Microinjection of a cyclooxygenase inhibitor into the anteroventral preoptic region attenuates LPS fever

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Scammell, Thomas E., John D. Griffin, Joel K. Elmquist, and Clifford B. Saper. Microinjection of a cyclooxygenase inhibitor into the anteroventral preoptic region attenuates LPS fever. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R783–R789, 1998.—Considerable evidence supports the role of prostaglandins in fever production, but the neuroanatomic sites of prostaglandin synthesis that produce fever remain unknown. With the use of a novel microinjection technique, we injected the cyclooxygenase inhibitor ketorolac into the preoptic area (POA) to determine which preoptic regions produce the prostaglandins required for fever. Initial experiments demonstrated that intravenous ketorolac blocked the fever normally produced by lipopolysaccharide (LPS) 5 μg/kg i.v. Microinjection of ketorolac into the POA had no effect on body temperature, and injection of artificial cerebrospinal fluid into the POA did not alter LPS fever. Injection of ketorolac into the anteroventral POA markedly decreased the fever produced by LPS, compared with injections into more rostral, caudal, or dorsal locations. These observations indicate that prostaglandin synthesis in the anteroventral preoptic region is necessary for the production of fever.

MATERIALS AND METHODS

Animals. Pathogen-free, male Sprague-Dawley rats (Taconic, Germantown, NY) weighing 280–350 g were used in this study. Rats were housed individually in a pathogen-free barrier facility with unrestricted access to food and water in a room maintained at 21.5–22.5°C. Lights were turned on at 0700 and off at 1700. All protocols were approved by the Institutional Animal Care and Use Committees of Beth Israel Hospital and Harvard Medical School. Each rat was used only once. Eighty-seven rats were used in these experiments, and seven others were excluded because of postoperative bleeding (4 rats), delayed recovery from propofol anesthesia (2 rats), or preexperiment fever (1 rat).

Materials. Salmonella typhimurium LPS (lot 23H4047, Sigma) was diluted in pyrogen-free saline (Sigma) to a concentration of 5 μg/ml. Artificial CSF (aCSF) consisted of (in mM) 124 NaCl, 2 KCl, 1.25 KH2PO4, 2 CaCl2, 2 MgSO4, 26 NaHCO3, and 3.5 glucose. A 1% dilution of 0.1 μm rhodamine-
labeled latex microspheres (Molecular Probes, Eugene, OR) was added to the aCSF for injection site identification. Ketorolac tromethamine (a kind gift from Jeff Kitson, Syntex) was dissolved in aCSF to a final concentration of 1.0 mM. The pH of the aCSF and ketorolac-aCSF solution was 8.0. The concentration of the intravenous ketorolac solution was 80 mM and was administered at 10 mg/kg (0.33 ml/kg). Ketorolac was chosen for COX inhibition because it is among the most water-soluble, thoroughly studied COX inhibitors available (21) and is an effective antipyretic (25). We have found that the febrile response to microinjection of 100 ng PGE_2 into the POA is unaltered by simultaneous administration of ketorolac 10 mg/kg iv (unpublished results), suggesting that ketorolac produces antipyresis by acting prior to PGE_2, most likely via COX inhibition. COX catalyzes the formation of PGs and thromboxanes, collectively referred to as prostanoids. Because thromboxanes are thought not to contribute to fever, we refer to the relevant products of COX as PGs.

Procedures and analysis. In experiment 1, we injected ketorolac 10 mg/kg iv alone or in combination with LPS 5 µg/kg iv to determine whether ketorolac could effectively block LPS fever. In our model, this low LPS dose consistently produces a moderate biphasic fever, peaking 2–3 and 5–6 h after injection (9). We chose to focus our remaining experiments on the first peak of LPS fever at 2–3 h, as we found that this initial fever could be blocked by ketorolac and we were concerned that drugs would diffuse throughout the POA over time, limiting the anatomic specificity of the injections.

In experiment 2, we microinjected 100 nl aCSF into the POA under brief propofol anesthesia to establish whether this microinjection technique altered normal body temperature (T_b). In a separate group of rats, we microinjected 100 nl of ketorolac-aCSF to determine if preoptic COX inhibition altered normal T_b.

In experiment 3, we microinjected 100 nl aCSF into the POA to determine whether this injection technique altered the fever normally produced by LPS 5 µg/kg iv. These injections were targeted at the anteroventral preoptic region because our prior work with PGE_2 indicated that this should be a critical site of PG synthesis.

In experiment 4, we injected LPS 5 µg/kg iv and 100 nl of ketorolac-aCSF into a variety of hypothalamic sites to identify the regions that produce the PGs necessary for LPS fever.

Placement of intravenous catheters and telemetry devices. As we have done previously (9, 26, 27), telemetry was used for monitoring T_b, and chronic intravenous catheters were used for systemic drug injections. All temperature transmitters (type VM-FH, Mini-Mitter, Sunriver, OR) were calibrated in a warm water bath at temperatures between 35 and 39°C. The temperature transmitter was placed into the peritoneal cavity via a midline incision. Body temperature signals were received by an antenna below the rat's cage and relayed to a signal processor connected to a Compaq 486 personal computer. Monitoring of T_b began at least 12 h before drug injection to assess baseline T_b. Average baseline T_b was 37.1°C, and baseline T_b values did not differ significantly between groups. T_b data are presented as the mean change in temperature (±SE) in each 10-min interval relative to baseline (average temperature over the hour preceding injection).

POA injections. On the day of the experiment, each rat was anesthetized with the short-acting general anesthetic propofol 10 mg/kg iv (Zeneca, Wilmington, DE) (13) between 0900 and 1000. The rat was placed in a stereotaxic frame on a 36°C heating pad, and 100 nl aCSF or ketorolac-aCSF was microinjected into the POA using a previously described air pressure injection apparatus (1). In brief, solutions were injected using a silane-coated glass pipette drawn to a tip diameter of 25–30 µm. The meniscus within the pipette was directly visualized with an operating microscope, and fluid was ejected using brief puffs of air at 50 lb/ft². These air puffs were regulated by an electronically controlled air switch that opened for 5–10 ms every 0.5–1 s. These variables were adjusted for each pipette such that 100 nl was typically injected over a 2-min period. The injected volume was determined by directly measuring movement of the meniscus within the pipette with a reticle on the microscope. The pipette was introduced at a 7° angle off vertical to avoid the sagittal sinus, and injection coordinates varied from 0.25 mm anterior to bregma to 0.6 mm posterior to bregma, from 0.75 to 2.5 mm lateral to the midline, and from 6 to 8 mm below the dural surface. To maintain deep anesthesia (as judged by withdrawal to paw pinch), some animals received a small supplemental dose of propofol (2.5–5 mg/kg) 7–10 min after injection. Therefore, the total dose of propofol varied between animals, but there was no correlation between total propofol dose and the subsequent T_b response. After the brain injection, some animals received LPS 5 µg/kg iv or an equivalent volume of saline (1 ml/kg). The intravenous catheter then was flushed with 0.1 ml heparinized saline, the scalp wound was closed with surgical clips, and the rat was returned to its cage. The entire procedure was completed in 10–15 min, and rats recovered rapidly, usually resuming normal activity and grooming within 5–10 min after returning to their cages. T_b was recorded over the next 4 h.

Perfusion and histology. After the 4-h monitoring period, animals were deeply anesthetized with chloral hydrate (500 mg/kg ip) or propofol (20 mg/kg iv) and perfused transcardially with 0.9% saline for 5 min followed by 500 ml of phosphate-buffered 4% paraformaldehyde (pH 7.0). The brains were removed, stored in the same fixative for 4 h, and submerged in 20% sucrose, and coronal sections were cut at 40 µm on a freezing microtome. Serial sections through the POA were examined under fluorescence microscopy. The center of the injection site was defined by the cluster of fluorescent spheres and drawn with a camera lucida. Latex beads adhere to brain, and even with injections into the subarachnoid space or third ventricle, beads could be identified adhering to the meninges or ependyma close to the pipette track.

Statistical analysis. LPS produces an initial fever in rats 2–3 h after injection (9, 27), and the average change in T_b over this interval was chosen as the endpoint for statistical analysis in all experiments. The results of experiments 1, 2, and 3 were analyzed using independent-samples t-tests without assuming equal variances (Systat, SPSS, Chicago, IL); P was considered significant if <0.05.

To determine whether intraperitoneal ketorolac blocked LPS fever in experiment 4, the febrile responses of 15 rats that received ketorolac in the anteroventral preoptic region were compared with those of the five rats that received aCSF in this same region using an independent-samples t-test. This anteroventral preoptic region encompasses the preoptic nuclei most sensitive to PGE_2: the OVLT, ventromedial POA, median preoptic nucleus, anteroventral periventricular nucleus, the meninges of the preoptic recess just above the
optic chiasm and rostral to the OVLT, and the cell-sparse parenchyma immediately adjacent to these meninges (26). To determine which preoptic injection sites were most effective in blocking fever, injection sites were grouped into one of three possible regions (see Fig. 5): 1) the anteroventral preoptic region (n = 15); 2) more lateral sites adjacent to the anteroventral preoptic region, including the nucleus of the diagonal band of Broca and the medial and lateral preoptic areas (n = 14); and 3) other sites >1 mm from the OVLT, including the periventricular preoptic nucleus, perifornical region, medial septum, third ventricle, and other sites (n = 17). Febrile responses after ketorolac injections into these regions were compared using a one-way analysis of variance (ANOVA) for repeated measures with a post hoc Tukey’s honestly significant difference (HSD) procedure. Data are presented as the mean change in Tb (±SE) in each 10-min interval relative to baseline (average temperature over the hour preceding injection).

RESULTS

In all four experiments, mean baseline Tb ranged between 36.4 and 37.7°C, and no group's baseline differed significantly from another.

Experiment 1: Effect of intravenous ketorolac on LPS fever. Injection of LPS 5 µg/kg iv into awake rats produced a biphasic fever, with the first peak occurring 2–3 h after injection and a second peak at 5–6 h (Fig. 1). Ktorolac 10 mg/kg iv abolished the first fever peak (P < 0.01 during the 2- to 3-h period after injection), but Tb began to rise 4–5 h after the injection. Occasionally, either injection was followed by a small, transient rise in Tb that has been attributed to the momentary stress of handling during the intravenous injection (14). Neither injection of ketorolac 10 mg/kg iv nor an equal volume of saline (data not shown) had any lasting effect on Tb, and 2–3 h after the injections the ketorolac group did not differ significantly from the saline group.

Experiment 2: Effect of intrapreoptic microinjection of aCSF or ketorolac on Tb. Microinjection of 100 nl aCSF into the POA had little effect on Tb (Fig. 2); Tb did not differ from baseline 2–3 h after injection (P > 0.5). Although the rats appeared to behave normally within 10 min of the procedure, a slight drop in Tb (0.1–0.2°C) for 10–20 min followed the microinjection procedure in some animals and may have been due to lingering effects of the propofol anesthesia. Intrapreoptic microinjection of 100 nl ketorolac-aCSF (1.0 mM) also had little effect on Tb; 2–3 h after the microinjection, Tb did not differ from the aCSF group (P = 0.5).

Experiment 3: Effect of intrapreoptic microinjection of aCSF on LPS fever. Intrapreoptic injection of 100 nl aCSF had no effect on the fever produced 2–3 h after LPS 5 µg/kg iv (P > 0.5) (Fig. 3). These five injections were centered on the anteroventral POA (see Fig. 5). As

Fig. 1. Effect of intravenous injection of ketorolac on lipopolysaccharide (LPS) fever. LPS 5 µg/kg iv produces a biphasic fever with the first peak at 2–3 h and a second fever beginning at 4 h. Coinjection of ketorolac 10 mg/kg iv with LPS abolishes the first fever, but body temperature (Tb) begins to rise after 4 h. Injection of ketorolac alone has no lasting effect on Tb, n = 5 in each group.

Fig. 2. Effect of preoptic (POA) microinjections on Tb. Microinjection of 100 nl artificial cerebrospinal fluid (aCSF) or 100 nl ketorolac-aCSF (1.4 mM) does not alter Tb. Note that the data are now presented on a 4-h time axis. n = 5 in each group.

Fig. 3. Effect of preoptic microinjection of aCSF on LPS fever. Propofol anesthesia may cause a transient drop in Tb after microinjection of aCSF, but the fever amplitude 2–3 h after injection of LPS is indistinguishable from that seen with intravenous injection of LPS alone. n = 5 in each group.
seen in experiments 1 and 2, animals that received only
an intravenous injection tended to have a brief rise in
T_b 30 min later, but the animals that received aCSF
microinjections occasionally had transient drops in T_b.
After this first hour, the thermal responses between the
groups did not differ.

Experiment 4: Effect of intrapreoptic microinjection of
ketorolac on LPS fever. Microinjection of ketorolac into
the anteroventral preoptic region markedly decreased
the fever produced 2–3 h after LPS 5 µg/kg iv (Fig. 4).
Five rats that received LPS intravenously and aCSF
into this region had large fevers (1.4°C). In contrast, 15
rats with ketorolac injections near the OVLT and
leptomeninges of the preoptic recess generally had very
small fevers (0.53°C) that were significantly less than
those seen with aCSF microinjections (P < 0.001).

The most striking attenuations of LPS fever followed
injections of ketorolac in this anteroventral region
(region A), but injections into other preoptic sites also
attenuated fever, although to a lesser degree. Injections
near the horizontal nucleus of the diagonal band or
medial POA (region B) moderately reduced fever
(0.76°C). More distant preoptic injections, generally >1
mm from the OVLT (region C), modestly decreased
fever (0.82°C). Comparison of the febrile responses
after injections in these three regions with an ANOVA
revealed a significant difference between groups
(F = 3.95, P < 0.05). A post hoc Tukey HSD comparison
demonstrated a significant difference between regions
A and C (P < 0.05). Injections into the anatomically
intermediate region B did not differ significantly from
either A or C.

The anatomic specificity of the ketorolac injections is
best appreciated in the coronal diagrams of Fig. 5 and
in Table 1. The most strikingly effective sites were
clustered in the anteroventral aspects of the POA; these injections typically decreased fever by
at least three standard deviations below that seen in
the LPS-aCSF group (<0.68°C). These highly effective
sites included the OVLT, the ventromedial POA, the
anterior preoptic nucleus, the median preoptic
nucleus, and the meninges of the preoptic recess.
Microinjections into the vertical limb of the diagonal
band of Broca, medial septum, bed nucleus of the stria
terminalis, medial POA, or lateral POA generally at-
tenuated fever to a lesser degree.

### DISCUSSION

Intrapreoptic microinjection of the COX inhibitor
ketorolac markedly decreased the fever produced by
intra prefers LPS. The most effective antipyretic sites
were clustered in the anteroventral aspect of the POA,
surrounding the OVLT. These observations indicate
that PG synthesis in the anteroventral preoptic region
is necessary for the production of fever.

Methodological considerations. We chose to use a
novel microinjection technique to administer drugs into
the POA, which avoids many of the technical problems
inherent with chronic cannulas. This technique has
several advantages, but the acute anesthesia with
propofol can briefly disrupt thermoregulation, result-
ing in small, transient drops in T_b once the rat is
returned to its cage. This disturbance is brief, and rats
readily produce fever after intrapreoptic injection of
PGE_2 (26). Of importance to the current study, T_b 2–3 h
after microinjection of aCSF or ketorolac is unchanged
from baseline, and the amplitude of LPS-induced fever
after intrapreoptic injection of aCSF is identical to that
seen with LPS alone. Thus our injection technique has
little lasting effect on normal T_b or LPS-induced fever.

Our aCSF and ketorolac-aCSF solutions were slightly
alkaline (pH 8), and the pH of injected solutions can
influence preoptic physiology. Sehic and colleagues (30)
have shown that acidic solutions can hinder the preop-
tic synthesis of PGE_2 and blunt fever. However, injec-
tion of a mildly alkaline aCSF (pH 7.8) does not alter
the rise in PGE_2 after LPS (29). Our slightly alkaline
aCSF also failed to alter normal T_b or LPS fever. Thus
we feel it is unlikely that the alkalinity of the aCSF
significantly altered preoptic PGE_2 synthesis.

To minimize disturbance of the animals, we chose to
administer LPS immediately after intrapreoptic injec-

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**Table 1. Effect of intrapreoptic ketorolac or aCSF on body temperature 2–3 h after injection of intravenous LPS**

<table>
<thead>
<tr>
<th>Ketorolac Injection Sites</th>
<th>n</th>
<th>Rise in T_b °C</th>
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<tbody>
<tr>
<td>Peri-OVLT region</td>
<td>8</td>
<td>0.48 ± 0.06*</td>
</tr>
<tr>
<td>Preoptic recess</td>
<td>7</td>
<td>0.60 ± 0.08*</td>
</tr>
<tr>
<td>Nucleus of the diagonal band</td>
<td>8</td>
<td>0.68 ± 0.10*</td>
</tr>
<tr>
<td>Other preoptic sites</td>
<td>15</td>
<td>0.84 ± 0.10†</td>
</tr>
<tr>
<td>Medial and lateral preoptic areas</td>
<td>6</td>
<td>0.88 ± 0.09</td>
</tr>
<tr>
<td>Third ventricle</td>
<td>2</td>
<td>0.68 ± 0.38</td>
</tr>
<tr>
<td>aCSF into anteroventral POA</td>
<td>5</td>
<td>1.40 ± 0.11</td>
</tr>
</tbody>
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Values are means ± SE. LPS, lipopolysaccharide; T_b, body temperature; OVLT, organum vasculosum of the lamina terminalis; POA, preoptic area. *P < 0.01 and †P < 0.05 compared with intrapreoptic artificial cerebrospinal fluid (aCSF) injection.
tion of ketorolac. PGE$_2$ begins to produce fever within 20 min of injection (26), but the kinetics of LPS fever suggest that PG synthesis probably begins ~100 min after intravenous LPS. Thus ketorolac had 100 min to diffuse throughout the POA, lessening the anatomic resolution of these injections compared with the PGE$_2$ injections of our previous study.

In our final experiment, preoptic microinjection of ketorolac markedly attenuated fever, but a modest rise in $T_b$ still occurred. These microinjections contained roughly $10^{-5}$ less ketorolac than was required systemically for antipyresis; most likely, a higher preoptic concentration or bilateral administration of ketorolac would be needed for complete antipyresis. LPS also might induce PG synthesis in other potentially chemosensory sites, such as the ventrolateral medulla or area postrema, and complete antipyresis could require COX inhibition in both the POA and medulla. Alternatively, the residual fever could have occurred through pathways independent of preoptic PG synthesis; other paracrine mediators such as nitric oxide could stimulate preoptic thermoregulatory circuits (27) or ascending

Fig. 5. Composite diagram of the effect on LPS fever of preoptic microinjections of ketorolac or aCSF. Each symbol represents the injection site and corresponding change in temperature 2–3 h after injection of LPS. The most highly effective ketorolac injections attenuated fever by 3 standard deviations below that seen with LPS and aCSF (<0.68°C). These most effective injections are clustered in anteroventral preoptic regions including the organum vasculosum of the lamina terminalis (OVLT), anteroventral preoptic nucleus (AVPV), ventromedial POA (VMPO), median preoptic nucleus (MnPO), and the preoptic recess. Less effective injections are scattered around this region and include the vertical limb of the diagonal band of Broca (DBB), medial POA (MPO), and lateral POA (LPO). LV, lateral ventricle; ox, optic chiasm; ac, anterior commissure; 3V, third ventricle.

<table>
<thead>
<tr>
<th>Rise in temperature</th>
<th>LPS 5 µg/kg &amp; ketorolac</th>
<th>$\times &lt; 0.68$ °C</th>
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<tbody>
<tr>
<td></td>
<td>LPS 5 µg/kg &amp; aCSF</td>
<td>$\times &gt; 0.68$ °C</td>
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vagal signals could directly stimulate preoptic fever-producing neurons (2, 23). These alternative pathways may influence fever, but our observation that fever is markedly attenuated after ketorolac injections into anteroventral preoptic sites demonstrates that preoptic PG synthesis clearly plays an important role.

Our results fit well with the previous work on COX inhibition in the POA. As demonstrated in several prior studies (6, 19, 34), we have shown that inhibition of hypothalamic COX significantly attenuates LPS fever. Our observations confirm and extend those of Vaughn and colleagues (34), showing that the most effective sites for COX inhibition are in the same preoptic region that responds best to PGE$_2$, suggesting that PGE$_2$ may act very close to its site of synthesis. In contrast to these prior studies, which were performed in rabbits and guinea pigs, we chose to perform our experiments in rats, because the neuroanatomy and physiology of the rat brain is well understood, providing an opportunity to identify specific fever-producing pathways. Finally, all prior studies used chronic cannulas for preoptic injections of COX inhibitors. These cannulas disturb local architecture and produce gliosis with microglial proliferation, which may act as an additional source of PGE$_2$, altering preoptic physiology. Acute microinjection with glass pipettes such as we used generally produces less trauma and inflammation in the region being studied.

Mechanisms of PG synthesis in fever. The PGs mediating fever are produced by COX, but the specific isozyme is unknown. We chose ketorolac for these experiments because it is among the most watersoluble COX inhibitors available and it inhibits both the constitutive form of COX, COX-1, and the inducible form, COX-2 (21). Recent evidence suggests that LPS fever may be mediated predominantly by COX-2 as selective COX-2 inhibitors block LPS fever (5). COX-2 is constitutively expressed in neurons of several brain regions including the anteroventral POA (3). LPS rapidly induces COX-2 in microglia surrounding vessels within the brain and in meningeal macrophages, especially within the leptomeninges just above the optic chiasm (7). Cao and colleagues (4) have demonstrated that LPS also induces COX-2 in endothelial cells. The presence of induced COX-2 in microglia, macrophages, and endothelium suggests that these barrier cells may play a key role in the response to cytokines.

The afferent signals that drive preoptic PGE$_2$ synthesis are the subject of considerable debate. Several researchers have proposed that the vagus nerve may mediate fever and other responses to systemic inflammation. Vagal signals to the medulla may be conveyed to the hypothalamus via sensory roots or interfere with the function of perivascular or meningeal cells to activate fever-producing circuitry in nearby thermoregulatory areas. Ascending vagal signals may also drive PG synthesis, and future experiments that lesion vagal pathways (8). The paraventricular nucleus is well positioned to produce fever by regulating autonomic activity that increases brown fat thermogenesis and diverts blood from superficial (e.g., tail vein) to deep vascular beds to limit heat loss.

Perspectives

Our observations indicate that LPS fever requires production of PGs within the anteroventral region of the POA. Most likely, these PGs act as paracrine signaling molecules, diffusing outward from cytokine-stimulated perivascular or meningeal cells to activate fever-producing circuitry in nearby thermoregulatory areas. Ascending vagal signals may also drive PG synthesis, and future experiments that lesion vagal sensory roots or interfere with the function of perivascular microglia and meningeal macrophages may help distinguish between these hypotheses. In addition, we know little about the preoptic location and type of PGE$_2$ receptors mediating the subsequent febrile response. Oka and Hori (20) have presented convincing evidence that EP$_1$ receptors may mediate fever, although recent reports suggest a role for the EP$_2$ and EP$_3$ receptors as well (22). We have recently identified the EP$_1$ and EP$_3$ types of PGE$_2$ receptor in the POA using the reverse transcriptase-polymerase chain reaction (unpublished results), and EP$_3$ receptors have been localized in the POA using in situ hybridization histochemistry (33). Which of these receptors are present on thermoregulatory neurons remains to be determined. Molecular studies such as these will help define the preoptic mechanisms governing fever and provide greater insights into how LPS and cytokines influence the brain.

The authors are grateful to Quan Ha for technical assistance. Jeff Kitson of Syntex kindly provided ketorolactromethamine.
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