Indomethacin impairs LPS-induced behavioral fever in toads

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Bicego, K. C., A. A. Steiner, J. Antunes-Rodrigues, and L. G. S. Branco. Indomethacin impairs LPS-induced behavioral fever in toads. J Appl Physiol 93: 512–516, 2002; 10.1152/japplphysiol.00121.2002.—We tested the hypothesis that PGs mediate lipopolysaccharide (LPS)-induced behavioral fever in the toad Bufo paracnemis. Measurements of preferred body temperature (Tb) were performed with a thermal gradient. Toads were injected intraperitoneally with the cyclooxygenase inhibitor indomethacin (5 mg/kg), which inhibits PG biosynthesis, or its vehicle (Tris) followed 30 min later by LPS (0.2 and 2 mg/kg) into the lymph sac. LPS at the dose of 0.2 mg/kg caused a significant increase in Tb from 7 to 10 h after injection, and then Tb returned toward baseline values. LPS at the dose of 2 mg/kg produced a different pattern of response, with a longer latency to the onset of fever (10th h) and a longer duration (until the end of the experiment at the 15th h). Tris significantly attenuated the fever induced by LPS at 0.2 mg/kg, but not at 2 mg/kg. Moreover, indomethacin completely blocked the fever evoked by LPS (2 mg/kg). These results indicate that the behavioral fever induced by LPS in toads requires the activation of the COX pathway, suggesting that the involvement of PG in fever has an ancient phylogenetic history and that endogenous PGs raise the thermoregulatory set point to produce fever, because behavioral thermoregulation seems to be related to changes in the thermoregulatory set point.

FEVER IS THE MOST OUTSTANDING component of a complex host response to invading agents called “acute-phase response” (3), which also includes neutrophilia, activation of the hypothalamic-pituitary-adrenal axis, and changes in serum metal levels (9). By definition, fever is a regulated increase in body core temperature (Tb) characterized by a raised thermoregulatory set point (14).

A number of studies have reported that, besides endothermic species, a variety of ectothermic vertebrates (as well as invertebrates) develop fever in response to injections of exogenous pyrogens, such as lipopolysaccharide (LPS; endotoxin), viruses, gram-positive bacteria, and yeast. LPS, which is the most purified form of a compound from the cell wall of gram-negative bacteria, usually Escherichia coli, has been extensively used to induce fever in experimental animals (for a review, see Ref. 14). In mammals, it is generally believed that LPS-induced fever is mediated by stimulation of immune cells to produce and release a complex variety of soluble mediators, called endogenous pyrogens. Among these, the cytokines interleukin (IL)-1β, IL-6, interferons, and tumor necrosis factor (TNF) (13, 14) are thought to convey the pyrogenic message to the brain region where fever is regulated, namely the preoptic area of the anterior hypothalamus (POAH) (3).

Classically, PGE2 is considered to be the most proximal mediator of fever, acting on the POAH (16, 17). Evidence in favor of this hypothesis is that the levels of PGE2 raise in the POAH in conjunction with the generation of fever (25), and administration of PGE2 into the POAH causes a rise in Tb (21, 23). Moreover, pretreatment of humans and experimental animals with inhibitors of cyclooxygenase (COX), the key enzyme in PG biosynthesis, attenuates fever (17, 33, 34), and COX inhibitors do not affect fever induced by the PGE series (18). However, not all fevers are mediated via PGs, because, in rats, some endogenous pyrogens, such as macrophage inflammatory protein-1 (8), IL-8 (34), and endothelin-1 (10), have been reported to evoke PG-independent fever, i.e., a fever that is not blocked by a COX inhibitor.

Amphibians, as well as all ectothermic species, rely essentially on behavior for Tb regulation (11), which is usually related to changes in the thermoregulatory set point (5, 6, 31). This characteristic and their phylogenetic position certainly make these animals interesting models to study thermoregulation and evolution of fever. In fact, it has been reported that amphibians exhibit a behavioral fever in response to bacterial or endogenous pyrogenic agents (for a review, see Ref. 11). Recently, we demonstrated that the toad Bufo paracnemis develops behavioral fever after LPS injection into the lymph sac (2), but whether this response is dependent on PG has not been assessed.

To our knowledge, only one study demonstrated that the synthesis of PG is necessary for the development of fever in an ectothermic vertebrate. Bernheim and Kluger (1) reported that the COX inhibitor sodium
salicylate attenuates the behavioral fever induced by the bacteria Aeromonas hydrophila in the lizard Dip- sosaurus dorsalis. As to amphibians, PGs have already been suggested to act as pyretic mediators in the central nervous system (12, 19), but no data exist on the role of endogenous PGs in the development of fever in these animals.

In view of these considerations, we tested the hypothesis that PGs are involved in LPS-induced behavioral fever in the toad Bufo paracnemis. To this end, we measured the effect of the COX inhibitor indomethacin on preferred $T_b$ of toads injected with LPS.

MATERIALS AND METHODS

Animals. Toads (Bufo paracnemis, Lutz) of both sexes, weighing 150–250 g, were collected in the vicinity of Ribeirao Preto, Sao Paulo state, Brazil. The toads were maintained in containers with free access to water and basking area. All animals were fed cow liver twice a week until at least 5 days before experiments. The experiments were performed during spring (from October to December 2000) and summer (from January to March 2001), which are the rainy seasons in Brazil.

Measurement of preferred $T_b$. Preferred $T_b$ was determined in a thermal gradient chamber (1.50 m long, 0.15 m high, and 0.20 m wide) with an aluminum floor. One end of the floor was cooled to 10°C by a copper pad connected to a refrigerated water bath (1160A, VWR Scientific, Niles, IL). The other end was heated to 38°C by another copper pad connected to another water bath (310A, Barnstead/Thermolyne, Dubuque, IA). Petri dishes filled with tap water throughout the chamber provided access to water at all temperatures, and the chamber was continuously flushed with humidified room air at a rate of 1.5 l/min. An animal with a temperature probe, which was secured 2 cm into the cloaca with skin sutures, was placed in the center of the thermal gradient, and the thermistor output was continuously displayed on a chart recorder (LR93125, Barnstead/Thermolyne). Cold and warm water were used to calibrate the temperature probes before each experiment.

Experimental procedure. Experiments were performed on unanesthetized, unrestrained, and undisturbed toads. One toad was placed in the middle of the thermal gradient and left there for a period of ~8 h. Saline or 0.2 or 2 mg/kg body wt of LPS (from Escherichia coli, serotype 0111:B4, Sigma Chemical) were injected into the dorsal lymph sac of the animals, and preferred $T_b$ was monitored for an additional 15 h. The doses of LPS were chosen based on a previous study from our laboratory (2) and on pilot experiments. To verify the effect of the COX inhibitor, the same protocol was performed except that, 30 min before LPS injection, 5 mg/kg body wt of indomethacin or its vehicle, Tris·HCl buffer (0.2 M Tris; pH 8.3), were injected intraperitoneally. This dose of indomethacin was chosen on the basis of previous studies in rabbits (33) and rats (26). Even though no report exists about the appropriate dose of indomethacin in amphibians, the same dose used in rats and rabbits (5 mg/kg) was shown to be effective in affecting fever in our experiments. Indomethacin was dissolved in pyrogen-free Tris just before the injections.

Calculations and statistical analysis. Mean preferred $T_b$ was determined every hour in all experiments from individual chart paper recordings based on a previous calibration. Two-way ANOVA was used for data analysis followed by a point-by-point unpaired t-test to assess differences between groups. Tukey’s test was applied as a post hoc test to find differences over time. All values are reported as means ± SE. Values of $P < 0.05$ were considered to be significant.

RESULTS

During the control period (5 h before injections), preferred $T_b$ ranged from 20 to 29°C in all protocols. Pretreatment with Tris did not change the preferred $T_b$ of saline-treated toads (Fig. 1A).

Figure 1B shows that LPS at the dose of 0.2 mg/kg caused a significant increase in preferred $T_b$ from 7 to 10 h after injection ($P < 0.05$), followed by the return of $T_b$ toward control values. However, pretreatment with

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**Fig. 1.** Effect of saline or lipopolysaccharide (LPS) alone or combined with indomethacin vehicle (Tris) on the preferred body temperature ($T_b$) of toads. Tris was injected intraperitoneally 30 min before saline or LPS injection into the lymph sac. Arrows indicate time of injections. Preferred $T_b$ values recorded between the 2 injections are not plotted. Values are means ± SE. *Significant effect compared with time 0, $P < 0.05$. †Significant difference between groups at the same time points, $P < 0.05$. Nos. in parentheses, no. of animals (A: 6 male, 5 female; B: 8 male, 5 female; C: 4 male, 7 female).
Tris significantly attenuated the behavioral fever induced by injection of 0.2 mg/kg of LPS (P < 0.05; Fig. 1B). When the higher dose (2 mg/kg) of LPS was used, significant increases in preferred T_b were observed from 10 to 15 h after injection (P < 0.05; Fig. 1C). Because Tris did not affect this response (Fig. 1C), the dose of 2 mg/kg LPS was chosen for further experiments.

Indomethacin caused no change in the preferred T_b of saline-injected toads, and no significant difference was observed between the indomethacin-saline and Tris-saline groups (Fig. 2). As can be seen in Fig. 3, pretreatment with indomethacin abolished the increase in preferred T_b induced by injection of 2 mg/kg LPS. The preferred T_b of the Tris-LPS group was significantly higher than that of the indomethacin-LPS group from 11 to 15 h after LPS injection (P < 0.05; Fig. 3).

DISCUSSION

The present study provides evidence that the synthesis of PGs is necessary for the development of LPS-induced behavioral fever in toads, because this response was abolished by the COX inhibitor indomethacin in Bufo paracnemis.

As reviewed by Kluger (14), fever results from a rise in the thermoregulatory set point. In mammals, which use autonomic and behavioral mechanisms to maintain T_b, fever is functionally expressed as increases in metabolic heat production and decreases in heat loss, besides behavior (7). However, thermoregulation in ectotherms is primarily behavioral, and thus preferred T_b has been shown to be directly related to changes in the thermoregulatory set point (5, 6, 31). This characteristic certainly makes ectothermic species an interesting model to study thermoregulation.

With few exceptions, ectothermic vertebrates develop fever in response to injections of endotoxin or other substances pyrogenic to mammals (14). In a recent study, we demonstrated that LPS induces behavioral fever in an anuran amphibian, the toad Bufo paracnemis (2). In that study, T_b was monitored for 11 h, and it was observed that LPS at the dose of 0.2 mg/kg body wt caused a significant increase in the preferred T_b of toads from 8 to 11 h after injection into the lymph sac. In the present study, we measured preferred T_b for 15 h after LPS injection and noticed that, after the 11th h, the T_b of toads injected with 0.2 mg/kg LPS returned toward baseline. The reason why Tris caused a reduction in fever induced by the lower dose of LPS is not clear to us. As far as we know, no study has ever tested the effects of Tris on LPS-induced fever, even though Tris was used as vehicle (32, 33).

Similar to our data, a recent study (27) found that DMSO, a vehicle frequently used to increase the solubility of lipophilic drugs, reduced LPS fever in guinea pigs. Interestingly, DMSO has been shown to inhibit LPS-induced TNF-α and nitric oxide production and to reduce TNF-α mRNA levels in Kupffer cells of rats. DMSO also suppresses the LPS-induced nuclear factor-κB activation, an important signaling factor for LPS-induced effects, in a murine macrophage-like cell line (20). However, the mechanisms by which Tris attenuates fever in toads remain to be determined. However, the fact that Tris impaired the behavioral fever induced by 0.2 mg/kg LPS (Fig. 1B) precluded the use of this dose of LPS in further experiments.

Thus we tested the higher dose of LPS, 2 mg/kg. As expected, this dose of LPS produced a longer fever, which lasted until the end of the experiment, but this fever presented longer onset latency (10 h). The reason for this longer latency for the onset of fever is unclear, even though it may reside in differences among the febrile responsiveness of toads during spring and summer. In fact, the fever curves for LPS at 0.2 and 2 mg/kg were obtained in the spring and summer, respectively. However, this fact does not complicate the interpretation of data because all of the experiments involving the dose of 0.2 mg/kg were performed during the spring, whereas all of the experiments that used

![Fig. 2. Effect of indomethacin on preferred T_b of toads. Tris or indomethacin was injected intraperitoneally 30 min before saline injection into the lymph sac. The Tris + saline curve is the same as that plotted in Fig. 1A. Arrows indicate time of injections. Preferred T_b values recorded between the 2 injections are not plotted. Values are means ± SE. Nos. in parentheses, no. of animals (3 male, 8 female).](http://jap.physiology.org/DownloadedFrom/10.1152/jappl.01238.2001)

![Fig. 3. Effect of indomethacin on LPS-induced behavioral fever in toads. Tris or indomethacin was injected intraperitoneally 30 min before LPS injection into the lymph sac. The Tris + LPS 2 mg/kg body wt curve is the same as that plotted in Fig. 1B. Arrows indicate time of injections. Preferred T_b values recorded between the 2 injections are not plotted. Values are means ± SE. *Significant effect compared with time 0, P < 0.05. †Significant difference between groups at the same time points, P < 0.05. Nos. in parentheses, no. of animals (5 male, 5 female).](http://jap.physiology.org/DownloadedFrom/10.1152/jappl.01238.2001)
LPS at 2 mg/kg were done during the summer. Moreover, differences in the pharmacokinetics (distribution and clearance) between the lower and the higher dose of LPS might also help to explain the longer latency of 2 mg/kg LPS fever. Anyhow, because pretreatment with Tris did not affect the behavioral fever induced by 2 mg/kg LPS (Fig. 1C), this dose was chosen to study the effect of indomethacin on the febrile response of toads.

Besides the fact that exogenous pyrogens elicit behavioral fever in amphibians (2, 11, 14), to our knowledge only one study demonstrated a role of endogenous pyrogens in febrile frogs. *Rana esculenta* injected intraperitoneally with plasma from frogs previously injected with killed *Mycobacterium ranae* select warmer temperatures, elevating their colonic temperature by 2–5.3°C (19). Injection of frogs with plasma from normal, untreated frogs does not affect preferred Tb. These results, associated with the fact that the latency of the febrile response of frogs that received plasma of infected animals was shorter than the response of frogs that were injected directly with killed bacteria, led the authors to suggest that the blood of donor frogs contained pyrogenic substances different from bacterial endotoxins. However, the nature of these endogenous pyrogens has not been assessed.

As to the role of PG in the development of fever in amphibians, these agents have already been suggested to act as pyretic mediators in the central nervous system (12, 19). PGE₁ injected into the third ventricle of the salamander *Necturus maculosus* results in a relatively long-lasting behavioral fever (12). As to anuran amphibians, Myhre et al. (19) observed an increase in preferred Tb of *Rana esculenta* injected with a solution of sodium PGE₁ into the diencephalon. However, the method used by Myhre et al. deserves some comments. One problem with this protocol is that the increase in preferred Tb of frogs observed after PGE₁ injection may have occurred because of manipulation and surgical stress because the frogs were placed in the thermal gradient immediately after surgery. Another problem is that two of the five frogs used for those experiments died ~30 min after PGE₁ injection. Furthermore, because no saline injection was made just after surgery, it is difficult to conclude that the increase in preferred Tb was a specific effect of PGE₁. Despite these methodological considerations, the results obtained with exogenous PGE are in accordance with the role of PG in inducing fever in mammals by acting on the POAH (23, 25).

It is important to point out that the present study is the first to suggest a role of endogenous PGs in the development of behavioral fever in amphibians. The nonselective COX inhibitor indomethacin administered intraperitoneally caused no change in preferred Tb of nonfebrile toads (Fig. 2), whereas it blocked LPS-induced behavioral fever (Fig. 3), which is in agreement with previous results obtained for mammals (17, 33). As to other ectothermic species, Bernheim and Kluger (1) reported that, in the lizard *Dipsosaurus dorsalis*, the COX inhibitor sodium salicylate attenuates behavioral fever induced by *Aeromonas hydrophila* bacteria.

Although our data indicate that indomethacin plays an antipyretic role in toads by inhibiting PG synthesis, we did not establish the exact site at which indomethacin acts to evoke antipyresis. At least in mammals, intraperitoneal injection of indomethacin has been shown to inhibit peripherally and centrally the two isoforms of the enzyme COX, i.e., the inducible (COX-2) and constitutive (COX-1) isoforms (29, 30). In rats, injection of ketorolac, a water-soluble COX inhibitor, into the anterovenal POAH markedly attenuates the fever produced by LPS, indicating that PG synthesis in this area is necessary for the production of fever (24). As to amphibians, it is known that the brain tissue of frogs is able to produce PGE₂, which is markedly reduced by incubation with indomethacin (15). Taken together, these data would point at the central nervous system as a target for the antipyretic effect of indomethacin in toads. However, a role of blood-borne PGE₂ in the genesis of fever may not be excluded, because intravenous injection of albumin-bound PGE₂ induces fever in rabbits (22). Anyway, even though the source of febrigenic PGE₂ is controversial, this molecule seems to cause fever by acting on the POAH of mammals (21, 23, 25, 28) and amphibians (19).

Because indomethacin inhibits the behavioral fever of toads, whose changes in preferred Tb are thought to be related to changes in the thermoregulatory set point, our data reinforce the idea that PG induces fever by raising the thermoregulatory set point. In fact, sheep infused with PGE₁ into a lateral cerebral ventricle develop fever at ambient temperatures of 10, 18, and 40–45°C with the use of different heat loss and heat production effectors (4), which indicates that PGE₁ affects the thermoregulatory set point rather than a specific thermoregulatory mechanism.

In conclusion, our data support the fact that, similar to mammals, LPS-induced fever in toads is dependent on the synthesis of PGs, indicating that this mechanism has an ancient phylogenetic history.

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