The effects of selective and nonselective cyclooxygenase inhibitors on endothelin-1-induced fever in rats

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Submitted 5 August 2004; accepted in final form 10 November 2004

IT IS NOW WELL ESTABLISHED that the PGs represent the final mediators of fever induced by exogenous and endogenous pyrogens through an action on PG receptor-expressing neurons in the preoptic area (POA) of the anterior hypothalamus (33). In fact, PGE2 induces fever when injected centrally (13, 33, 38), and its levels in the cerebrospinal fluid (CSF) and in the POA rise in parallel with the generation of fever caused by several stimuli (9, 12, 16, 19, 23, 27, 35, 42). Moreover, the inhibition of PG synthesis by cyclooxygenase (COX) inhibitors attenuates fever in humans and experimental animals, whereas these drugs do not affect fever induced by the administration of PGs (4, 7, 12, 13, 37, 39). PGF2α also induces fever when injected centrally (13, 34), and its levels are increased in rat CSF in response to LPS (10).

We have previously observed that endothelin-1 (ET-1), a member of the ET family of peptides (29), acts as a mediator of LPS-induced fever (15). In fact, intracerebroventricular (icv) pretreatment with the selective ETB receptor antagonist BQ-788 blunted fever induced by intravenous LPS or intracerebroventricular ET-1 (15). The rise in core temperature induced by intracerebroventricular ET-1 was accompanied by such thermoeffector response as cutaneous vasoconstriction, measured as a decrease in tail skin temperature (Fabricio, unpublished observation). The simultaneous occurrence of increased core temperature and cutaneous vasoconstriction defines the effect of ET-1 as a real fever, as the elevation in core temperature accompanied by tail skin vasodilation is defined rather as hyperthermia (2).

Fever induced by intracerebroventricular injection of 100 fmol ET-1 in the rat is not modified by the pretreatment with a nonselective COX inhibitor, indomethacin, whereas the ETB receptor blockade does not affect fever induced by centrally administered IL-1β or TNF-α (15), whose pyrexic effects are effectively suppressed by indomethacin (13). Taken together, these findings led us to propose that, similar to corticotropin-releasing hormone, macrophage inflammatory protein-1, preformed pyrogenic factor, and IL-8 (13, 31, 46, 47), ET-1 may act as a mediator of a putative COX-independent pathway of fever triggered by LPS in rats (15).

Another line of evidence shows that COX-2 isoform plays a crucial role in fever. The deletion of genes encoding COX-2 (26) or PGE receptor subtype EP3 (44) prevents febrile response to LPS. Selective COX-2 inhibitors suppressed both LPS-induced fever (7, 45, 48) and the rise in PGE2 levels in the CSF (45). Furthermore, the induction of COX-2 was closely correlated with fever in terms of both timing and magnitude (7). Although ETs were shown to increase COX-2 expression and PG production in a variety of cells, including rat astrocytes (24), to our knowledge, no study has been carried out to investigate the effects of selective COX-2 inhibitors, also referred to as coxib, on ET-1-induced fever. Here, we investigated the effect of two coxib agents, celecoxib and lumiracoxib, on fever induced by centrally injected ET-1, and compared these effects with those of indomethacin. The relationship between fever and the levels of pyrogenic prostaglandins, PGE2 and PGF2α, in the CSF were also investigated following the above treatments. LPS was taken as a positive control in this experimental paradigm.

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METhODS

Animals. Experiments were conducted using male Wistar rats weighing 180–200 g, housed individually at 24 ± 1°C under a 12:12-h light-dark cycle (lights on at 0600) with free access to food and tap water until the day of the experiment, when only water was made available. All experiments were previously approved by the ethical committee for research on laboratory animals of the University of São Paulo and were performed in accordance with Brazilian ethical committee for research on laboratory animals of the Institute for Laboratory Animal Research (20).

Intracerebral cannula implantation. Under anesthesia with pentobarbital sodium (40 mg/kg ip), a permanent 22-gauge stainless steel guide cannula (0.7 mm OD, 10 mm long) was stereotaxically implanted into the right lateral ventricle at these coordinates: 1.6 mm lateral to the midline, 1.5 mm posterior to bregma, and 2.5 mm under the brain surface of the brain (incisor bar was lowered 2.5 mm below the horizontal zero) (36). Cannulas were fixed to the skull with jeweler’s screws embedded in dental acrylic cement. Animals were then treated with oxytetracycline hydrochloride (400 mg/kg im) and allowed to recover for 1 wk before the experiments. After each experiment, the animals were anesthetized (as before), and the location of the cannula track was verified histologically. Animals showing cannula misplacement, blockage upon injection, or abnormal weight gain patterns during the postimplantation period were excluded from the study.

Temperature measurements. Rectal temperature was measured in conscious and unrestrained rats for 1 min every 30 min for up to 6 h, in most cases, by gently inserting a small thermistor probe (model 402 coupled to a model 46 telethermometer, Yellow Springs Instruments, Yellow Springs, OH) 4 cm into the rectum, without removing them from their home cages. Experimental measurements were conducted at the thermonutral zone for rats (17) in a temperature-controlled room (28 ± 1°C), after the animals adapted to this environment for at least 1 h. After this period, baseline temperature was determined 4 times at 30-min intervals before any injections, and only animals displaying mean basal rectal temperatures between 36.8 and 37.4°C were selected for the study. To minimize core temperature changes due to handling, animals were conditioned to this environment and procedure twice on the preceding day. The experiments involving indomethacin were conducted essentially as described above, except that core body temperature was measured by using battery-operated biotelemetry transmitters (Data Science, St. Paul, MN) implanted in the peritoneal cavity at the same time as intracerebroventricular biotelemetry transmitters (Data Science, St. Paul, MN) implanted in the brain surface of the brain (incisor bar was lowered 2.5 mm below the horizontal zero) (36). Cannulas were fixed to the skull with jeweler’s screws embedded in dental acrylic cement. Animals were then treated with oxytetracycline hydrochloride (400 mg/kg im) and allowed to recover for 1 wk before the experiments. After each experiment, the animals were anesthetized (as before), and the location of the cannula track was verified histologically. Animals showing cannula misplacement, blockage upon injection, or abnormal weight gain patterns during the postimplantation period were excluded from the study.

CSF sampling and determination of PG levels in the CSF. A single sample of CSF was collected from each animal according to the method described by Consiglio and Lucion (11) for estimation of immunoreactive PG levels. Briefly, each rat was anesthetized with pentobarbital sodium (40 mg/kg) and fixed to the stereotaxic apparatus, with its body flexed downward. Thereafter, the skin covering the base of the skull and back of the neck was incised and wet with a cotton swab soaked with ethanol to reveal a small depression between the occipital protuberance and the atlas. A scalp cannula connected to a syringe was then inserted through the depression into the cisterna magna to collect 60- to 100-μl samples, which were centrifuged at 1,300 g for 15 min, frozen immediately and kept at −20°C until assayed for PG immunoreactivity. Samples contaminated with blood were discarded. After dilution (1:5) of the CSF samples, PGE2 or PGF2α was measured using ELISA kits from Cayman Chemical (Ann Arbor, MI) following the procedures detailed in the instructions. Detection limits of PGE2 and PGF2α ELISA assays were 15 and 8 pg/ml, respectively. The intra- and interassay coefficients of variation were <10% for both assays. Cross-reactivity data were as follows: 43% with PGE3, 18.7% with PGE1, 1% with 6-keto-PGF1α, 0.25% with 8-iso-PGF2α, and <0.01% with all other prostanoids tested (including PGF2α) in the PGE2 assay; 51% with PGD2, 1% with 6,15-diketo-13,14-dihydro PGF1α, 0.4% with PGF2α, and 0.2% with 11-dehydro-thromboxane B2, and <0.01% with all other prostanoids tested in the PGF2α assay.

Experimental protocols. In a first set of experiments, we tested the effect of a nonselective inhibitor of COX, indomethacin (2 mg/kg ip) or its vehicle (Tris·HCl, pH 8.2), 30 min before injection, on changes of temperature induced by Escherichia coli LPS (5 μg/kg iv), ET-1 (1 pmol icv), or their vehicles, sterile saline (1 ml/kg iv; A) or artificial cerebrospinal fluid (aCSF icv; B). Values represent means ± SE of the changes in rectal temperature (∆T) of 4 to 7 animals. *P < 0.05 compared with corresponding values of the LPS-treated group.

Fig. 1. Effect of indomethacin (Indo) on fever induced by LPS or endothelin-1 (ET-1) in rats. Indo (2 mg/kg ip) or Tris·HCl (Tris, pH 8.2) was injected 30 min before LPS (5 μg/kg iv; A) or ET-1 (1 pmol icv; B). Control animals received sterile saline (Sal, 1 ml/kg iv; A) or artificial cerebrospinal fluid (aCSF icv; B). Values represent means ± SE of the changes in rectal temperature (∆T) of 4 to 7 animals. *P < 0.05 compared with corresponding values of the LPS-treated group.

In another series of experiments, we analyzed the changes in the PG2α and PG2β levels in the CSF of rats 3 h after the intravenous injection of 5 μg/kg LPS or the central injection of ET-1 (1 pmol icv). We have chosen a 3-h interval between pyrogen administration and collection of CSF for PG determination, as this allows sufficient time for transcription (25) and translation of the COX-2 protein (3), as well as for maximal rise of both PGs in CSF after LPS injection (19). Finally, we investigated the effect of indomethacin (2 mg/kg), celecoxib (1, 2.5, and 5 mg/kg) or their vehicles on the increase in CSF PG levels induced by ET-1.
For intracerebroventricular injections of ET-1, a 31-gauge needle, connected by polyethylene tubing to a 25-μl Hamilton gas-tight syringe (Hamilton, Birmingham, UK), was lowered into the guide cannula so that it protruded 2.5 mm beyond its tip into the ventricle, and a volume of 3 μl was slowly infused over 1 min to avoid abrupt increases in CSF volume. LPS (5 μg/kg, 200 μl/rat) or the corresponding vehicle (sterile saline) was administered to the rats by intravenous injection via a lateral tail vein. Pyrogenic stimuli were always injected between 1000 and 1100 to minimize possible diurnal variability.

Drugs. The following drugs were used: ET-1 from Research Biochemicals International (Natick, MA); LPS (E.coli 0111:B4) and pentobarbital sodium from Sigma (St. Louis, MO); oxytetracycline hydrochloride (Terramycin) from Pfizer (São Paulo, Brazil); indomethacin, a gift from Merck, Sharp & Dohme (São Paulo, Brazil); celecoxib (Celebra) from Pharmacia (São Paulo, Brazil); and lumiracoxib, kindly provided by Novartis Pharma SpA (Origgio, Varese, Italy).

Statistical analysis. All variations in rectal temperature were expressed as changes from the mean basal value (i.e., ΔT, in °C). Values are presented as means ± SE, and statistical comparisons were performed by one-way ANOVA followed by Tukey’s test or by Student’s t-test for comparison between group means, when appropriate, using a SPSS statistical software (SPSS, Chicago, IL). Differences were considered significant when P < 0.05.

RESULTS

Experiments on fever. Intravenous injections of 5 μg/kg LPS produced a fever with a rapid rise, peaking at 2–3 h after administration; the rectal temperature remained elevated throughout a 6-h observation period (Figs. 1A, 2A, and 3A). The central injection of ET-1 (1 pmol icv) caused a slowly-developing and long-lasting fever (Figs. 1B, 2B, and 3B). The pretreatment of rats with indomethacin (2 mg/kg ip) significantly reduced, but by no means abolished, the pyrogenic response to intravenous injection of LPS (Fig. 1A). In sharp contrast, indomethacin did not affect fever induced by ET-1 (Fig. 1B).

On the other hand, both celecoxib (5 and 10 mg/kg) and lumiracoxib (5 mg/kg) prevented fever induced either by LPS or ET-1, whereas they did not modify the rectal temperature of control rats (Figs. 2 and 3). The effect of celecoxib or lumiracoxib on LPS- or ET-1-induced fever was also expressed as the

Fig. 2. Effect of celecoxib on fever induced by LPS or ET-1 in rats. Animals were pretreated with celecoxib (1, 2.5, or 5 mg/kg) or sterile water (H2O, 1 ml/animal) by oral gavage 1 h before injection of LPS (5 μg/kg iv; A) or ET-1 (1 pmol icv; B). Control animals received Sal (1 ml/kg iv) or aCSF icv. A dose of 10 mg/kg celecoxib completely prevented LPS- and ET-1-induced fever (results not shown). Values represent means ± SE of the changes in ΔT of 5–10 animals. *P < 0.05 compared with the corresponding value of the LPS-treated group (A) or ET-1-treated group (B).

For intracerebroventricular injections of ET-1, a 31-gauge needle, connected by polyethylene tubing to a 25-μl Hamilton gas-tight syringe (Hamilton, Birmingham, UK), was lowered into the guide cannula so that it protruded 2.5 mm beyond its tip into the ventricle, and a volume of 3 μl was slowly infused over 1 min to avoid abrupt increases in CSF volume. LPS (5 μg/kg, 200 μl/rat) or the corresponding vehicle (sterile saline) was administered to the rats by intravenous injection via a lateral tail vein. Pyrogenic stimuli were always injected between 1000 and 1100 to minimize possible diurnal variability.

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Statistical analysis. All variations in rectal temperature were expressed as changes from the mean basal value (i.e., ΔT, in °C). Values are presented as means ± SE, and statistical comparisons were performed by one-way ANOVA followed by Tukey’s test or by
percentage of inhibition of the total response (Fig. 4). In LPS-induced fever (Fig. 4A) the estimated dose that resulted in a 50% inhibitory effect (ID$_{50}$) was 3.4 mg/kg (8.89 µmol/kg) for celecoxib and 1.26 mg/kg (4.29 µmol/kg) for lumiracoxib. In ET-1-induced fever, the estimated ID$_{50}$ was 4.3 mg/kg (11.3 µmol/kg) for celecoxib and 1.6 mg/kg (5.45 µmol/kg) for lumiracoxib (Fig. 4B). Thus on a molar basis, lumiracoxib was twice as potent as celecoxib as an antipyretic on both LPS and ET-1-induced fever.

**Experiments on CSF PG levels.** Cisternal CSF of the animals was collected 3 h after injection, when both LPS and ET-1 have induced significant increases in core temperature (Fig. 5A).

Baseline CSF levels of PGE$_2$ and PGF$_{2\alpha}$ were 138.7 ± 55.3 and 70.5 ± 17.5 pg/ml, respectively, in animals receiving intravenous saline, and 212.4 ± 87.25 and 89.4 ± 25.05 pg/ml, respectively, in animals receiving intracerebroventricular aCSF (Fig. 5, B and C). LPS induced a fourfold increase in PGE$_2$ levels and a 3-fold increase in PGF$_{2\alpha}$ levels in the cisternal CSF compared with control animals. ET-1 induced a similar increase in PGF$_{2\alpha}$ (~3-fold) but was more effective than LPS in increasing CSF PGE$_2$ levels (~6-fold) compared with animals receiving aCSF (Fig. 5, B and C). There appears to be no correlation between temperature and PGE$_2$ levels in CSF: LPS-induced fever is almost twice the magnitude of ET-induced fever, while PGE$_2$ levels in LPS-injected animals are lower than those of animals receiving ET-1.

**DISCUSSION**

The present study confirms previous observations by our group that exogenous ET-1 induces fever in rats (15) and...
reveals that this effect is accompanied by an increase in PG levels in the CSF. Furthermore, it shows that selective COX-2 inhibitors reduce fever caused by intracerebroventricular ET-1 in the rat; this effect is accompanied by a reduction in ET-1-stimulated PG levels in the CSF. These findings suggest that ET-1 induces fever via an increase in COX-2-generated PGs.

The COX-2 isoform has been localized at the level of different cell types in the rat brain; first, COX-2 is inducible in endothelial cells of brain vasculature, where its expression can be stimulated by LPS and pyrogenic cytokines (3–7, 30), as well as in glial cells, where COX-2 is also sensitive to induction by endotoxin, proinflammatory cytokines, and other factors, notably including ET-3 (14, 24, 32). Second, COX-2 gene expression is constitutive in neurons of various brain areas, including the hypothalamus (1). Because selective COX-2 inhibitors in this study were always given before the administration of pyrogenic stimuli, this experimental design does not allow us to distinguish whether they act on constitutive COX-2 in neurons, the inducible isoform in endothelial and glial cells, or both targets.

Findings presented here provide pharmacological evidence that COX-2 and its PG products are involved in the mechanism of ET-1-induced fever. Indeed, the antipyretic actions of lumiracoxib and celecoxib in our model correlate well with their selectivity in inhibiting COX-2 activity (8) and differs from currently available selective COX-2 inhibitors, including celecoxib (28), in a number of important structural aspects. In our experimental paradigm, lumiracoxib proved to be a more potent antipyretic than celecoxib on fever induced by LPS or ET-1, which reflects different selectivity of the two drugs in inhibiting COX-2 in vitro: IC₅₀ ratio COX-1/COX-2 of 400 for lumiracoxib compared with an IC₅₀ ratio COX-1/COX-2 of 30 for celecoxib (8). Thus our results confirm and extend previous observations supporting a role for COX-2 in the induction of fever (3–7, 26, 30, 45, 48).

Here, we show that the ET-1-induced fever is accompanied by an increase of PG levels in the CSF in the rat. It is well known that a number of physiological and pathophysiological actions of ETs in the body are mediated through the activation of COX pathway (40, 41). That occurs in most, albeit not in all, cases after the activation of pyrogenic stimuli, this experimental design does not allow us to distinguish whether they act on constitutive COX-2 in neurons, the inducible isoform in endothelial and glial cells, or both targets.

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Here, we show that the ET-1-induced fever is accompanied by an increase of PG levels in the CSF in the rat. It is well known that a number of physiological and pathophysiological actions of ETs in the body are mediated through the activation of COX pathway (40, 41). That occurs in most, albeit not in all, cases after the activation of ETₐ receptor subtype (18, 24, 43), and we have previously shown that such receptor is specifically involved in fever (15). ET-1 and ET-3, which shares with ET-1 the ability to activate ETₐ receptors (29), increase COX-2 expression via ETₐ receptors in macrophages and astrocytes, respectively, to which a specific increase in PGE₂ production ensues (24, 43); PGE₂ production in ET-1-stimulated macrophages is almost completely inhibited by NS-398, a COX-2 selective inhibitor (43).
Taken collectively, our data and the evidence discussed above would fit into a simple theoretical model, according to which ET-1 induces fever in the rat through an increase in brain PGs generated by the COX-2 isomerase. However, we also found (confirming our previous observations) that the nonselective COX inhibitor indomethacin, given at doses that blunt PG levels in the CSF, does not prevent fever induced by ET-1. This finding is difficult to explain, as one expects that indomethacin, insofar as it also blocks COX-2, should mimic all of the effects of selective COX-2 inhibitors in this paradigm. Discrepancies between the effects of indomethacin and coxib drugs on ET-1-induced fever might reflect distinct effects of these drugs on PG-synthesizing enzymes, as well as PG transporters and PG catabolism in fever-related sites, as recently reported (21, 22). One other discrepancy in our study concerns the different potency of celecoxib in reducing ET-1-induced fever, starting from 5 mg/kg. Thus the inhibitory effect on fever, beginning from 5 mg/kg, is apparently at odds with the inhibitory effect on fever, starting from 5 mg/kg. Thus the above discrepancies leave the space open to alternative action mechanisms of ET-1-induced fever, possibly involving COX-2-independent pathways.

ACKNOWLEDGMENTS

We are very grateful to M. C. C. Melo and J. A. Vercesi for expert technical assistance.

GRANTS

This study was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (grants 97/09837-6 and 98/09738-0) and Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil. A. S. C. Fabrício is the recipient of a fellowship from the Istituto Giuseppe Toniolo di Studi Superiori.

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