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RESEARCH PAPER

The antipyretic effect of dipyrone is unrelated to inhibition of PGE_2 synthesis in the hypothalamus

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BACKGROUND AND PURPOSE
Bacterial lipopolysaccharide (LPS) induces fever through two parallel pathways; one, prostaglandin (PG)-dependent and the other, PG-independent and involving endothelin-1 (ET-1). For a better understanding of the mechanisms by which dipyrone exerts antipyresis, we have investigated its effects on fever and changes in PGE_2 content in plasma, CSF and hypothalamus induced by either LPS or ET-1.

EXPERIMENTAL APPROACH
Rats were given (i.p.) dipyrone (120 mg·kg^{-1}) or indomethacin (2 mg·kg^{-1}) 30 min before injection of LPS (5 μg·kg^{-1}, i.v.) or ET-1 (1 pmol, i.c.v.). Rectal temperature was measured by tele-thermometry. PGE_2 levels were determined in the plasma, CSF and hypothalamus by ELISA.

KEY RESULTS
LPS or ET-1 induced fever and increased CSF and hypothalamic PGE_2 levels. Two hours after LPS, indomethacin reduced CSF and hypothalamic PGE_2 but did not inhibit fever, while at 3 h it reduced all three parameters. Three hours after ET-1, indomethacin inhibited the increase in CSF and hypothalamic PGE_2 levels but did not affect fever. Dipyrone abolished both the fever and the increased CSF PGE_2 levels induced by LPS or ET-1 but did not affect the increased hypothalamic PGE_2 levels. Dipyrone also reduced the increase in the venous plasma PGE_2 concentration induced by LPS.

CONCLUSIONS AND IMPLICATIONS
These findings confirm that PGE_2 does not play a relevant role in ET-1-induced fever. They also demonstrate for the first time that the antipyretic effect of dipyrone was not mechanistically linked to the inhibition of hypothalamic PGE_2 synthesis.

Abbreviations
4-AA, 4-aminoantipyrine; 4-MAA, 4-methylaminoantipyrine; aCSF, artificial cerebrospinal fluid; AVP, arginine-vasopressin; BBB, blood–brain barrier; BCSFB, blood–CSF barrier; BK, bradykinin; COX, cyclooxygenase; CRF, corticotrophin-releasing factor; CSF, cerebrospinal fluid; ET-1, endothelin-1; IL, interleukin; LPS, lipopolysaccharide; NSAID, nonsteroidal anti-inflammatory drugs; PFPF, pre-formed pyrogenic factor; PG, prostaglandin; POA/AH, preoptic area of the anterior hypothalamus; TNF, tumour necrosis factor; Tsv, *Tityus serrulatus* venom

Introduction

Fever, a characteristic consequence of infection and an important host defence response, is triggered by a variety of exogenous pyrogens, including the so-called pathogen-associated molecular patterns, such as lipopolysaccharide (LPS) produced by Gram-negative bacteria (Roth *et al.*, 2006; 2009). This response is mediated by several endogenous pyrogens, such as tumour necrosis factor-α, interleukin (IL)-1β, IL-6, corticotrophin-releasing factor (CRF), endothelin-1 (ET-1), pre-formed pyrogenic factor (PFPF), bradykinin and prostaglandins (PG) (Roth and De Souza, 2001; Roth *et al.*, 2009).

Previous data from our group (Fabricio *et al.*, 1998; 2005a,b; 2006a) and others (Strijbos *et al.*, 1992) have suggested that two pathways running in parallel are responsible for the development of fever induced by LPS. One of them is PG-dependent and requires peripheral/central cytokine synthesis/release and subsequent PG synthesis (via COX-2) in the preoptic area of the anterior hypothalamus (POA/AH) (Nakamura *et al.*, 2005; Roth *et al.*, 2006; 2009; Lazarus *et al.*, 2007) and is sensitive to blockade by indomethacin. The other is a PG-independent pathway (insensitive to indomethacin), which involves PFPF derived from LPS-stimulated macrophages, CRF and ET-1 (Zampronio *et al.*, 2000; Fabricio *et al.*, 2005a,b; 2006a).

The mechanisms involved in the antipyretic action of nonsteroidal anti-inflammatory drugs (NSAIDs) have generally been ascribed to their ability to inhibit COX-1 and/or COX-2 in the CNS (Botting, 2006; Roth *et al.*, 2009). However, some NSAIDs also seem to display antipyretic properties unrelated to COX inhibition. For example, the antipyretic effect produced by a high dose of indomethacin (8 mg·kg^{-1}) is mediated to a substantial extent via vasopressin V_1 receptor activation by arginine–vasopressin, as it can be blocked by an antagonist of this receptor (Wilkinson and Kasting, 1989; De Souza *et al.*, 2002).

Dipyrone (also known as metamizol) is a potent antipyretic and analgesic pyrazolone derivative (Lorenzetti and Ferreira, 1985; Levy *et al.*, 1995) widely used in clinical practice in several countries. Unlike the other NSAIDs, dipyrone has pronounced analgesic and antipyretic effects, but very weak anti-inflammatory effects (Lorenzetti and Ferreira, 1985; Tatsuo *et al.*, 1994; De Souza *et al.*, 2002). Although Hinz *et al.* (2007) have shown that this drug blocks peripheral COX-1 and COX-2, its mechanism of antipyretic action is not yet entirely clear. Whereas some studies have reported that the antipyretic effect of dipyrone depends on PGE_2 synthesis inhibition (Shimada *et al.*, 1994; Kanashiro *et al.*, 2009), others suggest that it does not (De Souza *et al.*, 2002; Pessini *et al.*, 2006).

The current study aimed to clarify if the antipyretic effect of dipyrone was mechanistically related to the inhibition of PG synthesis. To this end, we have compared the effects of dipyrone and indomethacin on both fever and changes in PGE_2 levels in the CSF and the hypothalamus induced by LPS and ET-1 injection. In addition, we investigated the influence of pretreatment with dipyrone on the relationship between the plasma, CSF and hypothalamic PGE_2 levels following LPS injection. Our results strongly support the view that the fever induced by i.c.v. injection of ET-1 is PGE_2 independent. In addition, they show for the first time that even though dipyrone reduces PGE_2 concentration in the plasma and CSF, it does not inhibit hypothalamic PGE_2 synthesis, unlike indomethacin. These data suggest that, at the dose used here, the antipyretic effect of dipyrone is unrelated to the inhibition of hypothalamic PGE_2 synthesis.

Methods

Animals

Care and use of the animals were in full compliance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and Guide for the Care and Use of Laboratory Animals of the Institute for Laboratory Animal Research (National Research Council, 1996), and the study was previously approved by the Animal Research Ethics Committee of the Faculty of Medicine of Ribeirão Preto, University of São Paulo (Protocol no. 136/2007). Experiments were conducted on 246 male Wistar rats weighing 180–200 g, housed individually at 24 ± 1°C under a 12:12 h light–dark cycle (lights on at 06:00 AM) with free access to food and tap water until the night before the experiment, when only water was made available. Each animal was used only once.

Temperature measurements

The rectal temperature was measured in conscious and unrestrained rats every 30 min for 6 h by gently inserting a vaseline-coated thermistor probe (model 402 coupled to a model 46 telethermometer, Yellow Springs Instruments, Yellow Springs, OH, USA) 4 cm into the rectum, without removing the animal from its cage. Experimental measurements were conducted in a room with the temperature controlled at 27 ± 1°C, the thermoneutral zone for rats (Gordon, 1990). Baseline temperatures were determined three to four times and at 30 min intervals prior to any injection treatment (and always up to 10:00 AM). Only animals displaying mean basal rectal temperatures between 36.8 and 37.2°C were selected for the study. In order to minimize core temperature changes due to handling, animals were habituated to this environment and procedure twice on the preceding day.

Intracerebral cannula implantation

Under anaesthesia induced by a mixture of ketamine and xylazine (60 mg·kg^{-1} and 20 mg·kg^{-1}, respectively, i.p.), 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 (the incisor bar was lowered 2.5 mm below the horizontal zero) (Paxinos and Watson, 1986). Cannulae were fixed to the skull with jeweler's screws embedded in dental acrylic cement. Animals were then treated with oxytetracycline hydrochloride (400 mg·kg^{-1}, i.m.) and allowed to recover for 1 week before the experiments. After each experiment, the animals were anaesthetized as described 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 post-implantation period were excluded from the study.

CSF and venous blood sampling: determination of PGE_2 concentration

A single CSF sample was collected from each animal according to the method described by Consiglio and Lucion (2000). Briefly, just prior to CSF collection, each rat was anaesthetized as described before and fixed to the stereotaxic apparatus, with its body flexed downward. The top and back of the head were trichotomized and moistened with a cotton swab soaked in ethanol to facilitate the visualization of a small depression between the occipital protuberance and the atlas. A 25-gauge needle connected to a 1 mL syringe was then inserted vertically and centrally through this depression into the *cisterna magna* and a gentle aspiration caused the CSF to flow through it, resulting in 50 to 100 µL samples. Gentle movements of the needle are necessary during collection in order to prevent bleeding. The collected CSF samples were placed in Eppendorf tubes containing indomethacin (10 µM) to prevent PG production, *ex vivo*. Samples were maintained in the dark and on ice until centrifugation at 1300× *g* for 15 min at 4°C, and the supernatants were immediately frozen to –70°C until analysis. Samples contaminated with blood were discarded.

For blood collection, animals were anaesthetized and single blood samples of the abdominal vena cava (3 mL) were collected at 2 and 3 h after LPS, placed in tubes containing indomethacin (10 µM) and heparin, cooled on ice and protected from light, centrifuged at 1300× *g* for 15 min at 4°C, and the supernatants were immediately frozen to –70°C until analysis. We chose to determine the venous plasma PGE_2 concentration because arterial plasma PGE_2 levels are very low due to extensive metabolism after passage through the pulmonary circulation (Piper *et al.*, 1970; Steiner *et al.*, 2006).

PGE_2 levels were measured using ELISA kits from Cayman Chemical (Ann Arbor, MI, USA) following the procedures detailed in the instructions, with a detection limit of 7.8 $pg \cdot mL^{-1}$. Cross-reactivity data were as follows: 17.5% with PGE_3, 11.9% with PGE_1, 7% with $PGF_{1\alpha}$, 6% with $PGF_{2\alpha}$, 2.5% with 6-oxo-$PGF_{1\alpha}$ and less than 0.1% with all other prostanoids tested. Intra- and inter-assay coefficients of variation were <11%. All samples were assayed according to the manufacturer's instructions.

Dissection of hypothalamus and determination of PG levels in the hypothalamus

Immediately after CSF collection, the animals were killed by decapitation and their brains rapidly removed. The entire hypothalamus was dissected from the brain using the following limits: the anterior border of the optic chiasma, the anterior border of the mammillary bodies and the lateral hypothalamic sulci, with a depth of 2 mm. The total dissection time elapsed from decapitation was <2 min (Fabricio *et al.*, 2006b), and the hypothalami were immediately frozen to –70°C until analysis.

Each hypothalamus (~100 mg) was homogenized in 1 mL of RPMI medium containing indomethacin (2 $mg \cdot mL^{-1}$) using a Digital 600-w ultrasonic microprocessor cell disrupter (Virsonic 100® – VirTis, Gardiner, NY, USA) and then acidified with HCl (1 N) to pH = 3.5–4.0. Samples were maintained in the dark on ice until centrifugation at 20 000× *g* for 15 min at 4°C. The resulting supernatant was applied to a minicolumn (Sep-Pak® Classic C18 cartridge 360 mg, Waters Corporation, Milford, MA, USA) and PGE_2 was eluted using 2 mL of ethanol. The sample was dried using a speed vacuum (Hetovac® model CT110, Birkercd, Denmark) for 18 h. The following day, the dry sample was resuspended in enzyme immunoassay (EIA) buffer and the levels of PGE_2 were measured using a PGE_2 Express EIA Kit from Cayman Chemical according to the manufacturer's instructions.

Pretreatment and treatment protocols

Rats were pretreated with indomethacin (2 $mg \cdot kg^{-1}$, i.p.), dipyrone (120 $mg \cdot kg^{-1}$, i.p.) or vehicle (in both cases Tris-HCl in saline, i.p.; see Materials). Thirty minutes later, animals were given an i.v injection of *Escherichia coli* LPS (5 $\mu g \cdot kg^{-1}$), or an i.c.v. injection of ET-1 (1 pmol), or identical injection of their respective vehicles, that is, sterile saline (0.2 mL, i.v.) or artificial cerebrospinal fluid (aCSF; 3 µL, i.c.v.). The doses of indomethacin, dipyrone, ET-1 and LPS were selected based on previous studies from our group (De Souza *et al.*, 2002; Fabricio *et al.*, 2005a).

For i.c.v. 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. Intravenous injections of LPS or the corresponding vehicle were given via a lateral tail vein. Both pyrogenic stimuli were always injected between 10:00 and 11:00 h to minimize variability due to potential diurnal fluctuations in responsiveness.

Data analysis. For data analysis, the baseline temperature prior to any injection was determined for each animal and all subsequent rectal temperatures were expressed as changes from the mean basal value. Data are reported as mean ± SEM. Mean baseline temperatures did not differ significantly among the groups included in any particular set of experiments. The levels of PGE_2 were analysed by one-way ANOVA followed by Tukey's test. The changes in rectal temperature were compared across treatments and time points by two-way ANOVA for repeated measurements followed by Bonferroni's test. All data were analysed using Prism 5 computer software (Graph-Pad, San Diego, CA, USA). Differences were considered significant when $P < 0.05$.

Materials

The following compounds were used: ET-1 from Research Biochemicals International (Natick, MA, USA), LPS (*E. coli* 0111:B4) from Sigma (St Louis, MO, USA), indomethacin from Merck, Sharp & Dohme (São Paulo, Brazil), dipyrone (sodium metamizol) from Aventis Pharma Deutschland GmbH (Berlin, Germany), ketamine (Ketamina Agener®) from União Química Farmacêutica Nacional S.A. (São Paulo, Brazil), xylazine (Dopaser®) from Calier Laboratories S.A. (Barcelona, Spain), oxytetracycline hydrochloride (Terramicina®) from Pfizer (São Paulo, Brazil).

Indomethacin was initially dissolved in 1 mL of sterile Tris-HCl (0.2 M, pH 8.2) and subsequently diluted further with 9 mL of sterile saline. Dipyrone was first dissolved in 9 mL of sterile saline and further diluted with 1 mL of sterile

Tris-HCl, pH 8.2, to ensure that both drugs were administered in identical vehicles. LPS was diluted in saline and ET-1 in aCSF (composition mM: 138.6 NaCl, 3.35 KCl, 1.26 $CaCl_2$ and 11.9 $NaHCO_3$).

Results

Effect of indomethacin and dipyrone on fever induced by LPS or ET-1

Under our experimental conditions, i.v. injection of LPS (5 μg·kg^{-1}) elicited a marked rectal temperature elevation that started at 90 min, peaked between 2 and 3 h, and persisted up to 6 h (Figure 1A,C). On the other hand, i.c.v. administration of ET-1 (1 pmol) also caused a long-lasting fever, but the onset of this response was faster than that seen for LPS (Figure 1B,D).

Pretreatment of rats with indomethacin (2 mg·kg^{-1}, i.p.) significantly reduced the pyrogenic response to LPS from 3 to 6 h after its i.v. injection (Figure 1A). As expected (Fabricio *et al.*, 1998; 2005a), indomethacin did not affect the febrile response induced by ET-1 (Figure 1B). Furthermore, indomethacin did not modify the basal rectal temperature of control rats (Figure 1A,B).

Pretreatment of rats with dipyrone (120 mg·kg^{-1}, i.p.) significantly reduced the pyrogenic response to LPS from 2.0 to 3.5 h after its injection (Figure 1C) and abolished the fever induced by ET-1 (Figure 1D). Dipyrone did not modify the basal rectal temperature of control rats (Figure 1C,D).

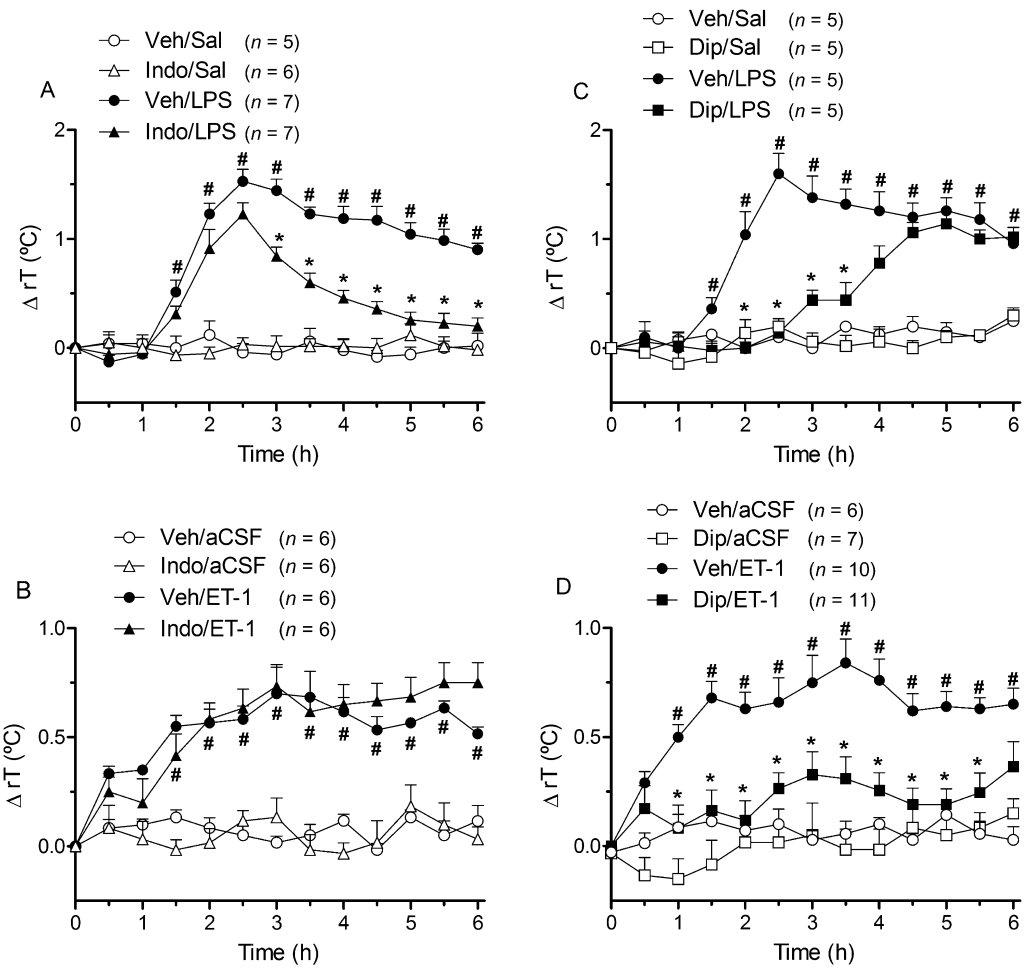

Figure 1

Effect of indomethacin (A and B) or dipyrone (C and D) on fever evoked by lipopolysaccharide (LPS; A and C) or endothelin-1 (ET-1; B and D). Rats received i.p. injections of indomethacin (Indo, 2 mg·kg^{-1}), dipyrone (Dip, 120 mg·kg^{-1}) or vehicle (Veh, 10% Tris-HCl in saline, 0.5 mL) 30 min prior to LPS (5 μg·kg^{-1}, i.v.), ET-1 (1 pmol, i.c.v.) or sterile saline (Sal)/aCSF (0.2 mL and 3 μL, respectively, as controls). Values represent the means ± SEM of the changes in rectal temperatures (ΔrT, °C) of the animals. *$P < 0.05$ compared with the groups treated with vehicle/LPS or vehicle/ET-1; #$P < 0.05$ compared with the groups treated with Veh/Sal or Veh/aCSF. Basal rectal temperatures of each group were as follows: (A) Veh/LPS = 36.99 ± 0.05; Veh/Sal = 36.96 ± 0.07; Indo/LPS = 37.00 ± 0.05; Indo/Sal = 36.95 ± 0.04; (B) Veh/ET-1 = 36.95 ± 0.06; Indo/ET-1 = 37.00 ± 0.08; Veh ET-1 = 36.90 ± 0.04; Indo/aCSF = 37.00 ± 0.04; (C) Veh/LPS = 36.92 ± 0.04; Veh/Sal = 37.00 ± 0.07; Dip/LPS = 37.02 ± 0.06; Dip/Sal = 36.96 ± 0.05; (D) Veh/ET-1 = 36.96 ± 0.04; Veh/aCSF = 37.02 ± 0.05; Dip/ET-1 = 36.95 ± 0.04; Dip/aCSF = 37.00 ± 0.06.

Effect of indomethacin or dipyrone on changes in PGE_2 concentration in the CSF and hypothalamus induced by LPS or ET-1

In order to measure the PGE_2 content in cisternal CSF and hypothalamus, samples were collected 2 and 3 h after LPS injection and 3 h after ET-1 injection. These times were selected to compare the CSF and hypothalamic PGE_2 concentration with the antipyretic effect because at 2 h indomethacin does not reduce fever to LPS, while at 3 h it does (Fabricio *et al.*, 2005a).

Under our experimental conditions, PGE_2 levels in the CSF of control animals treated with vehicle alone (saline or aCSF) were below the detection limit of the assay (Figures 2B and 3B), unlike the PGE_2 content in hypothalami collected from such animals, which was clearly detectable (Figures 2C and 3B). As expected, LPS or ET-1 treatment increased PGE_2 content in both the CSF (Figures 2B and 3B) and the hypothalamus (Figures 2C and 3C).

Confirming the results shown in Figure 1A,B, indomethacin (2 mg·kg^{-1}, i.p.) reduced LPS-induced fever at 2 and 3 h (Figure 2A), but failed to modify the fever induced by ET-1 at 3 h (Figure 3A). More importantly, at these time points, indomethacin reduced the increase in CSF and hypothalamic PGE_2 content induced by LPS (Figure 2B,C). Likewise, given prior to ET-1 injection, indomethacin also reduced PGE_2 content in CSF (Figure 3B) and hypothalami (Figure 3C), to values below those of vehicle/aCSF controls (Figure 3C). Additionally, after intravenous saline or i.c.v. aCSF injection, indomethacin did not modify the basal rectal temperature but reduced the hypothalamic PGE_2 content to values below those of vehicle-treated animals (Figures 2C and 3C).

Dipyrone (120 mg·kg^{-1}, i.p.), which inhibited the fever induced by both LPS (Figures 1C and 2A) and ET-1 (Figures 1D and 3A), also inhibited the increase in PGE_2 levels in CSF induced by these stimuli (Figures 2B and 3B). In sharp contrast to indomethacin, however, dipyrone did not change the hypothalamic PGE_2 content in LPS- (Figure 2C) or ET-1-stimulated rats (Figure 3C), but substantially reduced the hypothalamic PGE_2 content in animals that received i.v. saline (Figure 2C) or i.c.v. aCSF (Figure 3C) injections.

Effect of dipyrone on the change in venous plasma PGE_2 concentrations induced by LPS

Venous plasma PGE_2 concentrations were augmented 2 and 3 h after intravenous injection of LPS. Pretreatment with dipyrone fully blocked this increase in plasma PGE_2 concentrations (Figure 4).

Discussion

These findings constitute solid evidence that fever induced by i.c.v. ET-1 is PGE_2 independent and shows, for the first time, that dipyrone blocks fever and PGE_2 synthesis in the CSF induced by LPS and ET-1 without altering the content of this prostanoid in the hypothalamus. Also of significance was the finding that dipyrone inhibited the increase in PGE_2 content in the blood induced by LPS. Altogether, these *in vivo* findings open a new view about the mechanism involved in the

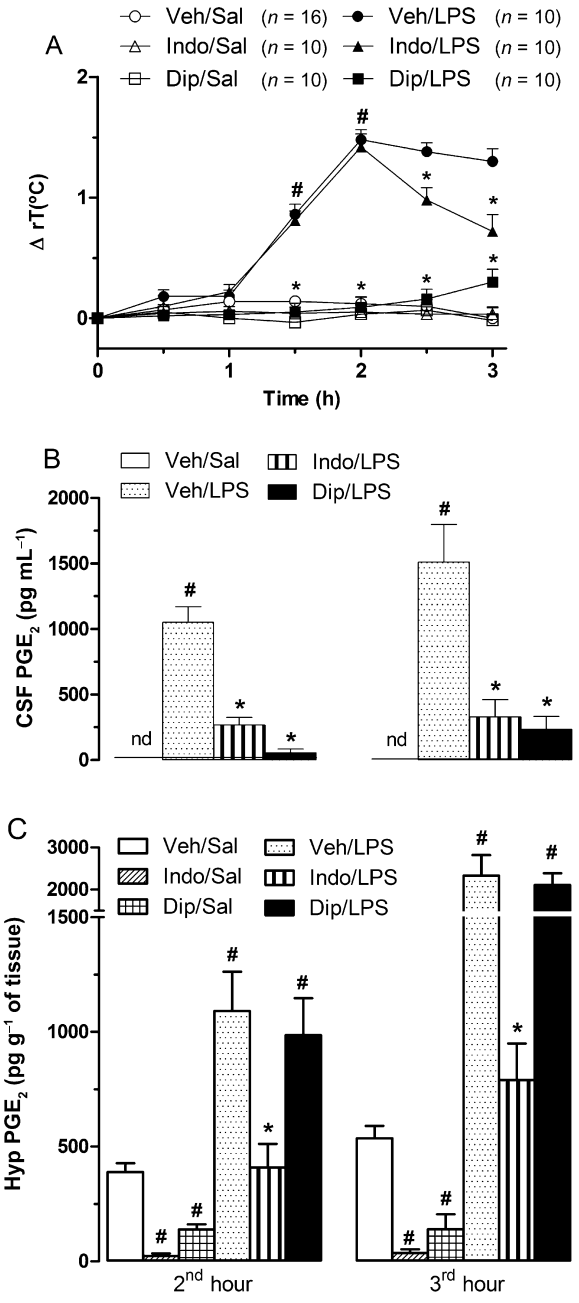

Figure 2

Effect of indomethacin (Indo) or dipyrone (Dip) on changes in rectal temperatures (A), CSF (B) and hypothalamic (C) PGE_2 concentration after lipopolysaccharide (LPS) injection in rats. Indo (2 mg·kg^{-1}, i.p.), Dip (120 mg·kg^{-1}, i.p.) or vehicle (Veh, 10% Tris-HCl in saline, 0.5 mL) was administered 30 min prior to LPS (5 μg·kg^{-1}, i.v.) or sterile saline (Sal, 0.2 mL, control) injection. The CSF and hypothalamus were collected 2 and 3 h after LPS or saline injection. PGE_2 concentration was determined by ELISA. Values represent means ± SEM of the variation in rectal temperature (ΔrT, °C) and the PGE_2 levels in the CSF (pg·mL^{-1}) and hypothalamus (Hyp; pg·g^{-1} of tissue). #,*P < 0.05 compared with the groups treated with Veh/Sal or Veh/LPS respectively. Basal rectal temperatures of each group were as follows: Veh/Sal = 37.01 ± 0.04; Indo/Sal = 36.89 ± 0.03; Dip/Sal = 36.87 ± 0.03; Veh/LPS = 36.98 ± 0.04; Indo/LPS = 37.03 ± 0.04; Dip/LPS = 36.99 ± 0.06.

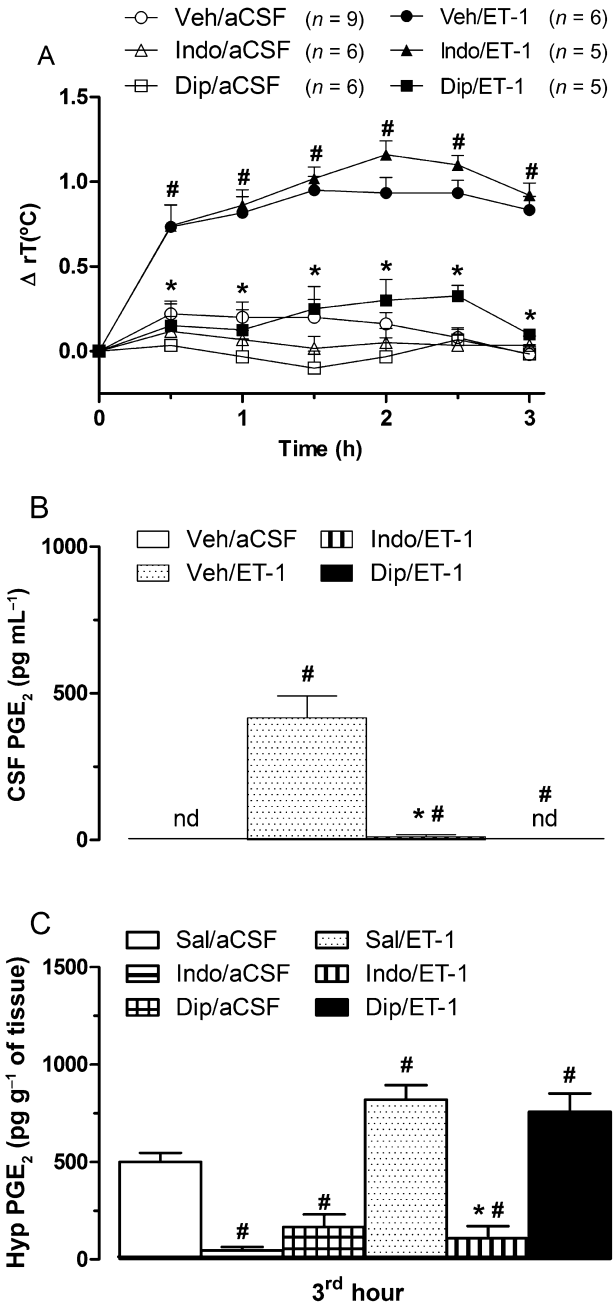

Figure 3

Effect of indomethacin or dipyrone (Dip) on changes in rectal temperature (A), CSF (B) and hypothalamic (C) PGE_2 concentration after endothelin-1 (ET-1) injection in rats. Indomethacin (Indo, 2 mg·kg^{-1}, i.p.), Dip (120 mg·kg^{-1}, i.p.) or its vehicle (Veh, 10% Tris-HCl in saline) was administered 30 min prior to ET-1 (1 pmol, i.c.v.) or sterile aCSF (3 µL, control) injection. The CSF and hypothalamus were collected 3 h after ET-1 or aCSF injection. PGE_2 concentration was determined by ELISA. Values represent means ± SEM of the variation in rectal temperature (ΔrT, °C) and the PGE_2 levels in the CSF (pg·mL^{-1}) and hypothalamus (Hyp; (pg·g^{-1} of tissue). #,*$P < 0.05$ compared with the groups treated with Veh/aCSF or Veh/ET-1 respectively. Basal rectal temperatures of each group were as follows: Veh/aCSF = 37.0 ± 0.05; Indo/aCSF = 36.89 ± 0.03; Dip/aCSF = 36.87 ± 0.03; Veh/ET-1 = 36.91 ± 0.06; Indo/ET-1 = 36.96 ± 0.05; Dip/ET-1 = 37.14 ± 0.07.

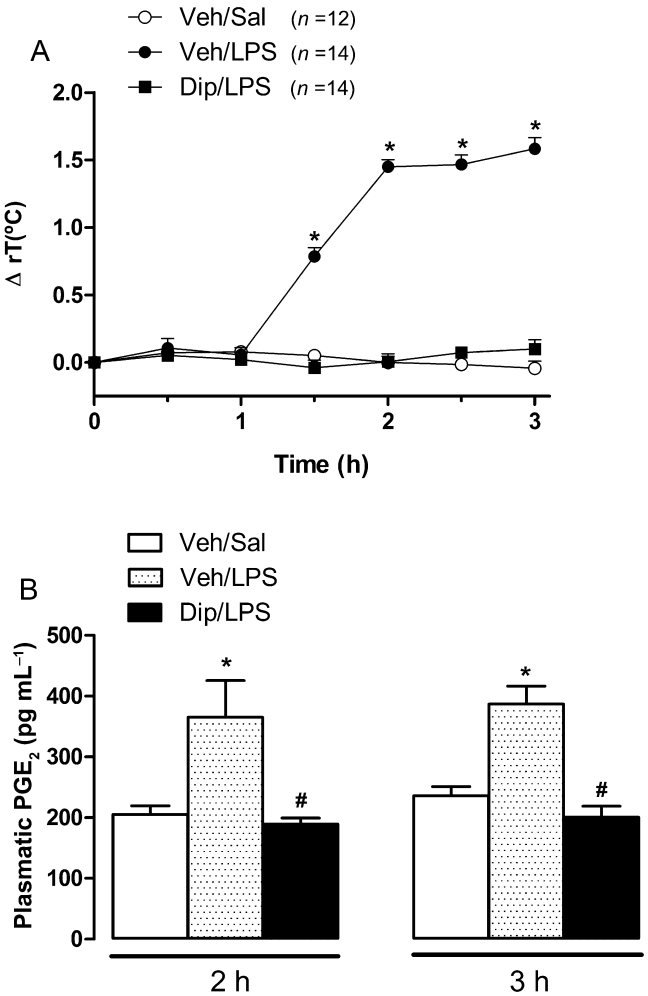

Figure 4

Effect of dipyrone on changes in rectal temperatures (A) and plasma (B) PGE_2 concentration after lipopolysaccharide (LPS) injection in rats. Dipyrone (Dip, 120 mg·kg-1, i.p.) or vehicle (Veh, 10% Tris-HCl in saline, 0.5 mL) was administered 30 min prior to LPS (5 µg·kg^{-1}, i.v.) or sterile saline (Sal, 0.2 mL, control) injection. Blood was taken 2 and 3 h after LPS or saline injection and plasma prepared. PGE_2 concentration was determined by ELISA. Values represent means ± SEM of the variation in rectal temperature (ΔrT, °C) and the PGE_2 levels in the plasma (pg·mL^{-1}). #,*$P < 0.05$ compared with the groups treated with Veh/Sal or Veh/LPS respectively. Basal rectal temperatures of each group were as follows: Veh/Sal = 36.90 ± 0.03; Veh/LPS = 36.97 ± 0.05; Dip/LPS = 36.96 ± 0.03.

antipyretic effect of dipyrone, which differs from that of indomethacin in that dipyrone blocks fever and PGE_2 synthesis in CSF but not in hypothalamus, regardless of the involvement of PGE_2 in the febrile response.

It is widely accepted that PGE_2 generated in the POA/AH is the main mediator of fever induced by LPS, by acting on prostaglandin EP_3 receptors expressed on thermoregulatory neurons located in the POA/AH (Engblom *et al.*, 2003; Oka *et al.*, 2003; Nakamura *et al.*, 2005; Roth *et al.*, 2006; 2009; Lazarus *et al.*, 2007). In agreement with this view, our present results show that fever induced by LPS was accompanied by increases in PGE_2 content in the CSF and also in the hypo-

thalamus (Sehic *et al.*, 1996; Matsumura *et al.*, 1997; Fabricio *et al.*, 2005a). As expected, indomethacin reduced the fever (Figures 1A and 2A) and the increase of PGE_2 in the CSF (Figure 2B), and abolished the increase of PGE_2 in the hypothalamus (Figure 2C) 3 h after LPS administration, demonstrating the relevance of PGE_2 to the mediation of LPS-induced fever. However, it is interesting to note that while the PGE_2 levels in the CSF (Figure 2B) and the hypothalamus (Figure 2C) of indomethacin-treated rats were clearly reduced to basal levels 2 h after LPS administration, the intensity of fever was unaffected at that time point (Figure 2A). In this context, Feleder *et al.* (2004; 2007) have shown that at this time point, hypothalamic PGE_2 is not essential for the expression of fever after LPS in guinea pigs and that it may result from α_1-adrenoceptor activation by noradrenaline (see Blatteis, 2007 for review).

Additionally, as mentioned in the Introduction, there are studies showing the existence of several pathways, running in parallel, during the development of fever induced by LPS (Strijbos *et al.*, 1992; Fabricio *et al.*, 1998; 2005a,b; 2006a; Zampronio *et al.*, 2000; Feleder *et al.*, 2004). The PG-independent (indomethacin-insensitive) pathway involves PFPF, which in turn depends on CRF release and activation of the endothelin system in the CNS, via ET_B receptors to produce fever (Zampronio *et al.*, 2000; Fabricio *et al.*, 2005a,b; 2006a).

ET-1 is considered to be one of the central mediators of febrile response induced by i.v. LPS, as this response is associated with increased levels of the peptide in the CSF, and BQ-788, an ET_B receptor antagonist, reduces the fever induced by LPS (Fabricio *et al.*, 1998; 2005b). Confirming previous findings from our laboratory, indomethacin did not reduce fever induced by ET-1, even though it fully blocked the increases of PGE_2 content in the CSF promoted by this peptide (Figures 1B and 3A,B) (Fabricio *et al.*, 2005a). However, several studies have clearly established that it is the PGE_2 content in the hypothalamus, rather than that in the CSF, that is relevant to the development of fever (Scammell *et al.*, 1998; Okumura *et al.*, 2006; Futaki *et al.*, 2009). Our current study demonstrated, for the first time, that indomethacin effectively inhibited the increase in PGE_2 content in the hypothalamus (Figure 3C) induced by ET-1 without affecting the febrile response. These results clearly dissociate fever induced by ET-1 from its enhancing effects on PGE_2 content.

The prodrug dipyrone is a potent antipyretic and analgesic pyrazolone derivative and several studies have proposed that these effects depend on its conversion to at least two active metabolites, 4-methylaminoantipyrine and 4-aminoantipyrine (Levy *et al.*, 1995; Hinz *et al.*, 2007). These metabolites indeed have been shown to inhibit COX either *in vitro* (Abbate *et al.*, 1990; Campos *et al.*, 1999; Pierre *et al.*, 2007) or *ex vivo* (Hinz *et al.*, 2007). Dipyrone decreases fever induced by IL-1β, a known PGE_2-dependent pyrogen, but not by PGE_2 itself (Shimada *et al.*, 1994; De Souza *et al.*, 2002). It also inhibits fever and the increase of PGE_2 levels in CSF which accompany zymosan-induced knee inflammation (Kanashiro *et al.*, 2009), suggesting that the antipyretic effect of dipyrone is related to the inhibition of PGE_2 synthesis. However, previous studies by our group found that dipyrone, at the same dose used here, blocked indomethacin-resistant (PG-independent) fever induced by several endogenous pyrogens, including $PGF_{2\alpha}$ (De Souza *et al.*, 2002), or by *Tityus serrulatus* venom (Pessini *et al.*, 2006), suggesting that dipyrone also has antipyretic properties unrelated to COX inhibition.

As expected, in the present study, dipyrone reduced LPS-induced fever (Figure 1C). In addition and more importantly, it shows for the first time that this drug also abolished the PGE_2-independent fever induced by ET-1 (Figure 1D), which strengthens considerably the idea that dipyrone has antipyretic properties unrelated to COX inhibition. Unexpectedly, although dipyrone reduced the febrile response (Figure 2A), as well as the increase in PGE_2 concentration in the CSF (Figures 2B and 3B), it did not show any effect in the hypothalamic (Figures 2C and 3C) PGE_2 content after LPS or ET-1 injection, even though it reduced the basal hypothalamic PGE_2 content in vehicle-injected control animals (Figures 2C and 3C). This may suggest that the amounts of dipyrone or of its metabolites that reached the hypothalamus were insufficient to inhibit the enhanced local PGE_2 synthesis promoted by these two inducers of COX-2 expression. In order to act in the CNS, systemically administered drugs must cross the blood–brain barrier (BBB) and/or blood–CSF barrier (BCSFB), where their distribution depends on the direction of gradients between CSF and interstitial cerebral fluid. Thus, the extent of permeability of each drug and its accessibility to different areas of the CNS is clearly compound dependent (Ghersi-Egea *et al.*, 2009). Cohen *et al.* (1998) showed that after oral administration of dipyrone, its metabolites were found in the CSF, demonstrating that dipyrone and/or its metabolites can cross the BBB or BCSFB. However, there are no studies measuring levels of these metabolites in the hypothalamus or any other brain areas. Taken together, these results suggest that the antipyretic effect of dipyrone is not dependent on inhibition of PGE_2 synthesis in the hypothalamus, considered the main brain area for the PGE_2 synthesis/effect during the development of fever (Scammell *et al.*, 1998; Nakamura *et al.*, 2005; Okumura *et al.*, 2006; Lazarus *et al.*, 2007; Futaki *et al.*, 2009).

Peripherally generated PGs could also be important for fever development (Steiner *et al.*, 2006; Blatteis, 2007). Thus, we also measured the effects of dipyrone on venous plasma concentrations of PGE_2 in rats given i.v. LPS. We found that dipyrone blocked the increase in plasma PGE_2 concentration induced by LPS (Figure 4), which could suggest that part of its antipyretic effect against LPS-induced fever might also result from decreased delivery of peripheral (blood) PGE_2 to the CSF and hypothalamus. However, as dipyrone reduced the PGE_2 levels in CSF (Figure 2B), but not in hypothalamus (Figure 2C), it is possible that peripheral PGE_2 might be a relevant source for the PGE_2 found in the CSF, but that neither PGE_2 generated in the periphery nor that contained in the CSF contributed to its levels in the hypothalamus. Finally, as i.c.v. injection of dipyrone abolished fever induced by LPS in mice, this drug is able to exert a central antipyretic effect, at least in this species (Souza *et al.*, 2002).

It is important to mention that many studies have dissociated the analgesic effects of dipyrone from an action on PGE_2 synthesis (Sachs *et al.*, 2004; Siebel *et al.*, 2004; Rezende *et al.*, 2008). Therefore, further studies are necessary to define fully the PG-independent mechanisms underlying the

antipyretic and analgesic properties of dipyrone or its metabolite(s).

In conclusion, these findings demonstrate unequivocally that PGE_2 does not play a relevant role in ET-1-induced fever. Moreover, our results from dipyrone-treated animals provide evidence that neither PGE_2 present in the CSF nor that synthesized in the periphery contributes to the levels of this eicosanoid found in the hypothalamus. The fact that dipyrone can block both PG-dependent and PG-independent pathways of the fever induced by LPS suggests that this drug has a distinct profile of antipyretic action from that of other COX inhibitors, which could be advantageous in treating fever.

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Conflict of interest

None.

References

Abbate R, Gori AM, Pinto S, Attanasio M, Paniccia R, Coppo M *et al.* (1990). Cyclooxygenase and lipoxygenase metabolite synthesis by polymorphonuclear neutrophils: in vitro effect of dipyrone. Prostaglandins Leukot Essent Fatty Acids 41: 89–93.

Blatteis CM (2007). The onset of fever: new insights into its mechanism. Prog Brain Res 162: 3–14.

Botting RM (2006). Inhibitors of cyclooxygenases: mechanisms, selectivity and uses. J Physiol Pharmacol 57 (Suppl. 5): 113–124.

Campos C, de Gregorio R, Garcia-Nieto R, Gago F, Ortiz P, Alemany S (1999). Regulation of cyclooxygenase activity by metamizol. Eur J Pharmacol 378: 339–347.

Cohen O, Zylber-Katz E, Caraco Y, Granit L, Levy M (1998). Cerebrospinal fluid and plasma concentrations of dipyrone metabolites after a single oral dose of dipyrone. Eur J Clin Pharmacol 54: 549–553.

Consiglio AR, Lucion AB (2000). Technique for collecting cerebrospinal fluid in the cisterna magna of non-anesthetized rats. Brain Res Protoc 5: 109–114.

De Souza GE, Cardoso RA, Melo MC, Fabricio AS, Silva VM, Lora M *et al.* (2002). A comparative study of the antipyretic effects of indomethacin and dipyrone in rats. Inflamm Res 51: 24–32.

Engblom D, Saha S, Engstrom L, Westman M, Audoly LP, Jakobsson PJ *et al.* (2003). Microsomal prostaglandin E synthase-1 is the central switch during immune-induced pyresis. Nat Neurosci 6: 1137–1138.

Fabricio AS, Silva CA, Rae GA, D'Orléans-Juste P, Souza GE (1998). Essential role for endothelin ET(B) receptors in fever induced by LPS (*E. coli*) in rats. Br J Pharmacol 125: 542–548.

Fabricio AS, Veiga FH, Cristofoletti R, Navarra P, Souza GE (2005a). The effects of selective and nonselective cyclooxygenase inhibitors on endothelin-1-induced fever in rats. Am J Physiol Regul Integr Comp Physiol 288: R671–R677.

Fabricio AS, Rae GA, D'Orléans-Juste P, Souza GE (2005b). Endothelin-1 as a central mediator of LPS-induced fever in rats. Brain Res 1066: 92–100.

Fabricio AS, Rae GA, Zampronio AR, D'Orléans-Juste P, Souza GE (2006a). Central endothelin ET(B) receptors mediate IL-1-dependent fever induced by preformed pyrogenic factor and corticotropin-releasing factor in the rat. Am J Physiol Regul Integr Comp Physiol 290: R164–R171.

Fabricio AS, Tringali G, Pozzoli G, Melo MC, Vercesi JA, Souza GE *et al.* (2006b). Interleukin-1 mediates endothelin-1-induced fever and prostaglandin production in the preoptic area of rats. Am J Physiol Regul Integr Comp Physiol 290: R1515–R1523.

Feleder C, Perlik V, Blatteis CM (2004). Preoptic alpha 1- and alpha 2-noradrenergic agonists induce, respectively, PGE2-independent and PGE2-dependent hyperthermic responses in guinea pigs. Am J Physiol Regul Integr Comp Physiol 286: R1156–R1166.

Feleder C, Perlik V, Blatteis CM (2007). Preoptic norepinephrine mediates the febrile response of guinea pigs to lipopolysaccharide. Am J Physiol Regul Integr Comp Physiol 293: R1135–R1143.

Futaki N, Harada M, Sugimoto M, Hashimoto Y, Honma Y, Arai I *et al.* (2009). The importance of brain PGE2 inhibition versus paw PGE2 inhibition as a mechanism for the separation of analgesic and antipyretic effects of lornoxicam in rats with paw inflammation. J Pharm Pharmacol 61: 607–614.

Ghersi-Egea JF, Mönkkönen KS, Schmitt C, Honnorat J, Fèvre-Montange M, Strazielle N (2009). Blood-brain interfaces and cerebral drug bioavailability. Rev Neurol 165: 1029–1038.

Gordon CJ (1990). Thermal biology of the laboratory rat. Physiol Behav 47: 963–991.

Hinz B, Cheremina O, Bachmakov J, Renner B, Zolk O, Fromm MF *et al.* (2007). Dipyrone elicits substantial inhibition of peripheral cyclooxygenases in humans: new insights into the pharmacology of an old analgesic. FASEB J 21: 2343–2351.

Kanashiro A, Pessini AC, Machado RR, Malvar Ddo C, Aguiar FA, Melo Soares D *et al.* (2009). Characterization and pharmacological evaluation of febrile response on zymosan-induced arthritis in rats. Am J Physiol Regul Integr Comp Physiol 296: R1631–R1640.

Lazarus M, Yoshida K, Coppari R, Bass CE, Mochizuki T, Lowell BB *et al.* (2007). EP3 prostaglandin receptors in the median preoptic nucleus are critical for fever responses. Nat Neurosci 10: 1131–1133.

Levy M, Zylber-Katz E, Rosenkranz B (1995). Clinical pharmacokinetics of dipyrone and its metabolites. Clin Pharmacokinet 28: 216–234.

Lorenzetti BB, Ferreira SH (1985). Mode of analgesic action of dipyrone: direct antagonism of inflammatory hyperalgesia. Eur J Pharmacol 114: 375–381.

Matsumura K, Cao C, Watanabe Y (1997). Possible role of cyclooxygenase-2 in the brain vasculature in febrile response. Ann N Y Acad Sci 813: 302–306.

Nakamura Y, Nakamura K, Matsumura K, Kobayashi S, Kaneko T, Morrison SF (2005). Direct pyrogenic input from prostaglandin EP3 receptor-expressing preoptic neurons to the dorsomedial hypothalamus. Eur J Neurosci 22: 3137–3146.

National Research Council (1996). Guide for the Care and Use of Laboratory Animals. Institute for Laboratory Animal Research. National Academy Press: Washington, DC, pp. 21–66.

Oka T, Oka K, Kobayashi T, Sugimoto Y, Ichikawa A, Ushikubi F *et al.* (2003). Characteristics of thermoregulatory and febrile responses in mice deficient in prostaglandin EP1 and EP3 receptors. J Physiol 551: 945–954.

Okumura T, Murata Y, Hizue M, Matsuura T, Naganeo R, Kanai Y *et al.* (2006). Pharmacological separation between peripheral and central functions of cyclooxygenase-2 with CIAA, a novel cyclooxygenase-2 inhibitor. Eur J Pharmacol 539: 125–130.

Paxinos G, Watson C (1986). The Rat Brain in Stereotaxic Coordinates, 2nd edn. Academic Press: New York.

Pessini AC, Santos DR, Arantes EC, Souza GE (2006). Mediators involved in the febrile response induced by *Tityus serrulatus* scorpion venom in rats. Toxicon 48: 556–566.

Pierre SC, Schmidt R, Brenneis C, Michaelis M, Geisslinger G, Scholich K (2007). Inhibition of cyclooxygenases by dipyrone. Br J Pharmacol 151: 494–503.

Piper PJ, Vane JR, Wyllie JH (1970). Inactivation of prostaglandins by the lungs. Nature 225: 600–604.

Rezende RM, França DS, Menezes GB, dos Reis WG, Bakhle YS, Francischi JN (2008). Different mechanisms underlie the analgesic actions of paracetamol and dipyrone in a rat model of inflammatory pain. Br J Pharmacol 153: 760–768.

Roth J, De Souza GE (2001). Fever induction pathways: evidence from responses to systemic or local cytokine formation. Braz J Med Biol Res 34: 301–314.

Roth J, Rummel C, Barth SW, Gerstberger R, Hübschle T (2006). Molecular aspects of fever and hyperthermia. Neurol Clin 24: 421–439.

Roth J, Rummel C, Barth SW, Gerstberger R, Hübschle T (2009). Molecular aspects of fever and hyperthermia. Immunol Allergy Clin North Am 29: 229–245.

Sachs D, Cunha FQ, Ferreira SH (2004). Peripheral analgesic blockade of hypernociception: activation of arginine/NO/cGMP/protein kinase G/ATP-sensitive K+ channel pathway. Proc Natl Acad Sci USA 101: 3680–3685.

Scammell TE, Griffin JD, Elmquist JK, Saper CB (1998). Microinjection of a cyclooxygenase inhibitor into the anteroventral preoptic region attenuates LPS fever. Am J Physiol 274: R783–R789.

Sehic E, Székely M, Ungar AL, Oladehin A, Blatteis CM (1996). Hypothalamic prostaglandin E2 during lipopolysaccharide-induced fever in guinea pigs. Brain Res Bull 39: 391–399.

Shimada SG, Otterness IG, Stitt JT (1994). A study of the mechanism of action of the mild analgesic dipyrone. Agents Actions 41: 188–192.

Siebel JS, Beirith A, Calixto JB (2004). Evidence for the involvement of metabotropic glutamatergic, neurokinin 1 receptor pathways and protein kinase C in the antinociceptive effect of dipyrone in mice. Brain Res 1003: 61–67.

Souza FR, Souza VT, Ratzlaff V, Borges LP, Oliveira MR, Bonacorso HG *et al.* (2002). Hypothermic and antipyretic effects of 3-methyl- and 3-phenyl-5-hydroxy-5-trichloromethyl-4, 5-dihydro-1H-pyrazole-1-carboxyamides in mice. Eur J Pharmacol 451: 141–147.

Steiner AA, Ivanov AI, Serrats J, Hosokawa H, Phayre AN, Robbins JR *et al.* (2006). Cellular and molecular bases of the initiation of fever. Plos Biol 4: e284 (1517–1524).

Strijbos PJ, Hardwick AJ, Relton JK, Carey F, Rothwell NJ (1992). Inhibition of central actions of cytokines on fever and thermogenesis by lipocortin-1 involves CRF. Am J Physiol Endocrinol Metab 263: E632–E636.

Tatsuo MA, Carvalho WM, Silva CV, Miranda AE, Ferreira SH, Francischi JN (1994). Analgesic and antiinflammatory effects of dipyrone in rat adjuvant arthritis model. Inflammation 18: 399–405.

Wilkinson MF, Kasting NW (1989). Central vasopressin V1-receptors mediate indomethacin-induced antipyresis in rats. Am J Physiol Regul Integr Comp Physiol 256: R1164–R1168.

Zampronio AR, Melo MC, Hopkins SJ, Souza GE (2000). Involvement of CRH in fever induced by a distinct pre-formed pyrogenic factor (PFPF). Inflamm Res 49: 473–479.