothermia in mice. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R52–R58, 1999.—The aim of the present study was to examine a possible involvement of leukotrienes (LTs) in lipopolysaccharide (LPS)-induced body temperature (Tb) response. We examined the effect of MK-886, an inhibitor of LT synthesis, on changes in Tb, plasma tumor necrosis factor-α (TNF-α), hypothalamic LT, and PGE2 production. Intraperitoneal injection of LPS (50 μg/mouse) led to a decrease in Tb starting 1 h after the injection. The hypothermic effect of LPS was accompanied by a significant elevation in TNF-α level in plasma and in LT and PGE2 production by ex vivo-incubated hypothalamus. MK-886 (1 mg/kg ip) administered 4 h before LPS efficaciously prevented LPS-induced hypothermia in mice. Pretreatment of mice with MK-886 did not alter the LPS-stimulated increase in plasma TNF-α. MK-886 significantly inhibited LT and enhanced PGE2 production in hypothalamus compared with LPS alone. These results suggest that 1) LPS-induced hypothermia may be mediated by LTs and 2) the antihypothermic effect of MK-886 is not associated with TNF-α bioactivity.

LPS induces a variety of metabolic, cellular, and regulatory effects known as acute phase response (APR) in mammals (1). One of the most prominent manifestations of APR is the thermoregulatory alteration referred to as fever (1). LPS exerts its pyrogenic effects by inducing the synthesis of endogenous cytokines, such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, interferon, etc., which in turn stimulates the synthesis of arachidonic acid (AA) products, eicosanoids (4, 10, 39). AA cascade includes two main metabolic pathways: 1) a cyclooxygenase (COX) pathway, which leads to generation of PGs, prostacyclin, and thromboxane, and 2) a lipooxygenase pathway, which leads to generation of LTs via 5-lipoxygenase (5-LO).

Accumulated evidence indicates the role of PGs of E series (mainly, PGE2) as the final central mediator of fever responsible for the elevation of the set point in the hypothalamic thermoregulatory center (10, 30, 40). Another large group of eicosanoids, leukotrienes (LTs), was mainly investigated with respect to its role in the mechanisms of anaphylaxis and inflammation. The LTs appear in two structural subtypes, differing in regard to the presence (LTC4, LTD4, LTE4, LTF4; peptido-LTs) or absence (LTB4) of peptide chain. The peptido-LTs (further referred to as LTs) undergo enzymatic transformations proceeding from LTC4 to LT4 via LTE4 and LTD4. The LTB4 is a potent chemotactic agent promoting the migration and aggregation of leukocytes, whereas other LTs are important mediators of asthma, cause contraction of bronchial and microvascular smooth muscles, and increase vascular permeability. These effects of LTs have been examined on numerous animal and human models of bronchial anaphylaxis and inflammation (3). Although the inflammation is often accompanied by the development of fever, much less attention was focused on the possible involvement of LTs in mechanisms of the febrile response to pyrogens. Several studies conducted in this area in the past have led to rather conflicting results showing the lack of changes in Tb (28, 34, 43) or the hypothermic effect of centrally injected LTs (6). Additionally, it was reported that propyl gallate, a 5-LO inhibitor, or LY-171883, a LT receptor antagonist, did not alter fever elicited by intravenous or intracerebroventricular injection of IL-1 (7), whereas another 5-LO inhibitor, BW-A797C, attenuated yeast-induced pyrexia in rats (3).

On the other hand, it should be noted that LPS administration is not obligatorily accompanied by elevation in Tb. In particular, small rodents, mice and rats, in response to systemic injection of LPS, responded with hypothermia, which may be followed by a subsequent rise in Tb (9, 23). The sustained hypothermia is one of the important characteristics of the endotoxic shock (37). Using 5-LO inhibitor (AA-861) or LT receptor antagonist (ONO-1078), Ogata et al. (33) showed that these agents significantly attenuated the symptoms of endotoxemia, such as leukopenia, thrombocytopenia, and hemoconcentration, and decreased mortality in mice. However, the role of LTs in LPS-induced hypothermia has not yet been established. Recent studies on the mechanisms of hypothermia caused by LPS have dealt mainly with TNF-α as a putative endogenous cryogen (8, 18, 22).

The purpose of the present study was to examine the role of LTs in LPS-induced hypothermia in mice with the use of MK-886, a selective inhibitor of 5-lipoxygenase-activating protein (FLAP). FLAP is an 18-kDa membrane protein required for the membrane translocation of 5-LO and for the subsequent cellular activation of synthesis and generation of all 5-LO products (11, 12). MK-886 was shown to inhibit LT biosynthesis in different in vivo and in vitro models (16). After the antihypothermic effect of MK-886 in LPS-treated mice was determined, we examined whether MK-886 may
influence TNF-α plasma level, in view of its cryogenic properties. We also assayed LTs and PGE₂ production by hypothalamus after LPS and MK-886 administration. Part of this data has been presented in a preliminary form at the International Symposium on Thermal Physiology (Copenhagen, July 8–12, 1997).

METHODS

Animals. Male CD/1 mice weighing 28–32 g were obtained from Harlan Laboratories (Israel) and allowed to acclimate at least 14 days before the experiments were commenced. The animals were housed in plastic cages (6 or 7 animals per cage) under controlled environmental conditions: ambient temperature of 22 ± 1°C, relative humidity of 45–55%, 12:12-h light (0600–1800)-dark (1800–0600) photoperiod cycle. Food and water were provided ad libitum. Experiments were carried out between 0830 and 1530, and mice were used only once for each experiment.

Temperature measurement. Tb (colonic) was measured with a plastic-coated thermocouple probe (type K) connected to an HL-600 (Anritsu, Anritsu Meter) digital thermometer and inserted 2.5 cm into the rectum. Tb was measured before the injection and every hour during the postinjection period. To minimize stress, animals were handled and adapted to temperature measurement on a daily basis at least 7 days before the experiment. Repeated Tb measurements with intervals of 15 min did not significantly affect values of Tb.

Drug administration. LPS from Esherichia coli (0127:B8, Sigma, St. Louis, MO) was dissolved in pyrogen-free sodium chloride (0.2% glucose) in an incubation medium, and injected at a dose of 50 μg/mouse. MK-886, 3-(1-(4-chlorobenzyl)-3-butyloxy-isopropylidene)-2-yl)-2,2-dimethyl propanoic acid (lot P1833, Biomol Research Laboratories) was diluted in 10% DMSO. MK-886 (1 mg/kg) or 10% DMSO was administered 1–4 h before LPS. All injections were made intra-peritoneally in a volume of 0.1 ml.

Blood sample collection. Trunk blood was collected at different times (15, 30, 60, 120, 180, and 240 min) after LPS injection in heparin-coated Eppendorf tubes and centrifuged (1,800 rpm, 10 min). The plasma was separated and stored at −20°C pending analysis of TNF-α.

Incubation of hypothalamus. Animals were decapitated at 1, 2, or 4 h after LPS administration, and the whole hypothalamus was quickly excised by tweezers. Its landmarks were defined according to Franklin and Paxinos (15): the mouse hypothalamus is located on the ventral surface of the brain behind the optical chiasmas and is visible distinct from surrounding tissues by color and slightly prominent margins. To avoid the inclusion of extrahypothalamic structures in the dorsoventral direction, hypothalamus (ventral part) of about 1.5 mm in thickness was used (the thickness of the adult mouse hypothalamus is 1 mm; Ref. 15). Each hypothalamus was incubated for 60 min at 37°C in a vial containing 1 ml of Krebs-Henseleit solution (0.2% glucose) in an environment of 95% O₂ and 5% CO₂ adjusted to pH 7.35–7.40. At the end of incubation, medium (1 ml) was collected from each vial. The weight of each hypothalamus was measured by a precision balance and stored at −20°C until determination of LTs and PGE₂.

Determination of LTC₄/LTD₄/LTE₄/LTF₄ (total LTs). Total content of LTs was measured in unextracted samples of incubation medium by enzyme immunoassay kits (PerSep-tive Diagnostics, Framingham, MA). The LTD₄ antibody cross-reacted with LTF₄ 66.7%, LTC₄ 55%, and LTE₄ 51%, whereas it showed nonsignificant cross-reactivity with LTB₄ 0.25%, arachidonic acid 0.12%, and PGs <0.01%. The detection limit of the kit was 7.62 pg/ml. The content of LTs in individual incubation medium samples was then plotted per weight of the correspondent hypothalamus.

Determination of PGE₂. PGE₂ that accumulated in the incubation medium was measured in unextracted samples by single antibody RIA with dextran-coated charcoal precipitation. PGE₂ for standard curve and rabbit antisera to PGE₂ were purchased from Sigma. Tritium-labeled PGE₂ (160 Ci/mmol) was obtained from The Radiochemical Center (Amersham, UK). The sensitivity of the assay was 0.15 ng/ml. The RIA was performed in duplicate for each sample. The content of PGE₂ in individual incubation medium samples obtained by RIA was then plotted per weight of the hypothalamus. The PGE₂ antisera serum cross-reacted with the following PGs (at 50% displacement): PGA₁, 3%; PGA₂, 1.2%; PGF₁, 7.7%; PGF₂ 6.8% other PGs <1%.

TNF-α ELISA. Plasma samples were assayed for the TNF-α content using an ELISA that specifically detects murine TNF-α (Pharmingen, San Diego, CA). Purified anticytokine capture monoclonal antibody in coating buffer (0.1 M NaHCO₃, pH 8.2) was added to wells of an enhanced protein ELISA plate and incubated overnight at 4°C. After washing twice with PBS-0.05% Tween, the plate was blocked with PBS-10% fetal calf serum for 2 h to reduce nonspecific binding. The standards and samples were added and the plate was incubated overnight at 4°C. The plate was washed four times and biotinylated anticytokine detection monoclonal antibody was added. The plate was incubated at room temperature for 45 min and then washed six times before adding streptavidin peroxidase in PBS-10% serum. After a 30-min incubation at room temperature, ABTS substrate ([2,2-azino-bis(3-ethylthiazoline-6-sulfonic acid)] with H₂O₂ was added and the plate was read at optical density 405 nm. The detection limit of the assay was 31 pg/ml.

Statistical analysis. Results are presented as means ± SE. Statistical evaluation was carried out using factorial analysis (ANOVA) and Student’s t-test (2 tailed) to test for differences between the control and experimental groups. Values of P < 0.05 were considered statistically significant.

RESULTS

Effect of LPS on Tb. The initial values of Tb in the control and LPS groups were similar (36.8 ± 0.1 and 36.9 ± 0.1°C, respectively). Injection of saline in the control group led to a transient increase in Tb (0.6°C at 0.5 h after injection) that returned to the pretreatment level and did not differ from the controls (Fig. 1). Tb in the LPS-treated group was apparently due to the stress induced by the injection procedure. In contrast to the control group, mice injected with LPS displayed a significant drop in Tb within 1 h postinjection (37.4 ± 0.2°C in saline-treated and 35.4 ± 0.3°C in LPS-treated mice at 1 h) without initial elevation in Tb. The hypothermic values of Tb in LPS-treated group were observed during the next 3 h and by 5–6 h postinjection Tb returned to the pretreatment level and did not differ from the controls (Fig. 1). Tb reached its minimal value (35.1 ± 0.2°C) 2 h after LPS injection.

Effect of MK-886 on Tb of LPS-treated mice. The effect of MK-886 at a dose of 1 mg/kg injected 1, 3, or 4 h before LPS is shown in Fig. 2. Pretreatment with MK-886 1 h before LPS did not prevent the hypothermic effect of LPS (35.1 ± 0.2°C), whereas it was mitigated when MK-886 was administered 3 or 4 h before LPS (35.4 ± 0.2°C)
0.3°C in MK-886 + LPS-treated mice). \( T_b \) decrease due to LPS was partially prevented by MK-886 administered 3 h before and completely blocked by MK-886 injected 4 h before LPS. In the next set of experiments, we compared the effect of three doses (0.1, 0.5, and 1 mg/kg) of MK-886 injected 4 h before LPS administration on \( T_b \) (Fig. 3). MK-886 at doses of 0.1–1 mg/kg did not influence the normal values of \( T_b \) measured for 4 h, except for a transient initial elevation of 0.4–0.6°C. One-week observation of MK-886-treated mice revealed normal patterns of their \( T_b \) and behavior. The lowest dose of MK-886 did not alter \( T_b \) response to LPS until 4 h postinjection. \( T_b \) of the LPS-treated mice pretreated with 0.5 mg/kg of MK-886 was significantly higher than that of mice injected with LPS alone (P < 0.05 at 1, 2, 3, and 4 h), however it was still lower compared with the saline-treated controls. In contrast to LPS alone, no decrease in \( T_b \) was observed in the group pretreated with 1 mg/kg of MK-886, i.e., the temperature changes seen in the MK-886 + LPS group did not differ from those seen in the saline-injected group at any of the four time points. Hence, administration of MK-886 at a dose of 1 mg/kg 4 h before LPS was sufficiently potent to prevent LPS-induced hypothermia in CD/1 mice.

### Effect of LPS and MK-886 on hypothalamic LT production.

The LT release by ex vivo-incubated hypothalamus was examined at 1, 2, or 4 h after the LPS administration. LPS injection was accompanied by approximately twofold elevation in LTs compared with the control group at each point measured (P < 0.05, data are not shown). MK-886 at a dose of 1 mg/kg completely inhibited the elevation in hypothalamic LTs due to LPS administration (10 ± 1 vs. 20 ± 4 pg·mg tissue\(^{-1}\)·h\(^{-1}\), at 2 h post-LPS injection; P < 0.05). The mean value of LTs in the MK-886 + LPS group did not differ significantly from that of the control group (Fig. 4A).

### Effect of LPS and MK-886 on hypothalamic PGE\(_2\) production.

As illustrated in Fig. 4B, intraperitoneal injection of LPS caused a considerable elevation in PGE\(_2\) release by ex vivo-incubated hypothalamus (P < 0.05 vs. control at 2 h postinjection). Pretreatment of mice with MK-886 4 h before LPS led to a lower hypothalamic PGE\(_2\) level than in mice injected with LPS alone (390 ± 41 vs. 282 ± 30 pg·mg tissue\(^{-1}\)·h\(^{-1}\); P < 0.05), i.e., MK-886 at a dose of 1 mg/kg enhanced LPS-stimulated elevation in PGE\(_2\).

### Effect of MK-886 on LPS-induced changes in plasma TNF-\(\alpha\).

Injection of saline in the control group did not cause a detectable TNF-\(\alpha\) concentration in plasma. The
TNF-α values in mice treated with MK-886 alone were also undetectable. Administration of LPS led to a rapid (within 30 min) increase in TNF-α production: plasma TNF-α level reached its maximal value at 1 h, thereafter gradually decreased, and by 4 h was hardly detectable (Fig. 5, inset). The peak TNF-α level was not altered by MK-886 administered 4 h before LPS (LPS 1,628 ± 189 vs. MK-886 + LPS 1,454 ± 232 pg/ml) (Fig. 5).

**DISCUSSION**

The major result of the present study is that MK-886, an inhibitor of LT synthesis, effectively prevents the decrease in T₀ and blocks the elevation in hypothalamic LT production caused by LPS. This fact may point to the involvement of LTs in endotoxin-induced hypothermia in mice. At the same time, an interpretation of results obtained by applying inhibitors, even selective ones, for elucidating a functional role of a system should be made with caution due to possible indirect effects. In this context, the possible effect of MK-886 on TNF-α should be excluded.

The role of TNF-α in the mechanisms of fever is still debatable, because both pyrogenic and antipyretic properties of this cytokine have been demonstrated (21, 38). However, a growing body of data indicates that TNF-α is probably the most appropriate candidate for the role of endogenous cryogen, at least in the case of LPS administration in rats and mice. Kozak et al. (22) reported that two agents that inhibit TNF-α bioactivity, TNF soluble receptor and antiserum to TNF-α, blocked LPS-induced hypothermia in mice. Recently, Leon et al. (25) demonstrated the exacerbated febrile response to LPS in TNF-α double-knockout mice. In rats, hypothermia induced by intravenous injection of LPS was also markedly attenuated by antiserum to TNF-α (8). On the other hand, the administration of neutralizing antiserum or polyclonal antibody to TNF-α significantly enhanced fever response to LPS in rabbits and rats (27, 29), whereas human recombinant TNF-α prolonged hypothermia and abolished fever in response to LPS in mice (22). We have found that LPS caused a dramatic increase in plasma TNF-α level, which preceded the development of hypothermia in mice (Fig. 5, inset). A similar time course of LPS-induced alterations in TNF-α level was demonstrated in mice (23, 45) and in rats (27). Therefore, we examined the effect of MK-886 on LPS-stimulated TNF-α level in plasma.
Pretreatment of mice with MK-886, at least in the given dose, which prevented the development of hypothermia, did not alter the LPS-induced elevation in TNF-α concentration in plasma. This observation confirmed the results of previous studies that demonstrated that LPS-stimulated TNF-α synthesis by cultured human monocytes (17), isolated rabbit lung (5), murine macrophages, or in murine plasma (41) was not influenced by MK-886. MK-886 also did not affect TNF-α level in response to other stimuli, such as concanavalin A, phorbol ester, or zymosan, as well as the synthesis of other cytokines (17). Moreover, other specific 5-LO inhibitors, CGS-8515 or AA-861, and a selective LTC4D4 receptor antagonist, ONO-1078, also failed to modulate LPS-induced TNF-α production in murine macrophages or in sera (33, 41). Hence these data taken together give evidence that the antihypothermic effect of MK-886 observed in the present study is not associated with TNF-α. Although we examined the effect of MK-886 only on TNF-α, the possible influence of MK-886 on other endogenous cryogens, such as arginine vasopressin, α-melanocyte-stimulating hormone, etc. (for review, see Ref. 21), should also be taken into account. Further investigations are needed to test this possibility.

In accordance with the present data, LPS-induced hypothermia was accompanied by elevation in LT production by the hypothalamus, started at least 1 h after the injection, and lasted at least 3 h more, i.e., an elevated level of LT was observed during the whole period of hypothermia. Both effects of LPS were prevented by the inhibitor of LT synthesis, MK-886.

It seems that LTs may induce hypothermic or, alternatively, restrict hyperthermic responses to pyrogens. Yet the effects of pyrogens on Tb response and LT production may be species specific. These suggestions gained certain support by the study of Hynes et al. (20) on the different models of fever in cats. The authors observed that intracerebroventricularly injected LPS or IL-1 caused elevation in Tc without any significant effect on LT level in cerebrospinal fluid (CSF). At the same time, intracerebroventricular administration of U-60,257, an inhibitor of 5-LO, enhanced hyperthermia and reversed LT elevation due to platelet-activating factor. On the contrary, the Tc decrease after concomitant administration of platelet-activating factor and indomethacin was associated with an enhanced LT level in CSF. The exacerbation of hypothermia due to indomethacin in LPS-treated mice was also observed in our previous study (14). A similar result was reported by Kozak et al. (23), which, however, suggested that this phenomenon was attributed to enhanced TNF-α bioactivity due to indomethacin. It should also be taken into account that alterations in the activity of two main enzymes of AA cascade may lead to the redistribution of AA between 5-LO and COX pathways. Hence it may be supposed that thermoregulatory effects of the inhibitors are partially associated with the alterations in the balance between different eicosanoids. Whereas indomethacin, a COX inhibitor, was shown to promote indirectly LT synthesis in the central nervous system (20), in the present study MK-886, in parallel to the inhibition of LT production, promoted the LPS-stimulated PGE2 production in the hypothalamus. Although the pyrogenic role of PGE2 in mice remains unclear (14) and its additional elevation due to MK-886 was relatively small, compared with LPS alone, the involvement of PGE2 in the antihypothermic effect of MK-886 cannot be excluded. Combinations of the COX and 5-LO inhibitors or the use of dual inhibitors of PG and LT synthesis may help to elucidate this issue.

On the basis of the results of the present study and data existing in the literature, the involvement of both LTs and TNF-α in LPS-induced hypothermia may be suggested. One of the possibilities may consider the role of LTs as mediators of the cryogenic effect of TNF-α. Several pieces of indirect evidence support this speculation. 1) TNF-α stimulates LT production, both in vivo and in vitro (19, 36), and this stimulation was similar to that evoked by an infusion of LPS (19). 2) As we found in the present study, an elevation in the LT synthesis by the hypothalamus was preceded by an increase in TNF-α activity (Figs. 4A and 5). A similar result was also observed in rat air pouch model of inflammation (13). 3) Different effects of TNF-α were prevented by using the LT inhibitors (2, 19, 32). On the other hand, the existence of two different regulatory pathways for LTs and TNF-α cannot be excluded.

In summary, we have found that FLAP inhibitor MK-886 concomitantly prevented an elevation in hypothalamic LT production and hypothermia caused by LPS. MK-886 did not alter the effect of LPS on plasma TNF-α level, and hence antihypothalamic action of MK-886 was not associated with its effect on TNF-α.

Tb is a result of a controlled balance between heat production (thermogenesis) and heat loss (mainly, vascular tone) (40). Provided that LTs possess a cryogenic feature, the question arises: how do LTs exert the hypothalamic effect, peripherally or centrally? It is known that LTs produce vasoconstriction in most vascular beds (35, 42, 44). Hence it seems doubtful that LTs promote heat loss. There is no evidence in regard to a possible direct effect of LTs on thermogenesis. As we demonstrated in the present study, LPS increased LT production in mouse hypothalamus. It should, however, be noted that our ex vivo model cannot specifically confirm the origin of the LTs released by the hypothalamus. Their elevation in incubation medium may reflect either LT synthesis de novo in hypothalamus or the release of nonhypothalamic LTs, which entered the hypothalamus, or both. It has been shown that several types of cells can produce LTs in brain, including neurons, glial cells, and probably the cells of the vessel wall (20). Noticeably, among different brain regions the hypothalamus was found to produce the greatest amount of LTs (26, 31). Hence it seems likely that a suggested hypothetic effect of LTs is attributed to their central action rather than to a peripheral effects. If so, it may be reasonable to assume that LTs, at least in mice, could be considered as endogenous cryogens. However, further investigations are required to validate this hypothesis.
A careful examination of centrally injected LTs accompanied by monitoring of body temperature should be carried out in mice to elucidate whether LTs meet the criteria for endogenous cryogen described by Kluger (21). Additionally, for evaluation of the role of LTs in thermoregulatory responses to bacterial endotoxin, the use of transgenic, LT-deficient mice may be beneficial.

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