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Cytokines and neutrophils as important mediators of platelet-activating factor-induced kinin B_1 receptor expression

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1 PAF injection into the rat paw is accompanied by the concomitant activation of NF-κB and neutrophil influx, which appears to be relevant to the up-regulation of kinin B_1 receptors. Herein, we analyse the role of TNF-α and IL-1β production for PAF-induced B_1 receptor upregulation in the rat paw. Additionally, we evaluate how cytokine production and neutrophil migration fit into the temporal sequence of events leading to PAF-induced B_1 receptor upregulation.

2 In our experiments, treatment with PAF resulted in a marked increase of B_1 receptor-mediated paw oedema and *in situ* production of TNF-α at 1 h and IL-1β at 3 and 6 h later. B_1 receptor-mediated paw oedema was significantly inhibited by anti-TNF-α antibody and by interleukin-1 receptor antagonist (IRA).

3 TNF-α was necessary for the local PAF-induced IL-1β production. NF-κB blocker PDTC prevented the production of both TNF-α and IL-1β, indicating that cytokine production is NF-κB dependent.

4 Depletion of neutrophils with an anti-PMN antibody prevented IL-1β, but not TNF-α, production. Although both TNF-α and IL-1β are relevant to functional B_1 receptor upregulation, PAF-induced increase in B_1 receptor mRNA was markedly suppressed by anti-TNF-α and, to a lesser extent, by IRA. B_1 receptor mRNA expression was also prevented by the anti-PMN antibody.

5 In conclusion, the activation of the TNF-α/neutrophil axis by PAF seems to be sufficient for B_1 receptor mRNA production. However, the TNF-α/neutrophil axis is also necessary for IL-1β production. These two processes might lead to the appearance of functional kinin B_1 upregulation receptors *in vivo* after PAF treatment.
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Abbreviations: DABK, des-Arg^9-bradykinin; HTAB, hexadecyltrimethyl ammonium bromide; i.d., intradermal; IRA, interleukin-1 receptor antagonist; PAF, platelet-activating factor; PDTC, pyrrolidinedithiocarbamate; PMN, polymorphonuclear cells; MPO, myeloperoxidase; nm, nanometers

Introduction

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent phospholipid messenger with several physiological activities (Hanahan, 1986; Ishii & Shimizu, 2000; Ishii *et al*., 2002). PAF is produced by various tissues and cell types, including platelets, neutrophils, macrophages, leucocytes, eosinophils and epithelial and endothelial cells (Chao & Olson, 1993; Korth *et al*., 1995; Ishii & Shimizu, 2000). The actions of PAF are mediated by the interaction with a specific seven-transmembrane G-protein-coupled receptor (Ishii *et al*., 1998; Ishii & Shimizu, 2000). Among its several effects, PAF induces activation and chemoattraction of neutrophils (Zhou *et al*., 1994; Franciose *et al*., 1996). Stimulation of PAF receptors leads to an increase in diacylglycerol, inositoltriphosphate (IP_3) and calcium (Ca^{2+}) with the activation of many types of kinases (MAPK, PKC and tyrosine kinase) and phospholipases (PLA_2, PLCβ) (Shimizu *et al*., 1992; Richardson *et al*., 1996; Ishii & Shimizu, 2000). Moreover, PAF is capable of activating NF-κB, which is a key regulator of many molecules involved in the inflammatory process such as receptors, enzymes and cytokines (De Plaen *et al*., 1998; 2000; Fernandes *et al*., 2003). Recently, the presence of functional PAF receptors at the nuclear membrane of endothelial cells and neurons has been reported (Marrache *et al*., 2002). In this context, PAF can be generated locally and may also act on nuclear PAF receptors (Marrache *et al*., 2002).

Kinins are another group of important mediators involved in several biological and pathophysiological conditions (Bhoola *et al*., 1992; Gabra *et al*., 2003; Ueno & Oh-ishi, 2003). Their effects can be amplified by release of, and/or interaction with, other inflammatory mediators (Sharma & Buchanan, 1994; Campos & Calixto, 1995; Böckmann & Paegelow, 2000). The

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actions of kinins are mediated by the activation of two distinct receptors, B_1 and B_2 (Marceau & Bachvarov, 1998; Calixto *et al*., 2000; Couture *et al*., 2001). B_2 receptors are generally constitutive and are responsible for most of the physiological actions of kinins (Calixto *et al*., 2000; 2001; Newton *et al*., 2002), whereas B_1 receptors are usually upregulated during inflammatory processes, after tissue injury or by way of some inflammatory cytokines and bacterial products (Marceau *et al*., 1998; Calixto *et al*., 2000; 2001; 2004).

It has been demonstrated that functional upregulation of kinin B_1 receptors induced by LPS and IL-1β in the rat paw involves the production of PAF and the activation of its receptors (Campos *et al*., 2002; Passos *et al*., 2004). In addition, we have shown that PAF may induce *in vivo* the expression of kinin B_1 receptors in the rat paw. The effects of PAF are dependent on PAF receptor activation resulting in neutrophil migration, NF-κB activation and *de novo* synthesis of B_1 receptors (Fernandes *et al*., 2003). In the sequence of events described previously by Fernandes *et al*. in 2003, it was not determined whether proinflammatory cytokine production occurred after PAF administration or whether it was necessary for NF-κB activation, neutrophil influx and B_1 receptor upregulation. In the present study, we sought to investigate the possible involvement of the secondary synthesis of TNF-α and IL-1β in the sequence of events leading to the upregulation of B_1 receptors after PAF treatment.

Methods

Measurement of rat paw oedema

Nonfasted male Wistar rats (140–180 g) kept in a controlled room temperature ($22 \pm 2^{\circ}$C) and humidity of around 60%, under a 12 : 12 h light-dark cycle (lights on 06 : 00 h), were used. All experiments were conducted according to the procedures described previously (Campos & Calixto, 1995). The animals received a 0.1 ml intradermal (i.d.) injection of phosphate-buffered saline (PBS, composition mmol/l^{-1}: NaCl 137, KCl 2.7, phosphate buffer 10) containing des-Arg^9-bradykinin (DABK, 100 nmol paw^{-1}) in one hind paw (right paw). The contralateral paw (the left) received 0.1 ml of PBS and was used as the control. Oedema was measured by the use of a plethysmometer (Ugo Basile) at several time points (10, 20, 30, 60 and 120 min) after injection of DABK. Oedema is expressed in ml as the difference between the right and left paws. Most animals were treated with PAF 6 h before the i.d. injection of DABK (Fernandes *et al*., 2003). All experimental procedures were performed under light anaesthesia with 2,2,2 tribromo-ethanol (0.125 g kg^{-1}, intraperitoneally (i.p.)). The reported experiments were carried out in accordance with current Guidelines For the Care of Laboratory Animals and Ethical Guidelines for Investigations of Experiments in Conscious Animals (Zimmermann, 1983).

Analysis of the involvement of secondary cytokine synthesis in B_1-receptor-mediated paw oedema in rats pretreated with PAF

To assess the contribution of secondary cytokine synthesis in the upregulation of B_1 receptors by PAF treatment, the animals were locally treated with the anti-TNF-α antibody (50 ng paw^{-1}). To evaluate the participation of the IL-1β receptor activation, the animals received a coinjection of the interleukin-1 receptor antagonist (IRA) (100 μg paw^{-1}) or anti-IL-1β antibody (50 ng paw^{-1}) with PAF. DABK-caused oedema was evaluated 6 h after PAF treatment as described before.

Measurement of the IL-1β and TNF-α levels in the rat paw

Tissue cytokine levels were measured according to the protocol described by Passos *et al*. (2004). The animals were treated with PAF (10 nmol paw^{-1}) and the subcutaneous tissue of the right hind paw was removed at different intervals of time (1–12 h). Tissues were placed on PBS containing Tween-20 0.05%, phenylmethylsulphonyl fluoride 0.1 mM, benzethonium chloride 0.1 mM, EDTA 10 mM and aprotinin A 20 KIU, homogenized, centrifuged at $3000 \times g$ for 10 min and stored at -70°C until further analysis. In another group of experiments, to assess the participation of NF-κB activation in cytokine production, the animals received pyrrolidinedithiocarbamate (PDTC) (100 mg kg^{-1}, i.p.) 30 min prior to PAF injection. The role of neutrophil migration in IL-1β production was evaluated in the animals pretreated with anti-PMN (34 μg kg^{-1}, i.p.) administered 30 min before PAF treatment. To analyse whether the IL-1β production was dependent on TNF-α synthesis, another group of animals received anti-TNF-α (50 ng paw^{-1}) together with the PAF injection. PBS-injected paws were used as the control. Cytokine levels were evaluated using an ELISA kit according to the manufacturer's recommendations (R&D Systems).

The role of cytokine synthesis in the increase of myeloperoxidase (MPO) activity after PAF treatment

Neutrophil recruitment to the rat paw was measured by means of tissue MPO according to the method described previously (Souza *et al*., 2000; Fernandes *et al*., 2003). The animals received an i.d. injection of PAF (10 nmol paw^{-1}) alone or coinjected with the anti-TNF-α antibody (50 ng paw^{-1}). In another group, the animals received PAF in association with IRA (100 μg paw^{-1}). Paws treated with PBS were used as the control. After 6 h, the subcutaneous tissue of the paws was removed, homogenized at 5% (w v^{-1}) in EDTA/NaCl buffer (pH 4.7) and centrifuged at 10,000 r.p.m. 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 r.p.m., 15 min, 4°C) and 25 μl of the supernatant was used for the MPO assay. The enzymatic reaction was assessed with 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 per milligram of tissue.

Assessing the participation of cytokine synthesis and neutrophil migration in expression of B_1 receptor mRNA in the rat paw

Functional evidence was extended by assessing whether the increase of B_1 receptor mRNA expression after PAF treatment was dependent on secondary cytokine synthesis and neutrophil

migration. For this purpose, the animals received PAF (10 nmol paw^{-1}) alone or coinjected with anti-TNF-α (50 ng paw^{-1}) or IRA (100 μg paw^{-1}). In another set of experiments, the animals received anti-PMN (34 μg kg^{-1}, i.p., 30 min) prior to PAF administration. PBS-treated paws were used as the control. Experiments were conducted as described previously (Fernandes *et al*., 2003). Briefly, 3 h after PAF treatment, the animals were killed and the subcutaneous tissue of the right paws was removed and immediately frozen in liquid nitrogen. The samples were homogenized and the total RNA was extracted using TRIzol Reagent (Gibco BRL® Rockville, MD, U.S.A.). Of the total RNA, 1 μg was reverse transcribed (RT) using oligo-dT as the primer (25 μg ml^{-1}) and 200 U of reverse transcriptase (Gibco BRL®) in 20 μl of PCR buffer containing (mM): dNTP 0.5, DTT 10, $MgCl_2$ 2.5, KCl 50 and Tris-HCl 20 (pH 8.4). The samples were incubated for 50 min at 42°C, heated for 15 min at 70°C and cooled in ice. After treatment with 2 U of RNAse H (20 min, 37°C), cDNA amplification of a specific sequence of rat B_1 receptor and β-actin was performed by PCR using the following primers: sense TGAAGCTGTGAGCTCTTTG and antisense GCCAG TTGAAACGGTTCCC for B_1 receptor, and sense GTTCC GATGCCCCGAGGATCT and antisense GCATTTGC GGTGCACGATGGA for rat β-actin. β-actin cDNA was used for standardization of the amount of RNA. An aliquot of 5 μl of RT samples was mixed in a 20 mM Tris-HCl buffer (pH 8.4) containing dNTP 300 μM, $MgCl_2$ 1.5 mM, 25 μg ml^{-1} of each primer, and 50 U ml^{-1} of *Taq* polymerase (Gibco BRL®), in a final volume of 100 μl. The cycling protocol used was the following: 4 min at 94°C, 36 cycles of 35 s at 94°C/45 s at 60°C/45 s at 72°C, and finally 7 min at 72°C. Aliquots of 25 μl were analysed on a 20% Tris/borate/EDTA polyacrylamide gel and were stained by ethidium bromide. The size of the products was 450 bp for B_1 receptor and 600 bp for β-actin.

Drugs and reagents

The following drugs and reagents were used: aprotinin A, benzethonium chloride, BSA, EDTA, HTAB, hydrogen peroxide, PDTC, PMSF, tetramethylbenzidine, 2,2,2, tribromoethanol and Tween-20 (all from Sigma Chemical Company, St Louis, MO, U.S.A.); DABK and PAF (from Bachem Bioscience Inc., King of Prussia, PA, U.S.A.); NaCl, $NaPO_4$ and KCl (from Merck, Germany). Anti-murine neutralizing antibody anti-TNF-α (lot CT101) and human recombinant glycoprotein IRA were obtained from R&D Systems (Minneapolis, MN, U.S.A.). Anti-rat neutrophil antiserum was obtained from Accurate Chemicals (San Diego, CA, U.S.A.).

Stock solutions of DABK were prepared in PBS, and PAF was prepared in a BSA 0.1% solution. All were stocked in siliconized plastic tubes and maintained at −18°C. They were then diluted to the desired concentration just before use. The other drugs were prepared daily in 0.9% (w v^{-1}) NaCl solution.

Data analysis

The results are presented as the mean ± s.e.m. of four to six animals. The percentages of inhibition are reported as mean ± s.e.m. of inhibitions obtained in each individual experiment at the peak of the oedema (20 min after injection of DABK) or 1–12 h after PAF injection depending on the experimental protocol. Statistical comparison of the data was performed by analysis of variance (ANOVA) followed by Dunnett's test or Student's unpaired *t*-test. P values <0.05 were considered significant.

Results

Cytokine participation in PAF-induced functional B_1 receptor upregulation

We have reported previously that PAF treatment was able to increase the functional response induced by the selective kinin B_1 receptor agonist DABK in the rat paw (Fernandes *et al*., 2003). Hence, the i.d. injection of DABK (100 nmol paw^{-1}) in naïve paws induced a very slight increase in rat paw volume (0.13 ± 0.007 ml), whereas in animals treated with PAF (10 nmol paw^{-1}, 6 h prior), i.d. injection of DABK produced a marked increase in oedema formation (0.33 ± 0.015 ml). To assess the contribution of the secondary TNF-α synthesis in the functional upregulation of B_1 receptors induced by PAF, animals were treated with the anti-murine antibody anti-TNF-α (50 ng paw^{-1}). The role of IL-1β production was also evaluated by the coinjection of the recombinant interleukin receptor antagonist IRA (100 μg paw^{-1}) or anti-IL-1β antibody (50 ng paw^{-1}). The paw oedema induced by DABK in rats pretreated with PAF was significantly reduced by the coinjection of anti-murine antibody anti-TNF-α (45 ± 2%) (Figure 1a). Similar inhibition was obtained as a result of treatment with IRA (59 ± 9%) or the anti-IL-1β antibody (56 ± 2%) (Figure 1b and c).

Importance of cytokine synthesis and neutrophil migration for B_1 receptor mRNA expression in the rat paw

We have also reported previously that functional upregulation of B_1 receptors following PAF treatment (10 nmol paw^{-1}) was accompanied by a marked increase in kinin B_1 receptor mRNA expression in the rat paw, which peaked at 3 h (Fernandes *et al*., 2003). B_1 receptor mRNA expression after PAF treatment was totally prevented by treatment with the anti-murine antibody anti-TNF-α (50 ng paw^{-1}) (Figure 2). Otherwise, IRA treatment caused a smaller, but significant, reduction of B_1 receptor mRNA expression after PAF injection (29 ± 11%). Interestingly, anti-PMN treatment abolished the B_1 receptor mRNA expression completely (Figure 2).

Cytokine levels in the rat paw

TNF-α and IL-1β production was assessed in the subcutaneous tissue of the rat paw after PAF treatment (10 nmol paw^{-1}, 1–12 h). The results demonstrated that PAF treatment induced a marked increase in both TNF-α and IL-1β levels (6 to 11 and 3 to 18-fold increase, respectively) in the rat paw (Figures 3a and 4a). TNF-α release peaked at 1 h after PAF treatment, diminished after 3 h, and was undetectable 6 h later (Figure 3a and results not shown). Maximal IL-1β formation was observed 3 h after PAF injection and was maintained up to 6 h, returning to basal levels at 12 h (Figure 4a).

To investigate whether the activation of the transcription factor NF-κB was required for TNF-α and IL-1β production following PAF treatment, the animals received the NF-κB blocker PDTC (100 mg kg^{-1}, i.p., 30 min). The results indicate

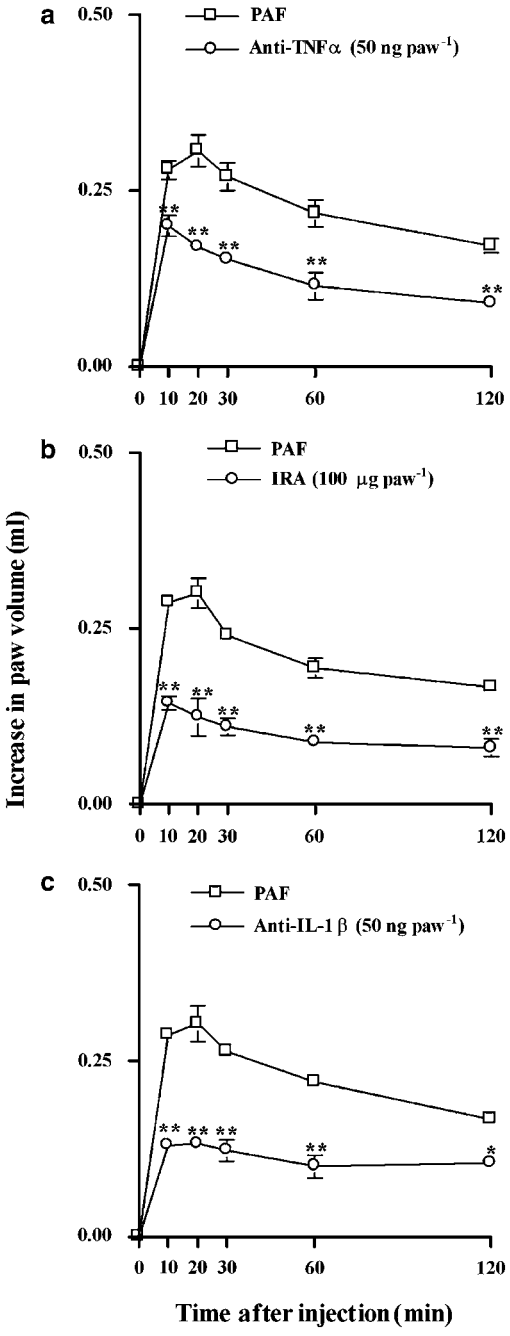

Figure 1 Effect of Abs anti-TNF-α or IRA on DABK-induced paw oedema. The anti-murine Abs anti-TNF-α (50 ng paw^{-1}; a), recombinant human IRA (100 μg paw^{-1}; b) or anti-murine Abs anti-IL-1β (50 ng paw^{-1}; c) were coadministered with PAF (10 nmol paw^{-1}) 6 h before the injection of DABK. Values represent the differences between volume (in ml) of vehicle-injected (0.1 ml of PBS solution) and drug-injected paws. Each point represents the mean ± s.e.m. of four to six animals. In some cases, error bars are hidden within the symbols. Significantly different from control values. $^{**}P<0.01$.

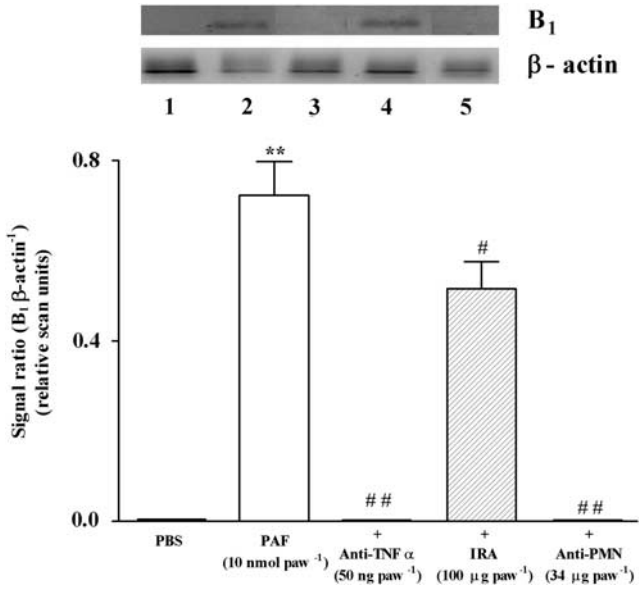

Figure 2 Effect of Abs anti-TNF-α, IRA or Abs anti-PMN on kinin B_1 receptor mRNA expression induced by PAF. The anti-murine anti-TNF-α (50 ng paw^{-1}) or recombinant human IRA (100 μg paw^{-1}) was coadministered with PAF injection (10 nmol paw^{-1}). The anti-rat anti-PMN (34 μg kg^{-1}, i.p.) was administered systemically 30 min before PAF injection (10 nmol paw^{-1}). Bottom, Graphic representation of B_1/β-actin signals ratio. Lane 1, PBS; lane 2, PAF 3h; lane 3, anti-TNF-α; lane 4, IRA; lane 5, anti-PMN. Number of replicates = 3. Significantly different from PBS $^{**}P<0.01$ or PAF-injected paws $^{\#}P<0.05$, $^{\#\#}P<0.01$ values.

that both TNF-α and IL-1β levels were highly reduced (86 ± 5 and 64 ± 12%, respectively) by the PDTC treatment (Figures 3b and 4b). We also investigated to what extent the IL-1β release induced by PAF was dependent on TNF-α production. Interestingly, IL-1β levels were greatly reduced (89 ± 2%) by the treatment with anti-TNF-α antibody (50 ng paw^{-1}) (Figure 4b). To evaluate the role of neutrophil migration in the IL-1β production, animals were treated with anti-rat neutrophil antiserum (anti-PMN, 34 μg kg^{-1}, i.p., 30 min). Anti-PMN treatment displayed a significant reduction of the IL-1β levels in the rat paw (55 ± 13%) (Figure 4b).

Role of cytokine synthesis in the increase of MPO activity

It was demonstrated that the i.d. injection of PAF (10 nmol paw^{-1}) induced a sustained and time-related neutrophil accumulation in the rat paw (3–12 h), as assessed by MPO activity measurement (Fernandes *et al*., 2003). Accordingly, local treatment with PAF induced a marked increase of MPO activity in the rat paw (about 22-fold increase) 6 h after treatment, in comparison to PBS-treated paws. The role of TNF-α release for neutrophil migration induced by PAF injection was assessed by treatment of animals with anti-TNF-α (50 ng paw^{-1}), while the requirement of IL-1β production was assessed by administration of IRA (100 μg paw^{-1}). Treatment with anti-TNF-α or IRA did not significantly alter the neutrophil influx induced by PAF (Figure 5a and b).

Discussion

The identification of molecules and cells involved in the inflammatory process is a very attractive field of investigation. In recent years, many pieces of evidence have indicated that the inflammatory process is orchestrated by the synthesis/upregulation of several mediators and/or receptors. Kinin B_1

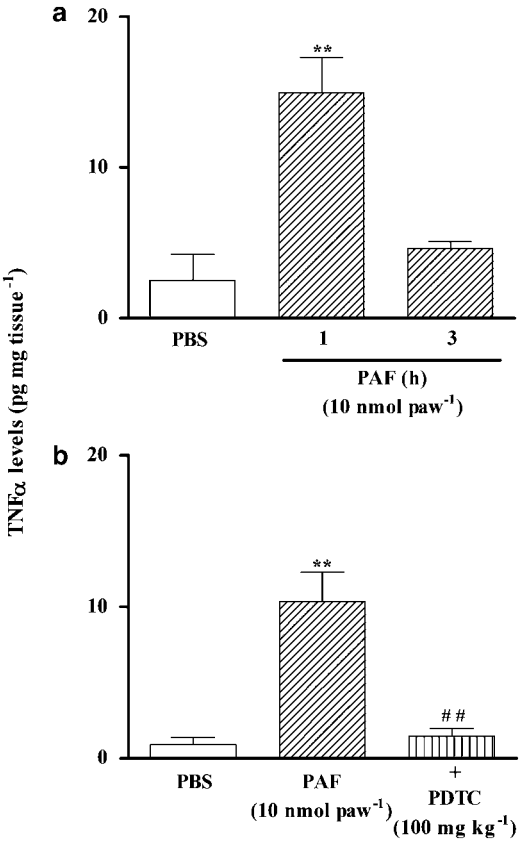

Figure 3 Time-dependent effect of PAF treatment on TNF-α levels and effect of PDTC on TNF-α production. (a) Effect of PAF injection (10 nmol paw^{-1}, 1–3 h) on TNF-α levels in the rat paw. (b) Effect of PDTC (100 mg kg^{-1}, i.p.) administered 30 min before PAF treatment on TNF-α production. Each column represents the mean$\pm$s.e.m. of four to six animals. Significantly different from PBS $^{**}P<0.01$ or PAF-injected paws ($^{\#\#}$) values.

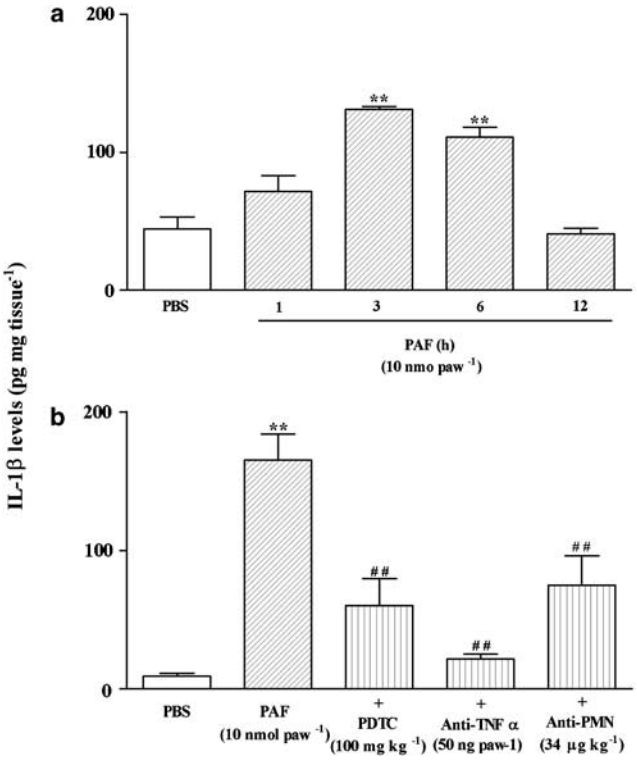

Figure 4 Time-dependent effect of PAF treatment on IL-1β levels and effect of PDTC, Abs anti-TNF-α or anti-PMN on IL-1β production. (a) Effect of PAF injection (10 nmol paw^{-1}, 1–12 h) on IL-1β levels in the rat paw. (b) Effect of PDTC (100 mg kg^{-1}, i.p.), anti-PMN (34 μg kg^{-1}, i.p.; administered 30 min before PAF) or anti-murine anti-TNF-α (50 ng paw^{-1}; coadministered with PAF) on IL-1β production (3 h after PAF injection). Each column represents the mean$\pm$s.e.m. of four to six animals. Significantly different from PBS $^{**}P<0.01$ or PAF-injected paws ($^{\#\#}$) values.

receptors represent an atypical class of G-protein-coupled receptors, which are not commonly expressed constitutively under normal conditions, but may be greatly upregulated under certain inflammatory stimuli or following tissue trauma. The participation of proinflammatory cytokines and inflammatory cells for the modulation of responses mediated by kinin B_1 receptors has been widely demonstrated (Larrivé *et al*., 1998; Campos *et al*., 1999; 2002; Phagoo *et al*., 2000; Fernandes *et al*., 2003; Gama Landgraf *et al*., 2004; Passos *et al*., 2004). Nevertheless, the precise mechanisms involved in the process of kinin B_1 receptor functional upregulation still remain to be better clarified. Here, we sought to investigate the role of local TNF-α and IL-1β production in the process of PAF-induced upregulation of kinin B_1 receptors in the rat paw. Additionally, we evaluated how the production of these proinflammatory cytokines fitted into the temporal sequence of events leading to B_1 receptor upregulation after PAF.

We have previously demonstrated that PAF was able to directly regulate the expression of B_1 receptors in the rat paw (Fernandes *et al*., 2003). More recently, we have also shown that upregulation of kinin B_1 receptors by LPS involves the participation of PAF receptors (Passos *et al*., 2004). The results of the present study demonstrate that B_1 receptor-mediated oedema formation induced by DABK after injection of PAF involves the secondary synthesis of TNF-α and IL-1β. Indeed, there was a marked production of TNF-α and IL-1β after injection of PAF in the rat paw. These results are consistent with others showing that cytokine synthesis induced in models of pulmonary infection with Gram-negative bacteria and colitis is broadly dependent on PAF (Soares *et al*., 2002; Galvez *et al*., 2003; Hirayama *et al*., 2003; Souza *et al*., 2003; Ferreira *et al*., 2004). Whereas TNF-α peaked at 1 h after PAF injection, levels of IL-1β were elevated at 3 and 6 h after PAF. Interestingly, the anti-TNF-α antibody significantly prevented the PAF-induced increase of IL-1β in the rat paw. Thus, IL-1β synthesis in response to PAF injection is TNF-α dependent. The relevance of IL-1β for the functional upregulation of B_1 receptors is provided by the ability of IRA to consistently prevent the oedema induced by DABK in animals previously treated with PAF. A similar inhibition was observed when rats received topical injection of the antibody anti-TNF-α. Hence, the functional response mediated by kinin B_1 receptors after PAF treatment seems to be dependent on the sequential production of the proinflammatory cytokines TNF-α and IL-1β. It is interesting to observe that upregulation of kinin B_1 receptors by IL-1β involves the participation of PAF receptors (Campos *et al*., 2002). Considering that kinin B_1 receptor

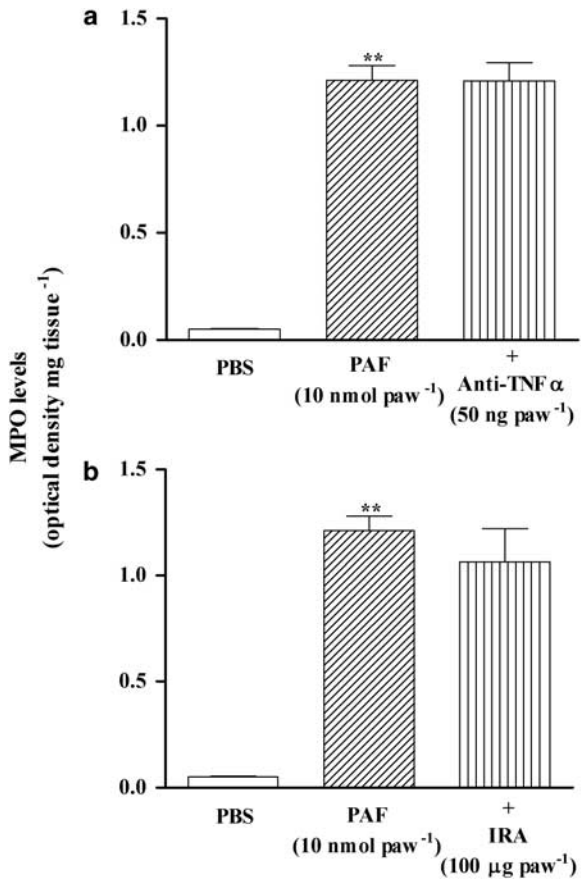

Figure 5 Effect of Abs anti-TNF-α or IRA on increase of MPO levels induced by PAF. (a) Effect of anti-murine anti-TNF-α (50 ng paw^{-1}; a) or recombinant human IRA (100 µg paw^{-1}; b) both co-administered with PAF (10 nmol paw^{-1}, 6 h prior) on MPO levels in the rat paw. Each column represents the mean ± s.e.m. of four to six animals. Significantly different from PBS values $^{**}P<0.01$.

upregulation after IL-1β treatment involves PAF receptor activation, and since PAF is capable of inducing IL-1β release, we might speculate that both mediators are able to influence each other. This hypothesis is supported by our data and by data reported in the literature that PAF production after treatment with LPS is dependent on IL-1β synthesis (Han *et al*., 2002). Besides, IL-1β production in the rat paw after IL-1β injection seems to be dependent on PAF receptor activation, as demonstrated by reduction in the release of this cytokine by the PAF receptor antagonist WEB2086 (results not shown). Therefore, it is certainly possible that the activated loop PAF-IL-1β-PAF is responsible for maintaining the long-lasting upregulation (up to 24 h) of B_1 receptor inflammatory responses observed after local treatment with PAF.

Another participant involved in the mechanisms whereby PAF produces upregulation of B_1 receptors is NF-κB, a pivotal transcriptional factor responsible for controlling the expression of more than 200 genes, including those involved in the pro-inflammatory cytokines and many other inflammatory molecules (Barnes & Karin, 1997; Ghosh *et al*., 1998; Liu *et al*., 1999; Li & Verma, 2002; Chen *et al*., 2003). It has been demonstrated that PAF is able to induce the activation of NF-κB in several models of inflammation (De Plaen *et al*., 1998; 2000; Choi *et al*., 2000; 2003; Fernandes *et al*., 2003; Passos *et al*., 2004). In addition, NF-κB activation has been well correlated with the upregulation of kinin B_1 receptors by several mediators, including that induced by PAF and by LPS (Campos *et al*., 1999; Fernandes *et al*., 2003; Passos *et al*., 2004). Indeed, we have recently shown that local treatment with PAF results in a marked increase in the NF-κB activation as early as 1 h after administration and that it persists for up to 12 h in the rat paw (Fernandes *et al*., 2003). In the present study, we investigated to what extent the synthesis of pro-inflammatory cytokines TNF-α and IL-1β induced by PAF was dependent on the NF-κB activation. Our data strongly suggest that both TNF-α and IL-1β production are resultant from NF-κB activation, since the levels of both cytokines were greatly reduced by the NF-κB blocker PDTC. These results confirm and also extend the notion that NF-κB activation is extremely important in the upregulation of kinin B_1 receptors and demonstrate that the production of the proinflammatory cytokines TNF-α and IL-1β is secondary to NF-κB translocation. These results are also in good agreement with literature data indicating that proinflammatory actions of the chemotatic factor PAF may be mediated by direct activation of NF-κB (De Plaen *et al*., 1998; 2000; Choi *et al*., 2000; 2003).

In our previous study (Fernandes *et al*., 2003), we found that neutrophil influx was necessary for full translocation of NF-κB. Here, we have shown that depletion of neutrophils by anti-PMN also fully inhibits the increased B_1 receptor expression induced by PAF. As the production of proinflammatory cytokines induced by PAF was dependent on NF-κB translocation, we further evaluated whether or not the neutrophil influx was also a necessary event for proinflammatory cytokine production. Our results clearly show that neither the blockade by IL-1β nor TNF-α antibodies significantly altered the increase of MPO activity observed in PAF-treated animals. This is consistent with the ability of PAF to cause profound effects on neutrophils, including $[Ca^{+2}]_i$ increase, respiratory burst, degranulation, adhesion to the endothelium and chemotaxis (Evangelou, 1994; Zhou *et al*., 1994; Franciose *et al*., 1996; Ishii *et al*., 1998; Ishii & Shimizu, 2000). However, these results disagree somewhat with the previous idea postulated by Campos *et al*. (2002) and Passos *et al*. (2004) who have suggested that TNF-α and/or IL-1β release could play a pivotal role in the neutrophil migration induced by IL-1β or LPS to the rat paw. Thus, it is suggested that activation of PAF receptors may induce neutrophil migration directly by the activation/induction of adhesion molecules, or *via* the production of other inflammatory mediators such as chemokines, possibly from resident cells such as endothelial cells, mast cells, fibroblasts, keratinocytes and epithelial cells, among others. Once migrated, neutrophils appear to have two distinct effects: enhancement of the translocation of NF-κB and induction of the production of IL-1β (see Figure 6). In contrast, TNF-α production does not appear to be dependent on neutrophil migration, as the maximal TNF-α production occurs as early as 1 h after PAF treatment, whereas increase in MPO levels begins only after 3 h. Despite the role of neutrophils in IL-1β production, we cannot fully discard the involvement of other cells, even resident or other migrated cells, in TNF-α and IL-1β synthesis.

As mentioned above, both neutrophil influx and TNF-α production were essential for the production of IL-1β, as the

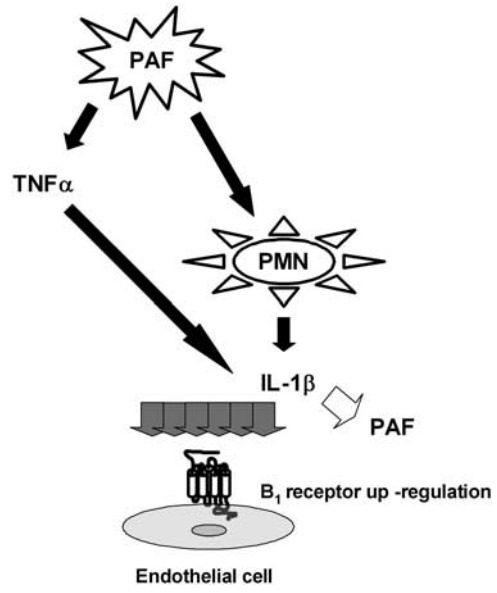

Figure 6 Possible sequence of events leading to B_1 receptor expression after local PAF administration *in vivo*.

blockade of either of these two events prevented IL-1β production. In addition, IL-1β appears to be relevant to the functional upregulation of B_1 receptors, as demonstrated by the ability of IRA to significantly prevent the oedema induced by DABK. Nevertheless, blockade of IL-1β was accompanied by a minor, albeit significant, inhibition of the expression of B_1 receptor mRNA. Although the putative mechanisms for these actions of IL-1β still need to be determined, it is tempting to speculate that IL-1β is responsible for the maintenance of kinin B_1 receptor-mediated inflammatory responses. A possible explanation of the latter results is that IL-1β could play a more relevant role in the post-transcriptional processes such as translocation, translation, homo-oligomerization and maturation, than it does in the production of kinin B_1 receptor mRNA. In fact, recent evidence has suggested that all of these processes might be implicated in the mechanisms of B_1 receptor upregulation (Zhou *et al*., 1999; Haddad *et al*., 2000; Marceau *et al*., 2002; Kang *et al*., 2005).

In conclusion, the results of the present study in conjunction with those of our previous study (Fernandes *et al*., 2003) suggest a sequence of events leading to B_1 receptor expression after injection of PAF into the rat paw (Figure 6). There is a rapid increase in TNF-α production and TNF-α-dependent IL-β formation. Concomitantly with the synthesis of cytokines, PAF induces the migration of neutrophils. The migrated neutrophils seem to facilitate the production of (or themselves produce) IL-1β. The activated TNF-α/neutrophil axis is sufficient for B_1 receptor mRNA production. However, the TNF-α/neutrophil axis is also necessary for IL-1β production, and the latter cytokine appears to provide a further signal necessary for B_1 receptor upregulated responses. These events then result in the functional upregulation of kinin B_1 receptors *in vivo* after treatment with PAF. It is possible to suggest that NF-κB activation participates of one or more of these processes. Finally, it is tempting to speculate that resident cells (e.g. endothelials) and/or migrated cells (e.g. neutrophils) could interact for upregulating B_1 receptors on the inflammatory scene, although this matter remains to be further investigated.

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