Effect of a PAF antagonist, BN52063, on PAF-induced bronchoconstriction in normal subjects

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1 The effects of an oral platelet activating factor (PAF) antagonist, BN52063, or matched placebo on inhaled PAF challenge were assessed in a double-blind study in eight normal subjects.

2 PAF (24 µg) induced an immediate bronchoconstriction, with a maximum fall in flow at 30% of vital capacity from a partial flow volume manoeuvre (V_{p30}) of $47.1 \pm 7\%$ (mean $\pm$ s.e. mean) 5 min after inhalation following placebo treatment. Repeated PAF challenges at 15 min intervals resulted in tachyphylaxis of the bronchoconstrictor response.

3 Two hours after the ingestion of BN52063 (120 mg) the maximum bronchoconstriction induced by inhaled PAF was attenuated ($35.9 \pm 9\%$ fall at 5 min) with a significant reduction ($P < 0.05$) in response after the first and second inhalations.

4 Inhaled PAF induced an immediate neutropenia ($73.2 \pm 9\%$ fall 5 min after inhalation) followed by a rebound neutrophilia, which were unaffected by pretreatment with BN52063.

5 Oral ingestion of a dose of BN52063 which is effective in reducing skin responses to PAF gave partial protection against the bronchoconstrictor effect of inhaled PAF in normal subjects.

Keywords platelet activating factor BN52063 bronchoconstriction PAF antagonist

Introduction

Platelet activating factor (PAF) is an ether linked phospholipid, produced by a variety of inflammatory cells which has several properties relevant to a mediator of allergic inflammation (Braquet *et al.*, 1987; Barnes *et al.*, 1988). PAF increases vascular permeability in a number of tissues including the airways (Evans *et al.*, 1987), and is the most potent chemoattractant for eosinophils described to date (Wardlaw *et al.*, 1986). More significantly, intradermal injection of PAF in man results not only in an acute weal and flare reaction (Archer *et al.*, 1984), but also in a late reaction characterised by cell accumulation which differs according to atopic status. Neutrophils predominate at sites injected with PAF in normal subjects (Archer *et al.*, 1985), whereas eosinophils, comparable in number and state of activa-

tion to that induced by antigen are observed in atopic subjects (Henocq & Vargaftig, 1986). Furthermore, PAF induces acute bronchoconstriction in several species including man (Cuss *et al.*, 1986). PAF inhalation also causes a profound but transient neutropenia in man (Chung *et al.*, 1987b).

The role of PAF in asthma is still not certain, but the recent development of selective PAF antagonists may now make it possible to evaluate the contribution of endogenous PAF to asthma. We have previously reported that one such compound, BN52063, which is a mixture of ginkgolides containing ginkgolides A, B and C in a ratio of 1:2:1 respectively (Braquet *et al.*, 1985) possesses selective antagonistic activity in man. BN52063 inhibits PAF-induced weal and flare

responses in skin and platelet aggregation *ex vivo* in a dose-related manner, with almost complete inhibition of the response to 100 ng of PAF 2 h after oral administration of 120 mg (Guinot *et al.*, 1986; Chung *et al.*, 1987a). Furthermore, the same dose of BN52063, administered 2 h prior to antigen challenge in atopic subjects inhibits the late cutaneous response to antigen, but not the early weal and flare (Roberts *et al.*, 1988), suggesting that PAF may be an important mediator of cellular infiltration in the skin. However, to date, although a number of PAF antagonists have been demonstrated to inhibit PAF-induced bronchoconstriction in experimental animals (Braquet *et al.*, 1987), no comparable study has been undertaken in man.

In the present study, we have investigated whether orally administered BN52063, in a dose which is effective against PAF- and allergen-induced skin responses, is able to inhibit the acute bronchoconstrictor response and the acute transient neutropenic response to inhaled PAF in normal subjects.

Methods

Subjects

Eight normal, non-atopic, healthy, non-smoking volunteers (aged 21–33 years, two females) gave informed written consent to participate in this study which was approved by the Brompton Hospital Ethics Committee. All subjects had been free of upper respiratory tract infection for at least 4 weeks, and were taking no medication other than the oral contraceptive pill (one subject).

Protocol

Atopy was excluded by skin prick testing to a variety of common allergens. Airway responsiveness to methacholine was measured prior to entry into the study to exclude hyper-responsiveness and repeated prior to the second PAF challenge to ensure return of reactivity to baseline, since PAF has previously been shown to increase responsiveness to methacholine in normal subjects for up to 4 weeks (Cuss *et al.*, 1986).

Subjects were randomised to receive matched capsules of either placebo or BN52063 (120 mg as a single dose; Ipsen International, London) in a double-blind fashion 2 h prior to PAF challenge. Study days were separated by at least 1 month.

A 19G intravenous cannula was inserted in an antecubital vein for collecting blood samples (in seven of the eight subjects), for evaluation of the effects of BN52063 ingestion on routine haematological and biochemical parameters prior to commencing the study, and for full blood counts and differential white cell counts 5 and 15 min after the first three inhalations of PAF and after completing the PAF challenge.

Airway function was measured using volume-standardized partial expiratory flow volume curves. This method has the advantage of sensitivity (Bouhuys *et al.*, 1969), so that small doses of PAF were sufficient to produce measurable changes in lung function, and cardiovascular effects of PAF were minimal. The reproducibility of this parameter, is of the order of 9–12% in normal subjects (Barnes *et al.*, 1981), increasing to 14% after bronchoconstriction has been induced (Michoud *et al.*, 1982; Eiser, 1987).

Flow volume curves were measured using a wedged spirometer (Vitalograph, UK) and were analyzed using a Hewlett-Packard microcomputer (Collingwood Measurements, Leicester, UK). Vital capacity (VC) was determined before each challenge and subsequent measurements were made from total lung capacity which does not change significantly during induced bronchoconstriction. The peak expiratory flow volume manoeuvre consists of expiration from 50–60% of VC, followed by a full inspiration to total lung capacity and determination of the expiratory flow at 30% of VC (V_{p30}). At least 1 min was allowed between inspiration to TLC and the next partial flow volume manoeuvre, since this allows recovery of bronchial tone (Barnes *et al.*, 1981).

PAF inhalation challenge

PAF (Novabiochem, Switzerland) was stored in 100% ethanol at -80°C at a concentration of 10 mg ml^{-1} . On the day of the study, a 1.5 mg ml^{-1} solution was prepared in 0.9% sterile saline containing a final concentration of 0.03% heat-treated human serum albumin (Immuno Ltd, UK). Aerosols were generated using a breath-activated dosimeter (Mefar, Brescia, Italy) driven by compressed air at a pressure of 22 psi. Subjects were instructed to inhale for 3 s from functional residual capacity to total capacity and breath hold for 7 s before exhaling. The output of the nebuliser was $24\text{ }\mu\text{l}$ per breath (median mass aerodynamic diameter $4\text{ }\mu\text{m}$).

Lung function measurements were made 1, 3, 5, 10 and 15 min after each inhalation of PAF, which was repeated five times.

Figure 1 Time course of PAF induced bronchoconstriction, indicated by changes in flow at 30% vital capacity, following pretreatment with placebo (●) and BN52063 (○) 120 mg given orally 2 h before challenge. Arrows indicate repeated inhalations of 24 μ g PAF. Means $\pm$ s.e. mean in eight normal subjects are shown. The bronchoconstrictor response was significantly inhibited following pretreatment with BN52063 after the first and second but not after subsequent inhalations of PAF ($P < 0.05$, t -test of differences between means).

Methacholine inhalation challenge

Doubling concentrations of methacholine chloride (Sigma, UK) were prepared in 0.9% NaCl (2–128 mg ml⁻¹). Following three satisfactory flow volume manoeuvres, subjects inhaled four breaths of sterile saline from the dosimeter. V_{p30} was measured at 90 and 150 s after inhalation and the mean V_{p30} calculated. Starting at 2 mg ml⁻¹, doubling concentrations of methacholine (four breaths) were administered until the 90 and 150 s values of V_{p30} were approximately 50% below post-saline values. The provocative concentration of methacholine needed to cause a 40% fall in V_{p30} (PC₄₀) was then computed.

Measurement of circulating cells

Samples of venous blood (2 ml) were taken prior to and 5 and 15 min after the first three inhalations of PAF. These were collected into tubes containing disodium EDTA.

Total white cell and platelet counts were measured on a Coulter Counter 880 (Coulter Electronics, Hialeah, Florida, USA). Blood smears were made from each sample and stained with May-Grunwald-Giesma stain. Differential cell counts were performed on 100 cells from each smear by an independent observer unaware of the experimental protocol. The number of

neutrophils, eosinophils, and lymphocytes/mm³ of blood were estimated by multiplying the percentage of each cell type by the total white cell count.

Statistical analysis

All values are given as mean $\pm$ s.e. mean. To assess the effect of BN52063 on PAF induced bronchoconstriction the mean percentage change in V_{p30} over the course of the study was calculated for each subject and a Student's t -test of the differences between the means on the 2 study days calculated. Similar analyses were performed for the time course of each inhalation. This analysis avoids the problems of performing repeated t -tests on a large number of time points. In addition the area under the curve was calculated using the trapezoid method for the first inhalation of PAF, and analysed using the Wilcoxon test for paired differences. A value of $P < 0.05$ was considered significant.

Pre challenge PC₄₀ values were compared using a paired t -test, after logarithmic transformation.

Results

BN52063 was well tolerated by all subjects, who reported no side effects. Routine biochemical and haematological tests were all within normal

limits after ingestion of 120 mg BN52063.

PAF caused acute bronchoconstriction maximal 5 min after the first inhalation ($47.1 \pm 7\%$) and gradually reversing over the next 10 min. Tachyphylaxis to repeated PAF challenges was observed (Figure 1) as previously reported (Cuss *et al.*, 1986). Pretreatment with BN52063 did not result in a significant reduction in the bronchoconstrictor response to PAF when the entire time course was analysed (Figure 1), but significantly inhibited the response to the first and second inhalations ($P < 0.05$) when assessed by the difference between the means. Additionally the area under the curve was reduced significantly by pretreatment with BN52063 ($P < 0.05$, first inhalation).

Five minutes after PAF inhalation, a marked neutropenia ($1.13 \pm 0.47 \times 10^9 \text{ l}^{-1}$ compared with $3.69 \pm 0.53 \times 10^9 \text{ l}^{-1}$ prior to the administration of PAF) was observed followed by a rebound increase ($5.35 \pm 0.77 \times 10^9 \text{ l}^{-1}$) in neutrophil counts which persisted for at least 1 h. No significant changes were observed in lymphocyte, eosinophil, red cell or platelet counts. After BN52063 there was a similar neutropenia ($4.11 \pm 0.77 \times 10^9 \text{ l}^{-1}$ before PAF to $2.19 \pm 0.99 \times 10^9 \text{ l}^{-1}$ 5 min after PAF), which was less marked, but not significantly different from control studies.

PC_{40} values determined by methacholine challenges prior to the second PAF challenge did not differ significantly from baseline values (geometric mean PC_{40} prior to placebo 12.6 mg ml^{-1} (range 2.7 to 69.2 mg ml^{-1}), and prior to BN52063 13.2 mg ml^{-1} (range 4.3 to 30.8 mg ml^{-1})).

Discussion

This study confirms earlier investigations demonstrating the potent bronchoconstrictor effects of inhaled PAF in normal subjects as determined by changes in partial flow volume curves (Cuss *et al.*, 1986). However, a dose of the PAF antagonist, BN52063, which is known to inhibit cutaneous effects of PAF (Chung *et al.*, 1987a) had only a partial inhibitory effect on the bronchoconstrictor response to inhaled PAF and failed to modify the neutropenic response to PAF.

In vitro investigations have shown that PAF contracts human parenchymal strips (Stimler *et al.*, 1983) but not human bronchial smooth muscle (Cerrina *et al.*, 1983), although platelets activated with PAF can induce a contraction of human bronchi (Schellenberg *et al.*, 1983). It is therefore of interest that intratracheal administration of PAF to baboons is associated with bronchoconstriction and a fall in peripheral

blood platelet counts (Denjean *et al.*, 1981). In humans exhibiting cerebral death significant decreases in blood platelet and leucocyte counts were observed following intratracheal administration of large doses of PAF ($0.12\text{--}1.2 \text{ mg kg}^{-1}$) (Gateau *et al.*, 1984).

In this study the bronchoconstriction induced by $24 \mu\text{g}$ of PAF was associated with a profound neutropenia, but there was no significant fall in the platelet count. The dose used in this study is much lower than that used by Gateau *et al.* (1984) which may explain the lack of thrombocytopenia, especially since it has been shown in experimental animals that although only modest amounts of PAF are needed to induce thrombocytopenia in anaesthetised guinea pigs (Vargaffig *et al.*, 1980) much higher doses are required in conscious animals (Demopoulos *et al.*, 1981). It is also possible that this dose did induce a fall in platelet count which may have recovered by the time of sampling since animal studies have suggested that platelet accumulation in the thoracic region of experimental animals given PAF intravenously is maximal at 60 s (Page *et al.*, 1983).

Human neutrophils have been shown to have specific high affinity binding sites for PAF (Valone *et al.*, 1982) and PAF induces chemotaxis and release of lysosomal enzymes from neutrophils (Czarnetzki & Benveniste, 1981) as well as aggregation (Cammussi *et al.*, 1981) *in vitro*. Intradermal injection of PAF in normal volunteers is associated with a neutrophil influx suggesting that PAF is chemotactic for neutrophils *in vivo* (Archer *et al.*, 1985). In addition, PAF has been shown to promote adhesion of neutrophils to vascular endothelium in culture (Ingraham *et al.*, 1982) and intravascular platelet aggregation and neutrophil margination as well as platelet diapedesis and extravascular degranulation have been observed following intravenous administration of PAF to guinea pigs (Lellouch-Tubiana *et al.*, 1985). When administered intratracheally to rabbits, PAF induces lung inflammation with accumulation of macrophages in the alveolar spaces and an increase in neutrophils and platelets in the alveolar capillary lumen (Cammussi *et al.*, 1983). Thus, although there is no direct evidence of neutrophil accumulation in human lungs exposed to PAF it is possible that the peripheral neutropenia reflects increased margination and adhesion of neutrophils in the pulmonary circulation. Although it is possible that the changes in neutrophil counts are secondary to mechanical changes in the pulmonary vasculature consequent upon bronchoconstriction this seems unlikely since the kinetics of the responses differ, especially with repeated PAF exposure.

Repeated challenge with the same dose of

PAF (24 µg) resulted in smaller changes in lung function. This phenomenon of tachyphylaxis to the bronchoconstrictor response to PAF has previously been described in guinea pigs (Lefort *et al.*, 1984) and man (Cuss *et al.*, 1986). Similarly, the neutrophil changes were tachyphylactic, but in contrast to the bronchoconstrictor response which was still detectable after five inhalations of PAF, neutropenia was observed only after the first inhalation, and was followed by a rebound neutrophilia. The response of blood elements to PAF *in vitro* has been extensively studied. Following exposure to PAF, rabbit platelets neither release nor aggregate when subsequently challenged with PAF (Lalau-Keraly & Benveniste, 1982) yet retain their reactivity to arachidonate. Similar desensitisation has been described in human platelets (Chesney *et al.*, 1985) and human neutrophils (O'Flaherty *et al.*, 1981). Although the mechanism of desensitisation is uncertain, exposure of human platelets to ^3H -PAF results in internalisation of PAF (and its proposed binding site) thus reducing the effective number of binding sites available for binding further PAF (Valone *et al.*, 1982). If PAF induced bronchoconstriction in man is platelet dependent as it is *in vitro*, and as has been described in guinea pigs and rabbits *in vivo* (Halonen *et al.*, 1981; Vargaftig *et al.*, 1980) it is plausible that internalisation of platelet PAF receptors results in desensitisation to the bronchoconstrictor response to PAF.

This study aimed to assess the ability of 120 mg of BN52063 to protect against the bronchoconstrictor effect of PAF. The dose of BN52063 used is the maximum for which toxicological data is available and has previously been shown to have selective PAF antagonistic activity in man against cutaneous PAF (Chung *et al.*, 1987a) when administered orally 2 h prior to skin testing, inhibiting the response to 200 ng of PAF by 60%. Data regarding the bioavailability of BN52063 after oral administration are difficult to obtain since no specific assay is available at present. The inhibition of PAF induced cutaneous responses in human skin is maximal 2 h after pretreatment with BN52063, but is still evident 8 h after pretreatment (Guinot *et al.*, 1987). It therefore seemed reasonable to administer the drug 2 h prior to challenge with PAF in this study.

Following pretreatment with BN52063 the responses to repeated challenges with PAF were partially inhibited (Figure 1). In view of the multiple measurements made following each PAF challenge, the data has been analysed using a *t*-test for the differences between the means for each subject following pretreatment with placebo

and BN52063, providing a simple measure of the differences between the time courses. This analysis reveals a significant difference in the response to the first and second inhalation but not in the responses to subsequent inhalations. Although the trend is maintained in the latter half of the study and given the small number of volunteers studied may have achieved statistical significance had a larger number of subjects been studied, the clinical significance of such changes are questionable since there is marked tachyphylaxis to the PAF response by this time and the observed differences in V_{p30} (about 10%) would not be associated with symptomatic improvement.

In addition the area under the curve has been calculated following the first inhalation, but not for subsequent inhalations, since the analysis of subsequent inhalations is complicated by the fact that pre-challenge V_{p30} is not basal, nor equal on each study day. This analysis confirms a significant inhibition of the response to the first inhalation of PAF.

Pretreatment with BN52063 did not inhibit the neutrophil response to PAF (Figure 2). This supports the hypothesis that the bronchoconstrictor and neutrophil responses are dissociated, since there was a significant inhibition of the bronchoconstrictor response to PAF at the time of neutropenia, yet this was unaltered. Although this dose of BN52063 has previously been shown to inhibit platelet aggregation *ex vivo* (Chung *et al.*, 1987a) such studies were not performed in these subjects so that no estimate can be made of the plasma level of BN52063 achieved in these subjects. BN52021 is the most active constituent of the ginkgolide mixture BN52063 (Braquet *et al.*, 1987) and has been shown to inhibit PAF binding to human neutrophils *in vitro* with an IC_{50} of 10^{-7}M and to reduce aggregation and degranulation of human neutrophils (Braquet *et al.*, 1985).

The dose of BN52063 used in this study has previously been shown to inhibit the effects of PAF in human skin (Chung *et al.*, 1987a). However, whilst this study was conducted with the same oral dose of BN52063, the PAF doses were in the range of 50–400 ng whereas in this study a total dose of 120 µg of PAF was delivered. Thus it is likely that the differences are due to the difference in dose of PAF used, although metabolism of BN52063 over the course of the study, so that insufficient drug was available to inhibit further challenges of PAF cannot be excluded, especially since it has been suggested that it is not possible to achieve adequate concentrations of drug in the airway lumen (Braquet, personal communication) with an oral formulation which

Figure 2 Time course of PAF-induced changes in neutrophil counts following pretreatment with placebo (●) and BN52063 (○) in seven normal subjects. Arrows indicate repeated inhaled challenges with 24 µg PAF.

is presumably where the PAF bronchoconstrictor response is initiated. Since the mechanism of neutropenia is not known it is difficult to speculate as to the reason for the lack of protection afforded by BN52063 of this phenomenon.

It is possible that an inhaled preparation may be more useful in studying the role of PAF in airway disease. Alternatively, it may be necessary to use a higher oral dose, more frequent dosing or a more potent antagonist.

In summary, although BN52063 does clearly have PAF antagonistic activity at this dose and given by this route the degree of protection afforded against inhaled PAF at a dose of 24 µg is

not sufficient to justify using this drug as a tool to investigate the role of endogenous PAF in the responses to inhaled antigen in human lungs. Thus in contrast to human skin where it has been possible to demonstrate a role for PAF in the late response to allergen challenge using this drug (Roberts *et al.*, 1988) further investigation of the role of PAF in airway allergic reactions will require more active PAF antagonists.

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