THE ROLE OF PLATELET ACTIVATING FACTOR IN AIRWAY HYPERRESPONSIVENESS

by

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A thesis submitted to the
University of Glasgow
in candidature for the degree of
Master of Science
in the
Faculty of Medicine

Department of Respiratory Medicine,
Western Infirmary, Glasgow, U.K.
November, 1991.

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SUMMARY

This thesis consists of two parts , the first part is a study of the effects of inhaled PAF on the bronchial airway responsiveness . PAF also activates eosinophils to release major basic proteins including eosinophil cationic protein(ECP) which when measured in the bronchial lavage fluid can provide an indirect assessment of airway damage. The second part of the project is concerned with whether serum levels of ECP can also be correlated with airway damage.

We have examined the effect of inhaled PAF(96 ug) on the bronchial airway responsiveness to methacholine at day 1, day 3 and day 7 after PAF challenge, in 6 nonatopic (mean age 29.7 yrs) and seven atopic (mean age 29.3 yrs) healthy volunteers.

After PAF inhalation, plasma ECP levels (ug/l) were measured over a 3 hour period using a Pharmacia radioimmunoassay.

PAF challenge and methacholine responsiveness were repeated on 2 occasions allowing at least 4 weeks between cycles. Airway responses were assessed by measuring specific airways conductance (SGaw) in a constant volume body plethysmograph. The maximum mean(sem) % falls in SGaw in the 1st, 2nd and 3rd PAF challenge in nonatopic subjects were 47(8.7), 49.5(9.3) and 47.2(6.3) respectively and in atopic subjects 41.7(6.2), 48.0(6.1) and 49.3(6.3) respectively. The changes in SGaw were comparable in 3 cycles in both groups. The geometric mean PC 35 SGaw to methacholine was 6.51 mg/ml in nonatopic and 0.99 mg/ml in atopic subjects before PAF inhalation. PAF did not significantly alter the mean PC35 SGaw in either group. One subject did show >2 fold decrease in PC35 SGaw but this was not reproducible in other cycles.

In the second part of the project, the effect of PAF challenge on serum ECP level was compared in nonatopic and atopic subjects. Seven nonatopic and eight atopic subjects participated in this study. Blood was collected for three hours post PAF challenge for ECP assay. The mean

(sem)serum eosinophil cationic protein (ECP) level in nonatopic subjects before PAF challenge was 8.3 (3.6) ug/L, while at 15, 30, 60, 120, and 180 minutes post PAF challenge the levels were 5.4 (2.1), 5.6(1.3), 5.7(1.2), 5.6(1.2), and 4.6(0.97)ug/L respectively. There was no statistically significant difference before and after PAF challenge in the serum ECP levels. Among the atopic subjects the mean(sem) ECP level before PAF challenge was 4.9(0.7) while at 15, 30, 60, 120, and 180 minutes post PAF challenge the mean levels were 3.5(1.0), 4.6(0.7), 4.2(0.7), 4.6(0.8) and 4.6(0.9) ug/L respectively. There was no statistically significant difference in serum ECP level before and after PAF challenge. Our results suggest that PAF is a potent bronchoconstrictor but it does not induce airway hyperresponsiveness in either nonatopic or atopic subjects. Although PAF inhalation may activate airway eosinophils in both atopic and nonatopic subjects, it does not produce a rise in the plasma ECP levels.

TABLE OF CONTENTS

INDEX OF TABLES

INDEX OF FIGURES

ACKNOWLEDGEMENTS

I wish to acknowledge my gratitude to Dr.K.R.Patel, Consultant Physician in Respiratory Medicine and to Professor John Reid, Professor of Medicine and Therapeutics , Western Infirmary Glasgow. The former encouraged and guided me throughout my research project and the latter inspired me with full zeal towards this study. I am also grateful to Dr S. K. Ghosh, who stimulated my interest in research work, and to rest of the Medical Staff who has been very helpful. I also wish to thank Dr Kennedy R. Lee Lecturer in Clinical Pharmacology who also kindly agreed to supervise my work.

The work described in this thesis was carried out while I was working as a Research Fellow in the Department of Respiratory Medicine , Western Infirmary Glasgow. Most of the work was done in the Respiratory Department. Part of the work, involving measurement of serum eosinophil cationic protein levels (ECP), was carried out in the department of Immunology, Western Infirmary Glasgow. I am also grateful to Dr C. McSharry for analysing the blood for ECP levels.

I wish to thank Mrs. Rita Jack and her colleagues in the Respiratory Laboratory, for the care and enthusiasm they showed in the work. Finally , I must thank all those who volunteered to participate, specially to mention the technical staff of Cardiology and Respiratory Departments Western Infirmary Glasgow, who took part in the experimental work described in the thesis.

AUTHOR DECLARATION

I hereby declare that this thesis "The Role of Platelet Activating Factor in Airway Hyperresponsiveness", is part of the on-going work on platelet activating factor which is being carried out in the Department of Respiratory Medicine, Western Infirmary, Glasgow. The thesis has been composed by myself under the supervision of Dr. K. Patel, Consultant Physician in Respiratory Medicine and a Honorary Senior Lecturer to the University of Glasgow. I also declare that this work has not been submitted in any previous application for degree. The work has been carried out by myself and the General methods and Composition were discussed with my supervisor beforehand. I also acknowledge my thanks to all sources of information which helped me in my work.

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PART ONE

CHAPTER ONE.

ASTHMA

1.1 HISTORICAL BACKGROUND.

Asthma is one of the classic diseases recognised by Hippocrates over 2,000 years ago. Aretaeus (81-138 AD), and Galen (139-199 AD) used the term asthma to describe any condition associated with dyspnoea. The great mediaeval physician Maimonides (1135-1204), in the Treatise of Asthma (1190)(1), also tended to confuse asthma with other pulmonary disorders. Nearer to the time of Floyer, Jean Baptiste van Helmont (1597-1644) and Thomas Willis (1621-1675) had distinguished asthma from other varieties of dyspnoea, but they regarded the condition as a kind of nervous or convulsive fit akin to epilepsy. It was Sir John Floyer(2) who clearly defined asthma (derived from 'Greek' meaning breathless), separating it from other pulmonary disorders. He also considered the cause of dyspnoea was bronchial constriction, due to spasm and he regarded the spasm to be tonic, more akin to catalepsy than to the clonic convulsion of epilepsy.

It is noteworthy that Floyer was able to achieve this merely by careful clinical observation alone. Though his galenic ideas of pathogenesis and medical treatment do not stand up to modern inquiry , nevertheless he was familiar with the multifactorial basis of asthma i.e. heredity, occupation, exercise, and psychological influences. The importance of Floyer's work was also appreciated by later writers on asthma, e.g., John Millar(1735-1805) in 1769(3). Later on Henry Salter (an asthma sufferer ,1823-71) in his book on Asthma : Its Pathology and treatment(4) defined asthma as 'Paroxysmal dyspnoea of a peculiar character , generally periodic with intervals of healthy respiration between attacks.

The condition from which Floyer and Salter suffered is so distinct that it may be diagnosed by non-medical people, and its name is used in common place. The common denominator underlying asthmatic

diathesis is a non-specific hyperresponsiveness of the tracheobronchial tree. This increased airway bronchial responsiveness can be familial or acquired and is materially worsened by events that promote airway inflammation. The stimuli that increase airway responsiveness and incite acute episodes of asthma can be grouped into seven major categories : allergic, pharmacological, environmental, occupational, infectious, exercise related, and emotional.

1.2 DEFINITION OF ASTHMA

Asthma is defined as a disease characterised by periodicity of symptoms of cough and wheezing, reversible obstructive ventilatory defect and increased airway responsiveness (5). The changes in severity of airway narrowing can occur spontaneously or as a result of therapy, and my be measured by forced expiratory volume in the first second (FEV1), peak expiratory flow rate per minute(PEF), airway resistance (Raw), or specific airway conductance(SGaw). In addition asthmatic subjects show an increased responsiveness of the tracheobronchial tree to a variety of antigenic stimuli(6).

The airway narrowing that occurs in asthma is intermittent and variable. Complete remission can occur in between attacks, although some abnormality of function is often detectable with sensitive tests(7). During attacks, widespread narrowing of bronchi results in diffuse wheezing, often associated with dyspnoea, even at rest. Although the reversibility of airway may be suspected from the history, it should always be evaluated objectively by measurement of airway function after administration of a bronchodilator(8). Asthma can generally be divided into two major categories, extrinsic and intrinsic, but there may be some overlap between the two.

1.2.1 EXTRINSIC ASTHMA

Extrinsic asthma occurs in patients who are atopic, a term used to describe a genetic predisposition to respond to antigenic challenge with the

formation of immunoglobulin anti_body of IgE type. The inheritance is complex but usually incomplete, increasing greatly if both parents are atopic. For example , of 13 children born of two allergic parents, 11 developed atopy over a 4 years follow up period.Viral upper respiratory tract infections frequently predated the onset of allergic manifestations, suggesting that interaction of viral infection with genetic predisposition may be important(9).

The prevalence of atopy increases until approximately age 20, when it gradually declines. Peak IgE levels occur at age 14. In infants and young children, atopy and asthma are twice as common in males as in females (10, 11). Besides demonstrating increased blood IgE levels, atopic individuals are characterised by immediate skin test responses to a variety of antigens and a high incidence of eczema, rhinitis and asthma. However, atopy is not synonymous with asthma.The former occurs in 30 per cent of population, whereas the incidence of asthma is less than 5 per cent. Although affected identical twins invariably develop atopy, their allergic manifestations and non-specific bronchial responsiveness are discordant(12, 13). Patients with extrinsic or atopic asthma are distinguished by ,(a) family history, (b) onset in the first three decades of life, (c) seasonal symptoms, (d) elevated blood levels of IgE and (e) positive skin and bronchial challenge tests to specific allergens (13).

1.2.2 INTRINSIC ASTHMA

Intrinsic asthma refers to patients in whom atopy or specific external triggers of bronchoconstriction cannot be identified. The term intrinsic was initially coined because it was believed that these patients were responding to antigens free microbial agents released in their tracheobronchial tree. Patients with intrinsic asthma are characterised by : (a) being of elder age group, (b) having no family history of asthma or allergic diseases, (c) an absence of elevated blood levels of immunoglobulin IgE, or positive skin or bronchial responses to allergen challenge, (d) increased blood and sputum eosinophil counts, (e)

responsiveness to therapy and (f) a tendency to persistent progressive diseases resulting in fixed airflow obstruction (14, 15).

Exercise induced asthma is not a separate category because the majority of patients with asthma develop bronchoconstriction during exercise. It is believed that the stimulus for exercise induced asthma is not exercise but the cooling or drying of the airway mucosa that occurs on inhalation of incompletely conditioned air.

In some patients exposure to a specific external agent can be clearly shown to be the cause of reversible bronchoconstriction and there is no tendency for excessive IgE production. Patients in this category are known to suffer from occupational (16) asthma.

1.3 BRONCHIAL HYPERRESPONSIVENESS.

Bronchial hyperresponsiveness is considered by some as a sine qua non of asthma (17). The nonspecific bronchial hyperresponsiveness represents the exaggerated airway narrowing that occurs in response to inhalation of a variety of non-allergenic, usually pharmacologic, stimuli(18). Although all the stimuli used to demonstrate bronchial hyperresponsiveness result in some degree of narrowing in normal subjects, it is the excessive narrowing at very much lower dose or concentration that characterises nonspecific bronchial hyperresponsiveness. Exaggerated bronchial narrowing in response to pharmacologic agents was described many years ago, but only during the last 10 years, has the importance of nonspecific bronchial hyperresponsiveness, been recognised and techniques to demonstrate and quantify it, been developed. In 1921 Alexander and Paddock reported that pilocarpine resulted in asthmatic breathing in asthmatic patients , but not in normal subjects. They also observed exaggerated vagal effects such as salivation and sweating, and suggested that asthma might be secondary to increased vagal tone. In 1929 and 1932, Weiss and his associates demonstrated a decrease in vital capacity in response to intravenous histamine in emphysematous and asthmatic subjects at a concentration that had no effect on normal subjects (19, 20). In

1949, Curry (21) administered histamine and acetylcholine by both inhaled and intravenous routes to normal subjects and patients with rhinitis and asthma.In addition to showing an exaggerated response in asthmatics, he demonstrated concordance of the response to both agents and was therefore the first to note the nonspecific nature of the hyperresponsiveness. Despite these important advances it was Tiffeneau in the 1950s who first recognised the potential importance of nonspecific bronchial hyperresponsiveness and who systematically and quantitatively began to study nonspecific bronchial hyperresponsiveness in patients with asthma and allergic rhinitis, employing acetylcholine and histamine as provocative agents (22). Since the mid 1970s, interest in nonspecific bronchial hyperresponsiveness has increased dramatically and the condition has been discussed extensively in the literature (23, 24). Its nonspecific nature has become increasingly recognised and the list of substances to which asthmatics respond excessively is continually enlarging. The pharmacological agents include histamine, pilocarpine, methacholine, carbachol, acetylcholine, serotonin, bradykinin, prostaglandin F2α, leukotriene C4, D4, adenosine (25, 26) and platelet activating factor. Asthmatics also show excessive airway narrowing in response to inhalation of atmospheric pollutants, dusts cold, dry air and to certain respiratory manoeuvres such as deep inspiration or forced expiration.

The fact that nonspecific bronchial hyperactivity is such a characteristic feature of asthma raises the question of whether it represents a basic defect in the control of bronchial calibre that precedes and predisposes to the development of asthma, or that it is a consequence of it. The demonstration that the bronchomotor response to pharmacological agents by various nonasthmatic animal species as well as man is highly variable raises the possibility that the exaggerated airway narrowing in asthmatics simply represents one end of a wide biological susceptibility (27, 28, 29). Certain canine species such as the Basenji-greyhounds exhibit markedly increased nonspecific bronchial hyperresponsiveness(30), whereas some strains of rats can be bred to manifest exaggerated bronchoconstriction(31).Also a percentage of clinically healthy first degree

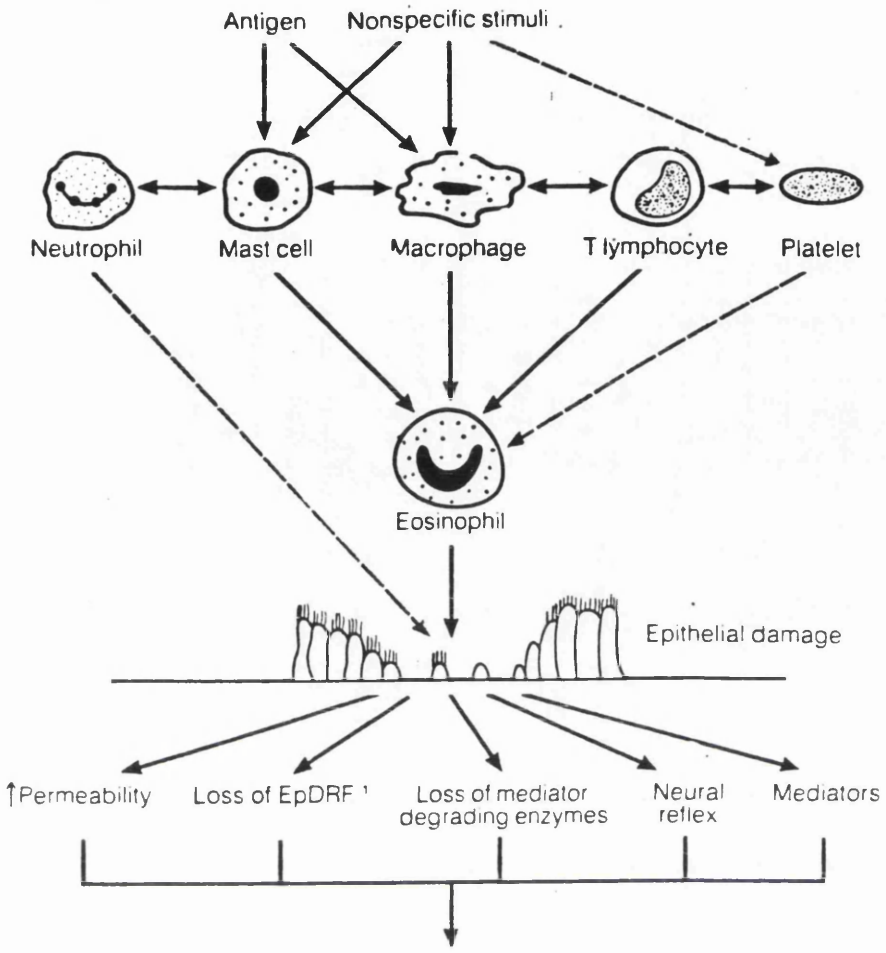
relatives of children with asthma demonstrate nonspecific bronchial hyperresponsiveness (10). These observations support the concept that a genetically determined airway responsiveness might predispose to asthma. However studies of monozygotic and dizygotic twins have shown similar inter-twin variability of response to methacholine, supporting the possible role of environmental factors as determinant of nonspecific hyper-responsiveness (32).

Non-specific bronchial hyperresponsiveness is not a static phenomenon, an individual's responsiveness can change considerably with the duration of exposure to infectious agents (33), environmental pollutants (34), and specific antigens or sensitising agents (35). For example in a group of patients with occupational asthma secondary to western red cedar exposure, nonspecific airway responsiveness decreased gradually over a period of months following cessation of exposure and increased again following re-exposure (36).

Non-specific bronchial hyperresponsiveness is so characteristic of asthma that it is questionable whether a diagnosis can be made in its absence(17). Rarely , patients with occupational asthma or non-occupational allergic asthma do not show increased nonspecific bronchial hyperresponsiveness at the time of diagnosis, but develop increased responsiveness with prolong exposure (37-40). Although nonspecific bronchial hyperresponsiveness is virtually 100 per cent sensitive in the diagnosis of asthma, it is far from being specific for example, it has been demonstrated in patients with sarcoidosis(41), extrinsic allergic alveolitis(42, 43) and chronic obstructive airway disease(44, 46, 47). Although in these condition it appears to be related to a baseline decrease in airway calibre(40, 41). In a comparison study of patients with asthma and chronic obstructive airway disease, nonspecific bronchial airway hyperresponsiveness was found to be unrelated to baseline forced expiratory volume in one second (FEV1) in the former but to be significantly related to pre challenge forced expiratory volume in one second (FEV1) in the latter(45). Similarly, in occupational surveys of airway responsiveness, the incidence of nonspecific bronchial

hyperresponsiveness is higher than that of clinically diagnosed asthma, suggesting an appreciable false positive rate (39).

Bronchial hyper-reactivity is quantified by measuring the dose or concentration of inhaled histamine or methacholine that causes a 20 per cent decrease in FEV1 . Rather than being a congenital defect that increases the risk of developing asthma, nonspecific bronchial hyperresponsiveness is probably an acquired abnormality;however its exact pathogenesis remains unknown. The most plausible theory is that it is a consequence of the chronic inflammatory reactions in the airway mucosa of asthmatic patients. Inflammation may cause hyperresponsiveness by epithelial damage, excessive mediator release, loss of a putative epithelial derived relaxing factor (EpDRF), increasing airway permeability leading to oedema and airway wall thickening and by altering the amount and contractility of airway smooth muscle (see Figure 1).



EpDRF: a putative epithelial derived relaxant factor

Figure 1 Cellular interactions leading to eosinophil infiltration and epithelial injury. (reproduced from Asthma: Basic Mechanisms and Clinical Management. Editors Barnes PJ, Rodger IW & Thomson NC. Academic Press, London, 1988; p.430 with authors permission)

1.4 MEDIATORS IN ASTHMA.

Many mediators have been implicated in asthma. Several can produce many of the features suggestive of asthma including; smooth muscle contraction, mucus hypersecretion, extravasation of plasma leading to bronchial oedema and inflammatory cell chemotaxis.

The complex interaction between many mediators may potentiate their effect, thus antagonism of a single mediator is unlikely to result in significant clinical improvement.

1.4.1 HISTAMINE

Histamine is released from airway mast cells and may have several local effects. It was first to be observed by Dale and Laidlaw in 1911 (48) as a potent vasoactive substance but its capacity to induce asthma was first noted by Weiss in 1929 (46) and described in more detail by Curry in 1946 (21). Histamine can cause bronchial obstruction by a direct effect on airway smooth as well as through vagal reflex action, neuropeptide release and potentiation of adrenergic responses. It also increases mucus secretion and causes oedema of the airway by increased permeability. The actions of histamine are mediated through two distinct receptors, H1 and H2, defined by the action of their respective agonist and antagonist. It is the H1 receptor which causes increased contractility of muscle, vascular permeability and prostaglandin generation and activation of airway vagal afferent nerves. Although anti-histamines are not useful in the treatment of asthma, but specific H1 antagonists such as terfenadine and cetirizine have certainly been shown to cause bronchodilation(49).

1.4.2 LEUKOTRIENES

Leukotrienes are derived from arachidonic acid by the action of lipoxygenase. The substances that fulfil the functional characteristics of slow releasing substance-A (SRS-A) are the leukotrienes C4, D4 and E4 (LTc4, LTD4 and LTE4). They are produced by mast cells, macrophages and

eosinophils and cause bronchial smooth contraction, increased vascular permeability and increased mucus production (50, 51). Leukotrienes C4 and D4 are potent bronchoconstrictors of the airways while LTB4 is a powerful chemotaxin and can attract both eosinophils and neutrophils to the site of its release(52). When administered by inhalation to human subjects leukotrienes cause dose dependent narrowing that is maximum at 3 minutes and resolves over 1 to 3 hours i.e., longer than that produced by histamine(53).

1.4.3 PROSTAGLANDINS

Prostaglandins PGD2, PGF2α and thromboxane are bronchoconstrictors produced from arachidonic acid by the action of cyclo-oxygenase in several inflammatory cells (mast cells, macrophages, eosinophils). However, the inhibition of this enzyme by aspirin and other non-steroidal anti inflammatory drugs has no clear beneficial effect in asthma, (54) in fact in approximately 3% of cases the asthma worsens.

OTHER MEDIATORS.

Bradykinin is an inflammatory peptide formed by the action of kininogenase and kallikrein on a plasma precursor. It acts as a potent bronchoconstrictor in asthmatics, probably by activating airway sensory nerves which are sensitized by inflammatory reaction.

Cytokines are peptide mediators released from several types of inflammatory cells(lymphocytes, macrophages, mast cells) which may be involved in coordinating the chronic inflammatory process in asthma. Similarly neutrophil chemotactic factor of anaphylaxis (NCFA)(55) and eosinophillic factor of anaphylaxis (ECFA) (55) and eosinophillic factor of chemotaxins derived from lipoxygenase metabolism of arachidonic acid may be important in the induction of late asthmatic response.

1.4.4 P.A.F..

Platelet activating factor (PAF) has recently assumed an important role in the pathogenesis of asthma (57), particularly because PAF has been demonstrated to increase nonspecific bronchial hyperresponsiveness in animals (58), and humans (59, 60).

CHAPTER TWO.

2.1 PLATELET ACTIVATING FACTOR.

Platelet Activating Factor (PAF), is a biologically active phospholipid mediator with potent inflammatory properties. It is derived from eosinophils (61), neutrophils (62), macrophages (63), basophils (64), endothelial cells (65) and platelets (66). It has been reported to possess many pharmacological actions pertinent to asthma, such as bronchoconstriction, non-specific bronchial airway hyperresponsiveness and increased capillary permeability with chemotactic stimulation of inflammatory cells infiltration.

The chemical structure of PAF was identified in 1979 by three independent groups as 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (67) (Fig 2). The active material is referred to as PAF-acether. Each feature of the structure is important for its optimal activity. The presence of ether linkage at position 1 and the length of alkyl side chain are critical determinants for biological activity, whereas the alkyl side chain at position 2 of the molecule is less critical. PAF derived from biological origin, including human skin (68) is a mixture of mainly C16 and C18 types (69). The biological activity of C16 PAF and C18 PAF do not appear to be qualitatively or quantitatively different.

Human eosinophils (61) and alveolar macrophages (63) which are extremely good sources of PAF, release PAF in response to activation by an IgE-dependent mechanism. Alveolar macrophages and eosinophils have IgE receptors on their surface and these cells are present in the airways of asthmatics and are activated following antigen provocation in sensitised asthmatics (70). Human : are reported to be heterogeneous consisting of normal density and light density cells that differ in their functional and morphological properties (71). The light density cells (rich in PAF) that are found in patients with an eosinophilia are generally regarded as being more activated than normal density cells (72).

The molecular mechanism by which PAF produces its various effect on target cells is still debated, but stereoselectivity of its effects, its high biological potency and development of tachyphylaxis, all suggest that membrane receptors are involved and a putative structure of the binding

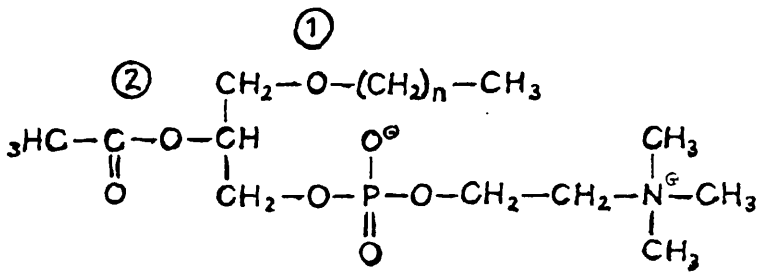


Figure 2 Diagrammatic representation of platelet-activating factor (PAF), illustrating the location of the ether bond (position 1) and the acetyl side chain (position 2). Modification of the molecule at either of these carbon atoms results in reduction or loss of biological acitvity.

site for PAF can be put forward (Fig 3). These receptors have been described in platelets (73), neutrophils (74), macrophages (75) and lung tissue (76). Valone et al. have isolated a PAF binding receptor from human platelets which appears to be protein (77). Howang et al found out that the rank order of potency for several antagonists was different in human neutrophils and platelets and that monovalent cations had different effects on binding to the two cells. From Hwang results and those of Lambrecht (75) it appears likely that there are at least two types of receptors.

Following binding of PAF to its receptor there is subsequent internalization of the PAF-receptor complex(78), which probably explains the rapid desensitization of PAF induced responses in a variety of tissues(79,80). Several biochemical changes are known to accompany the occupation of PAF-receptors by PAF(Fig 3). There is activation of phospholipase C, triggering the degradation of phosphoinositides to inosine triphosphate (IP3) and diacylglycerol (DG) both substances are known to be able to act as second messengers in bringing about a variety of intracellular events (81,82). For instance, DG is able to activate proteinkinase C, leading to the phosphorylation of specific intracellular protein involved in physiological processes such as secretion or contraction(83). IP3 is able to release intracellular Ca++ (84) from internal stores which may in turn regulate other intracellular events such as Ca++ dependent K+ channels (85). In some cell types such as the human platelet, PAF activation will inhibit the formation of cyclic AMP by other endogenous agents such as prostaglandins (86). PAF also stimulates cell to release free arachidonic acid and metabolize it to eicosanoids, which are responsible for some of the actions of PAF (87).

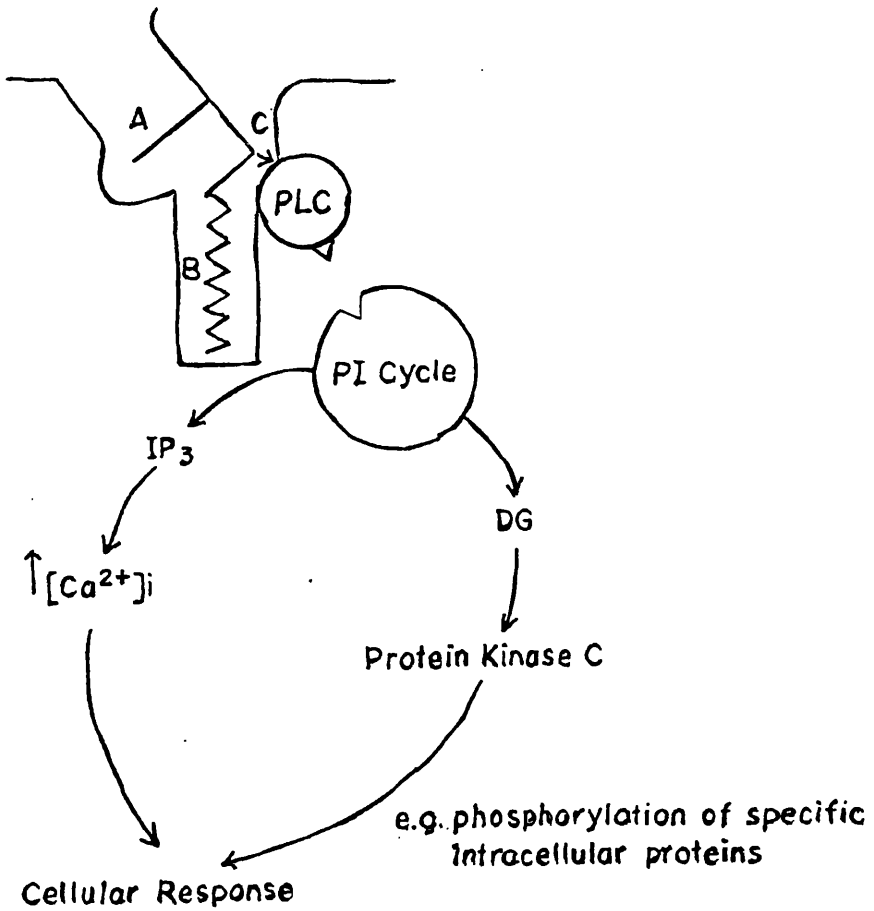


Figure 3. Interaction of PAF with its putative receptor site results in activation of membrane associated phospholipase C, leading to stimulation of the phosphotidylinositol(PI) cycle. Inositol triphosphate (IP 3) and diacylglycerol (DG) are generated from the PI cycle and act as second messenger in bringing about an increase in intracellular Ca++ and activation of protein kinase C. PAF is suggested to interact with the putative receptor at three distinct points in the molecule : A, acetyl group at position 2; B, C16-C18 backbone; C, ether link.

PAF produces acute reversible bronchocostriction in experimental animals (88). Repeated aerosol administration of PAF results in tachyphylaxis. In man following inhalation by normal healthy volunteers (59,60) PAF causes bronchoconstriction with a rapid onset of action (2-3 min) and a short duration (15-45min).

The mechanism by which PAF produces bronchocostriction is not exactly known. Patterson et al (90) have suggested that the bronchoconsrtriction caused by PAF in human is mediated at least in part, by histamine release not by cholinergic or cyclo-oxygenase dependent mechanism. This is partly supported by Chung et al(91) who reported that bronchoconstriction caused by PAF is partially inhibited by salbutamol. A direct effect of PAF on airway smooth muscle may exist but its importance appears to be minor. An indirect effect is mediated via generation of other biologically active mediator such as sulphidopeptide leukotrienes (89).

PAF is reported to be one of the most potent agents in inducing increased vascular permeability(92) thus causing oedema formation and activate inflammatory cells chemotaxis, including platelets(96), neutrophils(97), macrophages (98), monocytes (99) and eosinophils(100). In human skin PAF induces an acute weal and flare response(93) that can be potentiated by concomitant administration of vasodilator prostaglandins such as PGE 1 (94). The oedema formation seems to be independent of cyclo-oxygenase production or histamine, as it cannot be prevented by indomethacin or the histamine H1 antagonist mepyramine (95). On the other hand prior treatment with glucocorticoid significantly reduces the oedema formed after intracutaneous PAF infiltration. Following local administration of PAF to the skin of normal volunteers there is infiltration of inflammatory cells notably neutrophils,4-6 hours after treatment and a mixed cellular infiltration comprising neutrophils and mononuclear cells at 24 hours(101). In contrast local administration of PAF to the skin of atopic subjects results in selective eosinophil infiltration very reminiscent of antigen induced eosinophil infiltration in the same subjects (102).

CHAPTER THREE

PROJECT

The main aim of this work is as follows:-

1. To study the bronchoconstrictor effect of Platelet activating factor given by inhalation in atopic and nonatopic subjects.

2. To study the effects of Platelet activating factor on bronchial responsiveness to methacholine in atopic and nonatopic subjects.

3. Platelet activating factor has been shown to produce bronchial hyperresponsiveness both in animals and humans. To determine whether this bronchial hyper-responsiveness is reproducible.

3. 1 PLATELET ACTIVATING FACTOR & BRONCHIAL HYPERRESPONSIVENESS.

Platelet activating factor is a biologically active inflammatory mediator like histamine , prostaglandin & leukotrienes. It has been demonstrated to play an important role in allergic diseases(57). Lung seems to be the main target organ for its action where it causes bronchoconstriction (91, 103), induces bronchial epithelial damage (104) and increases bronchial pulmonary vascular permeability. PAF when administered either by inhalation or intravenously causes increase in bronchial hyperresponsiveness (100) which may last from 24 hours to several days. Cuss et al (59) have demonstrated increased hyperresponsiveness to methacholine in normal subjects for several weeks after PAF inhalation. Also Rubin et al (60) has demonstrated an enhanced bronchial reactivity in normal subjects but failed to show any increase in bronchial reactivity in asthmatics one hour after PAF challenge. Chung and Barnes (105) have reported that subjects with mild asthma do respond to PAF inhalation but to a similar degree as normal subjects. However several of the subjects did have increased reactivity at 3 days post PAF. Though it has been reported that PAF does increases bronchial responsiveness to methacholine in normal as well as asthmatic subjects, some studies (106,107,108,114) have failed to confirm this finding. In this study we have investigated the effect of inhaled PAF in inducing bronchial hyperresponsiveness by comparing atopic and non-atopic asymptomatic subjects. Furthermore we have studied whether this phenomenon is reproducible.

3. 2 SUBJECTS.

Thirteen subjects participated in the study which was approved by the West of Scotland Hospital Ethical Committee, Western Infirmary Glasgow. Written consent was obtained in each case. The subjects were all non smokers and were divided into two groups. The first group comprised, seven of them, who were atopic. In the second group there were six nonatopic healthy volunteers. The atopic subjects were all female age 21-38 (average 29. 3 yr), height between 157-169 (average 164 cm) and weight 58-68 (average 64. 7 kg). They were all skin tested to standard antigens, i. e. , house dust, house dust mites, cat, dog, feather, aspergillus, grass, pollens and a negative control. The atopy was defined by a positive

skin reaction of greater than 3 mm diameter to at least two antigens. The atopic subjects were asymptomatic at the time of the study which was carried out before the pollen season. The demographic data is given in detail in Table 1.

The six nonatopic (negative skin reaction to the above mentioned antigens) subjects comprised three males and three females, age 22-38 (average 29. 7 yr) ; height 163-187 (average 170. 7 cm) and weight (60-73 average 66. 9 kg). The demographic detail as shown in Table 2.

3. 3 CHALLENGE MATERIAL & METHOD

3. 3. 1 METHACHOLINE

Methacholine chloride solutions (Sigma Chemical Company Limited , Fancy Road Poole Dorset BH17 7NH) were prepared in phosphate buffer saline Ph 7. 4 and stored at -4 C . Before use , solutions were allowed to warm to room temperature. Increasing doubling concentrations , ranging from o. 0625 mg/ml to 64 mg/ml, were used.

3. 3. 2 NEBULISER

Methacholine solution was given via Wright's nebuliser which is a Jet nebuliser driven by compressed air & generating aerosol with a mass median aerodynamic diameter between 1 and 5 mm . The aerosol was passed directly into a face mask held over the mouth and clipped nose, and was inhaled by tidal breathing. The reservoir volume was 3 ml and the air flow of 9 L/min (pressure 3. 5 Kpa) delivered an output of 0.13ml/min. With 2 minutes inhalation time using tidal breathing, 0. 26 ml was delivered to the mouth. The nebuliser was calibrated by using 3ml of normal saline and operated for two total time samples. The output was determined by measuring the change in weight using A SARTORIUS GMBH GOTTINGEN balance (range 0 to 500 g)

Bronchial responsiveness was calculated from a series of methacholine challenge tests, starting with a smallest concentration of 0. 0.0625 mg/ml of methacholine given via the Wright's nebuliser. Successively greater concentration in two fold increments were used to the maximum concentration when SGaw (specific airway conductance) fell by 35 % of the lowest post saline starting value. The dose response curve was

plotted on a semilog paper and the concentration of methacholine that decreased SGaw to 35 % (PC 35)was determined by linear interpolation.

3. 3. 3 PAF & ITS INHALATION

Synthetic PAF c-16 (Cascade Biochem Limited , The Innovation Centre University of Reading Berkshire) was dissolved in chloroform/methanol (9:1) and stored at -20 C until required. Just before use 5 mg was dissolved in 2. 5 ml of phosphate buffer saline Ph 7. 4 to provide concentration of 2mg/ml. This solution was given as an aerosol delivered by the Acorn nebuliser attached to a dosimeter. This is a breath actuated device (Nebuchek P. K. Morgan Gillingham Kent), driven by compressed air at a pressure of 2. 5 Kpa and an output of 12 ug/breath 8 successive breaths were made and a total PAF dose of 96 ug was inhaled by each subject at one sitting. The response to PAF was measured by measuring specific air way conductance (SGaw by the Master Lab Body Plethysmography) before and at 0, 1, 2, 3, 5, 7, 10, 15, 20, and 45 minutes post PAF challenge.

3. 3. 4 SPECIFIC AIRWAY CONDUCTANCE & BODY PLETHYSMOGRAPHY

Plethysmography is the most accurate method of measuring absolute lung volume. Plethysmograph (Body Box) also provides a method for measuring airway resistance (Raw), which along with pulmonary tissue resistance (R ti)and chest wall resistance (R cw), make up the total respiratory resistance (RL).

Airway resistance in a normal individual is known to decrease with increasing lung volume. To provide a volume standard airway resistance measurement, the conductance (Gaw, the reciprocal of resistance) is divided by the TGV (thoracic gas volume) at which the Raw is measured, to yield the specific conductance SGaw.

SGaw is expressed as s^{-1} Kpa^{-1} (normal value is 1. 12-4. 00 $s^{-1}Kpa^{-1}$).

Airway resistance can be measured rapidly and non-invasively with standard Plethysmographic equipment. We used The MasterLab Body Plethysmograph Jaeger (Medical Electronics and Data Processing system, Leics) a computerised system working according to the constant volume, variable pressure system. It consists of a large chamber, a

pneumotachograph, and three transducers, which measure changes in the box pressure (Δ P box), mouth pressure (Δ P mo), and flow at the mouth (V). It is a very sensitive programme and needs volume and box calibration before use.

The subject is seated in a closed chamber breathes through a special mouth piece shutter assembly. At the end expiration, the shutter is closed to occlude the mouth piece, and the subject is asked to rhythmically compress and decompress the thorax by panting lightly against the closed shutter. While the shutter is closed, no airflow occurs within the airway, so mouth pressure changes (Δ Pmo) are equal to alveolar changes (Δ P alv). Also during this manoeuvre, the changes in box pressure reflect the changes in thoracic volume, and are proportional to the changes in alveolar gas pressure. Boyle's Law (P1V1=P2V2) can be applied to these pressure-volume changes to calculate the volume being compressed i. e, the subject's thoracic gas volume.

For measurement of airway resistance, the same shallow technique is employed, in order to keep the subjects glottis open and prevent respiratory temperature artifacts in the box. While the subject pants through the open mouth piece , flow at the mouth and corresponding cyclical changes in box pressure are recorded. The shutter is then closed briefly for TGV measurement. The ratio of alveolar pressure to thoracic compression (Δ Pmo/Δ P box shutter closed) is divided by the ratio of airflow at the mouth to thoracic compression (V/ Δ P box). The quotient represents the airway resistance.

3. 4 PROTOCOL & METHOD

The protocol aimed to examine the effects of inhaled PAF, on the bronchial airway responsiveness to methacholine by checking specific airway conductance SGaw in the volume constant body plethysmograph and to see whether this phenomenon was reproducible in atopic and nonatopic subjects.

This involved three cycles at least four weeks apart in both groups. Each cycle consisted of three to five visits each lasting for about 45 minutes. At the beginning of each cycle a baseline bronchial responsiveness was performed by giving doubling concentration of methacholine chloride calculating the PC 35 i. e. , the concentration of

methacholine at which the SGaw (specific airway conductance) falls by 35 %. On the next day a fixed dose of 96 ug PAF was given by Acorn nebuliser as explained in the previous section. Specific airway conductance (SGaw) was measured by the plethysmograph before and at 0, 2, 3, 5, 7, 10, 15, 20, and 45, minutes after PAF inhalation. 24 hours later i. e. , On the 1st post PAF day, bronchial airway responsiveness was measured by methacholine challenge test. The same challenge test was repeated on the 3rd post PAF day, 7th post PAF day and so on until the bronchial reactivity to methacholine came back to baseline level. PAF challenge and methacholine responsiveness was repeated on 2 occasions allowing at least 4 weeks between cycles. (Table 3)

3. 5 STATISTICAL ANALYSIS.

All values are listed as mean and standard error of the mean(sem) unless stated as G Mean (geometric mean). The analysis was performed by the Minitab Statistics System Fundamental version. The PC 35 value was calculated by computerised programme for PC. The comparison between Pre PAF and Post PAF was performed by paired t test and a p value of < 0.05 was considered to be statistically significant.

3. 6 RESULTS :

3. 6. 1 PAF CAUSES BRONCHOCONSTRICTION IN BOTH ATOPIC AND NONATOPIC SUBJECTS.

PAF when given by inhalation in a fixed dose of 96 ug caused marked bronchoconstriction in both atopic and non atopic subjects, in each PAF day of the three cycles. The maximum mean(sem) percentage falls in SGaw from post saline baseline value in nonatopic subjects (Table 4) were 47. 3 (8. 67), 49. 5 (9. 26), and 47. 17 (8. 19) in the three cycles respectively. (Fig. 4) There was no significant difference between the three cycles, suggesting that the PAF was equally effective in all cycles. The mean baseline SGaw in the same subjects on the study days of the three cycles (Table 5. 1, 5. 2, 5. 3) were comparable with no statistically significant difference (Figure 5. 1, 5. 2, 5. 3).

A similar response to PAF was also seen in the atopic subjects with the maximum mean (sem) percent fall in SGaw 41. 7 (6. 15), 48. 0 (6. 12) and 49. 29 (6. 33) respectively in the three cycles (Table 6) and (Fig. 6).

The mean Pre and Post saline baseline SGaw on the Methacholine challenge days were comparable and there was no significant difference (Table 7. 1, 7. 2, 7. 3), (Figure. 7. 1, 7. 2, 7. 3). Subjectively both atopic and nonatopic subjects who had shown a significant fall in SGaw also became wheezy. Two of the nonatopic subjects who were less wheezy were moderately flushed after PAF inhalation. After PAF inhalation neither atopic nor nonatopic subjects developed excessive airway secretions. Other characteristics of the aerosolised PAF included a rapid onset of action (1-3 min) and short duration (15-45 min).

3. 6. 2 EFFECT OF PAF ON METHACHOLINE RESPONSIVENESS:

There was no statistically significant difference between the mean PC 35 SGaw Methacholine before and after PAF challenge on day 1, day 3 and day 7 in the three cycles in nonatopic subjects (Table 8. 1, 8. 2, 8. 3)). However two subjects(JM Figure 8. 1, DS Figure 8. 5) of the nonatopic healthy volunteers did show increased bronchial responsiveness(Fig. 8).

Amongst the atopic asymptomatic subjects there was no statistically significant difference in PC 35 SGaw of methacholine before and after PAF challenge on day 1, day 3 and day 7 of the three cycles (Table 9. 1, 9. 2, 9. 3).

One subject (CJ) show some increase bronchial responsiveness (Figures. 9. 1-9. 7).

The mean baseline SGaw and geometric mean PC35 SGaw Methacholine before and after PAF challenge on the study days of the three cycles in both nonatopic (Table 12) and atopic (Table 13) subjects were comparable. The PC35 SGaw Methacholine in the three cycles in nonatopic subjects were 6. 51, 8. 16 and 8. 1 respectively. The PC35 SGaw methacholine in the three cycles of atopic subjects were o. 99, 0. 67 and 1. 15 respectively. These values were much smaller than that of nonatopic subjects. This suggests that bronchial airway in atopic subjects is more hyper-responsive to methacholine than nonatopic subjects(109,110).

3. 6. 3 WHETHER BRONCHIAL AIRWAY RESPONSIVENESS IS REPRODUCIBLE?

Our study failed to show statistically significant increase in bronchial responsiveness to methacholine after PAF challenge. However the two nonatopic subjects (JM, DS), who showed a moderate degree of increase (<2 fold increase) in bronchial responsiveness in the two cycle, the temporal relationship in the subsequent cycles was not clear (Fig. 8. 1, 8. 5)

3. 7 DISCUSSION.

Although the aetiology of increased bronchial responsiveness is not well defined, its presence as a component of clinical asthma is firmly established . It has also been established that changes in airway reactivity, either through seasonal allergen exposure or when attenuated by therapy(115), are closely related to the clinical expression of asthma. What is not certain, however, is what mediators are involved in the increase in nonspecific airway responsiveness. Recent evidence has given PAF as a unique role in this regard.

Cuss et al(59) have suggested that PAF may be involved in the changes in bronchial responsiveness. They demonstrated enhanced airway responsiveness in normal subjects for several weeks after PAF inhalation. This was in part confirmed by Rubin et al(60) who reported an increase in bronchial airway responsiveness in normal subjects, but not asthmatics 1 hour after PAF challenge. Although nonspecific bronchial responsiveness is increased in normal subjects, the fact that Rubin et al showed that asthmatic patients did not have increased bronchial responsiveness following PAF inhalation makes it unique as a mediator. All other known mediators cause immediate bronchoconstriction in asthmatic patients but in few normal subject. Importantly it has been reported to induce increase in nonspecific bronchial airway responsiveness, but mainly in nonasthmatic subjects. Stenton et al (113) also reported an increase in airway responsiveness following PAF inhalation,which were poorly sustained and not reproducible.

Our study showed contrasting evidence to the concept that PAF can increase bronchial responsiveness in normal subjects, as shown by Cuss et al (59) and Rubin et al (60). The fact that PAF inhalation had no effect on

bronchial airway responsiveness in our study supports the finding of Russell et al (107), Hopp et al (108), Jenkins et al (111) and Lai et al (114)

It is not known , however , if there is a threshold dose of inhaled PAF necessary to induce changes in airway responsiveness. For their study Russell et al (107) used five breaths of 200mg/L(30 ug delivered), Cuss et al (59) used a mean dose of inhaled PAF of 60 ug (27. 5-145 ug), given as five single breaths over 1 hour. Rubin et al(60) used a single breath of 1000 ug/L (delivered a dose of 23 ug). In our study all subjects inhaled eight breaths of 200mg/L (96 ug delivered). The difference in the results is not likely to be due to a discrepancy in the amount of inhaled PAF, as the dose was sufficient to cause marked bronchoconstriction. A recent report by Wardlaw et al (112) suggests that larger doses of inhaled PAF then used may be necessary to induce changes in nonspecific airway responsiveness.

It is also important to know that in the studies of Cuss et al(used pFEF 60-80 %) and Rubin et al(used SGaw & Vp30), the workers used a measurement of minimal changes in airway calibre to determine the changes in airway responsiveness. This is necessary because normal subjects often do not have marked changes to methacholine using the measurement of forced expiratory volume in one second. We used SGaw to measure the airway responsiveness and a similar measurement was used by Rubin et al . Although these tests are very similar, they also have a larger variability than FEV1.

Patient selection is another variable to be considered. All subjects studied by Cuss et al showed bronchoconstriction after PAF inhalation, with a greater than 40 % fall in Vp30. It is not clear whether their subjects were selected using these criteria. It is probable that not all subjects are similar in their response to PAF. The normal subjects studied by Rubin et al were less bronchial responsive to PAF compared to the subjects used by Cuss et al. It is conceivable that subjects with large airway response are more likely to have a prolonged change in airway responsiveness.

Our results on inhaled PAF in atopic subjects support the work of Rubin et al who failed to show an increase in bronchial responsiveness in asthmatics, 1 hour after PAF challenge. Chung and Barnes(115) have recently reported that in eight mild asthmatics there was no increase in airway responsiveness as a group, up to seven days following PAF inhalation. There were , however , selected asthmatic subjects who did have

increased airway responsiveness. In our study occasional atopic subjects had a moderate increase in bronchial airway responsiveness on post PAF day 1 and day 3 but a temporal relationship in subsequent cycles was less clear.

Airway hyperresponsiveness and airway eosinophilia are hallmarks of asthma. The study of PAF. induced bronchial hyperresponsiveness may give further insight into the pathogenesis of asthma,as PAF has many properties that make it a mediator of interest in the aetiology of asthma. The studies so far showed that inhaled PAF no doubt causes bronchconstriction but opinion still differs whether it cause increase bronchial airway hyperresponsiveness. Clearly further studies are required to clarify the potential role of PAF in the pathogenesis of hyperresponsiveness and to determine why there is difference in the results between various studies in this effect of PAF in normal healthy people,despite showing similar bronchoconstriction and cardiovascular responses. Also studies are needed to address the question whether the hyperresponsiveness is reproducible. Whatever conclusion is drawn from PAF challenge studies, its role in the pathogenesis of bronchial hyperresponsiveness and asthma awaits the bio-availability of potent PAF antagonist for clinical trials. It will then be possible to determine if specific PAF antagonist can inhibit specific allergens induced responses in the airway and increased reactivity resulting from such allergen challenge. Also their effects in clinical asthma can be assessed.

PART TWO

Aim: Platelet activating factor is a highly active mediator which has been implicated in allergic inflammation and bronchial asthma, possibly by interacting with eosinophils.

Eosinophils cause damage to the lung by releasing various proteins including eosinophilic cationic protein (ECP). Sputum levels of ECP have a positive correlation with the severity of the damage caused.

Whether measurement of the ECP in the serum has the same significance, is examined in the second part of the study.

CHAPTER FOUR.

SERUM EOSINOPHILIC CATIONIC PROTEINS IN NONATOPIC SUBJECTS BEFORE AND AFTER CHALLENGE.

4. 1. INTRODUCTION OF E. C. P.

The eosinophil was probably first observed in peripheral blood of humans in 1846 by Wharton Jones, an anatomist at Charing Cross Hospital. But it was Paul Ehrlich who discovered the best known characteristics of the cell. In 1879, Ehrlich described a leucocyte that avidly bound acidic dyes (116). He called this cell eosinophil because of the intense avidity of its granules for eosine, a brominated fluorescine derivative.

The presence of high numbers of eosinophils in diseases associated with parasite infection led to the widespread believe that the cell plays a unique and beneficial role in host defense against such organism. Eosinophils accumulate about parasites in vivo and deposit toxic granules contents on them (117). Both eosinophil granule proteins and oxygen metabolite have been shown to kill parasite worms(118). Similarly the high number of eosinophils in allergic diseases such as asthma coupled with knowledge that eosinophils associated enzymes can metabolize mediators of anaphylaxis including LTC4, histamine and platelet activating factor, led to the suggestion that one function of eosinophil was down regulation of the inflammation after immediate type hypersensitivity reaction (119). However as the knowledge of the toxicity of eosinophil to human tissue developed over the past decade, the view of the role in asthma changed. The eosinophil are now regarded by many as a potent pro-inflammatory cell with considerable tissue injuring potential and a primary mediator of epithelial injury and bronchial hyper-rectivity (120).

Eosinophils are rarely found in the normal human lower respiratory tract (121). However many inflammatory disorders of the lower respiratory tract are associated with an accumulation of eosinophils in the parenchyma. Eosinophil activity appears to be part of the inflammatory process in hypersensitivity pneumonitis histiocytosis X, eosinophilic pneumonia, idiopathic pulmonary fibrosis, sarcoidosis and the interstitial diseases associated with collagen-vascular or drug induced disorders (121,

122). Eosinophilia in bronchoalveolar lavage may be a marker of progressive lung damage in patients with idiopathic pulmonary fibrosis(123, 124). Eosinophil activation in the lung has also been related to the lung damage in adult respiratory distress syndrome(125). Rower and Colleagues(126) professed that activated eosinophils caused acute oedematous injury in isolated perfused rat lungs. Fujimoto and Co-worker (127) report on studies in a similar model that also involves the eosinophils participation in the production of microvascular injury. Activated eosinophils caused a biphasic pulmonary vascular response, an initial intense vasoconstriction was followed by increased pulmonary microvascular permeability that resulted in lung oedema.

How is the lung injury caused by the eosinophils and what process is involved to mediate lung injury?It is the identification and isolation of several highly cytotoxic secretory proteins, from the eosinophils(128, 129) which is mainly responsible for the tissue damage. The observation in several studies have shown a direct correlation between eosinophil number and activity on one hand and the severity of the disease such as asthma on the other(130-133)

Morphologically the human eosinophil is characterised by its content of eosine staining granules, some of which contain typical crystalloid formations, visible by electron microscopy. The granules contain four major proteins(128, 129), The eosinophil cationic proteins(ECP), Eosinophil peroxidase(EPO), Eosinophil protein X or eosinophil derived neurotoxin and major basic proteins make up to 90 % of all granule proteins. The major basic protein makes up the crystalloid in the granules, whereas the other proteins are located in the matrix of the granules . A further protein has been purified from human eosinophils (134-136). This protein is mainly found in the plasma membrane and forms the Charcot-Leyden crystals in tissue.

Eosinophils are one of the important cell type in asthma.When stimulated these cells produce several toxic proteins including major basic protein and eosinophilc cationic protein (ECP).In a recent study it has been reported that increased numbers of eosinophils are associated with

increasing severity of asthma.Furthermore ECP levels in bronchoalveolar lavage fluid is associated with the severity of symptoms(139).It has also been demonstrated that asthmatics with sputum eosinophilia are associated with increased levels of ECP in the sputum,thus measuring ECP in sputum is a good index of assessing the severity of asthma.This study was arranged to establish whether Inhaled PAF would have any effect on serum ECP levels in atopic and non-atopic subjects.

4.2 SUBJECTS DETAIL

Eight atopic and seven non-atopic asymptomatic subjects participated in this part of the study.The protocol was approved by the West of Scotland Hospital Ethical Committee Western Infirmary Glasgow.Written consent was obtained.All subjects were skin tested.

4.3 METHOD

The study was carried out concurrently with the PAF study previously described.Thirteen of the fifteen subjects (6 atopic & 7 non-atopic) were concurrent to both studies and 2 more atopic subjects were recruited.One visit to hospital was involved.Blood samples were taken during the first visit before and at 15, 30, 60, 120, and 180 minutes after a fixed dose of 96 ug of PAF was inhaled via Acorn nebuliser.Blood samples were collected by venepuncture and allowed to stand to clot at room temperature for 60 minutes.Serum was separated by centrifugation twice at 13050 g for 10 minutes.The sample was then stored at -20 C for analysis in batches.

The analysis was done by Pharmacia ECP RIA (Radioimmunoassay of eosinophil cationic protein).Pharmacia ECP RIA is a double antibody radioimmunoassay.In this process ECP in the sample competes with a fixed amount of 125-I labelled ECP for binding sites of specific antibody.Bound and free ECP are separated by the addition of a second antibody immunosobent followed by centrifugation and decanting.

The radioactivity in the pellet is then measured and is inversely proportional to the quantity of ECP in the sample.

4.3.1 REAGENTS

Each package of Pharmacia ECP RIA contains reagents for 50 assay tubes, sufficient for 19 samples and one standard curve in duplicate.

All reagents are ready for use and should be stored at 2-8 C until the expiry date on the labels.

Standard ECP (human) O ug/l, 5 ml	1 vial
2 ; 5 ; 15 ; 100 ; 200 ug/ml, 0.5 ml each	5 vials
Anti ECP (anti-serum raised in rabbit) 3 ml colour coded yellow	1 vial
ECP 125 I 12ug, 43.7kbq (1.2 uci) at date of manufacture, 3 ml colour coded blue	1 vial
Decanting suspension (sepharose anti-rabbit IgG raised in sheep), 220 ml 65	1 vial

4.3.2 TEST PROCEDURE :

Assay standards, control sera and unknowns in duplicate. Prepare a standard curve on each assay unit.

4.3.3 WORKING STEPS

UNKNOWNS/CONTROL		STEPS	STANDARDS
1.	Standards	50 ul	
2.	Unknown sample or control	-	50 ul
3.	ECP 125-I (c.Coded Blue)	50 ul	50 ul
4.	Anti-ECP(c.Coded Yellow)	50 ul	50 ul

CHECKPOINT content of all tubes should now be green.

5. Shake the rack to ensure mixing. Incubate for 3 hours at room temperature.

6. Decanting suspension 2 ml 2 ml

SHAKE THE VIAL TO MAKE THE SUSPENSION HOMOGENEOUS BEFORE USE

7. Incubate for 1/2 hour at room temperature.

8. Centrifuge for 10 minutes at 1500 * g.Decant the tubes immediately in one movement and let stand for 1/2 minute upside down on absorbent paper.

9. Determine the radioactivity.

CALCULATION OF RESULTS:

The result were calculated by a computerised programme called 2+2 logistic programme.

4.4 RESULTS :

The average ECP levels in non-atopic subjects before PAF challenge with mean (sem) was 8.3 (3.6) while the levels at 15, 30, 60, 120, and 180 minutes post PAF challenge were 5.4 (2.1), 5.6 (1.3), 5.7 (1.2), 5.6 (1.2), and 4.6 (0.97) ug/L (Table 10) respectively.There was no statistically significant difference in serum ECP levels before and after PAF inhalation.There were great individual variations in the ECP levels the highest being 28 and the lowest as 2.4 ug/L(Figure 10).

Amongst the atopic subjects the mean serum ECP level before PAF challenge mean(sem) was 4.9(0.7), while the mean(sem) levels at 15, 30, 60, 120, and 180 minutes post PAF challenge were 3.5(1.0), 4.6(0.7), 4.2(0.7), 4.6(0.9), and 4.6(0.9) ug/L(table 11) respectively.There was no statistically significant difference in serum ECP levels before and after PAF inhalation (Figure 11).

4.5 DISCUSSION:

PAF is a highly active mediator which has been implicated in allergic inflammation and asthma, possibly by interacting with eosinophils. Eosinophils can cause damage to lung tissue by releasing various proteins including ECP. The ECP has shown a positive correlation with late response after allergens challenge in asthma. It has been suggested that measurement of ECP may be used to predict the occurrence of a late asthmatic reaction(137)

A relation between airway hyper-responsiveness and activity of eosinophils is suggested by a study(137) in atopic subjects with seasonal allergic symptoms.In these patients there was a correlation between the rise in serum ECP concentration during pollen season and increased airway responsiveness(138).In the same study a group treated by immunotherapy had no changes in serum ECP levels and less responsiveness.Recently raised ECP levels in sputum has been demonstrated in asthmatics who had sputum eosinophilia(139), thus suggesting the importance of ECP levels in bronchoalveolar lavage fluid.This is an invasive procedure and is not always feasible.To see whether relatively easier measurement of ECP levels in blood serve the same purpose, we conducted this study to measure ECP levels in serum before and after PAF challenge up to three hours.We failed to show any significant change after PAF challenge in either atopic or non-atopic subjects studied up to three hours post PAF inhalation.It is possible that PAF may not be effective on the circulating eosinophils.This study suggested that serum ECP level is not a good index for assessing the effect of PAF on bronchial airway.A recent study by Mokte et al(140) also suggested that sputum ECP level and blood eosinophils may be more useful markers of expiratory lung flows than serum ECP in patients with chronic bronchial asthma.The importance of ECP in clinical practice needs further evaluation.

APPENDIX

Name	Age (yrs.)	Sex	Height (cm)	Wt (kg)	Skin test (+ to)
SG	32	F	168	64	Pol.HD.
MW	21	F	158	6	HD. Pol.Cat.Flower
JS	28	F	164	65	Cat. HD.
CR	38	F	165	66	HD.Cat.Pol.
CJ	31	F	167	68	Cat.Gr.Fea.H.D.M.Pol
MM	27	F	169	64	HDM.Gr.Hors.Cat.Asp.Pol
LM	28	F	157	58	Gr.HD.Fea.
Mean	29.3		164	64.7	

HD = House dust *Pol = Pollen* *HDM = House dust mites*
Gr = Grass *Asp = Aspergillous* *Fea = Feather*

TABLE 1. The demographic data of the atopic subjects

Name	Age (yrs.)	Sex	Height (cm)	Skin test
JM	38	F	170	-
EH	34	F	166	-
CO	22	M	170	-
JJ	27	F	163	-
DS	21	M	187	-
MI	36	M	168	-
Mean	29.7		170.7	

– = NEGATIVE

TABLE 2. Demographic data of the nonatopic subjects

Study day	PAF Inhalation	Methacoline Challenge
1	-	+
2	+	-
3	- Post PAF Day 1	+
5	- Post PAF Day 3	+
9	- Post PAF Day 7	+
12	- Post PAF Day 10	+
+/- =	Methacoline or PAF challenge given/not given	

TABLE 3. Showing detail about the protocol of PAF project

Name	CYCLE 1	CYCLE 2	CYCLE 3
JM	41	37	39
JJ	68	74	62
EH	20	27	26
CO	70	65	70
DS	58	70	62
MI	27	24	24
Mean	47.3	49.5	47.3
(sem)	8.67	9.29	8.19

TABLE 4 MAXIMUM PERCENTAGE FALL IN SGaw AFTER PAF CHALLENGE IN EACH OF THE THREE CYCLES IN NON-ATOPIC SUBJECTS

Name	Pre PAF	Post PAF		
		D1	D2	D3
JM	1.54	1.34	1.29	1.24
JJ	1.52	1.46	1.87	1.90
EH	1.42	1.22	1.33	1.34
CO	1.45	1.54	1.32	1.39
DS	1.31	1.34	1.34	1.29
MI	1.32	1.43	1.39	1.36
Mean	1.42	1.39	1.42	1.42
sem	0.03	0.04	0.09	0.098

TABLE 5.1 BASELINE SGaw IN NON-ATOPIC SUBJECTS ON STUDY DAYS OF THE FIRST CYCLE.

Name	Pre PAF	Post PAF		
		D1	D2	D3
JM	1.36	1.47	1.43	1.67
JJ	1.90	1.69	2.00	1.85
EH	1.34	1.23	1.22	1.32
CO	1.39	1.66	1.29	1.52
DS	1.29	1.61	1.09	1.27
MI	1.36	1.38	1.27	1.22
Mean	1.44	1.51	1.38	1.47
sem	(0.09)	(0.07)	(0.13)	(0.10)

TABLE 5.2 BASELINE SGaw IN NON-ATOPIC SUBJECTS ON STUDY DAYS OF THE SECOND CYCLE.

Name	Pre PAF	Post PAF		
		D1	D2	D3
JM	1.67	1.58	1.39	1.39
JJ	1.85	2.09	1.89	1.66
EH	1.32	1.22	1.11	1.15
CO	1.52	1.61	1.62	1.5
DS	1.27	1.36	1.14	1.41
MI	1.22	1.23	1.66	1.42
Mean	1.47	1.51	1.46	1.42
sem	(0.10)	(0.13)	(0.13)	(0.068)

TABLE 5.3 BASELINE SGaw IN NON-ATOPIC SUBJECTS ON STUDY DAYS OF THE THIRD CYCLE.

Name	CYCLE 1	CYCLE2	CYCLE3
CJ	50	45	42
SM	42	50	60
MM	27	65	48
JS	16	16	24
MW	42	44	40
CR	49	62	54
LM	66	54	77
Mean	41.7	48	49.3
(sem)	(6.15)	(6.12)	(6.33)

TABLE 6. MAXIMUM PERCENTAGE FALL IN SGaw AFTER PAF CHALLENGE IN EACH OF THE THREE CYCLES IN ATOPIC SUBJECTS.

Name	Pre PAF	Post PAF		
		D1	D3	D7
CJ	1.24	1.55	1.61	1.30
SM	1.45	1.00	0.82	0.89
MM	0.74	0.75	1.76	0.67
JS	1.82	1.76	1.79	1.79
MW	0.72	0.78	1.26	0.91
CR	1.13	1.26	1.19	1.29
LM	0.67	0.72	0.69	0.93
Avg	1.11	1.12	1.16	1.11
sem	(0.16)	(0.156)	(0.16)	(0.14)

TABLE 7.1 BASELINE SGaw IN ATOPIC SUBJECTS ON STUDY DAYS OF THE FIRST CYCLE.

Name	Pre PAF	Post PAF		
		D1	D3	D7
CJ	1.30	1.35	1.31	1.39
SM	0.89	1.20	1.28	1.49
MM	0.82	1.15	0.94	0.72
JS	1.79	1.40	1.78	2.10
MW	0.90	0.85	0.67	1.09
CR	1.29	1.39	1.13	1.20
LM	0.93	0.64	0.99	0.80
Avg	1.13	1.14	1.15	1.25
sem	(0.13)	(0.11)	(0.13)	90.17)

TABLE 7.2 BASELINE SGaw IN ATOPIC SUBJECTS ON STUDY DAYS OF THE SECOND CYCLE.

Name	Pre PAF	Post PAF		
		D1	D3	D7
CJ	1.39	1.46	1.29	1.45
SM	1.49	1.36	1.36	1.24
MM	0.72	0.75	0.68	1.18
JS	2.10	2.24	1.84	1.73
MW	1.09	0.99	0.99	1.16
CR	1.20	1.39	1.26	1.39
LM	0.80	0.63	0.80	0.64
Avg	1.25	1.26	1.17	1.25
sem	(0.17)	(0.20)	(0.15)	(0.13)

TABLE 7.3 BASELINE SGaw IN ATOPIC SUBJECTS ON STUDY OF THE THIRD CYCLE.

Name	Pre PAF	Post PAF		
		D1	D3	D7
JM	3.2	2.2	2.7	2.8
JJ	4.3	9.9	6.8	4.3
EH	9.7	14.1	8.3	22
CO	3.9	3.7	4.1	5.5
DS	5	3.6	5.4	5.5
MI	29.5	23.9	28.4	24.2
G.Mean	6.51	6.79	6.76	7.6

TABLE 8.1 PC 35 SGaw BEFORE AND AFTER INHALATION IN NON-ATOPIC SUBJECTS IN THE FIRST CYCLE.

$p > 0.05$ NS

Name	Pre PAF	Post PAF		
		D1	D3	D7
JM	4.3	3.4	3.9	4.8
JJ	4.3	7.9	4.5	3.2
EH	22	24.1	12.6	10.5
CO	5.5	4.6	6.6	3.04
DS	5.5	3.2	6.4	5.6
MI	24.5	30	17	25.8
G.Mean	8.16	8.1	7.36	6.42

TABLE 8.2 PC 35 SGaw BEFORE AND AFTER PAF INHALATION IN NON-ATOPIC SUBJECTS IN THE SECOND CYCLE.

$p > 0.05$ NS

Name	Pre PAF	Post PAF		
		D1	D3	D7
JM	4.8	3.4	3.7	5.7
JJ	8.4	4.2	4.01	3.7
EH	10.5	9.3	10.5	11
CO	4.6	4.3	4.03	5.9
DS	5.6	3.9	3.4	4.3
MI	25.8	27.1	31.1	28.2
G.Mean	8.1	6.3	6.4	7.4

TABLE 8.3 PC 35 $\overset{S}{A}$Gaw BEFORE AND AFTER PAF INHALATION IN NON-ATOPIC SUBJECTS IN THE THIRD CYCLE.

$p > 0.05$ NS

Name	Pre PAF	Post PAF		
		D1	D3	D7
CJ	3.8	3.4	2.3	4.4
SM	2.6	4.7	3.8	6.4
MM	0.9	1.3	2.3	0.8
JS	2.9	2.8	3.9	2.6
MW	0.4	0.3	0.1	0.3
CR	0.3	0.5	0.3	0.4
LM	0.3	0.1	0.3	0.13
G.Mean	0.99	0.98	0.95	0.99

TABLE 9.1 PC 35 ΔGaw BEFORE AND AFTER PAF INHALATION IN ATOPIC SUBJECTS IN THE FIRST CYCLE OF THE STUDY DAYS.

$p > 0.05$ NS

Name	Pre PAF	Post PAF		
		D1	D3	D7
CJ	2.8	2.8	3.5	3.8
SM	2.5	4.1	2.8	2.6
MM	0.9	1.3	0.7	0.5
JS	1.3	1.5	1.9	2.9
MW	0.2	0.2	0.4	0.4
CR	0.3	0.65	0.42	0.32
LM	0.13	0.12	0.08	0.33
G.Mean	0.67	0.86	0.78	0.89

TABLE 9.2 PC 35 ΔGaw BEFORE AND AFTER PAF INHALATION IN ATOPIC SUBJECTS IN THE SECOND CYCLE OF THE STUDY DAYS.

$p > 0.05$ NS

Name	Pre PAF	Post PAF			
		D1	D3	D7	D10
CJ	4.4	4.5	3.1	4.5	
SM	6.4	4.6	2	1.6	2.99
MM	0.8	0.4	0.2	0.5	
JS	2.6	2.9	3.8	3.4	
MW	0.3	0.1	0.1	0.4	
CR	0.4	0.31	0.14	0.2	0.38
LM	0.13	0.1	0.4	0.1	
G.Mean	1.15	0.79	0.75	0.89	

TABLE 9.3 PC 35 $\frac{S}{A}$Gaw BEFORE AND AFTER PAF INHALATION IN ATOPIC SUBJECTS IN THE THIRD CYCLE OF THE STUDY DAYS.

$p > 0.05$ NS

Name	Pre PAF	Post PAF				
		+15 min	+30 min	+60 min	+120 min	+180 min
JM	2.4	2.6	2.2	2	3	3
JJ	0	ND	2.2	3.2	2.4	2.2
EH	2.6	3.2	3.2	2.6	3.2	1.8
CO	9	7	7	10	6	5
DS	28	17	10	8	10	8
RJ	6	4	4.5	6	4.5	4.5
CD	10	4	10	8	10	8
Mean	8.3	5.4	5.6	5.7	5.6	4.6
(sem)	(3.6)	(1.3)	(1.3)	(1.18)	(1.22)	(0.97)

TABLE 10. SERUM EOSINOPHILIC CATIONIC PROTEIN LEVELS IN NON-ATOPIC SUBJECTS BEFORE AND AFTER PAF INHALATION.

$p > 0.05$ NS

Name	Pre PAF	Post PAF				
		+15 min	+30 min	+60 min	+120 min	+180 min
CJ	8.5	8.5	8	8.5	8.5	10
SM	3	3.2	3.2	3.2	3.2	1.8
MM	6	ND	6.5	6	5.5	5
JS	2.6	ND	2.4	2.6	2.6	3
MW	5.5	4.5	4.5	4	3.2	5
CR	6	6	5	4	7	6
IP	3.2	2.2	3	3	3	2.4
AJ	4.5	3.6	4.5	2.6	4	4
Mean	4.9	3.5	4.6	4.2	4.6	4.6
(sem)	(0.70)	(1.0)	(0.66)	(0.72)	(0.76)	(0.91)

TABLE 11. SERUM EOSINOPHILIC CATIONIC PROTEIN (ECP) LEVELS IN ATOPIC SUBJECTS BEFORE AND AFTER PAF INHALATION.

$p > 0.05$ NS

	Pre PAF	Post PAF		
		D1	D3	D7
CYCLE 1				
Mean Base line SGaw	1.42	1.39	1.42	1.42
(sem)	(0.03)	(0.04)	(0.09)	)0.09)
PC 35 SGaw (mg/ml)	6.51	6.79	6.79	7.6
CYCLE 2				
Mean Base line SGaw	1.44	1.51	1.38	1.47
(sem)	(0.09)	(0.07)	(0.13)	(0.10)
PC 35 SGaw (mg/ml)	8.16	8.1	7.36	6.42
CYCLE 3				
Mean Base line SGaw	1.47	1.51	1.46	1.42
(sem)	(0.10)	(0.13)	(0.13)	(0.07)
PC 35 SGaw (mg/ml)	8.1	6.3	6.4	7.4

TABLE 12. MEAN BASE LINE SGaw AND PC 35 SGaw BEFORE AND AFTER PAF INHALATION IN NON-ATOPIC SUBJECTS ON STUDY DAYS OF THE THREE CYCLES.

$p > 0.05$ NS

	Pre PAF	Post PAF		
		D1	D3	D7
CYCLE 1				
Mean Base line SGaw	1.11	1.12	1.16	1.11
(sem)	(0.16)	(0.15)	(0.16)	(0.14)
PC 35 SGaw (mg/ml)	0.99	0.98	0.95	0.99
CYCLE 2				
Mean Base line SGaw	1.13	1.14	1.16	1.25
(sem)	(0.13)	(0.11)	(0.13)	(0.17)
PC 35 SGaw (mg/ml)	0.67	0.86	0.78	0.89
CYCLE 3				
Mean Base line SGaw	1.25	1.26	1.17	1.25
(sem)	(0.17)	(0.020)	(0.15)	(0.13)
PC 35 SGaw (mg/ml)	1.15	0.79	0.75	0.89

TABLE 13 MEAN BASE LINE SGaw AND PC 35 SGaw BEFORE AND AFTER PAF INHALATION IN ATOPIC SUBJECTS ON STUDY DAYS OF THE THREE CYCLES.

$p > 0.05$ NS

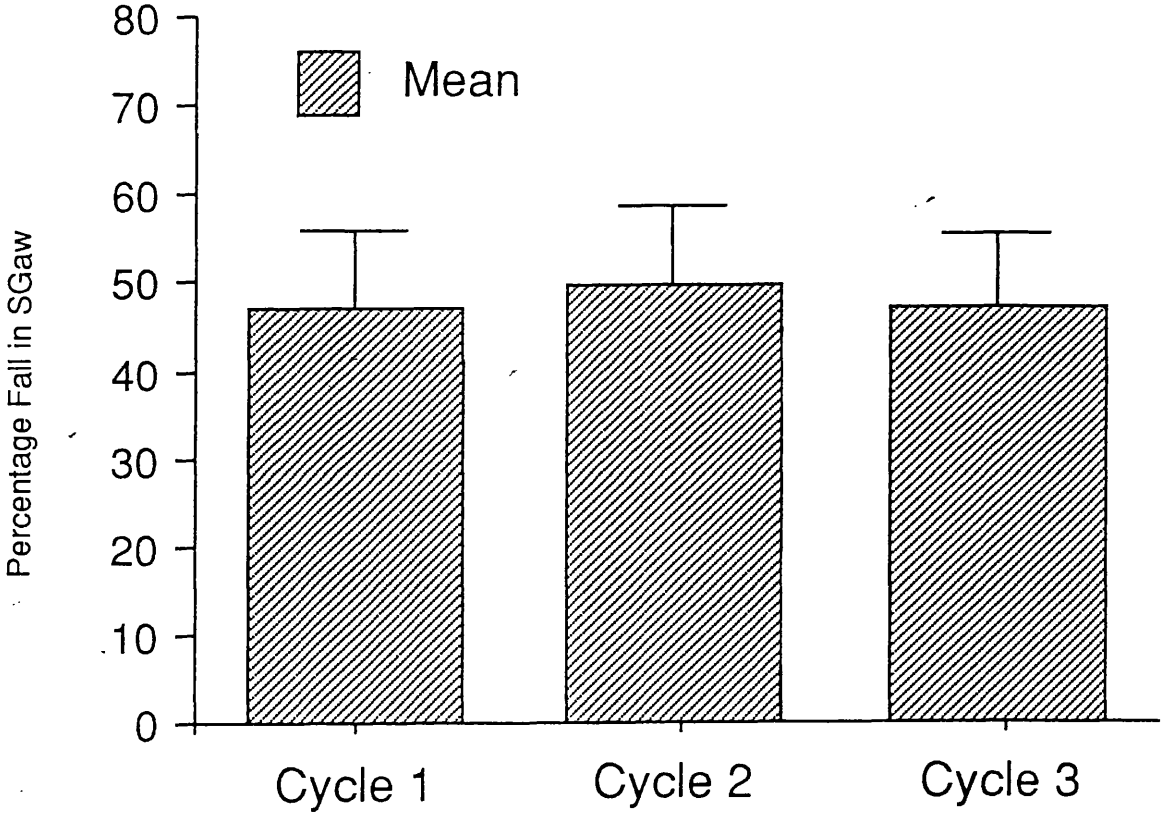


Figure 4 Mean maximum percent fall in SGaw after PAF challenge in each of the three cycles in non atopic subjects.

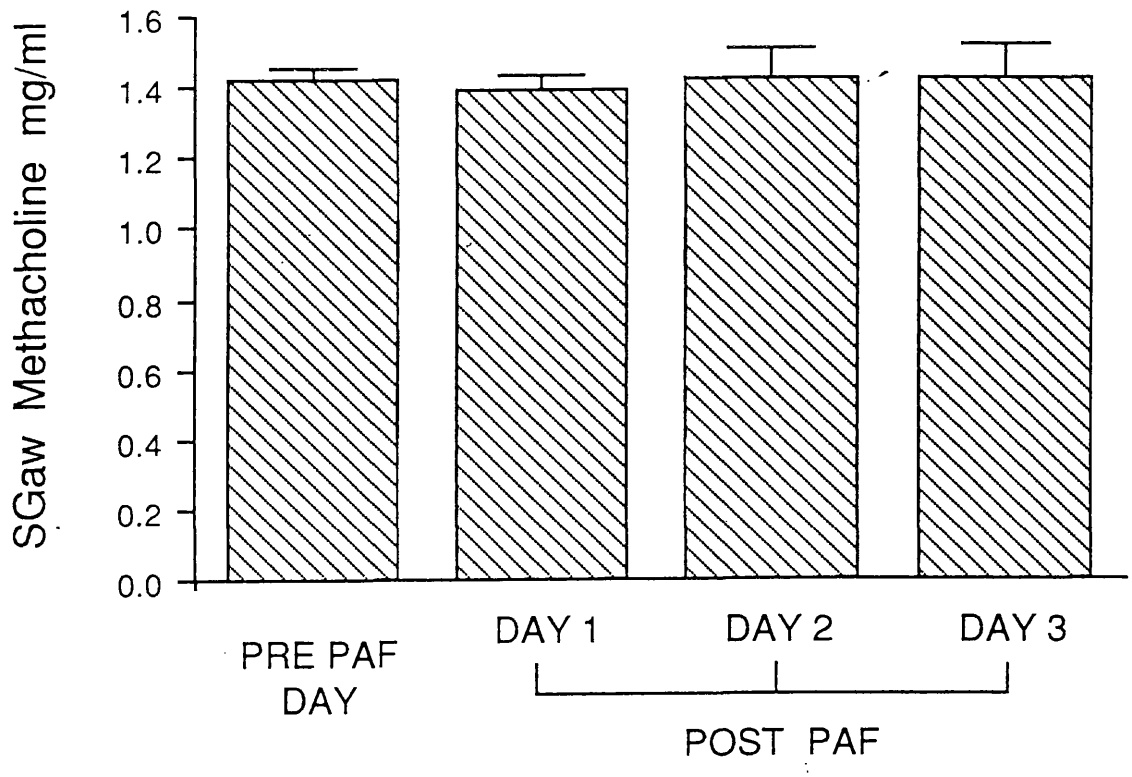


Figure 5.1 Mean baseline SGaw in non atopic subjects on study days of the first cycle

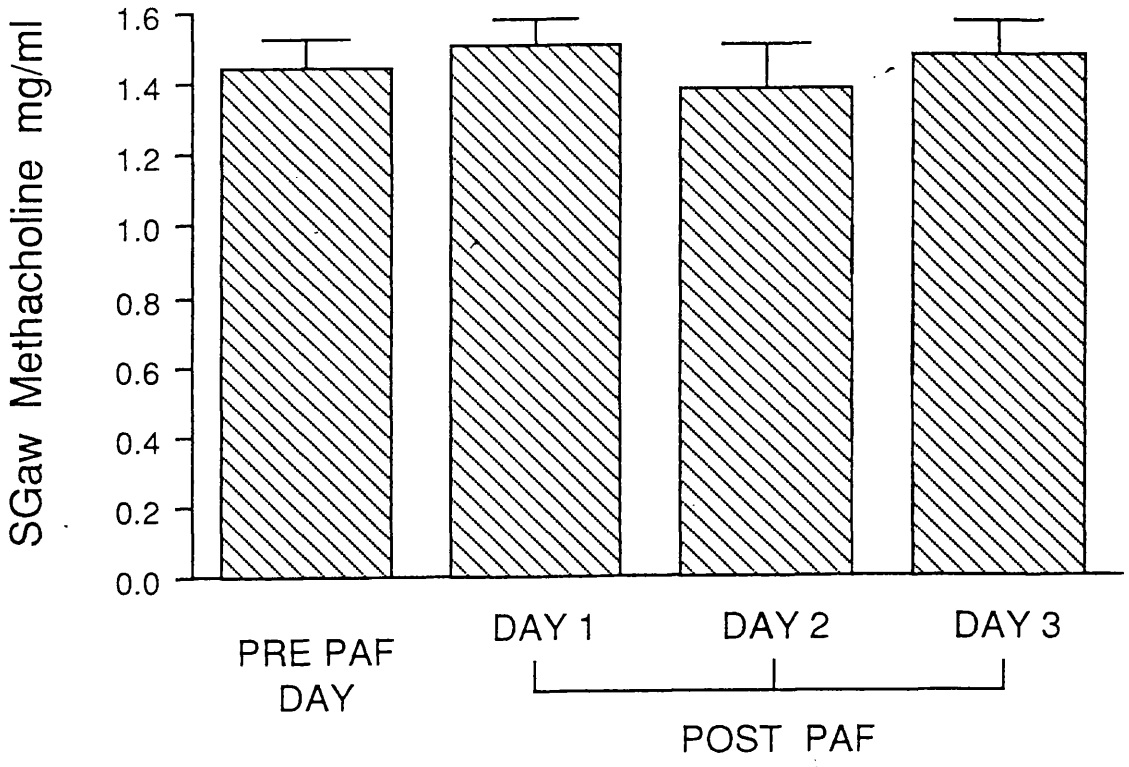


Figure 5.2 Mean baseline SGaw in non atopic subjects on study days of the second cycle

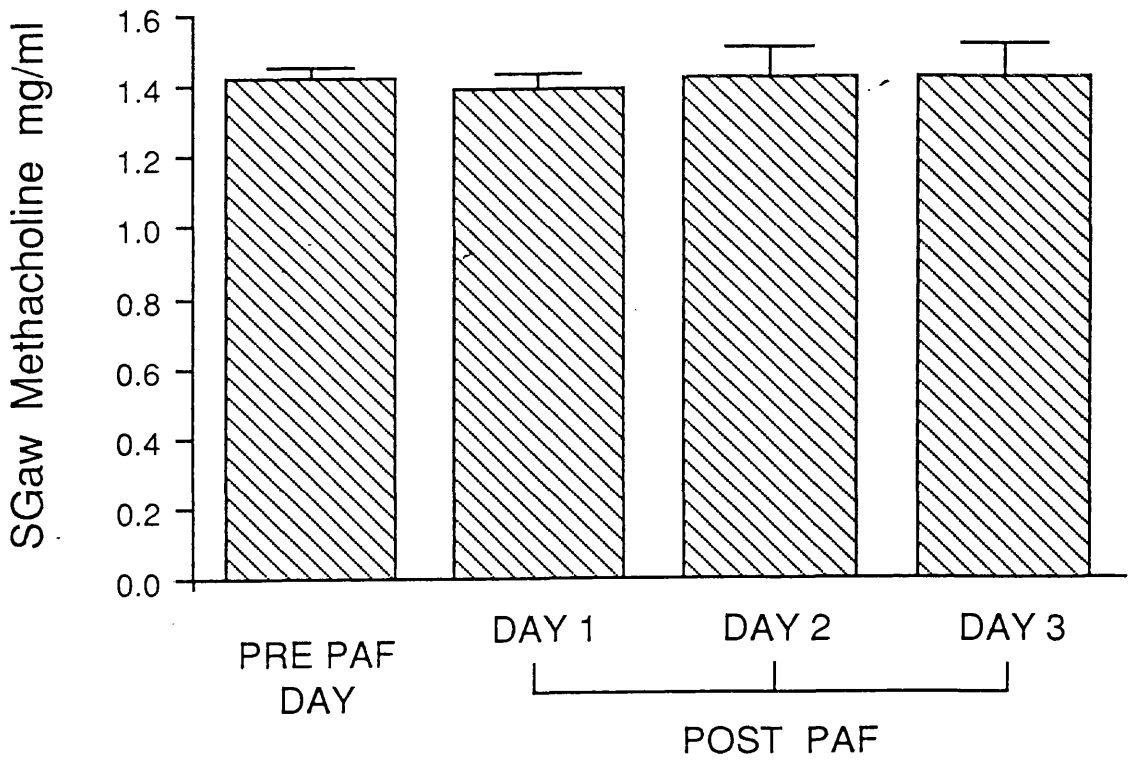


Figure 5.3 Mean baseline SGaw in non atopic subjects on study days of the third cycle

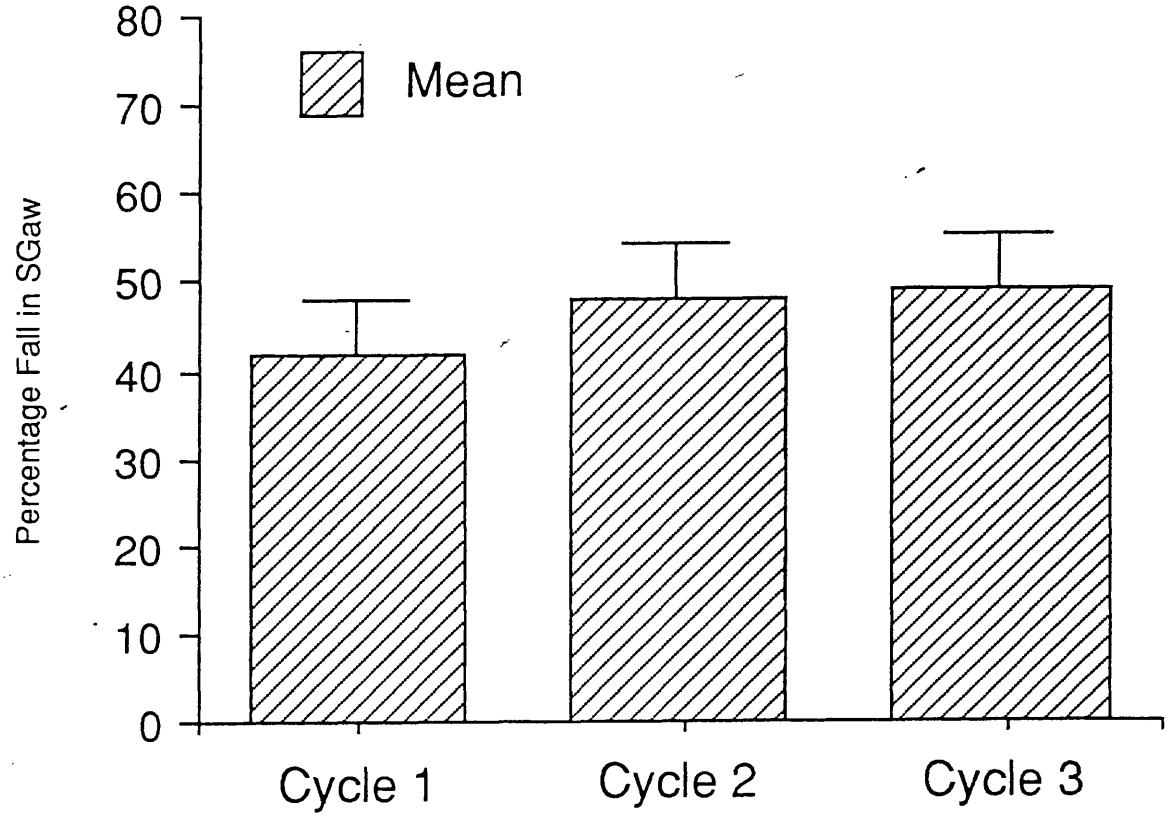


Figure 6 Mean maximum percent fall in SGaw after PAF challenge in each of the three cycles in atopic subjects.

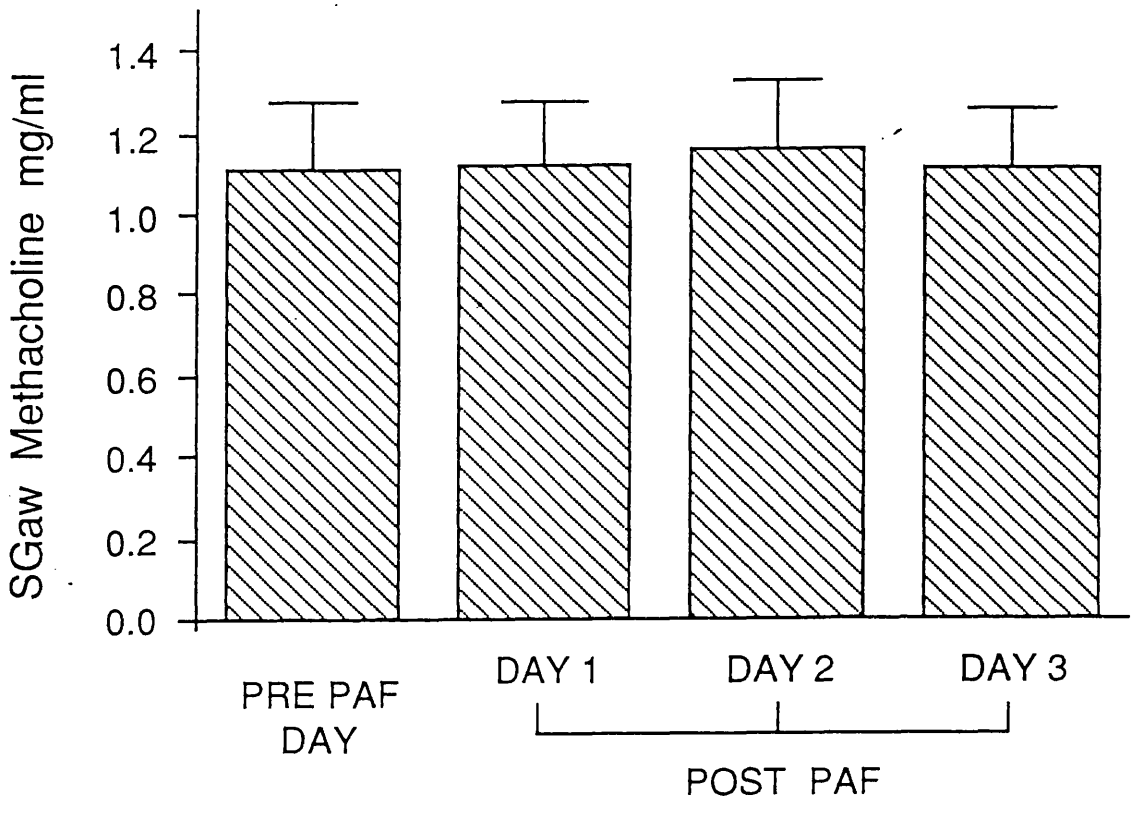


Figure 7.1 Mean baseline SGaw in atopic subjects on study days of the first cycle

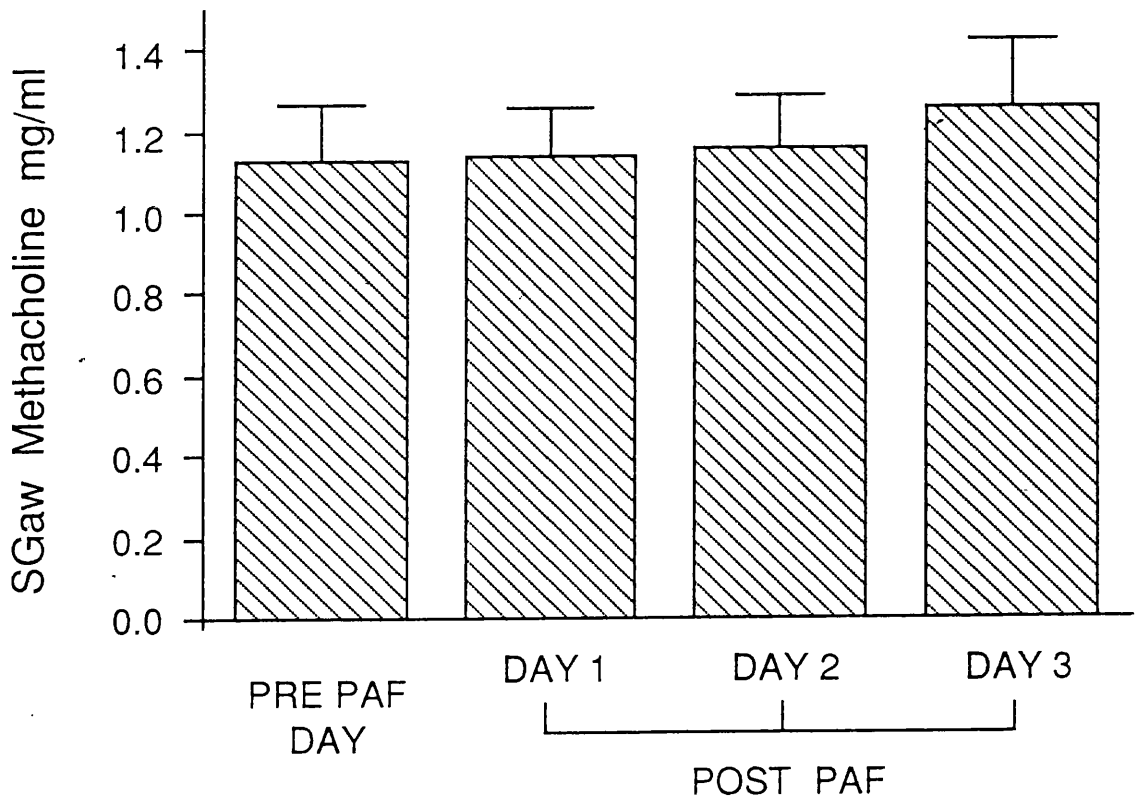


Figure 7.2 Mean baseline SGaw in atopic subjects on study days of the second cycle

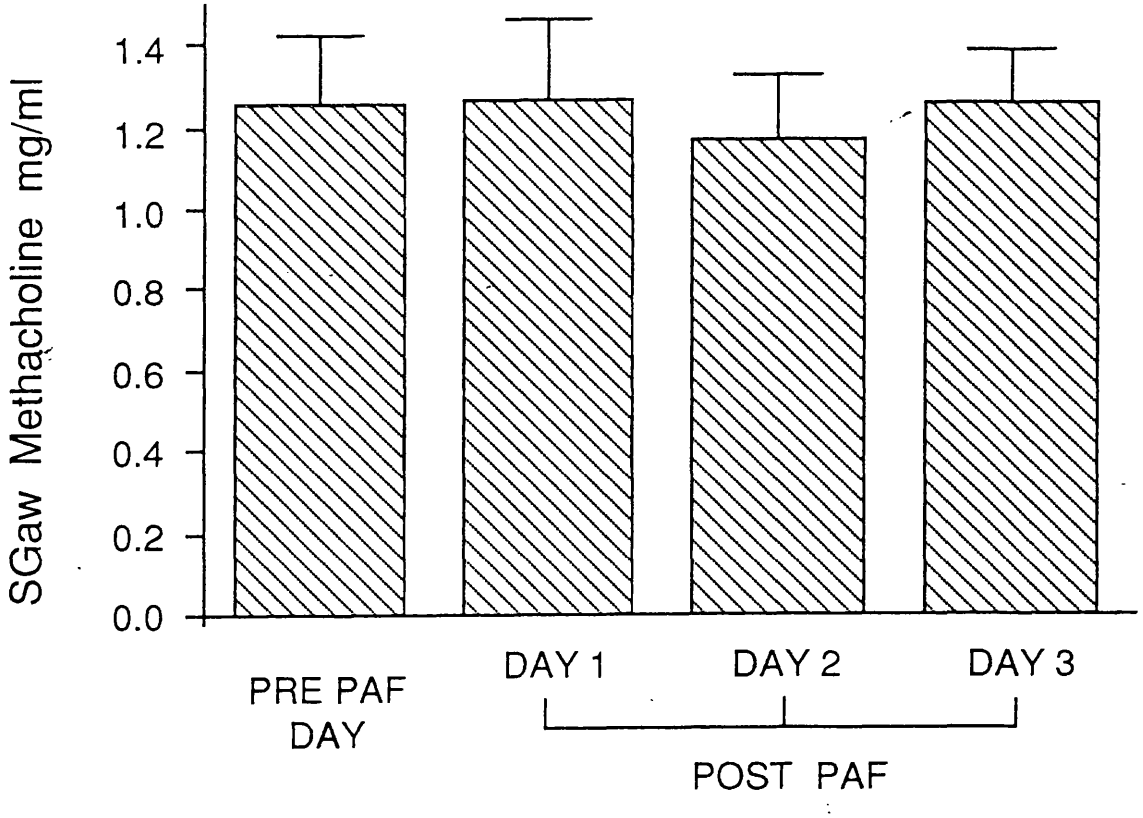


Figure 7.3 Mean baseline SGaw in atopic subjects on study days of the third cycle

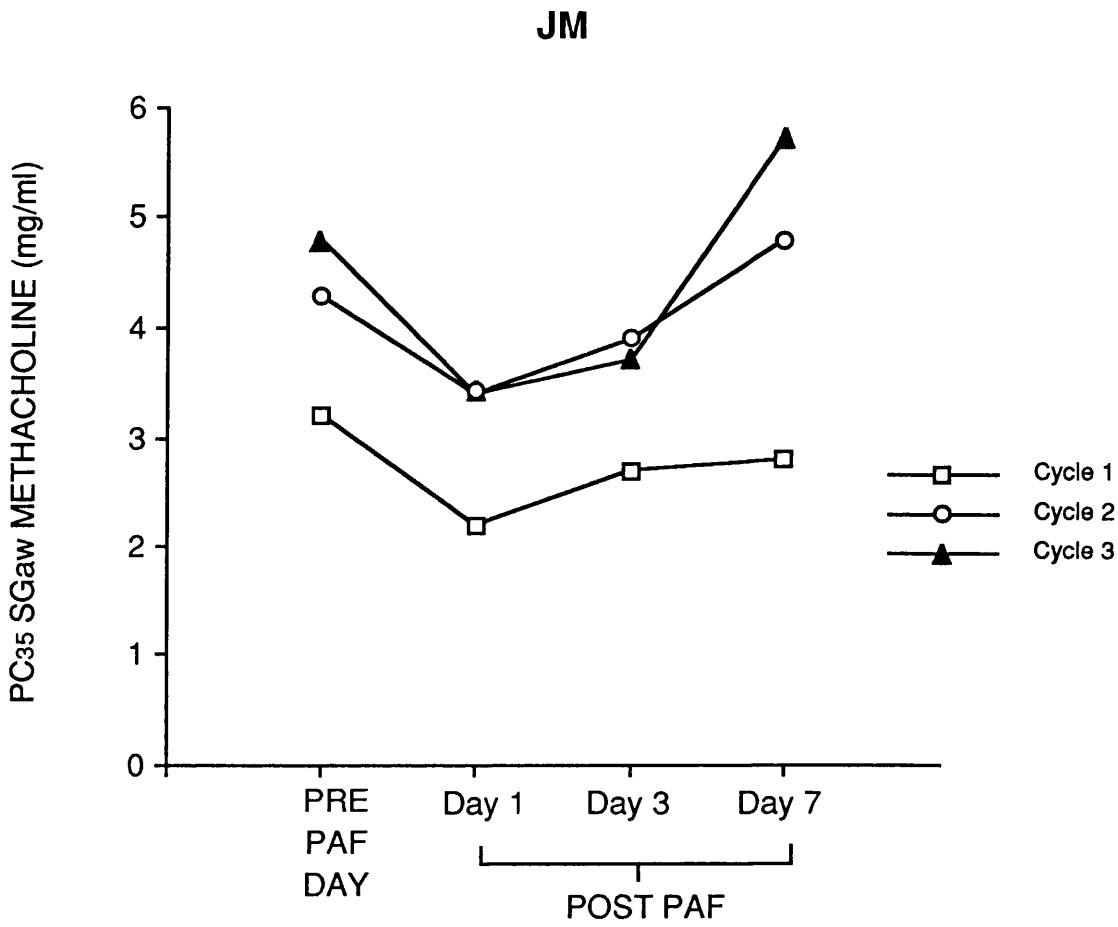


Figure 8.1 PC_{35} SGaw Methacholine before and after PAF challenge in non-atopic subject (JM) in the three cycles

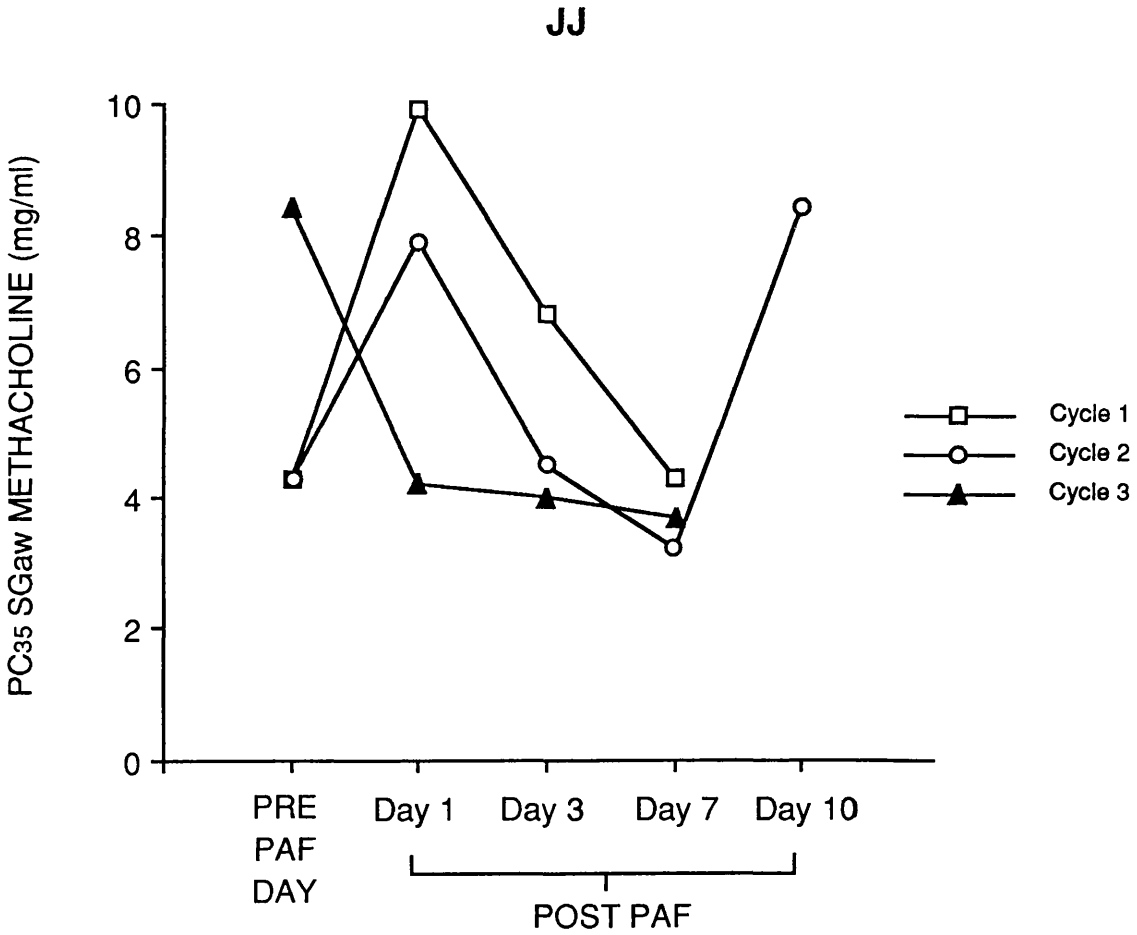


Figure 8.2 PC_{35} SGaw Methacholine before and after PAF challenge in non-atopic subject (JJ) in the three cycles

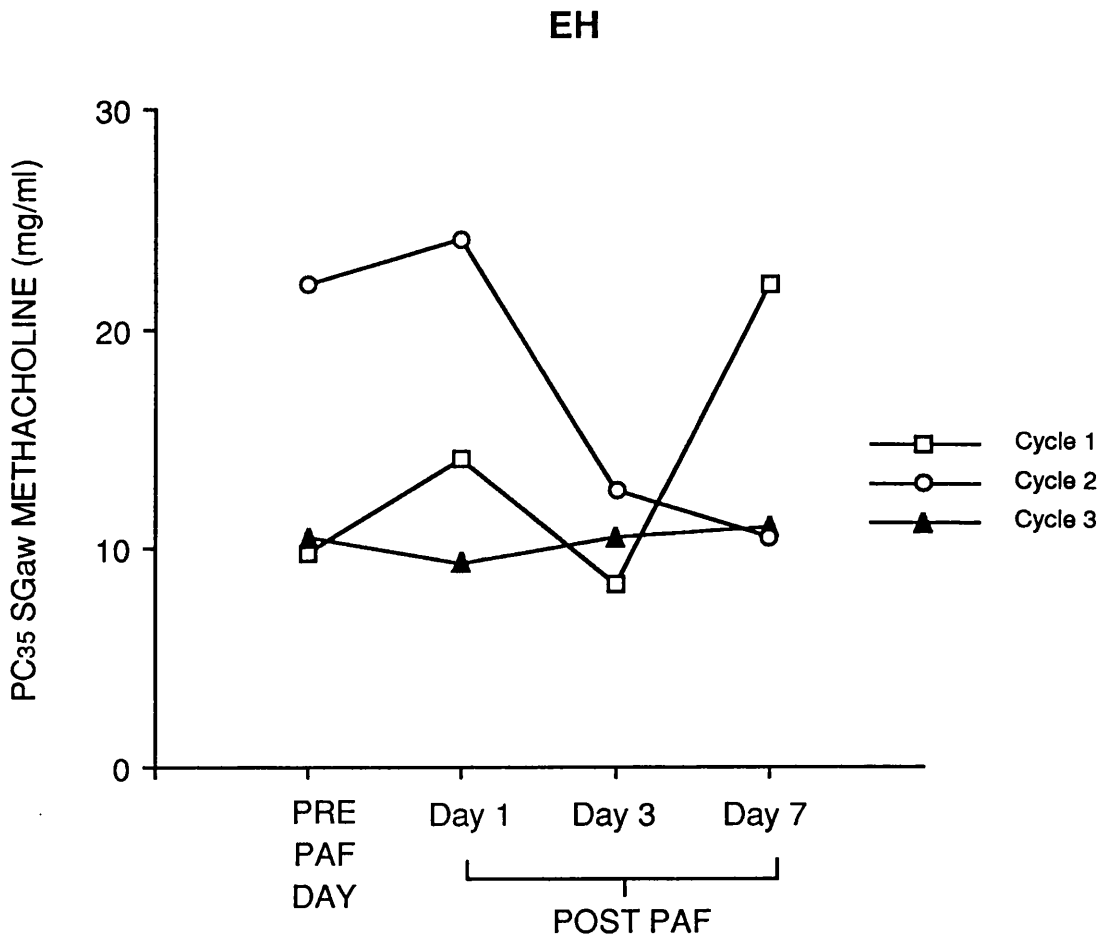


Figure 8.3 PC_{35} SGaw Methacholine before and after PAF challenge in non-atopic subject (EH) in the three cycles

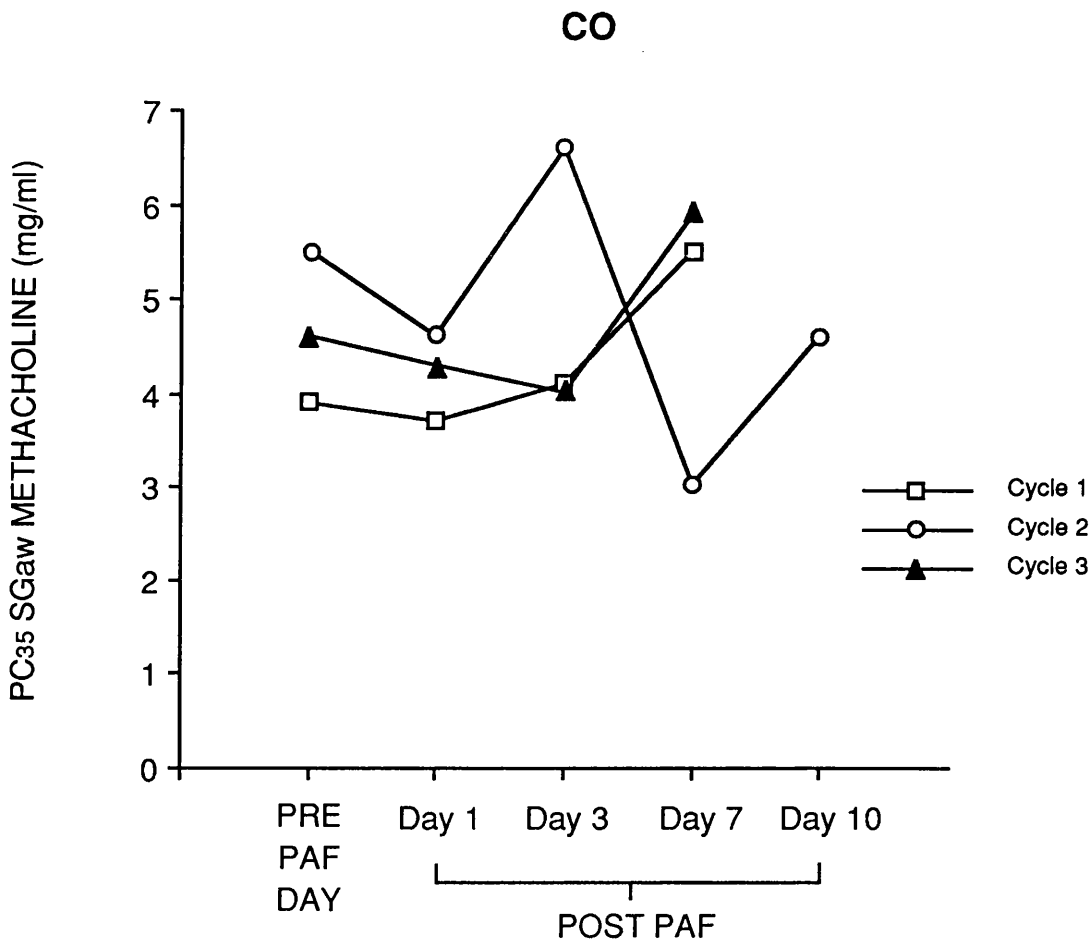


Figure 8.4 PC_{35} SGaw Methacholine before and after PAF challenge in non-atopic subject (CO) in the three cycles

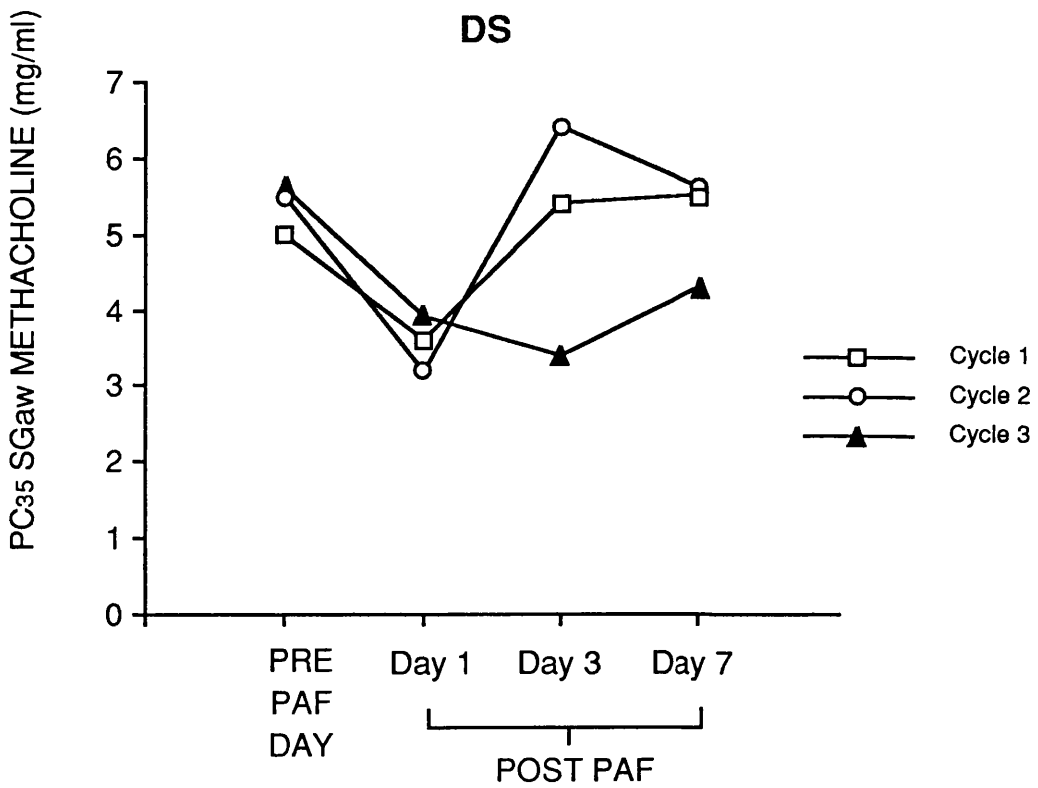


Figure 8.5 PC_{35} SGaw Methacholine before and after PAF challenge in non-atopic subject (DS) in the three cycles

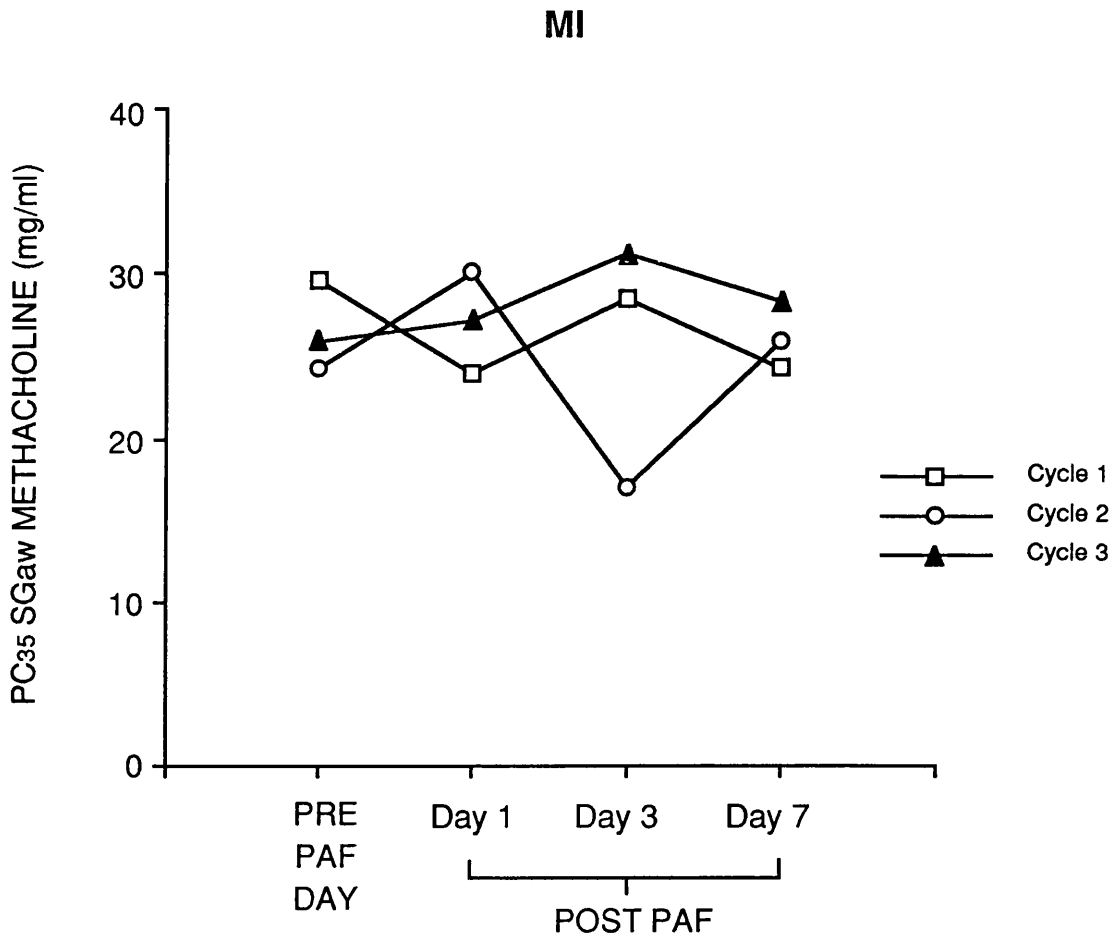


Figure 8.6 PC_{35} SGaw Methacholine before and after PAF challenge in non-atopic subject (MI) in the three cycles

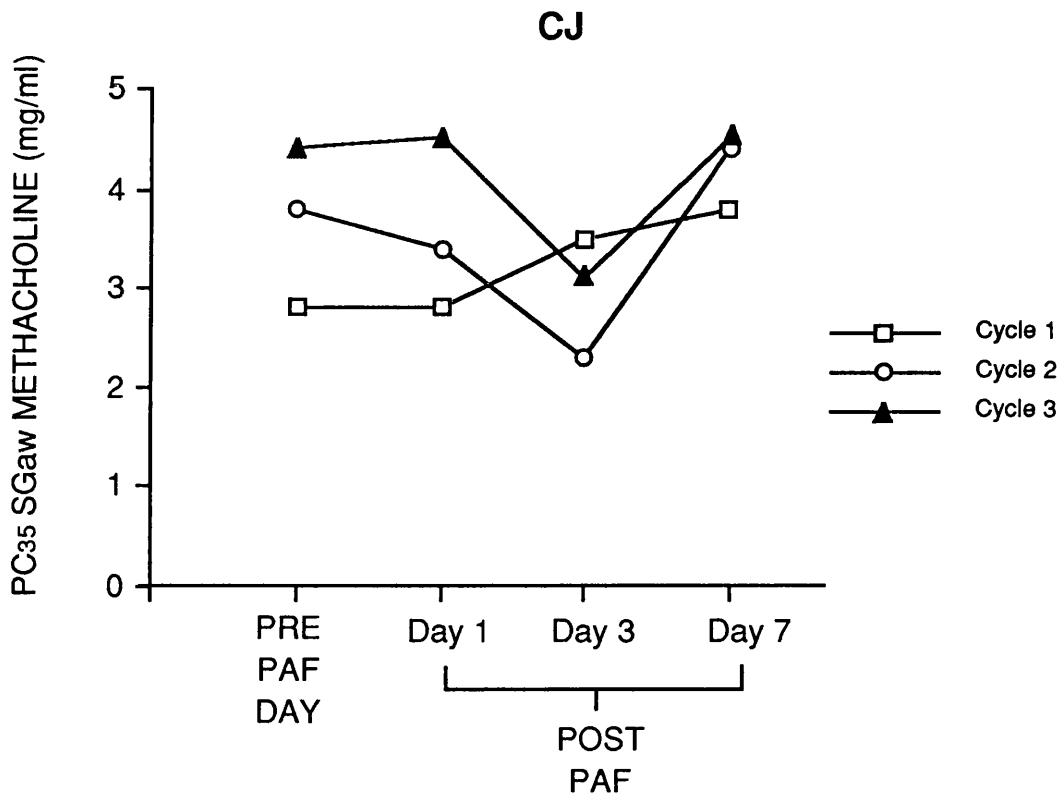


Figure 9.1 PC_{35} SGaw Methacholine before and after PAF challenge in atopic subject (CJ) in the three cycles.

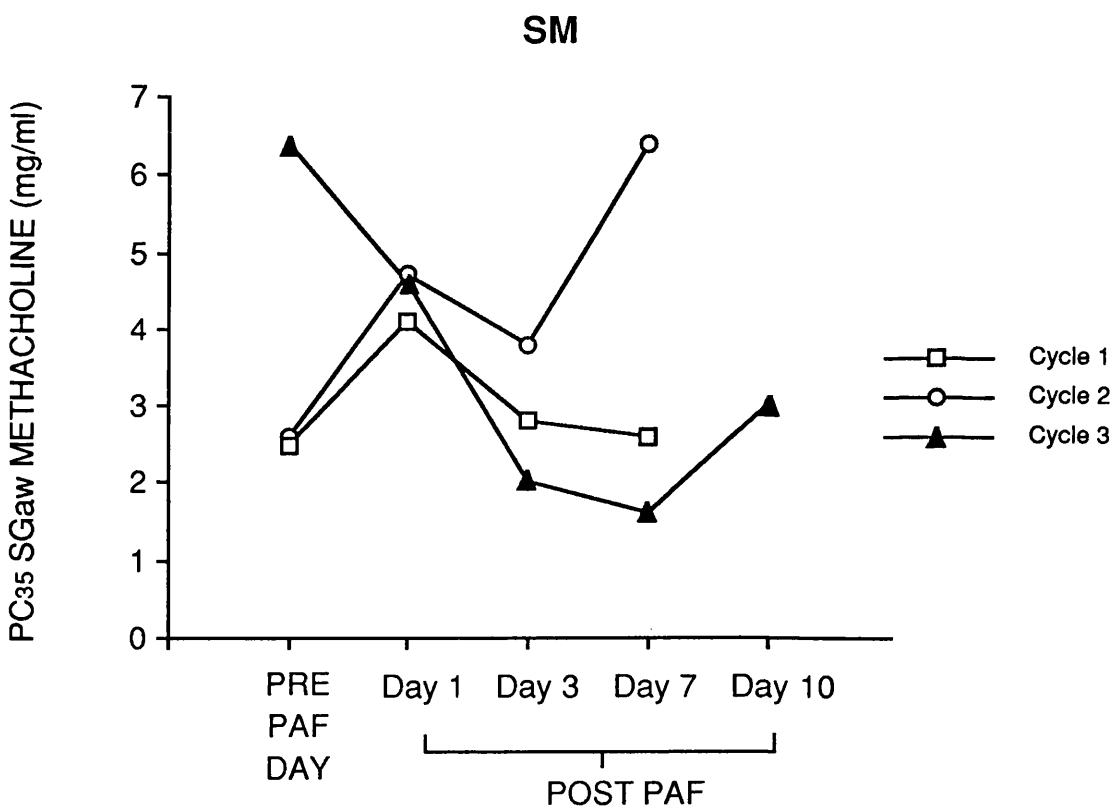


Figure 9.2 PC_{35} SGaw Methacholine before and after PAF challenge in atopic subject (SM) in the three cycles.

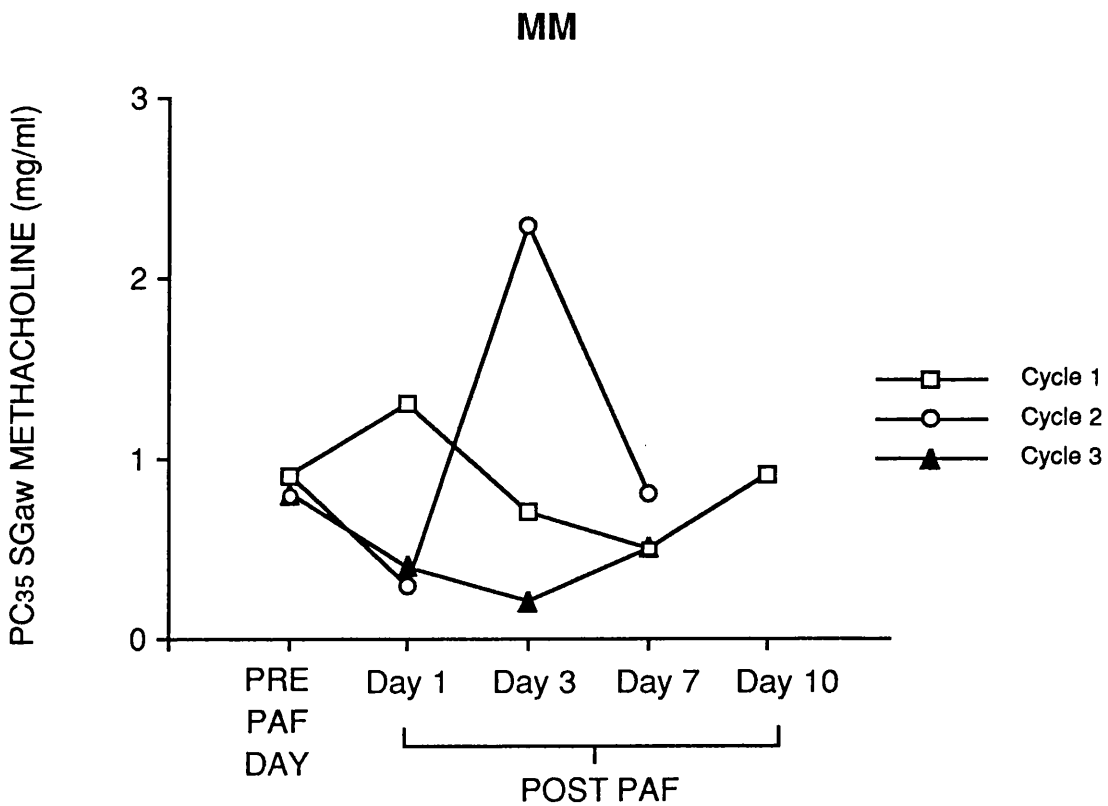


Figure 9.3 PC_{35} SGaw Methacholine before and after PAF challenge in atopic subject (MM) in the three cycles.

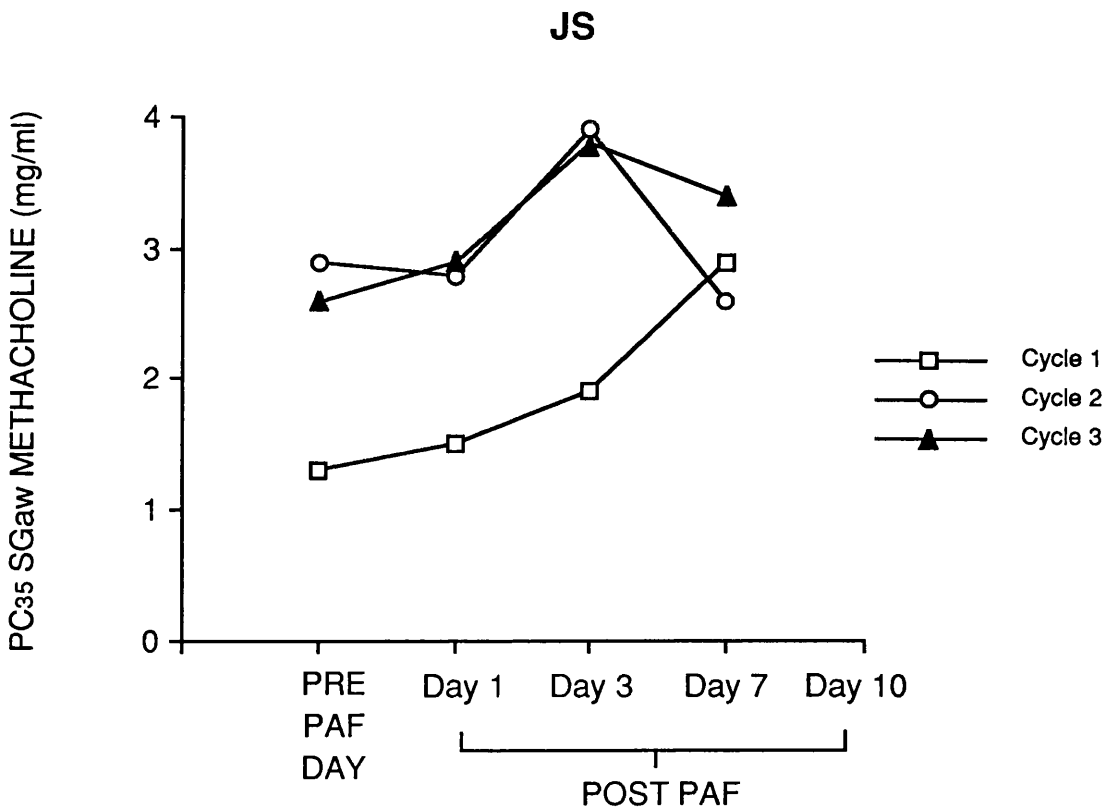


Figure 9.4 PC_{35} SGaw Methacholine before and after PAF challenge in atopic subject (JS) in the three cycles.

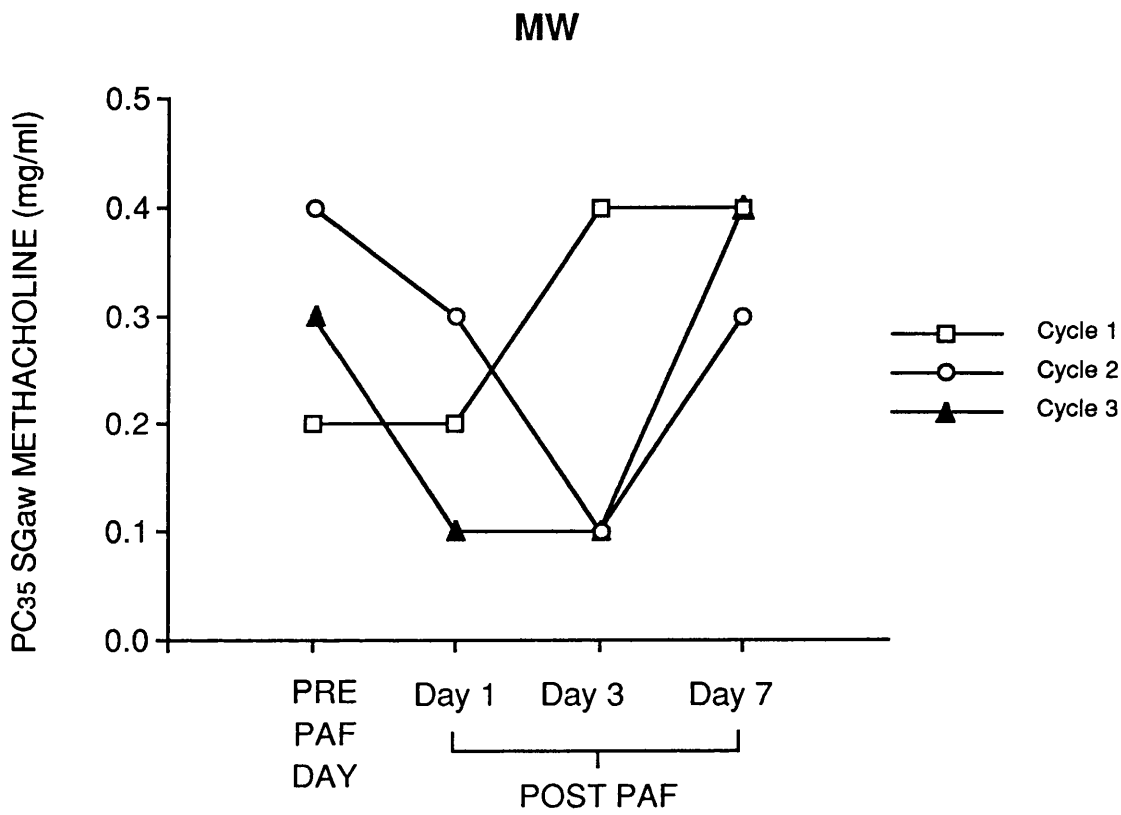


Figure 9.5 PC_{35} SGaw Methacholine before and after PAF challenge in atopic subject (MW) in the three cycles.

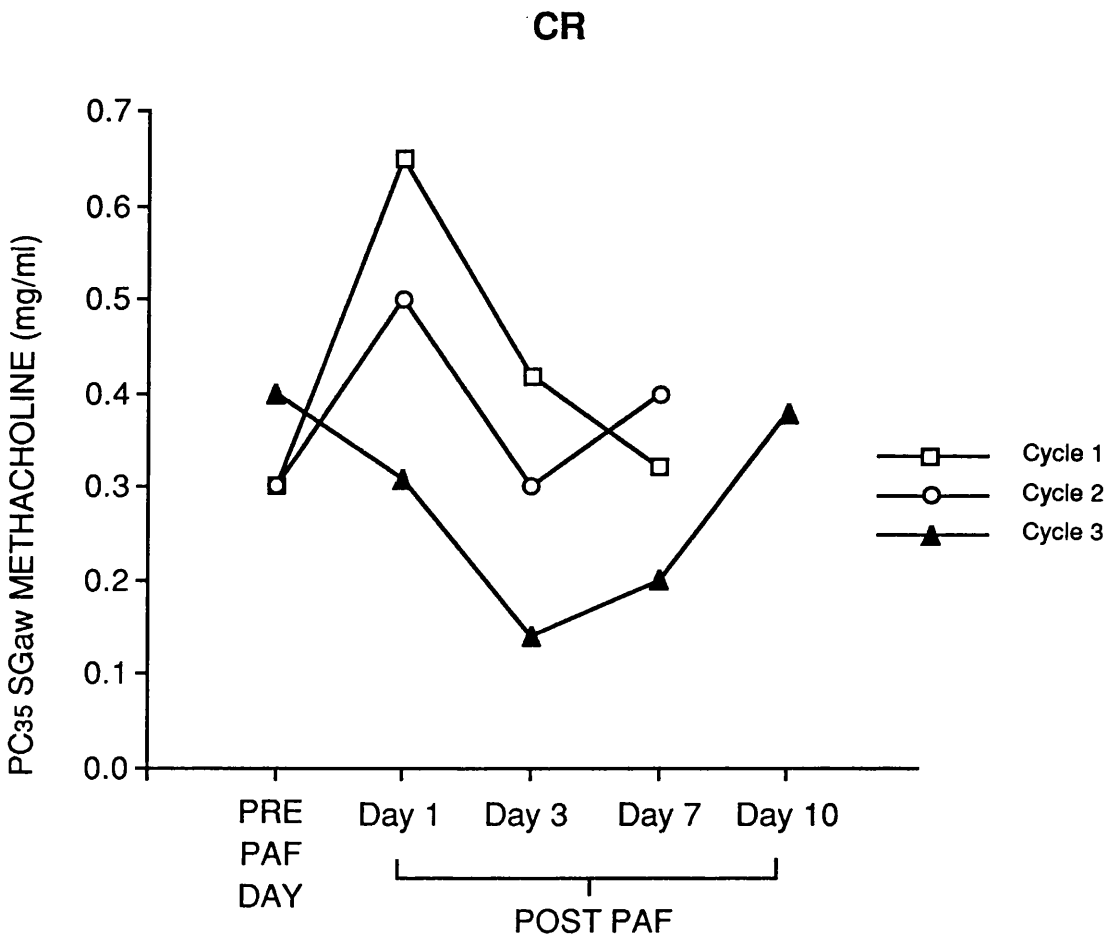


Figure 9.6 PC_{35} SGaw Methacholine before and after PAF challenge in atopic subject (CR) in the three cycles.

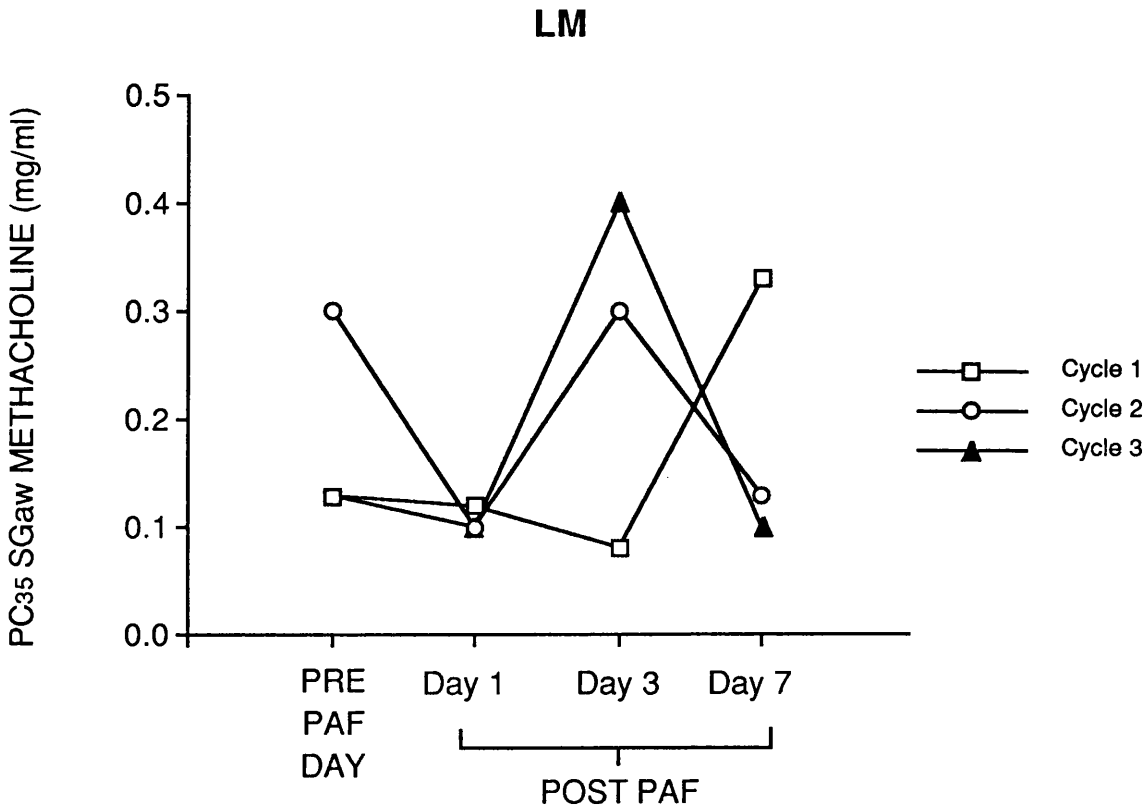


Figure 9.7 PC_{35} SGaw Methacholine before and after PAF challenge in atopic subject (LM) in the three cycles.

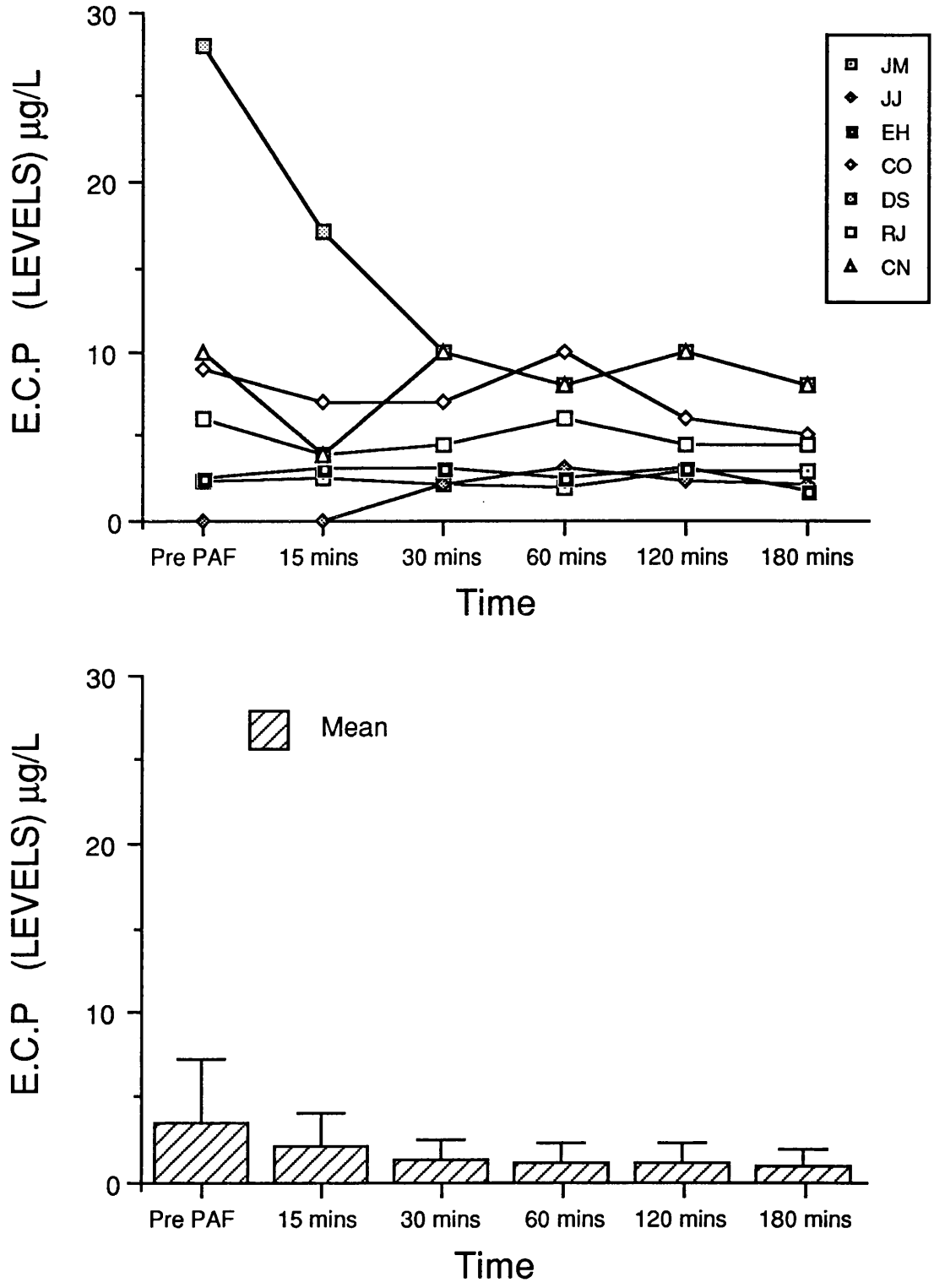


Figure 10 **Serum eosinophilic cationic protein (ECP) level in non atopic subjects before and after PAF inhalation**

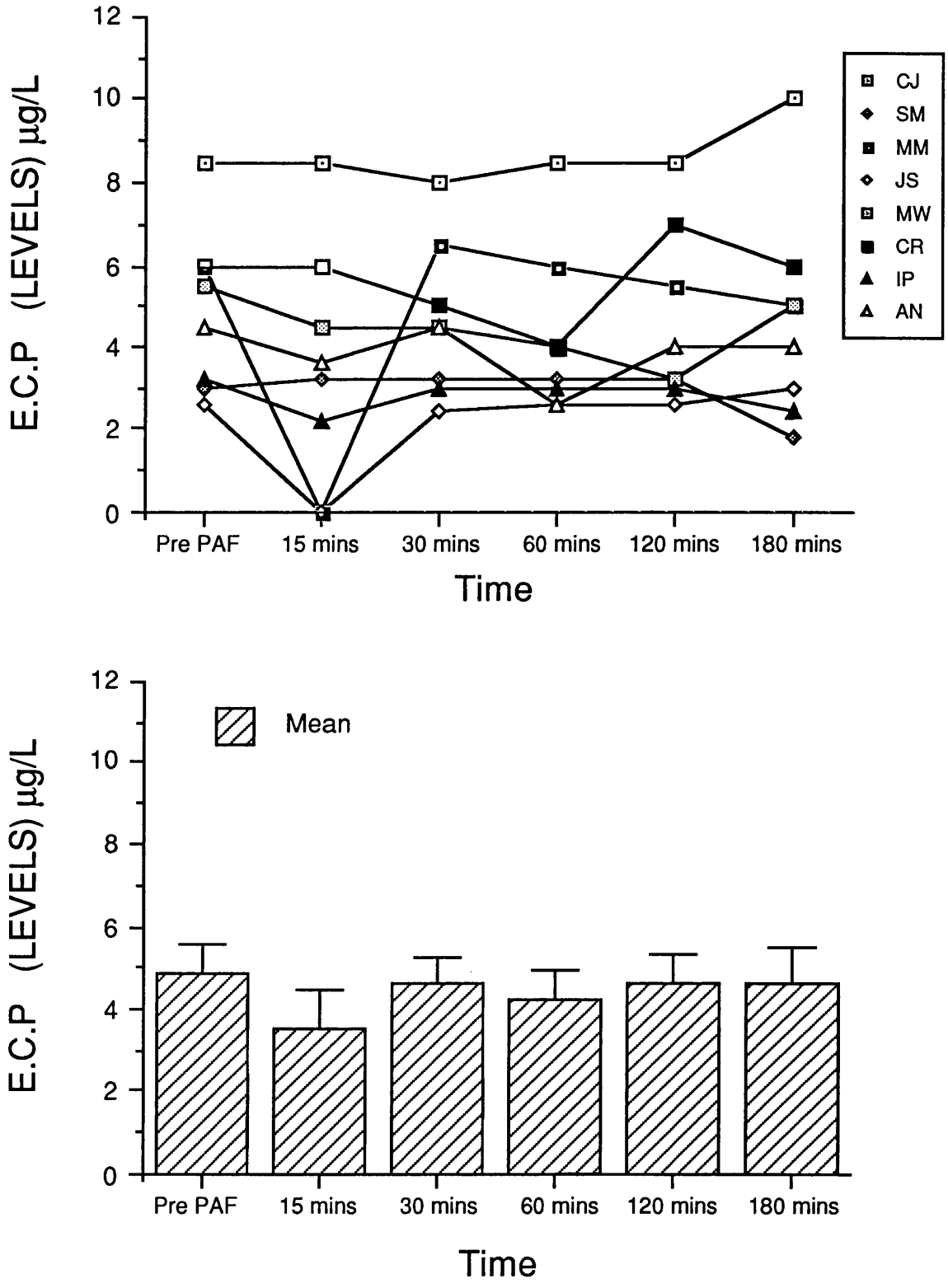


Figure 11 **Serum eosinophilic cationic protein (ECP) level in atopic subjects before and after PAF inhalation**

LIST OF ABBREVIATIONS USED.

PAF (PAF-acether)	Platelet activating factor
Post PAF D1	Post PAF DAY 1
Post PAF D3	Post PAF DAY 2
Post PAF D7	Post PAF DAY 3
PC 35 SGaw Methacoline	The concentration of methacoline in milligram/millitre which causes 35% fall in specific airway conductance.
SGaw	Specific airway conductance ($s^{-1}Kpa^{-1}$)
PC35 SGaw	PC35 SGaw Methacholine
G. Mean	Geometric mean
N.S.	Not Significant
IP3	Inosin triphosphate
DG	Diacylglycerol
PLA 2	Phospholipase A2
Pmo	Mouth pressure changes
Pbox	Changes in box pressure
TGV	Thoracic gas volume
ECP	Eosinophil cationic protein
RIA	Radioimmuno assay
Pesh	Peshawar, Pakistan

UNITS:

mg/ml	milligram/millitre
ug/L	microgram/litre
ml	millitre
uL	microlitre

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