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Effect of a Paf antagonist, WEB 2086, on airway microvascular leakage in the guinea-pig and platelet aggregation in man

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1 The triazolodiazepine WEB 2086 has been evaluated as an antagonist of platelet-activating factor (Paf) by studying its effects on Paf-induced human platelet aggregation and microvascular leakage in guinea-pigs.

2 WEB 2086 inhibited Paf-induced platelet aggregation in platelet-rich plasma *in vitro* (IC_{50} = 117 ± 35 nM, mean ± s.d.) but had no effect on adenosine 3',5'-diphosphate-induced aggregation.

3 Paf-induced microvascular leakage, measured by the extravasation of intravenously-injected Evans blue dye, was inhibited in a dose-related fashion in the airways and other tissues by WEB 2086, achieving a maximal inhibitory effect at 10 $\mu g\,kg^{-1}$, i.v.

4 However, WEB 2086 (10 $\mu g\,kg^{-1}$, i.v.) did not inhibit a comparable increase in vascular permeability induced by ovalbumin in sensitized guinea-pigs.

5 We conclude that WEB 2086 is a potent antagonist of Paf and that Paf does not appear to be responsible for antigen-induced microvascular leakage.

Introduction

Platelet-activating factor (Paf, 1-0-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) is a potent mediator of inflammation which increases microvascular permeability in systemic vascular beds (Humphrey *et al.*, 1982; Morley *et al.*, 1983; Evans *et al.*, 1987a; O'Donnell & Barnett, 1987) and which causes chemoattraction and activation of inflammatory cells such as eosinophils and neutrophils (Wardlaw *et al.*, 1986; Henocq & Vargaftig, 1986). In addition, Paf causes systemic hypotension (Bessin *et al.*, 1983), bronchoconstriction and bronchial hyperreactivity in several species including man (Mazzoni *et al.*, 1985; Chung *et al.*, 1986; Cuss *et al.*, 1986). In view of these properties, Paf has been implicated in several diseases including asthma and systemic anaphylaxis. The availability of Paf receptor antagonists makes it possible to evaluate its exact role as a mediator in the pathophysiology of these disease processes, since the measurement of Paf in body fluids remains technically difficult. We have studied the triazolodiazepine WEB 2086 (3-(4-(2-chlorophenyl)- 9-methyl-6H-thieno(3,2-f)(1,2,4) triazolo-(4,3-a)(1,4)diazepin- 2-yl)-1-(4-morpholinyl)-1-propanone) which has recently been shown to inhibit platelet aggregation induced by Paf *in vitro* (Casals-Stenzel *et al.*, 1987). We have also characterized its activity against Paf-induced human platelet aggregation *in vitro* and against Paf-induced microvascular leakage in the guinea-pig *in vivo*. Although it has been shown that Paf antagonists may prevent the hypotension and bronchoconstriction seen in antigen-induced anaphylaxis (Sanchez-Crespo *et al.*, 1985; Touvay *et al.*, 1985; Casals-Stenzels, 1987), it is not known whether the accompanying increase in microvascular leakage that occurs in the airways is also mediated by Paf. We therefore tested the effect of WEB 2086 on antigen-induced microvascular leakage in sensitized guinea-pigs.

Methods

Platelet aggregation

Venous blood from healthy volunteers was added to 3.28% trisodium citrate buffer solution (9 : 1 by

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volume) and platelet-rich plasma (PRP) obtained by centrifugation at 180 *g* for 10 min. The pellet was resuspended in buffer, centrifuged at 1100 *g* for 15 min and platelet-poor plasma (PPP) obtained. Aliquots (400 μl) of PRP were incubated at 37°C with 50 μl of WEB 2086 (10^{-10}–10^{-4} M) or vehicle for 1 min before the addition of Paf 10^{-7} M or adenosine 3',5'diphosphate (ADP) 10^{-5} M. Aggregation was measured in a dual channel aggregometer (Payton, Crawley, U.K.) using PPP as a blank and the inhibitory effect of WEB 2086 expressed as a % of aggregation in the presence of vehicle.

Paf-induced vascular permeability

Male Dunkin-Hartley guinea-pigs (300–450 g) were used throughout. They were premedicated with diazepam (5 mg kg^{-1} i.p.) and anaesthetized with 1 ml Hypnorm (0.31 mg fentanyl citrate and 10 mg fluanisone, i.m.). Microvascular leakage was assessed by extravasation of Evans blue dye (Saria & Lundberg, 1983) which has been shown to correlate well with extravasation of radiolabelled albumin (Udaka *et al.*, 1970). Paf was kept as a stock solution of 1 mg ml^{-1} in ethanol at −80°C and solutions of 10 and 100 ng ml^{-1} in 0.25% bovine serum albumin (BSA) freshly prepared on each experimental day. WEB 2086 in 1 ml distilled water at doses of 1 μg kg^{-1} ($n = 6$) or 10 μg kg^{-1} ($n = 6$); or water alone ($n = 5$) was injected into an internal jugular vein 4 min before i.v. Evans blue dye (30 mg kg^{-1}). After one minute, Paf (100 ng kg^{-1}) was injected intravenously. Control animals were injected with the inactive precursor and metabolite of Paf, lyso-Paf (100 ng kg^{-1}, $n = 6$). Five minutes later the thorax was opened and a blunt-ended 13 gauge needle passed through a left ventriculotomy into the aorta. The heart was clamped, the right atrium incised to allow outflow of perfusate, and the animal perfused with 100 ml of 1% paraformaldehyde in phosphate buffered saline (pH 3.5) at 120 mmHg pressure to remove intravascular dye and fix the tissues. The larynx, trachea, lungs, oesophagus, bladder and a portion of nasal mucosa were removed. The main bronchi were separated from the trachea and the intrapulmonary airways stripped of parenchyma and separated into central (first 3 mm) and peripheral components (Evans *et al.*, 1987a). Wet weights of all tissues were taken. Evans blue dye was extracted by incubating tissues in 2 ml of 100% formamide at 60°C for 24 h and its concentration determined by light absorbance at a wavelength of 620 nm (SP 1750 spectrophotometer, Pye Unicam, Cambridge, U.K.) and interpolated on a standard curve at Evans blue concentrations in the range 0.5–100 ng ml^{-1}. The Evans blue content from each sample was expressed as ng dye mg^{-1} wet weight of tissue.

Anaphylaxis

Guinea-pigs were sensitized three weeks prior to study according to the method of Andersson (1980) using a single i.p. injection of 0.5 ml saline containing 100 mg aluminium hydroxide and 20 μg ovalbumin. Control animals ($n = 5$) were injected with aluminium hydroxide alone. After anaesthesia, WEB 2086 (10 μg kg^{-1}; $n = 6$) or water ($n = 5$) was injected intravenously followed 1 min later by ovalbumin (200 μg in 1 ml saline, i.v.) and 3 min later by Evans blue dye. After a further 5 min the animal was perfused as described above.

Drugs and chemicals

Drugs and chemicals were obtained from the following sources: Evans blue, ovalbumin, bovine serum albumin, platelet-activating factor; Sigma Chemicals, St. Louis, MO, U.S.A. WEB 2086 was provided by Boehringer Ingleheim Ltd., West Germany.

Statistical analysis

Comparisons of median values between groups were made by use of the Mann-Whitney U-test. Results are presented as mean ± s.e. mean. P values of less than 0.05 were considered statistically significant.

Results

WEB 2086 significantly inhibited Paf-induced platelet aggregation *in vitro* at a concentration causing 50% inhibition (IC_{50}) of 116.8 ± 34.6 nM ($n = 9$) (Figure 1). ADP-induced aggregation was unaffected by WEB 2086 at concentrations up to 1 mM.

The effects of WEB 2086 on Paf-induced increases in vascular permeability in guinea-pig airways and other tissues are shown in Figure 2. WEB 2086 inhibited microvascular leakage in a dose-related manner, with complete inhibition at a dose of 10 μg kg^{-1}: i.e. the amount of Evans blue dye in tissues was not significantly different from that obtained in animals given the biologically inactive lyso-Paf. In previous studies we have shown that extravasation of Evans blue dye after lyso-Paf 100 ng kg^{-1} was not significantly different from that observed after 0.35% bovine serum albumin used as diluent (Evans *et al.*, 1987a). Sensitized guinea-pigs challenged with ovalbumin showed a significant increase in vascular permeability to Evans blue dye in all tissues studied (Table 1) with effects comparable to that of Paf 50 ng kg^{-1} (Evans *et al.*, 1987a). WEB 2086 (10 μg kg^{-1}) did not significantly inhibit the leakage of Evans blue dye induced by antigen in any tissues (Table 1).

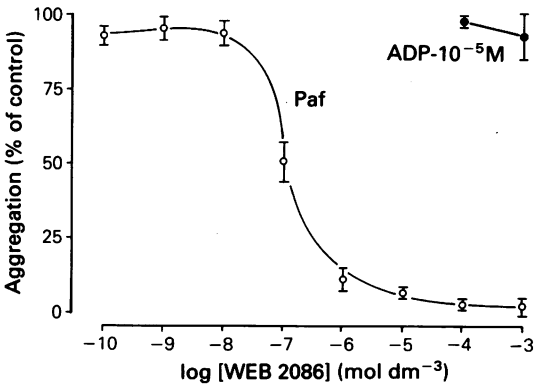

Figure 1 Effect of WEB 2086 on platelet aggregation in platelet-rich plasma induced by platelet activating factor (Paf, 10^{-7} M) (○) or adenosine 3',5'-diphosphate (ADP, 10^{-5} M) (●). Each point represents mean values ($n = 5$) and vertical lines s.e. mean.

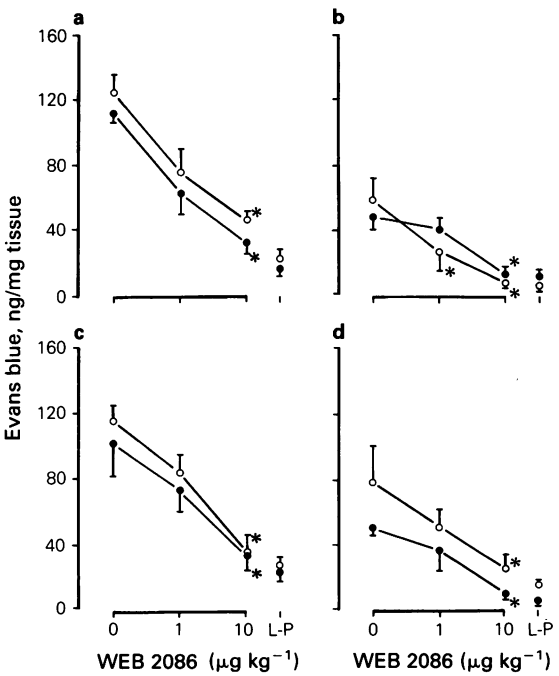

Figure 2 Effect of WEB 2086 or vehicle of extravasation of Evans blue dye induced by Paf 100 ng kg^{-1} in: (a) main bronchi (○) and trachea (●), (b) bladder (○) and nasal mucosa (●), (c) central (○) and peripheral (●) intrapulmonary airways, and (d) larynx (○) and oesphagus (●). * Denotes dose of WEB 2086 causing complete inhibition of effect (i.e. not significantly different from lyso Paf (L-P) 100 ng kg^{-1}. Values represent mean values for 5–6 animals. Vertical lines show s.e. mean.

Discussion

In agreement with the results of Casals-Stenzel *et al.* (1987), we have found WEB 2086 to be a potent inhibitor of Paf-induced platelet aggregation *in vitro* with a similar IC_{50} value. The lack of activity of WEB 2086 against ADP-induced aggregation suggests specificity for the Paf receptor. In a previous study we have shown that another class of Paf-receptor antagonist, the ginkgolide mixture BN 52063, also inhibited Paf-induced aggregation *in vitro* but with a higher IC_{50} of $2.76 \pm 1.34\,\mu$M (Chung *et al.*, 1987). The more than 50 fold difference in potency between these two Paf-antagonists is also reflected in their inhibition of Paf-induced microvascular leakage in guinea-pig airways *in vivo*. Thus, the dose of BN 52063 that completely inhibited the effect was 5 mg kg^{-1} (Evans *et al.*, 1987a), whilst the dose of WEB 2086 required in the present study was only 10 μg kg^{-1} and was, therefore, approximately 500 fold more potent. The greater potency of WEB 2086 compared with BN 52063 against the microvascular effects of Paf, compared with the inhibition of platelet aggregation, may be a reflection of differential receptor binding as demonstrated for other cells (Lambrecht & Parnham, 1986). Previous studies suggest that the microvascular leakage provoked by Paf is due to a direct effect on vascular endothelial cells (Evans *et al.*, 1987a; O'Donnell & Barnett, 1987), indicating that WEB 2086 may be more active against the receptors on endothelial cells compared to those on platelets.

Platelet-activating factor is one of the most potent inducers of microvascular permeability in the guinea-pig respiratory tract (Evans *et al.*, 1987a). Nevertheless, despite using the more potent Paf-antagonist, WEB 2086, there was no inhibition of antigen-induced microvascular leakage in the airways of sensitized guinea-pigs. This suggests that Paf is not the sole mediator of plasma exudation in this model. In a more recent study, we have shown partial inhibition of antigen-induced microvascular leakage using the antihistamines chlorpheniramine and cimetidine and the combined lipoxygenase and cyclo-oxygenase inhibitor, BW 755C (Evans *et al.*, 1987b). The antagonist of slow reacting substance of anaphylaxis, FPL 55712, partly inhibited antigen-induced microvascular leakage in the same model, further supporting a role for the leukotrienes. These results do not, however, deny a role for Paf in other manifestations of IgE-mediated systemic anaphylaxis. Antagonists of Paf such as L-652-731 and BN 52021 inhibit the cardiac impairment (Piper & Stewart, 1986) and bronchoconstriction (Braquet *et al.*, 1985) seen during anaphylaxis. In addition, Darius *et al.* (1986) found that the histamine- and leukotriene-independent contraction of lung parenchymal strips from sensitized animals challenged

Table 1 Effect of WEB 2086 on increase in vascular permeability to Evans blue dye induced by ovalbumin challenge of sensitized and unsensitized guinea-pigs

Treatment	Nasal mucosa	Oesophagus	Bladder	Larynx	Trachea	Main bronchi	Intrapulmonary airways Central	Intrapulmonary airways Peripheral
Unsensitized	7.4*	20.4*	7.2*	20.0*	23.6*	31.3*	34.6*	26.2*
($n = 5$)	± 0.5	± 6.4	± 1.9	± 4.8	± 6.6	± 12.2	± 11.2	± 6.8
Sensitized								
Distilled water	29.1	38.2	16.1	64.0	87.0	155.9	94.1	91.4
($n = 6$)	± 5.4	± 10.3	± 3.3	± 17.6	± 19.5	± 36.0	± 14.5	± 19.8
WEB 2086 ($10\,\mu g\,kg^{-1}$)	22.3	26.3	11.7	58.1	73.0	111.8	95.4	68.7
($n=5$)	± 4.5	± 5.4	± 2.9	± 12.7	± 15.3	± 24.8	± 24.3	± 11.6

Values are ng Evans blue mg^{-1} wet weight tissue, and are expressed as means ± s.e. mean.
* $P < 0.05$ compared with (sensitized) distilled water controls: WEB 2086 had no inhibitory effect.

in vitro was mediated by Paf. Taken together, these observations suggest that Paf may mediate some, but not all, aspects of the anaphylactic response.

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