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Involvement of endogenous leukotriene B_4 and platelet-activating factor in polymorphonuclear leucocyte recruitment to dermal inflammatory sites in rats

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Summary

A critical role for leukotriene B_4 (LTB_4) and/or platelet-activating factor (PAF) in regulating polymorphonuclear cell (PMN) trafficking to inflammatory sites has been reported in a number of experimental inflammatory models. *In vitro*, newly synthesized LTB_4 and PAF were shown to act in an autocrine/paracrine or intracrine fashion to enhance intracellular arachidonic acid availability and leukotriene biosynthesis. This suggested potentially cooperative effects of these lipid mediators in regulating PMN extravasation. The present study aimed to elucidate whether endogenous LTB_4 and PAF may both act to regulate plasma extravasation and PMN trafficking to inflammatory sites in experimental inflammation. With this aim, we have used selective and potent PAF and LTB_4 receptor antagonist pretreatments in dermal and pulmonary inflammation models in rats. Our results show additive inhibitory effects of dual LTB_4 and PAF receptor blockade in either PAF- or LTB_4-elicited cutaneous PMN accumulation compared to single-drug administration. Furthermore, the combined administration of the drugs inhibited the PMN accumulation induced by the chemically unrelated soluble agonists tumour necrosis factor-α and C5a. Finally, in a model of pulmonary inflammation induced by the intravenous injection of Sephadex beads, lung neutrophilia was reduced by 63% following the administration of LTB_4 and PAF antagonists, in contrast with the lack of effect of single drug administration. Our results strongly support a role of both endogenous LTB_4 and PAF in regulating PMN trafficking to inflammatory sites in various experimental conditions.

Keywords: extravasation; lipid mediator; leukotriene B_4; lung; migration; platelet-activating factor; skin; trafficking

Introduction

The generation and release of bioactive lipids after phospholipase A_2 (PLA_2)-mediated hydrolysis of membrane phospholipids is inherent in a wide range of physiological and pathophysiological processes pertaining to host defence and inflammatory diseases, encompassing phagocytosis, microbial killing, secretion, leucocyte trafficking, intercellular communication, etc.[1–3] Among oxygenated bioactive lipid metabolites, leukotriene B_4 (LTB_4) is one of the most potent chemoattractants and activators of polymorphonuclear leucocytes (PMN) generated from the cellular as well as transcellular metabolism of arachidonic acid.[4,5] Platelet-activating factor (PAF), a potent pro-inflammatory and chemotactic phospholipid, is concomitantly biosynthesized with eicosanoids within activated leucocytes[6,7] and endothelial cells.[8,9] Besides sharing many biological properties, LTB_4 and PAF were shown to act in an autocrine/paracrine[10] or intracrine[11] fashion to enhance intracellular arachidonic acid availability and LT biosynthesis. In addition, further *in vitro* studies showed that LTB_4 type 1 receptor (BLT1) signalling was involved in PAF-mediated PMN degranulation and chemotaxis, and that PAF-mediated calcium mobilization was partially desensitized by LTB_4.[12]

Previous *in vivo* studies supported a critical role for LTB_4 receptor engagement in regulating PMN trafficking to inflammatory sites elicited by various soluble chemoattractants,[13,14] including PAF.[15–17] Studies in inflammatory models underscored the central role of LTB_4 in

arthritis,[18,19] intestinal inflammation,[20] sepsis,[21] asthma,[22] ischaemia/reperfusion (I/R) tissue injury[23,24] and dermatitis.[25] Some studies provide evidence for a role of PAF as an upstream mediator of various inflammatory diseases.[26,27] Other studies underline a role for PAF as an early mediator in I/R injury,[28,29] acute pancreatitis,[30] airway hyperresponsiveness, asthma,[31] breast cancer proliferation,[32] intestinal inflammation[27,33] and sepsis.[34]

Against this background, and considering first, that LTB_4 and PAF are concomitantly generated in activated PMN, and second, that the two mediators show redundant (and complementary) proinflammatory properties, the present study aimed to elucidate whether LTB_4 and PAF may act in a cooperative manner to regulate plasma extravasation and PMN trafficking to inflammatory sites in experimental inflammation. Our results support a cooperative, central role of the lipid mediators in promoting the extravascular PMN accumulation elicited by various, chemically unrelated chemoattractants.

Materials and methods

Animals

Male Sprague-Dawley rats (100–150 g) were purchased from Charles River (St-Constant, Québec, Canada). All experimental protocols were performed in accordance with the Canadian Council for Animal Care guidelines and were approved by the institutional animal care and use committee.

Reagents

The (+)-1-(3S,4R)-[3-(4-phenyl-benzyl)-4-hydroxy-chroman-7-yl]-cyclopentane carboxylic acid (CP-105,696) was kindly provided by Dr D.W. Owens from Pfizer Corp. (Groton, CT), *N*-(2-dimethylaminoethyl)-*N*-(3-pyridinylmethyl)(4-(2,4,6-triisopropyl-phenyl)thiazol-2-yl]amine (SR-27417) was kindly provided by Dr J.-M. Herbert from Sanofi-Aventis (Toulouse, France) and UK-74,505 by Mrs S.D. Srodzinski from Pfizer Global Research & Development (Sandwich, Kent, UK). The 5(*S*),12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid (LTB_4) was purchased from Cayman Chemicals (Ann Arbor, MI). The stock solution of LTB_4 (200 μg/ml, in ethanol) was kept at –20° and diluted in vehicle immediately before use. Recombinant human methionyl granulocyte colony-stimulating factor (G-CSF) or filgrastim (Neupogen®) was obtained from Amgen Canada Inc. (Mississauga, Ontario, Canada). Bovine serum albumin (BSA; very low endotoxin), dimethyl sulphoxide, dimethylformamide (DMF), hexadecyltrimethylammonium bromide (HTAB), hydrogen peroxide (H_2O_2), PAF, prostaglandin E_2 (PGE_2) and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (St-Louis, MO). Tumour necrosis factor-α (TNF-α) was purchased from PeproTech Inc. (Rocky Hill, NJ). Modified Hanks' balanced salt solution (HBSS) and HEPES buffer were from Gibco Life Technologies (Grand Island, NY). Sephadex beads (G50 superfine) were obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). Ketamine (Ketalar®), xylazine (Rompun®) and pentobarbital (Euthansol) were obtained from CDMV Inc. (Ste-Hyacinthe, Québec, Canada). All solutions for parenteral administration were purchased from Baxter Corporation Laboratories (Toronto, Ontario, Canada).

Dermal inflammation

Rats were injected daily subcutaneously with filgrastim (5 μg/kg) for 9–11 days before experimentation; such pretreatment of animals was found to increase numbers of circulating PMN approximately five-fold and to proportionally enhance PMN accumulation at dermal inflammatory sites, thereby facilitating the assessment of drug effects on PMN trafficking. On the day of the experiment, rats were pretreated orally with SR-27417 (0·1–1 mg/kg) in sterile water, and/or CP-105,696 (1–30 mg/kg) in 0·5% carboxymethylcellulose (total volume for gavage, 0·5 ml), respectively, 2 and 16 hr before the intradermal (i.d.) injections of the soluble agonists. Vehicle-treated animals received orally the same excipients (and same volume) sequentially. Rats were placed on heating pads and the agonists under investigation, including LTB_4 (500 pmol/site), PAF (1·9 nmol/site), TNF-α (10 pmol/site) and 3% zymosan-activated plasma (ZAP), were injected at duplicate sites in 50 μl HBSS containing 0·1% BSA (HBSS-BSA) and 5 μM PGE_2 in shaved dorsal skin of rats anaesthetized with ketamine–xylazine (70–5 mg/kg). All the required control i.d. injections were also performed in duplicates, such as HBSS-0·1% BSA or HBSS-0·1% BSA containing $\leq$ 1% ethanol and 5 μM PGE_2. A maximum of 16 sites were injected per rat. Two hours later, the rats were killed with an overdose of pentobarbital, and skin biopsies (11-mm diameter) were punched out, weighed and frozen at – 80° until assayed for myeloperoxidase (MPO) activity for assessment of PMN accumulation (Fig. 1a). In another series of experiments aimed to assess vascular permeability, Evans blue (100 mg/kg in 0·9% NaCl) was injected intravenously (i.v.) through the jugular vein and agonists (PAF, LTD_4 and HBSS-BSA vehicle, without PGE_2) were injected i.d. Thirty minutes later, the thoracic aorta was cannulated and a 1-ml blood sample was obtained for total and differential circulating leucocyte counts, as well as for Evans blue quantification in both plasma and skin biopsies (Fig. 1b).

Pulmonary inflammation

In other experiments, pulmonary inflammation was elicited by the i.v. injection of 15 mg/kg Sephadex beads

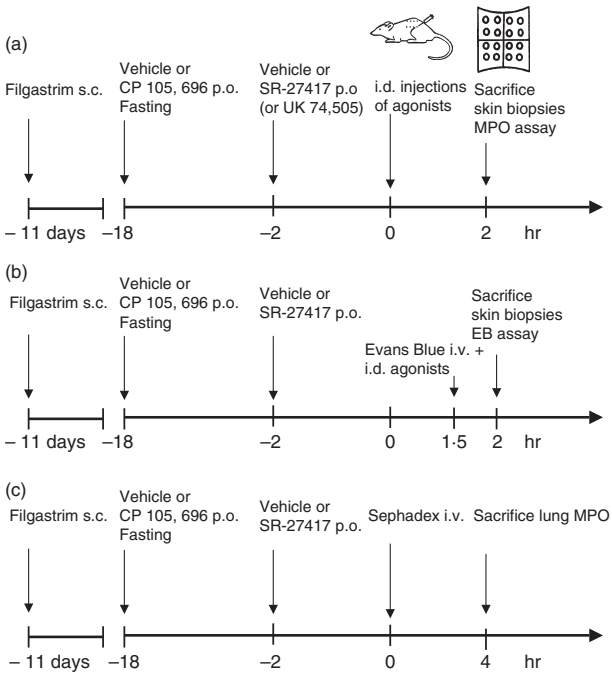

Figure 1. Schematic figure of experimental protocols for dermal (a and b) and lung (c) inflammation. Briefly, the rats were pretreated for 9–11 days before the experiments. On the day of the experiment, CP-105,696 and SR-27417 (or UK-74,505) were administered orally 16 hr and 2 hr before the intradermal (i.d.) injection of agonists in sedated rats (a). After a 2-hr accumulation period, the animals were killed and biopsies were taken for myeloperoxidase (MPO) assay. In additional experimental groups, Evans blue was injected intrvenously (i.v.), immediately followed by platelet-activating factor (PAF) and leukotriene D_4 (LTD_4), 30 min before killing, to assess vascular permeability. For Sephadex-induced lung inflammation, the animals were pretreated with the drugs as above, before i.v. Sephadex injection. The animals were killed 4 hr later and the lungs were kept for MPO assay.

through the subclavian vein of anaesthetized rats. Four hours later, blood (1 ml) was sampled as described above and the rats were killed with an overdose of pentobarbital (Fig. 1c). The heart was flushed with a solution of 0·9% NaCl containing 10 mM ethylenediaminetetraacetic acid (EDTA) and the heart and lungs were harvested *en bloc*. Lungs were dissected, weighed and frozen at −80° until assayed for MPO activity.

MPO assay

Tissue MPO activity was assayed in skin biopsies and lungs as previously described,[35] with some modifications. Briefly, lung tissues were homogenized in 1 ml phosphate-buffered saline and centrifuged. The lung tissue pellets and skin biopsies were homogenized in 1 ml acetate buffer (100 mM), pH 6·0, containing 0·5% HTAB and 20 mM EDTA. Lung homogenates were heated to 65° for 120 min in a water bath. The homogenates (lung and skin) were subjected to three freeze–thaw cycles and then centrifuged at 2000 ***g*** for 10 min. MPO (freed from PMN granules) was assayed by incubating supernatants with 3·2 mM TMB and 1·0 mM H_2O_2 for 5 min at 37°. The reaction was stopped by the addition of 100 µl of 0·2 M sodium acetate (pH 3·0). Calibration curves for PMN were prepared using rat peritoneal PMN (elicited by intraperitoneal injection of 10 ml per rat of a 5% casein solution in saline). The numbers of PMN per skin site or lung were calculated from the standard curves.

Evans blue assay

Plasma leakage in skin sites was assayed by measuring the absorbance (Evans blue) of DMF extracts (48 hr, room temperature) at 630 nm against a standard curve of Evans blue in DMF. Plasma extravasation was calculated as µl = (µg Evans blue per site)/(µg Evans blue per µl plasma).

Statistical analysis

All results are expressed as mean ± SEM, and statistical comparisons were performed using a one-way analysis of variance (ANOVA) followed by pair-wise multiple comparisons using the Student–Newman–Keuls methods using GraphPad Prism Version 4.0 (GraphPad Software, Inc., San Diego, CA). Differences were considered significant at $P < 0{\cdot}05$.

Results

Effect of BLT1 and PAF receptor antagonists on PMN accumulation in rat skin

To delineate the role of PAF and LTB_4 in inflammatory responses in rat skin, we have used selective and long-acting PAF (UK-74,505 and SR-27417) and BLT1 (CP-105,696) receptor antagonists administered orally to the animal before i.d. injections of soluble inflammatory agents in G-CSF (filgrastim) -pretreated rats. A first series of experiments was aimed at defining the dose–inhibition curves for the LTB_4 and PAF antagonists against LTB_4- and PAF-elicited PMN accumulation in the dermis. Pretreatment of rats with CP-105,696 (Fig. 2a) and SR-27417 (Fig. 2b) caused a dose-dependent inhibition of PMN accumulation in response to i.d. injections of LTB_4 (500 pmol/site) and PAF (1·9 nmol/site), respectively. Similar results were observed with UK-74,505, for which doses of 2·5 and 5 mg/kg reduced the number of PMN per skin site elicited by PAF by 28% and 62% ($P < 0{\cdot}05$), respectively (results not shown). Optimal doses were selected for combined administration of antagonists in PMN migration studies. For SR-27417, the optimal dose for combined drug administration was 0·3 mg/kg, a dose that inhibited PAF-elicited PMN recruitment by ∼ 40%

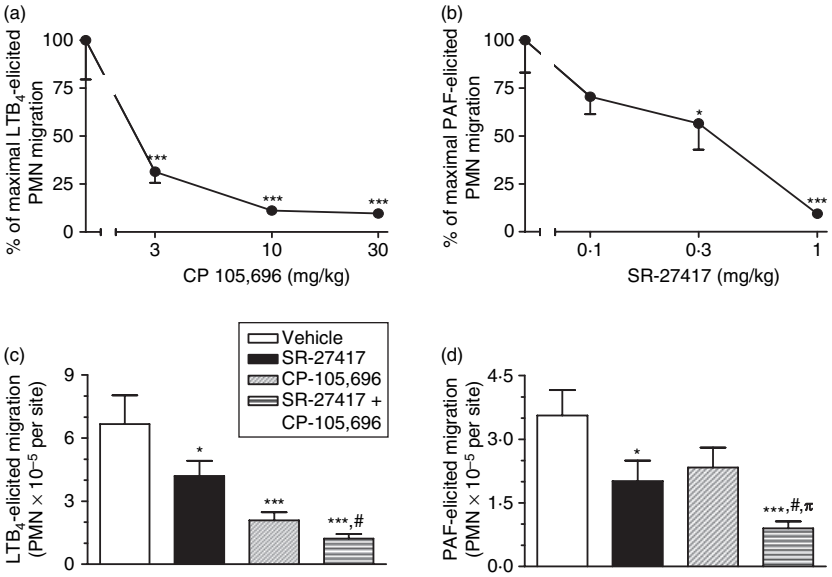

Figure 2. Inhibition of leukotriene B_4 (LTB_4)-induced (500 pmol/site) and platelet-activating factor (PAF) -induced (1·9 nmol/site) dermal accumulation of polymorphonuclear cells (PMN) using selective BLT1 and PAF receptor antagonists. Rats were pretreated orally with various doses of CP-105,696 and/or SR-27417, 16 and 2 hr, respectively before the intradermal (i.d.) injections of soluble agonists (50 μl/site) at duplicate sites in shaved dorsal skin. Two hours later, the rats were killed and skin biopsies were punched out. PMN accumulation at inflammatory sites was assessed by determination of myeloperoxidase (MPO) activity in skin biopsies. Dose-dependent inhibition of LTB_4- (a) and PAF- (b) elicited PMN accumulation by CP-105,696 (3–30 mg/kg) and SR-27417 (0·1–1 mg/kg), respectively. Effect of separate or combined administration of SR-27417 (0·3 mg/kg) and CP-105,696 (3 mg/kg) on LTB_4- (c) and PAF- (d) induced PMN accumulation. PMN migration values obtained from control sites injected with the vehicle (0·89 ± 0·14 PMN/site) were subtracted from PMN migration measurements obtained from LTB_4- or PAF-injected skin sites. Data are the mean ± SEM of 24 sites obtained from 12 rats. $^*P < 0{\cdot}05$ and $^{***}P < 0{\cdot}001$, compared to vehicle; $^{\#}P < 0{\cdot}05$ compared to SR-27417 and $^{\pi}P < 0{\cdot}05$ compared to CP-105,696-treated rats.

(Fig. 2b,d). For CP-105,696, the dose selected for combined administration was the lowest dose tested (Fig. 2a), 3 mg/kg, which provided ∼ 60–70% inhibition of LTB_4-elicited PMN accumulation. This dose is low compared to the dose-range utilized by others in rodents (10–100 mg/kg).[36–39]

Figure 2(c,d) show that whereas CP-105,696 (3 mg/kg) and SR-27417 (0·3 mg/kg) administered alone partially inhibited both LTB_4- and PAF-elicited PMN accumulation, additive effects were observed following combined drug administrations. The combined administration of CP-105,696 (3 mg/kg) with UK-74,505 (5 mg/kg) also showed an additive inhibitory effect on PAF-elicited PMN accumulation to skin sites with a maximal reduction of 72%, because the administration of UK-74,505 led to blockade of LTB_4- and PAF-induced PMN accumulation by 38% and 51% ($P < 0{\cdot}05$), respectively. None of the pharmacological treatment modulated blood leucocyte counts (results not shown).

We also tested whether CP-105,696 (3 mg/kg) and/or SR-27417 (0·3 mg/kg) could inhibit PMN accumulation elicited by chemically unrelated soluble agonists such as ZAP (a source of $C5a_{desarg}$) and TNF-α. Whereas single drug administration did not significantly block PMN migration to dermal sites treated with the inflammatory agents, the combined drug regimen significantly inhibited ZAP- (Fig. 3a) and TNF-α- (Fig. 3b) induced migratory responses by 72% and 68% ($P < 0{\cdot}05$), respectively, unravelling the cooperative effects of PAF and LTB_4 in mediating PMN accumulation in response to chemically unrelated inflammatory agents.

Effect of BLT1 and PAF receptor antagonists on plasma extravasation

In the next series of experiments, the effect of the PAF receptor antagonist SR-27417 (0·3 mg/kg) and of the LTB_4 receptor antagonist CP-105,696 (10 mg/kg) administered alone or in combination was tested on plasma extravasation elicited by PAF and LTD_4. Figure 4 shows that SR-27417 significantly reduced plasma extravasation induced by PAF and that CP-105,696 showed little effect on plasma extravasation induced by both agonists. In this experiment, the combined administration of both antagonists resulted in a stronger inhibitory effect (significant against the vehicle) for either PAF- or LTD_4-elicited plasma extravasation. In both cases, however, the effect of the combined drug administration was not significantly different from the effect of the PAF antagonist alone. Therefore, although the co-administration of the

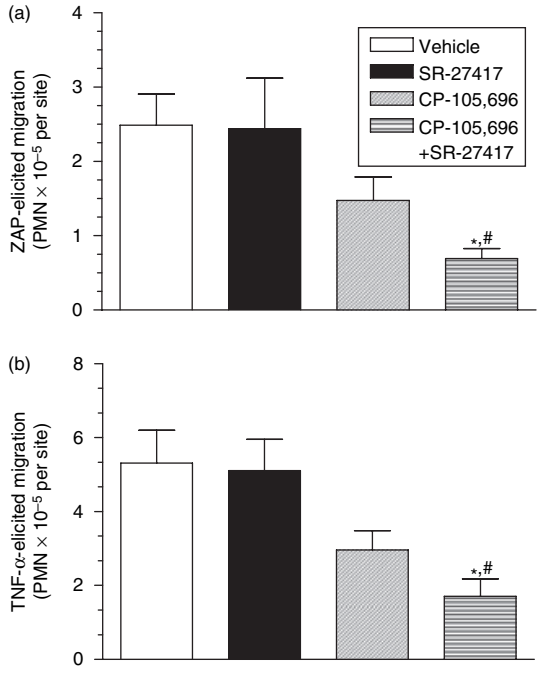

Figure 3. Inhibition of 3% zymosan-activated plasma (ZAP) (50 µl/site) (a) and tumour necrosis factor-α (TNF-α)(10 pmol/site) (b) -elicited PMN accumulation to dermal sites by pretreatment with platelet-activating factor (PAF; SR-27417, 0·3 mg/kg) and BLT1 (CP-105,696, 3 mg/kg) receptor antagonists in rats. Control values obtained from sites injected with Hanks' balanced salt solution/bovine serum albumin 0·1% were subtracted, as described in the legend to Fig. 1. Data are the mean ± SEM of 24 sites obtained from 12 rats. $^{*}P < 0{\cdot}05$ compared to vehicle, and $^{\#}P < 0{\cdot}05$ compared to SR-27417.

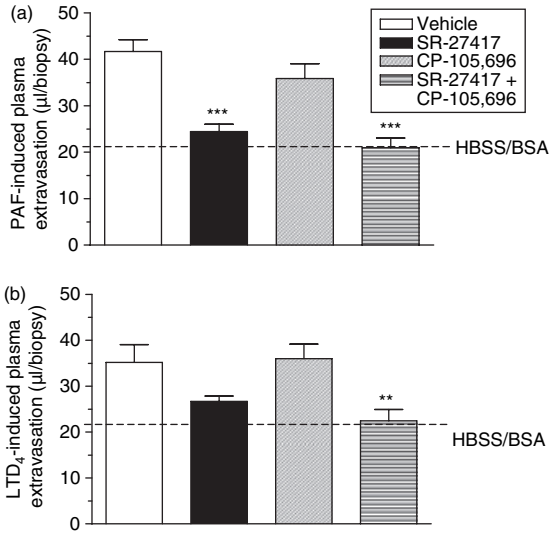

Figure 4. Effect of SR-27417 and CP-105,696 on soluble agonist-induced dermal oedema. Rats were pretreated orally with SR-27417 (0·3 mg/kg) and/or CP-105,696 (10 mg/kg). Thirty minutes before killing and 5 min before injecting agonists intradermally, Evans blue was injected intravenously (100 mg/kg) in sterile 0·9% NaCl through the jugular vein. Rats were killed and skin biopsies were punched out for measurement of Evans blue dye content and plasma extravasation. Effect of separate and combined administrations of SR-27417 (0·3 mg/kg) and CP-105,696 (10 mg/kg) on platelet-activating factor (PAF) (1·9 nmol/site) (a) and leukotriene D_4 (LTD_4) (500 pmol/site) (b) -elicited plasma extravasation. The dashed lines represent the mean plasma extravasation in Hanks' balanced salt solution/bovine serum albumin (HBSS/BSA) (vehicle)-injected sites. Data are the mean ± SEM of 8–12 sites obtained from four to six rats. $^{**}P < 0{\cdot}01$ and $^{***}P < 0{\cdot}001$, compared to vehicle.

antagonists showed a tendency toward increased inhibition of plasma extravasation (compared to the administration of the PAF antagonist alone), the data do not conclusively demonstrate a cooperation of PAF and LTB_4 in the regulation of PAF- and LTD_4-induced plasma extravasation. Reasons for that could be that PAF plays a much more important role than LTB_4 in regulating plasma extravasation, and/or that the agonists tested (PAF and LTD_4) elicited an only 1·6-2-fold increase of plasma extravasation over baseline, leaving little margin to generate statistically significant changes between the different experimental conditions.

Cooperative role of LTB_4 and PAF on PMN accumulation in lung inflammation

The role of LTB_4 and PAF in regulating PMN recruitment elicited by a wide range of inflammatory mediators generated within inflamed lungs was next investigated. Rats were pretreated with SR-27417 and/or CP-105,696 followed by the i.v. injection of Sephadex beads. The results show that either drug, administered alone, did not prevent PMN accumulation in lungs ($5{\cdot}6 \pm 0{\cdot}7 \times 10^6$ after Sephadex compared to $1{\cdot}8 \pm 0{\cdot}5 \times 10^6$ PMN/g lung in controls animals without Sephadex), whereas the combined administration of drugs reduced PMN accumulation in lungs by 63% ($P < 0{\cdot}05$) (Fig. 5a). Circulating PMN numbers were not altered by drug administration in Sephadex-treated rats (Fig. 5b).

Discussion

Both PAF and LTB_4 are potent activators of PMN functions. Whereas PAF has been shown to be involved in the regulation of events of the acute inflammatory response including vasodilation, vascular permeability and leucocyte transendothelial migration,[40] LTB_4 has mainly been associated with leucocyte activation, chemotaxis and phagocytosis and tissue repair.[3] In agreement with the autocrine/paracrine and juxtacrine effects of these mediators, our results support a role of both endogenous PAF and LTB_4 in the regulation of PMN trafficking to inflammatory sites.

The skin PMN migration assay used in the present studies allowed us to delineate the inhibitory effects of

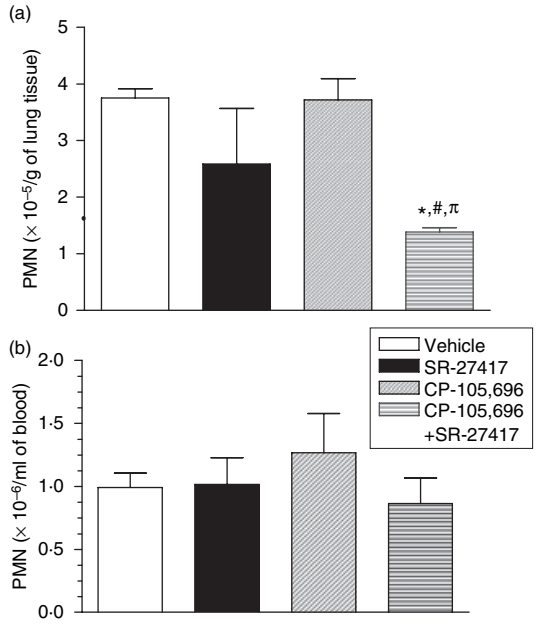

Figure 5. Effect of SR-27417 and/or CP-105,696 administration on pulmonary inflammation elicited by intravenous administration of Sephadex beads. Rats were pretreated orally with SR-27417 (0·3 mg/kg) and/or CP-105,696 (30 mg/kg), 2 and 16 hr, respectively, before the administration of Sephadex beads (15 mg/kg, in 0·9% NaCl) through the subclavian vein. Four hours later, the rats were killed, the heart and lungs were flushed with 0·9% NaCl/10 mM EDTA and harvested en bloc. Polymorphonuclear cell (PMN) accumulation in lungs was assessed by myeloperoxidase (MPO) activity assay. PMN accumulation data obtained from saline-injected animals ($1{\cdot}77 \pm 0{\cdot}45 \times 10^5$ PMN/g lung) were subtracted from values obtained from Sephadex-treated animals (a). Circulating blood PMN were also determined 4 hr after drug treatments (b). Data are the mean ± SEM of four to six rats. $^*P < 0{\cdot}05$ compared to untreated rats administered Sephadex intravenously; $^{\#}P < 0{\cdot}05$ compared to SR-27417-treated rats, and $^{\pi}P < 0{\cdot}05$ compared to CP-105,696-treated rats.

the pharmacological blockade of PAF and/or LTB_4 receptors on PMN accumulation at dermal sites in response to i.d. injected agonists in rats pretreated with G-CSF (filgrastim). The G-CSF was used to elevate circulating PMN counts and enhance recruitment to inflammatory sites, as previously reported.[41] Pharmacological blockade of LTB_4-elicited responses after the oral administration of CP-105,696, a selective and potent non-competitive antagonist of BLT1 receptors,[42] has been demonstrated in different species including mice,[36–39] guinea pigs,[43] rats,[24] primates[44] and humans[45] at doses ranging from 1 to 100 mg/kg. No side effects have been reported in the different species tested, after either acute or chronic administration of the compound.[38] In a similar manner, SR-27417, a selective and potent competitive PAF receptor antagonist, was shown to efficiently protect against PAF-induced bronchoconstriction, hypotension, oedema and leucopenia at doses up to 10 mg/kg per os in a number of species including rats.[46–50] As expected, we observed dose-dependent inhibitory effects of CP-105,696 and of SR-27417 on PMN migration elicited by the corresponding agonists (Fig. 2a and b). At the dose of 3 mg/kg, CP-105,696 inhibited LTB_4-induced PMN accumulation by 69% (Fig. 1c), while SR-27417, administered at a dose of 0·3 mg/kg, also elicited partial blockade (37%) of LTB_4-elicited PMN recruitment. Most interestingly, the concomitant administration of the LTB_4 and PAF antagonists elicited additive effects at inhibiting either LTB_4- or PAF-induced PMN migration, whether SR-27417 or UK-74,505 were used as PAF receptor antagonists, strongly suggesting that both PAF and LTB_4, when administered i.d., elicit the *de novo* formation of additional PAF and LTB_4, which significantly contributes to the stimulation of PMN extravasation. This hypothesis is supported by previous findings that PAF can trigger LTB_4 biosynthesis,[51] and that LTB_4 potently activates the $cPLA_2\alpha$ in PMN,[52] an enzyme directly involved in PAF biosynthesis.[53] This hypothesis is also in good agreement with our previous *in vitro* studies that unravelled the involvement of endogenous LTB_4 and PAF in a positive regulatory feedback loop on LT biosynthesis whereby newly synthesized PAF and LTB_4 enhance LT synthesis.[10] It was also shown that LTB_4 and/or PAF exerted their effects on LT synthesis through enhancing arachidonic acid availability. Additional evidence for a role of PAF in stimulating the biosynthesis of LTB_4 through autocrine and/or intracrine stimulation of 5-lipoxygenase (5-LO) activity and increased arachidonate availability has also been documented.[11,17] This is also in agreement with previous observations that PAF stimulates LTB_4 in human[11,51] and rat PMN.[54] Moreover, a role of endogenous PAF in eliciting intracellular calcium mobilization in response to LTB_4 has been demonstrated[55] and partial inhibition of LTB_4-elicited actions following PAF receptor blockade has also been observed *in vivo*. For example, partial inhibition of LTB_4-elicited chemotaxis by a PAF receptor antagonist (BN-52021) has been observed in a rat model of inflammation.[56] Conversely, LTB_4 was shown to mediate PAF-induced PMN degranulation and chemotaxis *in vitro*.[15] Other studies suggested a role for LTB_4 in mediating the actions of PAF in an immune-complex hypersensitivity model[57] and in an Arthus reaction[58] in the rat, and 5-LO inhibition was reported to inhibit PAF-induced hyperalgesia[59] and to partially inhibit rat PMN aggregation induced by PAF.[60] The present study shows cooperative behaviour between LTB_4 and PAF in promoting either LTB_4- or PAF-induced PMN recruitment *in vivo* (Fig. 2c,d), inasmuch as the partial (40–60%) inhibition observed with either antagonist alone was enhanced by the concomitant administration of both BLT1 and PAF receptor antagonists. These data clearly demonstrate that cross-talk between the two classes of lipid mediators exists and is physiologically significant.

The present results also show that PMN migration towards other soluble agonists chemically unrelated to LTB_4 and PAF, such as ZAP ($C5a_{desarg}$) and TNF-α, is more efficiently inhibited by the concomitant administration of PAF and LTB_4 receptor antagonists (Fig. 3), suggesting again that both PAF and LTB_4 mediate the stimulation of PMN extravasation by ZAP and TNF-α. This is in agreement with previous observations that C5a and TNF-α can promote LTB_4 biosynthesis in PMN[61,62] and that the administration of a 5-LO-activating protein (FLAP) inhibitor attenuated PMN recruitment to a variety of mediators including C5a and TNF-α.[13] In the present study, the putative effects of the PAF and LTB_4 antagonists used on MPO activity or expression have not been assessed. However, the inhibitory effects of the antagonists have been observed in similar studies (data not shown) where PMN migration was assessed by direct counting or by using a radiolabelled PMN methodology, as opposed to MPO assay. It is therefore unlikely that the effects of the antagonists on PMN migration reported herein, reflect unspecific effects on MPO activity.

The present studies did not specifically address the issue of a possible hierarchy in the formation and action of PAF and LTB_4 in response to soluble agonists. In fact, experimental evidence suggests that the biosynthesis of both lipid mediators is closely coupled, their precursors (arachidonic acid and lyso-PAF) being concomitantly generated by the action of the cytosolic $PLA_2\alpha$ on its phosphatidylcholine substrate.[63] Thus, in addition to the fact that there are redundancies in the biological properties of LTB_4 and PAF, their biosynthesis at inflammatory sites probably occurs simultaneously. It may be particularly important to recognize these features of the two lipid mediators inasmuch as failure to significantly modulate the pathological course of a disease by targeting a single lipid mediator might possibly be circumvented by dual blockade of PAF and LTB_4 actions. In this regard, it is interesting that attempts to treat patients suffering from rheumatoid arthritis or ulcerative colitis by targeting either LTB_4 or PAF have been unsuccessful.[64,65]

Our studies on the pulmonary inflammation model further support the concept that targeting both LTB_4 and PAF provides a much stronger anti-inflammatory effect. Indeed, lung inflammation elicited by intravenous Sephadex bead injection is characterized by a time-dependent accumulation in the lung tissues of a mixed population of neutrophil and eosinophil granulocytes.[66] In this model, granulocyte infiltration is accompanied by the local generation of inflammatory mediators, including PAF, LTB_4, bradykinin and C5a, which were shown to modulate granulocyte influx into lungs and bronchoalveolar lavage fluid.[67] Our results clearly show that only the dual blockade of both PAF and LTB_4 receptors elicited a significant reduction of lung PMN recruitment, in contrast to either drug alone (Fig. 4). These results support a cooperative role for LTB_4 and PAF in regulating PMN trafficking to inflammatory sites. Increased biosynthesis of the lipid mediators by inflammatory stimuli at the blood–endothelial interface involving autocrine/paracrine cross-regulation and transcellular biosynthesis of LT, may facilitate PMN transendothelial migration and their extravascular accumulation at inflammatory sites.

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