Br. J. Pharmacol. (1990), 99, 396–400

Prevention of non-specific airway hyperreactivity after allergen challenge in guinea-pigs by the PAF receptor antagonist SDZ 64-412

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1 Allergen challenge by aerosol in sensitized guinea-pigs elicited non-specific airway hyperreactivity assessed by reactivity to i.v. histamine or acetylcholine. Airway hyperreactivity to histamine persisted for at least 48 h and was accompanied by pulmonary eosinophilia as determined by bronchoalveolar lavage cell analysis.

2 Airway hyperreactivity was independent of vagal reflex mechanisms since it was not abrogated by bilateral vagotomy.

3 The novel platelet-activating factor (PAF) receptor antagonist SDZ 64-412 inhibited the development of airway hyperreactivity, as measured 24 h after aerosol allergen challenge, when given as a single treatment orally 2 h before allergen challenge. The PAF receptor antagonist WEB 2086 as well as methylprednisolone and ketotifen also showed efficacy in preventing development of airway hyperreactivity.

4 Neither the two PAF antagonists nor ketotifen had any effect on bronchoalveolar lavage (BAL) eosinophil numbers. Methylprednisolone was the only substance which readily prevented eosinophil recruitment in addition to airway hyperreactivity.

5 We conclude that allergen-induced airway hyperreactivity in guinea-pigs is inhibited by prophylactic anti-asthma drugs and specific PAF receptor antagonists, thus demonstrating a pivotal role of PAF in this response. There was a lack of correlation between airway hyperreactivity and the presence of BAL eosinophils.

Introduction

Among the functional impairments associated with allergic asthma are acute antigen-induced bronchoconstriction and increased non-specific airway hyperreactivity. Although many inflammatory mediators have been implicated in the pathogenesis of this disease by virtue of their bronchoconstrictive properties, platelet-activating factor (PAF) has recently attracted attention due to its ability to induce not only acute bronchoconstriction (Rubin *et al.*, 1987) but also sustained, non-specific airway hyperreactivity (Cuss *et al.*, 1986) in man. Airway hyperreactivity is a hallmark feature of asthmatics, and PAF is the only mediator to date which induces this response in a sustained manner in experimental animals and man.

Pathological features of asthma include pronounced pulmonary eosinophilia associated with epithelial cell damage (Laitinen *et al.*, 1985). Attention has focused on the role of the eosinophil in inducing much of the epithelial damage seen in asthma because it is a major source of PAF (Lee *et al.*, 1984). Furthermore, PAF is the most potent chemoattractant for human eosinophils yet described (Wardlaw *et al.*, 1986).

The purpose of the present studies was to examine the potential therapeutic benefit of a PAF antagonist in an animal model of allergic asthma, as determined by its ability to inhibit the development of non-specific airway hyperreactivity induced by aerosol allergen exposure in sensitized guinea-pigs. Furthermore, we also attempted to correlate the presence or absence of eosinophils in bronchoalveolar lavage fluid with airway hyperreactivity.

Methods

Sensitization procedure

Male Hartley strain guinea-pigs were used throughout the study. Animals were barrier bred and initially weighed 250 to 280 g. Animals were sensitized to ovalbumin (OA) by a series of three i.p. injections of ovalbumin precipitated with aluminum hydroxide ($Al(OH)_3$) in saline. Each animal received 10 μg OA and 10 mg $Al(OH)_3$ in a volume of 0.5 ml. The first injection was done 7 to 14 days after arrival of the animals. The second and third booster injections were performed 14 days apart. Animals were used between 14 and 21 days after the last injection. This procedure has previously been shown to result in the development of IgE-type antibody (Andersson & Bergstrand, 1981) and was verified by its ability to produce passive cutaneous anaphylaxis in naïve guinea-pigs.

Antigen challenge procedure

All animals were pretreated with 10 mg kg^{-1} diphenhydramine (DPH) i.p. 1 h before aerosol antigen exposure. This treatment was necessary to prevent acute, fatal anaphylaxis. Negative control animals (see below) were treated with DPH at a corresponding time. Those animals receiving additional drugs were dosed as described for each group below. Animals were then placed in a clear plastic chamber (approximate volume 40 l) which was connected to the output of a DeVilbiss ultrasonic nebulizer (Model 100 HD). The nebulizer chamber was filled with an OA solution (0.1% in saline). Nebulizer output was approximately 1.5 ml min^{-1}. The duration of the antigen challenge was 60 min.

Treatment groups

A negative control group consisted of animals ($n = 25$) which were sensitised to OA but not exposed to aerosol antigen. The positive control group consisted of animals ($n = 35$) which were sensitized and subsequently exposed to aerosol antigen without prior drug treatment. The above two groups were run concurrently with all subsequent drug treatment groups, which accounts for the higher number of animals in these two groups compared to the others.

The drug treatment groups consisted of animals that were sensitized and exposed to antigen in addition to drug treatment. 2,3-Dihydro-5-[4-[2-(3,4,5-trimethoxyphenyl)ethyl] phenyl] imidazo[2,1-α] isoquinoline hydrochloride (SDZ

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64-412)-treated animals received the PAF antagonist SDZ 64-412 at either 10 ($n = 8$) or 20 mg kg^{-1} ($n = 10$). The drug, dissolved in water, was given orally 2 h before antigen challenge. 4-[3-[6-(2-Chlorophenyl)-1-methyl-4H-1,2,4-triazolo[4, 3-a] thieno [3,2-f]-1,4-diazepin-8-yl]-1-oxopropyl-morpholine (WEB 2086)-treated animals received the structurally dissimilar PAF antagonist WEB 2086, 10 mg kg^{-1} ($n = 6$), orally 2 h before challenge. WEB 2086 was synthesized by the Medical Chemistry Department at Sandoz, E. Hanover, and its chemical purity and pharmacological activity assured as described previously (Handley *et al.*, 1988). Methylprednisolone (MP)-treated animals ($n = 9$) received MP, 37.5 mg kg^{-1} i.p., at 24 and 2 h before antigen challenge. Ketotifen-treated animals ($n = 7$) were given ketotifen (1 mg kg^{-1} orally) 2 h before antigen exposure. Since ketotifen has H_1 receptor antagonist properties, a drug control group ($n = 5$) was also included which received the same dose of ketotifen but without antigen challenge to test for residual histamine antagonist properties.

In all groups, oral dosing of the guinea-pigs was accomplished by inducing light CO_2 narcosis by exposure of the animals to dry-ice vapour in a closed container followed by gavage through a short length of polyethylene tubing. Animals fully recovered from this exposure within 15 s. The negative and positive control groups received distilled water as vehicle controls.

Bronchoprovocation testing

Except as noted, bronchoprovocation testing was performed 24 h after antigen challenge. Animals were anaesthetised with 45 mg kg^{-1} sodium pentobarbitone i.p. A tracheostomy was made and the animals ventilated with a Harvard rodent ventilator (tidal volume = 4 ml, rate = 50 breaths min^{-1}) through a heated Fleisch pneumotachograph (size 0000) to measure tracheal flow rate. A small incision was made in the right thorax and a side-holed polyethylene catheter was inserted into the pleural space. Transpulmonary pressure was measured as the difference between tracheal pressure and intrapleural pressure by a Validyne DP45 transducer. Flow and pressure signals were fed to a Buxco Model 6 respiratory analyser the output of which was in turn fed to a DL-16 data logger and an 80286-based microcomputer for visual display and data storage. The right jugular vein was cannulated for administration of acetylcholine and histamine. Gallamine (1 mg kg^{-1} i.p.) was given to prevent spontaneous respiratory efforts. In those animals in the vagal section group, a bilateral vagal section was performed just before baseline resistance measurements.

Baseline pulmonary resistance measurements were made after the lung had been inflated with 3 tidal volumes. There were no differences in baseline resistances between any of the groups. Animals first received an ascending i.v. acetylcholine (ACh) challenge consisting of 2, 5, 10, 20 and 40 μg kg^{-1} acetylcholine chloride in saline. Three minutes were allotted between doses and the lungs were inflated with 3 tidal volumes before each challenge. Ten minutes after the last ACh challenge baseline resistance was again measured and was within 10% of the starting value. The animals then received an ascending i.v. histamine challenge consisting of 2, 5, 10, 20 and 40 μg kg^{-1} histamine diphosphate in saline. In some groups, as noted below, the ACh bronchoprovocation was omitted. The maximum value of pulmonary resistance after each dose of spasmogen was recorded and expressed as a % of the respective baseline value to construct ACh and histamine dose-response curves.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed after the bronchoprovocation testing. The lungs were lavaged with 5 aliquots of 10 ml each of a modified Tris-Tyrode buffer solution. Samples were centrifuged and resuspended in 1 ml of buffer. Total white blood cell (WBC) counts were performed by an electronic cell counter (Sysmex model CC-180) and differential counts were done on slides stained with a modified Wright stain (Diff-Quik, American Scientific Products). A total of 200 cells was counted to determine the percentage of BAL cells that were eosinophils. Total BAL eosinophils were expressed as 10^6 cells recovered.

Analysis of data

Data are presented as mean ± s.e.mean. Resistance values are expressed as the % increase from the baseline value. To compare differences between treatments, we performed an analysis of variance. When an overall significant F value was obtained we performed a t test for unpaired data. Statistical significance was assumed when $P \leqslant 0.05$.

Results

The time course of airway hyperreactivity in antigen exposed guinea-pigs was examined at 24, 48 and 96 h after antigen exposure (Figure 1). At 24 h after antigen challenge, guinea-pigs were markedly hyperreactive to histamine. At 48 h after antigen challenge, animals were still hyperreactive to histamine. Although there is a suggestion that the magnitude of the response had diminished, there was no significant difference between the response at 24 and 48 h. By 96 h after antigen exposure histamine reactivity had returned to control. Therefore, this antigen challenge procedure elicited sustained airway hyperreactivity to histamine, lasting at least 48 h. Since the airway reactivity was maximal at 24 h post antigen challenge, this time point was used in all further experiments to examine drug effects.

The involvement of vagal reflexes in the development of airway hyperreactivity in experimental animals and man has been proposed (Barnes, 1986). To determine the role of vagal reflexes in this model, a set of experiments was performed in guinea-pigs which were subjected to bilateral vagal section before histamine bronchoprovocation. The results of these experiments are shown in Figure 2. In the negative control animals (sensitized but not antigen challenged), bilateral vagotomy resulted in a diminution of the baseline histamine response, as has previously been demonstrated (Mills & Widdicombe, 1970). However, vagotomy did not attenuate the development of airway hyperreactivity to histamine after antigen exposure in the positive control group, effectively ruling out a contribution of vagal reflexes in this model.

Figure 3 shows the effect of the various drug treatments on histamine reactivity as evaluated at 24 h after antigen chal-

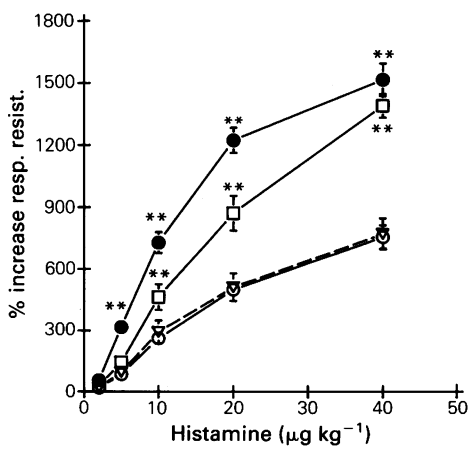

Figure 1 Airway reactivity to histamine at varying times after allergen challenge. (○) Negative control; (● positive control 24 h after allergen; (□) positive control 48 h after allergen; (▽) positive control 96 h after allergen. $^{**}P < 0.05$ vs negative control group. In this and subsequent figures each point represents the mean and vertical lines show s.e.mean.

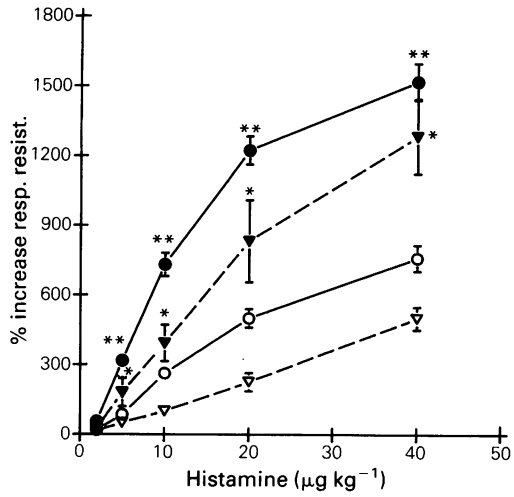

Figure 2 Effect of vagal reflexes on histamine hyperreactivity. Bilateral vagotomy was performed before histamine bronchoprovocation (24 h after allergen challenge). (○) Negative intact controls; (▽) negative vagotomized controls; (●) positive intact control group; (▼) positive vagotomized control group. $^{**}P < 0.05$ vs negative intact group; $^{*}P < 0.05$ vs negative vagotomized group.

lenge. The PAF antagonist SDZ 64-412 showed a modest effect at 10 mg kg^{-1}. However, at 20 mg kg^{-1} SDZ 64-412, histamine reactivity was significantly reduced at the three higher doses of histamine. A structurally dissimilar PAF receptor antagonist, WEB 2086 (Casals-Stenzel, 1986) was also used. At a dose of 10 mg kg^{-1}, this compound showed a variable response, with a statistically significant effect obtained at only one point on the histamine dose-response curve. Methylprednisolone and ketotifen also inhibited the airway hyperreactivity to histamine, with ketotifen being the only treatment that was able to abolish the response completely. Since the pharmacology of ketotifen includes H_1 antagonist properties (Phillips *et al.*, 1983), a seperate set of experiments was undertaken to eliminate the possibility that the results obtained with histamine as the bronchoconstrictor were merely the result of a sustained antihistamine action of ketotifen. The baseline histamine responses 24 h after H_2O vehicle or ketotifen (1 mg kg^{-1} p.o.) were identical (data not shown), eliminating the possibility that the histamine antagonist effects of ketotifen were responsible for the decreased reactivity to histamine.

Figure 4 shows the effects of these drugs on ACh reactivity. Similar results to those shown for histamine were obtained with this bronchoconstrictor. Acetylcholine reactivity was not done in the WEB 2086 group.

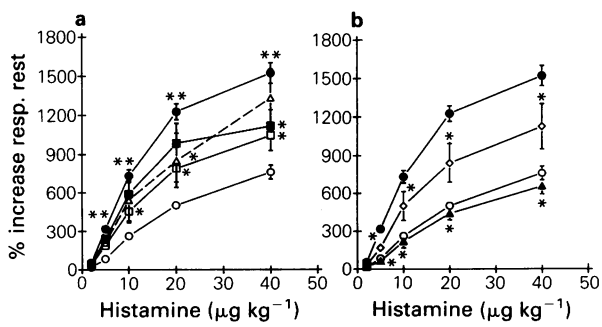

Figure 3 Effect of drug treatment on airway hyperreactivity assessed by histamine bronchoprovocation 24 h after allergen challenge. In (a), (○) negative control group; (●) positive control group; (■) SDZ 64-412 (10 mg kg^{-1}); (□) SDZ 64-412 (20 mg kg^{-1}); (△) WEB 2086 (10 mg kg^{-1}). In (b) negative and positive control groups are the same as in (a); (▲) ketotifen (1 mg kg^{-1}); (◇) methylprednisolone (75 mg kg^{-1} in 2 doses). $^{**}P < 0.05$ vs negative control group; $^{*}P < 0.05$ vs positive control group.

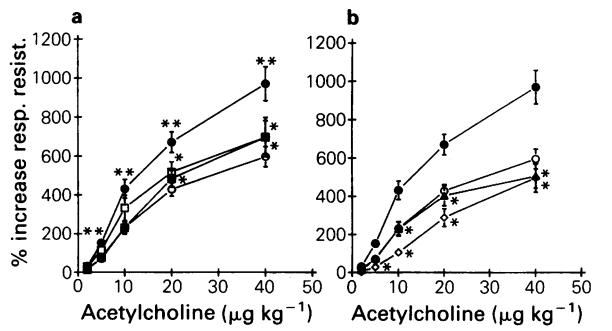

Figure 4 Effect of drug treatment on airway hyperreactivity assessed by acetylcholine bronchoprovocation 24 h after allergen challenge. Symbols are the same as in Figure 3.

Table 1 shows the number of eosinophils present in bronchoalveolar lavage from representative animals from the above experiments. There were no differences in recovery of BAL fluid between the groups (45 to 48 ml). At 24 h after antigen challenge there was a marked increase in the number of BAL eosinophils, which remained elevated at least until 96 h, the last time point studied. Neither of the PAF antagonists, SDZ 64-412 or WEB 2086 has any effect on eosinophil accumulation. Ketotifen also had no effect on BAL eosinophil accumulation. Methylprednisolone, the only treatment given twice, at 24 and 2 h before antigen, was unique in showing efficacy in preventing eosinophil accumulation.

Discussion

In these studies we have shown that the PAF receptor antagonist SDZ 64-412 inhibits the development of sustained airway hyperreactivity to histamine and acetylcholine which occurs in sensitized guinea-pigs after aerosol antigen challenge. SDZ 64-412 shares this property with two other clinically used drugs, methylprednisolone and ketotifen. Previously, Coyle and coworkers (1988) have shown inhibition of airway hyperreactivity after antigen challenge in guinea-pigs after treatment with the PAF receptor antagonist BN 52021. Effects on non-specific airway hyperreactivity have been proposed as the mechanism of action of prophylactic asthma drugs, especially steroids and disodium cromoglycate (Cockcroft & Murdock, 1987). Also, a central role for PAF in the exacerbation of asthma has been proposed (Page & Morley, 1986; Barnes & Chung, 1987); thus strengthening the concept that a PAF receptor antagonist may have therapeutic benefit as a prophylactic asthma drug.

The mechanism of action of ketotifen in inhibiting antigen-induced airway hyperreactivity in this animal model is not completely understood. Although it has been proposed that the clinical benefit of ketotifen derives from its ability to antagonize the action of PAF functionally (Morley, 1987),

Table 1 Eosinophils recovered from bronchoalveolar lavage fluid

Treatment	*Eosinophils* ($\times 10^6$ cells)
Negative control	2.7 ± 0.3
Positive control (24 h)	12.3 ± 3.5
Positive control (96 h)	13.3 ± 3.9
SDZ 64-412 (20 mg kg^{-1})	13.5 ± 1.5
WEB 2086 (10 mg kg^{-1})	15.0 ± 2.9
Ketotifen	14.8 ± 5.5
Methylprednisolone	2.1 ± 0.5*

Data shown are means ± s.e.mean. $^{*}P < 0.05$.

ketotifen is a relatively weak inhibitor of PAF-induced platelet aggregation (Criscuoli *et al.*, 1986; Casals-Stenzel *et al.*, 1987). Also, it is unlikely that it functions directly as a PAF receptor antagonist in the dose used here. Possibly, ketotifen acts proximally to the end organ effects of PAF by preventing the biosynthesis of PAF by inflammatory cells, as suggested by Joly *et al.* (1987), or distally to interfere with as yet unknown secondary mediators.

We are unaware of any studies that indicate that steroids are receptor antagonists to PAF. The effectiveness of steroids in inhibiting PAF-dependent responses may depend on the nature of the response studied. For example, at high doses steroids do not inhibit PAF-induced haemoconcentration in the guinea-pig (Handley *et al.*, 1985), but do protect against PAF-induced lethality in mice (Myers *et al.*, 1983). In the present experiments, steroid pretreatment may have inhibited the development of airway hyperreactivity by actions on cells that synthesize PAF in response to antigen exposure. Methylprednisolone was the only drug which we administered more than once in these studies (at 24 and 2 h before allergen challenge). It is well known that corticosteroids act through synthesis of secondary mediators, therefore sufficient time for protein synthesis must be allowed for steroids to exert an effect. We do not know what the effect of chronic treatment with a PAF antagonist would be. Perhaps repeated treatment would allow penetration of the drugs into compartments which are not accessible when given only 2 h before the allergen challenge.

Although PAF is a potent bronchoconstrictor in several species including man (Cuss *et al.*, 1986; Rubin *et al.*, 1987), the potential clinical utility of a PAF receptor antagonist may lie primarily in the prophylactic treatment of asthma, rather than the reversal of acute bronchoconstriction. Indeed, several studies have shown little effect of PAF antagonists in acute anaphylaxis. Pretolani *et al.* (1987) and Casals-Stenzel (1987) showed that WEB 2086 was not effective in blocking the bronchopulmonary responses to intravenous antigen in actively sensitized guinea-pigs, although there were modest effects in animals that had been passively sensitized. Also, WEB 2086 had no effect on the increased airway microvascular permeability in guinea-pigs after intravenous allergen challenge (Evans *et al.*, 1988). Danko *et al.* (1988) used the PAF receptor antagonists CV-3988 and L-652,731 and similarly showed no effects against intravenous antigen challenge in guinea-pigs, and concluded that PAF is not an important mediator of acute anaphylaxis in the guinea-pig. This is in accord with the work of Daffonchio *et al.* (1987) who demonstrated that bronchoconstriction after antigen challenge in the guinea-pig is due primarily to histamine and leukotrienes.

However, some studies have demonstrated an effect of PAF receptor antagonists in acute anaphylaxis. Cirino *et al.* (1986) showed that, in guinea-pigs passively sensitized with IgE-type antibody, the PAF antagonist BN 52021 inhibited the bronchoconstriction provoked by an aerosol of allergen. The PAF antagonist Ro 19-3704 was shown to be ineffective in blocking bronchoconstriction induced by intravenous antigen challenge, but showed effectiveness when tested against aerosol antigen exposure.

These findings suggest that the route of allergen presentation (i.e. inhaled vs intravenous) is an important determinant of the relative contribution of PAF to the acute response (Lagente *et al.*, 1988). Furthermore, we would also suggest that reversal or prevention of acute allergen-induced bronchospasm is not necessarily indicative of the therapeutic potential of prophylactic asthma therapy.

A prominent role for the eosinophil in the pathogenesis of asthma has been proposed (Durham & Kay, 1985; DeMonchy *et al.*, 1985). PAF has the potential for being important in eosinophil-mediated pathology for at least two reasons. Firstly, PAF is a potent chemoattractant for eosinophils (Wardlaw *et al.*, 1986), and secondly, eosinophils are a potential source of PAF (Lee *et al.*, 1984). Therefore, an interaction between PAF and eosinophils could include the possibility that PAF is one of the major signals responsible for pulmonary eosinophil recruitment, with subsequent tissue injury resulting from release of eosinophil granule proteins. Alternatively, eosinophils may enter the lung due to the presence of other chemoattractants and subsequently release PAF, resulting in tissue injury, airway inflammation and airway hyperreactivity.

The present study shows that three compounds that inhibited the development of airway hyperreactivity (SDZ 64-412, WEB 2086 and ketotifen) had no effect on BAL eosinophil recruitment (Table 1). These data argue that the presence of eosinophils is not sufficient in itself to cause airway hyperreactivity. Eosinophils may be required for the induction of airway hyperreactivity, but recruitment and activation may both be necessary before they exert their role. In this case, a PAF antagonist may exert its beneficial effect by blocking the end-organ effects of PAF released by activated eosinophils.

Inhibition of the functional manifestations of allergen exposure (airway hyperreactivity) did not correlate with the presence or absence of BAL eosinophils in our experiments. Similarly, other investigators have shown that the late-phase pulmonary obstruction, but not the accompanying pulmonary eosinophilia, following allergen challenge in guinea-pigs can be prevented by prior treatment with nedocromil sodium (Hutson *et al.*, 1988). Therefore, one final possibility which must be considered is that the eosinophil may not play a causative role in the development of airway hyperreactivity, but is recruited to the lung at a later time in response to tissue injury. Recent observations serve to support this hypothesis. Daffonchio *et al.* (1987) found that guinea-pigs were hyperreactive to 5-hydroxytryptamine (5-HT) as early as 1 h after aerosol antigen challenge, at a time when the prominent BAL cell is the neutrophil, not the eosinophil. Therefore, after antigen exposure, the early cellular response is a neutrophil infiltration which occurs during the time period consistent with development of airway hyperreactivity. Eosinophil recruitment is a relatively late occurrence (Dunn *et al.*, 1988) and may occur after airway hyperreactivity has already been established.

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(Received April 25, 1989
Revised September 8, 1989
Accepted October 11, 1989)