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Mechanisms underlying the nociceptive responses induced by platelet-activating factor (PAF) in the rat paw

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ABSTRACT

Platelet-activating factor (PAF) is an inflammatory mediator widely known to exert relevant pathophysiological functions. However, the relevance of PAF in nociception has received much less attention. Herein, we have investigated the mechanisms underlying PAF-induced spontaneous nociception and mechanical hypersensitivity in the rat paw. PAF injection (1–30 nmol/paw) resulted in a dose-related overt nociception, whilst only the dose of 10 nmol/paw produced a significant and time-related mechanical hypersensitivity. Local coinjection of PAF antagonist WEB2086 significantly inhibited both spontaneous nociception and mechanical hypersensitivity. Moreover, the coinjection of the natural IL-1β receptor antagonist (IRA) notably prevented both PAF-induced nociceptive responses, whilst these responses were not altered by anti-TNFα coinjection. Interestingly, pretreatment with the ultrapotent vaniloid agonist resiniferotoxin, coinjection of the TRPV1 receptor antagonist SB366791, or mast cell depletion with compound 48/80 markedly prevented PAF-induced spontaneous nociception. Conversely, PAF-elicited mechanical hypersensitivity was strikingly susceptible to distinct antineutrophil-related strategies, namely the anti-neutrophil antibody, the selectin blocker fucoidin, the chemokine CXCR2 receptor antagonist SB225002, and the C5a receptor antibody anti-CD88. Notably, the same antineutrophil migration strategies significantly prevented the increase of myeloperoxidase activity induced by PAF. The mechanical hypersensitivity caused by PAF was also prevented by the cyclooxygenase inhibitors indomethacin or celecoxib, and by the selective β_1 adrenergic receptor antagonist atenolol. Collectively, the present results provide consistent evidence indicating that distinct mechanisms are involved in the spontaneous nociception and mechanical hypersensitivity caused by PAF. They also support the concept that selective PAF receptor antagonists might constitute interesting targets for the development of new analgesic drugs.

1. Introduction

Platelet-activating factor (PAF, 1-O-hexadecyl-1-2-o-acetyl-sn-glycero-3-phosphocholine) is a potent phospholipid mediator involved in several pathophysiological events [1]. PAF interacts with a specific receptor that belongs to the superfamily of G-protein-coupled receptors. The activation of platelet-activating factor receptor (PAFR) can initiate and

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modulate several different intracellular signaling pathways, including the increase in diacylglycerol, inositoltriphosphate and calcium levels, with the stimulation of protein kinases (MAPK, PKC and tyrosine kinase) and phospholipases (PLA_2, PLCβ) [2–4]. Moreover, PAF is capable of activating NF-κB, a key regulator of the expression of many molecules involved in the inflammatory process [5–8].

PAF is involved in a series of functional cellular responses, including adhesion, activation and chemotaxis of leukocytes and gene expression [9,10]. It can be produced by a variety of cells, such as platelets, polymorphonuclear leukocytes, monocytes, mast cells, endothelial cells, and neuronal cells, among others [1,11–13]. Activation of PAFR stimulates the synthesis of various inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-8, IL-10, and eicosanoids, playing a key role in inflammatory processes [14–17]. More recently, the presence of functional PAFR has been reported at the nuclear membrane of endothelial cells and neurons [18], and PAF mRNA has been identified in mouse dorsal root ganglia and dorsal horn of the spinal cord [19].

So far, there are only a few pieces of evidence suggesting that PAF may play a role in pain signaling. For instance, it has been demonstrated that PAF elicits gene expression in neuronal and glial cell lines, leading to the release of PGE_2 from astrocytes [20]. Interestingly, it has been demonstrated that injection of PAF induces hyperalgesia in rats [21,22]. In addition, it has been suggested that the local accumulation of leukocytes induced by PAF could contribute to inflammatory pain [23,24]. Also, PAFR antagonists can reduce the hyperalgesia induced by *Bothrops jararaca* venom or formalin injection [25,26]. More recently, Tsuda et al. [27] established that mice lacking PAFR display a reduced hyperalgesia in response to the injection of formalin or capsaicin. Additionally, Zhang et al. [28] demonstrated that ultraviolet B radiation applied to the hindpaw caused an increased sensitivity to both mechanical and thermal stimulation in wild-type mice, but not in PAFR knockout mice.

It is not clear from the available published studies whether PAF-induced nociception is mediated by direct actions on sensory neurons, or instead, if the nociception is an indirect effect of PAF-induced inflammatory changes. In order to provide new evidence on the relevance of PAF in pain, the present study was addressed to further characterize PAF-induced nociceptive behaviors in the rat paw, and to investigate the possible mechanisms involved in the nociceptive responses induced by PAF in the rat hindpaw.

2. Materials and methods

2.1. Animals

Non-fasted male Wistar rats (150–220 g) housed under conditions of optimum light, temperature and humidity (12 h light-dark cycle, 22 ± 1 °C, 60–80% humidity), with food and water provided *ad libitum* were used. Experimental procedures were carried out in accordance with the National Institutes of Health Animal Care Guidelines (NIH publications) and were approved by the Ethics Committee of the Universidade Federal de Santa Catarina (protocol number PP00032). The number of animals and the intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects.

2.2. PAF-induced spontaneous nociception

Spontaneous nociception was evaluated according to the method described previously [29], with some modifications. Rats received a 100 μl i.pl. injection of either PAF (1–30 nmol) or vehicle (saline; 0.9% NaCl) into the ventral surface (footpad) of the right hindpaw. Each animal was then placed, immediately after the injection, into an individual Plexiglas observation chamber (30 cm × 30 cm × 30 cm), situated in front of a mirror (set at an angle of about 70° relative to the table) to enable full view of the paws at all times. The incidence of PAF-induced hindpaw licking, elevation or flinching was recorded cumulatively for 20 min, using a counter. Each individual paw flinch, or manifestation of licking bouts or hindpaw elevations above the floor level lasting at least 3 s, was considered to indicate nociceptive behavior.

2.3. PAF-induced mechanical hypersensitivity

The mechanical hypersensitivity was evaluated according to the method described previously [30,31]. The animals received a 100 μl i.pl. injection of saline solution (NaCl 0.9%) containing PAF (1–30 nmol/paw) or vehicle (100 μl of saline) into the right hindpaw. The nociceptive responses were evaluated at different time-points (1–24 h) following PAF injection. For that purpose, the rats were placed individually in clear Plexiglas boxes (13.8 cm × 18.0 cm × 68.2 cm), with three compartments (13.8 cm × 18.0 cm × 22.7 cm), on elevated wire mesh platforms (23.0 cm × 39.8 cm × 72.7 cm) to allow access to the ventral surface of the right hindpaw. The animals were acclimatized for 30 min prior to behavioral testing. The withdrawal response frequency (in %) was measured following 10 applications (with a duration of ~3 s each, and an interval of ~20 s among each) of VFH (von Frey Hairs, Stoelting, Chicago, U.S.A.). Stimuli were delivered from below to the plantar surface of the right hindpaw. The 4.0 g VFH filament produces a mean withdrawal frequency of about 20 %, which is considered an adequate value for the measurement of mechanical hypersensitivity. Hence, the 4.0 g VFH was used throughout this study. All the groups were evaluated before PAF injection, in order to determine the basal mechanical thresholds.

2.4. Measurement of myeloperoxidase activity

Neutrophil recruitment to the rat paw was assessed indirectly by means of tissue myeloperoxidase (MPO) activity, according to the method described by Fernandes et al. [7]. For this purpose, animals received an i.pl. injection of PAF (10 nmol/paw) into the right hindpaw and they were sacrificed 3 h later. Saline-treated paws were used as control. The paw subcutaneous tissue was removed, homogenized at 5% (w/v) in EDTA/NaCl buffer (pH 4.7) and centrifuged at 10,000 rpm for 15 min, at 4 °C. The pellet was resuspended in hexadecyltrimethyl ammonium bromide (HTAB) 0.5% buffer (pH 5.4) and the samples were frozen in liquid nitrogen and thawed three times. Upon thawing, the samples were recentrifuged

(10,000 rpm, 15 min, 4 °C) and 25 μl of the supernatant was used for the MPO assay. The enzymatic reaction was assessed in the presence of tetramethylbenzidine 1.6 mM, $NaPO_4$ 80 mM and hydrogen peroxide 0.3 mM. The absorbance was measured at 690 nm and the results are expressed in optical density (OD) per mg of tissue.

2.5. Pharmacological treatment protocols

This series of experiments was designed to evaluate some of the mechanisms possibly implicated in the nociceptive changes caused by the i.pl. injection of PAF into the rat paw.

To assess the contribution of PAFR activation, animals received a coinjection of the selective PAFR antagonist WEB2086 (33 nmol/paw). To evaluate whether TNFα or IL-1β might be implicated in the PAF-induced responses, other groups of animals received the monoclonal anti-TNFα antibody (50 ng/paw) or the natural interleukin-1β receptor antagonist IRA (100 μg/paw). To verify the relevance of neutrophil migration, different groups of rats were pre-treated with the selective CXCR2 antagonist SB225002 (1 mg/kg, i.p., 30 min), the non-selective selectin inhibitor fucoidin (10 mg/kg, i.v., 15 min), the C5a blocker anti-CD88 antibody (500 ng/paw), or the antineutrophil antibody (34 μg/kg, i.p., 30 min) [8,32,33]. The participation of macrophages in PAF-induced nociception was evaluated by pretreating animals with antimacrophage antibody (50 μg/kg, i.p., 24 h and 30 min).

In order to assess the role of mast cells in PAF-induced nociceptive responses, animals were treated daily by i.p. route with the mast cell-degranulating agent compound 48/80 (C48/80; 25, 60 and 125 μg/animal on the 1st, 2nd and 3rd days, respectively, and two injections of 200 μg/animal, on the 4th day) [34]. To verify the effectiveness of C48/80 pre-treatment, at the day of experiments, a group of animals received an i.pl. injection of C48/80 (10 μ/paw) or vehicle (100 μl of saline) into the right hindpaw. The incidence of C48/80-induced hindpaw licking, elevation or flinching was recorded cumulatively for 30 min, using a counter (data not shown) [34].

To determine the relevance of cyclooxygenase (COX) or adrenergic system activation, animals were pretreated with the COX inhibitors indomethacin (2.5 mg/kg, s.c.) and celecoxib (20 mg/kg, p.o.), or the selective β_1 adrenergic receptor antagonist atenolol (1 mg/kg, s.c.), administered 60 min before PAF injection. To explore the role of C-fibers and TRPV1 activation in the nociception induced by PAF, rats were systemically treated with the capsaicin analog resiniferatoxin (RTX, 300 μg/kg, s.c., 48 h before) [35] or they received locally the selective TRPV1 receptor antagonist SB366791 (10 nmol/paw). In an attempt to confirm the complete degeneration of the C sensory fibers after RTX treatment, the animals were submitted to an eye-wiping test. For this purpose, a 20 μl capsaicin solution 0.01% (w/v) was instilled on the eye and the number of wiping movements that occurred in 1 min was counted. The animals that wiped their eyes no more than 5 times were considered to be desensitized by RTX treatment (data not shown) [36].

PAF-elicited spontaneous nociceptive effects or mechanical hypersensitivity were measured as described in the above sections. The effects of antineutrophil strategies were also tested against the MPO activity. The control groups received the corresponding vehicle, at the same time-points and routes of administration, depending on the experimental protocol. Noteworthy, for every tested inhibitor, a parallel control group has been carried out. The doses of inhibitors, antagonists, antibodies, or the treatment protocols were chosen on the basis of pilot studies or previous publications.

2.6. Drugs and reagents

The following drugs and reagents were used: atenolol, benzethonium chloride, bovine serum albumine (BSA), compound 48/80, ethylene diamine tetracetic acid (EDTA), fucoidin, hexadecyltrimethil ammonium bromide (HTAB), hydrogen peroxide, indomethacin, resiniferotoxin (RTX), SB366791, tetramethylbenzidine (TMB), 2,2,2, tribromoethanol, Tween-20 and Tween-80 (all from Sigma Chemical Company, St Louis, MO, U.S.A.); Celecoxib (from Merck, Brazil); sodium chloride (NaCl) and sodium phosphate ($NaPO_4$) (from Merck, Germany); 1-O-hexadecyl-1-2-o-acetyl-sn-glycero-3-phosphocholine (PAF) (from Bachem Bioscience Inc., King of Prussia, PA, U.S.A.). Anti-CD88 antibody (catalog number: sc-25774) was purchased from Santa Cruz (Santa Cruz, CA, U.S.A.). Anti-murine neutralizing anti-TNFα antibody (catalog number: AF-510-NA) and the natural interleukin-1β receptor antagonist IRA (catalog number: 280-RA/CF) were obtained from R&D Systems (Minneapolis, MN, U.S.A.). Anti-rat neutrophil (catalog number: AIAD51140) and anti-rat macrophage (catalog number: AIAD51249) antibodies were obtained from Accurate Chemicals (San Diego, CA, U.S.A.). WEB2086 was a gift Boehringer-Mannheim, Germany. N-(2-hydroxy-4-nitrophenyl)-N*-(2-bromophenyl) urea (SB225002) was synthesized according to White et al. [37] with some modifications.

PAF was prepared in a BSA 0.1% solution, and stocked in siliconized plastic tubes at −20 °C. All the drugs, except indomethacin, atenolol, SB366791 and SB225002, were prepared daily in 0.9% (w/v) NaCl (saline) solution before use. Indomethacin, atenolol, and SB225002 were diluted in 5% sodium carbonate solution, in a 2% DMSO solution, and in 1% Tween-80 solution, respectively. SB366791 was diluted in a 2% ethanol solution (in saline).

2.7. Data analysis

The results are presented as the mean ± SEM of six to eight animals. The percentages of inhibition are reported as the mean ± SEM of inhibition obtained for each individual experiment at the spontaneous overt nociception (duration of 20 min after injection of PAF), or at 3 h (MPO experiments). For the mechanical hypersensitivity, the percentages of inhibition are reported as the mean ± SEM of the difference between the areas under the time–response curve (AUC) of the drug-treated group in relation to the corresponding vehicle-treated group. Statistical comparison of data was performed by one- or two-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's post hoc test, when appropriate. P-values of 0.05 ($P < 0.05$) or less were considered as indicative of significance.

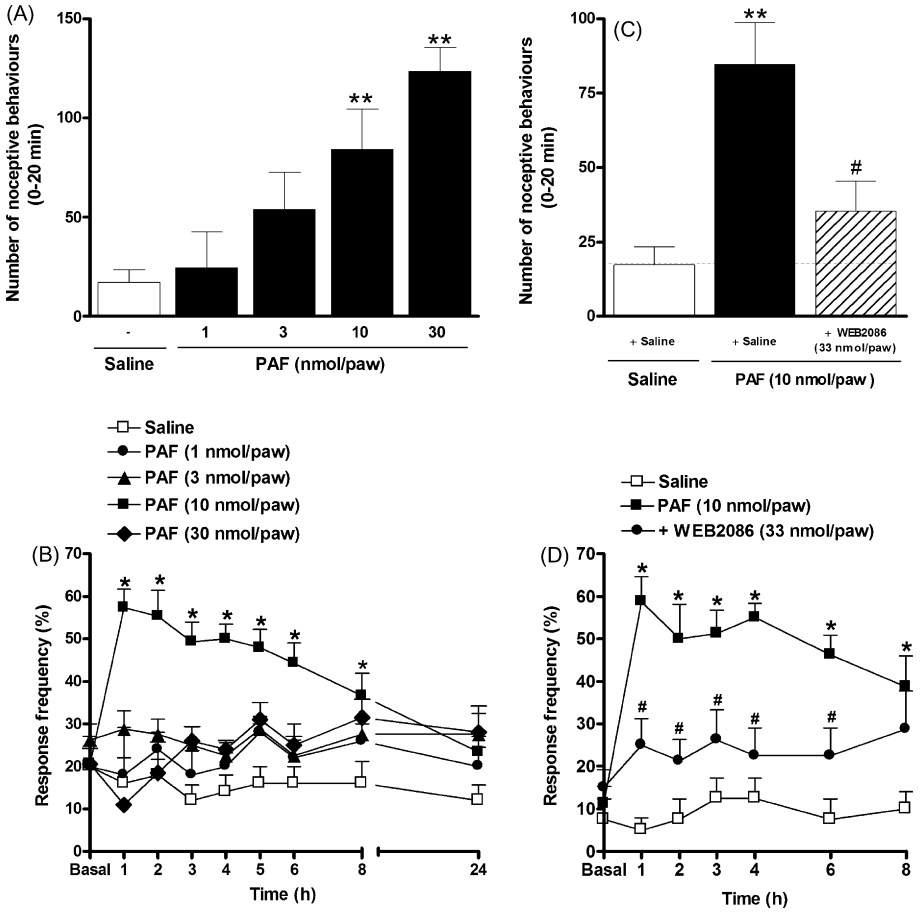

Fig. 1 – (A) Spontaneous nociception and (B) mechanical hypersensitivity induced by the intraplantar injection of PAF (1–30 nmol/paw) into the rat paw. Effect of coadministration of WEB2086 (33 nmol/paw) on the spontaneous nociception (C), or the mechanical hypersensitivity (D) induced by the injection of PAF (10 nmol/paw). Each group represents the mean of six to eight animals, and the vertical line indicates the SEM. Significantly different from saline ($^{*}P < 0.05$ or $^{**}P < 0.01$) or PAF-injected paw ($^{\#}P < 0.05$) values.

3. Results

3.1. *Characterization of PAF-induced nociceptive behavior*

We may observe from Fig. 1A that PAF injection into the rat hindpaw (1 to 30 nmol/paw) evoked a dose-dependent and marked spontaneous nociceptive response, in comparison to saline-injected paws, in a 20 min-period of evaluation. In addition, the i.pl. application of PAF caused a significant mechanical hypersensitivity, as indicated by a marked increase from baseline values in response to 4.0 g VFH stimulation. This effect was significant only for the dose of 10 nmol/paw, and it was observed as early as 1 h after PAF injection, remaining sustained for up to 8 h (Fig. 1B).

Predictably, either the spontaneous nociceptive response (Fig. 1C) or the mechanical hypersensitivity (Fig. 1D) induced by PAF (10 nmol/paw) was significantly inhibited by the coinjection of selective PAF receptor antagonist WEB2086 (33 nmol/paw). The percentages of inhibition for WEB2086 are shown in Table 1. Conversely, a partial, but not statistically significant inhibition of PAF-induced overt nociception was observed with the anti-TNFα antibody coinjection (50 ng/paw) (Fig. 2A). In a similar manner, this anti-TNFα strategy inhibited PAF-induced mechanical hypersensitivity only at 1 h after PAF injection (Fig. 2B and C). On the other hand, the coinjection of the natural antagonist of IL-1β receptor (IRA; 100 μg/paw) caused a prominent inhibition of both spontaneous nociceptive effect and mechanical hypersensitivity induced by PAF (Fig. 2; see Table 1 for the percentages of inhibition).

In order to assess the involvement of TRPV1-expressing afferent neurons in the nociceptive responses induced by PAF, we employed two different pharmacological tools. As shown in Fig. 3, the spontaneous nociception following PAF (10 nmol/paw) injection was broadly inhibited, in a significant manner, by RTX pretreatment (300 μg/kg, s.c., 48 h) or by the selective TRPV1 receptor antagonist SB366791 (10 nmol/paw) (Fig. 3A; Table 1). In contrast, both strategies failed to significantly affect the mechanical hypersensitivity in response to VFH stimulation in PAF (10 nmol/paw)-injected paws (Fig. 3B and C).

PAF has been demonstrated to activate and/or attract inflammatory cells to the sites of inflammation. To test whether this might be related to the nociceptive changes elicited by PAF, we employed different experimental

Table 1 – Effect of different classes of drugs on the overt nociception and mechanical hypersensitivity induced by PAF in the rat paw.

Drug	Site/mechanism of action	Dose	Inhibition (%)[a]		
			Overt nociception	Mechanical hypersensitivity[b]	MPO activity
WEB2086	PAF receptor antagonist	33 nmol/paw (co-injected)	73 ± 15*	63 ± 11**	70 ± 14**
Anti-TNF	Anti -tumor necrosis factor-α antibody	50 ng/paw (co-injected)	ns	ns	ne
IRA	Interleukin-1 receptor antagonist	100 μg/paw (co-injected)	82 ± 12**	79 ± 6**	ne
RTX	TRPV1 receptor agonist	300 μg/paw (24 h before)	104 ± 7**	ns	ne
SB366791	TRPV1 receptor antagonist	10 nmol/paw (co-injected)	54 ± 11*	ns	ne
Compound 48/80	Mast cell degranulator	25, 60, 125, 200 and 200 μg/animal (i.p., 4, 3, 2 and 1 day before, respectively)	76 ± 18*	ns	ne
Antimacrophage	Antimacrophage antibody	50 μg/paw (i.p., 24 h and 30 min)	ns	ns	ne
Antipolymorphonuclear	Antipolymorphonuclear antibody	34 μg/kg (i.p., 30 min)	ns	90 ± 10**	67 ± 18**
Fucoidin	Selectin inhibitor	10 mg/kg (i.v., 15 min)	ns	68 ± 8**	88 ± 11**
SB225002	CXCR2 receptor antagonist	1 mg/kg (i.p., 30 min)	ns	96 ± 10**	70 ± 12**
Anti-CD88	Anti-C5a receptor antibody	500 ng/paw (co-injected)	ns	110 ± 10**	63 ± 14**
Indomethacin	COX-1/COX-2 inhibitor	2.5 mg/kg (s.c., 1 h)	ns	32 ± 5*	ne
Celecoxib	COX-2 inhibitor	20 mg/kg (s.c., 1 h)	ns	62 ± 9**	ne
Atenolol	β_1-adrenergic receptor blocker	1 mg/kg (s.c., 1 h)	ns	37 ± 9*	ne

ns: No significant inhibition; ne: not evaluated. Asterisks denote the significance levels in comparison to control values.

[a] Mean ± SEM.

[b] For the mechanical hypersensitivity the inhibition is given as the difference (in percentage) between the mean area under the time-response curve (AUC; 1–8 h values) of the responses in drug-treated group and in relation to the vehicle-treated group.

* $P < 0.05$.

** $P < 0.01$.

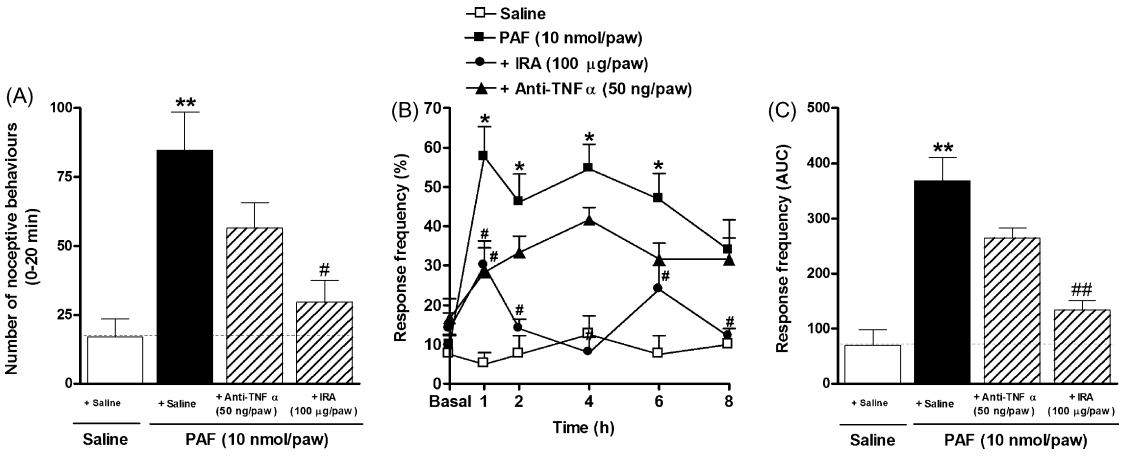

Fig. 2 – Effect of coadministration of anti-TNFα antibody (50 ng/paw) or the natural antagonist of IL-1β receptor (IRA; 100 μg/paw) on the spontaneous nociception (A), or the mechanical hypersensitivity (B and C) induced by the injection of PAF (10 nmol/paw). Each group represents the mean of six to eight animals, and the vertical line indicates the SEM. Significantly different from saline (*$P < 0.05$ or **$P < 0.01$) or PAF-injected paw (#$P < 0.05$ or ##$P < 0.01$) values.

approaches. Firstly, animals were treated with the mast cell-degranulating agent C48/80 (25, 60 and 125 μg/animal on the 1st, 2nd and 3rd days, respectively, and two injections of 200 μg/animal on the 4th day). Whereas the treatment with CP48/80 greatly prevented PAF-induced spontaneous nociception (Fig. 4A; Table 1), it was partially effective in the mechanical hypersensitivity, as this response was significantly blockaded only between 1 and 2 h after PAF injection (Fig. 4B and C). The treatment of animals with the anti-macrophage antibody (50 μg/kg, i.p., 24 h and 30 min) failed to

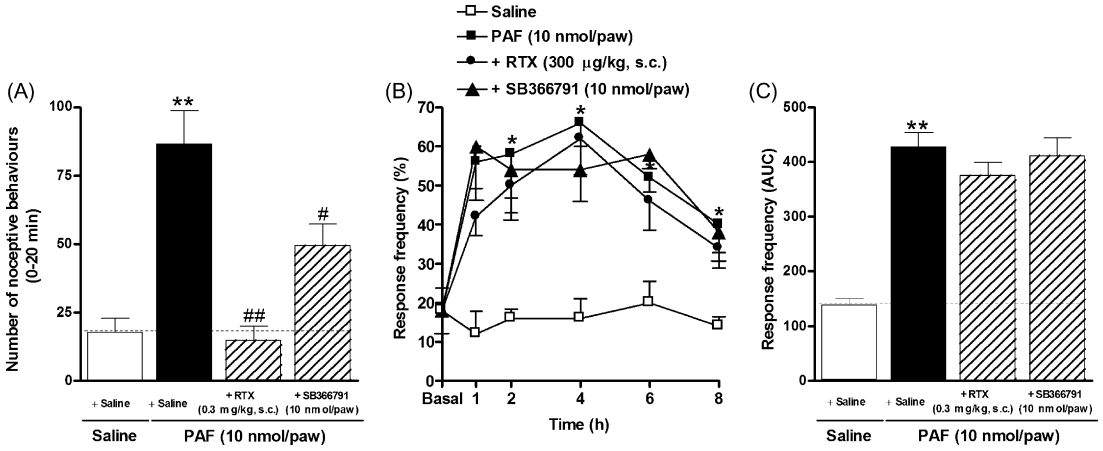

Fig. 3 – Effect of RTX pretreatment (300 μg/kg, s.c., 48 h) or SB366791 (10 nmol/paw) on the spontaneous nociception (A), or the mechanical hypersensitivity (B and C) induced by the injection of PAF (10 nmol/paw). Each group represents the mean of six to eight animals, and the vertical line indicates the SEM. Significantly different from saline ($^{*}P < 0.05$ or $^{**}P < 0.01$) or PAF-injected paw ($^{\#}P < 0.05$ or $^{\#\#}P < 0.01$) values.

significantly alter either the spontaneous nociceptive response (Fig. 4D) or the mechanical hypersensitivity (Fig. 4E and F) induced by PAF (10 nmol/paw). Of note, whilst the administration of antineutrophil antibody (34 μg/kg, i.p., 30 min) almost abolished the mechanical hypersensitivity following VFH application (Fig. 4H and I; Table 1), this antibody was not able to significantly affect the spontaneous nociception elicited by PAF (Fig. 4G).

Interestingly, the mechanical hypersensitivity evoked by PAF (10 nmol/paw) was also diminished by other pharmacological strategies directed to neutrophil migration, including: the non-selective selectin inhibitor fucoidin (10 mg/kg, i.v., 15 min), the anti-C5a receptor (anti-CD88) antibody (500 ng/paw), or the selective CXCR2 receptor antagonist (1 mg/kg, i.p., 30 min) (Fig. 5B and C; E and F; H and I; Table 1). On the other hand, none of these blockers succeeded in significantly changing the spontaneous nociception caused by PAF (Fig. 5A, D and E). It is worth mentioning that PAF (10 nmol/paw) injection produced a marked increase of MPO activity, according to assessment in the rat paw subcutaneous tissue 3 h after, and this parameter was almost completely inhibited by WEB2086, the antineutrophil antibody, fucoidin, SB225002, or anti-CD88 (Fig. 6; Table 1).

A final goal of our study was to evaluate the relevance of cyclooxygenase (COX) or adrenergic system activation for PAF-induced nociceptive changes. Systemic treatment with the COX inhibitors indomethacin (2.5 mg/kg, s.c.) or celecoxib (20 mg/kg, p.o.), or the β_1 adrenergic receptor antagonist atenolol (1 mg/kg, s.c.), given 60 min before PAF injection, produced a marked decrease of mechanical hypersensitivity (Fig. 7B and C; E and F; Table 1), without significantly altering the spontaneous nociception (Fig. 7A and D).

4. Discussion

It is widely known that PAF represents an important inflammatory mediator, which is involved in a series of biological events. Although PAF has been implicated in painful processes, additional studies are still required in order to characterize its nociceptive actions. In this study, we demonstrate the ability of PAF to evoke both spontaneous nociception and mechanical hypersensitivity when injected into the rat paw. Moreover, some of the potential mechanisms responsible for both nociceptive behaviors were evaluated. We have found that (i) both spontaneous nociception and mechanical hypersensitivity induced by PAF were greatly mediated by IL-1β production; (ii) PAF-induced spontaneous nociception, but not mechanical hypersensitivity, depends on the C fiber and TRPV1 activation and/or sensitization, as well as mast cell degranulation; and (iii) neutrophil recruitment, the release of prostanoids and the activation of adrenergic pathways are likely involved in the long-term mechanical hypersensitivity evoked by PAF.

Our first set of data confirms and extends previous literature evidence indicating that PAF might represent a pivotal mediator of inflammatory painful conditions [19,26]. Accordingly, PAF injection caused a marked spontaneous nociception in the rat paw, as observed in a 20 min-period of evaluation. In addition, PAF induced a significant and long-term (up to 8 h) mechanical hypersensitivity in response to VFH stimulation. The overt nociception was found to be a clearly dose-dependent event with an early onset. Conversely, the long-term increase of mechanical hypersensitivity was significant only at the dose of 10 nmol/paw. This might be related to the observed unspecific effects of higher doses of PAF (up to 10 nmol/paw) in the motor responses of some animals (data not shown).

Our next step was to verify, by employing different pharmacological tools, some of the mechanisms underlying both nociceptive behaviors in response to PAF injection. As an initial purpose, we analyzed the effects of PAF receptor blockage on the nociceptive responses induced by PAF. Not surprisingly, both spontaneous nociception and mechanical hypersensitivity were markedly inhibited by the coinjection of the selective PAF receptor antagonist WEB2086. This allows us to suggest that both nociceptive behaviors are dependent on PAF receptor activation.

It is reasonable to surmise that PAF binds to its receptor to induce nociception, and this is probably related to the

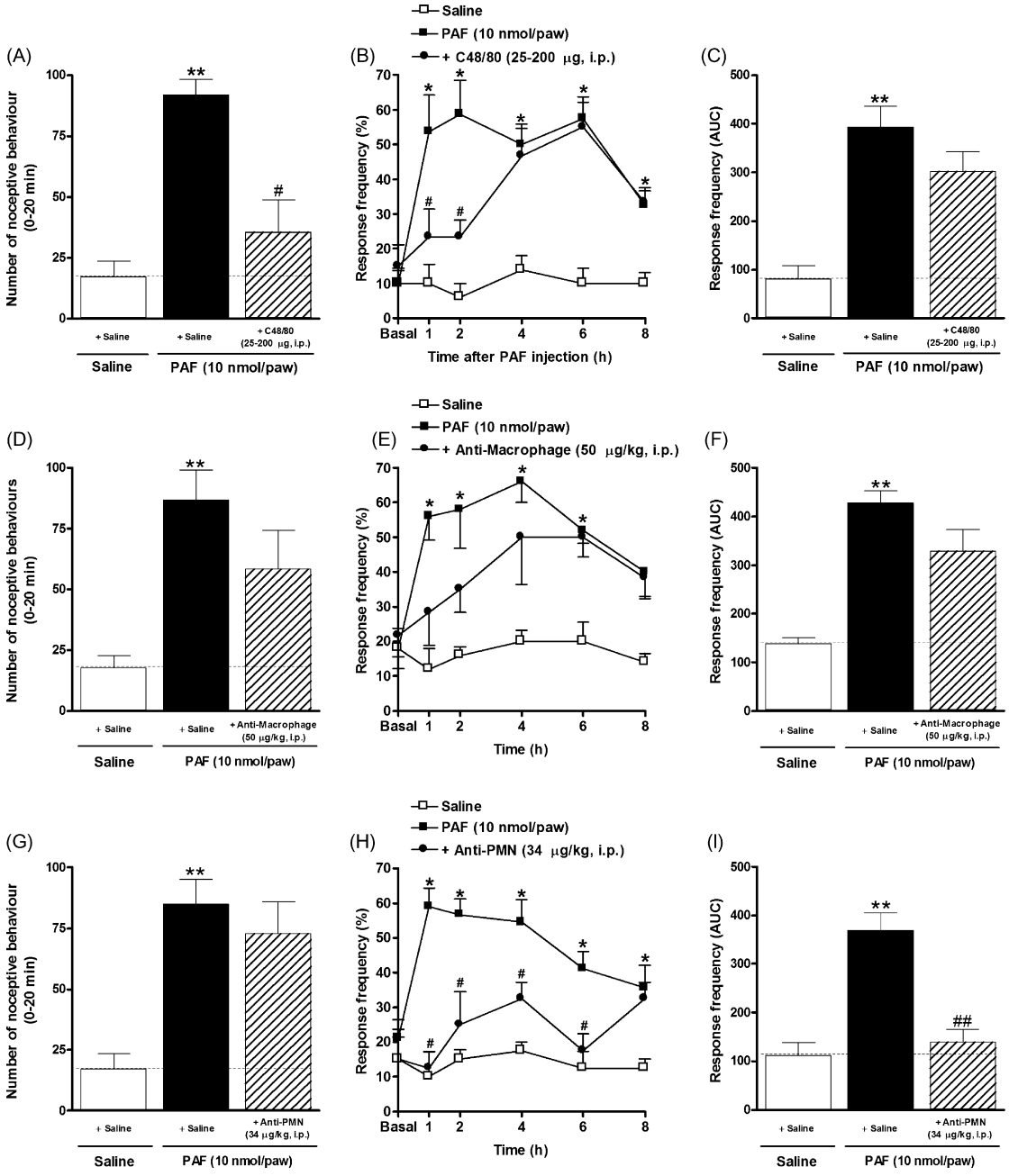

Fig. 4 – Effect of the mast cell-degranulating agent compound 48/80 (C48/80; 25, 60 and 125 μg/animal on the 1st, 2nd and 3rd days, respectively, and two injections of 200 μg/animal, on the 4th day, by i.p. route) on the spontaneous nociception (A), or the mechanical hypersensitivity (B and C) induced by the injection of PAF (10 nmol/paw). Effect of the antimacrophage antibody (50 μg/kg, i.p., 24 h and 30 min) on the spontaneous nociception (D), or the mechanical hypersensitivity (E and F) induced by the injection of PAF (10 nmol/paw). Effect of the antineutrophil antibody (34 μg/kg, i.p., 30 min) on the spontaneous nociception (G), or the mechanical hypersensitivity (H and I) induced by the injection of PAF (10 nmol/paw). Each group represents the mean of six to eight animals, and the vertical line indicates the SEM Significantly different from saline ($^{*}P < 0.05$ or $^{**}P < 0.01$) or PAF-injected paw ($^{\#}P < 0.05$ or $^{\#\#}P < 0.01$) values.

secondary release of other mediators. Following this reasoning, we next investigated whether PAF-induced pain behaviors would be allied to the release of proinflammatory cytokines. In fact, a growing amount of evidence suggests that cytokines might be implicated in nociceptive responses evoked by different inflammatory mediators [38]. The present results demonstrate that coinjection of anti-TNFα antibody was not able to significantly affect the spontaneous nociception or the mechanical hypersensitivity induced by PAF, whereas both nociceptive behaviors were greatly reduced by the local administration of the natural IL-1β antagonist IRA. In agreement with our data, a previous publication has demonstrated that hypernociception induced by the chemotatic factor KC in mice is sensitive to IL-1β, but not TNFα, inhibition

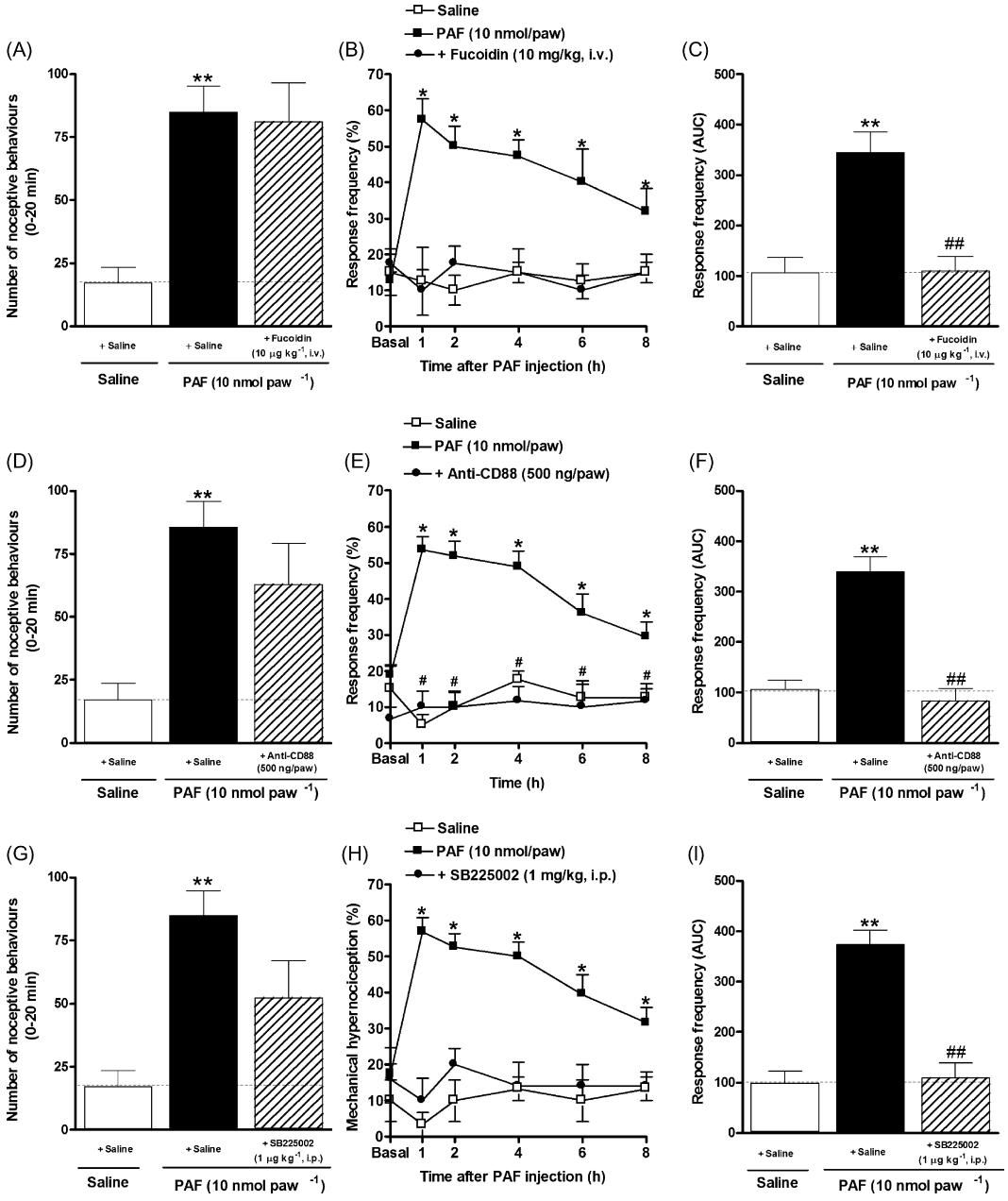

Fig. 5 – Effect of the non-selective selectin inhibitor fucoidin (10 mg/kg, i.v., 15 min) on the spontaneous nociception (A), or the mechanical hypersensitivity (B and C) induced by the injection of PAF (10 nmol/paw). Effect of the anti-C5a receptor (anti-CD88) antibody (500 ng/paw) on the spontaneous nociception (D), or the mechanical hypersensitivity (E and F) induced by the injection of PAF (10 nmol/paw). Effect of the selective CXCR2 receptor antagonist (1 mg/kg, i.p., 30 min before) on the spontaneous nociception (G), or the mechanical hypersensitivity (H and I) induced by the injection of PAF (10 nmol/paw). Each group represents the mean of six to eight animals, and the vertical line indicates the SEM Significantly different from saline ($^{*}P < 0.05$ or $^{**}P < 0.01$) or PAF-injected paw ($^{\#}P < 0.05$ or $^{\#\#}P < 0.01$) values.

[39]. IL-β is an important inflammatory mediator, which plays an essential role in pain sensitization [40–42]. For instance, IL-1β is able to induce mechanical hyperalgesia in rats, a response largely dependent on a direct activation of nociceptors, prostaglandin production and kinin B_1 receptor induction [43–45]. In addition, IL-β can modulate TRPV1 receptor sensitization; it induces the expression of pronociceptive genes, and increases the spontaneous activity in DRG neurons [46–49]. Also, increasing evidence suggests that IL-β enhance pain via central mechanisms; thus IL-β was found to be expressed in the spinal cord under different chronic pain conditions, especially in glial cells. Thus, spontaneous nociception and mechanical hypersensitivity induced by PAF injection appear to be dependent on IL-1β release; this might be either at peripheral or central sites.

It is broadly known that inflammatory mediators increase the sensitivity of sensory neurons, via mechanisms dependent on the activation of the capsaicin-gated channel TRPV1 [50,51].

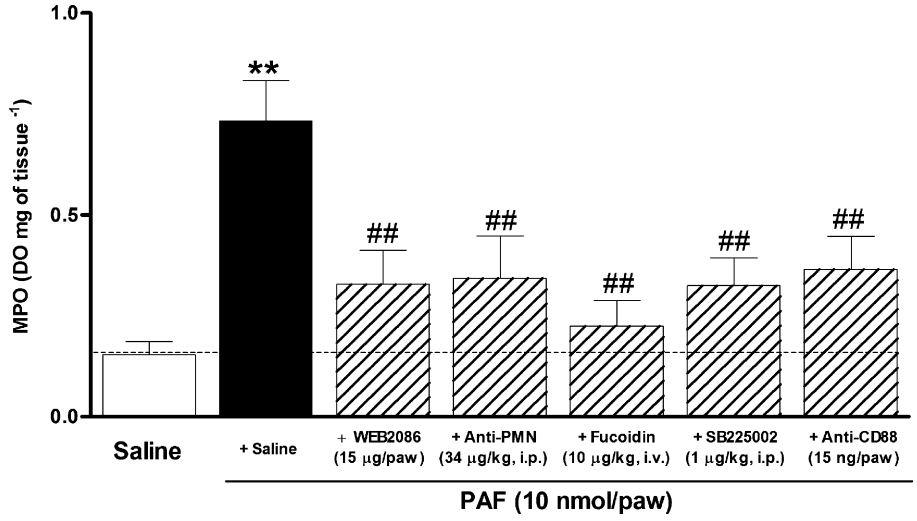

Fig. 6 – Effect of the treatment with WEB2086 (33 nmol/paw), fucoidin (10 mg/kg, i.v., 15 min before), anti-PMN (34 μg/kg, i.p., 30 min before), anti-CD88 (500 ng/paw) or SB225002 (1 mg/kg, i.p., 30 min before) on PAF-induced neutrophil influx (MPO activity) in the rat paw. Each column represents the mean ± SEM of six to eight animals. Significantly different from saline ($^{**}P < 0.01$) or PAF-injected paw ($^{\#\#}P < 0.01$) values.

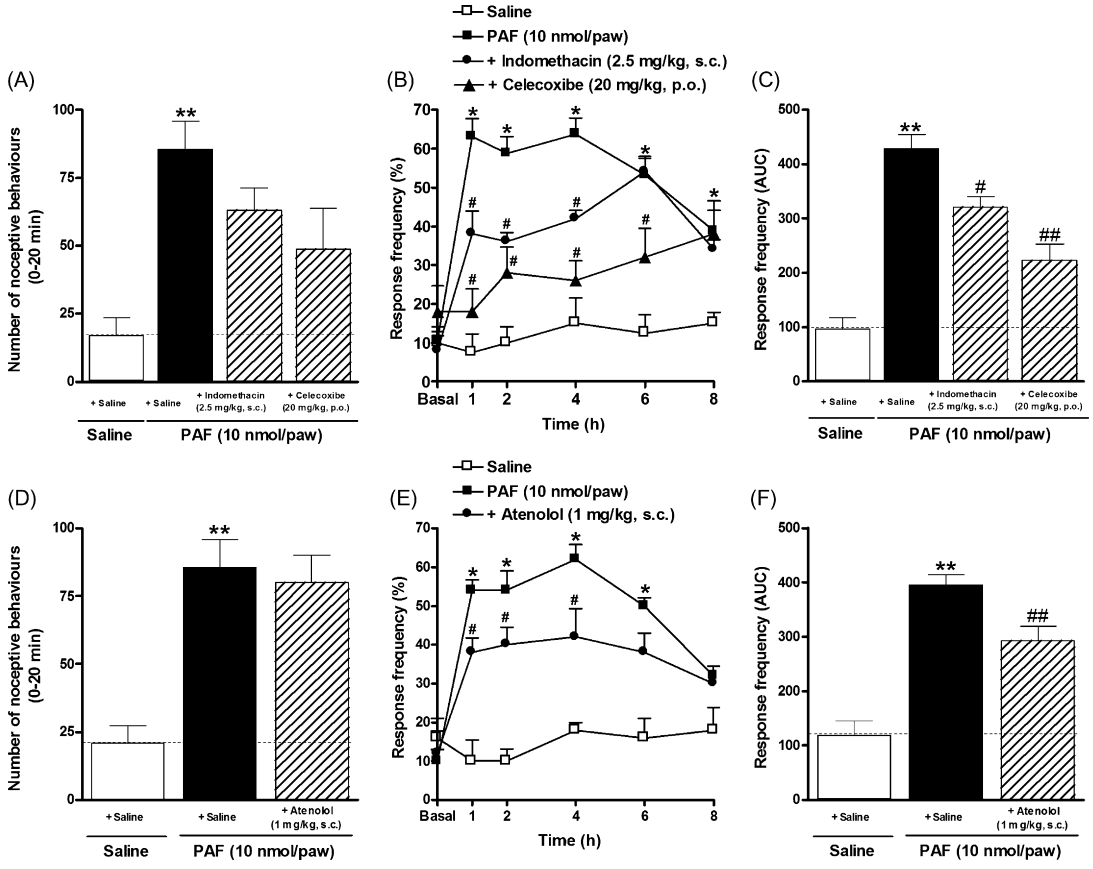

Fig. 7 – Effect of the COX inhibitors indomethacin (2.5 mg/kg, s.c., 1 h) and celecoxib (20 mg/kg, p.o., 1 h) on the spontaneous nociception (A), or the mechanical hypersensitivity (B and C) induced by the injection of PAF (10 nmol/paw). Effect of or the selective β_1 adrenergic receptor antagonist atenolol (1 mg/kg, s.c., 1 h) on the spontaneous nociception (D), or the mechanical hypersensitivity (E and F) induced by the injection of PAF (10 nmol/paw). Each group represents the mean of six to eight animals, and the vertical line indicates the SEM. Significantly different from saline ($^{**}P < 0.01$) or PAF-injected paw ($^{\#}P < 0.05$) values.

In an attempt to verify whether PAF would be able to induce pain via C fiber activation or TRPV1 sensitization, we assessed the effects of two different strategies: (1) systemic pretreatment of animals with the ultrapotent capsaicin analog resiniferatoxin, to promote C fiber destruction; (2) coinjection of the selective TRPV1 antagonist SB366791. Interestingly, both approaches expressively blocked the spontaneous nociception induced by PAF, although they did not significantly affect the mechanical hypersensitivity. This indicates that distinct mechanisms underlie the rapid spontaneous nociception and delayed mechanical hypersensitivity following PAF injection. In this context, our data undoubtedly shows a key role for C fibers and TRPV1 receptor in the overt nociception induced by PAF. Based on these results, we might suggest that PAF receptor activation, located in the C fibers, can lead to the activation and/or sensitization of TRPV1 receptors, confirming and extending previous findings [19].

Recent evidence has suggested that increased influx of cells to an injured site might be related to the onset of nociception processes [52]. Therefore, we decided to evaluate to what extent the blockage of different cell populations might prevent PAF-induced nociceptive responses. Mast cell activation has been correlated to nociceptive behavior. For instance, mast cell deficient rats display reduced visceral hypersensitivity caused by injection of 2,4,6-trinitrobenzene sulfonic acid (TNBS) into the proximal colon [53]. In addition, mast cell depletion has been found to be effective in preventing the spontaneous nociceptive behavior elicited by trypsin injection into the mouse paw [54]. In our study, the pharmacological depletion of mast cells, following pretreatment with the mast cell-degranulating agent C48/80, significantly diminished the spontaneous nociception induced by PAF, whilst the mechanical hypersensitivity was only partially altered. Literature evidence has indicated that mast cells within the normal skin express TRPV1 receptors [55], which might well support our results on PAF-induced spontaneous nociception. But on the contrary, our data revealed that a protocol designed to deplete macrophages, by systemically pretreating rats with an antimacrophage antibody, failed to significantly change either the spontaneous or the mechanical hypersensitivity induced by i.pl. injection of PAF. This series of data indicates that macrophages do not apparently display a relevant role in the nociceptive parameters analyzed in the present study.

The administration of an antineutrophil antibody markedly reduced the mechanical hypersensitivity induced by PAF, although this approach failed to reduce PAF-evoked spontaneous nociception. This is consistent with the long-term profile of the mechanical hypersensitivity in response to VFH stimulation, when compared to the spontaneous nociceptive behavior. One might suggest that reduced threshold to mechanical stimulation following PAF injection is secondary to polymorphonuclear neutrophil migration. To gain further insights into this hypothesis, we also tested additional tools that are known to interfere with the influx of neutrophils. Again, the mechanical hypersensitivity induced by PAF was significantly reduced by the selectin inhibitor fucoidin, by the selective CXCR2 chemokine receptor antagonist SB225002, and finally by the anti-C5a receptor antibody anti-CD88. On the other hand, the spontaneous nociception was not significantly altered by any of these treatments, thus further suggesting that distinct mechanisms underlie PAF-induced spontaneous nociception and mechanical hypersensitivity. The anaphylotoxin C5a is a chemotatic factor which interacts with a G-coupled seven transmembrane receptor called CD88. C5a is implicated in several pathophysiological events by driving neutrophil migration during the inflammatory scenario [56], also displaying a relevant role in nociceptive responses [57–59]. Likewise, some previous reports have suggested a role for CXCR2 in the nociception processes [60]. For instance, administration of the CXC chemokine CXCL8 (also named interleukin-8) into the rat paw causes a sympathetic-mediated hyperalgesia, an event which is shown to be independent of prostaglandin release [61]. Relevantly, a recent publication indicated that treatment with DF2162, a non-competitive allosteric inhibitor of CXCR1/2, consistently reduced the inflammatory nociception in mice [52]. Finally, it is now well known that adhesion molecules are not constitutively active, but they can become activated under stimulation by PAF and other locally secreted chemotatic factors [62,63]. Moreover, we have observed (results not shown) that i.pl. injection of PAF causes a time-related increase in the expression of the adhesion molecules β_2-integrin and P- and E-selectins in the rat paw tissue, at time-points that are perfectly related to the mechanical hypersensitivity.

PAF injection into the rat paw is able to induce a significant augmentation of MPO activity (an indirect measure of neutrophil influx) as early as 1 h following PAF injection, with a maximal increase between 3 and 6 h [7]. Extending this notion, we also provide evidence showing that PAF-induced increase in MPO activity was almost abolished by WEB2086, the antineutrophil antibody, fucoidin, SB225002, or anti-CD88. An overall analysis of our data, permit us to assume that PAF-induced mechanical hypersensitivity is greatly dependent on neutrophil migration, by mechanisms certainly involving the activation of CXCR2 chemokine receptors, C5a receptors, adhesion molecules and central sensitization mechanism (probably involving IL-1β actions). In this context, it is worth mentioning that IL-1β production after PAF injection into the rat paw was widely dependent on polymorphonuclear cells migration to the inflammatory site [8].

Prostaglandin release and activation of the adrenergic system have been pointed to as important events for the establishment of nociception in different models of inflammatory pain [64–66]. Furthermore, the adrenergic system has been implicated in the nociception responses induced by TNFα and carrageenan [67]. Herein, we observed a partial, though significant, contribution of these pathways to the mechanical hypersensitivity evoked by PAF. Thus, COX inhibitors indomethacin and celecoxib, as well as atenolol, significantly increased the mechanical threshold in PAF-injected paws. In contrast, indomethacin, celecoxib and atenolol all failed to significantly inhibit the spontaneous nociception induced by PAF, discarding the relevance of prostanoids and sympathetic activation for this nociceptive behavior.

Taken together, the present results throw new lights on the mechanisms underlying the nociceptive responses evoked by PAF. Firstly, both spontaneous nociception and mechanical hypersensitivity following PAF injection are extensively reliant on PAFR activation and IL-1β production. Secondly, spontaneous overt nociception is a rapid onset event,

mediated mainly by TRPV1 activation and mast cell stimulation; on the other hand, the long-term mechanical hypersensitivity is virtually dependent on neutrophil influx-related mechanisms, and it involves prostanoid and adrenergic pathways. Our data certainly contribute to paving the way for elucidating the nociceptive effects of chemotatic factors, such as PAF.

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