Altered platelet function associated with the bronchial hyperresponsiveness accompanying nocturnal asthma

Paolo Gresele, MD, PhD,[a, b] Maurizio Dottorini, MD,[b] Maria Laura Selli, MD,[a, b] Leonardo Iannacci, MD,[b] Silvio Canino, MD,[b] Tommaso Todisco, MD,[b] Salvatore Romano, PhD,[c] Paul Crook, BSc,[a, d] Clive P. Page, PhD,[a, d] and Giuseppe Giorgio Nenci, MD[a, b]

Perugia and Florence, Italy, and London, England

Background: *Nocturnal awakening is a common feature of bronchial asthma, and yet the mechanisms underlying this phenomenon are poorly understood. We investigated whether nocturnal awakening is associated with changes in platelet function with the use of a variety of markers of platelet activation.*

Methods: *Ten patients with a history of nocturnal asthma and 10 age- and sex-matched healthy control subjects were studied at 10:00 PM, 4:00 AM, and 10:00 AM on 2 consecutive days. The following parameters were tested: forced expiratory volume in 1 second (FEV_1), log dose of methacholine inducing a 20% fall in FEV_1, platelet count and volume, platelet aggregation induced by collagen or activating factor, and plasma and intraplatelet levels of β-thromboglobulin and platelet factor 4.*

Results: *We have demonstrated that altered platelet function and platelet activation occurs at 4:00 AM in patients with nocturnal asthma and is associated with the maximum increases in bronchial reactivity. Such changes were not observed in 10 control subjects. Platelet dysfunction has been detected as a reduced aggregatory response of platelets to collagen and platelet activating factor such that up to 5 times more platelet activating factor and 1.5 times more collagen were required to elicit a threshold aggregatory response in asthmatic subjects when compared with control subjects; this difference was evident at all time points tested. Furthermore, at 4:00 AM there were significantly lower levels of intraplatelet β-thromboglobulin corresponding to the maximum reduction in peak expiratory flow and to the maximal increase in bronchial responses to inhaled methacholine.*

Conclusions: *These results suggest that platelet activation accompanies nocturnal asthma and further suggest that platelets may play a role in this common clinical condition. (J ALLERGY CLIN IMMUNOL 1993;91:894-902.)*

Key words: *Platelet function, bronchial hyperresponsiveness, nocturnal asthma*

Nocturnal asthma is a major clinical problem that is believed to represent an exaggeration of the normal circadian variation in airway caliber.[1-4] Although circadian rhythms of respiratory function in asthma have not been found to be clearly related to the circadian variation of bronchial hyperresponsiveness to methacholine,[3] a close relationship between the amplitude of "morning dipping" (as a marker of nocturnal asthma) and bronchial responsiveness to inhaled histamine has been observed.[5] However, although various suggestions have been made as to the underlying cause of this exaggerated circadian variation in airway caliber, including changes in endogenous circulating corticosteroids,[3, 6] endogenous adrenaline levels,[1] and the influx of inflammatory cells into the lungs in the early morning,[7] the precise mechanisms underlying this phenomenon have yet to be determined. The observations on inflammatory cells are of particular interest because airway inflammation has been impli-

From [a]International Inter-University Centre on Thrombosis and Haemostasis; [b]Istituto di Medicina Interna e di Medicina Vascolare, University of Perugia, Italy; [c]Department of Physiopathology, University of Florence, Italy; and [d]Department of Pharmacology, King's College, University of London, England.

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Reprint requests: C. P. Page, PhD, Department of Pharmacology, King's College, London, Manresa Rd., London SW3 6LX, England.

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Abbreviations used

β-TG:	β-thromboglobulin
FEV_1:	Forced expiratory volume in 1 second
$FEF_{25\%}$:	Forced expiratory flow
PEF:	Peak expiratory flow
PAF:	Platelet activating factor
PD_{20}:	Log dose of methacholine inducing a 20% fall in FEV_1
PF_4:	Platelet factor 4
SEM:	Standard error of the mean
TAC:	Threshold aggregating concentration

cated in the induction of airway hyperresponsiveness induced by a variety of exogenous inciting agents including allergen, pollutants, and sensitizing chemicals associated with certain occupations.[8]

One of the plethora of inflammatory mediators implicated in the pathogenesis of such inflammatory changes is platelet activating factor (PAF) because this endogenous phospholipid has been demonstrated in both experimental and clinical studies to mimic many of the changes that accompany bronchial asthma.[9] Recent evidence from experiments with animals has suggested that the ability of PAF to induce bronchial hyperresponsiveness and eosinophil infiltration may involve the activation of platelets.[10, 11] The participation of platelets in clinical asthma has been described to accompany acute allergen-induced bronchospasm and exercise-induced bronchospasm,[12, 13] but very few studies have analyzed the behavior of platelets during spontaneous attacks of asthma such as nocturnal asthma, and certainly none has attempted to correlate such changes with changes in bronchial responsiveness.[14] In the present study we therefore sought to obtain evidence for platelet activation in parallel with measurements of airway responsiveness in patients who were undergoing clinical exacerbations of their nocturnal asthma. Part of this work was presented at the 48th Annual Meeting of the American Thoracic Society.[15]

METHODS

Subjects and study protocol

Patients were selected on the basis of (1) previous history of nocturnal asthma and (2) capability to perform autorhythmometry (i.e., self-measurements of peak flow at 4-hour intervals over a period of 24 hours) as discussed by others.[16] Four male and six female patients with allergic asthma (age range, 14 to 34 years) and four male and six female control subjects (age range, 22 to 34 years) were enrolled in this study. All asthmatic patients had a history of atopy and had a basal forced expiratory volume at 1 second (FEV_1) that ranged from 70% to 93% of predicted (mean ± SEM = 84.3% ± 2.8%).

All subjects were nonsmokers and refrained from any drug treatment for 2 weeks before and during this study. All subjects were hospitalized during the study and gave written informed consent to particpate in this study. The study was carried out under the principles of the Declaration of Helsinki and was passed by the ethical committee of the Regione dell'Umbria. Two hours before any procedure, the subjects were given a light, standardized meal to avoid dietary interference with the platelet function tests. No alcohol or methylxanthine-containing beverages were allowed throughout the study. Before each experiment was initiated, subjects lay down for at least 20 minutes, and a 19G buttefly needle was inserted in an antecubital vein of the arm for sampling blood. Immediately after this, bronchial responsiveness was assessed according to the technique of Chai et al.[17] as described below. Blood sampling and airway responsiveness tests were carried out at 10:00 PM, 4:00 AM, and 10:00 AM on 2 consecutive days.

Lung function tests

Lung function tests were assessed by the plethysmographic method (Body Star FG 90, Werner and Gut AG, Basel, Switzerland). The following parameters were measured: FEV_1, peak expiratory flow (PEF), forced expiratory flow (FEF 25%) and inspiratory and expiratory airway resistance (raw data). The reference values were those of the Communitee European for Coal and Steel.[18]

Bronchial hyperresponsiveness to methacholine

Bronchial responsiveness to methacholine was measured only if basal FEV_1 was greater than 70% of the predicted value. Lyophilized methacholine (Laboratorio Farmaceutico Lofarma, Milan, Italy), which was dissolved in distilled water, and its solvent as a control were used for this test. They were delivered by means of a dosimeter (Mefar, Bovezzo, Italy). This computerized device allows one to preset the duration of delivery. After the nose clips were applied, the subjects inhaled a standard dose of solvent followed by methacholine in doubling doses, from 15 to 1920 μg, or until a decrease of more than 20% in FEV_1 was observed. An interval of 3 minutes separated each dose. The concentration of methacholine was 1 mg/ml for doses up to 60 μg and 10 mg/ml for higher doses.

A dose-response curve was then obtained and the cumulative dose, which induced a 20% fall in FEV_1 (PD_{20}) was calculated by a linear interpolation of the last two points on semilogarithmetic paper.

Platelet aggregation

Twenty milliliters of blood was taken by clean venipuncture into tubes that contained 2 ml of 3.8% trisodium citrate. Platelet-rich plasma was prepared by centrifugation at 150 *g* for 15 minutes at room temperature, and platelet poor plasma was obtained from centrifugation of the blood that remained after removal of platelet-rich plasma for 20 minutes at 2000 *g*. The platelet count was adjusted to 250 × 10^9/L with the use of autologous platelet-poor plasma, and 250 μl aliquots were used to measure the threshold aggregating concentrations (TAC) to PAF and collagen as previously described.[19] Platelet aggregation was evaluated with the photometric method in an Elvi 840 dual channel aggregometer (Elvi Logos, Milan, Italy) as previously described.[20] TAC was defined as the minimal concentration of the stimulus that produced full irreversible aggregation (greater than 60% light transmission) starting within 2 minutes from the addition of the inducer.

The minimal incremental step used for the search of the TAC was 10 nmol/L for PAF and 0.2 μg/ml for collagen. A stock solution of PAF was prepared before the study, frozen at −20° C in separate aliquots, and used throughout the study to ensure maximal reproducibility. Dilutions of the collagen stock solution were freshly prepared on each study day. Platelet aggregation induced by PAF was carried out between 60 and 90 minutes after venipuncture, which is the period of maximal reactivity of platelets to this inducer (results not shown). Platelet aggregation induced by collagen was tested between 90 and 120 minutes after venipuncture. Each inducer was always tested in the same aggregometer channel.

Plasma β-TG and PF_4 determination

Four milliliters of blood was withdrawn into a precooled syringe and immediately transferred into a tube that was immersed in crushed ice and contained two-tenths volume of an anticoagulant mixture that consisted of ACD formula A; ethylenediaminetetracetic acid (EDTA) (10^{-3} mol/L in NaCl 0.15 mol/L), adenosine (10^{-3} mol/L in NaCl 0.15 mol/L) and prostaglandin E_1 (10^{-5} in ethanol). The blood-anticoagulant mixture was carefully mixed by gentle rotation and left immersed in crushed ice for 30 to 45 minutes. The samples were then centrifuged at 4° C for 45 minutes at 3000 *g*; 1 ml of the middle layer of the supernatant was collected and further centrifuged for 5 minutes at 12,000 *g*. Two 300 μl aliquots were finally taken from the resulting platelet free plasma and stored at −20° C for later measurement of β-TG and platelet factor 4 (PF_4) by commercial enzyme-linked immunosorbent assay tests from Boehringer Biochemica Robin (Milan, Italy) and Behring S.p.A. (Scoppito, L'Aquila, Italy), respectively, as described previously.[21]

Intraplatelet β-TG and PF_4 determination

Five microliters of Triton x100 was added to 250 μl of platelet-rich plasma, and the samples were vigorously shaken before centrifugation for 5 minutes at 12,000 *g*. The supernatant was stored at −20° C for later assay of β-TG and PF_4 as described previously.[21]

Assay of β-TG and PF_4

Plasma β-TG was assessed in samples diluted 1:11, and intraplatelet β-TG was measured in samples diluted 1:330 and 1:2000. Plasma PF_4 was assessed in undiluted plasma, and intraplatelet PF_4 was assessed at dilutions of 1:100 and 1:200. Intraassay and interassay reproducibility studies for measurements of β-TG and PF_4 gave the following results: β-TG = 5.5% and 14.8% coefficients of variability, respectively; PF_4 = 4.1% and 16.1 coefficients of variability, respectively.

Platelet count and volume

Two milliliters of blood was drawn into a tube that contained 60 μl of 1.85 × 10^{-3} mol/l EDTA, thoroughly mixed, and stored at 4° C for 4 to 10 hours before measurement of platelet count by an electronic counter (Model Autocounter H_1, Technicon Instruments, Tarrytown, N.Y.).

Statistical analysis

Autorhythmometric data concerning PEF were analyzed by the current chronobiologic methods referred to as a Single Cosinor and Mean Cosinor.[22-24] The first method uses a sinusoidal function time series by linear least squares method. A mesor (interpolated daily average), an amplitude (half interpolated daily range), and an acrophage (timing of interpolated maximum within the day) are gathered together with the probability *(p)* of acceptance of null amplitude hypothesis (i.e., of lack of individual sinusoidal circadian rhythm). The second method starts from individual single cosinor results, mesor (± SEM), and the average population amplitude and acrophase are estimated, together with their 95% confidence limits, when the probability of acceptance of null hypothesis (i.e., lack of population circadian rhythm) is $p < 0.05$. All other data, except PD_{20}, were analyzed by partially repeated measures analysis of variance (ANOVA).[25] The calculations were made with the SAS statistical package (SAS Institute Inc., Cary, N.C.).

Raw data have been used to construct tables and figures, but unfortunately, some occasionally missing

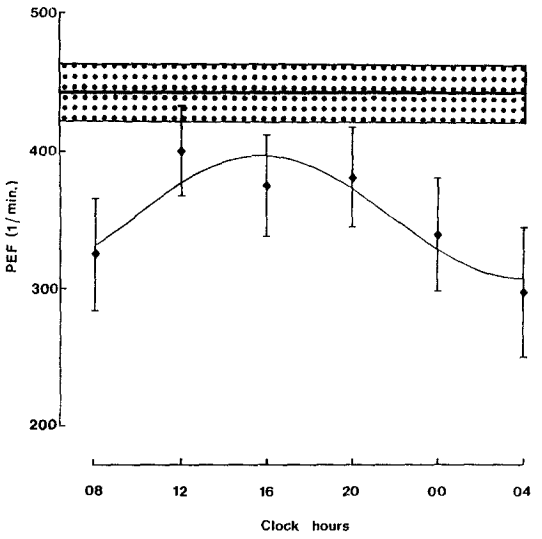

FIG. 1. Circadian changes in PEF over a 24-hour period in asthmatic subjects. Results are expressed as mean ± SEM at each clock hour. Data were analyzed by the Cosinor method; the average *(continuous line)*, and 95% confidence limits *(shaded area)* for control subjects are shown.

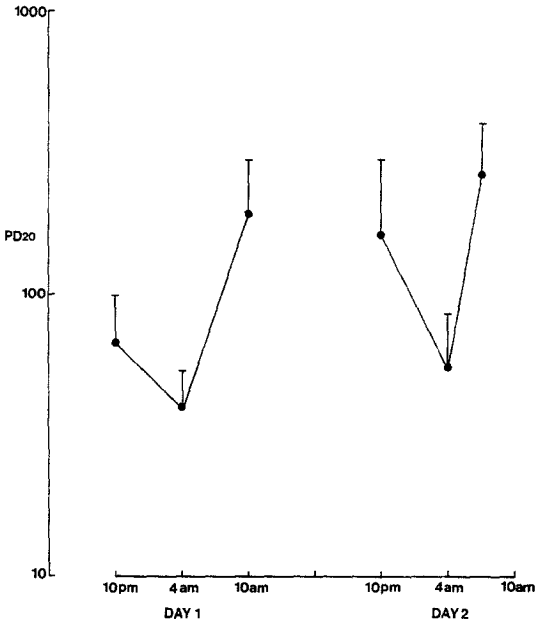

FIG. 2. Bronchial responsiveness assessed as the dose of inhaled methacholine required to induce a 20% decrease in FEV_1 (PD_{20}) at various time points over a 2-day period in patients with asthma. The results are expressed as mean ± SEM PD_{20} at each time point and show a typical diurnal variation with a nadir at 4 AM on both days of the study.

data would have reduced the number of subjects entering the statistical analysis. The occasional missing data have therefore been substituted with the corresponding within subject average; this was only in those cases in which only one missing point of six scheduled measurements was present, and the amount of substituted data was very low. In order to assess the impact of the substitution of the missing data, ANOVA was performed for every parameter by substituting the missing value with a random value chosen from a Gaussian distribution with an average of the available data of the subject and a variance identical to the variance of the average of that subject. Such analysis did not alter the outcome of the study. PD_{20} was analyzed by repeated measures of ANOVA.[24]

Homoscedasticity was checked by the Bartlett test, and equality of variance hypothesis was not rejected ($p > 0.25$ at least) only after log-transformation of the data for all variables but plasmatic β-TG, in which no significant effects were detected before and after transformation. ANOVA results are presented and discussed with reference to log-transformed data. With the aim of visual comparison means ± SEM of non-transformed data are presented.

RESULTS

Autorhythmometry

Autorhythmometry showed a circadian rhythm of PEF in patients with asthma which was highly significant ($p < 0.001$). The population mesor is 353 ± 37 L/min (SEM), the amplitude is 46 (with 95% confidence limits at 26 and 66), and the bathyphase corresponds to 3:52 AM (local time) with 95% confidence limits at 3:12 and 4:56. The means around the clock and the interpolated sinusoidal curve are shown in Fig. 1.

Bronchial hyperresponsiveness

Bronchial responsiveness to inhaled methacholine showed a typical daily variation with a significant maximum at 4:00 AM on both days of the study in subjects with asthma (shown in Fig. 2 as the nadir in the PD_{20} to methacholine). No abnormal bronchial responsiveness was detected in any of the normal subjects tested at any observation point ($PD_{20} > 1975$ μg).

Platelet studies

The responses of platelets to either collagen or PAF in individual normal and asthmatic subjects did not follow a statistically significant daily variation (Figs. 3 and 4). However, there was a significant difference in the TAC of PAF and collagen required to activate platelets from the two study groups. The overall mean difference between patients with asthma and control subjects was highly significant for TAC to PAF

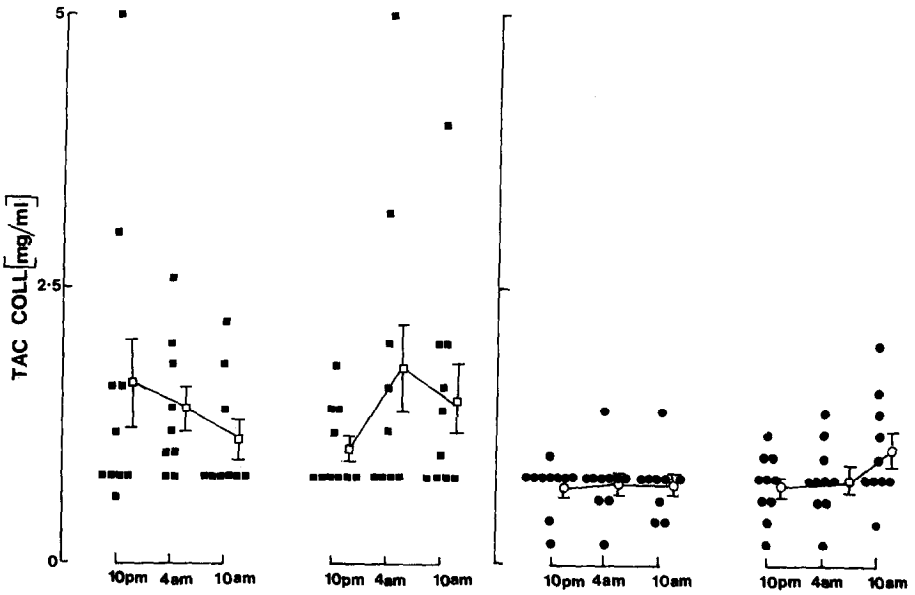

FIG. 3. The threshold aggregating concentration *(TAC)* to collagen required to aggregate platelets from patients with asthma (■) and normal subjects (●) at various time points over a 2-day study period. Results are expressed as mean ± SEM and show that 1.5 to 2 times more collagen is required to activate platelets from patients with asthma at each time point but without a significant diurnal variation.

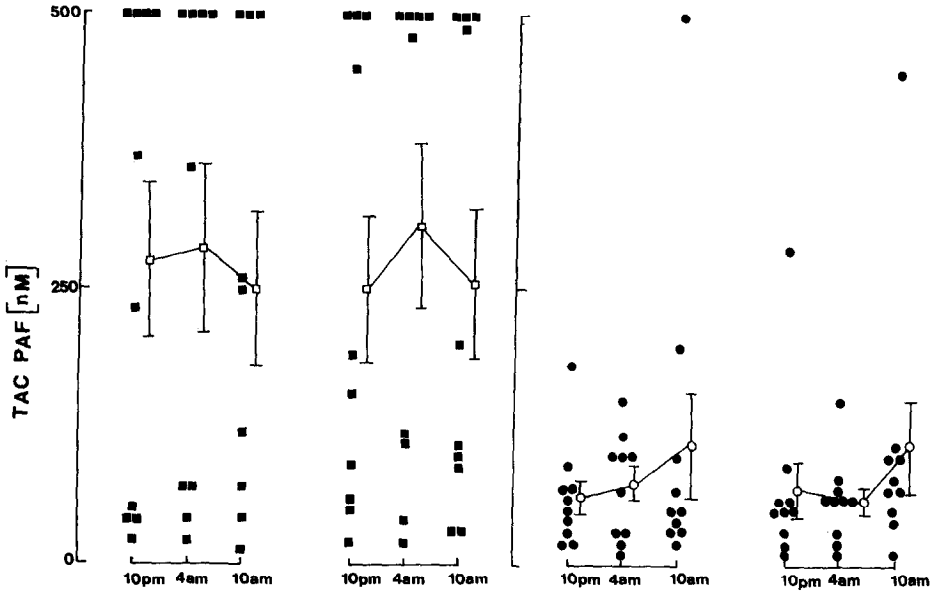

FIG. 4. The threshold aggregating concentration *(TAC)* to PAF required to aggregate platelets from patients with asthma (■) and normal subjects (●) at various time points over a 2-day study period. Results are expressed as individual data and as mean ± SEM and show that five times more PAF is required to activate platelets from patients with asthma at each time point, but without a significant diurnal variation.

($p < 0.03$) and significant for TAC to collagen ($p < 0.01$) (Fig. 5). At the different time points studied up to five times more PAF was required to activate platelets in the asthmatic group when compared with the control population, whereas a maximal increase of 1.5 to 2 times in TAC was noted for collagen in the patients with asthma at each time point studied when compared with normal subjects (Figs. 3 and 4).

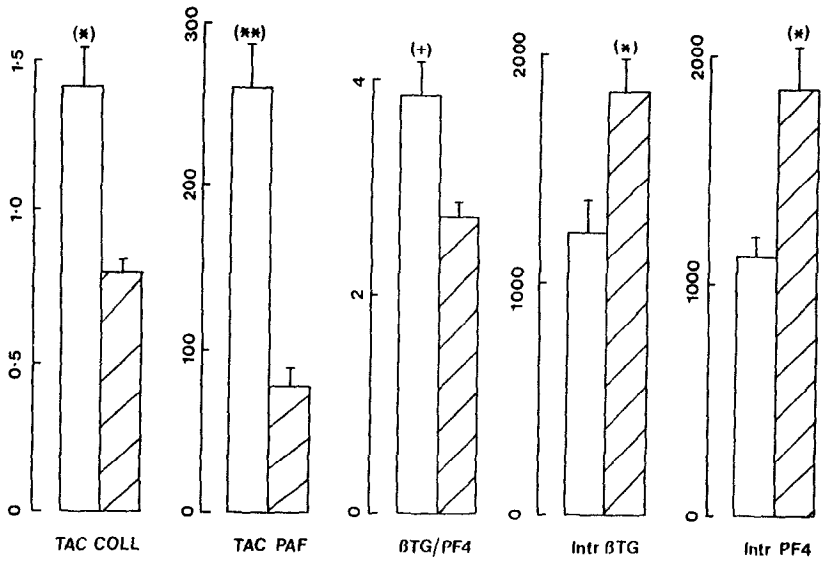

FIG. 5. The individual and overall mean difference in the threshold aggregating concentration of collagen *(TAC Coll)* or PAF (TAC PAF) required to activate platelets from normal *(dashed columns)* and asthmatic subjects *(empty columns)* is depicted, as well as the ratio of plasma β-TG/PF_4, intraplatelet β-TG, or intraplatelet PF_4 in normal or asthmatic subjects. The results are expressed as the mean ± SEM. ***$p < 0.01$; **$p < 0.03$; *$p < 0.04$; †$p < 0.07$.

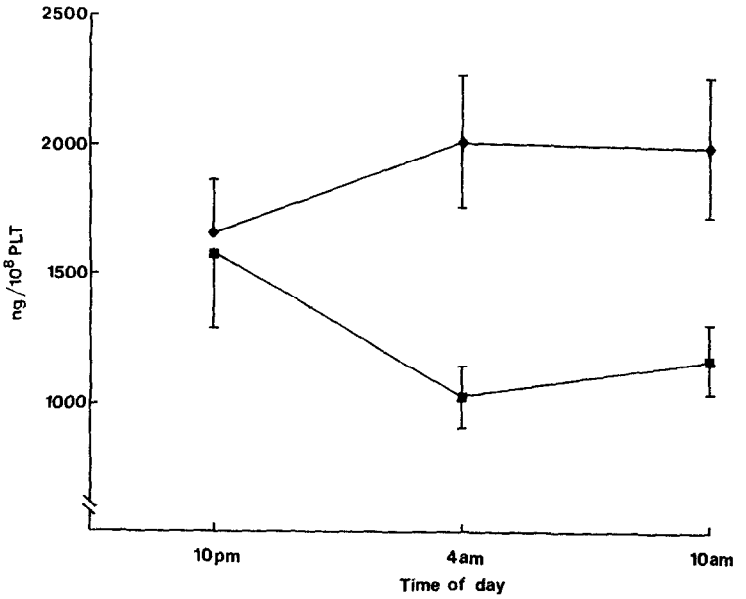

FIG. 6. The daily pattern of intraplatelet levels of β-TG expressed as mean ± SEM ng β-TG/10^8 platelets *(PLT)* in asthmatic (■) and normal (♦) subjects. A significant difference was observed in the daily pattern between asthmatic and control subjects ($p < 0.05$); the lowest values of intraplatelet β-TG were found at 4:00 AM in asthmatic subjects, which corresponds to the maximum increase in bronchial responsiveness to inhaled methacholine (see Fig. 2).

Platelet volume and number

No significant difference in the daily average number or volume of platelets was detected in the subjects with asthma when compared with the normal subjects. A significant daily variation was revealed in both of the variables ($p < 0.0007$ and $p < 0.006$, respectively), and there was no significant interaction with diagnosis factors.

TABLE I. Absolute levels of PF4 and BTG in patients with asthma and normal subjects

	Day 1					
	10 PM		4 AM		10 AM	
	Asth	Norm	Asth	Norm	Asth	Norm
Platelet number ($\times 10^3/\mu l$)	289 ± 23	249 ± 16	280 ± 25	252 ± 12	298 ± 28	271 ± 11
Platelet volume (fl)	8.8 ± 0.31	9.2 ± 0.22	9.1 ± 0.32	9.1 ± 0.28	8.4 ± 0.30	8.9 ± 0.33
Plasma β-TG (ng/ml)	15.93 ± 1.34	16.30 ± 2.19	18.63 ± 3.01	15.44 ± 1.94	15.54 ± 1.88	14.43 ± 1.82
Plasma PF_4 (ng/ml)	4.03 ± 0.87	6.02 ± 1.05	4.97 ± 0.80	6.33 ± 1.24	4.74 ± 0.64	6.74 ± 1.67
Intraplatelet β-TG (ng/10^8 platelets)	1832 ± 622	1816 ± 363	1055 ± 189	2210 ± 288	1266 ± 225	1837 ± 419
Intraplatelet PF_4 (ng/10^8 platelets)	1066 ± 316	1960 ± 431	908 ± 153	1953 ± 388	1092 ± 278	1608 ± 308

Values are expressed as mean ± SEM.
Asth, Patients with asthma; *Norm*, normal subjects.

Intraplatelet PF_4 and β-TG

A significant overall mean difference was observed in the intraplatelet levels of PF_4 or β-TG between the patients with asthma and control subjects ($p < 0.04$) (Fig. 5). There was also a significant difference in the daily pattern of intraplatelet β-TG between patients with asthma and control subjects ($p < 0.05$); in patients with asthma the lowest values of intraplatelet β-TG were at 4:00 AM (Fig. 6), which corresponded to the maximal reduction of PEF (Fig. 1) and to the maximal increase of bronchial responsiveness to methacholine (Fig. 2); no such time course was observed in control subjects (Fig. 6).

Plasma PF_4 and β-TG

No significant difference was observed in the absolute levels of PF_4 or β-TG detected in plasma between the patients with asthma and normal subjects at any time point tested (Table I). However, the β-TG-PF_4 ratio, an excellent indicator of in vivo platelet activation,[26] showed an overall mean difference between patients with asthma and control subjects; which was almost significant ($p < 0.07$) (Fig. 5).

DISCUSSION

The present study has confirmed that subjects with asthma who have symptoms of nocturnal asthma exhibit a clear circadian variation in airway responsiveness to inhaled methacholine, which showed the greatest response at 4:00 AM on the 2 study days. Platelets that were isolated from peripheral venous blood from patients with asthma exhibited signs of altered function in comparison with those that were isolated from normal subjects. In particular, a reduced reactivity to the aggregating agents, PAF and collagen, was noted when platelets were investigated in vitro with platelet aggregometry at all time points tested. Selective desensitization of platelets to PAF by prior exposure to this mediator has been used as a bioassay to provide evidence for the in vivo release of PAF in both experimental anaphylaxis[27] and clinical asthma.[28] However, in the present study the observed hypoaggregability of platelets in response to PAF cannot be considered conclusive evidence for the release of PAF in patients who exhibit nocturnal asthma because the platelets from these subjects also showed hyporesponsiveness to collagen, an agonist that is not normally desensitized by prior exposure of platelets to PAF.[29] Rather, we believe that our results provide further support for the idea of asthma being associated with chronic platelet activation that results in "exhausted platelets," which has been shown in other clinical conditions to be hyporesponsive when investigated in vitro.[12, 21] This suggestion is further supported by our observations that the intraplatelet content of the α-granule proteins β-TG and PF_4 are significantly reduced in the platelets that are isolated from patients with asthma at all points tested when compared with those of normal subjects. This has been suggested by other investigators[30] to also be a marker of chronic activation of platelets. In addition, the plasma β-TG-PF_4 ratio, another parameter of in vivo platelet activation,[26] was also greater in patients with asthma than in control subjects. Interestingly, the depletion of intraplatelet β-TG reached its maximum

Day 2					
10 PM		4 AM		10 AM	
Asth	Norm	Asth	Norm	Asth	Norm
285 ± 24	276 ± 12	268 ± 22	246 ± 9	293 ± 29	264 ± 12
8.8 ± 0.31	8.9 ± 0.34	9.0 ± 0.31	9.0 ± 0.31	8.3 ± 0.40	9.1 ± 0.30
14.54 ± 1.97	17.31 ± 3.45	15.93 ± 2.34	17.79 ± 3.78	15.06 ± 2.02	18.71 ± 2.35
5.64 ± 0.99	8.79 ± 2.69	4.77 ± 0.75	7.96 ± 2.82	5.82 ± 0.98	8.16 ± 2.50
1405 ± 209	1494 ± 218	1026 ± 187	1821 ± 437	1083 ± 161	2152 ± 407
1180 ± 226	1490 ± 270	1021 ± 199	1382 ± 303	820 ± 183	1655 ± 464

coincidently with the peak changes in bronchial hyperresponsiveness to methacholine in patients with nocturnal asthma. No daily variation of this parameter was observed in the normal control subjects who did not exhibit bronchial hyperresponsiveness to methacholine. The reduction of intraplatelet βTG in subjects with asthma was still present at 10:00 AM whereas the plasma β-TG-PF_4 ratio, which was "high" at 4:00 AM was normalized at 10:00 AM. It is possible that the repletion of the intraplatelet β-TG store may take longer than 6 hours and that it may reflect the appearance of new platelets within the circulation. In this regard, it is of interest to note that others have reported increased platelet turnover[31] in patients with asthma, which is also consistent with such a hypothesis. It cannot be excluded that platelets from subjects with asthma are abnormal as a result of abnormal platelet production.[32, 33] However, our results, which show a circadian variation in platelet granule content, together with previous observations on abnormal platelet turnover in asthmatic subjects[31, 34] are more consistent with the hypothesis that platelets undergo in vivo platelet activation during nocturnal asthma[14] and demonstrate for the first time that such activation is associated with the bronchial hyperresponsiveness that is typical of allergic asthma and more specifically of nocturnal asthma.

The relevance of such platelet dysfunction to the pathogenesis of the bronchial hyperresponsiveness associated with nocturnal asthma is not yet known, but it has recently been reported that platelet depletion in experimental animals results in a modification of the magnitude of the late-onset airway obstruction, bronchial hyperresponsiveness, and bronchial eosinophil infiltration that normally follows allergen challenge of allergic animals.[10, 11] Moreover, certain platelet products including PF_4,[35] the cytokine RANTES,[36] and platelet-derived histamine releasing factor[37] have all recently been shown to cause eosinophil chemotaxis, and the latter substance has been shown to induce both late-onset airway obstruction and bronchial hyperresponsiveness in experimental animals.[37] It is therefore plausible to suggest that the activation of platelets may participate in the direct recruitment of eosinophils into bronchoalveolar lavage fluid, which is known to accompany exacerbations of nocturnal asthma.[7]

In summary, we have produced evidence, with the use of well-controlled methodology for monitoring platelet activation, that mild platelet dysfunction is a feature of patients with nocturnal asthma. These observations further support the concept that platelets may participate in this common clinical problem.

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