**INTRODUCTION**

Fever is a state of elevated body temperature and a common symptom associated with systemic infectious diseases and inflammation. The most commonly used experimental model of fever comprises systemic administration of lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria. The injection of LPS induces the expression of cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) in peripheral and central cells including macrophages of the lung and liver, and endothelial cells of the hypothalamus. The COX-2 and mPGES-1 induction results in conversion of arachidonic acid to PGE2 (1-3). PGE2, in turn, induces fever by stimulating E type prostaglandin (EP) receptor subtype EP3 (and possibly EP1) in the thermoregulatory center of the hypothalamus (4, 5), thereby being the most important proximal mediator of the febrile response (6).

In contrast to PGE2, however, little is known about the role of other prostanoids in the fever reaction. PGD2 is the most abundant prostanoid in the CNS (7). It is produced from arachidonic acid by action of COX and PGD synthase (PGDS). Two distinct types of PGDS have been identified, lipocalin-type PGDS (L-PGDS) and hematopoietic PGDS (H-PGDS). L-PGDS is a secretory protein that is expressed in the brain and in male genital organs, while H-PGDS is localized in immune and inflammatory cells (8). There is considerable evidence that PGD2 is involved in the regulation of several physiological and pathophysiological processes such as sleep induction, attraction of inflammatory cells, allergic asthma, platelet aggregation, smooth muscle relaxation and hormone release (8, 9). An involvement of PGD2 in thermoregulation has been suggested, but the data obtained so far are rather controversial, since both hyperthermic and hypothermic effects have been observed after central administration of PGD2 in rats (10, 11). The purpose of this study was to more clearly describe the role of PGD2 in LPS-induced fever in rats. We investigated the content of PGE2 and PGD2 in cerebrospinal fluid (CSF), plasma and lungs of rats after systemic injection of LPS. The injection of LPS evoked fever and an increase of PGE2 in the CSF, while the CSF content of PGD2 was not significantly altered. However, both PGE2 and PGD2 levels were elevated in plasma and lungs after LPS injection. Interestingly, pretreatment with a novel selective inhibitor of hematopoietic prostaglandin D synthase (H-PGDS), EDJ300520 (10-40 mg/kg p.o.), selectively and dose-dependently prevented the LPS-induced increase of PGD2 in plasma and lungs but did not affect the PGE2 content. Most remarkably, EDJ300520 pretreatment led to an hypothermic response after LPS injection during the first 3 h and prevented fever induction. These data indicate that PGD2 produced peripherally by H-PGDS essentially contributes to LPS-induced fever.

**Key words:** body temperature, febrile response, lipopolysaccharide, prostaglandin E2, prostaglandin D2, hematopoietic prostaglandin D synthase, body temperature

## MATERIALS AND METHODS

**Animals**

The ethic guidelines for investigations in conscious animals were obeyed and all procedures were approved by the local...
Ethics Committee for Animal Research (Regierungspärisdium Darmstadt, Germany). Fifty-seven male Sprague Dawley rats (Charles River, Sulzfeld, Germany) weighing 250-300 g were used. Animals had free access to standard rat chow and tap water and were housed in a room maintained at 22 ± 1 °C with a 12:12-h light/dark cycle (lights on 07:00 am - 07:00 pm).

**Animal experiments**

Studies were performed on conscious, unrestrained rats at an ambient temperature of 22 ± 1 °C. One week before the experiment, animals were anesthetized with an isoflurane/carbogen mix and a radiotelemetry transmitter (ET-Mitter 4000 system, Minitrack, Orange, CA) was inserted into the intraperitoneal cavity for continuous recording of core body temperature (Tc) without stress to animals. One day before the experiment, each cage was placed on a receiver and radiotelemetry signals emitted by the implanted transmitter were continuously monitored in intervals of 5 min using the Vitalview software (Minitrack). At the day of the experiment (at 9:00 am), rats were shortly anesthetized with isoflurane and fever was induced by intraperitoneal (i.p.) injection of 50 µg/kg LPS (E. coli, O111:B4; Sigma-Aldrich, Seelze, Germany) dissolved at a concentration of 5 µg/ml in pyrogen-free 0.9% NaCl solution (B. Braun, Italy). In experiments with the PGD2 inhibitor Evir-M, rats were perorally (p.o.) treated with Evir-M (40 mg/kg) suspended in 0.5% methyl cellulose in water, and fever was induced 1 h thereafter by i.p. LPS injection. Three animals were investigated per experimental day with one animal receiving either vehicle, 10 mg/kg or 40 mg/kg Evir-M. Average Tc values for 20-min periods were computed from TC recorded at 5-min intervals. For each rat, a baseline temperature was defined as the mean Tc during the 60-min period immediately preceding fever induction. The change in Tc (∆Tc) was calculated by subtracting the baseline temperature from each recorded Tc value. The number of animals per group was 6-9.

For tissue harvesting, rats were deeply anesthetized with ketamine-midazolam. The neck muscles were rapidly reflected to gain access and to withdraw a cerebrospinal fluid (CSF) sample from the cisterna magna. CSF (~ 120 µl) was collected in an Eppendorf tube that was put immediately on dry ice and stored at -80 °C. The content of PGE2 and PGD2 in CSF, plasma and lungs was determined by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-MS/MS), a method that allows ultra-sensitive detection of prostaglandins (12). As PGE2 and PGD2, in vivo are rapidly inactivated by enzymatic degradation to metabolites (for instance, the half-life in peripheral circulation is ~ 20 sec) (13, 14)), we additionally determined the levels of metabolites of PGE2 and PGD2. The concentration of PGE2 was then calculated as sum of PGE2 and the PGD2 metabolites 15-keto-PGE2, 13,14-dihydro-15-keto-PGE2, 13,14-dihydro-15-keto-PGA2, bicyclo-PGE2 and tetrano-PGEM. The concentration of PGD2 was calculated as sum of PGD2 and the PGD2 metabolites 13,14-dihydro-15-keto-PGD2, 11-β-PGD2, 11-β-13,14-dihydro-15-keto-PGF2α, and PGI2.

Sample and standard preparation: CSF samples (100 µl) were added with 100 µl 0.15 M EDTA, 80 µl 45 mM H3PO4, and 20 µl methanol. Plasma samples (250 µl) were added with 100 µl 0.15 M EDTA, 600 µl 45 mM H3PO4, 10 µl 2,6-di-tert-butyl-4-methylphenol (2 mg/ml in methanol) and 20 µl methanol. Lung samples were cut into small pieces and homogenized thoroughly using a pellet pestle (Kontes Glass Company, Vineland, New Jersey, USA). Homogenates (25 mg) were suspended in 125 µl PBS. Then 100 µl 0.15 M EDTA, 80 µl 45 mM H3PO4, and 20 µl methanol were added. In samples for standard curves and quality control, the CSF, plasma or lung samples were replaced by 100 µl artificial cerebrospinal fluid, 250 µl PBS or 150 µl PBS, respectively, and the working and internal standards were dissolved in 20 µl methanol.

Prostaglandin extraction. Extraction of prostaglandins was performed with liquid-liquid-extraction (CSF and lungs) or solid-phase-extraction (plasma). For liquid-liquid-extraction, samples were incubated twice with 600 µl ethyl acetate. The organic phase was removed at 45 °C under a gentle stream of nitrogen. The residues were reconstituted with 50 µl of acetonitrile / water / formic acid (20:80:0.0025, v/v,v, pH 4.0), centrifuged for 2 min at 10,000 g and transferred to glass vials (Macherey-Nagel, Düren, Germany) prior to injection into the LC-MS/MS system. For solid-phase-extraction, 1 ml Chromabond HR-X cartridges (Macherey-Nagel) were washed with 2 ml of hexane / ethyl acetate / isopropanol (35:60:5, v/v), dried for 20 sec, conditioned with 1 ml of methanol and equilibrated with 1 ml of water. One milliliter of the prepared plasma / PBS was loaded onto the column and washed with 1 ml water and 1 ml methanol / water (10:40, v/v). The cartridges were then dried for 7 min and eluted with 1 ml of hexane / ethyl acetate / isopropanol (35:60:5, v/v), dried for 20 sec, conditioned with 1 ml of methanol and equilibrated with 1 ml of water. One milliliter of the prepared plasma / PBS was loaded onto the column and washed with 1 ml water and 1 ml methanol / water (10:40, v/v). The cartridges were then dried for 7 min and eluted with 1 ml of hexane / ethyl acetate / isopropanol (35:60:5, v/v). Removal of the organic phase and reconstitution of residues was performed as described above.

Prostaglandins were separated on a Syngery Hydro-HR column (ID 150 x 2 mm, particle size 4 µm and pore size 80 Å; phenomenex, Aschaffenburg, Germany) and determined with an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany) as described previously (12).

**Statistics**

The results are expressed as means ± SEM. Statistical analysis comprised Student’s t-test or analysis of variance with
subsequent Bonferroni post-hoc test. \( P < 0.05 \) was considered as statistically significant.

**RESULTS**

Changes of PGD\(_2\) and PGE\(_2\) levels in CSF, plasma and lung during LPS-induced fever

In order to test whether PGD\(_2\) might be involved in the LPS-induced fever response, the content of PGD\(_2\) and PGE\(_2\) in CSF, plasma and lungs at various time points after i.p. injection of LPS were determined by LC-MS/MS analyses. Both PGD\(_2\) and PGE\(_2\) were detectable in samples from saline-treated control rats (CSF: 159.7 ± 47.3 and 53.0 ± 8.0 pg/ml; plasma: 62.7 ± 10.2 and 432.4 ± 76.3 pg/ml; lung: 135.7 ± 30.3 and 36.6 ± 7.1 pg/mg tissue; PGD\(_2\) and PGE\(_2\), respectively). LPS injection (50 µg/kg i.p.) evoked the typical progressive rise in T\(_c\) during 120 to 300 min after injection, while in animals injected with saline T\(_c\) remained stable throughout the experiment (Fig. 1A). The initial slight elevation of T\(_c\) after saline or LPS injection was apparently due to the stress induced by the injection procedure.

The LPS-induced changes of PGD\(_2\) and PGE\(_2\) levels in CSF, plasma and lungs are summarized in Fig. 1B-G. PGD\(_2\) was significantly increased in CSF of LPS-treated animals as compared to saline-treated control animals at 1, 3 and 5 hours after LPS injection with maximal levels at 3 h (Fig. 1B). In plasma, PGE\(_2\) was also significantly elevated from 1 to 5 hours after LPS injection, however the maximum PGE\(_2\) concentration was already detected at 1 h after LPS (Fig. 1C). These data are consistent with recent reports indicating that circulating PGE\(_2\) may trigger the early phase of LPS-induced fever, while centrally produced PGE\(_2\) is crucial for later phases of fever (3, 15). We also investigated PGE\(_2\) production in the lungs, i.e. in a ‘LPS-processing’ organ (16). LPS injection slightly increased the PGE\(_2\) levels in lung tissue, being significant at 3 h after injection (Fig. 1D).

In contrast to PGE\(_2\), levels of PGD\(_2\) were not significantly altered in the CSF after LPS injection, although a tendency towards an increase at late time points was observed (Fig. 1E). However, in plasma and lungs PGD\(_2\) was elevated from 1 to 5 h (plasma) and at 3 h (lungs) after LPS injection (Fig. 1F,G). Notably, the time course of LPS-induced changes in PGD\(_2\) was similar to that of PGE\(_2\) with maximum levels being detected at 1 h (plasma) and 3 h (lungs) after LPS. All in all, these data confirm the pivotal role of PGE\(_2\) in the early phase of LPS-induced fever, while centrally produced PGE\(_2\) is crucial for later phases of fever (3, 15). We also investigated PGE\(_2\) production in the lungs, i.e. in a ‘LPS-processing’ organ (16).

Inhibition of peripheral PGD\(_2\) synthesis prevents the LPS-induced fever

We then analyzed the effect of peripheral PGD\(_2\) inhibition on the fever reaction by using a novel inhibitor of hematopoietic prostaglandin D synthase (H-PGDS), EDJ300520. Selectivity of EDJ300520 towards inhibition of PGD\(_2\) synthesis was first investigated in vitro. RAW 264.7 macrophages and HeLa cells were stimulated by cytokines and the content of PGD\(_2\) and PGE\(_2\) in the supernatant was determined after 16 h. As shown in Fig. 2, addition of EDJ300520 (0.5–50µM) to the cell culture medium concentration-dependently inhibited the cytokine-induced production of PGD\(_2\) (Fig. 2A), but PGE\(_2\) levels were not altered by EDJ300520 (Fig. 2B). These data confirm that EDJ300520 selectively inhibits the synthesis of PGD\(_2\) but not of PGE\(_2\) in vitro.

In order to inhibit H-PGDS, we applied EDJ300520 orally to rats 1 h prior to i.p. LPS injection and analyzed the changes in T\(_c\) as well as the content of PGD\(_2\) and PGE\(_2\) in CSF, plasma and lungs. Administration of EDJ300520 without any other stimulus did not change T\(_c\) as compared to vehicle treatment (data not shown). However, when EDJ300520 was administered 1 h before i.p. LPS, an unexpected hypothermic response to LPS occurred during the first 3 h with a maximal drop in T\(_c\) of ~0.7 °C at 100 min after LPS (Fig. 3A). Thereafter, T\(_c\) returned to baseline, but the typical LPS-induced febrile T\(_c\) rise did not occur (Fig. 3A). The contents of PGD\(_2\) and PGE\(_2\) were determined at the end of the observation period, i.e. 5 h after LPS injection. PGE\(_2\) levels in CSF, plasma and lungs were not affected by treatment with EDJ300520 (Fig. 3B-D), CSF levels of PGD\(_2\) were also not significantly altered after administration of the H-PGDS inhibitor (Fig. 3E). However, the content of PGD\(_2\) in plasma and lungs
was significantly reduced as compared to vehicle-treated animals (Fig. 3 F, G). These data show that (i) EDJ300520 in vivo selectively blocks the LPS-induced peripheral PGD2 synthesis, and that (ii) H-PGDS mediated PGD2 synthesis is required for a febrile response to i.p. LPS.

DISCUSSION

PGE2 has long been known as the principal mediator of the febrile response. In contrast, the role of PGD2 in LPS-induced fever is poorly understood. We here demonstrate that systemic LPS injection increases the production not only of PGE2 but also that of PGD2 in the periphery, i.e. in plasma and lungs. Interestingly, inhibition of LPS-induced, peripheral PGD2 production by a selective H-PGDS inhibitor prevents the fever reaction in response to LPS injection. Therefore, peripherally generated PGD2 seems to be implicated in the mechanisms underlying fever.

There is considerable evidence that the entire febrile course needs de novo synthesis of PGE2, and that the PGE2-synthesizing enzymes are induced in the brain and in peripheral organs. As a result, increased levels of PGE2 in both CSF and plasma have been detected after injection of LPS and other pyrogens (14, 16-21). Recent data indicate that LPS-induced PGE2 synthesis is more rapidly activated in peripheral tissues than in the brain. The peripherally generated, circulating PGE2 is thought to cross the blood-brain barrier and to cause the early phase of fever which occurs in a thermoneutral environment after i.v. LPS injection (3, 22). The LPS-induced changes of PGE2 content in CSF and plasma described in the present study agree well with this hypothesis, since the highest PGE2 levels were detected in plasma already 1 h after LPS injection, while the PGE2 levels in CSF peaked after 3 h. About the source of the rapid PGE2 increase in plasma one can only speculate. In the lungs, the PGE2 content was significantly increased only at 3 h after LPS and it is therefore unlikely that the lungs are the locus of rapid, LPS-induced PGE2 production. Other LPS-processing organs such as the liver might be involved, but this was not investigated in the present study.

Our data further demonstrate that, similar to PGE2, the synthesis of PGD2 is also increased in plasma and lungs in response to systemic LPS injection. However, in contrast to PGE2, the LPS injection did not affect the levels of PGD2 in the CSF. We concluded that peripherally generated PGD2 might modulate the febrile response and therefore we investigated the effects of an inhibitor of H-PGDS, which mainly mediates the synthesis of PGD2 in peripheral organs (8, 23). Most notably, oral pretreatment with the H-PGDS inhibitor EDJ300520 (i) prevented the LPS-induced PGD2 increase in plasma and lungs, (ii) prevented the LPS-induced fever response, and (iii) induced a hypothermia that started ~ 60 min and peaked ~ 100 min after LPS injection. The mechanisms of a hypothermic response to bacterial endotoxin are poorly understood. In general, LPS injection can evoke either fever or hypothermia in rats. Two critical factors that influence the outcome are the LPS dose and the thermal environment (24, 25). However, in our experiments all rats received the same dose of LPS and the ambient temperature was also the same. Thus, peripherally generated PGD2 is obviously an additional critical factor that decides whether systemic LPS injection induces fever or hypothermia.

In summary, our experiments demonstrate that peripherally produced PGD2 is involved in the mechanisms underlying fever. Additional studies are needed to explore the exact mechanism by which PGD2 modulates the fever response.

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