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RESEARCH PAPER

Dipyrone metabolite 4-MAA induces hypothermia and inhibits PGE_2-dependent and -independent fever while 4-AA only blocks PGE_2-dependent fever

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BACKGROUND AND PURPOSE

The antipyretic and hypothermic prodrug dipyrone prevents PGE_2-dependent and -independent fever induced by LPS from *Escherichia coli* and *Tityus serrulatus* venom (Tsv) respectively. We aimed to identify the dipyrone metabolites responsible for the antipyretic and hypothermic effects.

EXPERIMENTAL APPROACH

Male Wistar rats were treated i.p. with indomethacin (2 mg·kg^{-1}), dipyrone, 4-methylaminoantipyrine (4-MAA), 4-aminoantipyrine (4-AA) (60–360 mg·kg^{-1}), 4-formylaminoantipyrine, 4-acethylaminoantipyrine (120–360 mg·kg^{-1}) or vehicle 30 min before i.p. injection of LPS (50 µg·kg^{-1}), Tsv (150 µg·kg^{-1}) or saline. Rectal temperatures were measured by tele-thermometry and dipyrone metabolite concentrations determined in the plasma, CSF and hypothalamus by LC-MS/MS. PGE_2 concentrations were determined in the CSF and hypothalamus by ELISA.

KEY RESULTS

In contrast to LPS, Tsv-induced fever was not followed by increased PGE_2 in the CSF or hypothalamus. The antipyretic time-course of 4-MAA and 4-AA on LPS-induced fever overlapped with the period of the highest concentrations of 4-MAA and

4-AA in the hypothalamus, CSF and plasma. These metabolites reduced LPS-induced fever and the PGE_2 increase in the plasma, CSF and hypothalamus. Only 4-MAA inhibited Tsv-induced fever. The higher doses of dipyrone and 4-MAA also induced hypothermia.

CONCLUSIONS AND IMPLICATIONS

The presence of 4-MAA and 4-AA in the CSF and hypothalamus was associated with PGE_2 synthesis inhibition and a decrease in LPS-induced fever. 4-MAA was also shown to be an antipyretic metabolite for PGE_2-independent fever induced by Tsv suggesting that it is responsible for the additional antipyretic mechanism of dipyrone. Moreover, 4-MAA is the hypothermic metabolite of dipyrone.

Abbreviations

4-AA, 4-aminoantipyrine; 4-AAA, 4-acethylaminoantipyrine; 4-FAA, 4-formylaminoantipyrine; 4-MAA, 4-methylaminoantipyrine; aCSF, artificial CSF; CRF, corticotrophin-releasing factor; ET-1, endothelin-1; NSAID, non-steroidal anti-inflammatory drugs; PFPF, preformed pyrogenic factor; POA/AH, preoptic area of the anterior hypothalamus; rT, rectal temperature; Tsv, *Tityus serrulatus* venom

Introduction

Fever, an important brain-mediated response that is part of an acute-phase response, is traditionally defined as the elevation in body temperature in response to injury, trauma or invasion by infectious agents (Morrison and Nakamura, 2011). It is triggered by a variety of exogenous pyrogens, including the venom of poisonous animals, such as the *Tityus serrulatus* venom (Tsv) as well as the so-called pathogen-associated molecular patterns, such as LPS produced by Gram-negative bacteria (Pessini *et al.*, 2006; Roth *et al.*, 2009). The febrile response induced by exogenous pyrogens is mediated by several endogenous pyrogens, such as TNF-α, IL-1β, IL-6, corticotrophin-releasing factor (CRF), endothelin-1 (ET-1), preformed pyrogenic factor (PFPF), bradykinin and PGE_2 (Roth and Souza, 2001; Fabricio *et al.*, 2006a; Roth *et al.*, 2009).

Some authors have proposed that LPS induces fever via the PGE_2-dependent and -independent pathways (Strijbos *et al.*, 1992; Fabricio *et al.*, 2006a; Malvar *et al.*, 2011). The first pathway requires cytokine synthesis/release and subsequent PGE_2 synthesis (via COX-2) in the preoptic area of the anterior hypothalamus (POA/AH) (Lazarus *et al.*, 2007; Roth *et al.*, 2009; for review Morrison and Nakamura, 2011). The second pathway is PGE_2-independent and seems to be orchestrated by PFPF, CRF and ET-1 (Zampronio *et al.*, 2000; Fabricio *et al.*, 2006a). Although a PGE_2-independent pathway is involved in LPS-induced fever, non-steroidal anti-inflammatory drugs (NSAIDs) dose-dependently reduce this response (Fabricio *et al.*, 2005; Soares *et al.*, 2006; Malvar *et al.*, 2011). Moreover, mice deficient in prostanoid EP_3 receptors (for receptor nomenclature see Alexander *et al.*, 2013) do not develop fever in response to LPS (Oka *et al.*, 2003), suggesting a crucial role for PGE_2 in the development of fever induced by LPS.

It has been reported that i.p. injection of Tsv induces systemic and local alterations similar to those observed in the LPS-induced acute-phase response, such as plasmatic cytokines (IL-6, IL-1α and TNF-α), an increase in C-reactive protein and circulating leukocytes, fever, cell migration to the site of injection and pain in mice and rats (Pessini *et al.*, 2003; 2006; 2008).

A febrile response has been reported to occur in about 18% of human patients stung by *T. serrulatus* (Freire-Maia *et al.*, 1994). A previous study from our group showed that Tsv-induced fever is mediated by kinins, IL-1 and NO, but unaffected by NSAIDs, such as celecoxib and ibuprofen, suggesting the involvement of a PGE_2-independent mechanism. However, dipyrone, at doses that effectively reduce LPS-induced fever (Souza *et al.*, 2002), also reduce Tsv-induced fever (Pessini *et al.*, 2006).

Dipyrone is a prodrug with potent antipyretic and analgesic effects (Lorenzetti and Ferreira, 1985; Souza *et al.*, 2002). Following i.v. administration dipyrone is briefly detectable in the serum for about 15 min but it is not detectable after oral intake (Ergün *et al.*, 2001) as it is rapidly hydrolysed in the gastrointestinal tract to 4-methylaminoantipyrine (4-MAA) and absorbed as such. 4-MAA is further metabolized to 4-formylaminoantipyrine (4-FAA) and to 4-aminoantipyrine (4-AA), which is acetylated by a polymorphic N-acetyltransferase system to 4-acethylaminoantipyrine (4-AAA) (Cohen *et al.*, 1998).

There are few studies suggesting that dipyrone, like other NSAIDs, produces an antipyretic effect by PGE_2 synthesis inhibition (Shimada *et al.*, 1994; Kanashiro *et al.*, 2009). Corroborating with this idea, dipyrone has been shown to inhibit PGE_2 synthesis *in vivo* by COX inhibition through two of its metabolites, 4-MAA and 4-AA, indicating that these may be the active metabolites of dipyrone (Hinz *et al.*, 2007; Pierre *et al.*, 2007). However, a significant body of evidence from our group has demonstrated that the antipyretic mechanism of dipyrone does not only relate to PGE_2 synthesis inhibition (Souza *et al.*, 2002; Pessini *et al.*, 2006; Malvar *et al.*, 2011). In addition, unlike other NSAIDs, at high doses dipyrone induces hypothermia by an unknown mechanism.

Despite knowing that dipyrone is a prodrug, most of the *in vitro* experiments have been carried out using dipyrone itself. Consequently, these studies do not elucidate the mechanism/metabolite through which dipyrone exerts its antipyretic and analgesic effects. This study, for the first time, demonstrated that 4-MAA and 4-AA are the antipyretic metabolites of dipyrone on LPS-induced fever, while only 4-MAA was effective on Tsv-induced fever in rats. We also showed that 4-MAA is the only dipyrone metabolite that causes hypothermia.

Methods

Animals

Experiments were conducted on 246 male Wistar rats weighing 180–200 g. They were housed individually at 24 ± 1°C under a 12:12 h light–dark cycle (lights on at 06:00 h) 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. Care and use of the animals were in full compliance with the Ethical Principles in Animal Research adopted by the National Council for the Control of Animal Experimentation (CONCEA) and the Guide for the Care and Use of Laboratory Animals of the Institute for Laboratory Animal Research (Alvarez and Pardo, 1997). Also the study was previously approved by the Animal Research Ethics Committee of the Faculty of Medicine of Ribeirão Preto, University of São Paulo (Protocol nr. 200/2008). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Temperature measurements

The rectal temperature (rT) 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 rats from their cages. Experimental measurements were conducted in a temperature-controlled room at 27 ± 1°C, within the thermoneutral zone for rats (Gordon, 1990). Baseline temperatures were determined three to four times, and at 30 min intervals before i.p. injection of LPS or Tsv (and always before 10:00 h). Only animals displaying mean basal rTs between 36.8 and 37.2°C were selected for the study. In order to minimize core temperature changes because of handling, the animals were habituated to this environment by carrying out immobilization for i.p. injection and rT measurement procedures twice (at 30 min intervals) on the preceding day.

Plasma and CSF collection

For blood and CSF collection, animals were anaesthetized with a mixture of ketamine and xylazine (60 and 20 mg·kg^{-1}, respectively, i.p.). Single blood samples, which were collected by cardiac puncture, were placed in tubes containing heparin, cooled on ice and protected from light, centrifuged at 1300× *g* for 15 min at 4°C, and the plasma was collected and immediately frozen to −70°C until analysis. A single CSF sample was collected from each animal according to the method described by Consiglio and Lucion (2000). Briefly, just before CSF collection each rat was 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 (Fabricio *et al.*, 2005; Malvar *et al.*, 2011). A 25-G needle connected to a 1 mL syringe was then inserted vertically and centrally through this depression into the cistern magna and a gentle aspiration caused the CSF to flow through it, resulting in 50–100 µL samples. Gentle movements of the needle are necessary during collection in order to prevent bleeding. CSF samples contaminated with blood were discarded.

Dissection of hypothalamus

Immediately after CSF collection, the animals were killed by decapitation and their brains removed immediately. The entire hypothalamus was dissected from the brain using the following limits: the anterior border of the optic chiasm, 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 at −70°C until analysis.

Determination of PGE_2 concentration in the CSF and hypothalamus

The CSF samples were placed in Eppendorf tubes containing indomethacin (10 µmol·L^{-1}) to prevent PG production. Samples were maintained in the dark and under ice until centrifugation at 1300× *g* for 15 min at 4°C, and the supernatants were immediately frozen at −70°C until analysis.

Each hypothalamus (~100 mg) was homogenized in 1 mL of RPMI medium containing indomethacin (2 mg·mL^{-1}) using a Digital 600 W ultrasonic microprocessor cell disrupter (Virsonic 100®; VirTis, Gardiner, NY, USA) and then acidified with HCl solution (1 mol·L^{-1}) 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. On the following day, the dry sample was resuspended in enzyme immunoassay (EIA) buffer.

The PGE_2 contents in the CSF and hypothalamus samples were measured using a PGE_2 Express EIA *Kit* from Cayman Chemical (Ann Arbor, MI, USA) following the procedures detailed in the instructions, with a detection limit of 7.8 pg·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-keto-$PGF_{1\alpha}$, and less than 0.1% with all other prostanoids tested. Intra- and inter-assay coefficients of variation were <11%.

Determination of dipyrone metabolite concentrations in the plasma, CSF and hypothalamus by LC-MS/MS

The concentrations of the dipyrone metabolites 4-MAA, 4-AA, 4-FAA and 4-AAA were determined by LC-MS/MS according to the method described by Aguiar *et al.* (2013). Hypothalamus samples were submitted to ultrasonic disruption in 1 mL of water. Fifty microliters of NaOH solution (1.0 mol·L^{-1}) and dichloromethane (3 mL) were then added to the plasma, CSF and hypothalamic samples for the liquid–liquid extraction of the dipyrone metabolites. The resolution of the dipyrone metabolites and moclobemide (used as internal standard) was achieved using a C18 column (XTerra®, 100 × 39 mm, 3.5 µm particle size, Waters Corporation) with an isocratic elution profile of water : methanol (70:30, v v^{-1}) plus 0.5% glacial acetic acid as the mobile phase, at a flow-rate of

0.5 mL·min^{-1}. Mass analyses using positive electrospray ionization and multiple reaction monitoring modes were also conducted (Supporting Information Figs S1–S3).

Treatment protocols

Rats were pretreated with indomethacin (2 mg·kg^{-1}, i.p.), dipyrone (60–360 mg·kg^{-1}, i.p.), 4-MAA (60–360 mg·kg^{-1}, i.p.), 4-AA (60–360 mg·kg^{-1}, i.p.), 4-FAA (120–360 mg·kg^{-1}, i.p.), 4-AAA (120–360 mg·kg^{-1}, i.p.) or vehicle (saline). Thirty minutes later, LPS from *Escherichia coli* (50 μg·kg^{-1}), *T. serrulatus* venom (Tsv; 150 μg·kg^{-1}) or sterile saline (0.5 mL) were injected i.p. The doses of indomethacin, dipyrone, LPS and Tsv were established in previous studies from our group (Souza *et al.*, 2002; Pessini *et al.*, 2006; Malvar *et al.*, 2011).

Scorpion venom and drugs

Desiccated *T. serrulatus* scorpion venom was purchased from Phoneutria Biotechnology and Services (Belo Horizonte, MG, Brazil) and kept at –20°C until use. The following reagents were used: LPS (*E. coli* 0111:B4), dipyrone (sodium metamizol) and 4-aminoantipyrine (4-AA) from Sigma (St. Louis, MO, USA), indomethacin from Merck, Sharp & Dohme (São Paulo, SP, Brazil), ketamine (Ketamina Agener®) from União Química Farmacêutica Nacional S.A. (São Paulo, SP, Brazil), xylazine (Dopaser®) from Calier Laboratories S.A. (Barcelona, Spain), oxytetracycline hydrochloride (Terramicina®) from Pfizer (São Paulo, SP, Brazil). 4-MAA was synthesized from dipyrone while 4-FAA and 4-AAA were synthesized from 4-AA as described by Aguiar *et al.* (2013).

Statistical analysis

For data analysis, the baseline temperature before any injection was determined for each animal and all subsequent rTs were expressed as changes from the mean basal value (ΔrT). 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 rT and dipyrone metabolite concentrations were compared across treatments and time points by two-way ANOVA for repeated measurements followed by the Bonferroni's test. All data were analysed using Prism 5 computer software from Graph-Pad (San Diego, CA, USA). Differences were considered significant when $P < 0.05$.

Results

Antipyretic and hypothermic effect of dipyrone

I.p. injection of LPS (50 μg·kg^{-1}) elicited a marked elevation of rT that started at 2 h and persisted up to 6 h (Figure 1A). In contrast, i.p. administration of Tsv (150 μg·kg^{-1}) caused a high and short-lasting fever. Tsv increased rT after 1 h, peaked

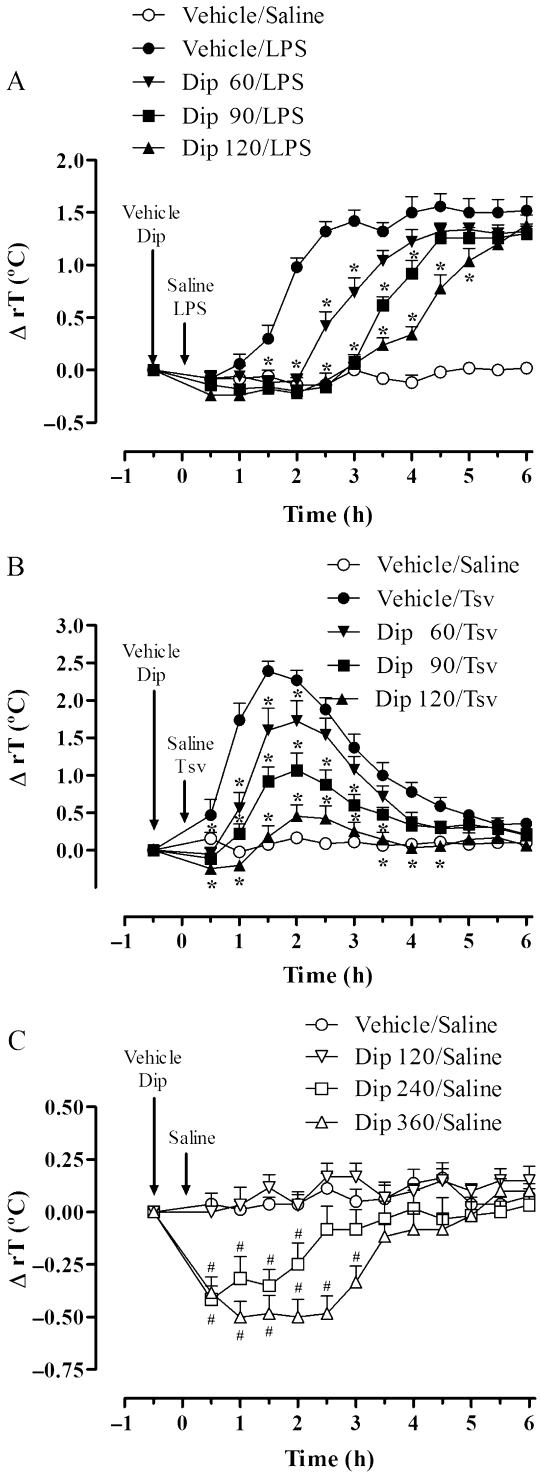

Figure 1

Antipyretic and hypothermic effect of dipyrone (Dip). In (A) and (B) rats received i.p. injections of dipyrone (60, 90 or 120 mg·kg^{-1}) or vehicle (saline) 30 min before LPS (A, 50 μg·kg^{-1}, i.p.), Tsv (B, 150 μg·kg^{-1}, i.p.) or sterile saline (0.5 mL, control). In (C) rats received i.p. injections of dipyrone (120, 240 or 360 mg·kg^{-1}) or vehicle (saline) 30 min before saline. Values represent the means ± SEM of the changes in rT (ΔrT, °C) of 6–10 animals per group. $^{\#,*}P < 0.05$ compared with the groups treated with vehicle/saline or vehicle/pyrogenic stimulus respectively. Basal rTs of each group were as follows: (A and B) vehicle/saline 36.96 ± 0.07; vehicle/LPS or Tsv 36.99 ± 0.05; dipyrone 60/LPS or Tsv 37.00 ± 0.05; dipyrone 90/LPS or Tsv 37.02 ± 0.06; dipyrone 120/LPS or Tsv 37.00 ± 0.05; (C) vehicle/saline 37.00 ± 0.07; dipyrone 120/saline 37.00 ± 0.04; dipyrone 240/saline 36.96 ± 0.05; dipyrone 360/saline 36.95 ± 0.04.

at 1.5 h by nearly 2.5°C, and decreased thereafter, until reaching basal values approximately 5.0 h later (Figure 1B).

Dipyrone (60–120 mg·kg^{-1}, i.p.) produced a dose-related antipyretic effect from 1.5 to 5.0 h after LPS (Figure 1A) and from 30 min to 4.5 h after TSV injection (Figure 1B). Moreover, at 120 mg·kg^{-1} dipyrone abolished the fever induced by Tsv (Figure 1B), but did not modify the basal rT of control rats. Importantly, at 240 and 360 mg·kg^{-1} (i.p.) dipyrone induced a dose-related hypothermic response (Figure 1C).

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

In order to measure the PGE_2 content in cisternal CSF and the hypothalamus, samples were collected 2 and 3 h after LPS injection and 1 and 2 h after Tsv injection. Under our experimental conditions, PGE_2 concentrations in the CSF of control animals treated with vehicle (saline) were below the detection limit of the assay (Figure 2B and E), while PGE_2 content in hypothalami collected from these animals was clearly detectable (Figure 2C and F). As expected, LPS induced fever (Figure 2A) and increased the PGE_2 content in both the CSF (Figure 2B) and hypothalamus (Figure 2C). Contrastingly, Tsv induced fever (Figure 2D), but without increasing the PGE_2 content in either the CSF (Figure 2E) or the hypothalamus (Figure 2F).

Indomethacin (2 mg·kg^{-1}, i.p.) reduced LPS-induced fever after 2.5 and 3.0 h, but failed to modify the fever induced by Tsv, while dipyrone (120 mg·kg^{-1}, i.p.) abolished the fever induced by both stimuli (Figure 2A and D, respectively). At these doses, indomethacin and dipyrone did not modify the basal rT of control animals (Figure 2A and D respectively).

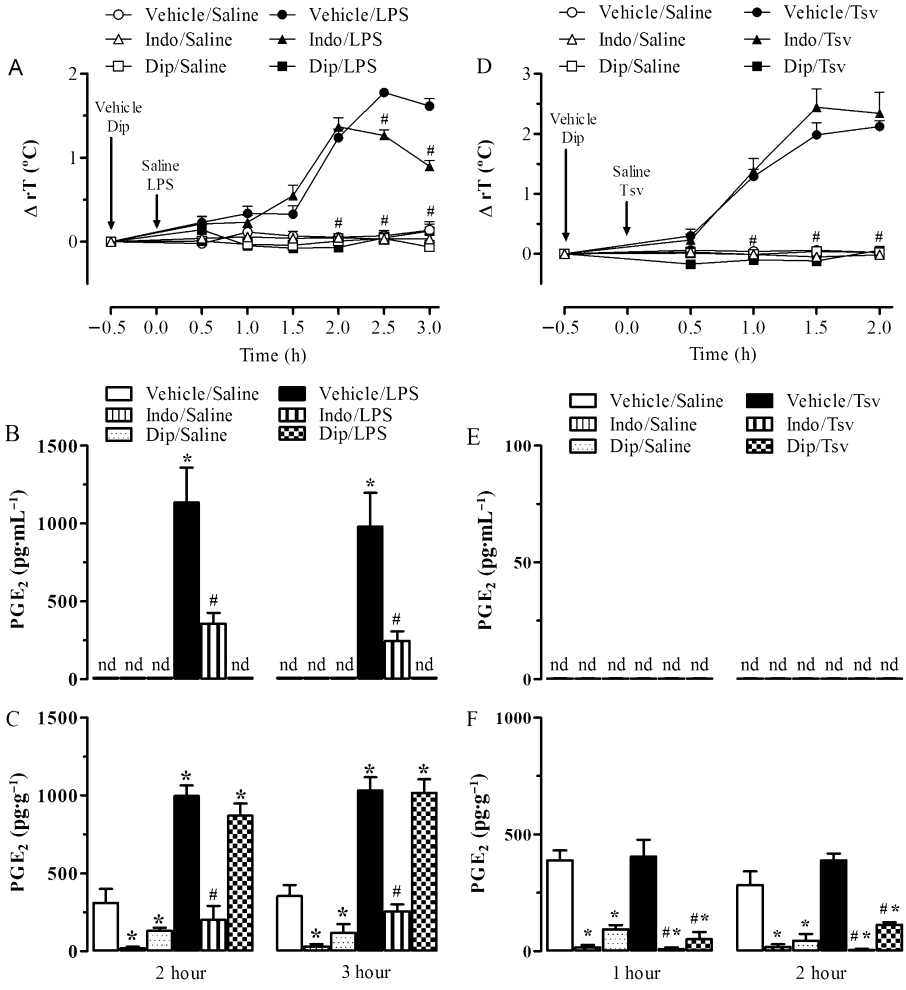

Figure 2

Effect of indomethacin (Indo) or dipyrone (Dip) on changes in rT (A and D), CSF (B and E) and hypothalamic (C and F) PGE_2 concentration after LPS (A–C) or Tsv (D–F) injection. Indomethacin (Indo, 2 mg·kg^{-1}, i.p.), dipyrone (Dip, 120 mg·kg^{-1}, i.p.) or vehicle (10% Tris-HCl in saline, 0.5 mL) was administered 30 min before LPS (50 μg·kg^{-1}, i.p.), Tsv (150 μg·kg^{-1}, i.p.) or sterile saline (0.5 mL, control) injection. The CSF and hypothalamus were harvested 2 and 3 h after LPS or saline injection and 1 and 2 h after Tsv or saline injection. PGE_2 concentration was determined by ELISA. Values represent means ± SEM of the variation in rT (ΔrT, °C) and the PGE_2 levels in the CSF (pg·mL^{-1}) and hypothalamus (pg·g^{-1} of tissue) of six animals per group. $^{\#,*}P < 0.05$ compared with the groups treated with vehicle/saline or vehicle/pyrogenic stimulus respectively. Basal rTs of each group did not differ significantly and were all around 37°C (see Figure 1 legend).

Moreover, indomethacin reduced the increase of PGE_2 concentration in both CSF and hypothalamus (Figure 2B and C) while dipyrone only reduced the increase of PGE_2 concentration in CSF (Figure 2B). Although dipyrone significantly reduced the basal amount of PGE_2 in the hypothalamus (Figure 2C and F), it did no modify the hypothalamic content of PGE_2 at 2 and 3 h after LPS (Figure 2C). Administration of indomethacin or dipyrone reduced the amount of PGE_2 in the hypothalami of animals treated with Tsv to values below those observed in the vehicle-treated animals (Figure 2C and F).

Antipyretic and hypothermic effect of dipyrone metabolites

I.p. treatment with 4-MAA, 4-AA (60–120 mg·kg^{-1}, i.p.) and 4-FAA, but not 4-AAA (both at 120–360 mg·kg^{-1}, i.p.), produced a dose-related antipyretic effect on LPS-induced fever (Figures 3A, C, 4A and C). The highest dose of 4-MAA (120 mg·kg^{-1}) abolished the febrile response induced by LPS for up to 6 h (Figure 3A). 4-MAA and 4-AA inhibited its onset, from 1.5 to 5.0 h (Figure 3A and C), while 4-FAA reduced the LPS-induced fever from 3 to 6 h (Figure 4A). Moreover, at these doses used, 4-MAA and 4-AA, but not 4-FAA, were more effective than dipyrone to reduce the fever induced by LPS.

4-MAA (60–120 mg·kg^{-1}, i.p., Figure 3B) produced a dose-related antipyretic effect on Tsv-induced fever while 4-AA (60–120 mg·kg^{-1}, i.p., Figure 3D), 4-FAA and 4-AAA (both at 120–360 mg·kg^{-1}, i.p., Figure 4B and D) were ineffective. It is important to note that, unlike what was observed for the LPS-induced fever, 4-MAA was less effective than dipyrone in reducing the fever induced by Tsv.

Like dipyrone (Figure 1C), 4-MAA at the i.p. antipyretic dose of 120 mg·kg^{-1} did not modify the basal rT of control rats, but at the highest doses (240–360 mg·kg^{-1}, i.p.) it induced a dose-related hypothermia in control animals (Figure 5A). Moreover, at the doses used here the hypothermia induced by 4-MAA was more pronounced than that induced by dipyrone. Finally, treatment with 4-AA, 4-FAA and 4-AAA (120–360 mg·kg^{-1}, i.p.) did not significantly change the basal temperature of animals (Figure 5B, C and D).

Effect of 4-MAA and 4-AA on changes in PGE_2 concentration in the CSF and hypothalamus induced by LPS

PGE_2 contents were measured in the CSF and hypothalamus samples collected 3 h after LPS injection. The amount of PGE_2 in the cisternal CSF of control (saline treated animals) was

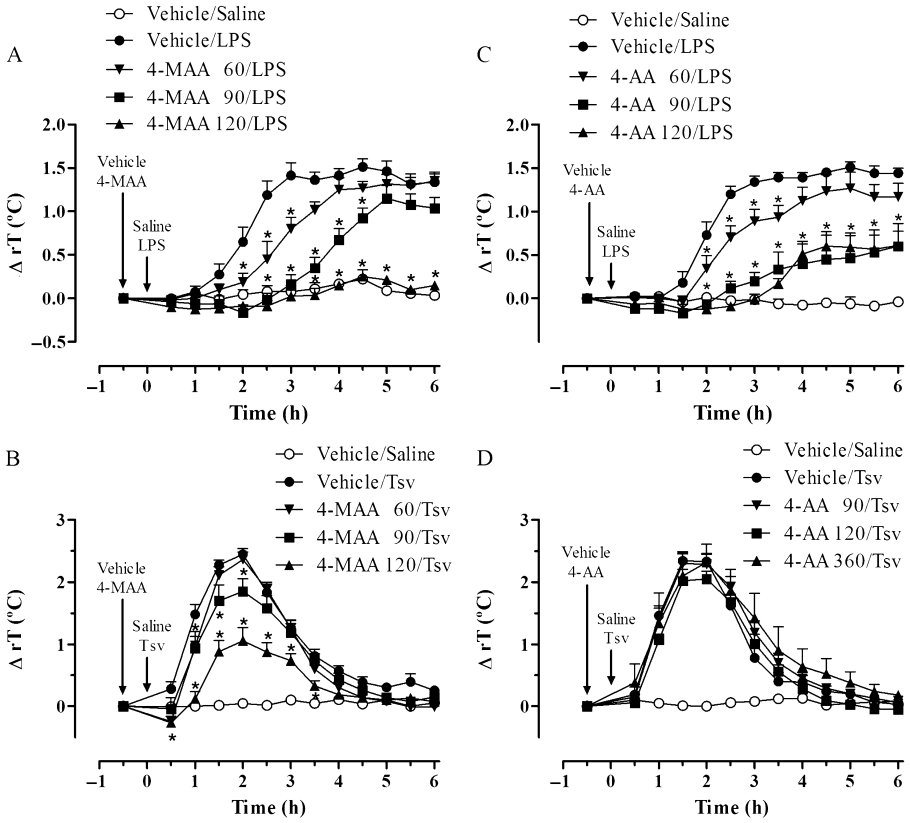

Figure 3

Antipyretic effect of 4-MAA (A and B) or 4-AA (C and D) on fever evoked by LPS (A and C) or Tsv (B and D). Rats received i.p. injections of 4-MAA (60–120 mg·kg^{-1}), 4-AA (60–360 mg·kg^{-1}) or vehicle (saline) 30 min before LPS (50 μg·kg^{-1}, i.p.), Tsv (150 μg·kg^{-1}, i.p.) or sterile saline (0.5 mL, control). Values represent the means ± SEM of the changes in rT (ΔrT, °C) of 6–15 animals per group. *, $P < 0.05$ compared with the groups treated with vehicle/LPS or vehicle/Tsv. Basal rTs of each group did not differ significantly and were all around 37°C (see Figure 1 legend).

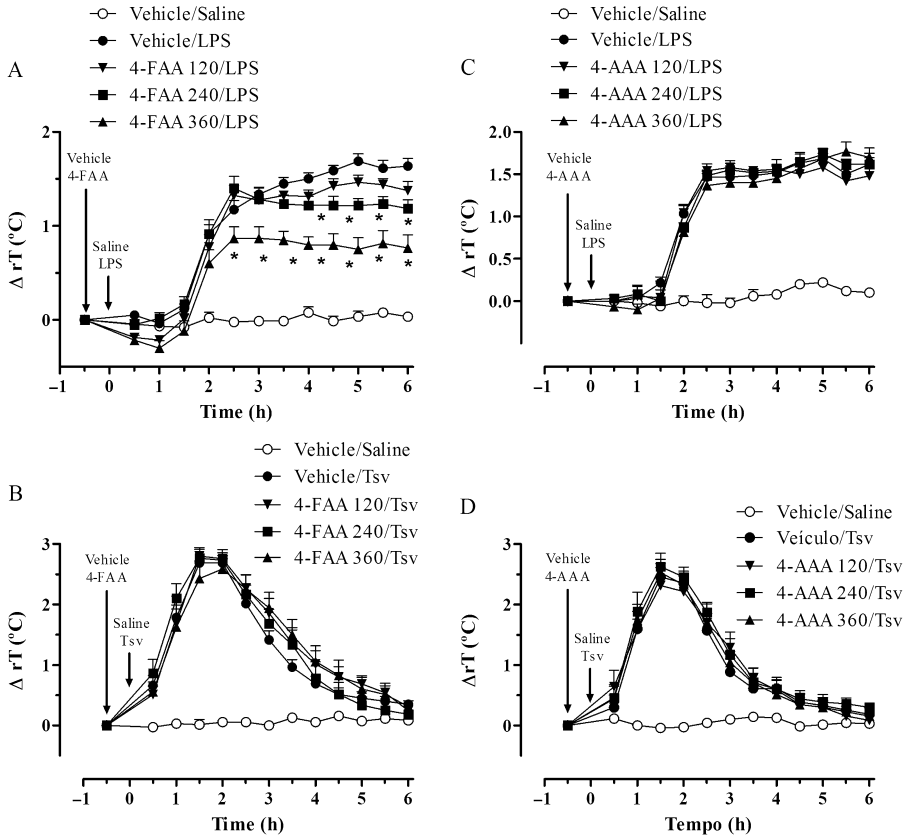

Figure 4

Antipyretic effect of 4-FAA (A and B) or 4-AAA (C and D) on fever evoked by LPS (A and C) or Tsv (B and D). Rats received i.p. injections of 4-FAA (120–360 mg·kg^{-1}), 4-AAA (120–360 mg·kg^{-1}) or vehicle (saline) 30 min before LPS (50 μg·kg^{-1}, i.p.), Tsv (150 μg·kg^{-1}, i.p.) or sterile saline (0.5 mL, control). Values represent the means ± SEM of the changes in rT (ΔrT, °C) of 6–10 animals per group. *, $P < 0.05$ compared with the groups treated with vehicle/LPS. Basal rTs of each group did not differ significantly and were all around 37°C (see Figure 1 legend).

below the detection limit of the assay (Figure 6B), while the PGE_2 content in hypothalami collected from these animals neared 420 pg·g^{-1} (Figure 6C) similar to the amount observed in the previous experiment (Figure 2C and F). As expected, LPS induced fever (Figure 6A) and increased the PGE_2 content in both the CSF (Figure 6B) and the hypothalamus (Figure 6C). 4-MAA and 4-AA (90 mg·kg^{-1}, i.p.) reduced the fever (Figure 5A) and inhibited the increase in the concentration of PGE_2 in the CSF and hypothalamus (Figure 6B and C).

Concentration of dipyrone metabolites in plasma, CSF and hypothalamus samples

In order to measure the dipyrone metabolite contents in the plasma, CSF and hypothalamus, samples were collected between 1.5 and 5.5 h after dipyrone (120 mg·kg^{-1}, i.p.), 4-MAA or 4-AA (90 mg·kg^{-1}, i.p.) treatments. The evaluated doses and times were selected for their effectiveness in producing similar duration and degree of antipyretic effect after LPS administration. All dipyrone metabolites were found in the plasma, CSF and hypothalamus after i.p. administration of dipyrone, 4-MAA or 4-AA and they presented a similar temporal distribution in these tissues (Figure 7A–I). The antipyretic period of dipyrone (from 1.5 to 5.5 h after its administration; Figure 1A) is associated with the period of high concentrations of 4-MAA and 4-AA and low concentrations of 4-FAA and 4-AAA in the plasma (Figure 7A, B and C), CSF (Figures 7D, 6E and F) and hypothalamus (Figures 7G, 6H and I). Compared with dipyrone (120 mg·kg^{-1}), treatment with 4-MAA (90 mg·kg^{-1}) promoted a higher concentration of this metabolite (Figure 8A, B and C) and 4-AA (Figure 8D and F) for up to 2.5 h in the plasma, 0.5 h in the CSF and 3.5 h in the hypothalamus after treatments. Similarly, compared with dipyrone, treatment with 4-AA (90 mg·kg^{-1}) also promoted a higher concentration of 4-AA for up to 2.5 h in the plasma, 3.5 h in the CSF and 5.5 h in the hypothalamus after the treatments (Figure 8B, D and F).

Aiming to evaluate if LPS or Tsv changed the metabolism of dipyrone, animals were pretreated with dipyrone (120 mg·kg^{-1}) 30 min before i.p. injection of saline, LPS (50 μg·kg^{-1}) or Tsv (150 μg·kg^{-1}) and samples of plasma, CSF and hypothalamus were collected at 1 and 3 h after pyrogenic stimuli or saline injection and the dipyrone metabolites were measured. LPS or Tsv injection did not change the amount of 4-MAA, 4-AA (Figure 9), 4-FAA or 4-AAA (data not shown) in the plasma, CSF or hypothalamus.

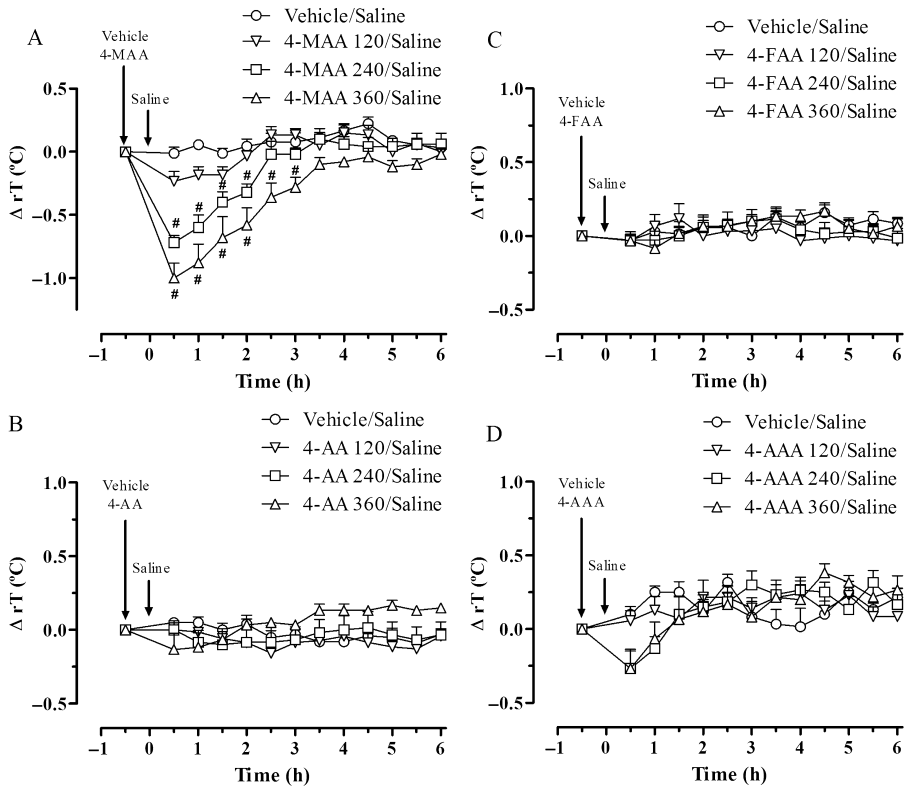

Figure 5

4-MAA (A) induces a dose-dependent hypothermia while 4-AA (B), 4-FAA (C) or 4-AAA (C) did not alter the basal temperature of rats. Rats received i.p. injections of 4-MAA, 4-AA, 4-FAA, 4-AAA at doses of 120–360 mg·kg^{-1} or vehicle (saline) 30 min before sterile saline (0.5 mL, control). Values represent the means ± SEM of the changes in rT (ΔrT, °C) of six to eight animals per group. $^{\#}$, $P < 0.05$ compared with the groups treated with vehicle/saline. Basal rTs of each group did not differ significantly and were all around 37°C (see Figure 1 legend).

Discussion

The current study showed for the first time that the fever induced by Tsv was not followed by an increase in PGE_2 concentration in either the CSF or hypothalamus. Corroborating with previous findings from our lab, dipyrone, but not indomethacin, inhibited Tsv-induced fever suggesting that Tsv induces fever through a PGE_2-independent pathway (Pessini *et al.*, 2006) and that dipyrone has additional antipyretic mechanisms (Malvar *et al.*, 2011). Moreover, among the dipyrone metabolites, 4-MAA was the only one able to inhibit the PGE_2-independent fever induced by Tsv, while 4-MAA and 4-AA were the main active antipyretic metabolites of dipyrone on LPS-induced fever. After i.p. administration of dipyrone (120 mg·kg^{-1}), the amount of 4-MAA and 4-AA in the plasma, CSF and hypothalamus is less in comparison with the amount found after 4-MAA (90 mg·kg^{-1}, i.p.), which could explain why 4-MAA, but not dipyrone, reduces PGE_2 synthesis in the hypothalamus at the doses used. At higher doses, dipyrone and 4-MAA (240–360 mg·kg^{-1}, i.p.) caused hypothermia in the saline-treated animals, suggesting that this metabolite is responsible for this effect of dipyrone.

As described in the introduction, dipyrone is a prodrug with potent antipyretic and analgesic effects (Lorenzetti and Ferreira, 1985; Souza *et al.*, 2002). Some studies suggest that dipyrone, like other NSAIDs, produces antipyresis by PGE_2 synthesis inhibition (Shimada *et al.*, 1994; Kanashiro *et al.*, 2009). Moreover, it has been shown that dipyrone inhibits PGE_2 synthesis through COX inhibition by two of its metabolites, 4-MAA and 4-AA, indicating that they may be the active metabolites of dipyrone (Hinz *et al.*, 2007; Pierre *et al.*, 2007). However, several studies from our group indicate that the antipyretic mechanism of dipyrone does not only involve PGE_2 synthesis inhibition (Souza *et al.*, 2002; Pessini *et al.*, 2006; Malvar *et al.*, 2011; Martins *et al.*, 2012).

In the present study, i.p. administration of dipyrone produced a dose-dependent antipyretic effect and inhibited the increase of PGE_2 content in the CSF, but not in the hypothalamus after i.p. injection of LPS. This result is in agreement with our previous studies that report these same dipyrone effects after intravenous injection of LPS, i.p. injection of live *Staphylococcus aureus* or i.c.v. injection of ET-1 (Malvar *et al.*, 2011; Martins *et al.*, 2012). As it is widely accepted that PGE_2 generated in the POA/AH appears to be the key mediator of the fever induced by LPS (Engblom *et al.*, 2003; Oka *et al.*, 2003; Lazarus *et al.*, 2007; Roth *et al.*, 2009; for review see Morrison and Nakamura, 2011), these results are in agreement with previous findings from our group (Malvar *et al.*, 2011) that dipyrone possesses an additional antipyretic mechanism that does not rely on hypothalamic PGE_2 synthesis inhibition.

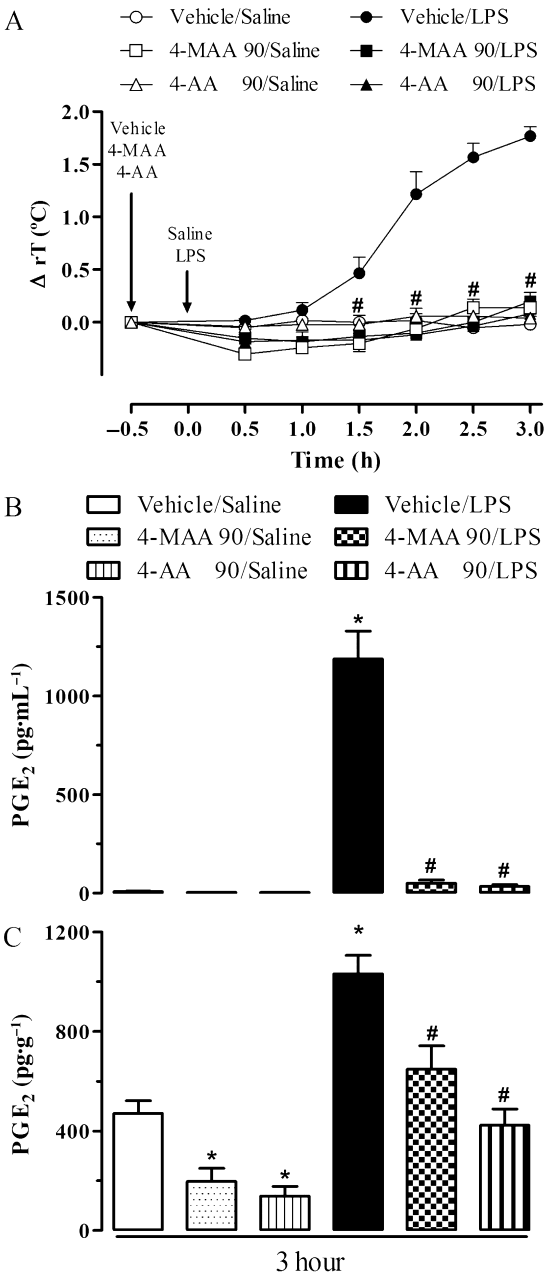

Figure 6

Effect of 4-MAA or 4-AA on changes in rTs (A), CSF (B) and hypothalamic (C) PGE_2 concentration after LPS injection. 4-MAA (90 mg·kg^{-1}, i.p.), 4-AA (90 mg·kg^{-1}, i.p.) or vehicle (saline, 0.5 mL) was administered 30 min before LPS (50 μg·kg^{-1}, i.p.) or sterile saline (0.5 mL, control) injection. The CSF and hypothalamus were harvested 3 h after LPS. PGE_2 concentration was determined by ELISA. Values represent means ± SEM of the variation in rT (ΔrT, °C) and the PGE_2 levels in the CSF (pg·mL^{-1}) and hypothalamus (pg·g^{-1} of tissue) of 6 animals per group. $^{\#,*}P < 0.05$ compared with the groups treated with vehicle/saline or vehicle/LPS respectively. Basal rT of each group did not differ significantly and were all around 37°C (see Figure 1 legend).

A previous study by our group showed that Tsv-induced fever is not inhibited by selective (celecoxib) and non-selective COX (ibuprofen) inhibitors, suggesting that Tsv-induced fever is PGE_2-independent (Pessini *et al.*, 2006). Corroborating these findings, the present study showed that Tsv-induced fever was not reduced by indomethacin, another non-selective COX inhibitor. Moreover, Tsv-induced fever was not followed by an increased PGE_2 concentration in the CSF or hypothalamus. Scorpion venoms are constituted by mucopolysaccharides, hyaluronidase, phospholipase, 5-HT, histamine, enzyme inhibitors and neurotoxic peptides (Petricevich, 2010). Similar to the LPS-induced fever (Morrison and Nakamura, 2011), the fever induced by Tsv (Pessini *et al.*, 2006) is related to sympathetic system activation (Petricevich, 2010), but the mechanism by which Tsv induces fever and the involvement of toxins in this response remain unclear. Toxins isolated from Tsv may depolarize excitable cells by directly interacting with ion channels (Arantes *et al.*, 1994; Possani *et al.*, 1999). Therefore, it is possible that toxins from Tsv may depolarize neurons involved in the thermoregulation by acting directly on ion channels, which would explain the PGE_2-independent fever induced by Tsv, but this hypothesis remains to be explored. Nevertheless, Tsv toxins induce synthesis/release of inflammatory mediators (Petricevich *et al.*, 2007; Petricevich, 2010) and a previous study from our group demonstrated that Tsv-induced fever is mediated by kinins, IL-1, NO and vagal neurotransmission (Pessini *et al.*, 2006). Thus, we cannot exclude the possibility that toxins from Tsv might change fire rate of thermoregulatory neurons by induction of endogenous pyrogens. Finally, as previously reported by Pessini *et al.* (2006), in the present study, dipyrone inhibits the PGE_2-independent fever induced by Tsv, confirming the additional antipyretic mechanism of dipyrone.

Considering the evidence discussed earlier, we thought it premature to assume that 4-MAA and 4-AA are the antipyretic metabolites of dipyrone relying only on their ability to inhibit PGE_2 synthesis. Therefore, a parallel study was performed in order to develop and validate an analytical method to measure dipyrone metabolites in the plasma, CSF and hypothalamus using LC-MS/MS (Aguiar *et al.*, 2013). Our results demonstrated that all dipyrone metabolites were found in measurable amounts in plasma, CSF and hypothalamus 15 min after dipyrone administration, except 4-AAA, which was detected only 30 min later in the CSF and hypothalamus. Moreover, significant relationships were observed among plasma, CSF and hypothalamic concentrations for all dipyrone metabolites (Aguiar *et al.*, 2013). In line with these results, dipyrone metabolites have been found in the CSF 30 min after oral administration of dipyrone (Cohen *et al.*, 1998). All together, these results demonstrated that all dipyrone metabolites easily cross from blood to the brain.

During inflammation, changes occur in the plasma protein bindings, in the hepatic and/or intestinal microsomal cytochrome P450 isoenzymes, and in the renal excretion of drugs, suggesting that LPS injection could affect the pharmacokinetic of drugs (Yang and Lee, 2008). However, in the present study the plasmatic, CSF and hypothalamic time-course content of all dipyrone metabolites were not changed by LPS or Tsv administration suggesting that the pyrogenic

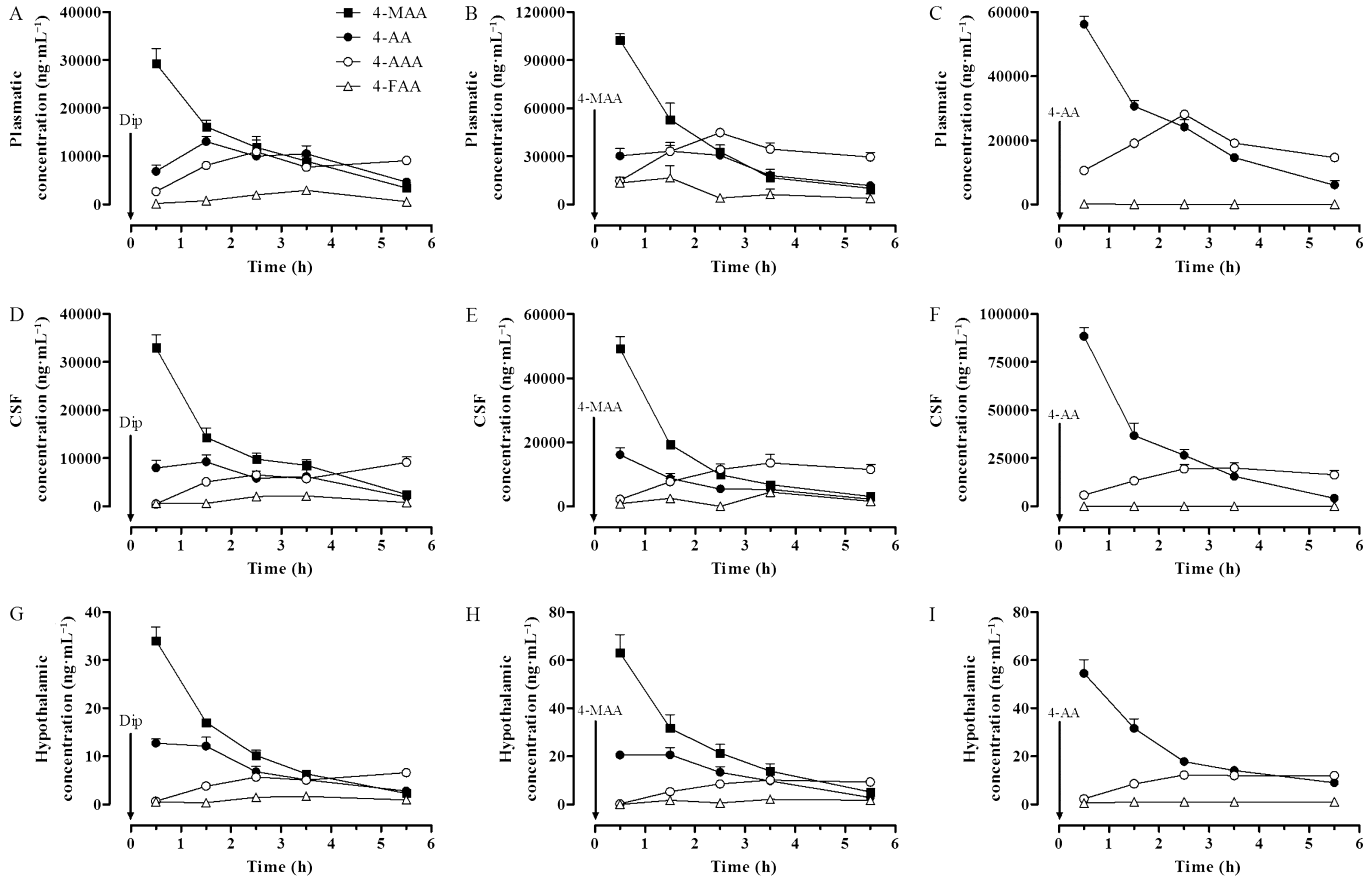

Figure 7

Concentration of dipyrone (Dip) metabolites in the plasma (A, B and C), CSF (D, E and F) and hypothalamus (G, H and I) after dipyrone (Dip; A, D and G), 4-MAA (B, E and H) or 4-AA (C, F and I) administration. Rats received i.p. injections of Dip (120 mg·kg^{-1}), 4-MAA (90 mg·kg^{-1}) or 4-AA (90 mg·kg^{-1}). The plasma, CSF and hypothalamus were harvested from 0.5 to 5.5 h after drug administration. Concentration of Dip metabolites was determined by LC-MS/MS. Values represents the means ± SEM of the concentration of dipyrone metabolites in the plasma (ng·mL^{-1}), CSF (ng·mL^{-1}) and hypothalamus (ng·g^{-1} of tissue) of six to eight animals per group.

dose of these stimuli do not modify the pharmacokinetic of dipyrone metabolites. Moreover, only high concentrations of 4-MAA and 4-AA in the plasma, CSF and hypothalamus matched with the antipyretic period of dipyrone on LPS- and Tsv-induced fever. Furthermore, 4-MAA, 4-AA and 4-FAA, but not 4-AAA, produced a dose-related antipyretic effect on LPS-induced fever. After LPS injection 4-MAA and 4-AA were more effective than dipyrone for inducing antipyresis while 4-FAA produced antipyresis only at the highest dose (360 mg·kg^{-1}). Taken together, these results suggest that 4-MAA and 4-AA are antipyretic metabolites of dipyrone and that the acetylation of 4-AA to 4-AAA abolishes its antipyretic effect. Furthermore, 4-FAA has little or no importance in the antipyresis promoted by dipyrone given that it was only effective at doses two and three times higher than the antipyretic dose of dipyrone (120 mg·kg^{-1}).

An important finding of the present study is that only 4-MAA inhibited the PGE_2-independent fever induced by Tsv, suggesting that 4-MAA or its other metabolites (not studied here) could be responsible for the dual antipyretic effect of dipyrone. In fact, in addition to the metabolites studied here, after oral administration of dipyrone several new metabolites derived from 4-MAA are formed in minor amounts such as 4-hydroxyantipyrin, oxamazide, 1-methyl-2-phenylacetohydrazide, N-methyloxamic acid, oxalic acid monohydrazide (Wessel *et al.*, 2006), arachidonoyl-4-methylaminoantipyrin and arachidonoyl-4-aminoantipyrin (Rogosch *et al.*, 2012). Moreover, unlike what was observed during LPS-induced fever, 4-MAA was less effective than dipyrone to reduce the fever induced by Tsv, which could suggest that 4-MAA is not the sole metabolite responsible for this effect.

Our results also demonstrated that pretreatment with 4-MAA or 4-AA (90 mg·kg^{-1}, i.p.) prevents the fever and the increase in PGE_2 in the CSF and hypothalamus induced by LPS, while dipyrone (120 mg·kg^{-1}) produces antipyresis without changing the PGE_2 increase in the hypothalamus. The amount of 4-MAA and 4-AA in the plasma, CSF and hypothalamus 3.5 h after dipyrone administration is minor in comparison with the amount found after 4-MAA which could explain why 4-MAA, but not dipyrone, reduces PGE_2 synthesis at 3 h after LPS administration in the hypothalamus at the doses used. Furthermore, at 3.5 and 5.5 h after treatment with 4-AA, its concentration in the hypothalamus

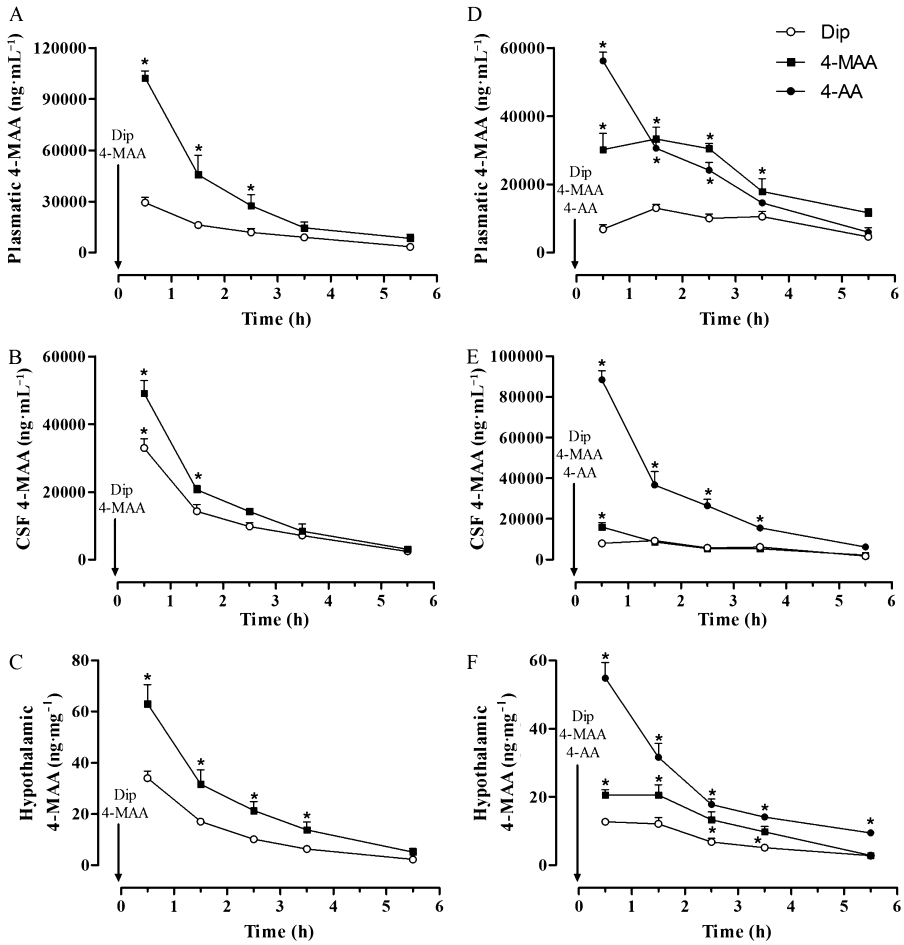

Figure 8

Concentration of 4-MAA (A, B and C) and 4-AA (D, E and F) in the plasma (A and D), CSF (B and E) and hypothalamus (C and F) after dipyrone (Dip), 4-MAA or 4-AA administration. Rats received i.p. injections of Dip (120 mg·kg^{-1}), 4-MAA (90 mg·kg^{-1}) or 4-AA (90 mg·kg^{-1}). The plasma, CSF and hypothalamus were harvested from 0.5 to 5.5 h after drug administration. Concentration of 4-MAA and 4-AA was determined by LC-MS/MS. Values represent the means ± SEM of the concentration of 4-MAA and 4-AA in the plasma (ng·mL^{-1}), CSF (ng·mL^{-1}) and hypothalamus (ng·g^{-1} of tissue) of six to eight animals per group.

was significantly higher than in animals treated with dipyrone. This finding may help to explain why, unlike dipyrone, treatment with 4-AA, reduced the PGE_2 synthesis in the hypothalamus and produced an antipyretic effect until the end of the experimental period.

Finally, some authors have reported that, unlike NSAIDs, high doses of dipyrone induce hypothermia (Souza *et al.*, 2002; Schlosburg *et al.*, 2012). In the present study, only high doses of dipyrone and 4-MAA induced hypothermia, demonstrating that 4-MAA is the hypothermic metabolite of dipyrone. It is well known that vasculature of the tail is important for thermoregulation and fever development (Romanovsky, 2007; Morrison and Nakamura, 2011). Dipyrone has been reported to induce relaxation by activating the ATP-sensitive potassium channel in rat thoracic aorta smooth muscle previously contracted with angiotensin II or noradrenaline (Valenzuela *et al.*, 2005). Thus, one can hypothesize that the hypothermic mechanism of high doses of dipyrone or 4-MAA may involve an increased heat loss through vasculature relaxation in the tail. However, more studies are needed to clarify if central or peripheral mechanisms are involved in this effect of dipyrone and 4-MAA.

In conclusion, this study endorses previous evidence that in addition to blocking PGE_2 synthesis, dipyrone possesses an another antipyretic mechanism, which seems to be exerted by 4-MAA. Moreover, 4-MAA and 4-AA are the main active antipyretic metabolites of dipyrone on LPS-induced fever and 4-MAA is the unique metabolite that inhibits the PGE_2-independent fever induced by Tsv and is responsible for the hypothermic effect of dipyrone.

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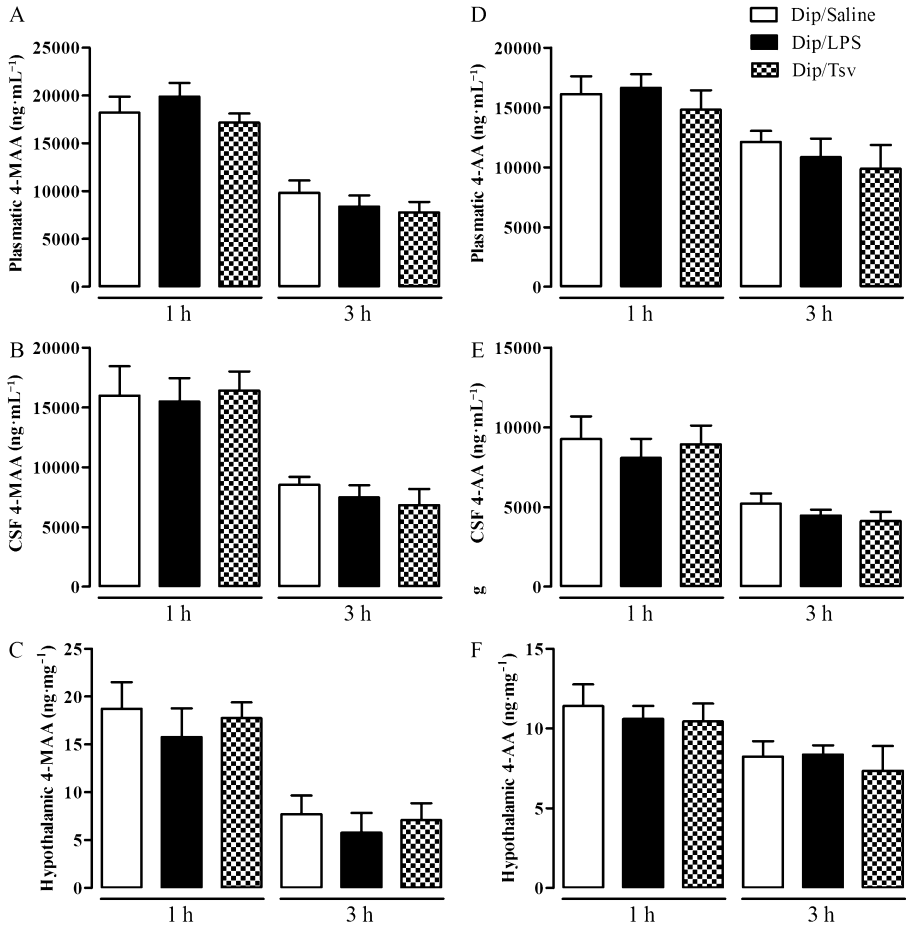

Figure 9

Concentration of 4-MAA (A, B and C) and 4-AA (D, E and F) in the plasma (A and D), CSF (B and E) and hypothalamus (C and F) after pretreatment with dipyrone (Dip) followed by saline, LPS or Tsv administration. Rats were pretreated with Dip (120 mg·kg^{-1}, i.p.) 30 min before i.p. injection of saline, LPS or Tsv. The plasma, CSF and hypothalamus were harvested at 1.0 and 3.0 h after saline or pyrogenic stimuli injection. Concentration of 4-MAA and 4-AA was determined by LC-MS/MS. Values represent the means ± SEM the concentration of 4-MAA and 4-AA in the plasma (ng·mL^{-1}), CSF (ng·mL^{-1}) and hypothalamus (ng·g^{-1} of tissue) of six animals per group.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12717

Figure S1 Chromatograms of dipyrone metabolites on plasma (a), hypothalamus (b) and CSF (c) from a rat treated with dipyrone. The samples were collected 1 h after dipyrone administration (120 mg·kg^{-1}). IS, internal standard; DIP, dipyrone.

Figure S2 Chromatograms of dipyrone metabolites on plasma (a), hypothalamus (b) and CSF (c) from a rat treated with 4-methylaminoantipyrine. The samples were collected 1 h after 4-methylaminoantipyrine administration (90 mg·kg^{-1}). IS, internal standard; DIP, dipyrone.

Figure S3 Chromatograms of dipyrone metabolites on plasma (a), hypothalamus (b) and CSF (c) from a rat treated with 4-aminoantipyrine. The samples were collected 1 h after 4-aminoantipyrine administration (90 mg·kg^{-1}). IS, internal standard; DIP, dipyrone.