

Platelet-activating factor is a potent pyrogen and cryogen, but it does not mediate lipopolysaccharide fever or hypothermia

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Abbreviations: COX, cyclooxygenase; i.p., intraperitoneal(ly); i.v., intravenous(ly); LPS, lipopolysaccharide; PAF, platelet-activating factor; T_a , ambient temperature; T_b , body temperature.

We examined whether platelet-activating factor (PAF) and its receptor mediate lipopolysaccharide (LPS)-induced fever and hypothermia in rats. Two highly potent, structurally distinct antagonists of the PAF receptor, CV6209 and WEB2086, were used. At a neutral ambient temperature (T_a) of 30°C, administration of LPS at a low (10 µg/kg, i.v.) or high (1,000 µg/kg, i.v.) dose resulted in fever. The response to the high dose was turned into hypothermia at a subneutral T_a of 22°C. Neither LPS-induced fever nor hypothermia was affected by pretreatment with CV6209 (5 mg/kg, i.v.) or WEB2086 (5 mg/kg, i.v.). However, both PAF antagonists were efficacious in blocking the thermoregulatory response caused by PAF (334 pmol/kg/min, 1 h, i.v.), regardless of whether the response was a fever (at 30°C) or hypothermia (at 22°C). Additional experiments showed that the thermoregulatory responses to LPS and PAF are also distinct in terms of their mediation by prostaglandins. Neither PAF fever nor PAF hypothermia was affected by pretreatment with the cyclooxygenase-2 inhibitor SC236 (5 mg/kg, i.p.), which is known to abrogate LPS fever. The responses to PAF were also unaffected by pretreatment with the cyclooxygenase-1 inhibitor SC560 (5 mg/kg, i.p.), which is known to attenuate LPS hypothermia. In conclusion, PAF infusion at a picomolar dose causes fever at thermoneutrality but hypothermia in a subthermoneutral environment, both responses being dependent on the PAF receptor and independent of prostaglandins. However, the PAF receptor does not mediate LPS-induced fever or hypothermia, thus challenging the dogma that PAF is an upstream mediator of responses to LPS.

Introduction

Platelet-activating factor (PAF) is a highly potent bioactive phospholipid with a diversity of physiological roles, most of which are dependent on the G-protein-coupled PAF receptor.^{1,2} Particular attention has been paid to the roles of PAF in sepsis and other systemic inflammatory conditions, and anti-PAF therapies to treat these deadly disorders have been proposed.^{3,4} Much of the knowledge about the roles of PAF in inflammatory disorders derives from the experimental model of lipopolysaccharide (LPS)-induced systemic inflammation. In this model, blood and organ levels of PAF are increased shortly after LPS administration.^{5–8} Furthermore, many pathophysiological alterations induced by LPS can be mimicked by the administration of PAF at picomolar doses.^{9–12} Moreover, structurally distinct PAF receptor antagonists block, or

at least attenuate, key components of the systemic inflammatory response, including circulatory shock² and pulmonary edema.¹³ PAF and its receptor have also been proposed to mediate the thermoregulatory manifestations of the systemic inflammatory response, but evidence in support of this notion is less compelling.

An alteration in deep body temperature (T_b)—either fever or hypothermia—occurs in all septic patients.^{14,15} Fever and hypothermia also occur in LPS-challenged laboratory rodents, with the development of fever versus hypothermia being a function of the LPS dose and the ambient temperature (T_a). A low dose and a warm environment favor the development of fever; a high dose and a cool environment favor the development of hypothermia (for review, see Romanovsky *et al.*¹⁶). The involvement of the PAF receptor in fever finds support in a single study,¹² in which the *Ginkgo biloba*-derived PAF antagonist, BN52021, attenuated

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all phases of the polyphasic fever induced by a low dose of LPS in rats kept at a neutral T_a . This compound, however, could exert nonspecific actions, as it is capable of activating PAF acetylhydrolases,¹⁷ catabolic enzymes with affinity to a broad spectrum of substrates other than PAF, *e.g.*, oxidized phospholipids.^{18,19} The participation of the PAF receptor in LPS-induced hypothermia is also supported by a single study.²⁰ Although that study involved a potent and selective PAF antagonist (CV3988), it did not control for fluctuations in T_a , which might have affected the results. To complicate the matter even further, there is evidence against the involvement of the PAF receptor in the T_b responses to LPS, at least when it comes to fever, as LPS fever was only marginally affected by a PAF antagonist in a study in horses²¹ and not affected at all by another PAF antagonist in a study in humans.²²

In view of these contradictions and experimental limitations, we reinvestigated the involvement of the PAF receptor in LPS-induced fever and hypothermia in rats. Two highly potent competitive antagonists of the PAF receptor were tested: CV6209 (a PAF structural analog)^{23,24} and WEB2086 (a triazolobenzodiazepine derivative).^{24,25} Both of these compounds also block the PAF receptor by acting as inverse agonists.²⁶ Importantly, neither compound activates PAF acetylhydrolases.^{27,28} To eliminate another shortcoming of the earlier studies described above, T_a was tightly controlled in our experiments. To avoid stress responses associated with the acute drug administration, LPS was administered via pre-implanted intravenous (i.v.) catheters.

Results

We initially tested the ability of CV6209 or WEB2086 to block PAF-induced T_b responses. Male Wistar rats were pretreated i.v. with CV6209 (5 mg/kg), WEB2086 (5 mg/kg), or the corresponding vehicle at -30 min. Each rat was then infused i.v. with PAF (334 pmol/kg/min) twice: at 0–60 min and 240–300 min. The two infusions were employed to assess the duration of PAF receptor antagonism by CV6209 and WEB2086. The doses of the PAF antagonists chosen have been shown to be efficacious in various *in-vivo* tests in rats.^{23,29–31} The dose of PAF and the form of its administration (a complex with albumin) were chosen based on our previous studies^{12,32} (also see *Materials and Methods*). At a neutral T_a of 30°C, vehicle-pretreated rats responded to both infusions of PAF with marked but transient increases in T_b ($F_{12,132} = 13.28$, $P < 0.001$ for the first infusion; $F_{12,156} = 15.62$, $P < 0.001$ for the second infusion); **Figure 1**. Exposure to a T_a of 22°C changed the response of the vehicle-pretreated rats from febrile to hypothermic ($F_{12,144} = 58.82$, $P < 0.001$ for the first infusion; $F_{12,144} = 19.80$, $P < 0.001$ for the second infusion), but the transient nature of the responses was preserved (**Fig. 2**). When the same 2 infusions of PAF were performed following a pretreatment with CV6209, there were no significant changes in T_b , regardless of T_a , meaning that the single pretreatment with CV6209 abolished the T_b responses to both PAF infusions (**Figs. 1 and 2**). A single pretreatment with

WEB2086 was similarly effective at eliminating the febrile responses to both PAF infusions at T_a of 30°C (**Fig. 1**). However, WEB2086 was partially effective against the hypothermic responses to both PAF infusions at T_a of 22°C (**Fig. 2**): this response was attenuated compared to the response of the vehicle-pretreated controls ($F_{1,12} = 19.97$, $P < 0.001$ for the first infusion; $F_{1,12} = 10.09$, $P = 0.008$ for the second infusion), but was not eliminated in relation to baseline T_b ($F_{12,72} = 62.40$, $P < 0.001$ for the first infusion; $F_{12,72} = 36.14$, $P < 0.001$ for the second infusion). These data clearly demonstrate that the pretreatment with CV6209 or WEB2086 was efficacious in blocking or attenuating responses to PAF for the entire duration of experiments, *i.e.*, for at least 6 h.

The next step was to evaluate how the same pretreatments affected T_b responses to LPS. As shown in **Figure 3**, fever was the prevailing response to LPS at T_a of 30°C, regardless of the dose used (10 or 1,000 $\mu\text{g}/\text{kg}$; $F_{80,2640} = 30.53$, $P < 0.001$ for both doses compared to

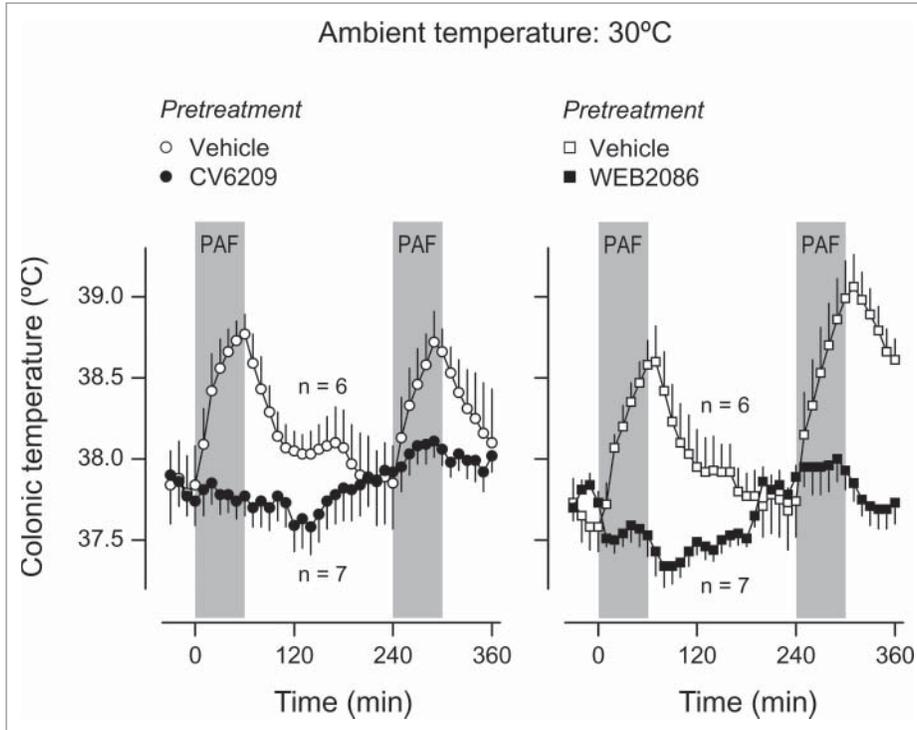


Figure 1. Effect of the i.v. pretreatment with CV6209 (5 mg/kg), WEB2086 (5 mg/kg), or the corresponding vehicle on the T_b responses to PAF at a neutral T_a (30°C). PAF was infused i.v. at a rate of 334 pmol/kg/min for 1 h twice; the two periods of infusion are marked in gray. Data are means \pm SEM. The number of animals in each group (n) is indicated.

saline). The lower dose elicited 3 well-defined febrile phases with T_b peaks at ~40, 120, and 300 min. The second and third phases were also present in the response to the higher dose of LPS, whereas the first phase was either small or absent. These fevers developed regardless of whether the rats were pretreated with a vehicle, CV6209, or WEB2086; no statistical differences were revealed between any of the pretreatment groups. When the same experiment was conducted at T_a of 22°C, hypothermia was the prevailing T_b response to LPS (Fig. 4). The biphasic hypothermic response was significantly different from the response to saline only at the higher LPS dose ($F_{80,2640} = 37.95$, $P < 0.001$). This hypothermic response was unaffected by pretreatment with either CV6209 or WEB2086.

Owing to the importance of prostaglandins in LPS-induced fever^{16,33-36} and hypothermia,³⁷⁻³⁹ the found noninvolvement of the PAF receptor in the responses to LPS raised the possibility that PAF administration could trigger T_b responses in a prostaglandin-independent fashion. To test this possibility, we pharmacologically targeted cyclooxygenase (COX), an important enzyme in prostaglandin biosynthesis. The intraperitoneal (i.p.) pretreatment with the COX-2 inhibitor, SC236, at a dose (5 mg/kg) known to be efficacious in suppressing LPS fever,^{37,40} exerted no effect on the fever induced by PAF at T_a of 30°C (Fig. 5). SC236 also failed to attenuate the hypothermia induced by PAF at T_a of 22°C (Fig. 6). Likewise, pretreatment with the COX-1 inhibitor, SC-560, at a dose (5 mg/kg) known to block LPS hypothermia,^{33,35} suppressed neither PAF-induced hypothermia (Fig. 6) nor PAF-induced fever (Fig. 5).

Discussion

We conducted a comprehensive study of the effects of PAF receptor blockade on the thermoregulatory responses to LPS, *viz.*, fever and hypothermia. Two potent, structurally unrelated PAF receptor antagonists (CV6209 and WEB2086) were used, and their efficacy in blocking the PAF receptor for the entire duration of experiments was confirmed by studying their effects on T_b responses caused by repeated infusions of PAF. We then studied the effects of these PAF antagonists on a broad spectrum of LPS-induced T_b responses; the febrile and hypothermic components of these responses were revealed to different extents by challenging rats exposed to a neutral or subneutral T_a with a low

or high dose of LPS. Last, but not least, the study circumvented 2 methodological pitfalls that frequently interfere with the outcome of thermoregulation experiments: (i) acute stress at the time of the LPS injection was avoided by using preimplanted venous catheters; (ii) fluctuations in T_a were minimized by placing the rats inside an environmental chamber (for a discussion, see Romanovsky *et al.*⁴¹).

The results of this investigation do not support the proposed involvement of the PAF receptor in the T_b responses to LPS, regardless of whether the prevailing response is fever or hypothermia. Although our findings do not exclude a receptor-independent role for PAF in the T_b responses to LPS, it should be considered that we found no indication that PAF could induce a T_b response independently of its receptor. Indeed, all thermoregulatory responses to PAF observed in this study were blocked completely by at least one of the PAF receptor antagonists used (CV6209).

A broader implication of the present findings is that they challenge the notion that PAF occupies a relatively upstream position in the LPS-induced inflammatory cascade, *i.e.*, serves as a common mediator of most, if not all, responses to LPS. Such an upstream position of PAF would agree with the early report that a PAF receptor antagonist (TCV-309) attenuated the surge in plasma tumor necrosis factor- α in endotoxemic chimpanzees.⁴² However, subsequent studies with PAF receptor antagonists of different classes failed to reproduce this observation in horses and

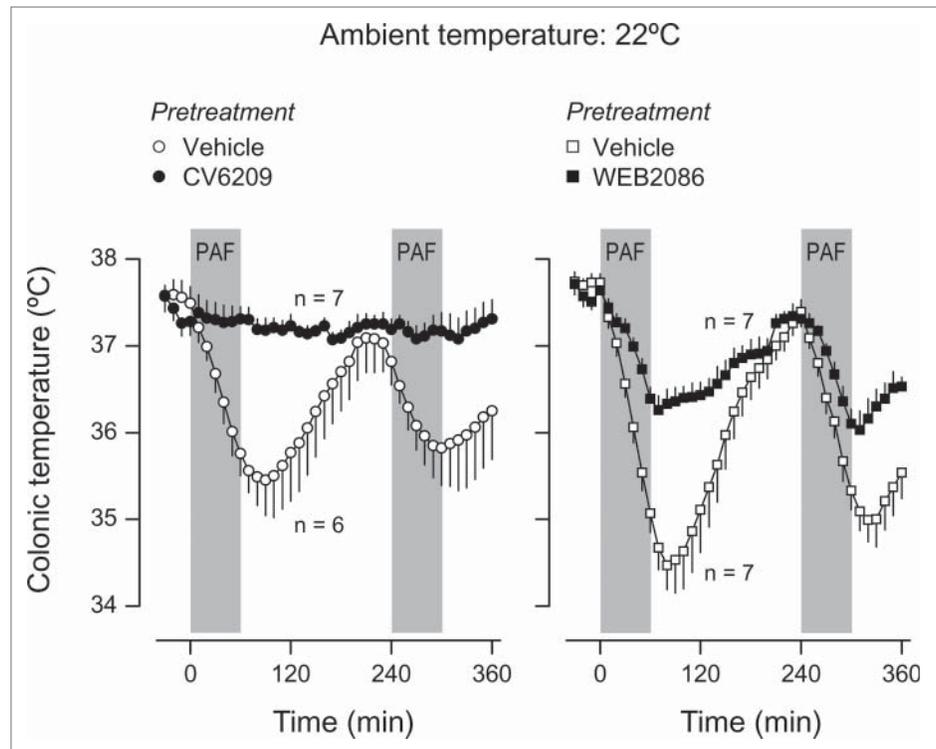


Figure 2. Effect of the i.v. pretreatment with CV6209 (5 mg/kg), WEB2086 (5 mg/kg), or the corresponding vehicle on the T_b responses to PAF at a subneutral T_a (22°C). PAF was infused i.v. at a rate of 334 pmol/kg/min for 1 h twice; the two periods of infusion are marked in gray. Data are means \pm SEM. The number of animals in each group (n) is indicated.

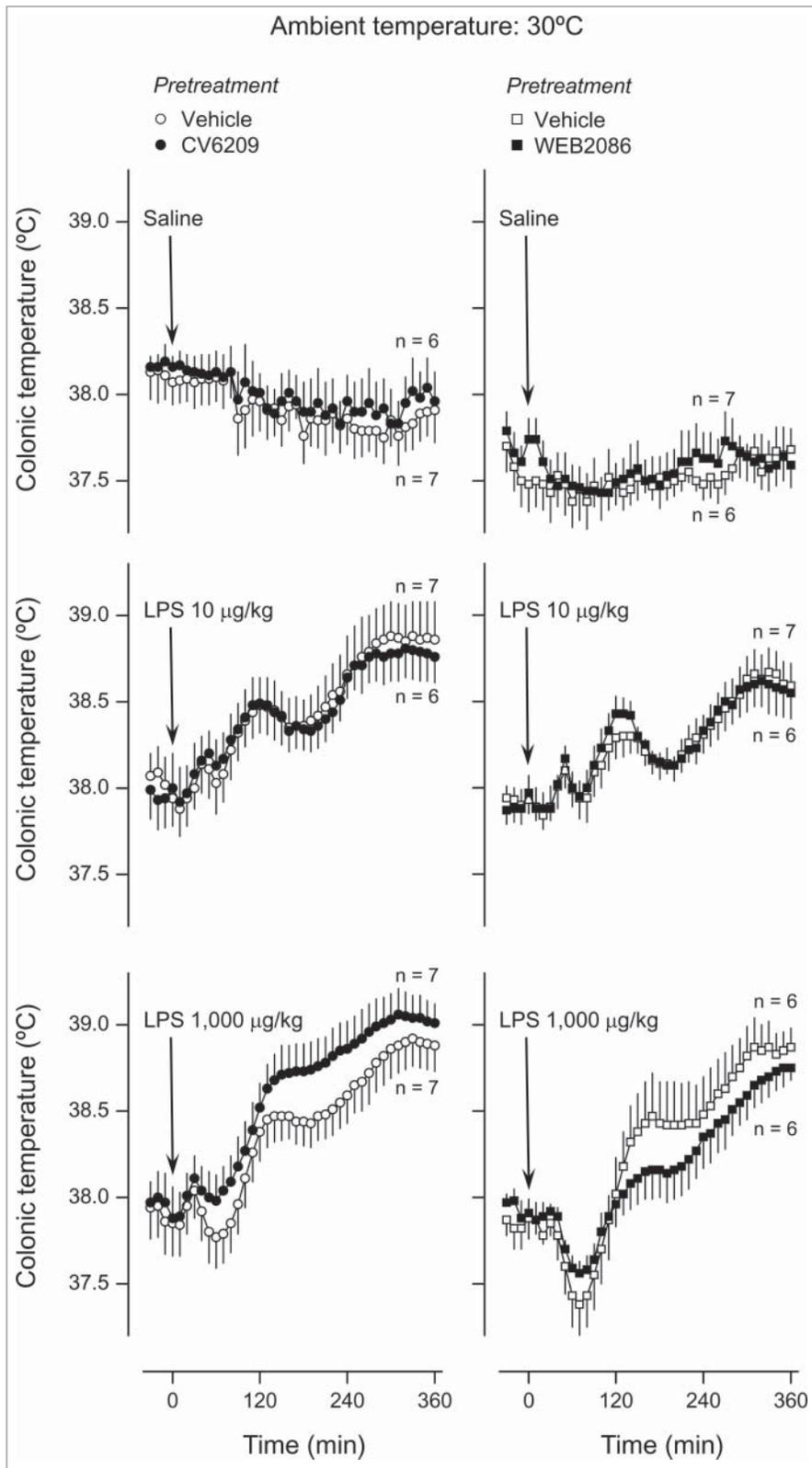


Figure 3. Effect of the i.v. pretreatment with CV6209 (5 mg/kg), WEB2086 (5 mg/kg), or the corresponding vehicle on the T_b responses to LPS at a neutral T_a (30°C). LPS or its vehicle (saline) was administered as an i.v. bolus injection at time zero; LPS doses are indicated. Data are means \pm SEM. The number of animals in each group (n) is indicated.

humans.^{21,22} Later on, other components of the inflammatory response (e.g., neutrophil recruitment^{43–45} and liver injury⁴⁶) were also found to occur independently of the PAF receptor. In this context, the noninvolvement of PAF in the T_b responses to LPS is not surprising.

While rejecting a role for PAF in the T_b responses to LPS, the present study confirmed the ability of PAF to affect T_b when administered i.v. at a picomolar dose in rats. As in previous studies,^{12,32} such a high potency was attained by delivering PAF in the form of a preformed complex with albumin. Ivanov *et al.*¹² have estimated that, in this form, i.v. PAF is at least 170 times more potent at inducing fever than i.v. prostaglandin E_2 , the most established lipid pyrogen. Such a low dose of PAF is sufficient to induce not only fever at a neutral T_a , but also hypothermia at a subneutral T_a ; this is noteworthy because hypothermia is usually a response to stronger inflammatory stimuli.^{47,48} It is possible that, while being irrelevant to LPS-induced thermoregulatory responses, PAF might be of relevance to thermoregulatory responses in other inflammatory conditions. An example is the prominent role of PAF as a mediator of hypothermia in models of IgG-dependent anaphylaxis.⁴⁹

Another important finding of the present study is that neither PAF-induced fever nor PAF-induced hypothermia was suppressed by COX inhibition, in spite of PAF's demonstrated ability to induce prostaglandin biosynthesis via the COX-2 pathway in cultured cells of different types.^{50–52} Therefore, PAF should be added to the list of inflammatory mediators capable of inducing T_b responses independently of COX and prostaglandins. To date, this list (which is based on a relatively small number of studies) includes macrophage inflammatory protein-1 (CCL3 and CCL4),^{53,54} interleukin-8 (CXCL8),⁵⁵ and carbon monoxide.⁵⁶ It should be considered, however, that the relevance of COX-independent fevers is a matter of debate, because both experimental LPS-induced fever^{57,58} and clinical, infection-associated fevers^{59,60} are usually quite dependent on COX-2. The relevance of COX-independent hypothermia to systemic inflammation is also unclear and does not agree with studies

suggesting that LPS-induced hypothermia is COX-1-dependent.^{37,39}

In conclusion, the present study shows that the PAF receptor does not play a role in LPS-induced fever or hypothermia, thus challenging the notion that PAF is an important upstream mediator in acute systemic inflammation. By the same token, the present study confirms that PAF is a potent pyrogen and cryogen. It also shows that both the febrile and hypothermic effects of PAF are dependent on the PAF receptor and independent of COX. Future studies are necessary to examine whether the pyrogenic and cryogenic properties of PAF are relevant to inflammatory conditions that are different from bacterial LPS-induced systemic inflammation.

Materials and Methods

Animals

Male Wistar rats obtained from Harlan (Indianapolis, IN, USA) were housed in a microisolator caging system with air temperature control (Allentown Caging Equipment, Allentown, NJ, USA). The temperature of the incoming air was maintained at 28°C. The holding room was on a 12:12 h light-dark cycle (lights on at 07:00 AM). Rats had free access to standard chow and tap water. The environment in the cages was enriched with artificial "rat holes" (cylindrical confiners made of stainless steel wire); the same confiners were used in the experiments. In addition to spending time in the confiners voluntarily, the rats were systematically habituated to them (7 daily training sessions, 4 h each). The rats weighed an average of 330 g (240–405 g) at the time of the experiments. Each rat was used in an experiment once and euthanized with sodium pentobarbital (100 mg/kg, i.v.) immediately thereafter. All protocols were approved by the St. Joseph's Hospital and Medical Center Animal Care and Use Committee.

Jugular catheterization

Each rat was anesthetized with ketamine-xylazine-acepromazine (56:6:1 mg/kg, i.p.), treated prophylactically with an antibiotic (enrofloxacin, 5 mg/kg, subcutaneously), and maintained on a heated

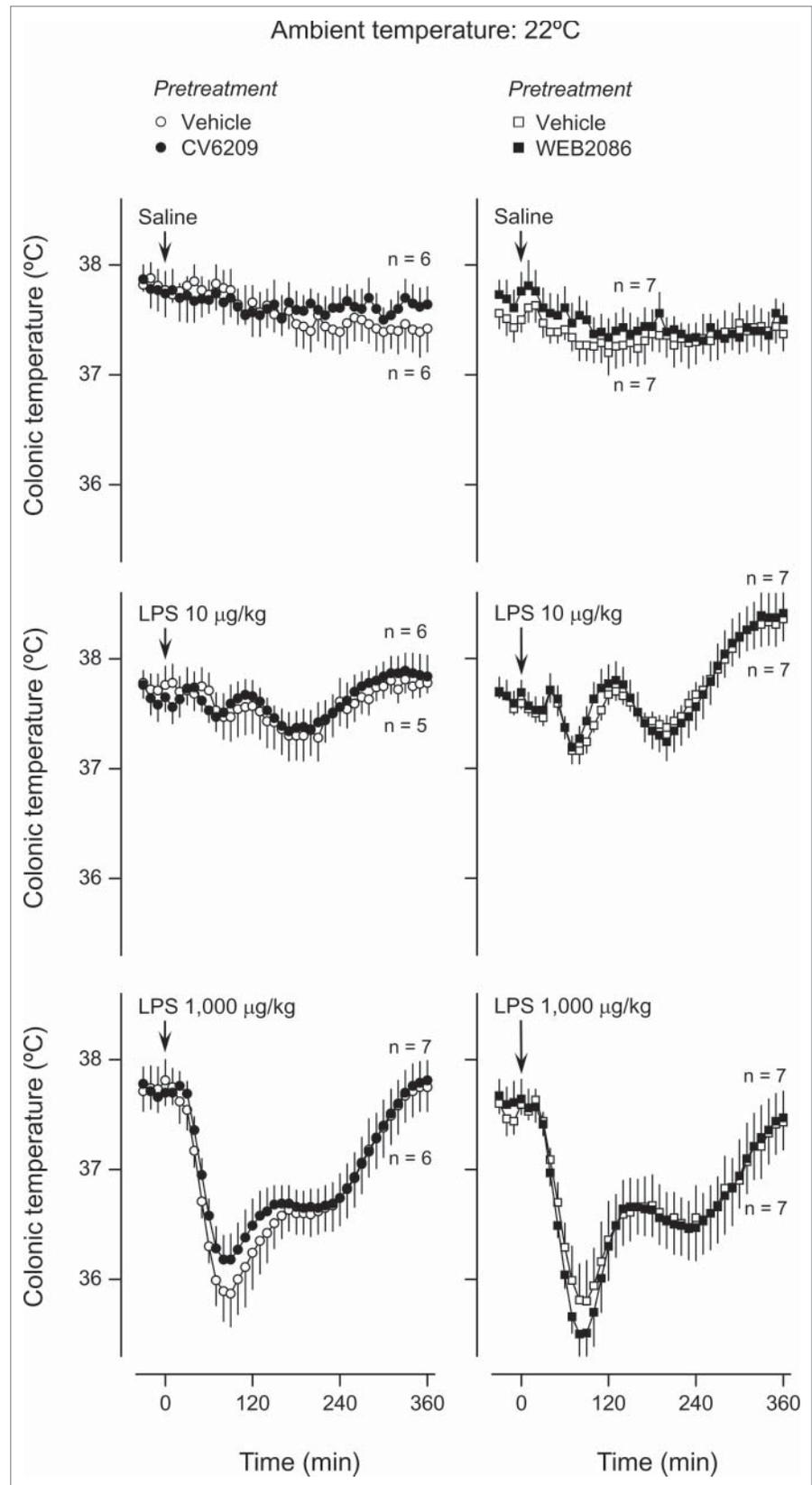


Figure 4. Effect of the i.v. pretreatment with CV6209 (5 mg/kg), WEB2086 (5 mg/kg), or the corresponding vehicle on the T_b responses to LPS at a subneutral T_a (22°C). LPS or its vehicle (saline) was administered as an i.v. bolus injection at time zero; LPS doses are indicated. Data are means \pm SEM. The number of animals in each group (n) is indicated.

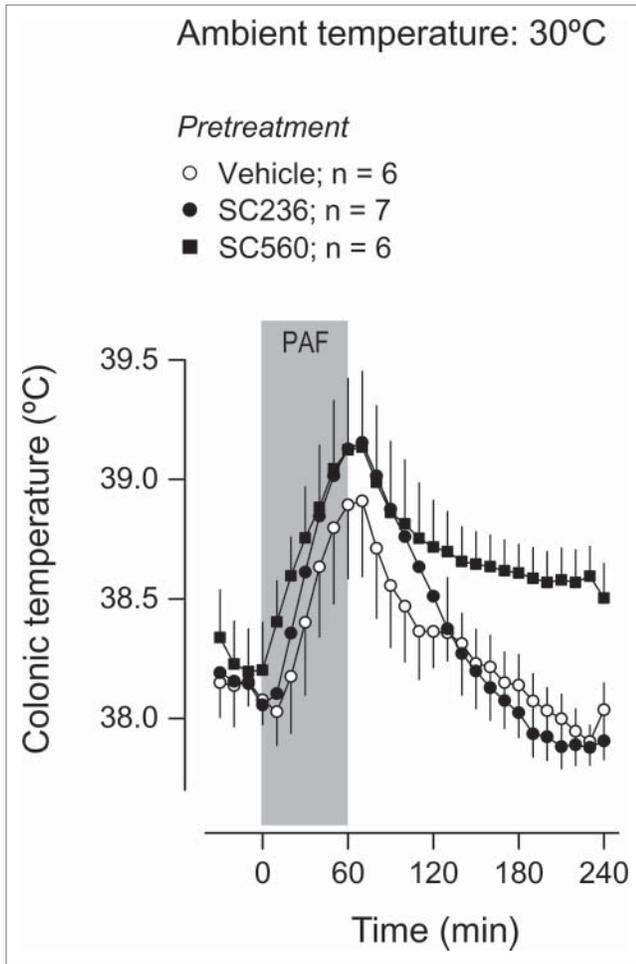


Figure 5. Effect of the i.p. pretreatment with SC236 (5 mg/kg), SC560 (5 mg/kg), or their vehicle on the T_b response to PAF at a neutral T_a (30°C). PAF was infused i.v. at a rate of 334 pmol/kg/min for 1 h; the period of infusion is marked in gray. Data are means \pm SEM. The number of animals in each group (n) is indicated.

(37–39°C) operating board. The left jugular vein was accessed via a longitudinal incision on the ventral surface of the neck. The vein was cleared from connective tissue and ligated, and a silicone catheter (ID 0.5 mm, OD 0.9 mm) filled with heparinized (10 U/ml) saline was inserted toward the superior vena cava. The catheter was secured in place with ligatures. The distal end of the catheter was passed under the skin and exteriorized at the nape. The skin incision was sutured. The rats were allowed to recover from surgery for 5–7 d before being taken in an experiment. During the recovery period, the catheters were flushed with heparinized saline every other day.

Experimental setup

Each rat was placed in a confiner and equipped with a colonic thermocouple, which was inserted 10 cm beyond the anal sphincter and fixed to the base of the tail with adhesive tape. The analog signals from thermocouples were converted to digital by a Digi-Sense multichannel thermometer (Cole-Parmer, Vernon Hills, IL, USA) and sent to a personal computer. Rats in their

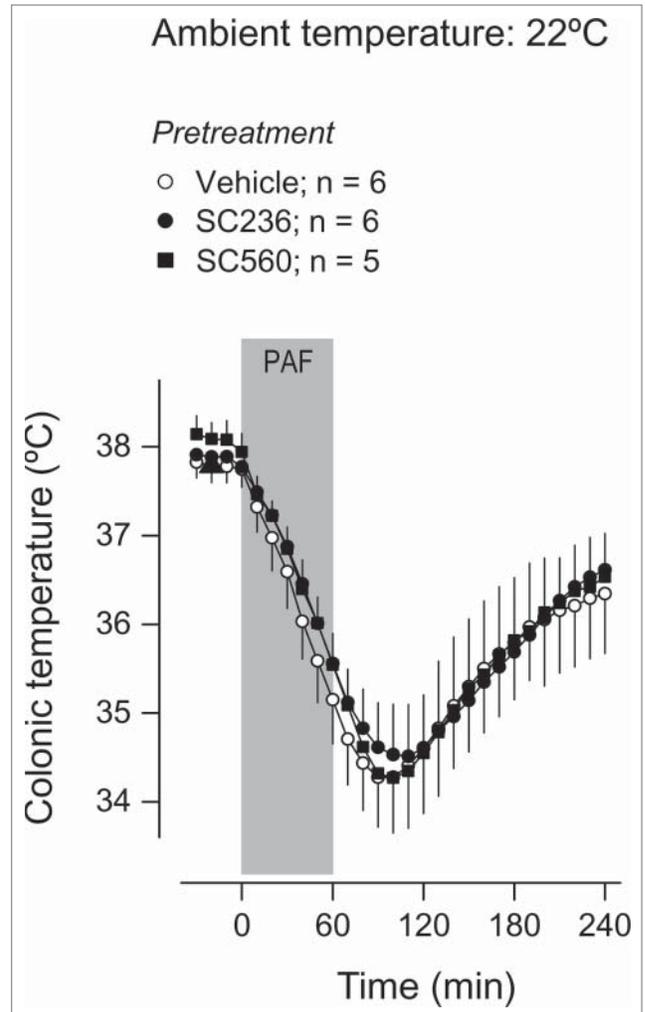


Figure 6. Effect of the i.p. pretreatment with SC236 (5 mg/kg), SC560 (5 mg/kg), or their vehicle on the T_b response to PAF at a subneutral T_a (22°C). PAF was infused i.v. at a rate of 334 pmol/kg/min for 1 h; the period of infusion is marked in gray. Data are means \pm SEM. The number of animals in each group (n) is indicated.

confiners were kept inside an environmental chamber (model 3940; Forma Scientific, Marietta, OH, USA) for the duration of the experiment. Inside the chamber, T_a was maintained at either $30.0 \pm 0.1^\circ\text{C}$ or $22.0 \pm 0.1^\circ\text{C}$; relative air humidity was maintained at $50 \pm 5\%$. A saline-filled PE-50 extension of the venous catheter was passed through a wall port to the outside of the chamber, from where drugs were administered with the help of a syringe pump (Stoelting, Wood Dale, IL, USA).

Drugs

PAF (β -Acetyl- γ -O-Alkyl-L- α -phosphatidylcholine, from bovine heart lecithin) and phenol-purified LPS (from *E. coli* 0111: B4) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The PAF receptor antagonists, CV6209 and WEB2086, were purchased from Enzo Life Sciences (Farmingdale, NY, USA) and Tocris (Minneapolis, MN, USA), respectively. Selective COX inhibitors, SC236 (COX-2) and SC560 (COX-1), were obtained

from Cayman Chemical (Ann Arbor, MI, USA). PAF was administered in the form of a 1:1 (molar ratio) complex with fatty acid-free bovine serum albumin (Sigma). To prepare the complex, a saline suspension of PAF (20 nmol/ml) and albumin was sonicated for 3 min and then incubated at 37°C for 1 h. The resulting preparation was administered as a 60-min i.v. infusion at a rate of 16.7 µl/kg/min. LPS was diluted in saline at a concentration of either 10 or 1,000 µg/ml, and injected i.v. in bolus at a volume of 1 ml/kg. Pretreatment with a PAF receptor antagonist (CV6209 or WEB2086) was performed at 30 min before either the LPS injection or the first infusion of PAF. CV6209 was dissolved in saline to a concentration of 5 mg/ml; it was bolus injected i.v. at a volume of 1 ml/kg. WEB2086 was dissolved in a 1:4 ethanol-saline solution to a concentration of 10 mg/ml; it was administered as a 10-min i.v. infusion at a rate of 50 µl/kg/min. COX-1 and COX-2 inhibitors (SC560 and SC236, respectively) were also administered as pretreatments, but at 90 min before the start of PAF infusion. Both COX inhibitors were dissolved in

DMSO to a concentration of 10 mg/ml; they were administered by i.p. injection at a volume of 0.5 ml/kg.

Statistical analyses

All T_b responses were evaluated for the effects of treatments and pretreatments over time by repeated-measures ANOVA followed, as necessary, by the Tukey least significant difference test. The calculations were performed using Statistica Advanced 8.0 (StatSoft, Tulsa, OK, USA), with the level of significance set at $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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