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**REGULATION OF β_2 -ADRENOCEPTORS
BY STEROID HORMONES
IN ASTHMA**

KIA SOONG TAN M.B. Ch.B. (Glas) M.R.C.P. (UK)

**Department of Clinical Pharmacology
Ninewells Hospital and Medical School
Dundee, Scotland**

**A thesis submitted to the University of Glasgow for the degree of
Doctor of Medicine.**

July 1998

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ABSTRACT

Previous studies have demonstrated the facilitatory effects of corticosteroids on the β_2 -adrenoceptor (β_2 -AR). Tolerance or subsensitivity with regular β_2 -agonist use is of concern in asthma therapy. As corticosteroids and β_2 -agonists are often prescribed together, the aim of these studies was to investigate their interaction on β_2 -AR function.

Our initial study showed that despite taking inhaled corticosteroids, asthma patients developed bronchodilator and systemic β_2 -AR subsensitivity following regular formoterol use. Administration of systemic corticosteroid rapidly restored β_2 -AR responsiveness. This reversibility did not appear to be related to β_2 -AR polymorphism. However, the development of formoterol-induced bronchodilator subsensitivity was influenced by β_2 -AR polymorphism, with glycine 16 homozygous patients developing greater desensitisation than those homozygous for arginine 16. We further demonstrated that inhaled corticosteroids failed to prevent the development of tolerance to the bronchoprotective effects of formoterol against methacholine and AMP, irrespective of formoterol dose and dosing frequency. β_2 -AR polymorphism did not appear to influence the development of bronchoprotective tolerance, in contrast to bronchodilator tolerance. Unlike systemic corticosteroid, high-dose inhaled corticosteroid, in single and repeated doses, showed no significant facilitatory effect on lymphocyte β_2 -AR parameters. In contrast, we demonstrated that low-dose systemic corticosteroid protected against the development of β_2 -AR subsensitivity induced by regular formoterol treatment.

Female sex steroid hormones also influence β_2 -AR function. Indeed, abnormal β_2 -AR regulation has been postulated as a cause of premenstrual asthma. We found that normal cyclical regulation of β_2 -AR function was lost in female asthmatic patients. In addition, there was greater airway hyperresponsiveness to AMP during the premenstrual period compared to the follicular phase. Our studies showed that exogenous progesterone, but not oestradiol, had facilitatory effects on lymphocyte β_2 -AR function in normal females. In contrast, exogenous progesterone had a 'paradoxical' down-regulating and desensitising effect on β_2 -AR function in female asthmatic patients. Unlike those with natural menstrual cycles, female asthmatics taking the oral contraceptive pill had attenuated cyclical changes in airway hyperresponsiveness and diurnal peak flow rates.

We therefore conclude that corticosteroids have significant facilitatory effects on β_2 -AR function in asthma. Our findings also suggest that there may be abnormal hormonal regulation of β_2 -AR in female asthma patients. Further studies examining the facilitatory effects of inhaled corticosteroid on β_2 -AR function in asthma are indicated. Research into the role of exogenous sex hormones on β_2 -AR function in premenstrual asthma, pregnancy and the menopause is also necessary.

ACKNOWLEDGEMENTS

I have great pleasure in thanking Dr Brian Lipworth for giving me the opportunity in working with him in this project. It would not have been possible without his tremendous enthusiasm and vision. He gave helpful constructive criticism for the papers arising out of this work and the drafts of this thesis. My thanks also extend to my research colleagues in the Department of Clinical Pharmacology: Alison Grove, Martin Devlin and Imran Aziz. All laboratory analyses including hormone assays and lymphocyte β_2 -adrenoceptor parameters were carried out by Lesley McFarlane who worked tirelessly through the studies. My thanks also go out to Dr Ian Hall and his team at University Hospital of Nottingham who carried out all β_2 -adrenoceptor polymorphism analysis in this thesis. I would like to take the opportunity to thank all the volunteers and asthma patients from Kings Cross Hospital and Ninewells Hospital who helped in the studies. I thank the National Asthma Campaign for providing the grant for my fellowship and to Astra Pharmaceuticals for part sponsorship of the studies in Chapter 3 and 5. Finally I thank my wife Linda who has borne all this with great forbearance and helped tremendously by typing the thesis.

DECLARATION

I am the sole author of this thesis and I have personally consulted all the references listed. This work was undertaken by myself in the Department of Clinical Pharmacology, Ninewells Hospital and Medical School, Dundee. The study in Chapter 5 was a collaborative work with the Department of Respiratory Medicine, Newcastle General hospital, Newcastle. The study in Chapter 6 was a collaborative work with my colleague Imran Aziz, where I designed the study protocol and helped in recruiting patients and data collection. This thesis has not been previously submitted for a higher degree.

ABBREVIATIONS

AMP	Adenosine monophosphate
AUC	Area under curve
β_2-AR	β_2 -adrenoceptor
B_{max}	Receptor density (as fmol/10 ⁶ cells)
cAMP	Cyclic adenosine monophosphate
DRC	Dose-response curve
E_{max}	Maximal cyclic AMP response to isoprenaline (as pmol/10 ⁶ cells)
FEV₁	Forced expiratory volume (in one second)
FEF₂₅₋₇₅	Forced expiratory flow (during the middle half of the forced vital capacity)
K_d	Dissociation constant (as nmol/L)
MDI	Metered dose inhaler
PEFR	Peak expiratory flow rate (as L/min)
SD	Standard deviation
SEM	Standard error of mean

CHAPTER 1

INTRODUCTION

1.1 PATHOGENESIS OF ASTHMA

There is at present no definition of asthma using unequivocal criteria. The majority of descriptions stem from the first CIBA symposium which concluded that 'asthma refers to the condition of subjects with widespread narrowing of the bronchial airways, which changes in severity over short periods of time either spontaneously or under treatment, and is not due to cardiovascular disease' (Fletcher, 1959). In addition to this variable airway obstruction, mucosal inflammation and hyperresponsiveness of the airways also have an important influence on asthma pathophysiology. Bronchial hyperresponsiveness can best be described as an exaggerated bronchoconstrictor response to a wide variety of exogenous and endogenous stimuli, both specific and non-specific. It underlies much of the symptomatology of asthma, as has been recognised in the description of the disease by the American Thoracic Society (1987a). Airway hyperresponsiveness is considered a hallmark of asthma and its extent correlates with its clinical severity and the need for treatment (Juniper, 1981). Over the last decade, asthma has been recognised as a chronic inflammatory condition of the airways with bronchial hyperresponsiveness being a manifestation of this inflammation. Intense airway inflammation has been noted in patients dying of severe asthma (Dunnill, 1969). With the advent of fiberoptic bronchoscopy, inflammatory changes in the bronchial mucosa have been confirmed as being characteristic even of mild disease, suggesting that they are fundamental to the pathophysiology of asthma (Djukanovic, 1990; Bradley, 1991).

1.2 β_2 -AGONIST TREATMENT IN ASTHMA

β_2 -adrenoceptor (β_2 -AR) agonists are the core of bronchodilator therapy in asthma. While the use of these agents has lessened with the emphasis on inhaled corticosteroids, they remain necessary for rapid symptomatic relief in asthma.

1.2.1 Mechanism of action

β -AR are subdivided into β_1 , β_2 and β_3 -subtypes. β_1 -receptors are preferentially activated by noradrenaline and regulated by sympathetic nerves. β_2 -receptors are preferentially regulated by circulating adrenaline. β_3 -receptors or 'atypical' β -receptors are involved in lipolysis. Although both β_1 and β_2 -AR are present in the lungs, bronchodilation appears to be entirely a function of β_2 -AR (Barnes, 1995a).

β_2 -AR agonists produce their effects through interaction with these specific β_2 -AR located on the plasma membrane of virtually all cell types. The receptor consists of a protein which traverses the cell membrane seven times, forming three extracellular and three intracellular loops. The receptor is linked to a stimulatory guanine nucleotide-binding protein (G_s). Binding of the agonist to the receptor results in a conformational change in the receptor leading to the activation of the α -subunit of G_s . This in turn activates adenylate cyclase, which catalyses the conversion of adenosine triphosphate (ATP) to cyclic adenosine-3'5'-monophosphate (cAMP). cAMP acts as an intracellular second messenger and is responsible for the physiological response, the nature of which differs with each cell type (Nijkamp, 1992). In airway smooth muscle, the intracellular mechanisms involved in mediating the relaxant effects of β_2 -agonists have been extensively investigated. The rise in

cAMP concentration activates protein kinase A (PKA) leading to phosphorylation of several proteins which results in smooth muscle relaxation. PKA inhibits myosin light chain phosphorylation and phosphoinositide hydrolysis and promotes $\text{Ca}^{2+}/\text{Na}^{+}$ exchange. These changes cause a fall in intracellular $[\text{Ca}^{2+}]$ and stimulate $\text{Na}^{+}/\text{K}^{+}$ ATPase. β_2 -agonists also give rise to smooth muscle relaxation by opening membrane potassium (maxi-K) channels either by increasing cAMP or by a cAMP-independent route through direct activation via the α -subunit of G_s (Barnes, 1995a).

1.2.2 Efficacy of β -agonists

β_2 -agonists can be administered orally, by inhalation, or by subcutaneous or intravenous injection. However, the inhaled route is by far the preferred way as side effects are fewer for any given degree of bronchodilation (Nelson, 1995). Inhaled β_2 -agonists can be divided into those with a short duration of action of 3-6h (eg salbutamol, terbutaline) and those with a long duration of action of greater than 12h (eg salmeterol, formoterol). The mechanism by which the long-acting β_2 -agonists achieve their prolonged duration of action differs. Salmeterol has a long side-chain which binds to a specific site within the β_2 -adrenoceptor allowing prolonged activation of the receptor (Johnson, 1993). Formoterol appears to enter and then gradually diffuses from the plasmalemma lipid bilayer and is available over a prolonged period to stimulate the β_2 -adrenoceptor (Anderson, 1993). In addition to their bronchodilating action, β_2 -agonists also demonstrate protection against a variety of stimuli such as exercise (Anderson, 1979), allergen (Cockcroft, 1987) and cold, dry air (O'Byrne, 1982), all of which cause bronchoconstriction in asthmatic patients. This property of 'functional antagonism' allows β_2 -agonists to inhibit or reverse a contractile response, irrespective of the constricting stimulus (Torphy, 1983). This is a

property of particular importance in asthma, since several spasmogens are likely to be involved (including histamine, leukotriene D₄, acetylcholine, bradykinin). β_2 -agonists also have a variety of other non-bronchodilator activities. These include enhanced mucociliary clearance (Devalia, 1992), inhibition of cholinergic neurotransmission (Rhoden, 1988), suppression of microvascular leakage (Erjefalt, 1991) and inhibition of mediator release from mast cells, basophils and other cells. *In vitro*, β_2 -agonists inhibit the release of histamine, leukotrienes and prostaglandins from human mast cells (Butchers, 1991). AMP is thought to cause bronchoconstriction indirectly through the release of mediators from mast cells (Cushley, 1985). Inhaled terbutaline confers greater protection against AMP than methacholine-induced bronchoconstriction in asthma suggesting that β_2 -agonists produce an inhibitory effect on mast cell mediator release (O'Connor, 1994). This difference was attributed to the combined effect of terbutaline on mast cell and smooth muscle β_2 -AR in protecting against AMP, and on smooth muscle β_2 -AR only in protecting against methacholine.

1.3 β_2 -ADRENOCEPTOR FUNCTION IN ASTHMA

Since Szentivanyi (1968) hypothesised that there may be an inherent defect in β_2 -adrenoceptor function in asthma, there has been intensive debate about the contribution of β_2 -AR dysfunction to the pathophysiology of asthma. This was reinforced by observations that patients with asthma, but not healthy subjects, had increased sensitivity to β_2 -AR inhibitors (Koeter, 1982). Several early studies had demonstrated reduced receptor density and adenylate cyclase activity in circulating leukocytes of asthmatic patients (Brooks, 1979; Kariman, 1980; Parker, 1973). It is difficult, however, to interpret these data because of inadequate washout periods for β -agonists and other anti-asthma medication leading to confounding drug effects. It is well recognised that β_2 -AR responsiveness is enhanced by systemic corticosteroids (Logsdon, 1972; Brodde 1985) and is depressed by β -agonists (Tashkin, 1982). In the study by Parker *et al* (1973), inhaled β -agonists were used by the patients but had no defined wash-out period. This was also the case when Brooks *et al* (1979) examined β_2 -AR on leukocytes when four subjects were taking oral prednisolone during the study period. Two subjects studied by Kariman *et al* (1980), were taking prednisolone up to 48h prior to the study and four subjects were taking theophyllines. It soon became clear that these abnormalities were likely to be due to tolerance or subsensitivity induced by the β -agonist therapy and could be reproduced in normal individuals given the same therapy. Furthermore, when β -agonist therapy was withdrawn, β_2 -AR responsiveness returned to normal (Conolly, 1976; Galant, 1978). This therefore implied that the β_2 -AR dysfunction reported in asthmatic patients was the result of subsensitivity following exposure to β -agonists and was not due to

intrinsic β_2 -AR dysfunction. Several studies have now shown that there is no difference in β_2 -AR density or adenylate cyclase activity when comparing leukocyte β_2 -AR from healthy controls and stable asthmatics not taking regular β -agonists (Connolly, 1976; Galant, 1978; Meurs, 1982; Newnham, 1993). Likewise, airway β_2 -AR assessed by salbutamol dose-response curves (as specific airways conductance) showed no difference between mild asthmatics and healthy controls (Tattersfield, 1983).

1.3.1 Desensitisation and down-regulation of β_2 -adrenoceptors

Reduced responsiveness occurs with most cell surface receptors when exposed continuously or repeatedly to an agonist. Tachyphylaxis refers to short-term desensitisation and tolerance after repeated exposure to an agonist. Homologous desensitisation refers to reduced responsiveness to β -agonists, as described above, while heterologous desensitisation describes desensitisation to other agonists and usually involves cAMP.

Homologous desensitisation has been the subject of extensive investigation (Barnes, 1995a) and involves several steps. In the short term, there is rapid phosphorylation-mediated uncoupling of the receptor from effector units. The two principle kinases involved are cAMP dependent protein kinase A (PKA) and β -adrenergic receptor kinase (β -ARK) (Inglese, 1993). β -ARK is activated by the binding of agonist to receptor. A co-factor termed β -arrestin is necessary for β -ARK inhibition of receptor function (Loshe, 1990). Phosphorylation of β -receptors by β -ARK enhances the ability of β -arrestin to bind and thereby to uncouple the receptor from Gs. Phosphorylation of the receptor may impair its ability to interact with Gs (biochemical

uncoupling) or it may trigger its sequestration away from Gs (physical uncoupling). There is rapid simultaneous sequestration or internalisation of the receptor from the cell surface which is an agonist-dependent process. This process has been demonstrated in frog erythrocytes (Chuang, 1980) and smooth muscle cells (Sher, 1984). Sequestration results in a decrease in the number of surface membrane β_2 -AR which is paralleled by an increase in cytosolic receptors. The uncoupled internalised receptor may return to the cell membrane once the β -agonist is removed (resensitisation). Following uncoupling and sequestration, down-regulation of the β_2 -AR occurs, usually after prolonged agonist stimulation. Down-regulation is defined as the loss of total receptor binding sites with an accompanying loss in effector stimulation. After down-regulation, responsiveness is only restored by the synthesis of new receptors. Receptor uncoupling and sequestration are rapid processes which take place within seconds to minutes following agonist exposure, whereas down-regulation occurs after more prolonged stimulation (>1 h). The more chronic and clinically relevant effects of β -agonists may be due to changes in β_2 -AR synthesis. Down-regulation may involve both proteolytic degradation of the receptor moiety and diminished *de novo* synthesis of the receptor (Bouvier, 1989). The molecular mechanisms underlying down-regulation are poorly understood. This process may involve receptor-Gs coupling and the PKA pathway. Using a hamster cultured smooth muscle line, it has been shown that prolonged exposure to β -agonists or cAMP analogues (such as forskolin) produce down-regulation which was accompanied by substantial decreases in β_2 -AR mRNA levels (Hadcock, 1988; Collins, 1989). Hadcock and Malbon (1988) observed that while agonist-promoted down-regulation of receptors was rapid preceding the decline in mRNA levels, cAMP resulted in a more modest decrease in β_2 -AR mRNA. The studies by Collins *et al* (1989) indicated that

decreases in β_2 -AR mRNA in response to agonist or cyclic AMP treatment, tended to precede the loss of receptor numbers. In addition, the treatment with forskolin produced an equally effective reduction in β_2 -AR mRNA levels, although the effect on receptor density was less dramatic. Since the purpose of agonist activation of the receptor is to stimulate adenylate cyclase and generate cAMP, the ability of cAMP to mimic the decrease in both receptor mRNA and in part receptor number, suggests intimate involvement of the PKA pathway. However, because agonists produce more rapid and more effective receptor down-regulation, an agonist-promoted, cAMP-independent mechanism may be responsible for receptor down-regulation. Down-regulation is achieved by inhibition of gene transcription of receptors or by increased post-transcriptional processing of mRNA. Using actinomycin D to inhibit transcription in a cultured hamster vas deferens cell line, the decline in the steady state level of β_2 -AR mRNA following agonist exposure was thought to be secondary to a reduction in β_2 -AR mRNA stability (Haddock, 1989). Molecular mechanisms of down-regulation have also been investigated in lung tissue after exposure to β -agonists. In a study with guinea pigs (Nishikawa, 1994), a seven day infusion of noradrenaline produced a marked reduction in β_2 -AR density in lung parenchyma which was associated with a similar reduction in steady state β_2 -AR mRNA levels. It was also found that different cell types have a differing capacity to down-regulate with greater down-regulation in alveolar wall than in airway smooth muscle. This may relate to an increased rate of gene transcription in airway epithelial cells compared with lung parenchyma.

1.3.2 Tolerance to β -agonists in asthma

Although the molecular and cellular aspects of β -agonist mediated β_2 -AR down-regulation and tolerance are accepted as described above, there continues to be controversy about such tolerance in asthma. The more recent introduction of long-acting β_2 -agonists has further fuelled this debate because their prolonged receptor occupancy may theoretically increase the propensity for down-regulation. Before discussing the subject, it is important to appreciate the methodology required to demonstrate such tolerance or subsensitivity. It is necessary to distinguish between effects on resting bronchomotor tone (ie: bronchodilator activity) and anti-bronchoconstrictor properties (ie: functional antagonism) as tolerance may develop preferentially to one property rather than the other.

Short-acting β_2 -agonists

Holgate *et al* (1977), in a uncontrolled study, showed that in healthy volunteers bronchodilator subsensitivity (measured as specific airways conductance) developed after 4 weeks of inhaled salbutamol 200 μ g four times a day. In healthy volunteers, there was rapid down-regulation of β_2 -AR density by 70% in bronchial epithelial cells and alveolar macrophages obtained by bronchoalveolar lavage after 24h of inhaled metaproterenol (Turki, 1995a). Maximal cAMP response to isoprenaline was likewise reduced by 48% and 86% in epithelial cells and alveolar macrophages respectively. Research to date has generally shown that it has not been possible to demonstrate bronchodilator tolerance in asthmatic airways. There have been few dose-response studies with placebo control to assess bronchodilator tolerance. In a study without placebo control, Repsher *et al* (1984) demonstrated tolerance to the duration but not the peak bronchodilator response to a single dose of salbutamol following regular

treatment with the drug. Other studies have failed to show such tolerance following oral and inhaled β_2 -agonists with bronchodilator responses to single and cumulative doses of inhaled, intravenous and subcutaneous β_2 -agonists (Larsson, 1977; Repsher, 1981; Tashkin, 1982; Lipworth, 1989). Lipworth *et al* (1989) performed a crossover study giving placebo, low-dose inhaled salbutamol (800 μg daily) or high-dose inhaled salbutamol (4000 μg daily) for two weeks respectively, with two week run-in and wash-out periods without β_2 -agonists. A cumulative dose-response curve to inhaled salbutamol performed after each treatment period showed a right-hand shift in extra-pulmonary, but not in airway β_2 -AR responses, even with the higher dose. This apparent tissue dissociation in β_2 -AR subsensitivity may reflect a high level of β_2 -AR gene expression or a high density of 'spare receptors' in the airway, so that even a marked reduction in the β_2 -AR density is not accompanied by a reduced response.

With regular use of β -agonists, it is important to distinguish between tolerance to bronchoprotective effects and the rebound increase in airway reactivity which occurs after stopping therapy. It is also necessary to highlight the different stimuli used to provoke bronchoconstriction. Histamine and methacholine are predominantly direct smooth muscle stimulants, while allergen and AMP (and possibly, exercise) act indirectly by stimulating mast cells to release bronchospastic mediators. There may be differential effects of β_2 -agonists on these stimuli with a consequent difference in susceptibility to tolerance. O'Connor *et al* (1992) assessed airway hyperresponsiveness to methacholine and AMP before and after one week of treatment with terbutaline 500 μg four times daily in 12 asthmatic subjects. Their study design did not include re-establishing the baseline PC_{20} values after each treatment. Terbutaline initially produced a 2.7 doubling dose shift of methacholine PC_{20} compared with a 3.8

doubling dose shift of the AMP PC₂₀. This difference was attributed to the combined effect of terbutaline on mast cell and smooth muscle β_2 -AR in protecting against AMP, and on smooth muscle β_2 -AR only in protecting against methacholine. After the one week treatment period, terbutaline shifted the methacholine PC₂₀ by 2.2 doubling doses (19% loss in protection). By contrast, there was a much greater loss of bronchoprotective effect against AMP as terbutaline only shifted the AMP PC₂₀ by 1.7 doubling doses, a reduction of 55% from the 3.8 doubling doses. The interpretation of this greater loss of protection against inhaled AMP, a mast cell stimulus, was that β_2 -AR on mast cells might be more susceptible to tolerance than are the β_2 -AR on airway smooth muscle. This differential effect of different bronchoconstrictor stimuli was again seen in the study by Cockcroft *et al* (1993). They studied the effect of inhaled salbutamol 200 μ g four times daily on airway hyperresponsiveness to methacholine and allergen. The baseline methacholine and allergen PC₂₀ values were re-established following each treatment period. Methacholine PC₂₀ was reduced to 2.5 doubling doses after two weeks treatment with salbutamol from an initial protection of 3.2 doubling doses (22% loss of protection). The protection against allergen was initially 3.7 doubling doses and it fell to 2.5 doubling doses (32% loss of protection). However, the most striking finding was a highly significant increase in airway responsiveness to allergen. There was almost a doubling of airway responsiveness to allergen following two weeks of salbutamol. Two additional studies showed that the late asthmatic response to allergen was almost double after a week of regular salbutamol treatment compared with placebo (Cockcroft, 1995; Gauvreau, 1995). There does not appear to be a loss in functional antagonist effect of salbutamol against exercise-induced bronchoconstriction (Inman, 1996). Studies using histamine as the bronchoconstrictor have been less consistent with some studies showing

tolerance while others have not (Tashkin, 1982; Van Shyack, 1990; Kraan, 1985; Kerrebijn, 1987; Waalkens, 1991). Vathenen *et al* (1988) also showed reduced protection of inhaled terbutaline against histamine-induced bronchoconstriction following two weeks of treatment. In addition, an increase in airway reactivity amounting to 1.5 doubling doses was found at 23h after stopping treatment. This rebound effect was also described by Larsson *et al* (1977) with a trend towards increased reactivity to allergen challenge at 48h after stopping treatment with oral terbutaline being demonstrated. When considering these studies, it is important to bear in mind the intrinsic variability of bronchial reactivity which can amount to one doubling dose. Hence, changes which are statistically significant may not be clinically relevant.

Long-acting β_2 -agonists

Early studies did not find evidence of bronchodilator tolerance to salmeterol or formoterol. Ullman *et al* (1990) compared two weeks of treatment with salmeterol 50 μg twice daily or salbutamol 200 μg four times daily in 12 asthmatic patients. There was no placebo control and no run-in or wash-out period without β_2 -agonists. A dose response curve to salbutamol (cumulative dose 900 μg) was constructed before and after each treatment period. The authors concluded that there was no evidence of tolerance after treatment with salmeterol. However, the baseline FEV₁ was significantly higher after than before salmeterol treatment. In addition, a ceiling was reached in the dose-response curve with salbutamol. This finding, together with the confounding effect of different baselines, made valid comparisons between the dose-response curves difficult. In a similar study (Arvidsson, 1989), formoterol 12 μg as twice daily dosing was compared with salbutamol 200 μg twice daily for two weeks in

a cross-over design. Again, there was no placebo control or run-in period. As in the study by Ullman *et al* (1990), baseline FEV₁ was significantly higher 12h after the last dose of formoterol, prior to the salbutamol dose-response curve, and together with a ceiling being reached in the dose-response curve, comparisons between treatment are difficult to make. Other studies have not found evidence of bronchodilator tolerance after treatment with salmeterol (Cheung, 1992; Pearlman, 1992), although the findings were based on responses to single doses of salmeterol and absence of a run-in period prior to the study. More recent work, employing a placebo-control period, a two week run-in period without β_2 -agonists and a longer washout phase prior to the dose response curve has shown evidence of tolerance to the bronchodilator effects of formoterol (Newnham, 1994a; Newnham, 1995). Four weeks of treatment with inhaled formoterol 24 μ g twice daily, as pressurised metered dose inhalers and dry powder preparations, was compared with placebo, with a dose-response curve to formoterol constructed (cumulative dose 126 μ g) at the end of each treatment period. This tolerance was manifested as a significant reduction in both peak bronchodilator response and a greater reduction at 6h after the final dose of the DRC. It is important to note that in contrast to the studies by Ullman *et al* and Arvidsson *et al*, there were no significant differences in baseline FEV₁ and no ceiling was reached in the dose-response curve. Because of equal baselines, delta responses from baseline could be used in comparing the dose-response curves. Predictably, in both of these studies by Newnham *et al*, formoterol produced subsensitivity of extrapulmonary β_2 -AR responses and down-regulation of lymphocyte β_2 -AR density and responses, compared to placebo. Yates *et al* (1995) showed evidence of bronchodilator subsensitivity following two weeks' treatment with formoterol 24 μ g twice daily, with a significantly reduced FEV₁ 36h after the last dose of formoterol, compared with the

first dose, but was not lower at other time periods. Using a similar design to Newnham *et al*, Grove *et al* (1995) compared salmeterol 50 µg twice daily with placebo in 17 asthmatic patients. A dose-response curve (cumulative dose 3200 µg) was constructed at the end of each treatment period with a 36h washout phase. There was a rightward shift in the salmeterol DRC following treatment. This bronchodilator subsensitivity equated to a 2.5 fold and 4 fold greater dose of salbutamol being required to produce a given FEV₁ or peak expiratory flow respectively. It is important to note that in these studies by Newnham *et al* and Grove *et al*, bronchodilator tolerance occurred after regular use of a long-acting β_2 -agonist despite the fact that most of the patients were receiving inhaled corticosteroids. In each of the three studies, (Newnham, 1994a; Newnham, 1995; Grove, 1995), peak expiratory flow rates improved significantly with formoterol and salmeterol compared with placebo, which may lead to patients being lulled into a false sense of security in the presence of bronchodilator tolerance.

Given that it is possible to demonstrate tolerance to the protective effects of short-acting β_2 -agonists, it is not surprising that the same phenomenon has been reported with long-acting β_2 -agonists. When considering tolerance to anti-bronchoconstrictor effects of long-acting β_2 -agonists, it is important to consider whether tolerance occurs not only at peak effect, but also towards the end of the action at 12h, given that these drugs are usually administered as a twice daily regimen. Cheung *et al* (1992), in a parallel placebo controlled trial, showed that salmeterol protection against methacholine challenge fell from tenfold on day 1 to only twofold protection after four weeks' treatment with salmeterol 50 µg twice daily (70% loss of protection), although no loss of bronchodilator effect was noted. The methacholine challenges

were performed 1h after receiving a dose of salmeterol, having withheld treatment for 36h previously. In this study, there was no rebound hyperresponsiveness after the eight week treatment was discontinued. The loss of bronchoprotective effect of salmeterol was also demonstrated in asthmatic children (Verberne, 1996). Another study (Bhagat, 1995), studied the time course of the development of tolerance to the bronchoprotective effect of salmeterol against methacholine. Methacholine PC₂₀ was measured 1h after the first, third, fifth and seventh doses of twice daily 50 µg salmeterol in double-blind, placebo cross-over design. Methacholine PC₂₀ was also measured 10 minutes after 200 µg salbutamol 24h after stopping salmeterol. By the third dose of salmeterol, the bronchoprotective effect was reduced from 3.3 to 2.4 doubling doses and continued to fall to a protection of only 1.5 doubling doses after the seventh dose. The PC₂₀ after salbutamol on day 5 was also reduced from a 3.7 doubling dose protection after placebo to a 1.9 doubling dose shift after the seventh dose of salmeterol. These studies may be criticised for selecting patients with very mild asthma, not on inhaled corticosteroids, in whom long-acting β-agonists would not have been indicated. Booth *et al* (1993) studied a group of mild to moderate asthma patients in whom 19 out of 26 were taking inhaled corticosteroids. In a parallel group study, patients received eight weeks' treatment, with either salmeterol 50 µg twice daily or placebo, with methacholine challenge performed at 12h after the first dose and at four and eight weeks of treatment. No significant difference was detected in the protection afforded by salmeterol, when comparing the first dose (0.9 doubling doses difference from baseline) with subsequent repeat dosing at four weeks (1.2 doubling doses) or eight weeks (0.6 doubling doses). However, the patients were allowed free access to inhaled β-agonists before and during the study as rescue medication, so any tolerance may have been present at the beginning of the study.

Therefore the negative results were likely to be caused by the pre-trial and continued use of rescue inhaled β -agonists. The same investigators have since repeated a study using the same design as Cheung *et al*, by performing the methacholine challenge 1h after inhalation of salmeterol following a 36h washout period (Booth *et al*, 1996). In this study, all patients received inhaled corticosteroid. It was found that the bronchoprotection against methacholine fell from 3.3 doubling doses after the first dose to 2 doubling doses after four and eight weeks' treatment. Regular dosing with formoterol 24 μ g twice daily in steroid-naïve asthmatic patients also resulted in a significant loss of protection against methacholine at 12h after the first dose (1.9 doubling dose protection) compared with two weeks' treatment (0.5 doubling dose protection) (Yates, 1995). This amounted to a 74% loss of bronchoprotection. There was no rebound increase in airway hyper-reactivity on stopping treatment. There is also evidence to show that tolerance develops to the protective effect of salmeterol against exercise-induced bronchoconstriction in a study comparing salmeterol 50 μ g twice daily with placebo given for two weeks, with an exercise challenge performed at 6h and 12h after the first dose and after four weeks' treatment (Ramage, 1994). Salmeterol protected against exercise-induced bronchoconstriction by 66% and 40% at 6h and 12h respectively compared with placebo after the first dose. After four weeks' treatment, the last dose produced only a 27% and 18% reduction in exercise-induced bronchoconstriction at 6h and 12h respectively which was not significantly different from placebo (55-60% loss of bronchoprotection). It is worth noting that the patients had mild asthma and only three out of 12 patients were taking corticosteroids.

Several of the studies described have shown that inhaled corticosteroids do not appear to prevent the development of anti-bronchoconstrictor tolerance. Kalra *et al* (1996) showed that there is a rapid onset of tolerance to the bronchoprotective effect of salmeterol in subjects using inhaled corticosteroids 500 to 1500 µg daily. Tolerance to the bronchoprotective effect against methacholine was significant after the first two days (at 24h) and was similar after seven doses. In addition, subjects showed less protection against challenge following administration of salbutamol 24h after the last dose of salmeterol, compared with placebo. This was further evaluated by Yates *et al* (1996) in a crossover study with twelve asthmatic patients who received inhaled budesonide (800µg twice daily) or placebo for two weeks. Salmeterol 50µg twice daily was then added for one week. The protection afforded by a single dose of salbutamol against methacholine challenge was evaluated after two weeks' treatment (without salmeterol) and after three weeks (with salmeterol), 12h after the last dose. There was a significant attenuation in the protection by salbutamol after treatment with salmeterol by 1.1 doubling doses in those receiving budesonide and by 1.4 doubling doses in those receiving placebo, with no significant difference in protection between the budesonide and placebo periods. Inhaled corticosteroids also do not appear to protect against the loss of functional antagonist effects of short-acting β -agonists. High dose budesonide (1600 µg per day) was compared with high dose budesonide plus salbutamol in a blinded cross-over trial (Cockcroft, 1995). An equivalent tolerance to bronchoprotection was found, with the initial protection falling from 2.9 doubling doses to 2.25 doubling doses (22% loss of protection), with no significant difference between placebo and salbutamol. Inhaled budesonide did not prevent the parallel increase in airway responsiveness to allergen when salbutamol and budesonide were administered concurrently.

In summary, regular use of long-acting β -agonists appears to result in bronchodilator subsensitivity although this does not seem to be the case with short acting β -agonists in patients with asthma. Tolerance to functional antagonism however, appears to be more readily demonstrable than bronchodilator subsensitivity. Significant tolerance can be seen at very low doses and can develop very rapidly, and occurs with all agents and to all stimuli. Corticosteroids do not seem to prevent development of such tolerance. The mast cell stimuli (AMP and allergen) appear to be more prone to tolerance than are the direct stimuli (methacholine and histamine). In addition, regular use of inhaled β -agonists increases baseline airway responsiveness to allergen (early and late) such that the post-salbutamol allergen PC_{20} after two weeks of salbutamol was almost two doubling doses lower than after placebo (Cockcroft, 1993). How this tolerance relates to the increased morbidity and mortality associated with the regular use of β -agonists is at present uncertain. However, as inhaled β -agonists are often used for their functional antagonist effect, it would seem likely that the significant loss of protection against allergen and exercise, seen particularly with long-acting β -agonists might have important clinical relevance.

1.4 β_2 -ADRENOCEPTOR POLYMORPHISMS IN ASTHMA

Although asthma is now regarded as a chronic inflammatory disorder of the airways, Szentivanyi's (1968) original hypothesis that the disease may be due to dysfunctional β_2 -AR has again become topical. The previous discussion on β_2 -AR tolerance and down-regulation as a result of chronic β -agonist effects assumes that the mechanisms underlying these effects are the same throughout the population. Since the cloning of the β_2 -AR on the long arm of chromosome 5 (Kobilka, 1987), a number of common variants (or polymorphisms) of the receptor have been identified which could alter the behaviour of the receptor (Reishaus, 1993). Reishaus *et al* compared genomic DNA from peripheral blood lymphocytes in 51 asthma patients and 56 normal volunteers, using the original cloned 'wild type' DNA as reference. Nine point mutations were found in the entire cohort. Of these, five were degenerate in that they did not result in a change in amino acid sequence. Four other point mutations resulted in amino acid substitutions. Before discussing these, it is important to describe the 'wild type' of the receptor which was originally sequenced by Kobilka *et al* (1987). It consists of arginine (Arg) at locus 16, glutamate (Glu) at locus 27, valine (Val) at locus 34 and threonine (Thr) at locus 164. The four point mutations identified by Reishaus resulted in substitution of glycine (Gly) for Arg at locus 16, glutamine (Gln) for Glu at locus 27, methionine (Met) for Val at locus 34 and isoleucine (Ile) for Thr at locus 164. The frequency of the polymorphisms at loci 16 and 27 was no different between the asthmatic and normal groups (the polymorphisms at loci 34 and 164 were too infrequent for analysis), indicating that polymorphisms of the β_2 -AR were not a primary cause of asthma. Further work investigating the functional significance of the polymorphisms was performed using site-directed mutagenesis and transfecting

Chinese hamster fibroblast cell lines (which do not normally express β -adrenergic receptors but contain G-proteins, adenylyl cyclase and other regulatory proteins necessary for receptor-promoted signal transduction) to express the different forms of the receptor (Green, 1993). It was found that the Met 34 polymorphism displayed normal agonist binding and functional coupling profiles, while the Ile 164 form displayed four times less affinity for certain agonists (Green, 1993). Loci 16 and 27 polymorphisms had normal agonist and antagonist binding and coupling to adenylyl cyclase (Green, 1994). However, after exposure to agonist, the Gly 16 polymorphism underwent enhanced down-regulation (41%) compared to the Arg 16 receptor (26%). In contrast, the Glu 27 receptor appeared to be refractory to down-regulation, displaying no significant change in receptor expression after prolonged agonist exposure. However, cells with the Gly 16 and Glu 27 polymorphisms had enhanced down-regulation, similar to those with Gly 16 polymorphism, indicating that effects of Gly 16 are dominant over those due to Glu 27. Similar findings also resulted from examination of these receptor polymorphisms on primary bronchial smooth muscle cell cultures (Green, 1995a).

Two clinical studies have suggested that the Gly 16 polymorphism may be associated with markers of more severe asthma. Holdroyd *et al* (1995) found Gly 16 to be associated with bronchial hyperresponsiveness in a group of Dutch asthmatic families. In addition, asthma patients experiencing nocturnal symptoms were more likely to have the Gly 16 polymorphism than those without nocturnal falls in peak flow rate (Turki, 1995b). The Gly 16 allele frequency was 80% in the nocturnal group compared to 52% in the non-nocturnal group. This finding could be explained by day-time β -agonist use by patients with Gly 16 polymorphism, enhancing β_2 -AR down-regulation and increasing susceptibility to nocturnal asthma. This hypothesis is supported by the

observation that in patients with nocturnal asthma, leukocyte β_2 -AR expression decreases at 0400h compared to 1600h (Szeffler, 1991). More recently, it was shown that wheezy children with the Gly 16 receptor were less likely to have a significant bronchodilator response to a single 180 μ g dose of salbutamol compared to those with Arg 16 (Martinez, 1997). No association was found between polymorphisms at locus 27 and response to salbutamol. The two groups used similar anti-asthma medication and thus had no difference in predisposition to β_2 -AR down-regulation prior to the study. When 65 mild to moderately severe asthmatics were assessed using methacholine challenge, those with the Glu 27 polymorphism had airways which were four times less reactive than those with the Gln 27 form (Hall, 1995). These workers also found a significant association between the Gln 27 form of the receptor and elevated IgE levels in 60 asthmatic patients (Dewar, 1997). This could be accounted for by linkage disequilibrium between the β_2 -AR gene and the gene for control of IgE production (that is, the two are not independently distributed in the general population) which is also located on the long arm of chromosome 5 (Postma, 1995). There was no association between Gln 27 and asthma or atopy. Recently, however, the same group has demonstrated an association between Gln 27 and reported asthma symptoms in 410 children (Hopes, 1998). The Gln 27 allele conferred an independent increased risk of reported asthma with an odds ratio of 2.18. Conversely, the Glu 27 homozygotes had a lower risk (odds ratio 0.46).

The evidence to date would indicate that polymorphisms at loci 16 and 27 may be relevant to the asthma phenotype. However, this is further complicated by the observation that the two loci are in linkage disequilibrium (Dewar, 1998). Although it is highly unlikely that β_2 -AR polymorphism is a cause of asthma, it might partly

account for an individual's propensity to develop asthma, when exposed to appropriate environmental stimuli. In particular, it may influence a patient's response to inhaled β -agonist treatment with regard to developing β_2 -AR down-regulation and tolerance.

1.5 REGULATION OF β_2 -ADRENOCEPTOR FUNCTION BY STEROID HORMONES

1.5.1 Corticosteroids

Corticosteroids are the most effective therapy available for patients with asthma, forming the cornerstone of anti-inflammatory treatment (Barnes, 1995b). The undisputed clinical efficacy of inhaled and oral corticosteroids in suppressing this airway inflammation has been substantiated by intensive research and will not be discussed further.

Corticosteroids have been known to have facilitatory effects on the β_2 -AR (Davies, 1984). Chronic administration of β -agonists leads to β_2 -AR down-regulation and subsensitivity as discussed above. As both corticosteroids and β -agonists are often prescribed together in asthma, it is therefore of particular interest to examine the interaction between 'up-regulating' corticosteroids and 'down-regulating' β -agonists on β_2 -AR function and responsiveness.

There are two major areas of action of corticosteroids on the β_2 -AR: receptor number and coupling to adenylate cyclase. In a number of early studies, hydrocortisone was found to increase isoprenaline-stimulated cAMP accumulation (Lee, 1977; Logsdon, 1972; Marone, 1980). Davies and Lefkowitz (1980) found that when corticosteroids were administered, adenylate cyclase activity increased by 93% in neutrophils. This effect has also been seen in mast cells (Tolone, 1979). The steroid-induced changes in adenylate cyclase activity are associated with increased receptor binding sites. Corticosteroids have been shown to increase β_2 -AR density in a number of different

tissues, including rat lung (Mano, 1979), cultured human lung cells (Fraser, 1980), cultured human astrocytoma cells (Foster, 1980) and rabbit foetal lung tissue (Cheng, 1980). Oral administration of cortisone in humans results in a 39% increase in neutrophil β_2 -AR density (Davies, 1980). Mano *et al* (1979) found that adrenalectomy in rats induced a 30% fall in the number of β_2 -AR and this change was reversed by the exogenous administration of hydrocortisone. In addition, oral cortisone administered to healthy volunteers increased the β_2 -AR proportion in the high affinity state (ie - increased efficiency in coupling to adenylate cyclase) from 54% to 80% of receptors. This was also repeated in human neutrophils exposed to hydrocortisone *in vitro* (Davies, 1980). It can therefore be concluded that corticosteroids regulate β_2 -AR function by increasing receptor number and the formation of the high-affinity coupled form of the receptor.

More recent works have shown that corticosteroids increase the transcription of the β_2 -AR gene amongst others (Barnes, 1992). The human β_2 -AR gene has several glucocorticoid-response element consensus sequences (GREs) in its 5' noncoding, coding and 3' noncoding regions (Emorine, 1987). The GREs in the 5' noncoding region are obligatory for corticosteroid responsiveness (Malbon, 1988). Corticosteroids increase the steady-state level of β_2 -AR mRNA in cultured hamster vas deferens muscle cells without any increase in mRNA stability, indicating that corticosteroids may elevate β_2 -AR density by increasing the rate of gene transcription (Collins, 1988). This is confirmed by an increased transcription rate in these cells recovered by nuclear run-on assay, which directly measures gene transcription (Hadcock, 1989). Preceding that of the β_2 -AR, the rate of mRNA increase was rapid (within 1h) and then declined to a steady-state level, twice normal. Similarly,

corticosteroids have also been shown to increase the β_2 -AR gene transcription in human lung tissue (Mak, 1995a). Following incubation with dexamethasone, an increase in β_2 -AR mRNA could be detected after only 15 min, whilst β_2 -AR density rose by 1.6 fold within 17-24h. Again, β_2 -AR mRNA stability was unchanged by dexamethasone and a doubling in the rate of β_2 -AR mRNA transcription led to a greater steady-state level of mRNA, confirmed by a nuclear run-on assay.

Although the effects of corticosteroids on β_2 -AR in normal tissues have been discussed, it is perhaps more relevant to review the effects of corticosteroids on desensitised tissues and in particular to understand the interaction of corticosteroids and β -agonists on β_2 -AR function. Early studies have examined the facilitatory action of corticosteroids on airway β_2 -AR function. As discussed previously, Holgate *et al* (1977) induced bronchodilator subsensitivity in healthy volunteers with inhaled salbutamol 200 μ g four times daily for four weeks. Following an intravenous injection of 200 mg hydrocortisone, bronchodilator response to salbutamol was completely restored by 6h. There was no change in bronchodilator responsiveness in those subjects who had not been receiving regular salbutamol following the steroid. In an earlier study (Ellul-Micallef, 1975), ten asthmatic patients who were declared resistant to bronchodilator therapy were studied. It was found that the bronchodilator response to a single 200 μ g dose of inhaled isoprenaline was significantly greater after an intravenous injection of 40 mg prednisolone compared to placebo injection. This improvement was maintained 8h after injection. Subsequent studies investigated the effect of corticosteroids on peripheral leukocytes. In healthy volunteers and mildly asthmatic patients receiving regular terbutaline, there was, as expected, down-regulation of β_2 -AR on lymphocytes (Hui, 1982; Brodde, 1985; Tashkin, 1982).

Following a single intravenous injection of corticosteroid, receptor numbers were restored to normal levels by 8-10h. Hui *et al* observed that corticosteroid had no effect on β_2 -AR density in subjects not exposed to β -agonists, nor was there any effect after *in vitro* incubation with corticosteroid. Interestingly, Brodde *et al* (1985) showed concomitant administration of ketotifen and oral terbutaline completely prevented the β -agonist induced decrease in β_2 -AR density and responsiveness, although this effect was not studied using corticosteroid. Brodde *et al* (1988) further studied asthmatic patients who had been desensitised due to regular β -agonist therapy. Although lymphocyte β_2 -AR density increased in 16h following a single dose of intravenous hydrocortisone (100mg), this was not accompanied by any improvement in bronchodilator responsiveness to an inhaled β -agonist. An *in vitro* study (Davies, 1983) examined neutrophils from healthy volunteers which were incubated with isoprenaline and hydrocortisone simultaneously. There was attenuation in the fall in cAMP responsiveness to catecholamine and the high-affinity state of the receptors was maintained compared with the group treated only with isoprenaline. However, hydrocortisone did not prevent the fall in β_2 -AR density. Another study also demonstrated that hydrocortisone could reverse prior agonist-induced down-regulation (Samuelson, 1984). Mak *et al* (1995b) showed that dexamethasone in rat lung prevented the down-regulation of β_2 -AR following a prolonged infusion of isoprenaline. This appears to be due to an increase in gene transcription, which counteracts the decrease in transcription due to chronic β -agonist exposure.

In summary, it would seem that simultaneous *in vivo* exposure of β_2 -AR to β -agonist and corticosteroid results in attenuation of the desensitisation process by altering the impaired coupling of the receptors, while consecutive *in vivo* exposure of β_2 -AR to β -

agonist then corticosteroid reverses the down-regulation. This latter process appears to be due to increased β_2 -AR mRNA transcription.

1.5.2 Female sex steroid hormones

Despite progesterone and oestradiol being steroid hormones and therefore likely to have facilitatory effects on β_2 -AR function, there has been relatively little research on this subject. In animal studies, oestrogen was found to increase α_2 -AR numbers (Hoffman, 1981), while progesterone reduced α_2 -AR numbers, but had no effect on β_2 -AR in rabbit myometrium (Roberts, 1981). Wagner *et al* (1979) found that chronic exposure of oophorectomised rats to oestrogen reduced β_2 -AR and cAMP responsiveness in the cerebral cortex. They also showed that β -AR and cAMP responsiveness to catecholamines in rat brain were reduced in those female rats with normal oestrous cycles compared with oophorectomised females or males (Wagner and Davis, 1980). It is not yet clear as to how this applies to β -AR in other tissues. The effects of sex steroids were examined in rabbit lung tissue (Moawad, 1982). β -AR numbers increased *in vivo* with the administration of oestrogen, but returned to control values with the subsequent addition of progesterone. This influence of sex steroids was further demonstrated by changes suggesting the dynamic regulation of ovarian β -AR numbers during the oestrous cycle of the rat (Jordan, 1981). The β -AR numbers fell by 50% during the oestrogen phase, compared to the progesterone phase.

Female sex steroid hormones have been shown to potentiate the vasorelaxant and bronchorelaxant effects of corticosteroids. Kalsner (1969) demonstrated a greater contractile effect of catecholamines on rabbit aortic strips when incubated with oestradiol, progesterone and deoxycorticosterone. This potentiating effect was greater for adrenaline than for noradrenaline. However, there was no facilitatory effect seen with sympathomimetic amines lacking the catechol nucleus (eg - phenylephrine) and the steroid potentiating effects were abolished by the addition of catechol-O-methyl

transferase (the major mechanism for inactivation of catecholamines). It was therefore concluded that these steroids potentiated the action of catecholamines by inhibiting their inactivation. A similar mechanism of action was also proposed for the potentiation by sex steroids and cortisol on the bronchorelaxant action of isoprenaline on pig bronchus (Foster, 1983).

Human studies show that female subjects exhibit greater systemic β_2 -AR responses to inhaled and intravenous β -agonists when compared to males (Johansson, 1988; Rahman, 1992) and that this enhanced response was independent of body weight (Johansson, 1988). Rahman *et al* observed that compared to males, females had significantly greater hypokalaemic, chronotropic and electrocardiographic (reduction in T-wave amplitude and prolongation of QT_c interval) changes. This research again suggests that sex steroid hormones may have a facilitatory effect on β_2 -AR function, although four out of ten subjects were taking the combined oral contraceptive pill and the subjects were studied during different phases of their menstrual cycle. To further investigate these findings, Wheeldon *et al* (1994) measured *in vitro* parameters of lymphocyte β_2 -AR function in nine non-asthmatic females during the follicular (day 2-4) and luteal (day 21-23) phases of the menstrual cycle. Compared to male controls, in whom there were no hormone changes, female β_2 -AR density and cAMP response to isoprenaline were significantly greater during the luteal phase and were mirrored by an increase in serum progesterone and oestradiol. However, when extra-pulmonary β_2 -AR responses to weight-adjusted intravenous infusion of salbutamol were studied, females had significantly greater *in vivo* responsiveness to salbutamol compared to males during the follicular phase of the menstrual cycle (Newnham, 1994b). This enhanced effect was again independent of weight, suggesting a heightened sensitivity

of extra-pulmonary β_2 -AR. This may initially appear surprising, as Wheeldon *et al* had shown greater β_2 -AR density and responsiveness during the luteal phase. Newnham concluded that β_2 -AR in different tissues may exhibit dissociated sensitivity to the facilitatory effects of oestradiol and progesterone. Up-regulation of the β_2 -AR in target tissues by the ovarian sex hormones may therefore take longer than would be expected from *in vitro* data and in effect, result in a time lag for *in vivo* β_2 -AR responses.

Research suggesting that a proportion of female asthmatic patients suffer a premenstrual exacerbation of their condition lends weight to the putative role of female sex steroid hormones in the regulation of β_2 -AR. Premenstrual asthma was probably first described by Frank (1931) in his original publication on premenstrual tension. This was followed by a study of 36 females with asthma who had perimenstrual worsening of asthma symptoms (Claude, 1938). Dawson (1969) further strengthened the evidence for a link between asthma and the menstrual cycle by observing a higher incidence of asthma in post-pubertal compared with pre-pubertal females. At the age of 10, the ratio of asthma in girls : boys was found to be 1:3, whereas at 14 years, the ratio became 1:1. More dramatically, Barkman (1981) reported asthmatic deaths occurring premenstrually in two sisters aged 13 and 14 years. Each had been menstruating for 12 months and in both instances, death occurred on the day preceding the onset of menses. Skobeloff *et al* (1992) found that 75% of patients admitted to hospital for exacerbations of asthma were female and that these patients required longer hospital stays than age-matched males. A recent survey by the same workers of female attendances to the Emergency Department for exacerbation of asthma, found that the majority (46%) occurred during the perimenstrual period,

(Skobeloff, 1996). Lenoir (1987) described three cases of severe acute asthma associated with menstruation which required ventilation in the intensive therapy unit. Improvement of asthma in each patient coincided with cessation of menstruation. Case reports document the decline in asthmatic symptoms following the withdrawal of a combined oral contraceptive in premenopausal women (Horan, 1968) and hormone replacement therapy in a post-menopausal female (Collins and Peiris, 1993). Two studies used hormonal manipulation in an attempt to treat premenstrual asthma. Beynon *et al* (1988) described three patients with severe premenstrual asthma, resistant to conventional anti-asthma medication including high-dose corticosteroid. The addition of intramuscular progesterone (100 mg daily in two cases and 600 mg twice weekly in one) eliminated the premenopausal dips in peak flow and allowed a significant reduction in the maintenance dose of prednisolone. Another patient with severe premenstrual asthma was successfully treated using gonadotrophin releasing hormone analogue with improvement in her spirometry and reduction in the use of systemic corticosteroids and β -agonist, resulting in fewer hospital admissions (Blumenfeld, 1994). Although there was little doubt that female sex steroid hormones played a role in premenstrual asthma, much of the data had been based on circumstantial evidence and anecdotal reports. Several studies attempted to investigate the subject more thoroughly. Rees (1963) asked 81 female premenopausal asthmatic patients to chart the subjective measures of frequency and severity of asthma attacks. He found that 27 patients (33%) had a 'clear tendency' for exacerbations to occur seven to ten days prior to menstruation. There was, however, no objective data regarding the severity of asthma and its relation to menstruation and he concluded that psychological factors were responsible. Three retrospective questionnaire-based studies were performed (Hanley, 1981; Gibbs, 1984; Eliasson, 1986). These estimated premenstrual asthma to be present in 33-40% of female asthmatic patients. These

studies could have been subject to recall bias, with the use of leading questionnaires resulting in false positive reporting. Gibbs *et al* and Hanley also asked their symptomatic patients to keep daily peak flow diaries. Although statistically significantly lower peak flow rates occurred premenstrually, the amplitude of these changes (16% in Hanley's, 5.3% in Gibb's cohort respectively) was small enough to be of doubtful clinical significance. Weinmann *et al* (1987) studied nine asthmatic females during two consecutive menstrual cycles. None of his subjects had symptoms of premenstrual asthma and despite appropriate changes in serum progesterone and oestradiol, there were no changes in spirometry and airway responsiveness to histamine (expressed as PC₁₅). A more detailed prospective study was carried out in 11 female asthmatics and 29 controls over three to four consecutive menstrual cycles (Pauli, 1989). Although there were increased asthma symptoms during the luteal period compared to the follicular, there were no significant changes in spirometry or airway hyperresponsiveness to methacholine. The authors noted that six out of 11 patients who were previously unaware of premenstrual asthma recorded worsening of asthma symptoms and had reduced peak expiratory flow rates seven days preceding menstruation, although again the amplitude of these peak flow changes was small. Juniper *et al* (1987) examined 17 female asthmatics, ten with natural menstrual cycles and seven receiving the combined oral contraceptive pill. No changes could be detected in spirometry and airway hyperresponsiveness to methacholine between the follicular and luteal phases in either group.

In summary, female sex steroid hormones, like corticosteroids have a facilitatory role in β_2 -AR function, in terms of regulating receptor number and its coupling to adenylate cyclase. Being a steroid hormone, like the corticosteroids, it might regulate the transcription of several genes including the β_2 -AR gene, although this is

speculative at present. How the sex steroid hormones interact with β_2 -AR in premenstrual asthma is unclear, although we postulate that there may be abnormal hormonal regulation of β_2 -AR in female asthmatics.

CHAPTER 2

GENERAL METHODS AND MEASUREMENTS

2.1 SUBJECTS

Patients with asthma were recruited from the out-patient clinic at King's Cross Hospital, Dundee and by advertisement. Healthy subjects were recruited from staff and students at Ninewells Hospital and Medical School, Dundee. All subjects gave written informed consent prior to enrolment to a study. All had a full physical examination and normal haematological and biochemical profile prior to inclusion into any study. All asthma patients were non-smokers and all had asthma according to American Thoracic Society criteria (American Thoracic Society, 1987a). None had had an exacerbation of asthma that required the use of oral corticosteroids or antibiotics in the preceding three months prior to a study. All healthy volunteers were not on any regular medication.

2.2 SPIROMETRY

The forced expiratory volume in one second (FEV_1) and the forced mid expiratory flow rate (FEF_{25-75}) are the most used parameters to detect airflow limitation in asthma. Spirometry was performed to criteria of the American Thoracic Society (1987b) using a Vitalograph Compact spirometer (Vitalograph Ltd, Buckingham, UK) with a pneumotachograph head and pressure transducer, and on-line computer-assisted determination of FEV_1 and FEF_{25-75} . Forced expiratory manoeuvres were performed from total lung capacity to residual volume. The best test FEV_1 value was taken from three consistent measurements (a coefficient of variation of less than 3% was considered acceptable).

The peak expiratory flow rate is a very useful and simple way for the patient with asthma to monitor disease control. This is done using a portable Wright peak flow meter (Airmed, London, UK) which the patient uses in the morning and evening, prior to taking any inhaled medication, recording the best of three consecutive manoeuvres.

2.3 BRONCHIAL PROVOCATION TESTS

Bronchial provocation tests have an established place in the investigation of asthma and have provided insight into the pathophysiology and pharmacology of asthma.

Bronchoconstrictor stimuli can be either direct-acting or indirect-acting. The methacholine inhalation test has been described since the 1940's and is an example of a direct-acting bronchoconstrictor whereby it induces bronchoconstriction by acting directly on bronchial smooth muscle to cause contraction. Adenosine monophosphate challenge, on the other hand is more recently described (Cushley, 1983) and is an example of an indirect acting bronchoconstrictor as it is thought to induce bronchoconstriction by stimulating mast cells to release histamine which in turn acts on bronchial smooth muscle cells to contract (Cushley, 1985). Results are expressed as either the provocation concentration or dose required to produce a 20% fall in FEV₁, the PC₂₀ or PD₂₀ respectively. Both of these bronchial provocation tests have been employed in the subsequent studies.

2.3.1 Adenosine monophosphate challenge

Bronchial challenge was performed using a Nebicheck nebuliser controller (PK Morgan Ltd) with a System 22 Acorn nebuliser (Medic-Aid Ltd) with a driving

pressure of 20 psi (138 kPa). The nebuliser was activated for 1.2 seconds from the initiation of inspiration. Fresh solutions of adenosine 5'-monophosphate (AMP; Sigma, Poole, UK) were made up in normal saline on each study day in a range of concentrations from 0.04 mg/ml to 400mg/ml. A mouthpiece was used with the nebuliser and the nose clipped during the procedure. The mouthpiece was placed between the teeth of the subject who exhaled to slightly below functional residual capacity and then inhaled slowly over one to two seconds toward total lung capacity, where the breath was held for three seconds before taking the next breath. Subjects inhaled five breaths of normal saline control solution followed by sequential doubling concentrations of AMP given at three minute intervals. FEV₁ was measured one minute after each administration of saline and AMP. The test was terminated when a 20% fall in FEV₁ from the post-saline value was attained. A log-dose response curve was constructed and PC₂₀ was calculated by linear interpolation. If the FEV₁ did not show a 20% fall after the maximum concentration had been given, a censored PC₂₀ value of 800 mg/ml (double of the maximum concentration) was assigned for that test for the purpose of statistical analysis (Chinn, 1991).

2.3.2 Methacholine challenge

In Chapter 5, a collaborative study with the Respiratory Unit at Newcastle General Hospital was carried out. This used a bronchoprovocation challenge previously validated by that Unit (Beach 1993; Connoll 1988) so that identical equipment and protocols were used in both centres. In this test, methacholine was administered in double cumulative doses from 3.125-6400µg using a microprocessor controlled dosimeter, and given at 5 minute intervals until a 20% fall in FEV₁ was recorded. The aerosol was released electronically using a pressure sensor in 10µl aliquots over approximately 2 seconds as the subject begins to inhale from functional residual

capacity. The patient continued to inhale for 5 seconds until an electronically controlled bleep indicated the inspiratory manoeuvre was completed. Five such aliquots were inhaled in immediate succession comprising a single dose. Each dose was administered immediately on completing the FEV₁ measurement, the start of spirometric manoeuvres governing the time sequence of the whole test. Spirometry involved 6 immediately consecutive forced expiratory manoeuvres and the mean of the 3 best FEV₁ readings is calculated at that time point. In order to avoid discomfort of completing the forced vital capacity manoeuvre, each forced expiration is discontinued after 1 second. All the nebulisers were calibrated individually prior to first use. The calculation of PD₂₀ was by computer assisted log linear interpolation from the dose-response curve. If a 20% fall in FEV₁ was not observed after the last dose, log linear extrapolation was applied. If the extrapolated PD₂₀ value was larger than twice the last dose, then the PD₂₀ was censored as twice the value of the last dose for the purpose of analysis. If a 20% fall in FEV₁ was obtained after the first dose, the PD₂₀ value was calculated using linear interpolation between baseline and the first dose. If the interpolated dose was smaller than half of the first given dose, the PD₂₀ value was censored as half of the first dose value.

2.4 LYMPHOCYTE β_2 -ADRENOCEPTOR PARAMETERS

Lymphocyte β_2 -adrenoceptor parameters including receptor density (B_{\max}), binding dissociation constant (K_d) and maximal cyclical AMP response to isoprenaline (E_{\max}) were evaluated. Forty ml whole blood was collected into tubes containing ethylene diamine tetraacetic acid, diluted to 50ml with phosphate buffered saline (PBS) and then two equal portions were centrifuged with 15ml Lymphoprep (Nycomed Pharma

AS, Oslo, Norway). The lymphocyte layer was removed and, after two further washes and centrifugation, the lymphocyte pellet was removed and resuspended in 5ml PBS prior to lymphocyte counting, with 5×10^6 cells being required for cyclic AMP stimulation by isoprenaline. Lymphocyte B_{\max} and K_d were determined with ^{125}I -iodocyanopindolol (ICYP, NEN-DuPont (UK) Ltd, Stevenage, UK) at eight concentrations between 5 and 160 pmol/l. CGP 12177 (Ciba-Geigy, Basle, Switzerland) was added to half the tubes to prevent ICYP binding to the receptor sites, thus allowing non-specific binding to be evaluated. After washing with assay buffer, the bound and unbound suspensions were aspirated onto filter paper using a Brandel Cell Harvester (Semat Technical [UK] Ltd, St Albans, UK). The resultant counts were determined with a gamma camera (LKB Wallac, Wallac OY Pharmacia, Turku, Finland) and specific binding was calculated from total binding minus non-specific binding. Receptor density was calculated from extrapolation from the specific and non-specific binding curves plotted for each concentration of ICYP. The inter-assay coefficients of variation for analytical imprecision were 10.3% for B_{\max} and 5.9% for K_d . A radioimmunoassay technique (Incstar, Stillwater, USA) was used to evaluate cyclic AMP levels (E_{\max}) following suspension in PBS containing theophylline (100 μg) and bovine serum albumin. It was stimulated with isoprenaline (10^{-4}M) during incubation at 37° C, before terminating the reaction by heating to 95° C. After centrifugation the supernatant was removed and cyclic AMP measured. The intra-assay and inter-assay coefficients of variation for analytical imprecision were 2.7% and 10.2% respectively.

2.5 β_2 -ADRENOCEPTOR POLYMORPHISM

A number of common variants (polymorphisms) of the β_2 -adrenoceptor have recently been described which alter the behaviour of the receptor following exposure to agonist. Four polymorphisms have been identified which result in single amino-acid substitutions in the receptor protein sequence; two of which have been studied in this thesis, codon 16 and 27. Analysis of β_2 -adrenoceptor genotype in this thesis was carried out at the Department of Medicine, University Hospital of Nottingham. β_2 -adrenoceptor genotype was assessed by allele-specific oligonucleotide hybridisation with methods adapted from those designed to study mutations in the α_1 -anti-trypsin gene and its promoter region. 5 ml whole blood was taken from each subject and genomic DNA extracted with the Nucleon system (Scot Lab). A 234 base pair fragment spanning the mutation of interest was generated by PCR from the 5' end of the β_2 -adrenoceptor gene. The primers used were: upstream, CCCAGCCAGTGCGCTTACCT, and downstream, CCGTCTGCAGCTCGAAC. One μ L PCR product was applied to duplicate Hybond N plus filters on a dot-blot apparatus. To assess genotype, each filter was hybridised as appropriate with excess 'cold' (unlabelled) followed by 'hot' (32 P-labelled) probes homologous with either the wild-type (Arginine 16, Glutamine 27) or Glycine 16 and Glutamate 27 forms of the receptor. The duplicate filter was treated identically but with the probes in reverse order. A random selection of PCR fragments was also directly sequenced to confirm the specificity of genotype identification by allele-specific oligonucleotide hybridisation.

2.6 HORMONE ASSAYS

Blood samples for hormone assays were centrifuged at 4° C and serum extracted and stored at -20° C till analysed in batches at the end of each study in duplicate. Serum oestradiol (Sorin Biomedica, Saluggia, Italy) and progesterone (Incstar, Stillwater, USA) were measured by radioimmunoassay. The intra-assay coefficients of variation for analytical imprecision were 2.9% and 6.3% respectively. Serum cortisol was also measured by radioimmunoassay (Sorin Biomedica, Saluggia, Italy). The intra-assay and inter-assay coefficients of variation for analytical imprecision were 9.4% and 6.65% respectively.

2.7 SERUM POTASSIUM

Serum potassium was measured by flame photometry (IL943 analyser, Instrumentation Laboratory Ltd, Warrington, UK) with analysis being performed in batches at the end of each study and samples being assayed in duplicate. The coefficients of variation for analytical imprecision within and between assays were 0.93% and 0.79% respectively. The normal reference range for our laboratory is 3.5 to 5.0 mmol⁻¹.

2.8 HEART RATE

Heart rate was calculated from the R-R interval of the electrocardiogram. The electrocardiogram was recorded on a standard lead II using a Hewlett-Packard (Palo

Alto, California, USA) monitor and printer with paper speed set at 50 mm/s and 0.5 mV/cm gain. The R-R interval was calculated from the mean of five consecutive complexes.

2.9 FINGER TREMOR

Finger tremor was recorded by a previously validated method (Lipworth, 1989) using an accelerometer transducer (Entran Ltd, Ealing, UK). This was taped to the distal phalanx of the middle finger. Postural tremor was measured with the fingers extended and the wrist in neutral position for 20 second periods. Five recordings were measured, and the results were stored on computer disc for subsequent spectral analysis of total tremor power of more than 2 Hz (units of mg^2/sec) using computer-assisted autocovariance. The mean of three lowest consistent readings was analysed.

2.10 STATISTICAL ANALYSIS

Data for finger tremor, receptor density B_{max} and PC_{20} and PD_{20} were transformed with logarithm to base 10 prior to analysis as these variables were not normally distributed. For dose-response curves, all parameters were analysed as delta responses from baseline. For all parameters comparisons were made by multifactorial analysis of variance (MANOVA) using subjects, treatments, visits and period as factors for analysis. When significant overall differences between treatments were found to occur with MANOVA, multiple-range testing was applied to identify where these differences occurred. A value of $p < 0.05$ (two-tailed) was considered as being

significant, and 95% confidence intervals (95% CI) for mean treatment differences were calculated where significant. Data were analysed with a Statgraphics Statistical software package (STSC Software Publishing Group, Rockville, USA).

2.11 DRUGS

Formoterol (Foradil®) pressurised metered dose inhaler, 12 µg per actuation.

Formoterol (Turbuhaler®) multi-dose dry powder inhaler and matching placebo, prepared by Astra Pharmaceuticals.

Terbutaline (Bricanyl®) multi-dose dry powder inhaler.

Ipratropium bromide (Atrovent Forte®) pressurised metered dose inhaler, 40 µg per actuation.

Prednisolone 25mg and 5mg tablets, with matching placebo tablets prepared by the Pharmacy Department, Ninewells Hospital.

Hydrocortisone 100mg ampoules for intravenous injection.

Fluticasone propionate (Flixotide®), pressurised metered dose inhaler, 250 µg per actuation with matching placebo inhaler prepared by the Pharmacy Department.

Salbutamol (Ventodisk®), dry powder inhaler, 400 µg per dose.

Salbutamol (Ventolin®) pressurised metered-dose inhaler, 100 µg per actuation.

Medroxyprogesterone (Provera®) 10mg tablets.

Ethinylestradiol (non-proprietary) 50 µg tablets.

2.12 ETHICAL APPROVAL

In all clinical studies, all subjects gave written informed consent prior to being evaluated. All studies had been approved by the Tayside Committee on Medical Research Ethics.

CHAPTER 3

SYSTEMIC CORTICOSTEROID RAPIDLY REVERSES BRONCHODILATOR SUBSENSITIVITY INDUCED BY FORMOTEROL IN ASTHMATIC PATIENTS

**(American Journal of Respiratory and Critical Care Medicine 1997;
156: 28-35)**

3.1 INTRODUCTION

There have been several studies demonstrating that bronchodilator subsensitivity develops after regular treatment with the long-acting β_2 -agonist, formoterol in asthma patients (Newnham 1994a; Yates 1995; Newnham 1995). This has also been demonstrated with salmeterol (Grove, 1995). The mechanism for this subsensitivity appears to be due to β_2 -adrenoceptor (β_2 -AR) down-regulation and desensitisation from prolonged β_2 -agonist exposure. Most of the patients involved in the above studies were receiving inhaled corticosteroid, indicating that perhaps inhaled corticosteroids do not protect against the development of β_2 -AR subsensitivity. There is some evidence to suggest that systemic corticosteroid may exhibit a facilitatory role in terms of reversing previously down-regulated β_2 -AR in normals and asthmatics (Hui, 1982; Brodde, 1985; Brodde, 1988). However, it is unclear as to the time-course for such effects *in vivo*. Also, it is unclear as to whether up-regulation of β_2 -AR *in vitro* correlates with reversal of *in vivo* responses.

In this initial study, we have investigated the acute facilitatory effects of systemic corticosteroid on *in vitro* and *in vivo* β_2 -AR function in asthmatic patients treated with regular inhaled formoterol, that is in the presence of established β_2 -AR subsensitivity.

3.2 METHODS

Patients

Twelve asthmatic patients (four female and eight male), with a mean age of 44 years (range: 20 - 63 yr) were recruited and completed the study. All gave written informed consent before being randomised according to a double blind, placebo controlled, cross-over protocol approved by the Tayside medical ethics committee. A full physical examination, 12-lead electrocardiogram, and measures of biochemical and haematological parameters were normal prior to inclusion of the patients. All had asthma according to the criteria of the American Thoracic Society (1987a), and all were non-smokers. At the initial screening visit, subjects were required to have an FEV₁ of 40-80% of predicted normal with at least 15% reversibility of FEV₁ with inhaled formoterol 24µg. The mean (SEM) FEV₁ in litres and percent predicted was 2.14 (0.15) and 66 (3)% predicted, range 1.34 to 2.97 L (% predicted range 47-75%). All patients were receiving inhaled corticosteroid (either budesonide or beclomethasone dipropionate; one patient received fluticasone propionate), median dose 1000 µg/day, range 400 - 1600 µg/day. All had been inhaling short-acting β₂-agonists as required prior to recruitment in doses of <800 µg/day. Five subjects were inhaling salmeterol 100 µg/day. In addition, 4 subjects were taking slow-release oral theophylline preparations. None had received oral corticosteroid for at least 3 months, and none had had a recent exacerbation of asthma in the month preceding the study. Before entry into the study, all subjects were supervised in the use of a metered-dose inhaler (MDI) using a Vitalograph aerosol inhalation monitor (Vitalograph Ltd, Buckingham, UK)

Protocol

After the initial screening visit, the subjects had a two week washout period without any β₂-agonists during which they used ipratropium bromide at 40µg per actuation (Atrovent Forte, Boehringer Ingelheim, Bracknell, UK) as a substitute for rescue

requirements. After two weeks, subjects attended for a randomisation visit, and FEV₁ was measured yielding a mean (SEM) value 2.20 (0.15)L or 67(3)% predicted. Subjects were then randomised to receive concurrent treatments in crossover fashion with either inhaled placebo or inhaled formoterol 24µg twice-daily (12µg per actuation; Foradil, Ciba-Geigy AG, Basel, Switzerland) both via a metered dose inhaler, for four weeks, whilst maintaining their inhaled steroid and other anti-asthma therapy at a constant dose (Figure 3.1). Six subjects received formoterol as first treatment and six received placebo first. During the treatment period, an ipratropium bromide inhaler was also available for rescue purposes in order to ensure that β_2 -agonists were not used during the placebo period. The study treatment was taken twice daily, between 7:00 A.M. and 9:00 A.M. and again between 7:00 P.M. and 9:00 P.M. The subjects were also asked to keep morning and evening peak expiratory flow readings, made with a Wright peak flow meter (Airmed, London, UK), in a diary.

Subjects attended the laboratory after three weeks and four weeks of treatment at 8:00 A.M., having withheld their study medication for 24 hours, ipratropium bromide for at least 8 hours and oral theophylline for 48 hours. At each visit, an intravenous cannula was inserted and kept patent by bolus injections of heparinised saline. A cannula dead space of 2ml was withdrawn prior to blood sampling. A dose-response curve (DRC) to inhaled formoterol was constructed one hour after ingestion of placebo tablets and intravenous placebo injection at the three week visit, and one hour after oral prednisolone 50mg and intravenous hydrocortisone 200mg at the four week visit. The rationale for the administration of corticosteroid was to mimic what would be normally given in an acute asthma attack ie. an initial bolus of hydrocortisone and a first dose of oral prednisolone to provide cover over the next 24 hours. The administration of placebo at week three and systemic corticosteroid at week four was done so as to avoid any possibility of carry-over from one dose-response curve to another.

The DRC was constructed with inhaled formoterol, given via a metered-dose inhaler, using doses of 12 µg, 24 µg, 24 µg and 48 µg - that is, a cumulative dose of 108 µg after the last dose, with the doses separated by 40 minutes. Measurements of FEV₁, FEF₂₅₋₇₅, serum potassium (K), heart rate (HR), and postural finger tremor (Tr) were undertaken over a 10-minute period at baseline (before placebo/steroid tablets and injection), one hour after placebo/steroid tablets and injection (that is, the start of the DRC) and 30 minutes after each dose, and repeated at 1, 2, 4 and 6 hours after the last dose. 40 ml of blood for lymphocyte β₂-AR parameters (B_{max}, E_{max} and K_d) was withdrawn at one and three hours post-administration of placebo/steroid. The next day, twenty-four hours after administration of placebo/steroid, subjects attended the laboratory for lymphocyte β₂-AR parameters, and measurement of FEV₁ and FEF₂₅₋₇₅ before and 30 minutes after 12µg formoterol. The purpose of the latter measurement was to observe whether the effects of the steroid were still present after 24 hours in terms of modulating *in vitro* and *in vivo* β₂-AR function, although a full DRC was not performed.

10 ml whole blood was also taken for β₂-AR polymorphism and stored in ethylenediamine tetraacetic acid at -20°C. Unfortunately, one sample was lost and we have analysed β₂-AR polymorphism in 11 of 12 subjects.

Measurements

FEV₁ and FEF₂₅₋₇₅ - see Chapter 2, Spirometry.

Serum potassium - see Chapter 2.

Heart rate - see Chapter 2.

Finger tremor - see Chapter 2.

Lymphocyte β_2 -adrenoceptor parameters - see Chapter 2.

Identification of β_2 -adrenoceptor polymorphisms - see Chapter 2.

Statistical analysis

All variables were analysed as delta responses from the baseline 1 hour after injection of placebo or hydrocortisone. Comparisons for the DRC were made as area-under-curve (AUC) in order to obviate multiple comparisons at several time points. For all parameters, comparisons were made by multifactorial analysis of variance (MANOVA) using subjects, treatments, visits and period as factors for analysis. When significant overall differences between treatments were found to occur with MANOVA, Duncan's multiple-range testing was applied to identify where these differences occurred. A value of $p < 0.05$ (two-tailed) was considered as being significant, and 95% confidence intervals (95% CI) for mean differences were calculated where significant.

3.3 RESULTS

Baseline values for airway and systemic parameters were not significantly different after treatment with either placebo or formoterol, either before or after administration of placebo/steroid tablets and injection (Table 3.1).

Bronchodilator and systemic DRC

The bronchodilator and systemic dose-response curves (as area-under-curve, AUC) after regular treatment with placebo and formoterol, with and without systemic steroid are summarised in Table 3.2, and shown graphically in Figures 3.2, 3.3 and 3.4.

The DRCs (as change from baseline) after pretreatment with either placebo or formoterol showed dose-dependent increases in delta FEV₁ and delta FEF₂₅₋₇₅ and a plateau in response was not attained within the dose range, 40 minutes after the last dose (ie. at t=2h 40min) (Figure 3.2). There was a rightward shift in the DRC for both delta FEV₁ and delta FEF₂₅₋₇₅ after treatment with formoterol compared with placebo: $p=0.01$ for dFEV₁ and dFEF₂₅₋₇₅. Following administration of systemic corticosteroid (S), there was significant reversal of bronchodilator tachyphylaxis for dFEV₁ ($p=0.03$) and dFEF₂₅₋₇₅ ($p=0.005$). There was no significant difference in response between formoterol plus steroid (FM+S) and placebo plus steroid (PL+S).

On the following day (ie. 24 hours after injection of placebo or steroid), the baseline FEV₁ and FEF₂₅₋₇₅ were lower following pretreatment with formoterol compared with placebo: $p=0.02$ for FEV₁ and $p=0.004$ for FEF₂₅₋₇₅ (Figure 3.3). Formoterol 12 μ g was then administered and the 30 minute post-bronchodilator values for FEV₁ and FEF₂₅₋₇₅ were not significantly different.

There were dose-dependent increases in finger tremor (Tr) and heart rate (HR), and decrease in serum potassium (K) (Figure 3.4). There were significant rightward shifts

in Δ Tr and HR after treatment with formoterol compared with placebo: $p < 0.001$ for HR and $p = 0.04$ for Tr. There was no significant rightward shift in Δ K ($p = 0.13$). After the administration of systemic corticosteroid, tachyphylaxis in HR response was reversed ($p < 0.001$). There was no significant reversal in DRC in Δ Tr response following FM with steroid compared with FM alone ($p = 0.20$). There was augmentation of the Δ HR response with placebo plus steroid compared with placebo alone ($p < 0.001$).

Lymphocyte β_2 -adrenoceptor parameters

There were no significant differences in $\log B_{\max}$ (fmol/ 10^6 cells) between any of the treatments one hour after injection of placebo or steroid (Figure 3.5). However, at 3 hours after injection, there was a significant increase in $\log B_{\max}$ comparing formoterol with steroid with formoterol alone ($p = 0.01$). There was also a significant increase with systemic steroid after placebo pretreatment ($p < 0.001$). After 24 hours, the effect of corticosteroid had diminished, and $\log B_{\max}$ had fallen to levels below baseline. The trend for time-profile changes in E_{\max} (pmol/ 10^6 cells) mirrored those of $\log B_{\max}$ in that there was a significant increase 3 hours post-corticosteroid administration in the placebo pre-treated group (E_{\max} at 1h vs E_{\max} at 3h ; $p = 0.003$). Values at 3 hours were: (PL+S vs PL) 4.62 vs 3.74, but these were not significantly different. For dissociation constant K_d , the time-profile changes were different to those of B_{\max} and E_{\max} . At one and three hours post-administration of corticosteroid, there were no significant differences in K_d . However, by 24 hours there was a significant ($p < 0.05$) decrease in K_d in subjects treated with formoterol or placebo, except in those given corticosteroid.

PEFR diary cards

Mean peak expiratory flow rates (L/min) in the morning were higher with formoterol than with placebo which was sustained for 4 weeks of treatment: (PL vs FM), for

week 1: ($p=0.05$); week 2: ($p=0.008$); week 3: ($p=0.008$) and week 4: ($p=0.005$). Likewise, evening PEF_R was higher with formoterol than with placebo throughout the 4 weeks of treatment. (Figure 3.6)

β_2 -adrenoceptor polymorphism

Eleven subjects were analysed for β_2 -AR polymorphism at codon 16 and 27. Seven subjects were homozygous Gly 16, three were heterozygous Arg 16/Gly 16 and one was homozygous Arg 16; three were homozygous Glu 27, six were heterozygous Gln 27/Glu 27 and two were homozygous Gln 27. The individual responses for AUC FEV₁ and AUC FEF₂₅₋₇₅ in terms of β_2 -AR polymorphism at codon 16 and 27 are shown in Figures 3.7 and 3.8 respectively. The data were not analysed in terms of differing subsensitivity because of insufficient numbers of subjects with different polymorphisms. Hence, only a qualitative assessment can be made of these results. After the development of bronchodilator subsensitivity following treatment with formoterol, the administration of systemic corticosteroid produced reversal in the majority of subjects irrespective of β_2 -AR polymorphism.

		PL	PL+S	FM	FM+S	'p' value
Baseline(pre- injection)	FEV ₁ (l)	2.18 (2.07-2.30)	2.11 (1.99-2.22)	2.00 (1.89-2.12)	2.01 (1.90-2.13)	0.10
	FEF ₂₅₋₇₅ (l/s)	1.41 (1.23-1.60)	1.25 (1.06-1.44)	1.27 (1.09-1.46)	1.37 (1.19-1.56)	0.53
	K (mmol/l)	3.91 (3.75-4.08)	3.86 (3.69-4.02)	3.84 (3.68-4.01)	3.97 (3.80-4.14)	0.69
	Tr (log units)	2.27 (1.97-2.57)	2.48 (2.20-2.76)	2.47 (2.19-2.75)	2.42 (2.16-2.68)	0.69
	HR (beats/min)	75 (71-78)	71 (67-74)	75 (72-79)	77 (73-80)	0.12
	FEV ₁ (l)	2.26 (2.15-2.38)	2.16 (2.05-2.27)	2.08 (1.96-2.19)	2.07 (1.95-2.18)	0.07
	FEF ₂₅₋₇₅ (l/s)	1.56 (1.41-1.71)	1.47 (1.32-1.62)	1.36 (1.21-1.51)	1.41 (1.26-1.56)	0.24
	K (mmol/l)	3.84 (3.70-3.98)	3.96 (3.82-4.10)	3.94 (3.80-4.08)	3.98 (3.84-4.12)	0.50
Baseline(1 hour post-injection)	Tr (log units)	2.08 (1.92-2.23)	2.05 (1.89-2.22)	2.15 (2.00-2.31)	2.32 (2.17-2.47)	0.09
	HR (beats/min)	68 (65-70)	66 (64-69)	69 (66-71)	67 (65-70)	0.69

Table 3.1 Mean (95% CI) baseline values measured before and one hour after administration of systemic corticosteroid (S), after regular treatment with placebo (PL) and formoterol (FM), before construction of the dose-response curve.

'p' value refers to overall comparisons between treatments by MANOVA.

	PL	PL+S	FM	FM+S
AUC FEV ₁ (L.h)	4.22 * (3.39-5.05)	4.26 * (3.74-4.78)	2.51 (1.68-3.34)	3.57 * (3.06-4.09)
AUC FEF ₂₅₋₇₅ (L×10 ³)	19.94 * (15.34-24.55)	20.05 * (17.47-22.63)	11.30 (6.72-15.92)	18.47 * (15.89-21.04)
AUC K (mmol.h/L)	2.92 (3.94-1.89)	3.55 * (4.57-2.52)	2.03 (3.11-0.96)	2.10 (3.18-1.03)
AUC Tremor (log units.h)	5.49 * (4.33-6.65)	6.00 * (3.52-8.49)	3.39 (1.85-4.92)	4.31 (3.08-5.54)
AUC HR (beats)	5200 * (3600-6900)	11400 *+ (9780-13080)	2700 (1320-4140)	9600 *+ (8200-11000)

Table 3.2 Mean (95% CI) airway and systemic dose-response curves (as area-under-curve, AUC) after treatment with placebo (PL) and formoterol (FM), with and without steroid (S). * denotes $p < 0.05$ compared with FM, + denotes $p < 0.05$ compared with PL

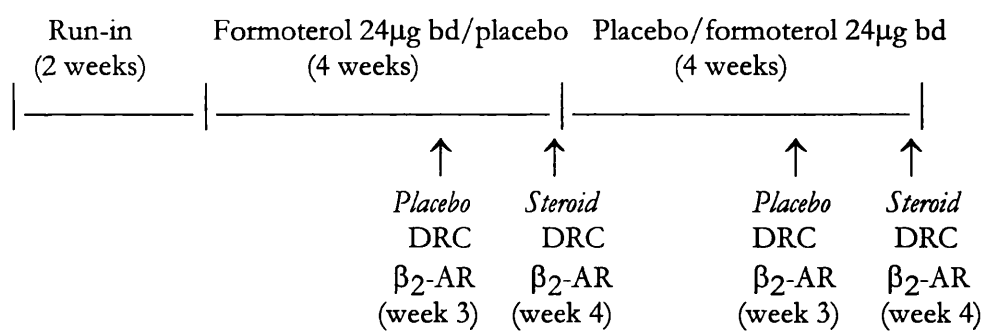


Figure 3.1 Overview of study design.

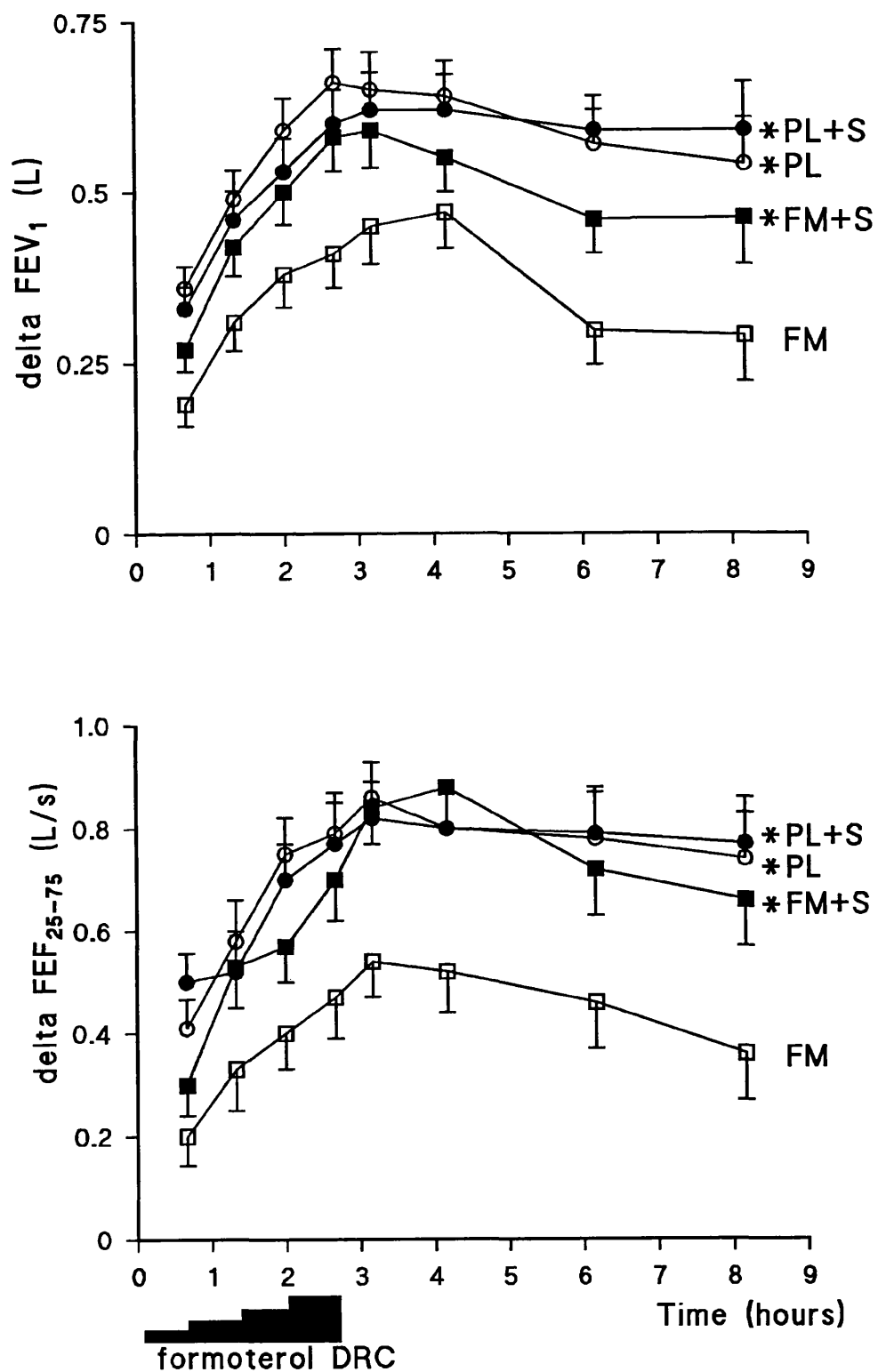


Figure 3.2 Dose-response curves and time-profile after last dose for FEV₁ and FEF₂₅₋₇₅ after regular treatment with placebo (PL) and formoterol (FM) with or without acute administration of systemic corticosteroid (S). Values are shown as mean (SEM) change from baseline. * $p < 0.05$ versus formoterol for AUC.

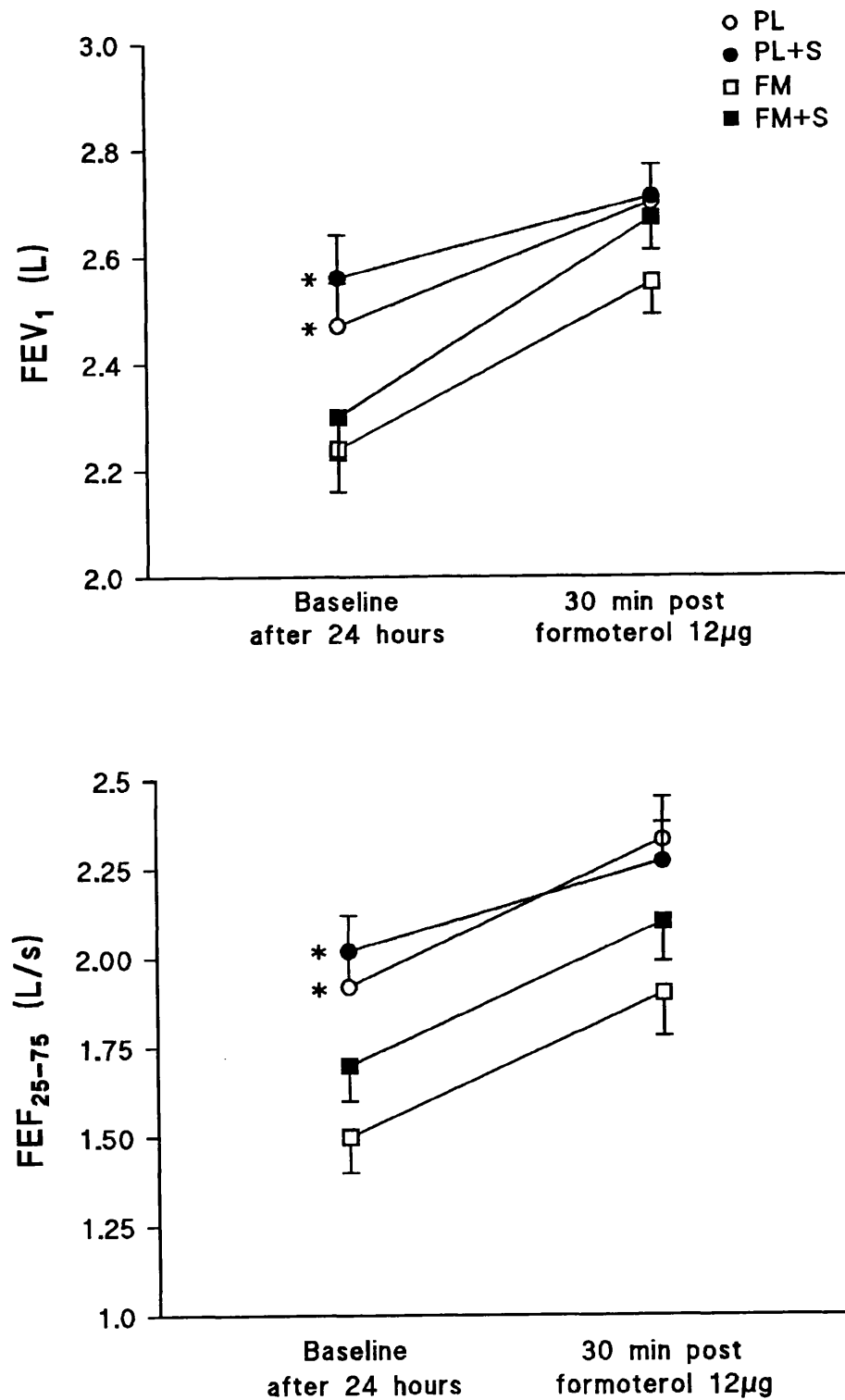


Figure 3.3 Absolute values for FEV₁ and FEF₂₅₋₇₅ at baseline and 30 min after a single 12μg dose of formoterol, with or without steroid, at 24h after administration, following regular treatment with formoterol (FM) and placebo (PL). * p<0.05 versus formoterol at baseline. No significant differences were seen after formoterol 12μg.

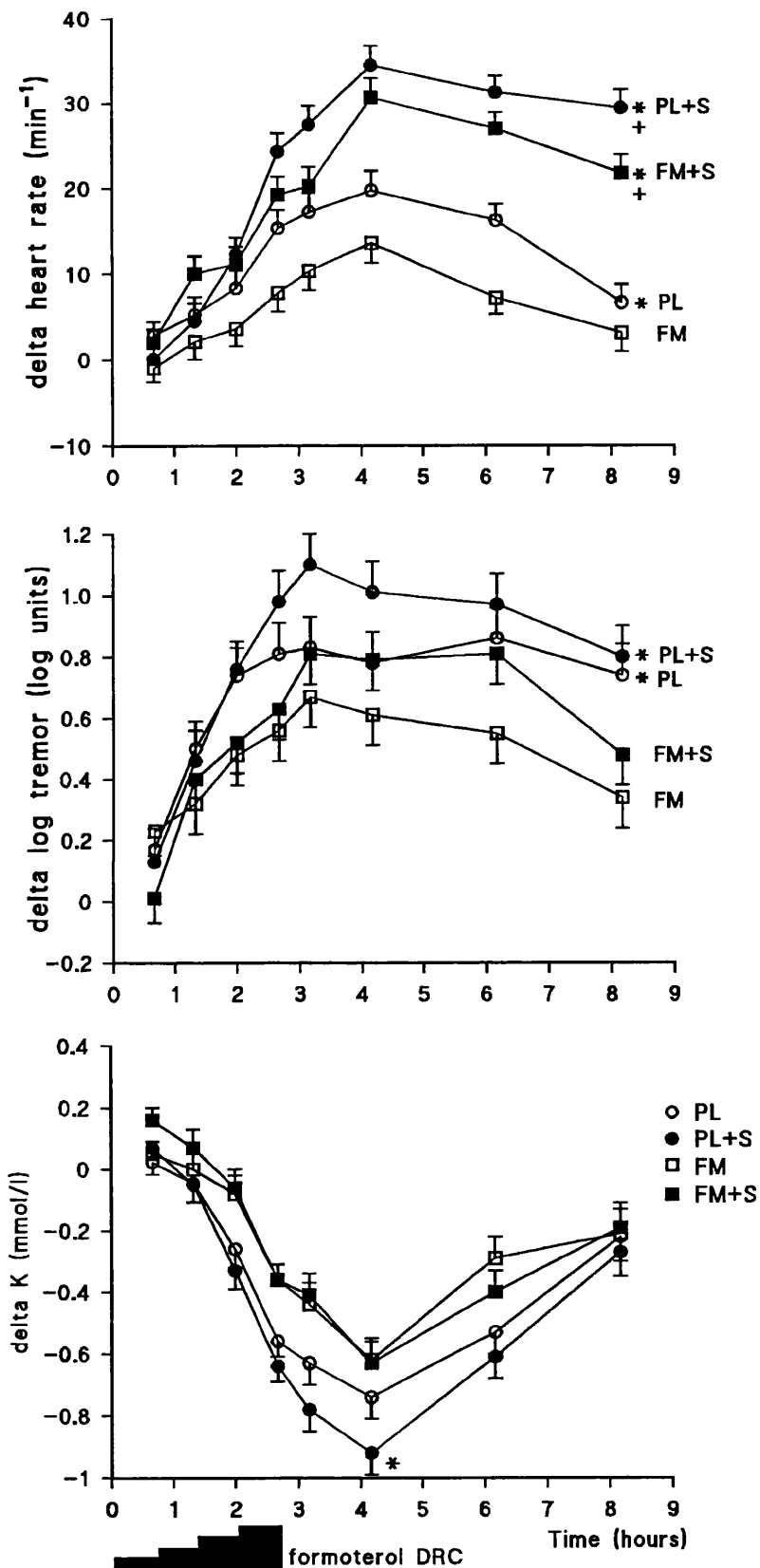


Figure 3.4 Dose-response curves and time-profile for heart rate, finger tremor and serum potassium (K) after last dose of regular treatment with placebo (PL) and formoterol (FM), with or without steroid (S). Values are shown as mean (SEM) change from baseline. * $p < 0.05$ versus formoterol; + $p < 0.05$ versus placebo for AUC.

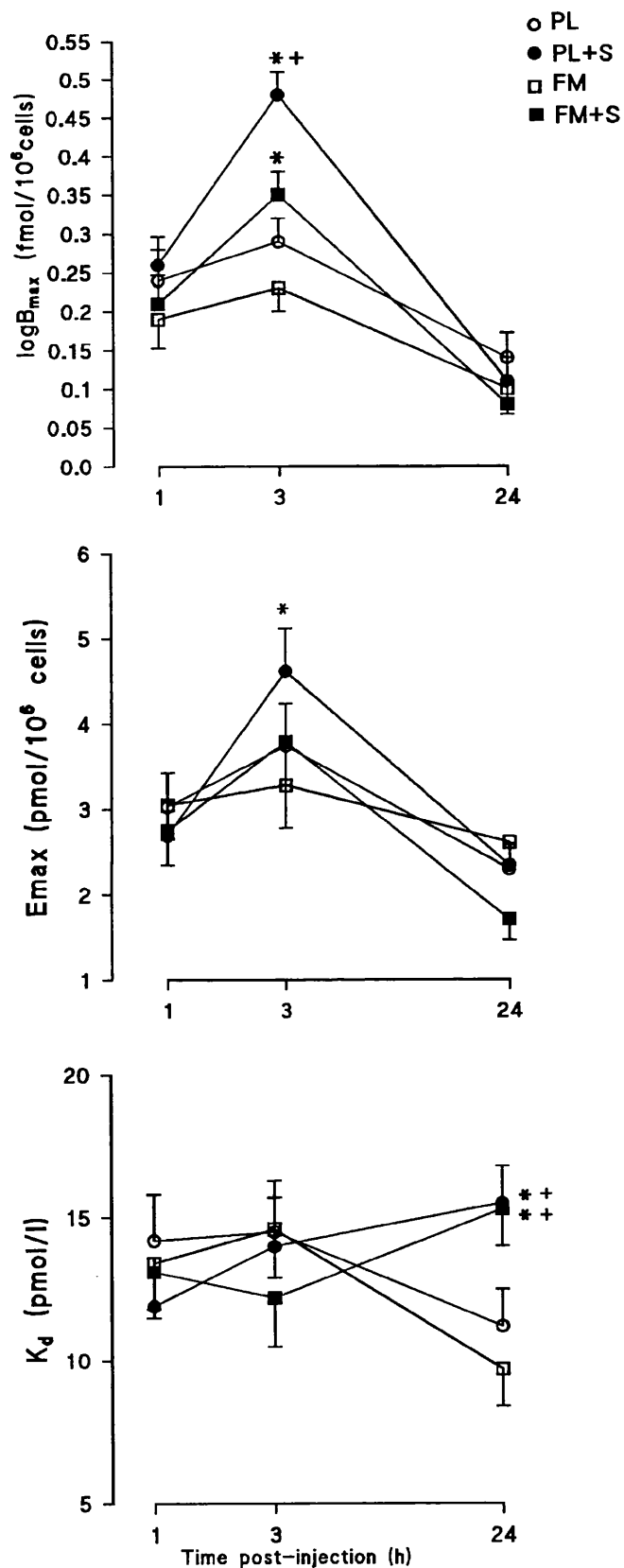


Figure 3.5 Lymphocyte β_2 -adrenoceptor parameters: B_{max} , E_{max} and K_d with or without steroid (S) at 1,3 and 24h after administration, following regular treatment with placebo (PL) and formoterol (FM). * $p < 0.05$ versus formoterol; + $p < 0.05$ versus placebo.

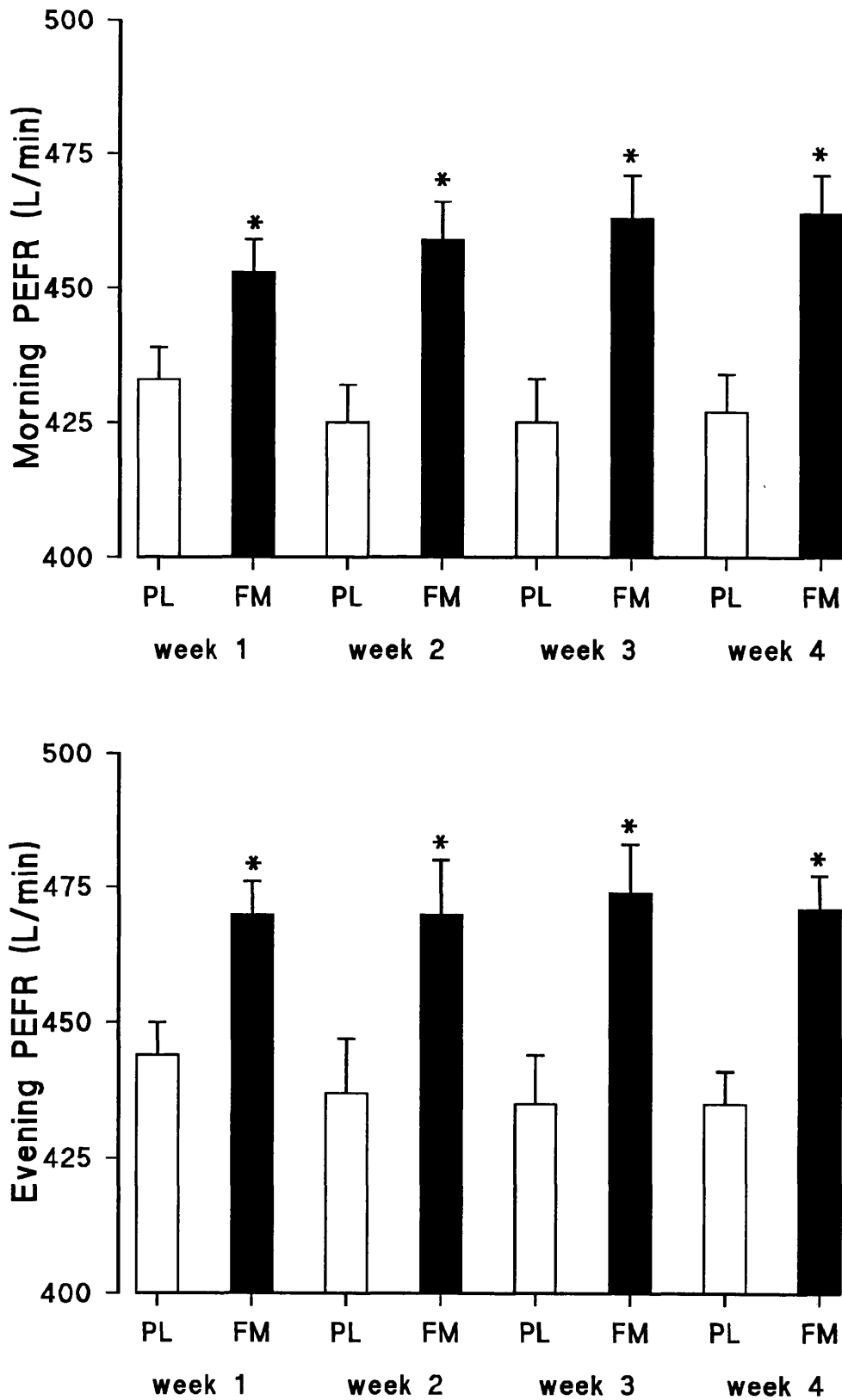


Figure 3.6 Mean (SEM) peak expiratory flow diary recordings (12h after each dose) in the morning and evening during 4 wk of treatment with placebo (PL) and formoterol (FM). * $p < 0.05$ FM versus PL.

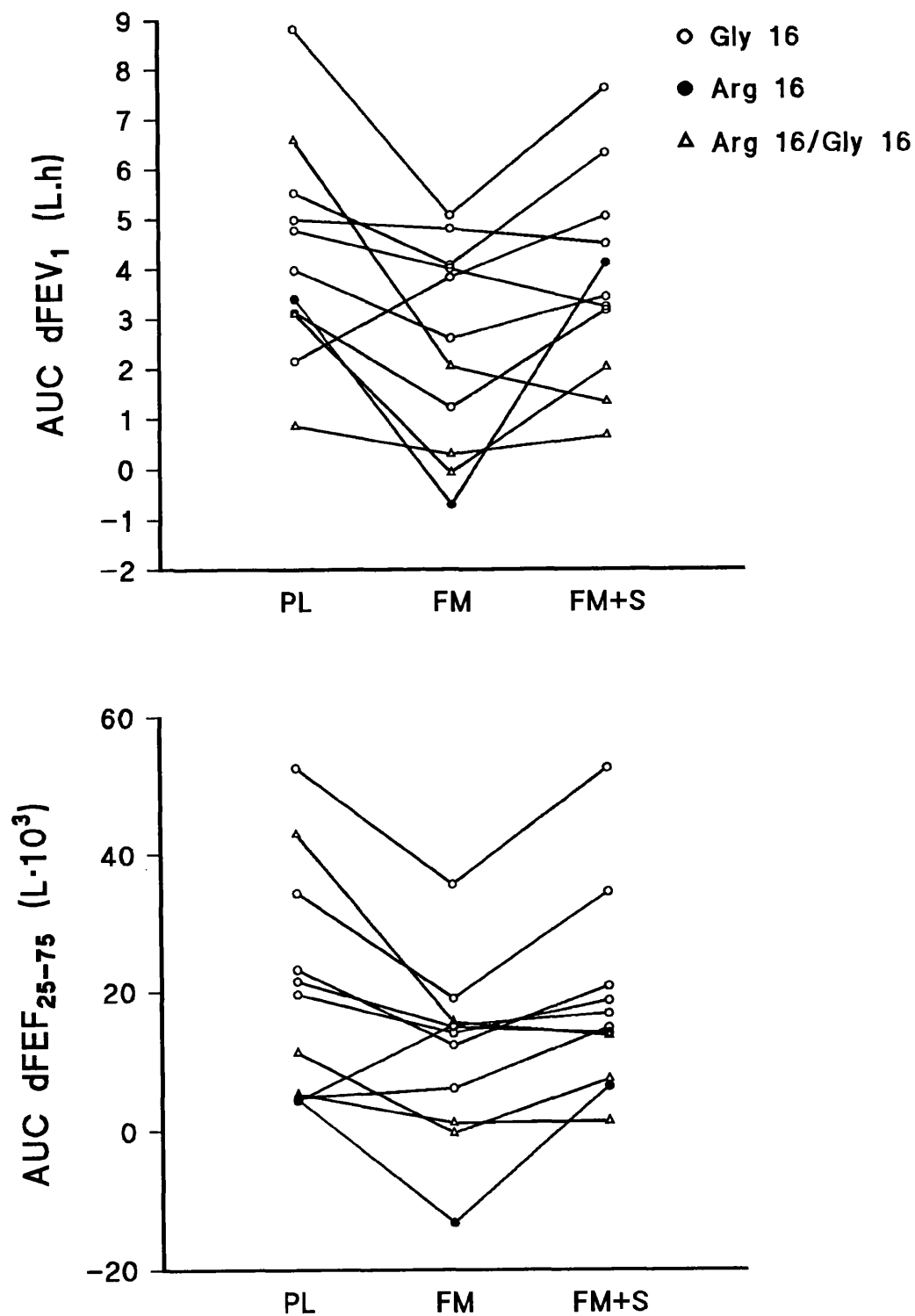


Figure 3.7 Individual responses for AUC FEV₁ and AUC FEF₂₅₋₇₅ after treatment with placebo (PL) and formoterol (FM), compared with reversibility following steroid (FM+S), according to β_2 -adrenoceptor polymorphism at codon16.

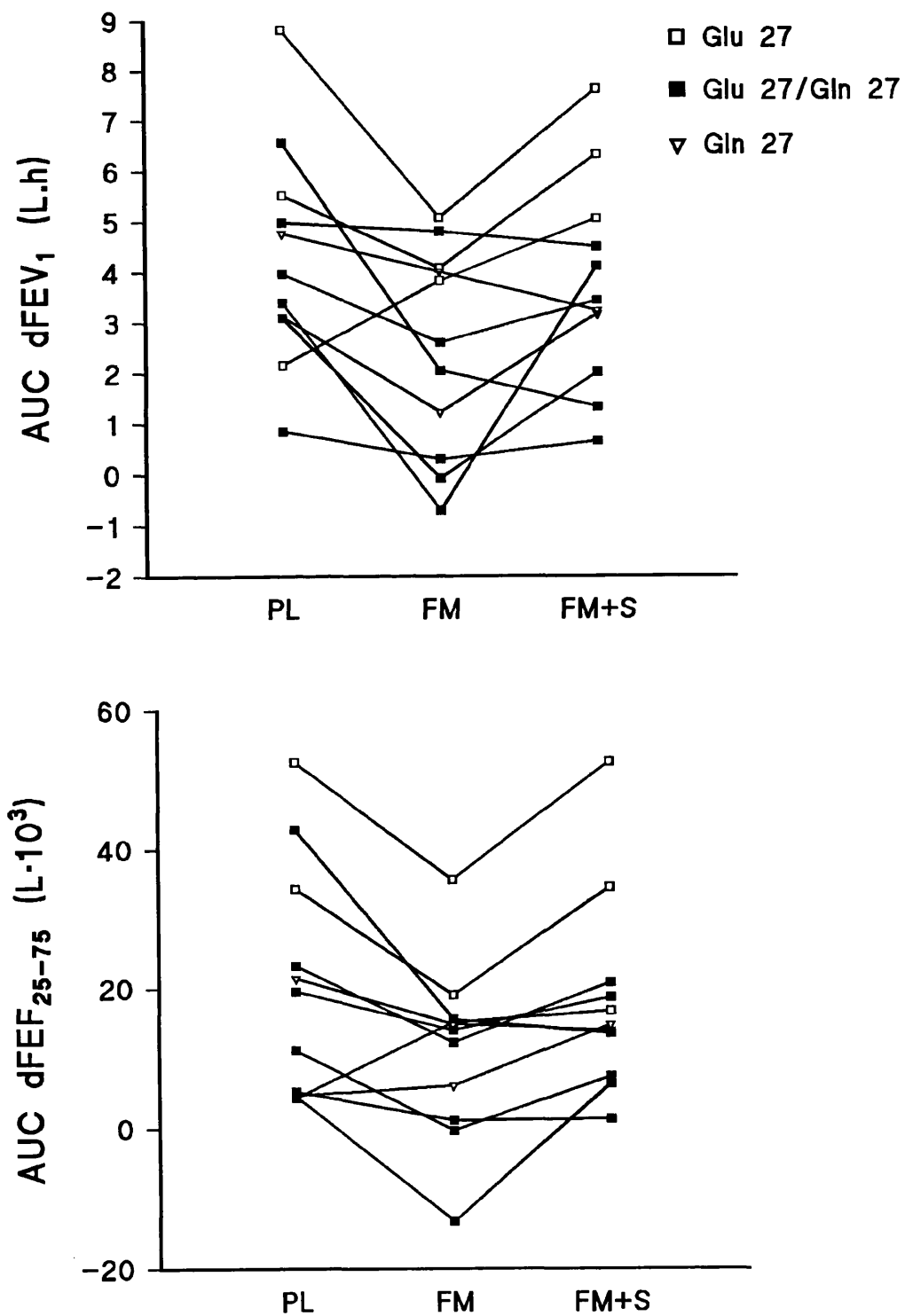


Figure 3.8 Individual responses for AUC FEV₁ and AUC FEF₂₅₋₇₅ after treatment with placebo (PL) and formoterol (FM), compared with reversibility following steroid (FM+S), according to β_2 -adrenoceptor polymorphism at codon 27.

3.4 DISCUSSION

Our results in this study have demonstrated that subsensitivity of bronchodilator and systemic β_2 -AR responses occurs after treatment with regular inhaled formoterol compared with placebo. These results are consistent with previous studies with long-acting β_2 -agonists with dry powder (Newnham, 1994a) and aerosol (Newnham, 1995) preparations of formoterol and salmeterol (Grove, 1995). In contrast, bronchodilator subsensitivity does not appear to occur with short-acting β_2 -agonists in placebo-controlled studies (Lipworth, 1989; Lipworth, 1990), although Turki *et al* (1995a) have reported down-regulation of lung cell β_2 -AR after 24 hours with metaproterenol.

It is important to note that baseline values for FEV₁ and FEF₂₅₋₇₅ were virtually identical between treatments for both pre and post injection of placebo or steroid, making it unlikely for differences between treatments to be due to baseline effects. There was no period effect between visits which excludes the possibility that the difference between formoterol alone and formoterol with steroid was a sequence phenomenon.

The use of formoterol in the present study in constructing dose-response curves has clinical relevance, in that formoterol is fast acting and it is conceivable that patients who are on regular therapy might also use it repeatedly for rescue purposes, as might occur during an acute attack of asthma.

As in previous studies (Newnham, 1994a; Newnham, 1995; Grove, 1995), morning and evening expiratory flow rates from diary cards at 12 hours after dosing were significantly greater with formoterol compared to placebo and this was sustained during the four week treatment period. This clearly illustrates the importance of constructing proper dose-response curves and ensuring at least 24 hours washout prior to DRC, in order to detect subsensitivity. It is, however, conceivable that

improved peak flow rates may lull patients into a false sense of security and delay seeking medical attention. It is also possible that regular long-acting β_2 -agonist therapy whilst improving symptoms and PEFr might result in the masking of deteriorating inflammation, as might occur leading up to an acute asthmatic attack. In this study, it is also relevant to note that bronchodilator subsensitivity has been demonstrated despite the fact that all the subjects were receiving regular inhaled corticosteroid, suggesting that inhaled corticosteroids do not protect against such tolerance.

Our results also showed rapid reversal of bronchodilator subsensitivity within one hour of injection of hydrocortisone. This finding is similar to that of a previous report (Ellul-Micallef, 1975). In an open placebo-controlled study, intravenous prednisolone restored the bronchodilator response to a single 200 μg dose of isoprenaline given 60 minutes after injection in 10 patients who had previously been shown to be non-responsive. Also, Holgate *et al*, in normal subjects, showed reversal of salbutamol DRC (sGaw) 16 hours after hydrocortisone (Holgate, 1977).

There is conflicting evidence as to whether lymphocyte β_2 -AR can be used as a surrogate for following changes in airway smooth-muscle β_2 -AR (Hauck, 1990; Hayes, 1996). In our subjects, systemic corticosteroid up-regulated lymphocyte β_2 -AR, in keeping with reversal of bronchodilator subsensitivity. This is in keeping with previous studies with lymphocytes, where up-regulation has been shown to occur in normal (Hui, 1982; Brodde, 1985) and asthmatic (Brodde, 1988) subjects with systemically administered corticosteroid. The mechanism of this effect by corticosteroids may involve an increase in the rate of synthesis of receptors by a process of increased β_2 -AR gene transcription (Mak, 1995a), or a reversal or inhibition of internalisation of receptors from the cell surface (Davies, 1984). Corticosteroids are also thought to promote the formation of the coupled, high-affinity state of the receptor, which in turn increases receptor function (Davies, 1980).

Systemic corticosteroid also up-regulated lymphocyte β_2 -AR which had not previously been down-regulated ie after placebo pretreatment. Mean values for $\log B_{\max}$ after placebo were similar to previously reported control values from normal volunteers (0.29 vs 0.28 fmol/ 10^6 cells) (Chapter 7). This up-regulation of lymphocyte β_2 -AR by corticosteroid was mirrored by its effect on heart rate where steroid administration augmented the heart rate response after placebo pretreatment. Indeed, even after formoterol, the presence of steroid increased the heart rate response compared to placebo alone. This therefore suggests that systemic corticosteroid is capable of supernormalising β_2 -AR density. This, along with the lack of protection against β_2 -AR down-regulation and subsensitivity by inhaled corticosteroid would therefore suggest that a facilitatory effect of corticosteroid is only conferred by the systemic route.

We studied a group of asthmatics who were heterogenous in terms of β_2 -AR polymorphism. In particular, it is known that homozygous Gly 16 and homozygous Glu 27 polymorphism confers susceptibility and protection respectively to the development of down-regulation to β_2 -agonists (Green, 1995b). The results in this study show that in general, systemic corticosteroid produced reversal of bronchodilator subsensitivity independent of β_2 -AR polymorphism. This is clinically relevant because homozygous Gly 16 subjects are most susceptible to desensitisation but appear to be responsive to the facilitatory effects of systemic corticosteroid. Our patients had stable, moderately-severe asthma and it is conceivable that even a small degree of subsensitivity would assume greater clinical importance in more severe asthmatics in the setting of an acute attack.

There were also some interesting findings which occurred 24 hours after administration of corticosteroid. The baseline level of FEV₁ at 24 hours was lower after pretreatment with formoterol compared with placebo, irrespective of administration of steroid.

However, this is unlikely to reflect persistent subsensitivity since the 30 minute response to formoterol 12 μg was preserved. This does not appear to relate to lymphocyte β_2 -AR density and isoprenaline cAMP response which were both diminished at 24 hours, irrespective of prior steroid administration. This fall in lymphocyte β_2 -AR density and cAMP responsiveness between 3 hours and 24 hours may represent the legacy of the repeated formoterol doses from the DRC. Lymphocyte receptor affinity as measured by the dissociation constant, K_d , showed a different pattern in that corticosteroid prevented a fall in K_d which occurred irrespective of formoterol or placebo pretreatment, inferring an effect of the previous formoterol DRC.

What might be the clinical relevance of our study? In acute asthma, where patients may be desensitised as a consequence of regular long-acting β_2 -agonist therapy, it demonstrates the importance of the early administration of systemic corticosteroid in order to restore normal airway β_2 -AR responsiveness. This suggests that systemic corticosteroid has a dual action in acute asthma in terms of an early effect on β_2 -AR response and a later effect on the inflammatory response.

Although the mean results showed significant bronchodilator subsensitivity, there were considerable inter-individual variation (Figures 3.7 and 3.8). It would be interesting therefore to investigate if these individual differences could be influenced by β_2 -AR polymorphism.

CHAPTER 4

ASSOCIATION BETWEEN β_2 -ADRENOCEPTOR POLYMORPHISM AND SUSCEPTIBILITY TO BRONCHODILATOR DESENSITISATION IN MODERATELY SEVERE STABLE ASTHMATICS

(Lancet 1997; 350: 995-999)

4.1 INTRODUCTION

In the previous study, it was shown that bronchodilator subsensitivity developed after regular inhaled formoterol in asthma patients. Although significant mean differences between treatments were demonstrated, there were quite marked inter-individual differences in the development of bronchodilator subsensitivity. We know from *in vitro* studies that genetic polymorphisms of the β_2 -adrenoceptor (β_2 -AR) might influence the development of β_2 -AR subsensitivity (Green, 1995b). The presence of glycine at codon 16 of the receptor predisposes to down-regulation following agonist exposure to a greater extent than the arginine 16 form of the receptor. Likewise, the presence of glutamate at codon 27 is thought to confer protection against such down-regulation compared to the glutamine 27 form.

In this study, we have performed a retrospective analysis of three similar randomised, placebo-controlled, double-blind, cross-over studies (Newnham, 1994a; Newnham 1995; Chapter 3) involving asthma patients receiving regular inhaled formoterol, including the previous study, to evaluate the relationship of β_2 -AR polymorphism to the development of bronchodilator subsensitivity.

4.2 METHODS

Patients

The three reference studies were done between 1992 and 1996. 35 patients were originally recruited to all three studies, which comprised 29 individuals. We were able to recall 22 (76%) of the 29 patients to the department to have a blood sample taken for genotype analysis. Of the remaining seven patients, one declined to return and six had moved from the area and could not be contacted. Of the 22 recalled patients, four participated in two studies, and one in three studies; these five patients had all participated in one particular study (Newnham, 1995), the data from which were thus included in analysis. A breakdown of the proportion of patients included from each of the three studies was five of seven (Newnham, 1994a), 11 of 16 (Newnham, 1995) and six of 12 (Chapter 3).

All participants had moderately severe asthma with a FEV₁ between 50-80% predicted with 15% reversibility to β_2 -agonist and 20 participants were receiving inhaled corticosteroid median dose 1000 μ g/day (range 200-2000 μ g/day). They were divided into groups depending on their polymorphism at codon 16: homozygous Arg 16 (n=4), heterozygous Arg 16/Gly 16 (n=8) and homozygous Gly 16 (n=10); and at codon 27: homozygous Gln 27 (n=5), heterozygous Gln 27/Glu 27 (n=11) and homozygous Glu 27 (n=6). β_2 -AR genotype was determined using previously described methodology (Hall, 1995). The characteristics of these patients are shown in Table 4.1. Baseline pulmonary function, as percentages of normal predicted FEV₁ and FEF₂₅₋₇₅ are not significantly different between the groups. Approval was obtained from the Tayside committee on medical research ethics, and all patients gave informed, written consent.

Protocol for studies

All three studies (Newnham 1994a; Newnham 1995; Chapter 3) had a similar protocol. In brief, subjects were randomised to receive either inhaled placebo or inhaled formoterol 24 µg twice daily for four weeks in double-blind, cross-over fashion. All inhaled corticosteroids were continued unchanged throughout the study. They were required to withhold all other β₂-agonists and were supplied with ipratropium bromide for rescue purposes during the run-in period and during each treatment. Bronchodilator dose-response curves (DRC) to inhaled formoterol (6-108 µg as cumulative doses) were then constructed at the end of each treatment period. FEV₁ and FEF₂₅₋₇₅ were measured 30 minutes after each dose and for up to 6 hours after the last dose. Mean (SD) baseline values for FEV₁ and FEF₂₅₋₇₅ (prior to DRC) were not significantly different for all 22 patients: 2.23 L (0.14) after placebo vs 2.30 L (0.14) after formoterol for FEV₁, and 1.57 L/s (0.23) after placebo vs 1.70 L/s (0.23) after formoterol for FEF₂₅₋₇₅.

Identification of β₂-adrenoceptor polymorphisms - see Chapter 2.

Statistical Analysis

Bronchodilator responses were measured after treatment with placebo or formoterol from the DRC, and defined as changes from baseline to the maximum and 6-h responses. The bronchodilator-desensitisation percentage was calculated with the following formula:

$$\% \text{ subsensitivity} = \frac{\text{delta response (placebo)} - \text{delta response (formoterol)}}{\text{delta response (placebo)}} \times 100$$

The percentage desensitisation for maximum and 6-h responses was calculated for both FEV₁ and FEF₂₅₋₇₅. For example, if the delta-FEV₁ response from the DRC after

placebo is a 1.00 L rise, and the delta-FEV₁ response from the DRC after formoterol is a 0.20 L rise, then the desensitisation percentage from the equation will be 80%. However, if the DRC after formoterol showed a fall rather than a rise from baseline, then the equation would produce a value for desensitisation exceeding 100%. For example, if the delta response from DRC after placebo is a 0.80 L rise, and the delta response from DRC after formoterol is a 0.20 L fall, then the desensitisation percentage will be 125%. Comparisons between groups were then made by two-way analysis of variance. A probability value of $p < 0.05$ (two-tailed) was considered as being of significance and 95% confidence intervals (95% CI) for mean differences were calculated where significant.

4.3 RESULTS

Individual and mean group bronchodilator responses are depicted in Figures 4.1 to 4.4. The mean values for percentage desensitisation for homozygous polymorphisms at codon 16 and 27 are summarised in Table 4.2. For codon 16 polymorphisms, maximum FEV₁ and FEF₂₅₋₇₅ responses showed a significantly greater degree of bronchodilator desensitisation with homozygous Gly 16 than with homozygous Arg 16. This result was mirrored by significantly greater desensitisation with homozygous Gly 16 than with homozygous Arg 16 for 6-h FEV₁ and FEF₂₅₋₇₅ responses. Mean values for the heterozygote form (Arg 16/Gly 16) were intermediate between homozygous Arg 16 and Gly 16, except for the maximum FEV₁ response, in which we found significantly greater desensitisation with Arg 16/Gly 16 than with Arg 16 (46% and -8%, respectively; 95% CI 10-98, $p < 0.05$).

For codon 27 polymorphism, there were significant differences in FEF₂₅₋₇₅ but not FEV₁ responses, with homozygous Glu 27 showing greater desensitisation than homozygous Gln 27 for maximum and 6-h FEF₂₅₋₇₅ responses. All patients who were homozygous for Glu 27 were also homozygous for Gly 16.

We found no evidence of genotype-related subsensitivity for bronchodilator response from the DRC after prior treatment with placebo, in terms of mean (SEM) maximum delta FEV₁ response (as % of predicted): Arg 16 19% (± 3), Arg 16/Gly 16 25% (± 5) and Gly 16 29% (± 5).

Genotype	Sex (M/F)	Age (years)	FEV ₁ (% pred.)	FEF ₂₅₋₇₅ (% pred.)	Inhaled steroid (µg/day)
Codon 16					
Arg 16/Arg 16 (n=4)	4/0	38 (3)	64 (15)	39 (10)	700 (1000)
Arg 16/Gly 16 (n=8)	4/4	31 (17)	69 (15)	46 (14)	1000 (1600)
Gly 16/Gly 16 (n=10)	5/5	44 (11)	57 (17)	30 (13)	1250 (800)
Codon 27					
Gln 27/Gln 27 (n=5)	5/0	40 (14)	63 (13)	38 (9)	1000 (600)
Gln 27/Glu 27 (n=11)	5/6	36 (16)	66 (14)	42 (15)	1000 (1200)
Glu 27/Glu 27 (n=6)	3/3	40 (10)	58 (22)	29 (16)	1150 (800)

Table 4.1 Patients' characteristics according to genotype at codon 16 and 27. Values are shown as means (SD) and median (interquartile range) for inhaled steroid dose. FEV₁ and FEF₂₅₋₇₅ values are shown as % predicted (% pred.).

Codon 16	Arg16/Arg16	Gly16/Gly16	'p' value	95% CI
Max FEV ₁	-8%	46%	p<0.05	15%-92%
Max FEF ₂₅₋₇₅	-32%	74%	p<0.05	49%-164%
6h FEV ₁	30%	81%	p<0.05	2%-105%
6h FEF ₂₅₋₇₅	21%	104%	p<0.05	38%-130%

Codon 27	Gln27/Gln27	Glu27/Glu27		
Max FEV ₁	2%	35%	p=0.08	-6%-73%
Max FEF ₂₅₋₇₅	-7%	68%	p=0.05	2%-147%
6h FEV ₁	46%	76%	p=0.24	-24%-83%
6h FEF ₂₅₋₇₅	43%	93%	p<0.05	5%-94%

Table 4.2 Degree of desensitisation for maximum (max) and 6h bronchodilator responses after treatment with formoterol for β_2 -adrenoceptor homozygous polymorphisms at codon 16 and codon 27. (Mean values for % desensitisation shown for each polymorphism alone with 95% CI for difference).

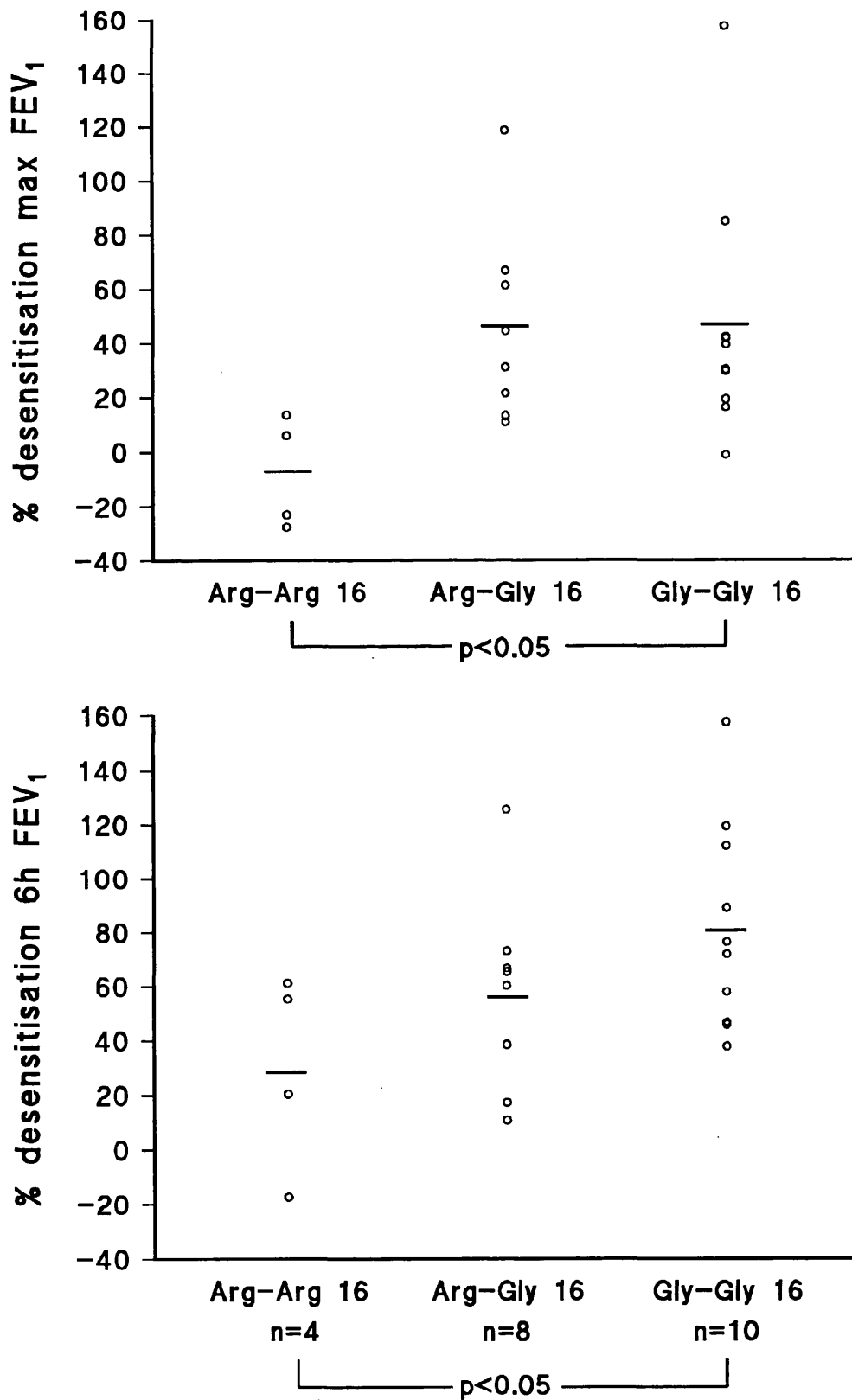


Figure 4.1 Degree of bronchodilator desensitisation after regular formoterol therapy for maximum and 6h FEV₁ responses at codon 16. (Mean values for each group indicated by horizontal lines).

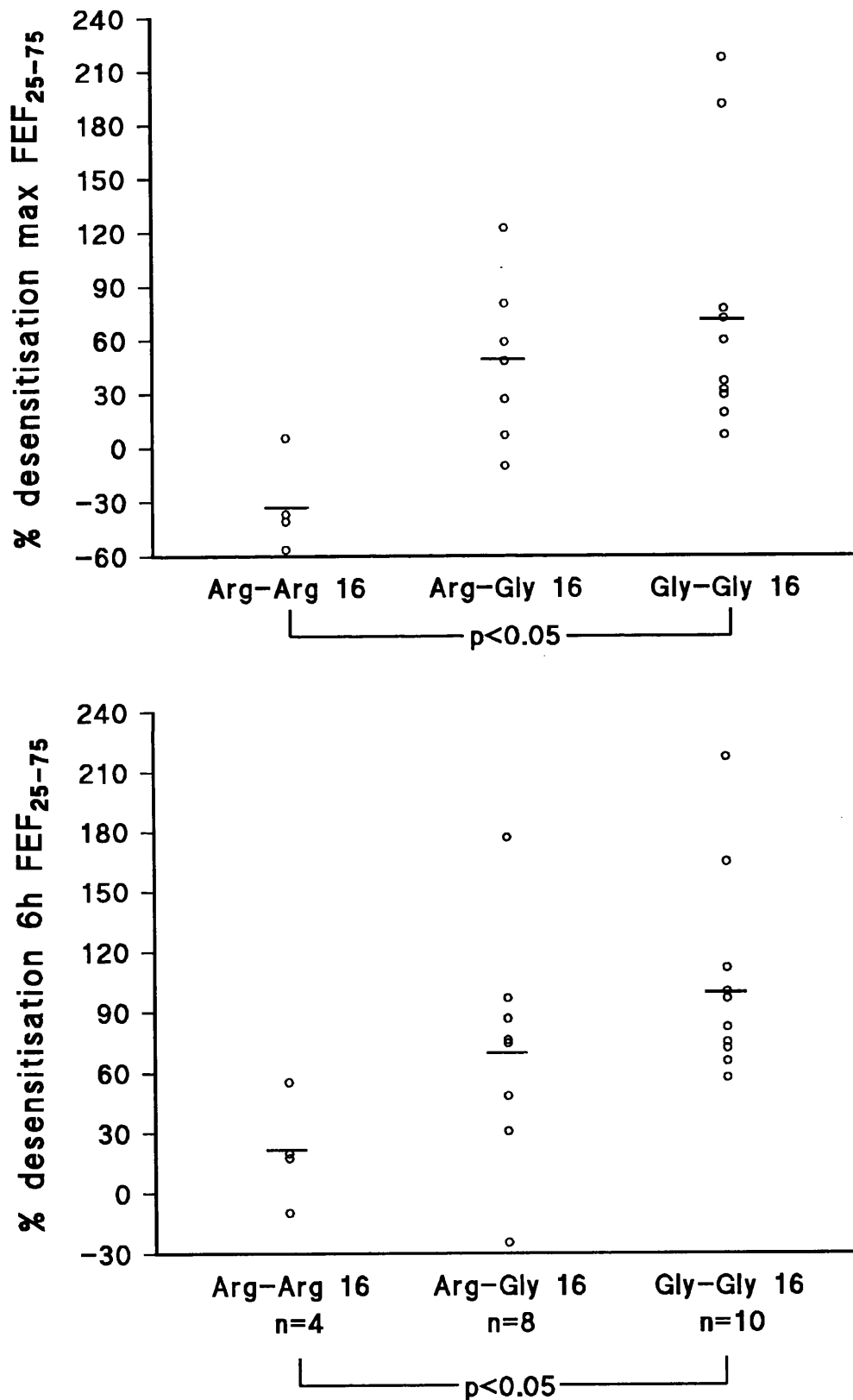


Figure 4.2 Degree of bronchodilator desensitisation after regular formoterol therapy for maximum and 6h FEF₂₅₋₇₅ responses at codon 16. (Mean values for each group indicated by horizontal lines).

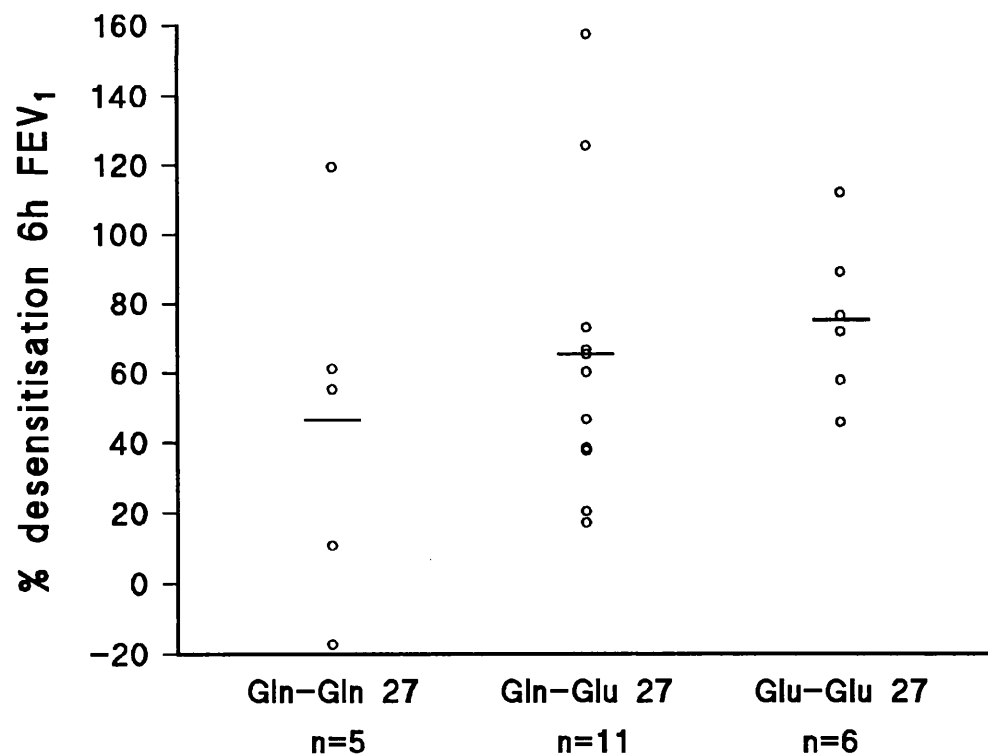
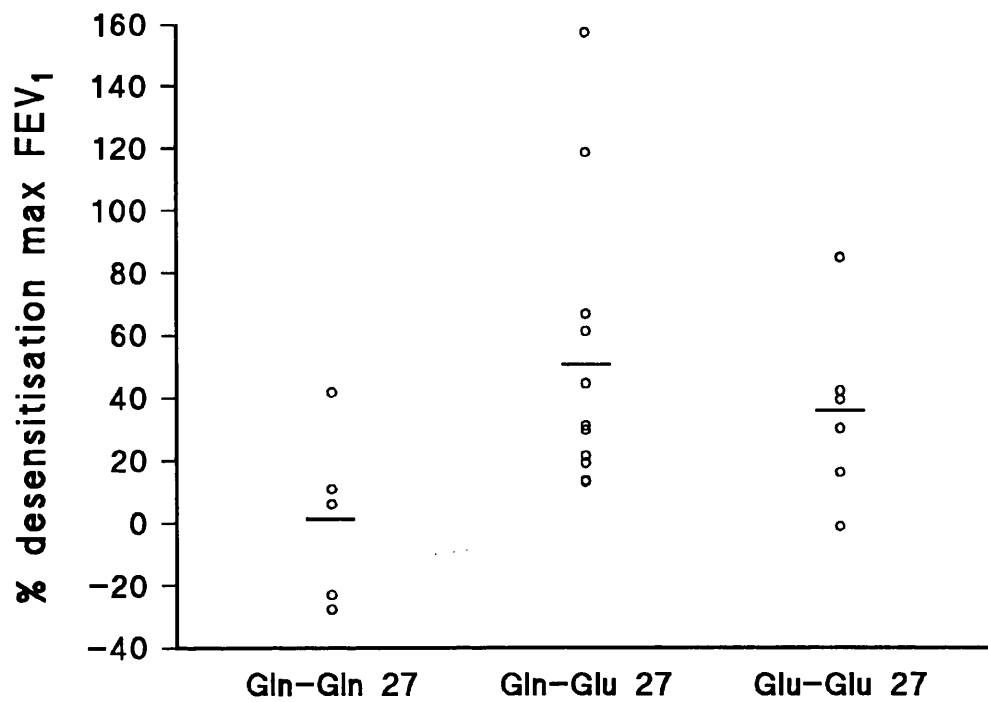


Figure 4.3 Degree of bronchodilator desensitisation after regular formoterol therapy for maximum and 6h FEV₁ responses at codon 27. (Mean values for each group indicated by horizontal lines).

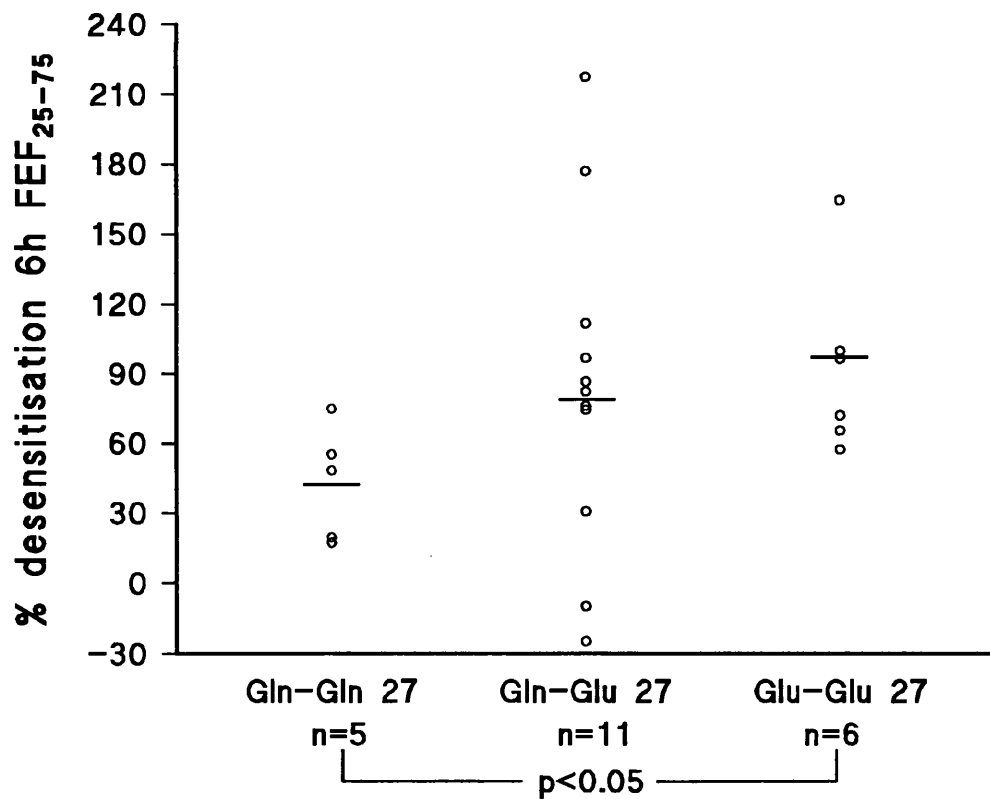
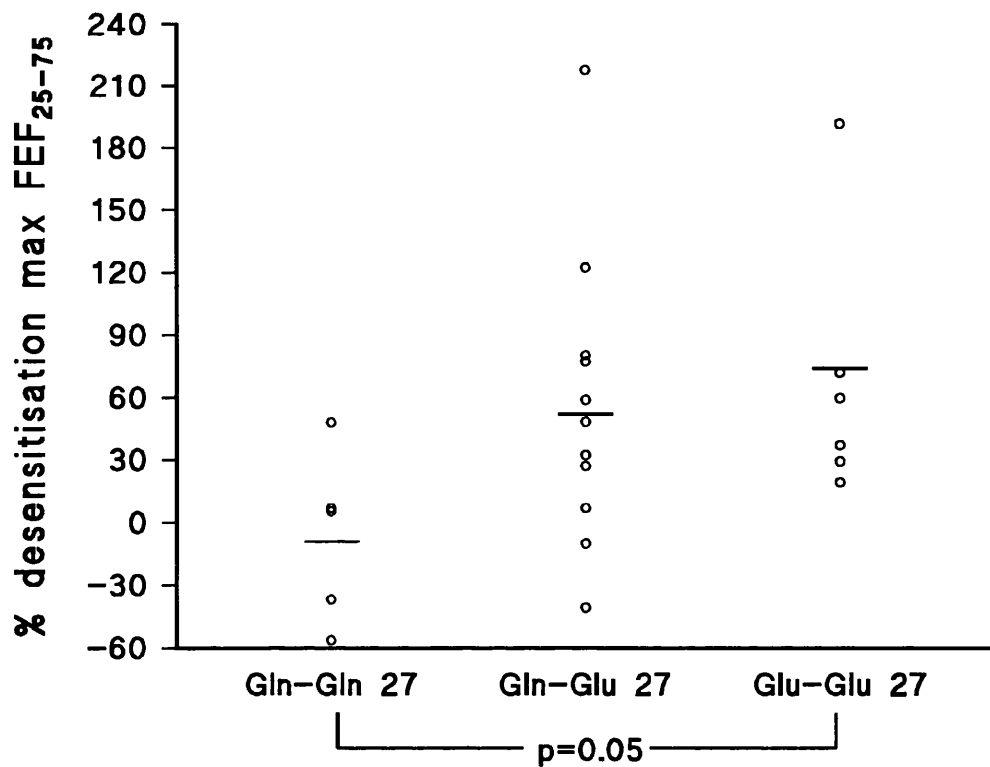


Figure 4.4 Degree of bronchodilator desensitisation after regular formoterol therapy for maximum and 6h FEF₂₅₋₇₅ responses at codon 27. (Mean values for each group indicated by horizontal lines).

4.4 DISCUSSION

Our results suggest that β_2 -AR polymorphism may be associated with altered β_2 -AR expression in asthmatic patients, in terms of susceptibility to bronchodilator desensitisation after chronic exposure to a long-acting β_2 -agonist. Those asthmatic patients who were homozygous Gly 16 developed significantly greater bronchodilator subsensitivity than those who were homozygous Arg 16; whereas heterozygote patients developed desensitisation to a degree intermediate between the two kinds of homozygote patients. These results are consistent with *in vitro* studies that have shown the Gly 16 form of the receptor to be more prone to down-regulate than the Arg 16 form (Green, 1994). We expected the Glu 27 homozygous form to protect against bronchodilator subsensitivity, as it did in *in vitro* studies (Green, 1994). However, we found that the Glu 27 group developed greater desensitisation than the Gln 27 group. This can be explained by the observation that all six subjects with homozygous Glu 27 were also homozygous Gly 16, which suggests that the effects of Gly 16 are dominant over those of Glu 27; this has been borne out in *in vitro* studies (Green, 1994). There is substantial linkage disequilibrium in the white population between these two polymorphisms as expected (Dewar, 1998).

We believe this is the first time the development of bronchodilator desensitisation to a long-acting β_2 -agonist in asthmatic patients has been associated with β_2 -AR polymorphism. All but two of our patients were receiving inhaled corticosteroid, which does not seem to protect against bronchodilator or bronchoprotective desensitisation with regular long-acting β_2 -agonist therapy (Grove, 1995; Kalra, 1996). Our results confirm some of Green and colleagues' (1995b) earlier predictions on the physiological implications of β_2 -AR polymorphism. The fact that bronchodilator responsiveness after pretreatment with placebo was not related to β_2 -AR genotype suggests that those patients with the Gly 16 polymorphism were not already desensitised before they received the chronic formoterol therapy.

We recognise and accept the limitations of a study with small numbers of participants. Our recalled sample represented 76% of all individual patients originally enrolled in the three reference studies. Criticism may be levelled, in particular at the small number of patients with homozygous Arg 16, even though this is believed to be the 'wild-type' of the receptor. The proportion of homozygous Arg 16 patients and other polymorphisms in this study is similar to that previously described (Reishaus,1993). The magnitude of the differences in our restricted sample suggest that their effects may be clinically - as well as statistically - relevant. Such effects are a basis on which to do a larger prospective study on groups of asthmatic patients with known β_2 -AR polymorphisms.

What are the clinical implications of our results? We recommend caution in the extrapolation of our findings to patients with more severe asthma, although even a small degree of desensitisation may be relevant in such cases. Our results may also have important consequences on research. Clinical studies of β_2 -AR function in normal and asthmatic people may have to be done in at-risk groups with known β_2 -AR polymorphism implicit in the study design - that is, with cohorts of homozygous Gly 16 patients. For the study of bronchodilator desensitisation due to long-acting β_2 -agonists in asthmatic patients, smaller groups of people homozygous for Gly 16 would be needed to give an accurate picture of the 'worst-case' scenario, since these patients are most likely to develop such desensitisation.

There has also been some concern about the safety of the regular use of long-acting β_2 -agonists, and it would have been interesting to know whether there was an excess of Gly 16 homozygotes in reports of those asthma deaths associated with the use of salmeterol (Clark, 1993; Castle, 1992; Finklestein, 1994). The Gly 16 β_2 -AR polymorphism may have predisposed patients with brittle asthma to develop bronchodilator desensitisation; hence the reduced efficacy of β_2 -agonists during worsening asthma. Another possible mechanism might be the loss, with chronic use,

of the bronchoprotective properties of β_2 -agonists. Previous studies have shown a development of tolerance to the bronchoprotective effects of long-acting β_2 agonists (Cheung, 1992; Ramage, 1994; Booth, 1996; Yates, 1996). However, we presume that groups who were heterogenous in terms of β_2 -AR polymorphism would have been evaluated in their studies, although β_2 -AR were not genotyped. We know already that the Gly 16 polymorphism is associated with increased bronchial hyperreactivity (Holroyd, 1995) and that the Glu 27 polymorphism is associated with lower hyperreactivity (Hall, 1995), though neither polymorphism seems to a risk factor *per se* for the development of asthma in the adult population. To investigate the possible association between β_2 -AR polymorphism and the development of tolerance to the bronchoprotective properties of β_2 -agonists would thus be worthwhile.

The recently revised British Thoracic Society guidelines recommended the earlier use of regular long-acting β_2 -agonists in Step 3, in addition to low dose inhaled corticosteroid (200-800 $\mu\text{g}/\text{day}$) (British Thoracic Society, 1997). Our preliminary data suggest there may be a subset of individuals in whom bronchodilator desensitisation occurs when long-acting β_2 -agonists are used on a regular basis. A larger prospective study is required to investigate this further.

CHAPTER 5

EFFECTS OF TREATMENT WITH FORMOTEROL ON BRONCHOPROTECTION AGAINST METHACHOLINE

(American Journal of Medicine 1998; 104: 431-438)

5.1 INTRODUCTION

β_2 -agonists exhibit protection against bronchoconstrictor stimuli such as methacholine, in addition to their bronchodilating properties. In the previous chapters, we have shown that the regular use of a long-acting β_2 -agonist, formoterol resulted in bronchodilator subsensitivity. It is also known that with regular β_2 -agonist treatment, subsensitivity develops to the functional antagonism against bronchoconstrictor stimuli (Cockcroft, 1996). The current role of regular twice-daily long acting β_2 -agonists is for patients whose asthma is not adequately controlled on inhaled corticosteroid therapy (British Thoracic Society, 1997). However, recent studies have shown that inhaled corticosteroids do not protect against the development of bronchoprotective tolerance induced by long-acting β_2 -agonists (Kalra, 1996; Yates, 1996). With regard to tachyphylaxis with regular long-acting β_2 -agonist therapy there remain some unresolved issues which have been addressed in the present study. Firstly, is the degree of tachyphylaxis determined by the total daily dose of long acting β_2 -agonist? Secondly, does the dosing frequency influence the development of subsensitivity, in terms of having a possible period of unoccupied airway β_2 -adrenoceptors (β_2 -AR) during a 24 hour dosing interval in association with once daily administration? Finally, how do long and short acting β_2 -agonists compare in their relative propensity for inducing bronchoprotective subsensitivity? We have attempted to resolve these questions in this study.

5.2 METHODS

Patients

The inclusion criteria for the study were as follows: (a) 16-65 years of age, (b) a diagnosis of asthma with minimum duration of 6 months according to American Thoracic Society criteria (American Thoracic Society, 1987a), (c) an FEV₁ equal to or greater than 60% of predicted normal value, (d) a methacholine PD₂₀ value equal to or less than 1000µg, and at least with a four-fold increase in PD₂₀ one hour after inhaling 24µg of formoterol from a Turbuhaler (Astra Draco, Sweden), (e) prior treatment with inhaled corticosteroids at a constant dosage level for at least 4 weeks prior to initial screening at visit 1 (up to 2000 µg/day of budesonide, beclomethasone dipropionate or fluticasone propionate).

Patients were excluded according to the following criteria: (a) treatment with oral corticosteroids within 4 weeks of visit 1, (b) a history of allergy during the relevant pollen seasons, (c) a lower respiratory tract infection within 4 weeks of visit 1, (d) cigarette smoking in the past year.

Study Design

This two-centre study was carried out at the Department of Clinical Pharmacology, Ninewells Hospital (Centre 1) and the Department of Respiratory Medicine, Newcastle General Hospital (Centre 2) using an identical protocol and equipment. The overall study design is summarised in the flow chart in Figure 5.1. In brief, eligibility for inclusion and enrolment was assessed at visit 1 on two successive days, with an unprotected challenge on day 1 and a protected challenge 1 hour after 24 µg of formoterol on day 2. This was followed by visit 2 with a baseline unprotected methacholine challenge performed 7-12 days after visit 1. Randomisation at visit 3 was performed 2-7 days after visit 2, 1 hour after the first dose of the study medication.

The duration between visits 1-3 was required to be no greater than 14 days. Visit 4 was performed after 7-9 days of study treatment, with a challenge done 1 hour after inhalation of the study medication. Visit 5 was performed after 14-16 days of study medication, with a challenge done 1 hour after the last dose of each treatment. The final visit (visit 6) was performed after a further 2-4 days of treatment, with a challenge performed 1 hour after inhalation with a reference 6 µg dose of formoterol (having stopped study medication 24 hours earlier). During the initial run-in period between visit 1 and visit 3 all β_2 -agonist therapy was withdrawn and substituted with ipratropium bromide MDI 2 puffs (as Atrovent forte, Boehringer Ingelheim, Bracknell, UK) which was used for rescue purposes. The same rescue medication with ipratropium was also available to use as required during each of the 2 week treatment periods. The patients were instructed to withhold rescue medication for at least 12 hours prior to any given methacholine provocation test.

Patients were randomised to receive one of the following five treatments:

(a) Formoterol fumarate (via Turbuhaler 6 µg per actuation metered dose, 4.5 µg per actuation delivered dose) 6 µg twice daily given morning and evening plus placebo Turbuhaler given midday and bedtime; (b) Formoterol fumarate (via Turbuhaler 12µg per actuation metered dose, 9 µg per actuation delivered dose) given 24 µg twice daily morning and evening, plus placebo Turbuhaler given midday and bedtime; (c) Formoterol fumarate (via Turbuhaler 12 µg per actuation metered dose, 9 µg per actuation delivered dose) 12 µg once daily in the morning plus placebo Turbuhaler at midday, evening and at bedtime; (d) Terbutaline sulphate (via Turbuhaler 500 µg per actuation metered dose) 500 µg four times daily given morning, midday, evening and bedtime ; (e) Placebo via Turbuhaler qid given morning, midday, evening and bedtime.

Patients were instructed to take the study medication at the same time in the morning (between 6 and 8 am), at midday (between 12 and 2 pm) in the evening (between 6

and 8 pm), and at bedtime at least 3 hours after the evening dose. All study medication was withheld for at least 12 hours prior to the methacholine provocation tests at chronic dosing (at visits 4 and 5). At visit 6 study medication was withheld for at least 24 hours prior to the methacholine provocation test.

Patients received written and verbal instructions on correct inhalation technique at each visit. At visit 1 patients were instructed in the correct use of Turbuhaler and pMDI to ensure good compliance using the Turbuhaler usage trainer (Astra Draco, Sweden) and the pMDI usage trainer (Vitalograph, Buckingham, UK). At this stage patients were also shown how to use a peak expiratory flow (PEF) meter properly and were required to measure the best of three morning and evening recordings in a diary card. As a measure of compliance the remaining doses in the study inhalers were checked during each treatment period and the number of doses taken were estimated. If less than 75% of the study medication was taken in the treatment period, the dose was considered to be non-evaluable. The patient was also asked to recall the time for study drug intake four times a day in a diary card.

The only other asthma medications except for the study treatment and rescue medication, which was allowed during the run-in and randomised treatment periods were: inhaled corticosteroids, sodium cromoglycate and nedocromil sodium, with the dose of these to be kept constant. All other treatments were withdrawn prior to visit 1 including: short and long-acting β_2 -agonists, theophylline, antihistamines and oral corticosteroids.

Measurements:

Methacholine Challenge - see Chapter 2

FEV₁ - see Chapter 2, Spirometry

Dormicillary peak expiratory flow - see Chapter 2, Spirometry

Statistical Analysis

Based on previous studies with a similar design it was calculated that the between subject standard deviation as change from baseline was 1.3-doubling doses of methacholine. Thus, with 12 patients in each group it would be possible to detect a 1.5-doubling dose (2.8-fold) difference in PD₂₀ with 80% power, assuming a significance level of 5% and a two-sided test. All values for log PD₂₀ were calculated as change from the initial unprotected baseline (i.e. visit 2).

The five treatments were compared in order to determine the following: (a) to compare the protection afforded by each of the active treatments compared with placebo, and to see whether there was a difference between first dose, 1 week and 2 weeks, (b) to assess whether there was a dose dependent difference between the low and high dose twice daily formoterol treatments (i.e. formoterol 6 µg bid versus formoterol 24 µg bid), (c) to assess whether there was a difference between the regular bid and od regimes of formoterol administration, and (d) to assess whether there was a difference between regular treatment with short-acting and long-acting β_2 -agonists (i.e. terbutaline vs formoterol).

The different time points were analysed as follows: (a) to assess whether there was any decrease in protective effect from the first dose to the last dose (after 14 days treatment), (b) to see if there was any additional decrease in protection between 7 and 14 days treatment, (c) to assess the residual protective effect of study treatment after 14 days, (d) to evaluate whether the protective effect of a single 6 µg dose of formoterol is affected by prior treatment with regular formoterol or terbutaline.

Within patient ratios for PD₂₀ values at each time point and the unprotected baseline at visit 2 were calculated. For example, a PD₂₀ protection ratio of 4-fold indicates that

a 4 times higher dose of methacholine is required after a given treatment as compared to baseline, in order to elicit a 20% fall in FEV₁. This would equate to a two doubling dose shift in the methacholine dose-response curve. These ratios were then compared using a multiplicative analysis of variance model with patient, centre and treatment as factors; and baseline PD₂₀ as a covariate for the analysis. 95% confidence intervals were calculated for comparisons with placebo.

Pre-challenge values for FEV₁ (1 hour after study medication) were also analysed in the same way as PD₂₀ using multiplicative analysis of variance models to compare treatment effects. Data for morning and evening PEF values from diary cards were analysed by calculating the average value for the last 7 days during the second treatment week as compared with the last 7 days of the run-in. The change from run-in to treatment was compared using an additive analysis of variance model with patient, centre and treatment as factors, and the run-in average as a covariate.

5.3 RESULTS

Patient demography

A total of 72 patients were randomised into the study of which 67 completed the full protocol. The demographic baseline data are summarised in Table 5.1 according to each of the 5 treatment groups. Mean demographic data at screening (visit 1) for the 72 randomised patients were: age 38 years, FEV₁ 87% of predicted, geometric mean methacholine PD₂₀ was 37 µg with geometric mean 30-fold protection after formoterol 24 µg. The geometric mean baseline unprotected PD₂₀ after the initial run-in, before randomisation (at visit 2) was 60 µg.

Methacholine challenge

The data for the methacholine protection with each of the treatment groups are shown in Figure 5.2 and Table 5.2. This illustrates the effects of methacholine challenge when unprotected at baseline, after the first dose of treatment, after repeated dosing at 7 days and 14 days, and finally after 15-17 days of treatment following a reference formoterol dose of 6 µg. Compared with placebo the first dose of active treatment produced significant protection. For the first dose of formoterol the degree of protection exhibited a dose-related pattern and the protection afforded by the first-dose of terbutaline was lower in comparison with the three formoterol treatments. For formoterol 24 µg twice daily this amounted to a 3.0-fold difference (95% CI 1.4-6.6).

After 14 days of repeated dosing, all three formoterol treatments exhibited a significant loss of protection as compared with the first-dose effect (as geometric mean and 95% CI for percentage loss between first and last dose vs placebo); formoterol 24 µg twice daily: a 87% loss (95% CI 69 - 94), 12 µg once daily: a 76% loss (95% CI 41 - 90), 6 µg twice daily: a 71% loss (95% CI 29 - 88). There was no

significant difference in the degree of protection loss between any of the formoterol treatment regimes. Regular terbutaline showed a smaller degree of protection loss between first-dose and 14 days and this was not significantly different compared with placebo: a 47% loss (95% CI -28 -78). The degree of protection loss between first-dose and 14 days was significantly less with terbutaline as compared to formoterol 24 µg twice daily, amounting to a 75% difference (95% CI 40 - 89).

For formoterol 6 µg and 24 µg twice daily, there were no further significant change in PD₂₀ after the first week of treatment, in contrast to 12 µg once daily where there was an additional decrease in protection compared to placebo, between day 7 and day 14: a 60% difference (95% CI 23-79). The protection exhibited by terbutaline reached a plateau after the first week.

In comparison with placebo after 14 days, there was no significant difference for any of the active treatments and there were no differences between the three formoterol treatments, or between formoterol and terbutaline.

The degree of protection conferred by formoterol 6 µg administered following 16 days of each active treatment (having stopped study medication 24 hours earlier), showed no significant difference as compared with after placebo treatment, although there was a trend towards lower responses after treatment with formoterol 12 µg once daily and formoterol 24 µg twice daily (as a percentage of placebo response and 95% CI): formoterol 24 µg twice daily: 59% (95% CI 24-143), 12 µg once daily: 53% (95% CI 21-130), 6 µg twice daily: 88% (95% CI 36-218) and terbutaline: 29% (95% CI 51-325).

Pre-challenge FEV₁

Values for FEV₁ one hour after inhalation (i.e. bronchodilator response prior to the methacholine challenge) after 14 days treatment were significantly greater than placebo with all three formoterol regimens but not with terbutaline (as percentage ratio vs placebo and 95% CI): formoterol 24 µg twice daily 109% (95% CI 113-116%), 12 µg once daily 111% (95% CI 105 - 118%), 6 µg twice daily 109% (95% CI 102-115%) and terbutaline 105% (95% CI 99 - 112%) (Figure 5.3). There were no significant differences between the active treatments and there was no sign of a decrease in FEV₁ response during the 2 week treatment period.

Peak expiratory flow

The diary card PEF data were calculated as the change from the last 7 days of run-in to the last 7 days of each treatment (Figure 5.4). Compared with placebo only formoterol 24 µg twice daily exhibited a significant difference for both morning PEF (as mean difference and 95% CI) 49L/min (95% CI 25-72) and for evening PEF 40 L/min (95% CI 15 - 66). Corresponding values for formoterol 12 µg once daily vs placebo were: morning PEF -4L/min (95% CI -29-20), evening PEF 12L/min (95% CI -14-38); and for 6 µg twice daily vs placebo were: morning PEF 16L/min (95% CI -9-40), evening PEF 34L/min (95% CI 7-60). There was also a significant difference in morning PEF when comparing formoterol 24 µg twice daily vs 6 µg twice daily: 33L/min (95% CI 9 - 57), and comparing formoterol 24 µg twice daily vs terbutaline for morning PEF: 47L/min (95% CI 22-71) and evening PEF: 27 L/min (95% CI 1-53). There was no sign of a diminution in morning or evening PEF values over the 2 week treatment period with formoterol 24 µg twice daily.

	F24µg bid	F12µg od	F6µg bid	T500µg qid	PL
Patient numbers	15	15	14	14	14
Mean age (yr)	33.3	35.5	39.4	41.7	38.1
Men/Women	7/8	8/7	10/4	7/7	6/8
Centre 1/Centre 2	7/8	7/8	6/8	6/8	6/8
Mean dose inhaled steroid (µg/day)	580	740	700	600	496
Mean FEV ₁ (L)	3.20	3.08	3.09	2.88	2.70
Mean FEV ₁ (% predicted)	90	87	85	88	86
Geom mean PD ₂₀ (µg)	30	29	44	61	30
Fold protection post formoterol 24µg	38	24	29	36	25

Table 5.1 Summary of demographic baseline data (visit 1)

Treatment	Mean	95% CI
Formoterol 24µg bid:		
First dose	10.2	4.6-22.5
14 days	1.4	0.6-3.4
Formoterol 12µg od:		
First dose	6.4	2.9-14.1
14 days	1.5	0.6-3.8
Formoterol 6µg bid:		
First dose	5.5	2.5-12.3
14 days	1.6	0.6-4.0
Terbutaline 500µg qid:		
First dose	3.4	1.5-7.8
14 days	1.9	0.8-4.9

Table 5.2 Methacholine protection ratios. Values shown are geometric means fold protection ratio and 95% confidence interval, versus placebo. (Confidence intervals excluding unity denote a significant difference from placebo)

STUDY FLOW CHART

Randomised parallel treatments (2 weeks)

Formoterol 24µg bid + placebo
Formoterol 6µg bid + placebo
Formoterol 12µg od + placebo
Terbutaline 500µg qid
Placebo qid

Run-in						
	X	X	X	X	X	X
Visit No:	1	2	3	4	5	6
Cumulative	1/2	7	14	21	28	30
days:						
Methacholine	Pre/post	Baseline	1 st dose of	1 week of	2 weeks of	Post F6µg
challenge:	F24µg	(unprotected)	study	study	study	
	(screening)		treatment	treatment	treatment	

Figure 5.1 Flow chart overview for study protocol. All protected challenges were performed 1h after inhalation of study drug. Prior to challenge at visits 4 and 5, study medication was withheld for at least 12h, and for at least 24h at visit 6. Prechallenge FEV₁ was recorded at each visit.

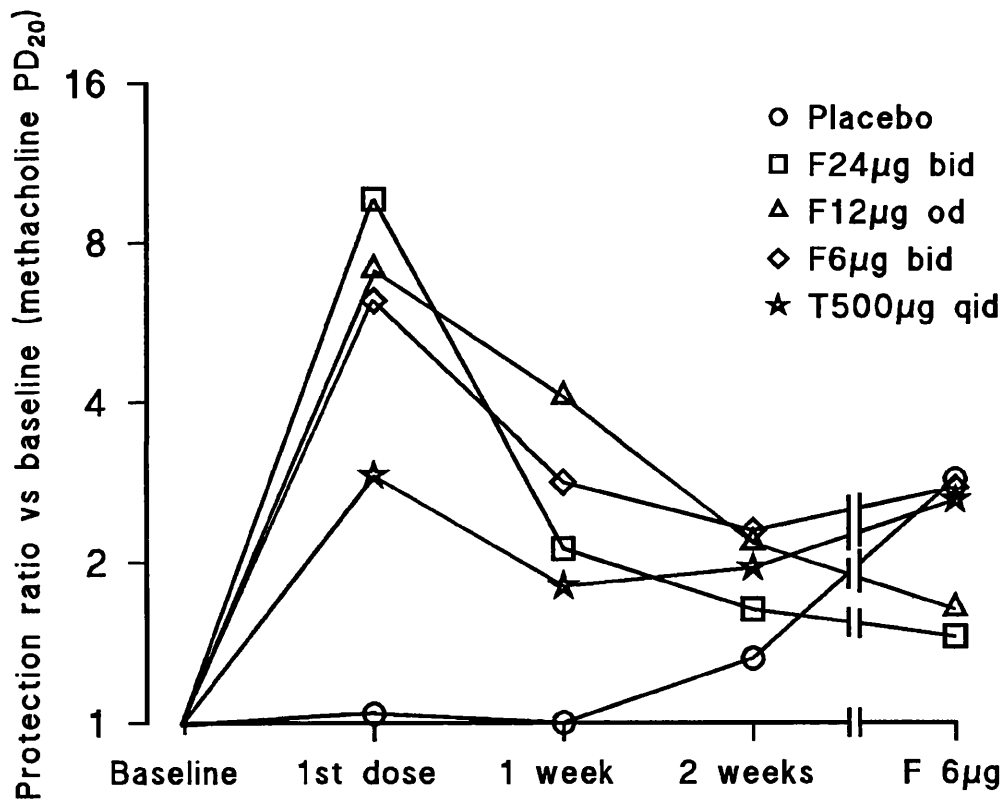


Figure 5.2 Effects of each of five treatments on methacholine challenge, performed at baseline (unprotected), 1h after the first dose, 1 wk and 2 wks, and after stopping treatment for 24h with a single 6 µg dose of formoterol (interrupted line). The protection ratio is calculated as the geometric mean for the fold-difference for each treatment versus unprotected baseline.

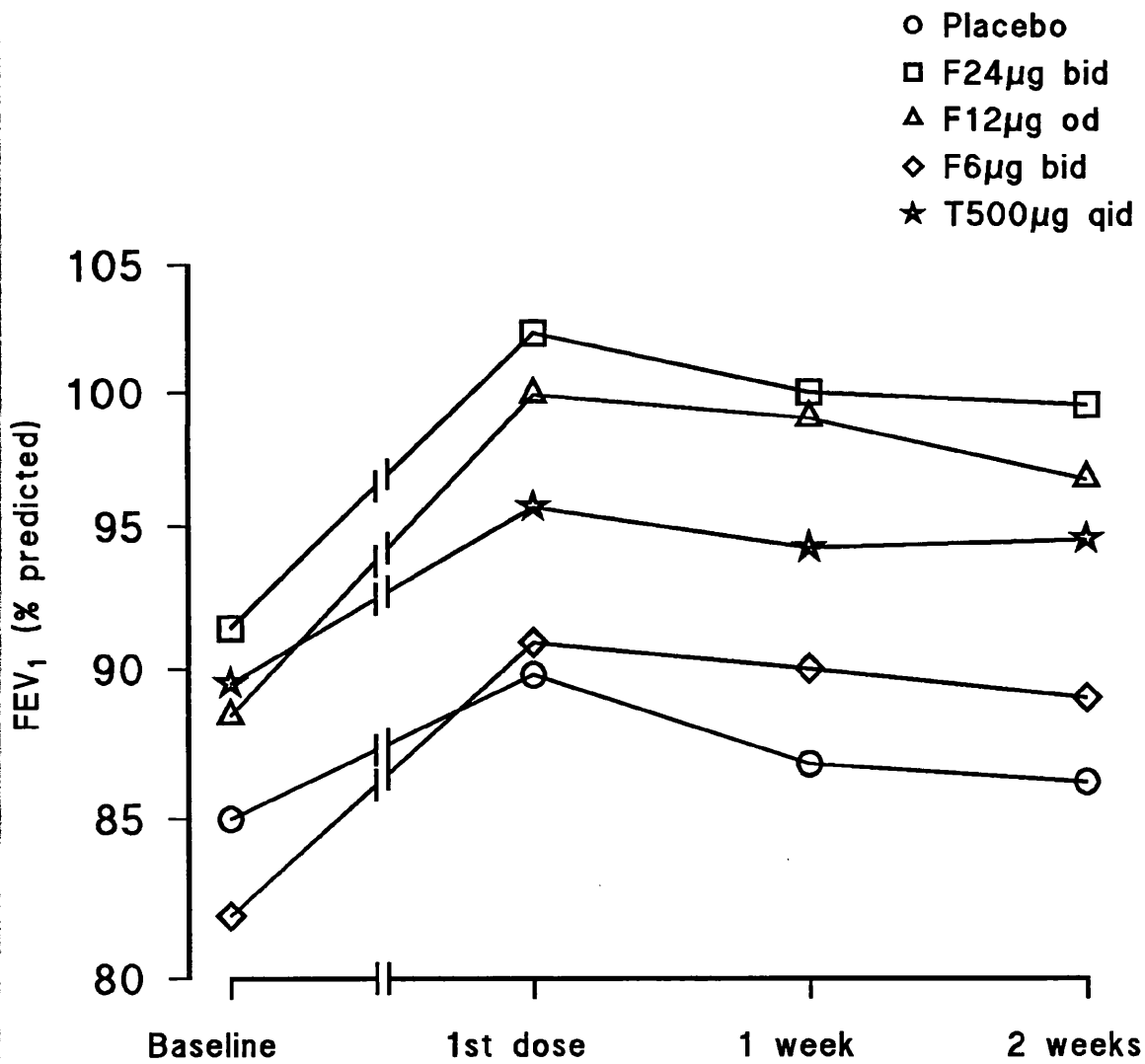


Figure 5.3 Mean values for pre-challenge FEV₁ as percentage predicted. Values were measured at 1h after inhalation of study for the first dose (visit 3), 1 wk (visit 4) and 2 wk (visit 5) of treatment. Baseline value (visit 2) was measured at the same time of day but after no study medication.

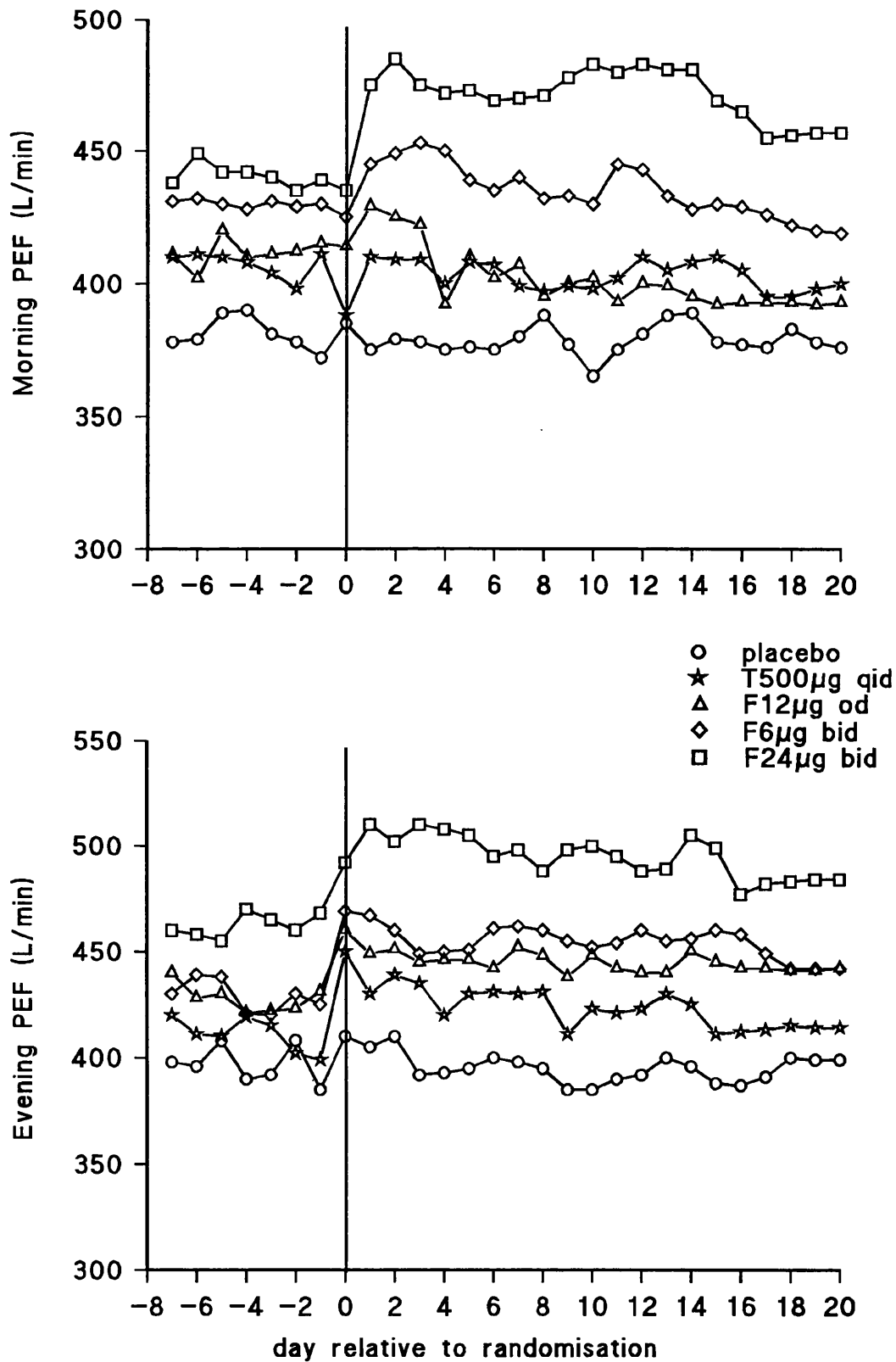


Figure 5.4 Effects of each of five treatments on morning and evening domiciliary peak expiratory flow (PEF) measurements. Values are shown for the run-in and treatment.

5.4 DISCUSSION

Summary of findings

There are three new findings which have emerged out of the results of the present study regarding the development of tachyphylaxis with long-acting β_2 -agonists. Firstly, we found that the degree of protection after 14 days was not dependent upon the total daily dose of formoterol, when comparing 6 μg twice daily and 24 μg twice daily. Secondly, the degree of tachyphylaxis occurred to a comparable degree with once daily and twice daily administration of formoterol. Thirdly, the residual degree of protection exhibited by regular formoterol was comparable to that observed with regular terbutaline. The degree of protection loss with regular formoterol appeared to reach a plateau level after one week of treatment when administered with a twice daily regime, in contrast to a once daily regime where there was a further loss of protection between 1 and 2 weeks of treatment.

The development of tachyphylaxis to the bronchoprotective effects of formoterol or terbutaline occurred despite concomitant administration of inhaled corticosteroid therapy. Despite the presence of bronchoprotective subsensitivity, diurnal control was better with twice daily formoterol compared to terbutaline four times daily, in terms of effects on morning and evening peak expiratory flow. The bronchodilator efficacy with all three formoterol treatments showed no evidence of subsensitivity in terms of maintained FEV₁ response 1 hour after inhalation (pre-challenge) as measured over the 2 week treatment period.

Interpretation of results

In evaluating the degree of tachyphylaxis, it is also important to look at the magnitude of protection exhibited by the first dose of each treatment regime. In this respect there appeared to be a dose-related protection exhibited by formoterol when comparing the

first dose effects of 6 µg, 12 µg and 24 µg. Furthermore, the first dose protection exhibited by all three formoterol treatment regimens was greater than with terbutaline 500 µg, although this was only significant in comparison with formoterol 24 µg. As a consequence of these differences in first dose protection, the absolute loss of protection between single and chronic dosing was greatest for formoterol 24 µg twice daily and was least for terbutaline 500 µg four times daily, with there being a significant difference in the protection loss between these two regimes from the first dose to 14 days. Nonetheless, in terms of the absolute fold protection ratio for PD₂₀ as compared with the initial unprotected baseline, there was a similar degree of residual antagonism with all four active treatments. In other words, the remaining degree of absolute protection seemed to be independent of the level of first dose protection.

It was particularly interesting to find that loss of protection after 14 days occurred to a similar degree in terms of comparing the same total daily dose of formoterol given once or twice daily (i.e. 12 µg once daily vs 6 µg twice daily). However, with the once daily regime but not the twice daily regime there was a further significant loss of protection between 7 days and 14 days of treatment. These findings with once daily administration of formoterol suggests that there must presumably be persistent occupancy of airway β_2 -AR even with a 24 hour dosing interval. In this respect we have previously shown no evidence of persistent bronchodilator activity for FEV₁ and FEF₂₅₋₇₅ at 24 hrs after stopping steady-state dosing with formoterol 24 µg twice daily (Newnham, 1995). The diurnal time profile after administration of a single 12 µg dose of formoterol (metered dose inhaler) has shown no significant difference from placebo at 24 hours for bronchodilator (FEV₁) or bronchoprotective (methacholine) activity (Rabe, 1993). This is supported by the present study where there was no evidence of any significant residual bronchodilator activity at 24 hours

after administration of formoterol 12 µg once daily at steady-state, as assessed by effects on morning peak expiratory flow.

The finding of tachyphylaxis with a once daily dosing regime for formoterol therefore implies that only a small degree of residual receptor occupancy is required towards the end of a 24 hour dosing interval, and that this cannot be detected in terms of bronchodilator activity. This would be consistent with the previous finding of persistent down-regulation of lymphocyte β_2 -AR 24 hours after stopping steady-state dosing with formoterol 24 µg twice daily, in the absence of detectable bronchodilator activity at the same time point (Newnham, 1995).

We chose to use terbutaline in a dose of 500 µg four times daily as this was the usual recommended dose for use on a regular basis. Although the residual degree of protection conferred by terbutaline 500 µg four times daily was comparable to that of formoterol 24 µg twice daily, the peak expiratory flow data clearly showed that diurnal control was superior with regular formoterol in terms of a significantly higher mean value for both morning and evening measurements, amounting to a mean treatment difference of 47L/min and 27L/min for morning and evening values respectively. This difference in morning and evening peak flow rates is not surprising given that the duration of action for formoterol is for up to 12 h as compared with the duration of terbutaline of up to 6 h. Furthermore these improvements in peak flow showed no sign of diminution over the 2 week treatment period. The bronchodilator response one hour after inhalation of active treatment (ie prior to methacholine challenge) showed that there was no evidence of any subsensitivity in terms of attenuation of the FEV₁ response with therapeutic doses of formoterol over the 2 week study period as compared with the first dose effects. It is well recognised that tachyphylaxis to bronchoprotective effects of β_2 -agonists occurs more readily than for bronchodilator effects (Lipworth, 1997). Our findings are in contrast to those of Pauwels *et al* (1997)

who showed a marked fall off in morning peak expiratory flow during the first two weeks of formoterol treatment, which occurred despite concomitant therapy with low or high dose inhaled corticosteroid.

Limitations of the study

The main limitation of this study is in terms of its power with a relatively small sample size in each treatment group. The study was powered at the 80% level such that it may have been possible to have missed a mean treatment difference in PD₂₀ of less than 1.5 doubling doses (i.e. a 2.8-fold difference in protection). However, it should be appreciated that a one doubling dose difference in methacholine PD₂₀ is within the limits of biological variability for the method. On inspecting the within-subject 95% confidence intervals for mean treatment differences after 14 days, it is evident that this amounts to approximately 2 doubling doses of methacholine. Another possible criticism of our methodology was the use of methacholine which acts directly on cholinergic receptors on bronchial smooth muscle, whereas in real life most bronchoconstrictor stimuli such as allergen, exercise or cold air act indirectly via inflammatory mediator release or neuronal stimulation. It would therefore be important to evaluate whether this loss of protection occurs with indirect-acting bronchoconstrictors such as AMP.

Clinical relevance of results

Our study was performed in patients with stable asthma of mild to moderate severity, all of whom were receiving concomitant therapy with inhaled corticosteroids. We felt it important to have the latter as an inclusion criteria because the present UK asthma management guidelines advocate the use of regular long-acting β_2 -agonists only for patients who are already receiving inhaled corticosteroids (British Thoracic Society, 1997). Our results should be taken in the context of the study of Pauwels et al (1997),

where formoterol showed additive effects to inhaled corticosteroid on asthma control over a 12 month period. In their study, the predominant effect on the rate of severe exacerbations occurred as a consequence of using a higher dose of inhaled corticosteroid rather than the addition of formoterol. The finding of tachyphylaxis to methacholine bronchoprotection with formoterol in our study is not surprising given that previous studies with salmeterol have also showed tachyphylaxis in patients taking inhaled corticosteroids (Booth, 1996; Grove, 1995; Kalra, 1996; Yates, 1996). It may not be possible to extrapolate our results to what happens in patients with more severe asthma, where tachyphylaxis may have more serious consequences, in that a given bronchoconstrictor response would occur from a lower starting baseline FEV₁ value.

Our results indicate that after two weeks there was a residual level of protection from baseline with all three formoterol regimes ranging from 1.6-fold to 2.3-fold. However, in view of the subsensitivity which occurs with chronic dosing, patients should be made aware that regular treatment with formoterol may not confer full protection against a given bronchoconstrictor stimulus, and additional β_2 -agonist rescue medication may therefore be needed in order to overcome an acute bronchoprovocation stimulus. With this in mind, studies with regular salmeterol have shown that cross-tolerance occurs in terms of producing subsensitivity to protection afforded by rescue therapy with salbutamol (Yates, 1996). Thus, it is likely that additional rescue therapy with short-acting β_2 -agonist would have to be given in higher doses in order to overcome this subsensitivity. Since formoterol unlike salmeterol, has a rapid onset action, it would therefore be possible to use additional doses of formoterol in this way in the event of being exposed to a bronchoconstrictor stimulus, although it is not currently licensed for this indication.

What is the rationale for using regular long-acting β_2 -agonists as suggested by the current asthma management guidelines? Studies with twice daily salmeterol and formoterol have shown that long-acting β_2 -agonists and inhaled corticosteroids have additive actions in terms of their long-term effects in improving asthma control and reducing exacerbation rates (Greening, 1994; Woolcock, 1996; Pauwels, 1997). However, there are many patients in whom adequate asthma control can be achieved with an optimised dose of inhaled corticosteroid as monotherapy, who only require occasional β_2 -agonists for rescue purposes for breakthrough episodes of bronchoconstriction. The question therefore arises in such patients as to whether a short or long-acting β_2 -agonists should be used on an as required basis for rescue therapy. Formoterol could in theory be used in this way because of its fast onset of action, although the results of our study would suggest that subsensitivity to protection occurs if it is taken at least once daily in a dose of 12 μg .

In the present study we also looked at whether the protection afforded by the single 6 μg dose of formoterol was influenced by prior treatment for two weeks with regular formoterol, having had a 24 h washout prior to the methacholine challenge. Our results suggest that after pretreatment with formoterol 24 μg twice daily for two weeks, there was persistent subsensitivity to the subsequent protection afforded by formoterol 6 μg , with a mean response which was 41% lower compared to placebo pretreatment, although this was not significant. Interestingly, prior treatment with formoterol 12 μg once daily also produced a similar degree of persistent subsensitivity, with the response to formoterol 6 μg being 47% lower compared to placebo pretreatment.

It would be interesting to know for the purposes of rescue therapy, whether the β_2 -AR down-regulation and associated subsensitivity could be overcome by using a

higher dose of formoterol, and whether it would be possible to achieve the same level of protection as with the first dose. In other words is there simply a parallel rightward shift in the formoterol dose-response curve for bronchoprotection, or is there an associated reduction in the maximal response which cannot be overcome by using a higher dose? Previous data from studies in asthmatic patients with lymphocyte β_2 -AR have shown that 24 hours after stopping regular treatment with formoterol 24 μg twice daily, a reduction in receptor binding density is accompanied by an attenuated maximal cAMP response to isoprenaline stimulation (Newnham, 1995). This therefore suggests that it may not be possible to achieve a normal maximal airway response by using higher doses of rescue β_2 -agonist therapy.

It is known from *in vitro* studies that corticosteroids exhibit a facilitatory effect on β_2 -AR in terms of preventing or reversing agonist induced down-regulation and associated desensitisation (Mak, 1995b; Samuelson, 1984). In Chapter 3, we have demonstrated in asthmatic patients, that airway β_2 -AR bronchodilator subsensitivity induced by regular formoterol 24 μg twice daily is rapidly reversed after administration of a bolus of systemic corticosteroid. In our study, the subsensitivity to bronchoprotection occurred despite concomitant inhaled corticosteroid therapy. It would therefore be interesting to know whether this subsensitivity could be overcome by giving a bolus of high-dose inhaled corticosteroid as an alternative to using systemic corticosteroid administration.

CHAPTER 6

SUBSENSITIVITY TO BRONCHOPROTECTION AGAINST ADENOSINE MONOPHOSPHATE CHALLENGE FOLLOWING REGULAR ONCE DAILY FORMOTEROL

(European Respiratory Journal, *in press*)

6.1 INTRODUCTION

In the previous chapter, we demonstrated that subsensitivity developed to the bronchoprotective effects of inhaled formoterol against methacholine; and that this tolerance was not dependent upon the total daily dose of formoterol nor on its dosing frequency (ie once versus twice daily). Inhaled corticosteroids did not appear to prevent against the development of subsensitivity. In this present study, we have compared the effects of once-daily versus twice-daily inhaled formoterol on bronchoprotection, but to render the study clinically relevant, we have used adenosine monophosphate (AMP) as the bronchoconstrictor stimulus, because it is an indirectly acting agent, which causes inflammatory mediator release from mast cells. This is likely to reflect other indirectly acting stimuli which occur in real life, such as allergen, cold air or exercise challenge. There are two other important differences from the previous study in Chapter 5. Firstly, the protocol was to perform the AMP challenge test twelve hours after the drug was administered, because the airways would be at their most vulnerable to the bronchoconstrictor stimuli at the time before the next dose is due. Secondly, we compared the dose of 24 μg formoterol once-daily against 24 μg twice-daily (in Chapter 5, we compared 12 μg once-daily versus 24 μg twice-daily).

6.2 METHODS

Subjects

Ten asthmatic subjects (5 male and 5 female), mean (SEM) age 30.8 (4.3), all taking inhaled corticosteroids median (range) dose 400 (200-2000) $\mu\text{g}/\text{day}$, all were using inhaled β_2 -agonists on as required basis for symptomatic relief (<4 puffs per day) were recruited to take part in the study. All had stable asthma according to the American Thoracic Society criteria (American Thoracic Society, 1987a) for at least three months prior to taking part in the study and no one had had oral steroids during this time. Baseline spirometry showed mean (SEM) FEV_1 2.95 (0.23) L, 82 (4.5) % predicted and mean (SEM) FEF_{25-75} 2.51 (0.30) L/s, 57.8 (5.9) % predicted. All the randomised patients had unknown genotype prior to entry into the study. Patients were subsequently typed for β_2 -adrenoceptor polymorphisms in order to assess whether there was a preponderance of a particular polymorphism which may have biased our sample. All gave written informed consent before being randomised in the double-blind, double-dummy crossover study, which was approved by the Tayside committee on medical research ethics. A full physical examination, haematological and biochemical parameters were normal prior to inclusion in the study.

Study Design

Subjects were randomised to receive one week treatment with either a) inhaled formoterol 24 μg twice daily, or b) inhaled placebo twice daily, or c) inhaled placebo (morning) and inhaled formoterol 24 μg (evening). Formoterol was delivered by dry powder capsules, 12 μg per capsule (Foradil: Geigy Pharmaceuticals, Horsham, UK). The doses were chosen to be within the manufacturer's recommended total daily dose

range of 24-48 µg. Placebo was delivered using identically prepared capsules containing lactose. The medication was taken between 8:00 P.M. and 9:00 P.M. for the evening dose and between 8:00 A.M. and 9:00 A.M. for the morning dose. The first and last doses were taken in the evening before each respective visit. There was a one week run-in period before randomisation and a minimum of a week washout between each of the randomised treatments. From the start of the run-in period till the end of the study, all β_2 -agonist therapy was stopped and was substituted with inhaled ipratropium bromide (Atrovent Forte; Boehringer Ingelheim, Bracknell, UK) for symptomatic rescue relief.

Subjects attended the laboratory for the first visit between 8:00 A.M. and 9:00 A.M. after taking the first dose on the previous evening and withholding the second dose, which was taken in the morning after the AMP challenge. The second visit was a week later, after seven days of each treatment, again twelve hours after the last evening dose, when the second AMP challenge was done. This was followed by the washout period before the next treatment cycle.

Measurements

FEV₁ - see Chapter 2, Spirometry

Airway reactivity to adenosine monophosphate - see Chapter 2, Bronchial Provocation Tests

β_2 -adrenoceptor polymorphism - see Chapter 2

Statistical Analysis

The study was powered at 80% to detect difference in AMP PC₂₀ of 1.5 doubling doses (within subject SD = 1.3 doubling doses). The data for PC₂₀ were log

transformed prior to analysis. The change in log PC₂₀ protection was calculated as the geometric mean (and SEM) fold difference, comparisons between the three treatments and for comparison of first versus the last dose. The comparisons were made by multifactorial analysis of variance (MANOVA) using subjects, treatments and visits as factors for analysis, followed by Bonferroni multiple range testing. A probability value of $p < 0.05$ (two-tailed) was considered as being significant and 95% confidence intervals (95% CI) for mean differences were also calculated. The genotype data was not subjected to statistical analysis because of small number of subjects within a given genotype for each locus.

6.3 RESULTS

Spirometry, genotype and demographic data on the patients at recruitment are shown in Table 6.1. All the subjects completed the study and no adverse effects were reported.

Pre-challenge FEV₁

The FEV₁ 12 hours after the first dose, prior to the AMP challenge during the active limbs of the trial, showed a small improvement as compared to placebo (PL: 2.85 L, F 24 µg bid : 3.22 L, F 24 µg od : 3.12 L) although this was not statistically significant (PL vs. F 24 µg bid : $p=0.06$). This effect was diminished after the last dose of regular treatment (PL : 2.88 L, F 24 µg bid : 3.00 L, F 24 µg od : 2.97 L). (Figure 6.1)

Airway reactivity to AMP

After the first dose there was significant difference in protection against AMP challenge between both active treatments and placebo: for formoterol 24 µg bid : a 6.7-fold difference (95% CI 2.5 to 18.0) $p=0.002$, and for formoterol 24 µg od : a 5.2-fold difference (95% CI 2.0 to 13.1) $p=0.003$, as compared to placebo. (Figure 6.2). There was no difference between the two active treatments, after the first dose. After the last dose there were no significant differences between active treatments and placebo : for formoterol 24 µg bid : a 1.7-fold difference (95% CI 0.9 to 3.3) and for formoterol 24 µg od : 1.7-fold difference (95% CI 0.6 to 5.0).

Within each treatment regimen there was a significant loss of protection between first and last dose protection: for formoterol 24 µg bid (geom. mean) PC₂₀ FEV₁ for first

versus last dose: 475 mg/ml vs. 129 mg/ml $p=0.006$, a 3.7-fold difference (95% CI 1.6 to 8.3); and with formoterol 24 od : 367 mg/ml vs. 127 mg/ml $p=0.005$, a 2.9-fold difference (95% CI 1.5 to 5.6). There was no significant difference between the first and last doses for placebo: 71 mg/ml vs. 75 mg/ml.

Individual responses for the loss of protection between first and last dose are depicted in Figure 6.3 according to genotype for β_2 -adrenoceptor at locus 16 and 27. All of the three subjects who were homozygous for Glu 27 were also homozygous for Gly 16. The data showed considerable inter-individual variation in the degree of protection loss for both once and twice daily formoterol. There appeared to be no particular relationship between genotype and degree of subsensitivity with either once or twice daily dosing.

Subject	Age	Sex	Genotype	Genotype	FEV ₁	FEV ₁	FEF ₂₅₋₇₅	FEF ₂₅₋₇₅
	(yrs)		Codon 16	Codon 27	(L)	% pred	(L/s)	% pred
1	34	F	Gly-Gly	Glu-Glu	2.38	82	2.33	62
2	29	M	Gly-Gly	Gln-Gln	2.84	74	2.31	49
3	21	F	Gly-Arg	Glu-Gln	2.59	93	3.15	79
4	60	M	Gly-Arg	Glu-Gln	2.02	63	1.29	37
5	49	M	Gly-Arg	Glu-Gln	2.65	63	1.14	27
6	20	M	Gly-Gly	Glu-Glu	3.85	79	2.59	49
7	28	F	Gly-Arg	Glu-Gln	3.50	105	2.58	63
8	25	F	Gly-Gly	Glu-Glu	2.85	95	3.09	75
9	20	M	Gly-Arg	Gln-Gln	4.35	92	4.5	86
10	22	F	Arg-Arg	Gln-Gln	2.47	74	2.1	51
Mean	30.8				2.95	82	2.51	57.8
SEM	4.3				0.23	4.5	0.30	5.9

Table 6.1 Spirometry, genotype and demographic data at recruitment

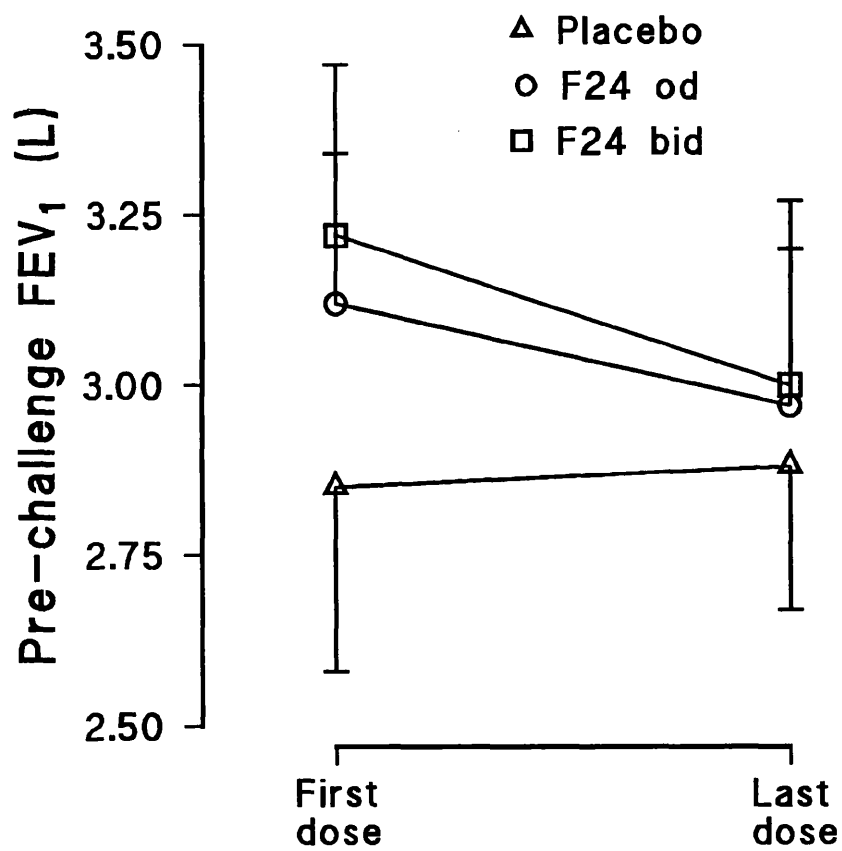


Figure 6.1 FEV₁ (L) before AMP challenge for each of the three treatments, after first and last dose. Results are expressed as mean (SEM).

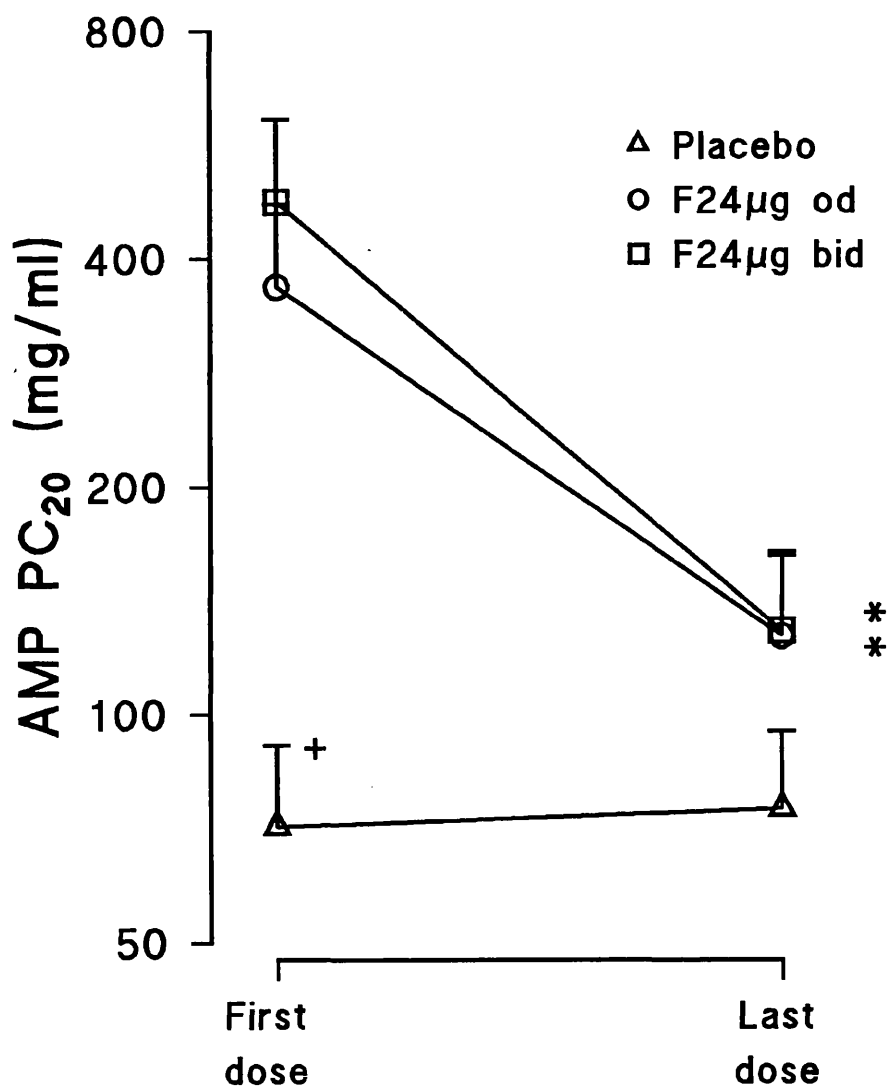


Figure 6.2 AMP PC₂₀ (mg/ml) for each of the three treatments, after first and last dose. Results are shown as mean (SEM). (* indicates p<0.01 between first and last dose; + indicates p<0.005 between active treatments and placebo) AMP PC₂₀ is plotted on a log₂ scale in order to show doubling doses.

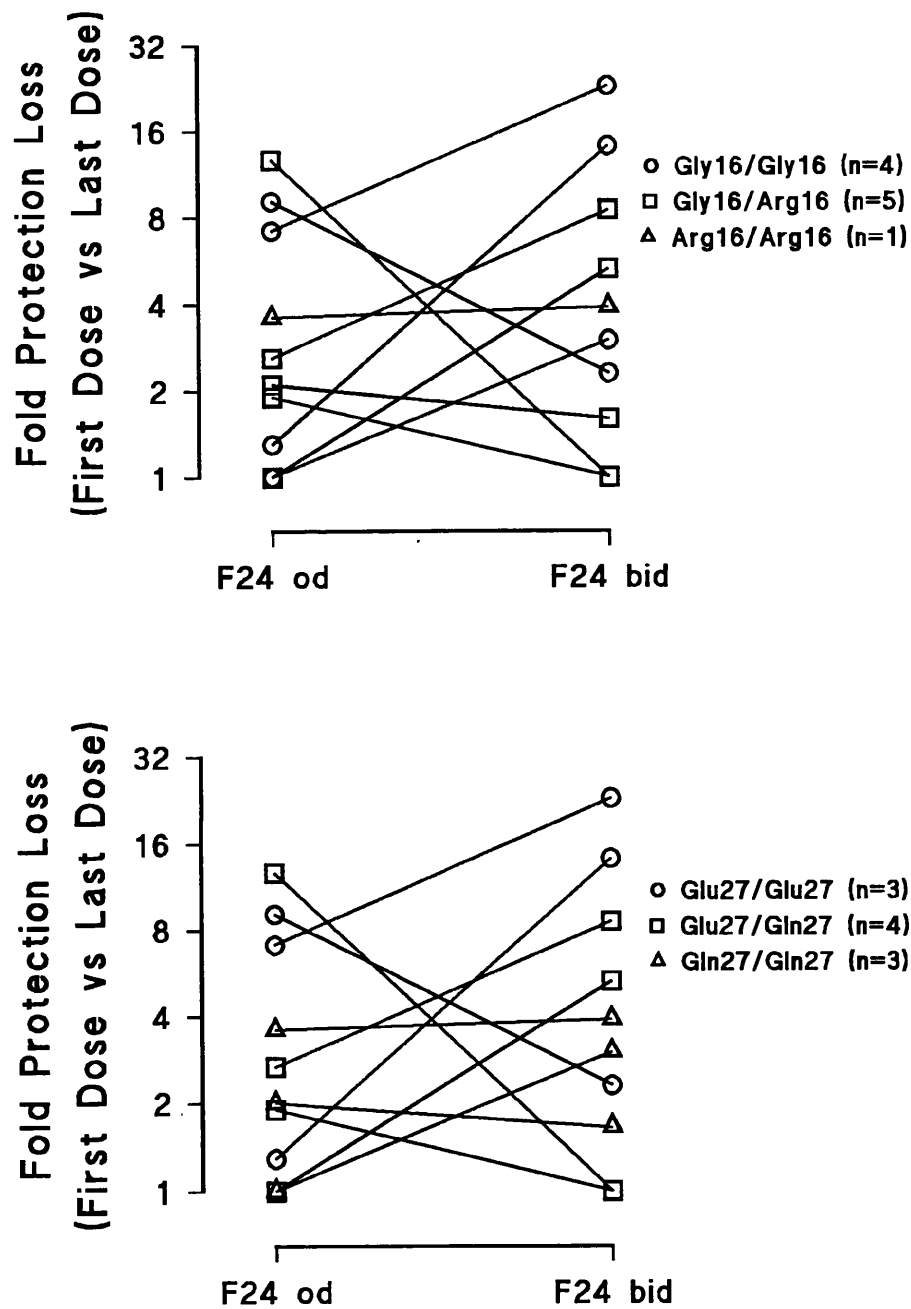


Figure 6.3 Individual data plotted to show the protection loss in AMP PC₂₀ between first and last dose for once daily (F24 od) and twice daily (F24 bid) treatment with formoterol. Fold protection loss is plotted on log₂ scale in order to show doubling doses. The results are depicted to show the individual's genotype for codon 16 and 27 of the β_2 -adrenoceptor. Data points for each individual with F24 od and F24 bid are joined by a solid line.

6.4 DISCUSSION

Our results showed that regular once daily treatment with formoterol leads to the development of subsensitivity to its bronchoprotective effect, which is comparable to the subsensitivity produced by twice daily therapy. This occurred despite the use of concomitant inhaled corticosteroid therapy. This is consistent with the findings in Chapter 5. The finding of tachyphylaxis with a once daily dosing regimen suggests that there must presumably have been a persistent degree of occupancy of airway β_2 -AR throughout the 24 hour dosing interval. We have previously shown that there is no detectable bronchodilator activity at 24 hours after stopping regular treatment with formoterol dry powder 24 μ g bid (Newnham, 1995). This in turn suggests that measuring effects on basal bronchomotor tone cannot be used as a sensitive surrogate for detecting persistent occupancy of airway β_2 -AR.

The doses of formoterol which we used were chosen to be within the manufacturer's recommended dose range of 24-48 μ g per day. Although there have been no previous studies looking specifically at protection loss with once daily long-acting β_2 -agonists therapy, previous studies with formoterol (Yates, 1995) and with salmeterol (Grove, 1995) had similarities in their design to the present study in their evaluating the bronchoprotective effect 12 hours after dosing. In the study of Yates *et al* with methacholine challenge on steroid naïve patients, there was a 2.7 fold protection loss between first and last dose of 24 μ g bid of formoterol dry powder given for two weeks. Values for first and last dose protection compared with placebo were 4.4 fold and 1.5 fold respectively. Grove *et al* reported only on the last dose protection against histamine in steroid treated asthmatics, after 50 μ g bid of salmeterol dry powder for 4

weeks, which showed a 1.5 fold residual protection compared with placebo. These data are similar to the results of the present study with AMP in terms of the degree of residual protection after the last dose, which was a 1.7 fold difference with both once and twice daily regimens. Taken together this suggests that the residual degree of protection is similar with either direct (methacholine and histamine) or indirect (AMP) challenge at 12 hours after dosing, with both salmeterol and formoterol given on a regular basis.

In a recent study, there was close agreement between the level of protection by terbutaline against AMP and histamine challenge, although only a single 500 µg dose of terbutaline was evaluated (Egbagbe, 1997). O'Connor *et al* (1992) showed that treatment with regular inhaled terbutaline produced a greater degree of subsensitivity for AMP than methacholine challenge. In their study there was a 4.2-fold loss of protection against AMP challenge after regular terbutaline, as compared to a 3.7-fold loss with twice daily formoterol in the present study.

Other studies (Cheung, 1992; Booth, 1996) have not shown any loss of bronchodilator effect after regular treatment with salmeterol 50 µg bid. In our study there was a trend towards a diminished pre-challenge FEV₁ value between the first and last dose, which is similar to findings reported by Yates *et al* (1995) with formoterol 24µg bid for two weeks. This could be due to a decrease in the duration of therapeutic bronchodilator effect of formoterol with regular treatment, as the FEV₁ was measured after one hour in the studies of Cheung and Booth, whilst in Yates's and our own study the FEV₁ was measured twelve hours after dosing. It is also important to mention that in the studies of Cheung *et al* and Booth *et al* there was a 36 hour washout after chronic dosing which might conceivably have restored β_2 -AR function and hence

bronchodilator response. It should be pointed out that our study was powered to detect bronchoprotection subsensitivity rather than bronchodilator subsensitivity and hence it is not possible to draw any firm conclusions on pre-challenge FEV₁ values with the present sample size. Furthermore a proper β_2 -agonist dose-response-curve is necessary in order to detect bronchodilator subsensitivity as demonstrated in Chapter 3. Studies with greater power are therefore required to investigate whether bronchodilator subsensitivity develops in the presence of regular once daily formoterol treatment.

We performed retrospective genotype analysis of β_2 -AR polymorphism in order to evaluate whether there was a preponderance of a particular polymorphism which may have biased our sample. From inspection of the individual data it was clear that the subsensitivity which developed with once or twice daily dosing regimens was independent of genotype at locus 16 and 27. In other words the finding of subsensitivity with once daily formoterol cannot be explained by a particular preponderance of a polymorphism in our sample, such as homozygous Gly 16 (Hall, 1996). Since bronchoprotection subsensitivity occurs more readily than bronchodilator subsensitivity, this may explain why there was no apparent relationship between the homozygous Gly 16 genotype and the degree of protection loss, even in association with formoterol 24 μ g twice daily. Larger prospective studies are underway to further investigate whether there is any association between β_2 -AR polymorphism and bronchoprotective subsensitivity.

In conclusion, regular treatment with inhaled formoterol induces subsensitivity to its bronchoprotective effect against AMP challenge, which is not influenced by the dosing frequency or by concomitant use of inhaled corticosteroids. Our research

disproves the hypothesis that the use of a 24 hour dosing interval may obviate the development of subsensitivity. This suggests that even with a once daily dosing regimen, there must presumably be inadequate recovery of β_2 -adrenoceptor down-regulation, due to prolonged receptor occupancy over a 24 hour period. It would therefore seem rational to suggest that long-acting β_2 -agonists could be used in the same way as short-acting β_2 -agonists, on an as required basis on top of optimised inhaled steroid therapy, up to a maximum recommended daily dosage. In this respect, formoterol has an advantage over salmeterol in that it has a fast onset of action and could therefore afford rapid relief of bronchospasm. For those patients with severe asthma who require regular formoterol, physicians should be aware that bronchoprotective subsensitivity occurs even with once daily administration, and hence higher doses of rescue β_2 -agonists might be required to obviate the effects of an acute bronchoconstrictor challenge.

CHAPTER 7

EFFECTS OF SINGLE DOSES OF INHALED FLUTICASONE PROPIONATE AND ORAL PREDNISOLONE ON LYMPHOCYTE β_2 -ADRENOCEPTOR FUNCTION IN ASTHMATIC PATIENTS

(Chest 1996; 109: 343-347)

7.1 INTRODUCTION

Inhaled corticosteroids are the most effective and widely used form of preventative anti-inflammatory treatment for use in bronchial asthma, and have been incorporated into national guidelines for this purpose (British Thoracic Society, 1997). In addition to potent anti-inflammatory properties, corticosteroids are known to play an important role in the regulation of β_2 -adrenoceptors (β_2 -AR). In Chapter 3, inhaled corticosteroids did not prevent the development of bronchodilator subsensitivity in patients with asthma when exposed to regular inhaled formoterol. Likewise, in Chapters 5 and 6, inhaled corticosteroids did not prevent the development of tolerance to the bronchoprotective effect of formoterol against methacholine and AMP respectively. On the other hand, in Chapter 3 we have shown that systemic corticosteroids rapidly reversed formoterol-induced bronchodilator subsensitivity. Hence, it would appear that inhaled corticosteroids did not have facilitatory effects on the β_2 -AR, in contrast to systemic corticosteroids. In this study, we have investigated the facilitatory effects of single doses of an inhaled corticosteroid compared with that of a systemic corticosteroid, which is known to possess significant facilitatory effects at the β_2 -AR (Hui, 1982; Brodde, 1985). In this respect, we chose to evaluate the effects of fluticasone propionate, a novel corticosteroid with enhanced glucocorticoid potency.

7.2 METHODS

Patients

10 (4 female) asthmatic patients were studied, with a mean (SEM) age of 28.6 (2.0) years, FEV₁ of 3.4 L (0.3), 79.9% (8.7) predicted and FEF₂₅₋₇₅ 3.0 L (0.4), 66.5% (9.7) predicted. Haematological and biochemical parameters were normal prior to inclusion. All gave written, informed consent before being randomised in a double-blind, placebo-controlled cross-over study approved by Tayside Medical Ethics Committee. All apart from one were non-smokers and all subjects had asthma according to the criteria of the American Thoracic Society (1987a). At the initial screening visit, subjects were required to have FEV₁>70% predicted and were inhaling less than or equal to 400ug corticosteroid per day. Of the 10 subjects, 9 were inhaling corticosteroid (beclomethasone dipropionate) in doses of 100 µg (one patient), 200 µg (three patients), 300 µg (one patient) and 400 µg (four patients). All patients were inhaling β₂-agonists as required, at a dose of < 200 µg salbutamol per day. In addition, one patient was taking beclomethasone nasal spray (100 µg daily). None had received oral corticosteroids within the preceding three months and none had had a recent exacerbation of asthma in the preceding three months.

For comparison as controls, we have also included data on lymphocyte β₂-adrenoceptor parameters for normal volunteers (n=59) and also asthmatic subjects (n=39), the latter having been off any β₂-agonist therapy for at least 4 weeks.

Protocol

Subjects attended the initial screening visit for measurement of FEV₁ using a Vitalograph Compact spirometer (Vitalograph Ltd, Buckingham, UK), having withheld inhaled bronchodilators for 8 hours and inhaled corticosteroid for 24 hours. Their inhaler technique was assessed using an aerosol inhalation monitor and instructions in

the correct usage were given. Subjects were then randomised to receive, via a pressurised metered dose inhaler, placebo, fluticasone propionate 1000 µg, fluticasone propionate 2000 µg (Flixotide®, Allen & Hanburys, Uxbridge, UK) or oral prednisolone 50 mg. Prednisolone was given as open medication within the randomisation sequence and inhaled treatments were given double-blinded. Fluticasone was delivered as 250 µg per actuation.

They had 4 study days separated by at least 3 days. Subjects were told to omit their morning dose of inhaled corticosteroid on the study day. At 10:00 PM on the evening prior to a study visit, subjects were instructed to take the appropriate dose from their study inhaler. They were instructed to use their bronchodilator inhaler 15 minutes before the study drug, and to take no further bronchodilator therapy overnight. In addition, they were instructed to mouth rinse after taking the inhaled steroid. They then attended the laboratory the following morning at 08:00 PM having fasted overnight, and withheld bronchodilators. After 30 minutes supine rest, 50 ml blood was taken from an antecubital vein for analysis of parameters of lymphocyte β_2 -adrenoceptor function and for serum cortisol.

Measurements

Lymphocyte β_2 -adrenoceptor parameters - see Chapter 2

Serum cortisol - see Chapter 2, Hormone Assays

Statistical analysis

Data for B_{\max} were transformed using logarithm to base 10, as it is not normally distributed. For all parameters, comparisons between treatments were made by

multifactorial analysis of variance (MANOVA), followed by Duncan's multiple range testing to identify where differences were significant. A probability value of $p < 0.05$ (two-tailed) was considered as being of significance. Values shown in the text as means for each treatment and pooled standard errors. 95% confidence intervals for the differences between treatments are shown for statistically significant differences.

7.3 RESULTS

Lymphocyte β_2 -adrenoceptor parameters

Parameters of receptor density (B_{\max}) and maximal cAMP responses to isoprenaline (E_{\max}) showed a significant ($p < 0.05$) increase after oral prednisolone, but not after inhaled fluticasone, with the mean (SEM) values for $\log B_{\max}$ (fmol/ 10^6 cells) being: placebo 0.27 (0.03), fluticasone 1000 μg 0.30 (0.03), fluticasone 2000 μg 0.32 (0.03) and prednisolone 0.48 (0.03) [95% CI prednisolone vs placebo: 0.075 to 0.341]. (Figure 7.1). The results for E_{\max} (pmol/ 10^6 cells) mirrored those for B_{\max} : placebo 4.00 (0.83), fluticasone 1000 μg 4.68 (0.83), fluticasone 2000 μg 4.26 (0.88) and prednisolone 7.46 (0.88) [95% CI prednisolone vs placebo: -0.01 to 6.91]. Dissociation constant (K_d : pmol/l) was not significantly altered by any treatment: placebo 13.44 (1.87), fluticasone 1000 μg 13.45 (1.48), fluticasone 2000 μg 14.04 (1.73) and prednisolone 16.75 (1.87).

Values of $\log B_{\max}$ for normal volunteers ($n=59$) and for asthmatics off β_2 -agonist therapy ($n=39$) were 0.28 (0.02) and 0.30 (0.02) respectively.

Serum cortisol

There was significant ($p < 0.05$) suppression of serum cortisol (nmol/l) following treatment with inhaled fluticasone propionate (2000 μg) and oral prednisolone compared with placebo: placebo 307.9 (26.75), fluticasone 1000 μg 323.2 (26.75), fluticasone 2000 μg 130.1 (26.75) [95% CI fluticasone 2000 μg vs placebo: 69.76 to 285.8] and prednisolone 51.8 (28.66) [95% CI prednisolone vs placebo: 144.11 to 368.01] (Figure 7.1).

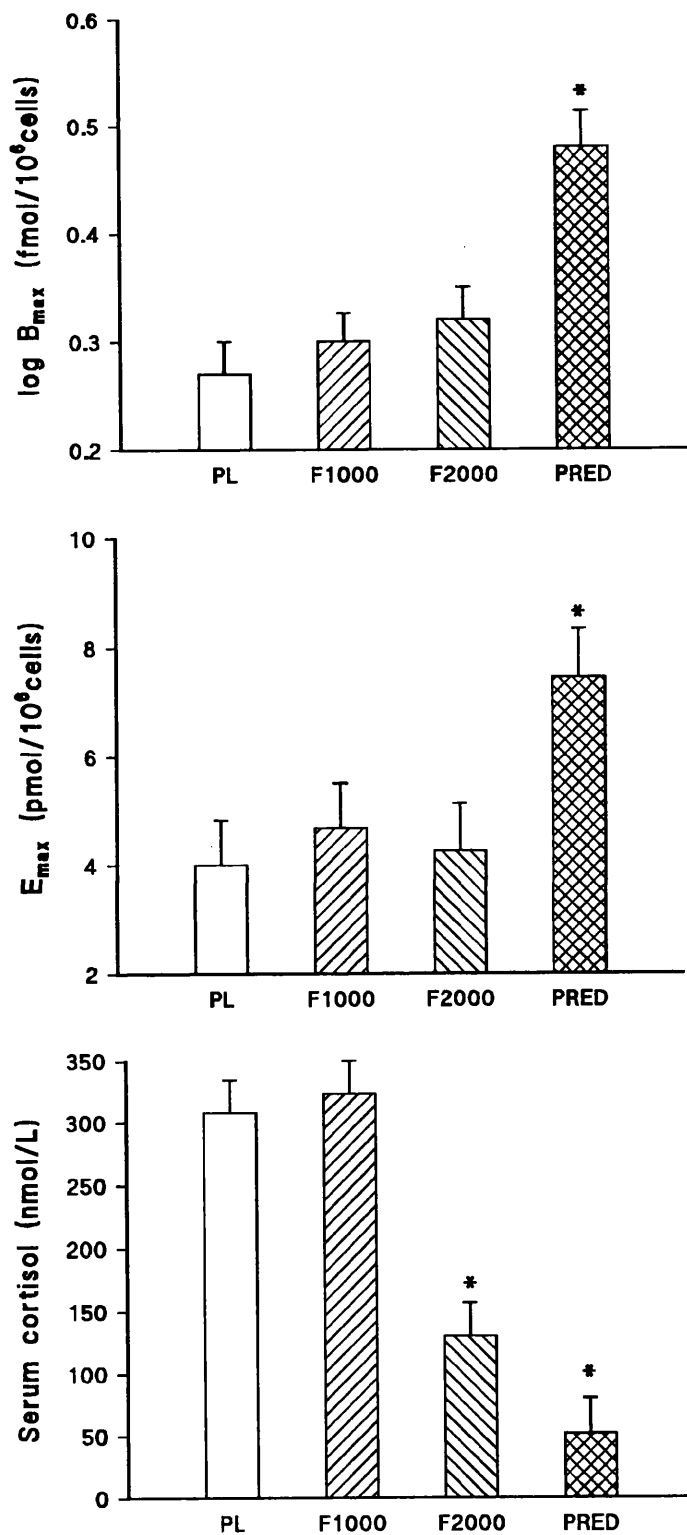


Figure 7.1 Values are shown for lymphocyte β_2 -adrenoceptor density ($\log B_{\max}$), cAMP response (E_{\max}), and serum cortisol after treatment with single doses of inhaled placebo (PL), inhaled fluticasone 1000 μ g (F1000), inhaled fluticasone 2000 μ g (F2000) and oral prednisolone 50 mg (PRED). (* $p < 0.05$ versus PL) Values are mean (SEM).

7.4 DISCUSSION

The results of this study indicate that high dose inhaled fluticasone, in a dose sufficient to suppress serum cortisol, did not have any significant facilitatory effects on lymphocyte β_2 -AR parameters. This is in contrast to oral prednisolone which up-regulated β_2 -AR density and increased cAMP responses. This would suggest a dissociation in systemic sensitivity between the effects of inhaled corticosteroid on adrenal suppression and lymphocyte β_2 -AR regulation.

Inhaled fluticasone was chosen in this study as it is a novel inhaled corticosteroid with a high degree of glucocorticoid potency. Using the McKenzie skin vasoconstrictor assay in humans, fluticasone showed approximately twice the topical anti-inflammatory activity in comparison to beclomethasone dipropionate (Phillips, 1990). The high intrinsic activity is probably due in part to the higher affinity of fluticasone to the glucocorticoid receptor (Hogger, 1994). Also, the oral bioavailability of fluticasone is virtually zero due to almost complete first-pass hepatic metabolism (Harding, 1990), and together with using mouth rinsing (Selroos, 1991; Pedersen, 1993) absorption of fluticasone from the gastrointestinal tract will be negligible. Hence, in the present study, any systemically bioactive drug would have been due to absorption via the lung-vascular bed, which would obviate first-pass hepatic metabolism (Lipworth, 1995). The finding of significant systemic bioactivity with a single 2000 μ g dose of fluticasone is not surprising, and consistent with recent data from other single and chronic dosing studies in normal and asthmatic subjects (Grahnen, 1994; Grove 1994; Boe, 1994).

The other finding of note is the lack of facilitatory effects of inhaled fluticasone on lymphocyte β_2 -AR function, in clear contrast to the effect of a single dose of prednisolone. Systemic corticosteroids, as well as having potent anti-inflammatory effects, have important regulatory functions affecting the β_2 -AR. This mechanism may

underlie some of their beneficial effect in the treatment of asthma. Corticosteroids increase the number of β_2 -AR and enhance the coupling of the receptor to the adenylate cyclase system (Davies, 1984). Oral prednisolone has been shown to rapidly restore previously down-regulated receptors after exposure to chronic β_2 -agonist treatment in normals and asthmatics (Hui, 1982; Brodde, 1985; Brodde, 1988). In Chapter 3, we also demonstrated the facilitatory effect of systemic corticosteroids in reversing bronchodilator subsensitivity induced by formoterol. It is therefore important to investigate if these facilitatory effects applied to inhaled corticosteroid as well. It is worth noting that the value for $\log B_{\max}$ observed in the present group of asthmatic patients is similar to values obtained from normal volunteers and from asthmatic subjects off β_2 -agonist therapy. This is in keeping with only minimal down-regulation in the present group of patients, as one would expect from their prior exposure to β_2 -agonists. The lack of effect of fluticasone may be due to the low level of β_2 -agonist consumed by the patients studied. This would not, however, explain why prednisolone had a positive effect and perhaps might infer that prednisolone 'super-normalised' β_2 -AR density, at least when compared with normal controls.

Another possibility is that the time-course of pharmacodynamic effects of fluticasone on lymphocyte β_2 -AR might be different to that on adrenocortical receptors. Our study compared single doses of fluticasone and it is conceivable that repeated doses might have up-regulated lymphocyte β_2 -AR. However, the long pharmacokinetic elimination half-life of fluticasone would be against this latter hypothesis.

In a study by Molema *et al* (1988), up-regulation of airway β_2 -AR does not appear to occur with inhaled beclomethasone dipropionate in doses up to 2000 μg per day given for four weeks in that there was no left-shift in the bronchodilator dose-response curves to inhaled salbutamol. However, *in-vitro* lymphocyte β_2 -AR parameters were not evaluated in that study. The findings of Molema *et al* probably reflect the absence of bronchodilator tolerance even in patients exposed to high doses of inhaled

salbutamol (Lipworth, 1989). In two studies by Newnham *et al* (Newnham, 1994a; Newnham, 1995) tachyphylaxis to bronchodilator and systemic β_2 -AR responses after treatment with formoterol mirrored down-regulation of *in vitro* lymphocyte β_2 -AR parameters, including a significant reduction in receptor density and cAMP response. Virtually all of the patients in these two studies were taking inhaled corticosteroids and it is interesting that the latter did not prevent β_2 -AR down-regulation. Likewise, we have demonstrated in Chapter 3, the development of bronchodilator subsensitivity in asthma patients on regular inhaled formoterol despite all patients receiving inhaled corticosteroids.

There is now accumulating evidence that regular inhaled β_2 -agonist treatment may have adverse effects on disease control in asthmatic patients (Sears, 1990; Van Schayck, 1990; Van Schayck, 1991). The question arises as to whether inhaled corticosteroid might protect against any potentially harmful effects of concomitant regular inhaled β_2 -agonist. Our study shows that lymphocyte β_2 -AR up-regulation does not occur, but this might not conceivably reflect effects on airway β_2 -AR or indeed effects on airway reactivity. There are studies (Paggiaro, 1991; Waalkens, 1991; Kerstjens, 1992) that have compared the combination of inhaled corticosteroid plus β_2 -agonist with β_2 -agonist alone. Such studies have shown that combined therapy with inhaled corticosteroid plus β_2 -agonist confers protection against bronchial hyperresponsiveness as well as against early and late allergen responses.

In summary, this study has shown that inhaled fluticasone propionate, given in a single dose of 2000 μg which suppressed adrenal function, had no significant facilitatory effects on lymphocyte β_2 -AR parameters in patients with asthma.

CHAPTER 8

EFFECTS OF REPEATED DOSES OF FLUTICASONE PROPIONATE AND ORAL PREDNISOLONE ON LYMPHOCYTE β_2 -ADRENOCEPTOR FUNCTION IN ASTHMATIC PATIENTS

(British Journal of Clinical Pharmacology 1997; 44: 565-568)

8.1 INTRODUCTION

Although the anti-inflammatory actions of inhaled corticosteroid are well known (Laitinen, 1992; Djukanovic, 1992; Jeffrey, 1992), the facilitatory effects on β_2 -adrenoceptor (β_2 -AR) function is less clear. Systemic corticosteroids have been shown to reverse β_2 -AR down-regulation in normal subjects (Hui, 1982; Brodde, 1985) and asthmatics (Brodde, 1988) who have been exposed to β_2 -agonists. Inhaled corticosteroids, however, do not appear to protect against the development of β_2 -AR subsensitivity. In Chapter 3, inhaled corticosteroid did not prevent the development of formoterol-induced bronchodilator subsensitivity; while in Chapters 4 and 5, inhaled corticosteroids did not protect against the development of tolerance to the bronchoprotective effect of β_2 -agonist against methacholine and AMP respectively. In Chapter 7, we had demonstrated that single inhaled doses of fluticasone propionate (1000 μg and 2000 μg) did not have any significant facilitatory effects on lymphocyte β_2 -AR in asthmatic patients, in contrast to oral prednisolone, which produced up-regulation. This begs the question as to whether this lack of effect with single doses of fluticasone may be due to pharmacokinetic properties and hence its time-profile for up-regulation. To address possible differences between single dose and steady-state time-profiles, we have performed a further study to see whether up-regulation might occur at steady-state with repeated dosing of fluticasone propionate.

8.2 METHODS

Patients

Ten asthmatic subjects (5 female), mean age 29 years (range 19-52) were recruited and completed this study. All gave written informed consent before being randomised into a double-blind, placebo controlled, cross-over study which was approved by the Tayside Committee on Medical Research Ethics. A full physical examination, haematological and biochemical profile were normal prior to inclusion into the study. All had asthma according to the criteria of the American Thoracic Society (1987a) and all were non-smokers. Mean (SEM) FEV₁ in litres and % predicted were 3.09(0.09) L and 89(5)% predicted, range 2.70 to 3.54 (% predicted range 65-108%). Nine subjects were inhaling corticosteroid (either budesonide or beclomethasone dipropionate) in a median dose of 400µg/day, range 200 to 800µg/day. All were inhaling a short-acting β₂-agonist as required in doses of <400µg/day. None had received oral corticosteroid for at least 3 months, and none had had a recent exacerbation of asthma in the past month.

Protocol

All usual inhaled corticosteroids were discontinued four days prior to the study and for the duration of the study. Subjects were randomised to receive either inhaled placebo, inhaled fluticasone 1000 µg/day (given as 500 µg twice daily; Flixotide®, Allen & Hanburys, Uxbridge, UK), inhaled fluticasone 2000 µg/day (given as 1000µg twice daily) and oral prednisolone 50mg. All inhaled medication was delivered by a pressurised metered-dose inhaler, which was taken at 08:00 AM and 10:00 PM with mouth rinsing after each dose. The study medication was taken for four days with a four-day washout between each treatment. Subjects attended the laboratory at 08:00 AM, with the last dose of study drug taken at 10:00 PM the previous night. Patients were instructed in the use of a metered-dose inhaler with the help of an aerosol inhalation monitor (Vitalograph Ltd, Buckingham, UK).

Oral prednisolone was given as open-medication but within the randomisation scheme. 50mg of oral prednisolone was taken at 10:00 PM and the subjects attended the laboratory at 08:00 AM the following day. At each visit, an intravenous cannula was inserted into an antecubital vein. After 15 minutes supine rest, 50ml of blood was withdrawn for plasma cortisol and lymphocyte β_2 -AR parameters (B_{\max} , E_{\max} and K_d).

Measurements

Lymphocyte β_2 -adrenoceptor parameters - see Chapter 2

Plasma Cortisol - see Chapter 2

Statistical analysis

Data for B_{\max} were log-transformed prior to analysis as it is not normally distributed. For all parameters, comparison between treatments was by multifactorial analysis of variance (MANOVA) with subjects and treatments as within factors for analysis. A probability value of $p < 0.05$ (two-tailed) was considered as being of significance and 95% confidence intervals (95% CI) for mean treatment differences were calculated where significant. The milligram equivalence ratio for fluticasone versus prednisolone was estimated by log-linear interpolation.

8.3 RESULTS

Lymphocyte β_2 -AR parameters

β_2 -AR density (B_{\max} ; fmol/ 10^6 cells) was significantly increased after oral prednisolone but not after inhaled fluticasone. The geometric mean values for B_{\max} being as follows: placebo (PL) 1.51, fluticasone 1000 μ g/day (F_{1000}) 1.20, fluticasone 2000 μ g/day (F_{2000}) 1.20 and prednisolone (PRED) 2.14 (a 1.4 fold difference PRED vs PL; 95% CI 1.05 to 1.95; $p < 0.001$) (Figure 8.1). There was no significant difference in K_d following any of the treatments. (Table 8.1). E_{\max} showed a similar trend to effects observed with B_{\max} but was not significantly different.

Plasma Cortisol

There was significant ($p < 0.001$) suppression of plasma cortisol (nmol/l) following F_{2000} and PRED compared with placebo: PL 393.8, F_{1000} 302.1, F_{2000} 205.0 (95% CI F_{2000} vs PL: 58.1 to 319.4) and PRED 87.0 (95% CI PRED vs PL: 176.2 to 437.5). There was suppression of plasma cortisol following F_{1000} (Figure 8.1) which was not significant. The estimated milligram equivalence ratio for adrenal suppression was calculated at 1:11 for fluticasone vs prednisolone.

	PL	F1000	F2000	PRED
K_d (pmol/L)	16.3 (11.7-20.9)	12.2 (7.3-17.1)	14.8 (10.2-19.4)	13.2 (8.6-17.8)
E_{max} (pmol/10⁶ cells)	4.85 (3.72-5.97)	4.64 (3.52-5.77)	4.39 (3.26-5.51)	5.91 (4.78-7.03)

Table 8.1 Receptor dissociation constant (K_d) and maximal cyclic AMP response to isoprenaline (E_{max}) after treatment with inhaled placebo (PL), inhaled fluticasone 1000 $\mu\text{g}/\text{day}$ (F1000), inhaled fluticasone 2000 $\mu\text{g}/\text{day}$ (F2000), each given for 4 days, and after a single dose of oral prednisolone 50 mg (PRED). Values are mean and 95% CI.

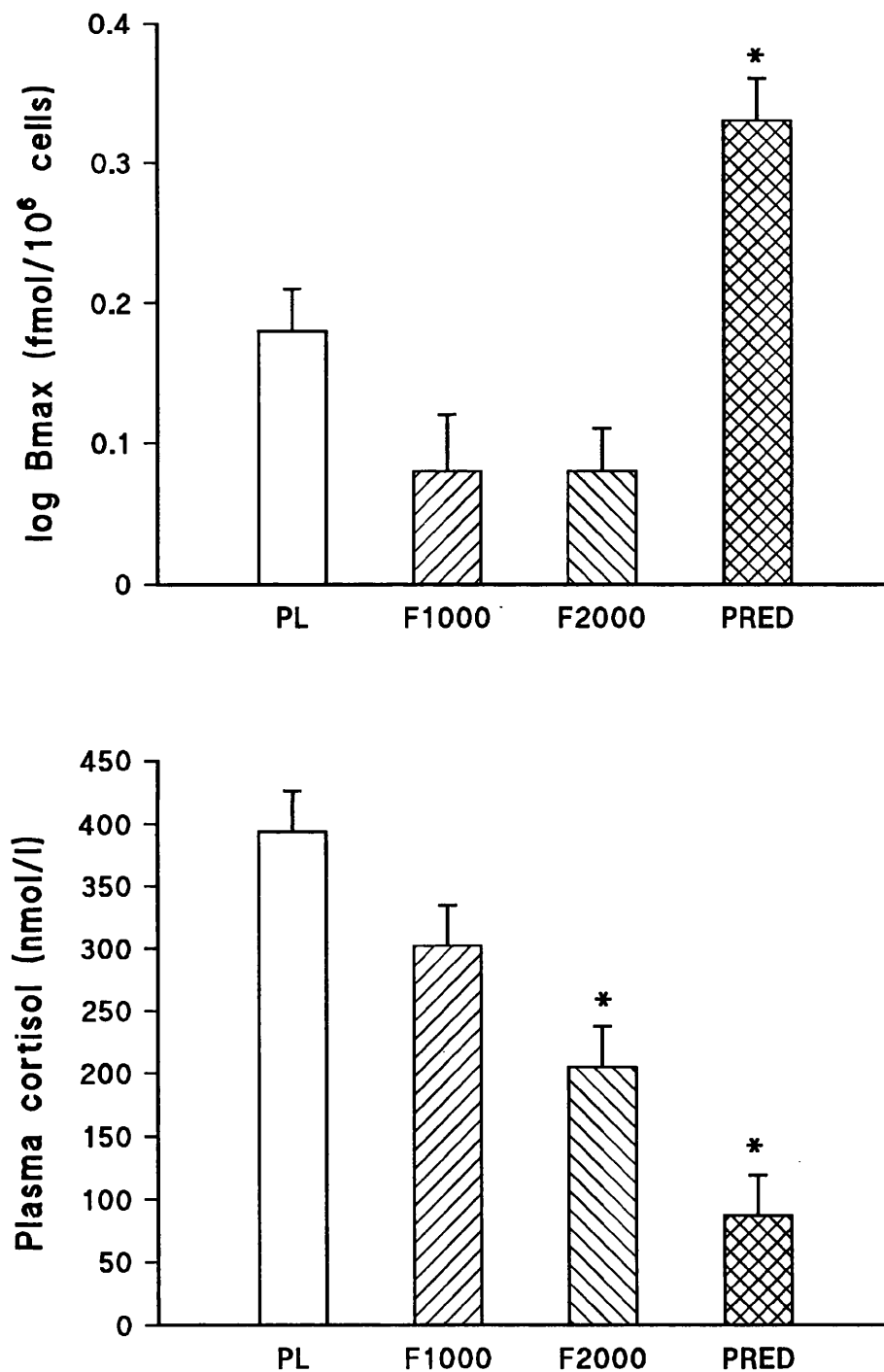


Figure 8.1 Values are shown for lymphocyte β_2 -adrenoceptor binding density ($\log B_{\max}$) and plasma cortisol after treatment with inhaled placebo (PL), inhaled fluticasone 1000 μ g/day (F1000), inhaled fluticasone 2000 μ g/day (F2000), each given for 4 days, and after a single dose of oral prednisolone 50 mg (PRED). Values are mean (SEM). (* denotes $p < 0.05$ versus PL)

8.4 DISCUSSION

The results of this study shows that repeated dosing with high dose inhaled fluticasone did not have any facilitatory effects on lymphocyte β_2 -AR despite having significantly suppressed early morning plasma cortisol with an estimated milligram equivalence ratio of 1:11 versus prednisolone. This result is in contrast to a single dose of oral prednisolone although suppressing plasma cortisol, significantly up-regulated β_2 -AR density. This study confirms our previous finding that single doses of inhaled fluticasone did not up-regulate β_2 -AR (Chapter 7). Both of these studies suggest a dissociation in sensitivity between effects of inhaled corticosteroid on adrenal suppression and lymphocyte β_2 -AR regulation. Our patients had relatively mild disease and had been exposed to small amounts of β_2 -agonists, mostly on an as required basis. Thus, it is conceivable that their β_2 -AR were not down-regulated prior to the study, and hence the lack of up-regulation with inhaled corticosteroid. This, of course, does not explain why prednisolone produced up-regulation of apparently naive β_2 -AR, as was the case in our previous study. It may be that higher doses of fluticasone (ie > 2mg) up-regulate β_2 -AR, although this would not be clinically relevant as the highest licensed dose is 2mg/day. Conversely, it would be interesting to know whether up-regulation occurs with lower doses of prednisolone (eg. 22mg) with equivalent adrenal suppression to fluticasone 2mg. Dose-ranging studies would be required to answer this important question.

We accept our study has its limitations as we used lymphocyte β_2 -AR as a surrogate for airway β_2 -AR, although there is some evidence to suggest that this may be valid (Hayes, 1996). One would expect that fluticasone given by the inhaled route would produce higher local concentrations in the airway than in the systemic compartment. Nonetheless, inhaled fluticasone is well absorbed with 16% bioavailability (Thorsson, 1997). Indeed, we showed evidence of systemic absorption in terms of adrenal suppression with an 11:1 milligram ratio versus oral prednisolone. Further studies are

required to look at possible differential effects of inhaled and oral corticosteroids on airway β_2 -AR function in asthmatic patients, in terms of bronchoprotective or bronchodilator effects of regular β_2 -agonist treatment.

Systemic corticosteroids, as well as having potent anti-inflammatory effects, have facilitatory effects on the β_2 -AR (Davies, 1980). Corticosteroids regulate β_2 -AR by increasing β_2 -AR gene transcription (Mak, 1995a). Likewise, systemic corticosteroid demonstrate significant facilitatory effects on β_2 -AR by reversing receptor down-regulation (Hui, 1982; Brodde, 1985; Brodde, 1988). However, our studies have demonstrated that inhaled high-dose corticosteroids do not appear to have facilitatory effects on the β_2 -AR. In this respect, inhaled corticosteroid therefore would not be expected to protect against β_2 -AR down-regulation and tachyphylaxis. This has been demonstrated in several studies involving formoterol (Newnham, 1994a; Newnham, 1995; Chapter 3) and salmeterol (Grove, 1995), where β_2 -AR down-regulation and bronchodilator subsensitivity developed despite most of the patients receiving inhaled corticosteroid.

In summary, this study has shown that repeated dosing with high-dose inhaled fluticasone had no significant facilitatory effects on lymphocyte β_2 -AR function in asthmatic patients, despite suppressing adrenal function with an estimated milligram equivalence ratio of 1:11 compared with prednisolone.

CHAPTER 9

LOW DOSE PREDNISOLONE PROTECTS AGAINST *IN VIVO* β_2 -ADRENOCEPTOR SUBSENSITIVITY INDUCED BY FORMOTEROL

(Chest 1998; 113: 34-41)

9.1 INTRODUCTION

In Chapter 3, we had demonstrated that bronchodilator and systemic β_2 -adrenoceptor (β_2 -AR) subsensitivity occurs after regular inhaled formoterol in patients with asthma. This β_2 -AR subsensitivity was reversed with a bolus of high-dose systemic corticosteroid, demonstrating the facilitatory effects of systemic corticosteroid on *in-vitro* and *in-vivo* β_2 -AR responses. In Chapters 7 and 8, we have shown that high-dose inhaled fluticasone propionate, in single and repeated doses, did not have significant facilitatory effects on lymphocyte β_2 -AR function in contrast to a single dose prednisolone (50mg).

Intravenous corticosteroid infusion in rats protects against down-regulation of pulmonary β_2 -AR induced by isoproterenol *in vivo* (Mak, 1995). Mak *et al* demonstrated that this protective effect of corticosteroid appears to be due to an increase in β_2 -AR gene transcription which counteracts the decrease in transcription due to chronic β_2 -agonist exposure. There have been no clinical studies investigating the protective effects of corticosteroids on β_2 -AR down-regulation induced by β -agonists in man.

In this study, therefore, we were interested to investigate whether the concomitant administration of systemic corticosteroid would protect against the development of β_2 -AR subsensitivity and down-regulation induced by continuous exposure to inhaled formoterol in humans.

9.2 METHODS

Subjects

Eleven healthy male subjects, mean (SEM) age 28 (2) years were recruited to completion in this study. All gave written informed consent before being randomised into the double-blind, placebo controlled, cross-over study which was approved by the Tayside Committee on Medical Research Ethics. A full physical examination, biochemical and haematological parameters and 12-lead electrocardiogram were normal prior to inclusion. All subjects were supervised in the use of a pressurised metered-dose inhaler using a Vitalograph aerosol inhalation monitor (Vitalograph Ltd, Buckingham, UK).

Protocol

Subjects were randomised to receive one week treatment with either inhaled formoterol 24 µg twice daily and placebo tablets or inhaled formoterol 24 µg twice daily and oral prednisolone 15 mg daily in double-blind, cross-over fashion. Formoterol was delivered by a pressurised metered-dose inhaler with 12 µg per actuation (Foradil®; Ciba-Geigy AG, Basel, Switzerland). Inhaled medication was taken twice daily at 08:00 AM and at 08:00 PM. Oral medication was taken once daily between 07:00 AM and 09:00 AM. Six subjects received placebo first and five subjects prednisolone first.

Subjects attended the laboratory at 08:00 AM prior to starting and one week after treatment, having withheld their study medication for the previous 24 hours on each occasion. At each visit, an intravenous cannula was inserted and kept patent by bolus injections of heparinised saline. Cannula dead space of 2 ml was withdrawn prior to blood sampling.

After 15 minutes supine rest, 50 ml blood was withdrawn for serum cortisol and lymphocyte β_2 -AR parameters. A dose-response curve (DRC) to inhaled salbutamol (Ventolin Diskhaler® 400 μg per actuation, Allen & Hanbury, Uxbridge, UK) was then constructed using doses of 800 μg , 800 μg and 1600 μg (i.e. cumulative doses of 800 μg , 1600 μg and 3200 μg respectively) with doses separated by 25 minutes. Measurements of serum potassium (K), heart rate (HR) and finger tremor (Tr) were undertaken over a 10 minute period at baseline and 15 minutes after each dose.

Measurements

Serum cortisol – see Chapter 2, Hormone Assays

In vitro lymphocyte β_2 -AR parameters – see Chapter 2

Heart rate - see Chapter 2

Finger tremor - see Chapter 2

Serum potassium - see Chapter 2

Statistical analysis

Data for finger tremor and B_{max} were transformed using logarithm to base 10, as both variables were not normally distributed. All systemic variables were analysed as delta responses from baseline. Comparisons for the DRC were made as area-under-curve (AUC) and as maximum responses. For all parameters, comparisons within and between treatments were made by multifactorial analysis of variance (MANOVA) using subjects, treatments, periods and visits as factors for analysis. A probability value of $p < 0.05$ (two-tailed) was considered as being of significance and 95% confidence intervals (95% CI) for mean differences were calculated where significant.

9.3 RESULTS

Early morning serum cortisol

There was no significant difference in 08:00 AM serum cortisol pre and post treatment with formoterol and placebo tablets (Table 9.1). As would be expected there was significant suppression of early morning serum cortisol after treatment with formoterol and prednisolone (pre vs post) 338.0 vs 194.9 (95% CI 50.0 to 236.2) $p < 0.01$.

In vivo systemic β_2 -AR responses

Baseline values for systemic parameters were not significantly different for each treatment period prior to each DRC (Table 9.1).

After treatment with formoterol, there were significant ($p < 0.05$) rightward shifts in the DRC (as AUC) for heart rate response (beats $\times 10^3$) (pre vs post), 0.76 vs 0.34 (95% CI 0.16 to 0.68), for tremor response (log units. h) 0.39 vs 0.18 (95% CI 0.01 to 0.41) and for potassium response (mmol.h/l) -0.34 vs 0.19 (95% CI -0.04 to -0.28). (Figure 9.1 and 9.2). Peak systemic responses were likewise significantly ($p < 0.05$) attenuated after treatment with formoterol (Table 2). Comparing pre vs post treatment values, for heart rate (beats/min) 28 vs 11 (95% CI 7.7 to 25.8); for tremor (log units) 0.72 vs 0.38 (95% CI 0.01 to 0.67); and for potassium (mmol/l) -0.81 vs -0.49 (95% CI -0.04 to -0.60). However, treatment with prednisolone protected against the development of subsensitivity induced by formoterol, in terms of AUC and peak response from the dose-response curves. Pre versus post values for AUC in presence of steroid were not significantly different: for heart rate (beats $\times 10^3$), 0.74 vs 0.64; for tremor (log units. h), 0.35 vs 0.34 and for potassium (mmol.h/l), -0.30 vs -0.25. (Figure 9.1 and 9.2)

Comparing values after formoterol treatment, the presence of steroid significantly increased heart rate and tremor response compared to formoterol alone, as AUC and peak responses. For heart rate, this was (placebo vs PRED) 0.34 vs 0.64 (as AUC, beats $\times 10^3$) (95% CI 0.07 to 0.50; $p < 0.05$) and 11 vs 22 (as peak, beats/min) (95% CI 1.6 to 18.6; $p < 0.05$). For tremor, this was 0.18 vs 0.34 (as AUC, log units.h) (95% CI 0 to 0.32; $p = 0.05$) and 0.38 vs 0.66 (as peak, log units) (95% CI 0.02 to 0.55; $p < 0.05$).

***In vitro* lymphocyte β_2 -AR parameters**

Following treatment with formoterol, there was significant down-regulation in lymphocyte β_2 -AR density (log B_{\max} ; fmol/ 10^6 cells) with placebo (pre vs post) 0.25 vs 0.11 (95% CI 0 to 0.22; $p < 0.05$) and with prednisolone (pre vs post), 0.21 vs 0.10 (95% CI 0.01 to 0.27; $p < 0.05$) (Figure 9.3). There was also a significant decrease in maximal cyclic AMP response to isoprenaline (E_{\max} ; pmol/ 10^6 cells) after treatment with formoterol (pre vs post) 6.21 vs 2.29 (95% CI 1.19 to 6.64; $p < 0.05$). However, treatment with prednisolone attenuated this fall in E_{\max} induced by formoterol and there was no significant difference in pre versus post values: 4.60 vs 3.28. E_{\max} values prior to each treatment were not significantly different. Comparing values after formoterol treatment, there was a trend towards a higher E_{\max} with formoterol and prednisolone than with formoterol alone: 3.28 vs 2.29 ($p = 0.06$) (Figure 9.3). There was no significant effect on dissociation constant, K_d (pmol/l) with either treatment (Table 9.1).

	FM+PL	FM+PL	FM+PRED	FM+PRED
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
HR (beats/min)	69 (61 to 71)	69 (66 to 72)	71 (69 to 74)	70 (68 to 73)
Tremor (log units)	2.29 (2.10 to 2.48)	2.37 (2.18 to 2.56)	2.36 (2.17 to 2.55)	2.24 (2.05 to 2.42)
K (mmol/l)	3.97 (3.82 to 4.12)	4.01 (3.86 to 4.16)	3.96 (3.81 to 4.11)	3.79 (3.64 to 3.94)
Cortisol (nmol/l)	358.9 (310.3 to 407.5)	296.2 (247.6 to 344.8)	338.0 (272.2 to 403.8)	194.9* + (129.1 to 260.7)
K_d (pmol/l)	18.1 (10.9 to 25.2)	21.2 (13.4 to 29.1)	21.1 (15.5 to 26.8)	21.1 (15.5 to 26.7)

Table 9.1 Mean (95% CI) baseline values (prior to dose-response curve) for systemic β_2 -AR parameters, early morning serum cortisol and lymphocyte β_2 -AR dissociation constant (K_d) before and after each treatment period.

(* indicates $p < 0.05$ for pre vs post treatment; + denotes $p < 0.05$ for post-treatment comparison)

	FM+PL	FM+PL	FM+PRED	FM+PRED
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
HR	28	11*	29	22 ⁺
(beats/min)	(22 to 35)	(5 to 18)	(23 to 36)	(15 to 28)
Tremor	0.72	0.38*	0.71	0.66 ⁺
(log units)	(0.48 to 0.95)	(0.14 to 0.61)	(0.51 to 0.91)	(0.47 to 0.86)
K	-0.81	-0.49*	-0.74	-0.60 ⁺
(mmol/l)	(-1.00 to -0.61)	(-0.68 to -0.29)	(-0.86 to -0.63)	(-0.72 to -0.48)

Table 9.2 Peak mean (95% CI) systemic β_2 -AR responses (as change from baseline) during the dose-response curve for salbutamol.

(* indicates $p < 0.05$ for pre vs post treatment; + denotes $p < 0.05$ for post-treatment comparison)

There were no differences between pre-treatment responses.

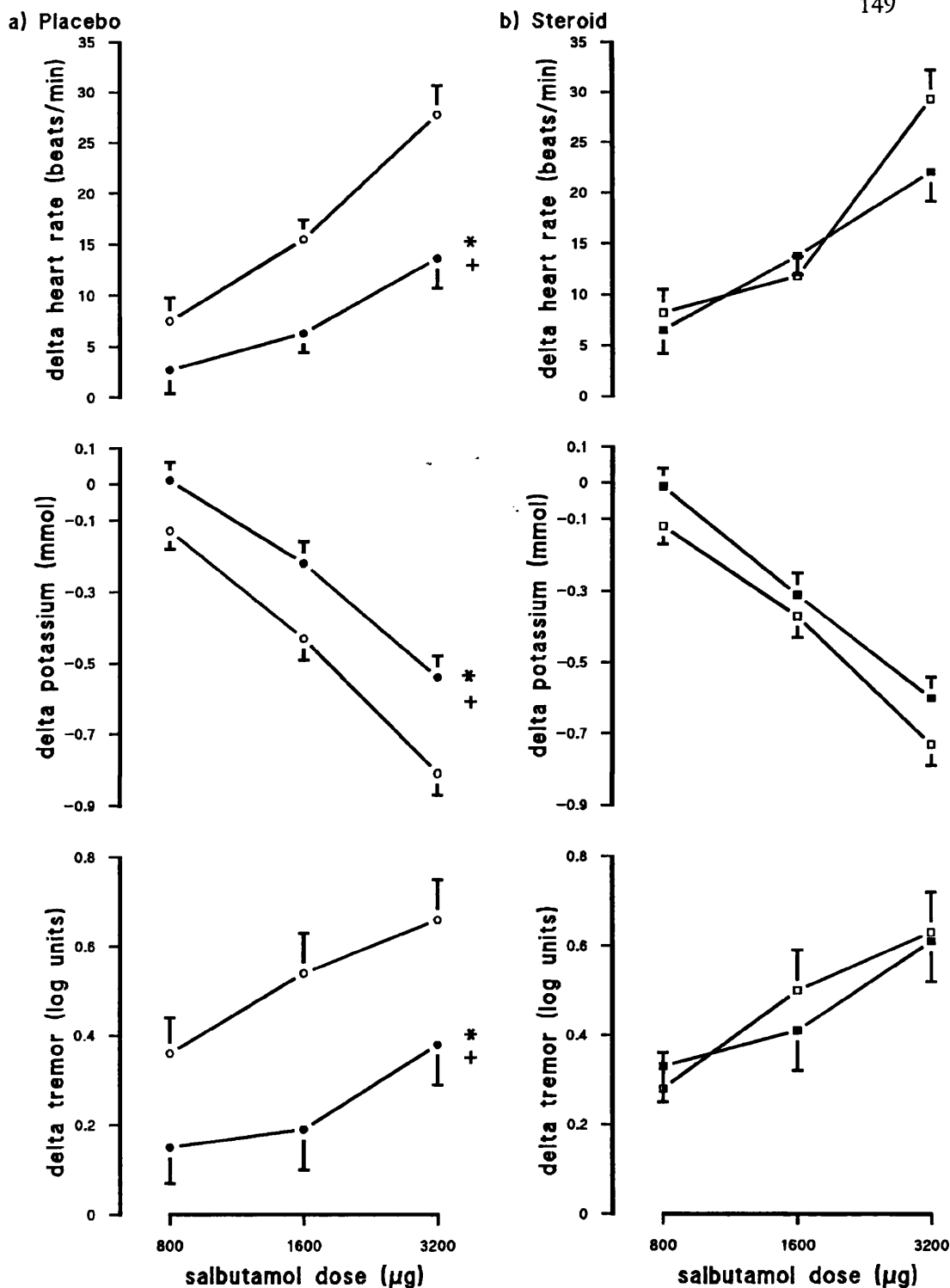


Figure 9.1 Dose-response curves for systemic β_2 -responses before and after regular treatment with formoterol and placebo (○ indicates pre-treatment and ● indicates post-treatment), and formoterol and prednisolone (□ indicates pre-treatment and ■ indicates post-treatment). For peak response: * indicates $p < 0.05$ for pre vs post treatment; + indicates $p < 0.05$ for post-treatment comparison (ie. FM+PL versus FM+PRED).

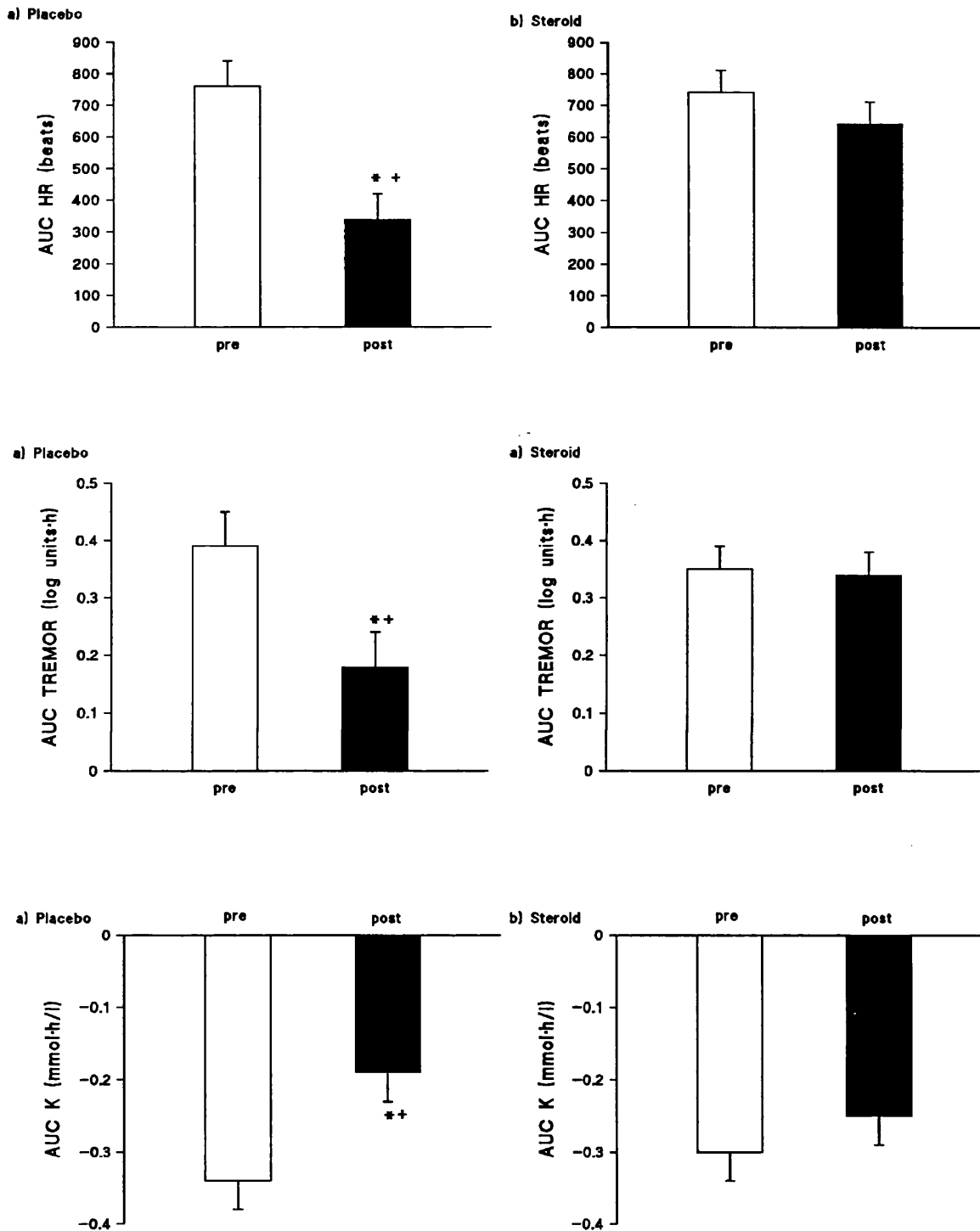


Figure 9.2 Dose-response curves for systemic β_2 -responses represented as mean values for area-under-curve (AUC) before and after regular treatment with formoterol with and without prednisolone. * indicates $p < 0.05$ for pre vs post treatment; + indicates $p < 0.05$ for post-treatment comparison (ie. FM+PL versus FM+PRED).

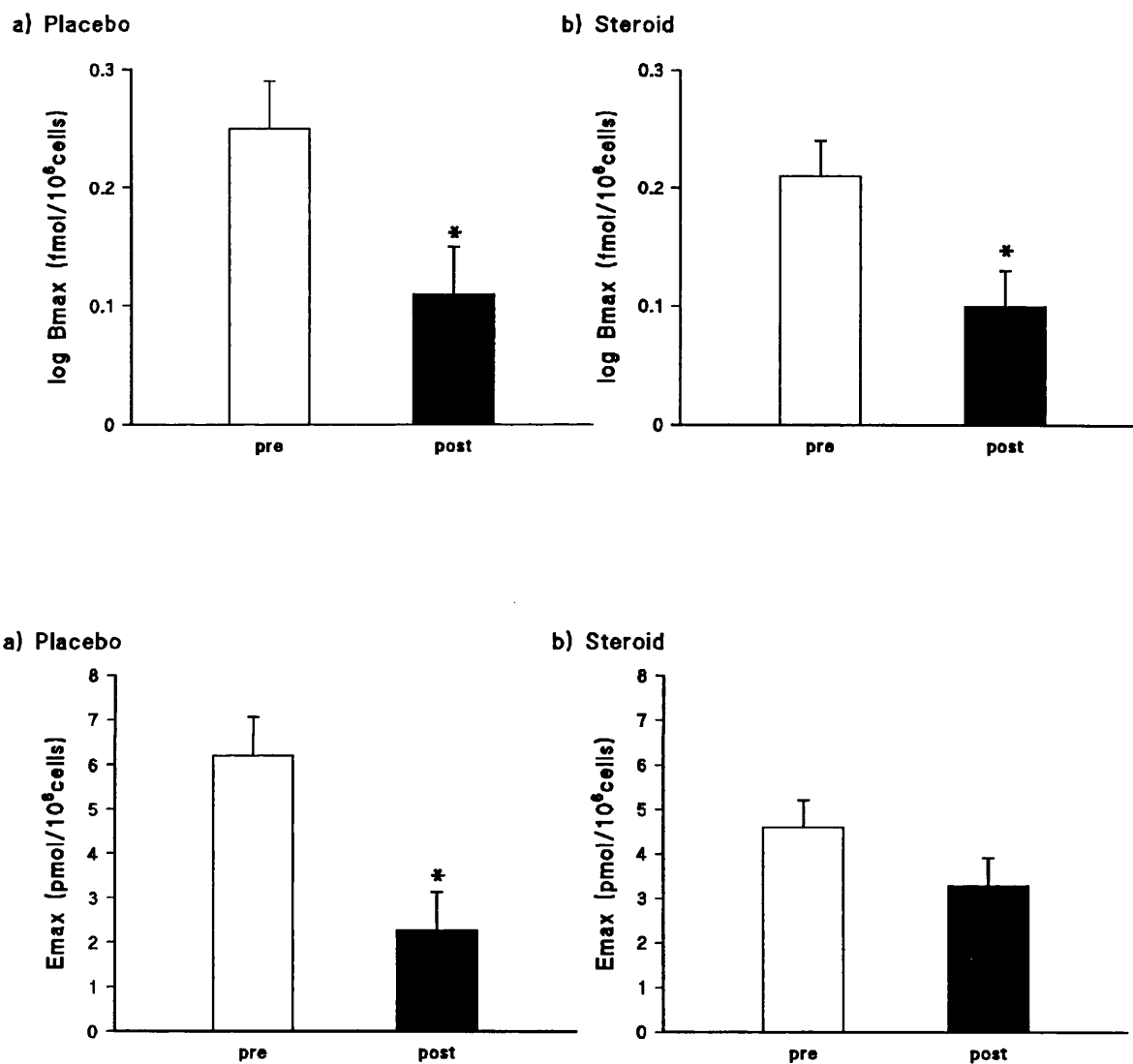


Figure 9.3 Mean values for lymphocyte β_2 -adrenoceptor density ($\log B_{\max}$) and maximal cyclic AMP response to isoprenaline (E_{\max}) before and after treatment with formoterol with and without prednisolone. * indicates $p < 0.05$ for pre versus post treatment.

9.4 DISCUSSION

We have demonstrated that regular treatment with inhaled formoterol produced *in vivo* systemic β_2 -AR subsensitivity associated with lymphocyte β_2 -AR down-regulation and desensitisation, consistent with previous studies (Newnham, 1994a; Newnham, 1995). However, the co-administration of low dose oral prednisolone protected against the development of *in vivo* β_2 -AR subsensitivity induced by formoterol. We believe that this is the first time the protective effect of corticosteroid on β_2 -AR desensitisation induced by long-acting β_2 -agonist has been demonstrated in humans. The finding that low dose prednisolone exhibited dissociation in protection against systemic *in vivo* β_2 -subsensitisation but not against *ex vivo* β_2 -AR down-regulation is particularly interesting. In this present study, systemic *in vivo* β_2 -AR subsensitivity induced by formoterol alone was followed by *ex vivo* lymphocyte β_2 -AR down-regulation and desensitisation. The reduction in lymphocyte β_2 -AR density induced by formoterol was not prevented by concomitant administration of prednisolone. In contrast, the presence of prednisolone attenuated the fall in E_{\max} caused by formoterol. Thus, the presence of corticosteroid attenuates the formoterol-induced desensitisation of β_2 -AR without altering the formoterol-induced down-regulation. Similar findings have been reported in a study in which healthy volunteers were given three days of oral terbutaline, followed by *in vitro* incubation of neutrophils with hydrocortisone (Samuelson, 1984). It was shown that hydrocortisone reversed terbutaline-induced desensitisation without affecting down-regulation.

There are several limitations to this study that we recognise. Firstly, we used healthy non-asthmatic subjects for evaluation. There were several reasons for this. We wanted to investigate the interaction between systemic corticosteroid and long-acting β_2 -agonist on β_2 -AR function in health first, and then to investigate this in patients with asthma to see if they behave differently. Also the administration of systemic corticosteroid to asthmatic subjects would tend to improve pulmonary function

making it difficult to compare bronchodilator dose-response curves due to confounding effect of systemic steroid on baseline airway geometry. Secondly, we investigated the effects on systemic β_2 -AR responses rather than airway β_2 -AR responses. However, in previous studies with long-acting β_2 -agonists (Newnham, 1994a; Newnham, 1995; Grove, 1995), airway β_2 -AR subsensitivity was accompanied by a similar degree of systemic β_2 -AR subsensitivity, making it valid, therefore, to look at systemic β_2 -AR responses as a surrogate for changes in airway β_2 -AR. Indeed, in Chapter 3, we found that a 200mg bolus of hydrocortisone produced rapid reversal of both airway and systemic β_2 -AR subsensitivity induced by formoterol in asthmatic patients. From a clinical point, though, systemic β_2 -AR tolerance may be seen as beneficial and augmented systemic β_2 -AR responses would be less desirable. Likewise, we have evaluated lymphocyte β_2 -AR parameters as a surrogate marker for airway β_2 -AR. Controversy exists as to whether it is valid to extrapolate from changes seen in circulating lymphocytes in vitro to changes in pulmonary receptors (Hauck, 1990; Hayes, 1996).

Subsensitivity of β_2 -AR caused by prolonged exposure to β_2 -agonists is due to a number of processes: reduction in receptor sites (due to internalisation and decreased production), desensitisation (fall in catecholamine-stimulated adenylate cyclase activity) and uncoupling (destabilisation of the high-affinity state of the receptor) (Davies, 1984). Corticosteroids are known to reverse these processes, in that they increase receptor numbers by a process of reversal of internalisation (Davies, 1984) and by increased β_2 -AR gene transcription (Collins, 1988; Mak, 1995a), and promote a high-affinity state of the receptor (Davies, 1980). That we have shown systemic corticosteroid protects against β_2 -AR subsensitivity is therefore, perhaps not surprising and in keeping with known acute facilitatory effects of systemic corticosteroid (Hui, 1982; Brodde, 1985; Brodde, 1988). The mechanism for this protective effect appears to be due to maintained sensitivity of the β_2 -AR via agonist-stimulated adenylate cyclase activity, at least in terms of effects on lymphocyte β_2 -AR

parameters. It may also be due in part to enhanced coupling or promotion of the high-affinity state of the receptor but this remains speculative as competition curves were not constructed to determine the ratio of the high to low affinity states of the receptor. This is in contrast to the findings of Mak *et al* (1995b) in rats where the protective effect of corticosteroid was due to increased receptor transcription such that there was no significant fall in receptor numbers after infusion with isoproterenol. This discrepancy may be due to cross-species comparison which may not be valid. In an *in vitro* study with human neutrophils (Davies, 1983), exposure to isoprenaline for 3h resulted in diminished cAMP response to isoprenaline-stimulation, whilst exposure to both isoprenaline and hydrocortisone resulted in a significantly greater cAMP response compared to isoprenaline alone. It is interesting that, as in our study, β_2 -AR density in the isoprenaline and hydrocortisone group was not different from that of the isoprenaline treatment group. The observed effect was due to an altered coupling state of the receptor, where corticosteroid attenuated the decrease in stability of the high-affinity state associated with desensitisation. In other words, it is possible for low dose systemic corticosteroid to protect against desensitisation by increased β_2 -AR coupling without necessarily producing up-regulation. In this respect we have shown that a high doses of prednisolone (50mg) appear to produce both up-regulation and increased cAMP response (Chapters 7 and 8).

The low dose of oral prednisolone (15mg) used in this study is equivalent to high dose inhaled corticosteroid in terms of systemic bioactivity. In Chapter 7 and 8, we have previously shown that inhaled fluticasone propionate had an estimated milligram equivalence of 1:10 as compared to prednisolone, in terms of suppressing early morning serum cortisol. Hence, 15mg oral prednisolone would be equivalent to 1500 μ g of inhaled fluticasone. In previous studies, single or repeated doses of up to 2000 μ g inhaled fluticasone had no facilitatory effects on lymphocyte β_2 -AR B_{max} or E_{max} in asthmatic patients in contrast to a 50mg dose of prednisolone. The lack of effect of inhaled fluticasone may be because the subjects were using minimal amounts

of β_2 -agonist and hence the receptors were not down-regulated. Whether doses greater than 15mg prednisolone in the present study would have protected against *ex vivo* lymphocyte β_2 -AR down-regulation as well as *in vivo* β_2 -AR desensitisation is unclear. The Tayside Committee for Medical Research ethics was not prepared to grant approval for higher doses of systemic steroid to be used in this study in view of the potential side effects associated with higher doses of systemic corticosteroid in normal volunteers. A proper dose-ranging study with prednisolone would therefore be needed to answer this question.

In conclusion, therefore, we have shown that low-dose oral prednisolone protects against formoterol-induced subsensitivity of *in vivo* systemic β_2 -AR as assessed by the response to inhaled salbutamol. This was associated with enhanced sensitivity but not density of *ex vivo* lymphocyte β_2 -AR. This has obvious implications for asthma treatment where glucocorticoids and long-acting β_2 -agonists are usually given together. Studies in normal and asthmatic subjects to investigate whether high-dose inhaled corticosteroid would have similar protective effects as low-dose prednisolone on β_2 -AR subsensitivity would therefore be required.

CHAPTER 10

LOSS OF NORMAL CYCLICAL β_2 -ADRENOCEPTOR REGULATION AND INCREASED PREMENSTRUAL RESPONSIVENESS TO ADENOSINE MONOPHOSPHATE IN STABLE FEMALE ASTHMATIC PATIENTS

(Thorax 1997; 52: 608-611)

10.1 INTRODUCTION

The role of female sex-steroid hormones in asthma is still unclear, although there is much circumstantial evidence to suggest that they may be important as discussed in Chapter 1. Up to 40% of female asthmatics report a premenstrual deterioration of their condition (Hanley, 1981; Gibbs, 1984; Eliasson, 1986; Pauli, 1989). However, the pathophysiology of this phenomenon remains unclear. Female sex-steroid hormones have a regulatory role on β_2 -adrenoceptor (β_2 -AR) function, and it has been postulated that abnormal β_2 -AR regulation may be a possible mechanism for premenstrual asthma. It has previously been demonstrated that cyclical changes in lymphocyte β_2 -AR function occur during the menstrual cycle in normal females with greater β_2 -AR density and isoprenaline responsiveness in the luteal phase during the premenstrual period (Wheeldon, 1994). Further support of this role is provided by *in vitro* studies which show that female sex-steroid hormones potentiate the bronchorelaxant effect of catecholamines (Foster, 1983).

In this study, we have investigated β_2 -AR regulation and airway responsiveness to AMP in female asthmatic subjects. AMP was used for two reasons. Firstly, previous work using direct-acting smooth muscle bronchoconstrictors such as histamine and methacholine have failed to show changes during the menstrual cycle (Weinmann, 1987; Pauli, 1989; Juniper, 1987). Secondly, it may be a better marker of airway inflammation as it induces bronchoconstriction via mediator release from mast cells and stimulation of sensory fibres (Polosa, 1991).

10.2 METHODS

Patients

15 stable well-controlled female asthmatics, mean age 25 years (range 18-39) and FEV₁ (SEM) 2.97L (0.11) (93.8% predicted), gave written informed consent to participate in this study which was approved by the Tayside Committee on Medical Research Ethics. All had asthma according to the criteria of the American Thoracic Society (1987a) and all were non-smokers. None had had a recent exacerbation of asthma in the preceding three months requiring the use of oral corticosteroids or antibiotics. None had any documented history of subjective premenstrual deterioration in asthma control. Median β_2 -agonist consumption was 0.8 puffs/day and ten subjects were receiving inhaled corticosteroid, median dose 800 μ g beclomethasone daily. (range 200-2000 μ g). Two subjects were on oral theophyllines (250mg and 600mg daily).

Protocol

Subjects were asked to keep a diary record of morning and evening PEF_R using a Wright's peak flow meter (Airmed, London, UK). The best of three successive readings was recorded. Subjects attended the laboratory on two separate days, during the follicular (day 1-6) and luteal (day 21-24) phases of the menstrual cycle. Day 1 being the first day of the menses. Before each visit, inhaled bronchodilators were withheld for eight hours, long-acting β_2 -agonists and oral theophyllines for 24 hours. Inhaled corticosteroids were continued unchanged. Both visits were made at the same time of day at 0900 AM.

At each visit, an intravenous cannula was inserted into an antecubital vein, and after 30 minutes rest, blood was removed for serum oestradiol and progesterone, serum eosinophil cationic protein (ECP) and lymphocyte β_2 -AR parameters. Airway responsiveness to AMP was evaluated using a standard challenge protocol. After

bronchial provocation, subjects were rested until FEV₁ returned to within \pm 5% baseline FEV₁. A dose-response curve (DRC) to inhaled salbutamol via a spacing device (Ventolin® pressurised metered-dose inhaler and Volumatic®, Allen & Hanburys, Uxbridge, UK) was then constructed by giving cumulative doubling doses of 100µg, 200µg, 400µg, 800µg and 1600µg. Measurements of FEV₁, FEF₂₅₋₇₅, plasma potassium and postural finger tremor were made at baseline and 20 minutes after each dose increment, with each dose increment given every 30 minutes.

Measurements

FEV₁ and FEF₂₅₋₇₅ - see Chapter 2, Spirometry

Airway hyperresponsiveness to adenosine monophosphate - see Chapter 2, Bronchial provocation tests

Plasma potassium

Postural finger tremor

Lymphocyte β_2 -adrenoceptor parameters

Serum oestradiol and progesterone

Serum eosinophil cationic protein (ECP)

Statistical analysis

PC₂₀, B_{max} and finger tremor were log-transformed for analysis as these are not normally distributed. Effects on salbutamol dose-response curves were analysed as peak responses and area under curve (AUC). Comparisons between the two phases were made by multifactorial analysis of variance (MANOVA). A probability value of $p < 0.05$ (two-tailed) was considered as significant.

10.3 RESULTS

The results of all parameters are summarised in Table 10.1. There were significant increases in both serum oestradiol (2.2-fold, $p < 0.001$) and progesterone (7.2-fold, $p < 0.05$) between the follicular and luteal phases. Mean differences between the follicular and luteal phases were 179.3 pmol/l for oestradiol (95% CI: 112.4 to 246.1) and 11.8 nmol/l for progesterone (95% CI: 5.1 to 18.6). Baseline FEV₁ (L) and FEF₂₅₋₇₅ (L/s) were not significantly different: 2.77 vs 2.78 and 2.16 vs 2.34 (follicular vs luteal) respectively. There were significant changes in AMP responsiveness during the menstrual cycle, with geometric mean PC₂₀ 19.0 mg/ml during the follicular phase and 7.6 mg/ml during the luteal phase ($p < 0.05$): a 2.51 fold difference (95% CI 1.19-5.30) amounting to 1.33 doubling doses. There was no significant correlation between the cyclical change in PC₂₀ AMP and the changes in oestradiol ($r^2 = 0.17$) or progesterone ($r^2 = 0.08$).

Lymphocyte β_2 -AR parameters and bronchodilator and systemic β_2 -AR responses to salbutamol (as peak or AUC) were not altered during the menstrual cycle. Serum ECP levels did not differ between the two phases of the cycle. Dormicillary peak flow recordings did not show any changes during the menstrual cycle.

	Follicular	Luteal
a) Progesterone (nmol/l)	1.9 (-2.9 to 6.6)	13.7 (8.9-18.5)*
Oestradiol (pmol/l)	151.4 (104.1-198.6)	330.6 (283.3-377.9)**
b) PC ₂₀ AMP (mg/ml) ⁺	19.0 (11.2-32.2)	7.6 (4.5-12.8)*
c) B _{max} (fmol/10 ⁶ cells) ⁺	1.70 (1.4-2.0)	1.80 (1.5-2.1)
K _d (pmol/l)	12.92 (8.35-17.49)	13.79 (9.22-18.37)
E _{max} (pmol/10 ⁶ cells)	6.65 (4.30-9.00)	5.69 (3.18-8.21)
d) ECP (µg/l)	4.43 (2.87-6.00)	4.78 (3.21-6.34)
e) Peak delta FEV ₁ (L)	0.48 (0.40-0.55)	0.43 (0.36-0.51)
Peak delta FEF ₂₅₋₇₅ (L/S)	1.31 (1.12-1.51)	1.10 (0.91-1.30)
Peak delta K (mmol/l)	-0.62 (-0.76 to -0.47)	-0.56 (-0.71 to -0.41)
Peak delta tremor (log units)	0.74 (0.50-0.98)	0.53 (0.28-0.79)
f) Morning PEFR (L/min)	402 (388-416)	409 (395-423)
Evening PEFR (L/min)	424 (410-437)	426 (412-439)
Diurnal variability (L/min)	21 (15-27)	18 (12-24)

Table 10.1 Values are shown for sex hormones, AMP airway reactivity, lymphocyte β 2-AR parameters, eosinophil cationic protein (ECP), peak responses for salbutamol DRC and peak flow readings, for follicular versus luteal phases. Values are mean (95% CI). (⁺ indicate geometric mean values) * denotes $p < 0.05$ and ** denotes $p < 0.001$.

10.4 DISCUSSION

Our study showed a 2.5-fold increase in airway responsiveness to AMP in the luteal phase, during the premenstrual period. Previous studies have not shown any changes in airway responsiveness during the menstrual cycle. These have used methacholine (Juniper, 1987; Pauli, 1989) and histamine (Weinmann, 1987) which are agents that act directly on airway smooth muscle. AMP, on the other hand, induces bronchoconstriction indirectly by activating mast cells to release bronchoconstrictive mediators (Cushley, 1985). It may be that increased AMP responsiveness during the luteal period may be due to sensitisation of adenosine receptors on mast cells by sex hormones, resulting in a lower threshold for mediator release in response to adenosine. Against this hypothesis is our finding of no correlation between hormone levels and PC₂₀. The influence of female sex hormones is not only confined to airway responsiveness changes but is also evident in skin-prick test reactions to histamine and allergen, with greater weal-and-flare reactions during the early luteal phase (Kalogeromitros, 1995). Also, it has recently been reported that hormone-replacement therapy may increase the risk of developing asthma in post-menopausal women and that seems to be related to the dose of the oestrogen component and duration of use (Troisi, 1995).

Our study has limitations which we recognise and accept. Firstly, we did not have a control group of non-asthmatics for comparison. The reason for this was it would not be possible to compare PC₂₀ in normal versus asthmatic airways because of confounding effects of airway geometry, even when using airways conductance. Secondly, our patients were stable and did not exhibit premenstrual asthma. Nonetheless, we felt at the outset that it would be valid to look at such patients as a way of investigating possible mechanisms for sex hormone modulation of airway and receptor function.

Despite an appropriate rise in luteal phase sex-hormones in our group of asthmatics, β_2 -AR parameters exhibited a loss of normal cyclical regulation previously described in normal females (Wheeldon, 1994). It is unlikely that this was due to the effects of exposure to exogenous β_2 -agonists as their consumption was minimal. This raises the possibility that in asthmatic patients there may be subsensitivity to the post-ovulatory rise in endogenous progesterone. The lack of difference in ECP probably reflects the low levels observed in this group of stable patients with mild disease.

What is the clinical implication of our findings? In stable asthma, it is important to appreciate that the intrinsic variability of bronchial hyperresponsiveness is such that PC_{20} values can vary by up to twofold ie one doubling dose (Juniper, 1978). Our findings in this study are therefore likely to have clinical relevance as it exceeds the limit of biological variability. Hence, this change in airway responsiveness from the follicular to luteal phase may be a possible mechanism to explain worsening symptoms during the premenstrual period, although the latter was not experienced by our group of mild asthmatics. It is conceivable that these changes may be more clinically significant in patients with more severe disease. Whether treatment for premenstrual asthma will involve drugs such as theophylline (Mann, 1985), sodium cromoglycate (Richards, 1988) or nedocromil sodium (Summers, 1990) which antagonise adenosine receptors would require further research.

CHAPTER 11

EFFECTS OF EXOGENOUS FEMALE SEX-STEROID HORMONES ON LYMPHOCYTE β_2 -ADRENOCEPTORS IN NORMAL FEMALES

(British Journal of Clinical Pharmacology 1996; 41: 414-416)

11.1 INTRODUCTION

Female sex-steroid hormones have a role in the regulation of β_2 -AR function. This has been demonstrated in a previous study where lymphocyte β_2 -AR density was significantly greater during the luteal phase as compared to the follicular phase, in association with raised premenstrual levels of progesterone and oestradiol (Wheeldon, 1994). However, in the previous chapter, we have shown that in female asthma patients, there is a loss of this normal cyclical regulation of β_2 -AR by ovarian sex-steroid hormones. Indeed, it has been postulated that abnormal premenstrual β_2 -AR regulation may be a possible mechanism for this phenomenon.

The aim of this present study was to determine which of the female hormones, progesterone or oestradiol, is responsible for the previously observed change in lymphocyte β_2 -AR density during the menstrual cycle. This was investigated by administering exogenous high doses of oestradiol and progesterone during the follicular phase when endogenous levels of both hormones are low.

11.2 METHODS

Subjects

Eight healthy female volunteers participated in this study, mean (SEM) age was 25 (3) years. All subjects had a normal full blood count and biochemical profile prior to entry.

None of the subjects had any significant past medical history, nor were they taking any regular medication. All but 1 (para 3) were nulliparous, with a normal menstrual history and regular cycles and none had recently (at least 3 months) taken oral contraceptives. All volunteers provided written informed consent prior to entry to the study which was approved by the Tayside Committee on Medical Research Ethics.

Protocol

Volunteers attended the laboratory during the follicular phase (day 1-6) on two successive menstrual cycles. Day 1 being the first day of the menstrual cycle. Baseline levels of serum progesterone and oestradiol were measured at each cycle. They were randomised to receive either progesterone (medroxyprogesterone 10mg, Upjohn Ltd) or oestrogen (ethinyloestradiol 50µg, Evans Medical Ltd) as single oral doses in cross-over fashion for the two menstrual cycles. Forty ml of peripheral blood was withdrawn, prior to the ingestion of the tablet, at baseline (T_0), and then at 24 hours (T_{24}) and 72 hours (T_{72}) after ingestion, for lymphocyte β_2 -AR assay on each of the two cycles.

Medroxyprogesterone was chosen as it is the least virilising of the progestones. The tablets are the highest dose licensed which are recommended for normal clinical practice. Two of the subjects experienced nausea with oestrogen and one had nausea after progesterone, indicating that higher doses would have been poorly tolerated.

Measurements

Lymphocyte β_2 -adrenoceptor parameters - see Chapter 2

Serum oestradiol and progesterone

Statistical analysis

The power of the study was 80% to detect a 25% difference in B_{\max} . For E_{\max} and K_d , comparisons between treatments were made by multifactorial analysis of variance (MANOVA) using subjects, treatments and time as within factors for the analysis. Duncan's multiple range test was then applied to ascertain at what times differences occurred within a given cycle for each treatment. For B_{\max} , which is not normally distributed, analysis was performed non-parametrically using Friedman's analysis of ranks. A probability value of less than 0.05 (two-tailed) was considered significant. Data for B_{\max} are shown as geometric means and non-parametric 95% CI. All other data are shown as means and 95% CI for within each treatment group.

11.3 RESULTS

Female sex steroid hormones

Baseline levels of oestradiol (pmol/l) and progesterone (nmol/l) were comparable for the two consecutive cycles (cycle 1 vs cycle 2) mean (95% CI): Oes 326.5(129.2 - 523.8) vs 252.1(54.8 - 449.4) and Prog 5.7(4.8-6.6) vs 6.1(5.7 - 7.0).

Lymphocyte β_2 -adrenoceptor parameters

The changes in lymphocyte β_2 -AR parameters following treatment with progesterone and oestradiol are summarised in Table 11.1. There were no significant differences in any parameter at T_0 when comparing the two treatments. There was a significant increase from baseline (T_0) in receptor density (B_{\max}) at T_{24} following treatment with progesterone but not oestradiol ($p < 0.01$ T_{24} vs T_0 : 1.39-fold geometric mean difference, 95% CI 0.97 - 2.00). By 72 hours, B_{\max} had fallen to approximately baseline (T_0) levels. The increase in B_{\max} following progesterone is greater than oestradiol at T_{24} ($p < 0.05$, Oes vs Prog at T_{24} 1.28 fold geometric mean difference 95% CI 0.95 - 1.73). There were no significant changes from baseline in K_d and E_{\max} following either treatment. Likewise there was no significant difference between the two treatments at each time point; for K_d , at T_0 (95% CI -8.9 to 5.4), at T_{24} (95% CI -10.8 to 7.1) and at T_{72} (95% CI -8.0 to 9.7). For E_{\max} , at T_0 , (95% CI -0.3 to 2.4), at T_{24} (95% CI -1.3 to 2.3) and at T_{72} (95% CI -2.1 to 2).

Statistical analysis did not show any evidence for order effect.

		T ₀	T ₂₄	T ₇₂
B _{max} (fmol/10 ⁶ cells)	Oes	1.40(1.16 - 1.69)	1.59(1.32 - 1.91)	1.43(1.19 - 1.72)
	Prog	1.45(1.19 - 1.77)	2.03(1.66 - 2.47)*†	1.56(1.28 - 1.90)
Kd (pmol/l)	Oes	10.85(4.22 - 17.48)	13.54(6.91 - 20.17)	10.65(4.02 - 17.28)
	Prog	9.11(5.87 - 12.35)	11.66(8.42 - 14.89)	11.50(8.26 - 14.73)
E _{max} (pmol/10 ⁶ cells)	Oes	5.53(4.55 - 6.51)	5.20(4.22 - 6.19)	5.68(4.70 - 6.67)
	Prog	6.60(5.26 - 7.93)	5.65(4.18 - 7.12)	5.61(4.28 - 6.95)

TABLE 11.1 Mean (95% CI) values for Kd and E_{max} following treatment with Oestrogen (Oes) and progesterone (Prog) at baseline (T₀), 24 hours (T₂₄) and 72 hours (T₇₂). Values for B_{max} are geometric means (non-parametric 95% CI).

(* indicates p<0.01 for T₂₄ versus T₀; † indicates p<0.05 Oes versus Prog at T₂₄)

11.4 DISCUSSION

This study has demonstrated that an exogenous high dose of oral progesterone but not oestradiol, given during the follicular phase, significantly increased lymphocyte β_2 -AR density. This would, therefore, suggest that endogenous progesterone is probably responsible for the previously observed increase in B_{\max} during the luteal phase of the female menstrual cycle (Wheeldon, 1994). A degree of caution is advisable in extrapolating our results with lymphocytes from normal subjects to changes in asthmatic airways. However, it has recently been shown with positron emission tomography that changes in lymphocyte β_2 -AR density closely mirror those on lung β_2 -AR, thus making comparisons between airway and lymphocyte β_2 -AR parameters seem valid (Hayes, 1996). The lack of difference in E_{\max} with progesterone probably reflects the relatively small difference in B_{\max} at T_{24} compared with T_0 . Whether a higher dose of progesterone would have increased E_{\max} as well as B_{\max} is unclear.

An alteration in normal cyclical regulation of β_2 -AR may be a possible mechanism for premenstrual asthma, as airway hyper-reactivity in response to both methacholine (Juniper, 1987; Pauli, 1989) and histamine (Weinmann, 1987) is unchanged in asthmatic subjects during the menstrual cycle. In keeping with this hypothesis is the observation that in a small number of patients with premenstrual asthma, intramuscular progesterone therapy prevented the falls in peak expiratory flow rates which occurred at this time, and allowed better control of these patients on smaller doses of systemic steroids (Beynon, 1988). In the previous chapter, we found loss of the normal cyclical β_2 -AR regulation in asthmatic women associated with increased premenstrual airway hyperresponsiveness to AMP. It may be that hormonal regulation of β_2 -AR in asthmatic women is abnormal. The findings of the present study showing that exogenous progesterone up-regulates β_2 -AR may suggest a possible mechanism for this therapeutic effect, and provide future therapeutic strategies for modulation of β_2 -AR in premenstrual asthma.

CHAPTER 12

PARADOXICAL DOWN-REGULATION AND DESENSITISATION OF β_2 -ADRENOCEPTORS BY EXOGENOUS PROGESTERONE IN FEMALE ASTHMATIC PATIENTS

(Chest 1997; 111: 847-851)

12.1 INTRODUCTION

It has been shown that lymphocyte β_2 -AR function in normal females is under the cyclical influence of ovarian sex-steroids with greater β_2 -AR density and cyclic AMP response to isoprenaline during the luteal phase as compared to the follicular phase, in association with raised post-ovulatory levels of progesterone and oestradiol (Wheeldon, 1994). In contrast, in Chapter 10, we demonstrated that in female asthma patients this cyclical pattern of β_2 -AR regulation is absent despite an appropriate post-ovulatory hormone response. In Chapter 11, we have observed that the administration of exogenous progesterone but not oestrogen in healthy females during the follicular phase produced up-regulation of lymphocyte β_2 -AR density. We postulate that there may be abnormal hormonal regulation of β_2 -AR in female asthmatics. The aim of this present study was to investigate how exogenously administered sex-steroid hormones might alter β_2 -AR regulation in female asthmatics.

12.2 METHODS

Subjects

Seven non-smoking female subjects with asthma, mean (SEM) age 26 (2) years participated in this study. Mean (SEM) FEV₁ was 3.03 (0.18) L, 94.7 (6.4)% of predicted and FEF₂₅₋₇₅ was 2.86 (0.30) L/s, 70.7 (7.4)% of predicted. All gave written informed consent before being randomised into a double-blind, cross-over study which was approved by the Tayside Committee of Medical Research Ethics. A full physical examination, biochemical and haematological parameters were normal prior to inclusion. All had asthma according to the criteria of the American Thoracic Society (1987a). Six subjects were receiving inhaled corticosteroid (beclomethasone or budesonide) in a median dose of 400µg/day (range 200-800µg/day). All subjects were taking inhaled β_2 -agonists on as required basis <200µg/day. One subject was receiving a sustained-release oral theophylline preparation.

Protocol

The protocol was identical to that in Chapter 11. Subjects attended the laboratory during the follicular phase (day 1-6) on two successive menstrual cycles, day 1 being the first day of the menstrual cycle. Baseline levels of serum progesterone and oestradiol were measured at each cycle. They were randomised to receive either progesterone (as medroxyprogesterone 10 mg, Upjohn Ltd) or oestrogen (as ethinyloestradiol 50 µg, Evans Medical Ltd) as single oral doses in double-blind, cross-over fashion for the two menstrual cycles. Peripheral blood was withdrawn, prior to the ingestion of the tablet, at baseline (T₀), and then at 1h (T₁), 24h (T₂₄) and 72h (T₇₂) after ingestion for serum sex hormones for each of the two cycles. Lymphocyte β_2 -AR parameters were measured at T₀, T₂₄ and T₇₂ for each of the treatments.

Medroxyprogesterone was chosen as it is the least virilising of the progesterones. The tablets are the highest dose licensed which are recommended for normal clinical practice.

Measurements

Lymphocyte β 2-adrenoceptor parameters - see Chapter 2

Serum sex-steroid hormones - see Chapter 2

Statistical analysis

In a previous study with $n=8$ females, it was possible to show a 40% increase in B_{\max} with progesterone (Chapter 11). Data for B_{\max} were log-transformed prior to analysis as it is not normally distributed. For all parameters, comparison between treatments were made by multifactorial analysis of variance (MANOVA) with subjects, treatments and visits as within factors for analysis. A probability value of $p<0.05$ (two-tailed) was considered as being of significance and Bonferroni multiple range testing was then applied to ascertain at what times differences occurred within a given cycle for each treatment. 95% confidence intervals (95% CI) for mean differences were calculated where significant.

12.3 RESULTS

Serum sex hormones

Baseline levels of oestradiol (pmol/l) and progesterone (nmol/l) were comparable for the two consecutive cycles (Table 12.1). After treatment with medroxyprogesterone, serum progesterone decreased significantly by T₁ compared to T₀: mean difference 0.77 nmol/l (95% CI: 0.03 to 1.52). Serum progesterone rose gradually to pre-treatment levels by T₇₂ which was significantly greater than T₁: mean difference 0.75 nmol/l (95% CI: 0.01 to 1.49). There were no significant differences in serum progesterone between the two treatments at the respective time points. Serum progesterone was not altered significantly after treatment with ethinyloestradiol. After treatment with ethinyloestradiol, serum oestradiol decreased significantly by T₂₄ compared to T₀: mean difference 70.91 pmol/l (95% CI: 3.24 to 138.57). Serum oestradiol levels increased by T₇₂ to baseline levels and significantly greater than T₂₄: mean difference 103.38 pmol/l (95% CI: 35.71 to 171.04). Comparing between treatments, serum oestradiol was significantly lower at T₁ after treatment with ethinyloestradiol: mean difference 49.72 pmol/l (95% CI: 22.32 to 77.12); and at T₂₄: mean difference 96.53 pmol/l (95% CI: 12.98 to 180.07). Serum oestradiol was not significantly altered after administration of medroxyprogesterone.

Lymphocyte β_2 -adrenoceptor parameters

Receptor density, B_{max}, was not significantly different at T₀ for both treatments (Figure 12.1). After administration of medroxyprogesterone, B_{max} decreased significantly ($p < 0.05$) by T₂₄ compared to T₀: amounting to a 1.34-fold geometric mean difference (95% CI: 1.01 to 1.78). By T₇₂, B_{max} had increased to baseline levels. There was no significant change in B_{max} following treatment with ethinyloestradiol. Comparing between treatments at T₂₄, B_{max} following medroxyprogesterone was significantly ($p < 0.05$) lower than after ethinyloestradiol: amounting to a 1.25-fold geometric mean difference (95% CI: 1.00-1.56). Data for

individual subjects are shown in Figure 12.2. Baseline values for maximal cyclic AMP response to isoprenaline (E_{\max}) and dissociation constant (K_d) were not significantly different before each treatment (Table 12.2). There was a trend toward a difference with E_{\max} being lower after medroxyprogesterone at T24 and increasing to baseline values by T72 ($p=0.06$). Receptor dissociation constant (K_d) was not significantly altered following either treatment.

Treatment	Time	Serum progesterone (nmol/l)	Serum oestradiol (pmol/l)
Medroxyprogesterone	T ₀	2.08 (1.71-2.45)	139.57 (103.07-176.07)
	T ₁	1.31* (0.93-1.68)	138.15 (101.65-174.65)
	T ₂₄	1.91 (1.53-2.28)	138.44 (101.94-174.94)
	T ₇₂	2.05 (1.68-2.43)	117.02 (80.52-153.52)
Ethinylestradiol	T ₀	1.79 (1.42-2.15)	112.82 (78.89-146.75)
	T ₁	1.29 (0.92-1.65)	88.43** (54.50-122.36)
	T ₂₄	1.59 (1.22-1.95)	41.91*+ (7.98-75.85)
	T ₇₂	1.66 (1.30-2.02)	145.29 (111.36-179.22)

Table 12.1 Serum progesterone and oestradiol levels at baseline (T₀), 1h (T₁), 24h (T₂₄) and 72h (T₇₂) after administration of medroxyprogesterone 10 mg and ethinylestradiol 50 µg. Values are mean (95% CI).

(* p<0.05 vs T₀; + p<0.05 medroxyprogesterone vs ethinylestradiol at T₂₄;

** p<0.05 medroxyprogesterone vs ethinylestradiol at T₁)

Treatment	Time	E_{\max} (pmol/ 10^6 cells)	K_d (pmol/l)
Medroxyprogesterone	T_0	5.20 (4.04-6.36)	13.79 (10.69-16.89)
	T_{24}	3.67 (2.51-4.83)	10.34 (7.24-13.45)
	T_{72}	5.58 (4.42-6.74)	15.44 (12.33-18.54)
Ethinylestradiol	T_0	4.29 (2.90-5.68)	17.42 (9.64-25.19)
	T_{24}	4.33 (2.94-5.74)	15.94 (8.17-23.72)
	T_{72}	4.90 (3.51-6.29)	14.72 (6.94-22.49)

Table 12.2 Maximal cyclic AMP response to isoprenaline (E_{\max}) and receptor dissociation constant (K_d) at baseline (T_0), 24h (T_{24}) and 72h (T_{72}) after treatment with medroxyprogesterone and ethinylestradiol. Values are mean (95% CI).

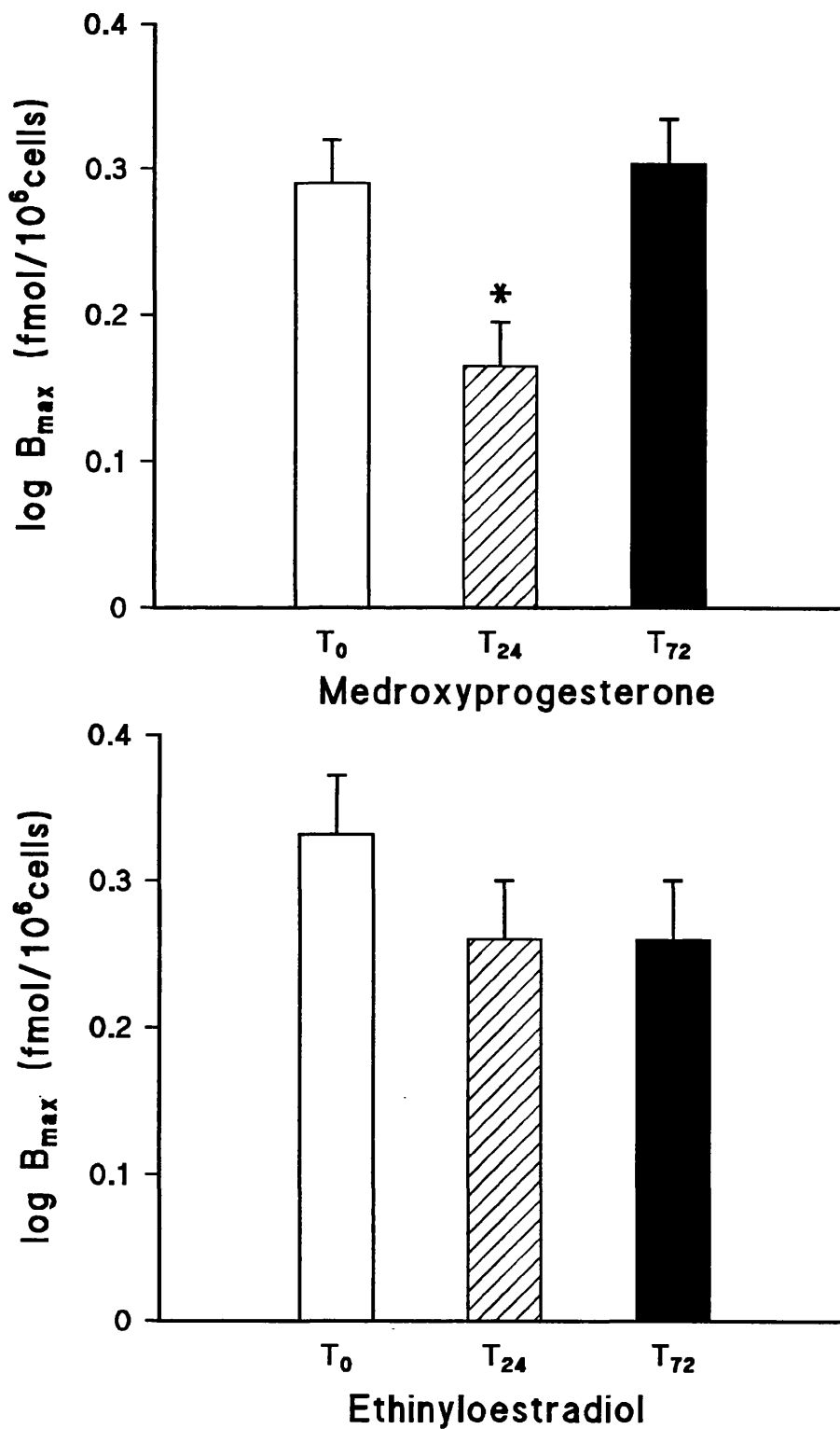
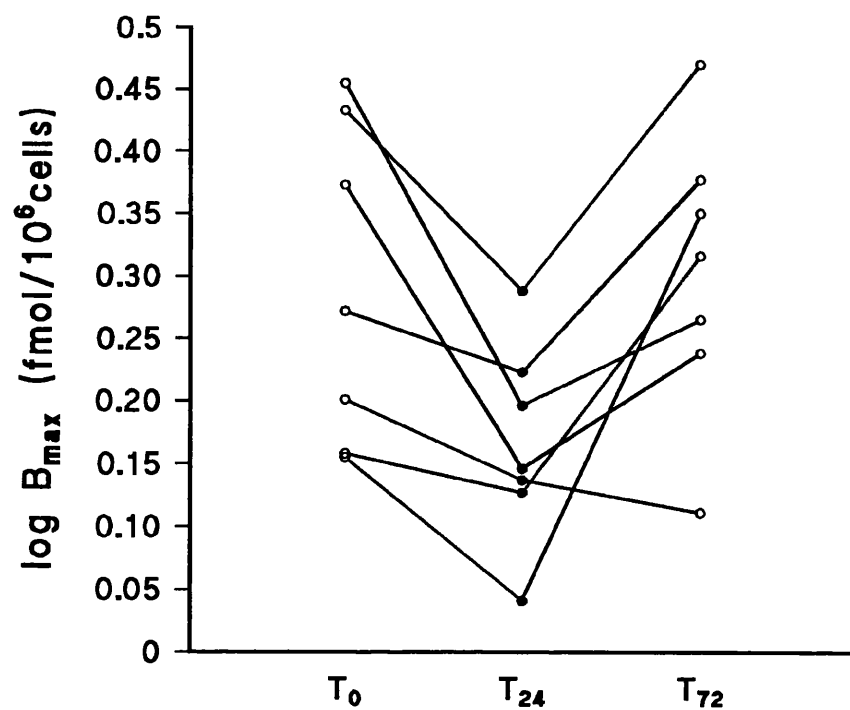
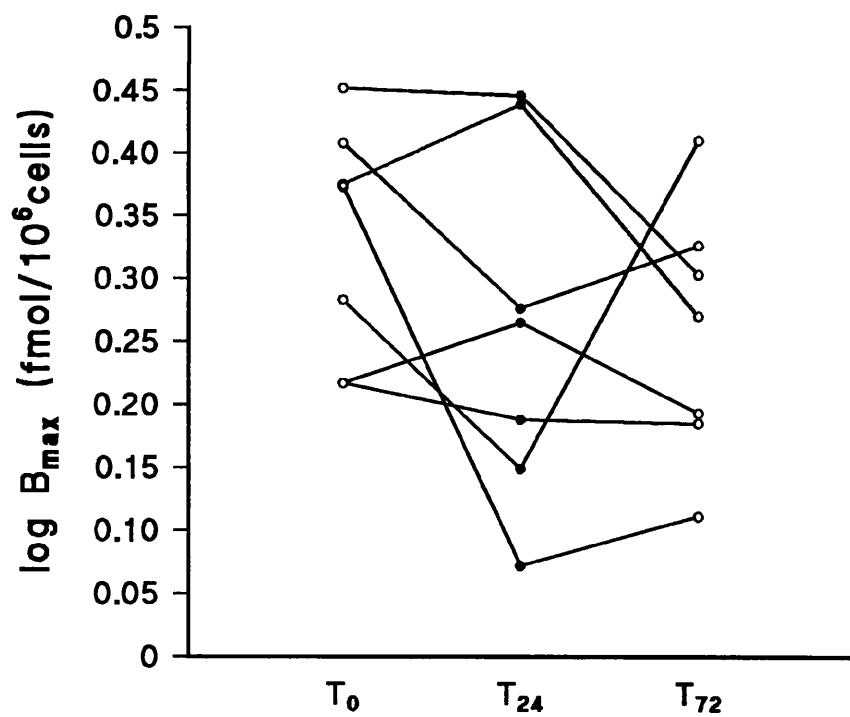


Figure 12.1 B_{max} at T₀, T₂₄ and T₇₂ after the administration of progesterone (as medroxyprogesterone, 10 mg) and oestrogen (as ethinyloestradiol, 50 µg). Values are mean log B_{max} (SEM). * denotes p<0.05 for medroxyprogesterone versus ethinyloestradiol at T₂₄.



Medroxyprogesterone



Ethinyloestradiol

Figure 12.2 B_{max} for individual subjects at T₀, T₂₄ and T₇₂ after the administration of progesterone (as medroxyprogesterone, 10 mg) and oestrogen (as ethinyloestradiol, 50 µg).

12.4 DISCUSSION

The results in this study show that the administration of exogenous progesterone but not oestrogen decreased lymphocyte β_2 -AR density and responsiveness in asthmatic females during the follicular phase. This is in contrast to our previous study in healthy females where the administration of progesterone (as medroxyprogesterone 10 mg) produced significant up-regulation of β_2 -AR density. Although we were not investigating airway β_2 -AR directly, it has been shown that changes in lymphocyte β_2 -AR density closely mirror those on lung β_2 -AR (Hayes, 1996), thus making comparisons between airway and lymphocyte β_2 -AR parameters seem valid. The change in E_{\max} followed that of B_{\max} although the trend was not statistically significant. This may be due to a relatively small difference in B_{\max} at T24 compared to T0. Whether a higher dose of medroxyprogesterone would have produced a greater fall in E_{\max} as well as B_{\max} is unclear. We also found that serum progesterone and oestradiol decreased significantly after the administration of medroxyprogesterone and ethinyloestradiol respectively. This is most likely due to the suppression of endogenous sex hormone production by the presence of synthetic sex hormones.

What are the implications of this study? Previous studies have not found any change in airway reactivity either to methacholine (Juniper, 1987; Pauli, 1989) or histamine (Weinmann, 1987) in female asthmatics during the menstrual cycle, and it has been postulated that abnormal hormonal regulation of β_2 -AR might be a possible mechanism for premenstrual asthma. This study has shown that instead of having a facilitatory effect on β_2 -AR as occurs in healthy females (Chapter 11), exogenous progesterone significantly decreased receptor density in female asthmatics. This would be in keeping with our previous observation in female asthmatics that despite an appropriate post-ovulatory hormone response, there is a loss of the normal cyclical pattern of β_2 -AR regulation (Chapter 10). It is therefore conceivable that during the premenstrual/luteal phase of the menstrual cycle when endogenous levels of

progesterone are raised, airway β_2 -AR may be attenuated and hence a loss of efficacy to endogenous catecholamines or exogenously administered β_2 -agonists, precipitating worsening asthma. However, this is not in keeping with a case report (Beynon, 1988) which found that the administration of intramuscular progesterone was beneficial in treating severe premenstrual asthma and therefore likely that other non- β_2 -AR mechanisms are implicated such as effects on smooth muscle (Fisher, 1978; Van Thiel, 1976), prostaglandins (Koullapis, 1980; Hyman, 1978) and microvascular leakage (Beynon, 1988).

In conclusion, this study has shown that the administration of exogenous progesterone but not oestrogen during the follicular phase decreased lymphocyte β_2 -AR density and responsiveness, in contrast to the up-regulating effect seen in healthy females with identical doses. This 'paradoxical' effect may represent abnormal hormonal β_2 -AR regulation in female asthmatics.

CHAPTER 13

MODULATION OF AIRWAY REACTIVITY AND PEAK FLOW VARIABILITY IN ASTHMATICS RECEIVING THE ORAL CONTRACEPTIVE PILL

**(American Journal of Respiratory and Critical Care
Medicine 1997; 155: 1273-1277)**

13.1 INTRODUCTION

Female sex-steroid hormones may play an important role in asthma. Previous studies have not demonstrated any change in airway reactivity during the menstrual cycle using methacholine (Pauli, 1987; Juniper, 1989) or histamine (Weinmann, 1987) challenges, which are direct bronchoconstrictor agents. In Chapter 10, we have shown that in female asthma patients, there was a significant increase in airway reactivity to AMP during the luteal phase of the menstrual cycle compared to the follicular. This increase in airway reactivity during the luteal phase was associated with the normal increase in serum sex-steroid hormones.

With such compelling evidence for hormonal influence on airways, we have therefore chosen to evaluate a group of female asthmatics with natural menstrual cycles and a group of female asthmatics on the oral combined contraceptive pill. Again in this respect, we have chosen to investigate airway reactivity using AMP, an indirect bronchoconstrictor which acts by stimulating the release of mediators from mast cells (Cushley, 1985).

13.2 METHODS

Patients

18 female subjects with stable asthma were evaluated in this study. In group 1, there were 9 subjects, mean (SEM) age 24 (6) years, FEV₁ in litres and percentage predicted was 2.93 (0.46) L and 93 (10) percent predicted, with natural menstrual cycles. In group 2, there were 9 subjects, age and severity matched, who were taking the oral combined contraceptive pill, age 24 (6) years with FEV₁ 2.98 (0.38) L and 93 (9) percent predicted. A full physical examination, biochemical and haematological parameters were normal prior to inclusion. All gave written informed consent before being evaluated in this study which was approved by the Tayside Medical Ethics Committee. All had asthma according to the criteria of the American Thoracic Society (1987a), and all were non-smokers. In the group with natural cycles, 7 subjects were receiving inhaled corticosteroid (either beclomethasone or budesonide), median dose 600 µg/day (range 200-2000 µg/day). One subject was taking inhaled salmeterol 50 µg bid and one was taking sustained-release theophylline. In the group on the OCP, 6 subjects were receiving inhaled corticosteroid, median dose 400 µg/day (range 200-1000 µg/day). One subject was also taking inhaled salmeterol 50 µg bid and one was taking sustained-release theophylline. All had been inhaling short-acting β_2 -agonists as required in doses of <400 µg/day. All medications were at stable dose for at least two months prior to the study and was taken at constant dosage throughout the study. None had received oral corticosteroids for at least three months, and none had had a recent exacerbation of asthma in the past month. All had stable asthma with no subjective history of premenstrual asthma.

In the group on the OCP, 6 subjects were taking monophasic and 3 subjects were taking triphasic preparations. Of the monophasic preparations, 5 were on ethinyloestradiol 30 µg/day and one was on 35 µg/day of ethinyloestradiol. There were different preparations and doses of progestogens.

Protocol

All subjects were evaluated at two visits in the laboratory. Group 1 subjects were evaluated during the follicular (visit 1) and luteal (visit 2) phases of the menstrual cycle. The follicular phase was defined as day 1-4 and luteal phase as day 21-24, with day 1 being the first day of the menses. Group 2 subjects were evaluated during the week while off the OCP (visit 1, during the week of menstruation) and at the end of the 21-day course of OCP (visit 2). At each visit, subjects attended the laboratory at 0900 AM, having withheld short-acting β_2 -agonists for 8 hours, long acting β_2 -agonists for 48 hours and theophylline preparations for 48 hours. After a 15 minute period of rest, 10ml of blood was withdrawn from a peripheral vein for serum oestradiol and progesterone. Then, airway reactivity to adenosine 5'-monophosphate (AMP; Sigma, Poole, UK) was determined. This was expressed as PC₂₀, the provocative concentration that causes a 20% fall in FEV₁.

During the study, subjects were asked to keep a diary of morning and evening peak expiratory flow readings using a Wright's peak flow meter (Airmed, London, UK) by recording the best of three consecutive readings. They were also asked to give a daily symptom score for wheeze (0-3) and for nocturnal symptoms (0-3), and the number of rescue puffs of β_2 -agonists needed.

Measurements

Female sex-steroid hormones - see Chapter 2, Hormone Assays

Airway reactivity to AMP - see Chapter 2, Adenosine monophosphate challenge.

Statistical analysis

PC₂₀ was log-transformed for analysis, as it is not normally distributed. For all variables, comparisons within groups for both visits were made by analysis of variance

(ANOVA) and where appropriate followed by Bonferroni multiple range testing. Comparisons between groups was by unpaired Student's t-test. A probability value of $p < 0.05$ (two-tailed) was considered as being of significance and 95% confidence intervals (95% CI) for mean differences were calculated where significant. The rescue requirements and symptom score were analysed non-parametrically using Kruskal-Wallis analysis of ranks.

13.3 RESULTS

Baseline airway calibre

Baseline FEV₁ for both groups did not differ between the two visits. For group 1, (visit 1 vs 2) FEV₁ was 2.74 vs 2.73 (p=0.90), and for group 2, FEV₁ was 2.85 vs 2.87 (p=0.77). Likewise, there was no significant difference between the groups at visit 1: 2.74 vs 2.85 (p=0.67) and at visit 2: 2.73 vs 2.87 (p=0.53).

Female sex-steroid hormones

In group 1, who had natural cycles, there was a significant increase in sex hormones (Figure 13.1), at visit 2; for progesterone (mmol/l), visit 1 vs 2: 2.5 vs 13.5 (95% CI 2.1 to 19.9; p<0.02) and for oestradiol (pmol/l): 152.3 vs 358.1 (95% CI 113.0 to 298.5; p<0.001). However, in group 2, who were on the OCP, there was no increase between visit 1 and visit 2 in female hormones; for progesterone (visit 1 vs 2), 0.9 vs 1.0 and for oestradiol 75.7 vs 21.8.

Airway reactivity to AMP

In group 1, there was a significant increase in airway reactivity during the luteal phase (visit 2) of the menstrual cycle (Figure 13.2). The geometric mean PC₂₀ was 18.8mg/ml at visit 1 and 4.7mg/ml at visit 2. This was a 4.0 fold difference (95% CI 1.25 to 13.03; p<0.03) which amounted to 2 doubling doses. One subject had shown a dramatic increase in airway reactivity from the follicular phase (log PC₂₀ 1.398) to the luteal phase (log PC₂₀ -0.903). When this 'outlier' is removed (ie n=8), and analysis performed with the 'trimmed' geometric mean, there is still a significant difference between the follicular (visit 1) and luteal (visit 2) phases of the cycle: 18.2mg/ml at visit 1 and 7.4mg/ml at visit 2. This is a 2.5 fold difference (95% CI 1.68 to 3.64; p<0.001) amounting to 1.3 doubling doses. In contrast, there was no change in airway reactivity in group 2 between the two visits: 23.5mg/ml at visit 1 and 21.4mg/ml at visit 2; a 1.06 fold difference (95% CI 0.41 to 2.78; p=0.83). There was

no difference in geometric mean PC₂₀ between the two groups at visit 1: 18.8 vs 23.5; a 1.25 fold difference (95% CI 0.3 to 5.1; $p=0.74$). There was a trend toward a difference between the two groups at visit 2: 4.7 vs 21.4, a 4.57 fold difference (95% CI 0.93 to 22.4; $p=0.06$).

Peak expiratory flow readings

In group 1, with natural cycles, there was a significant difference between morning and evening PEFr (L/min): at visit 1 (morning PEFr vs evening PEFr), 403 vs 430 (95% CI 5 to 50; $p<0.001$) and for visit 2, 415 vs 439 (95% CI 1 to 46; $p<0.001$) (Figure 13.3). In group 2, on the OCP, there was no significant difference in morning and evening PEFr at both visits; at visit 1, 411 vs 417 and at visit 2, 413 vs 427.

In both groups, there were no significant differences in symptom score and rescue requirements between visit 1 and 2.

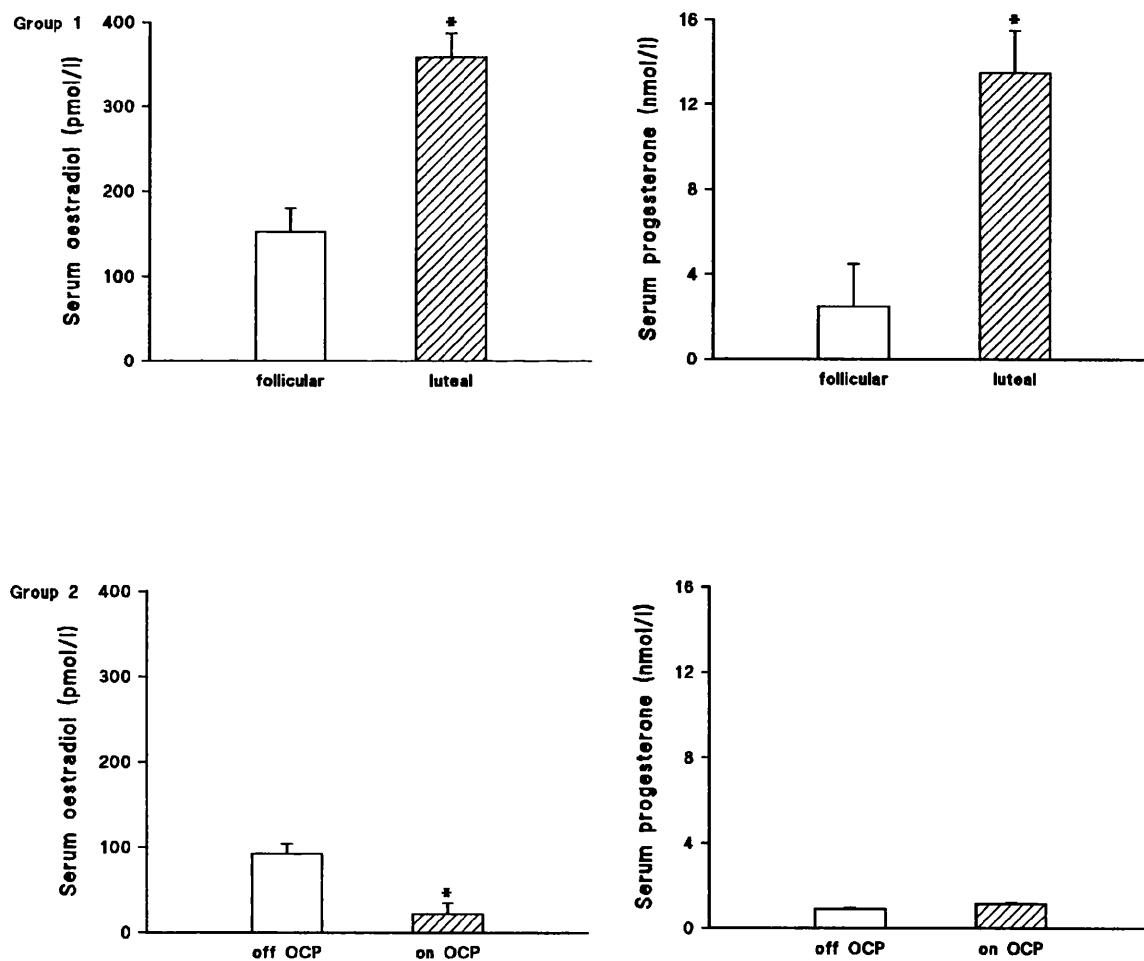


Figure 13.1 Female sex-steroid hormone levels at both visits in group 1 (with natural cycles) and group 2 (on the OCP). * indicates $p < 0.05$ for visit 1 versus 2.

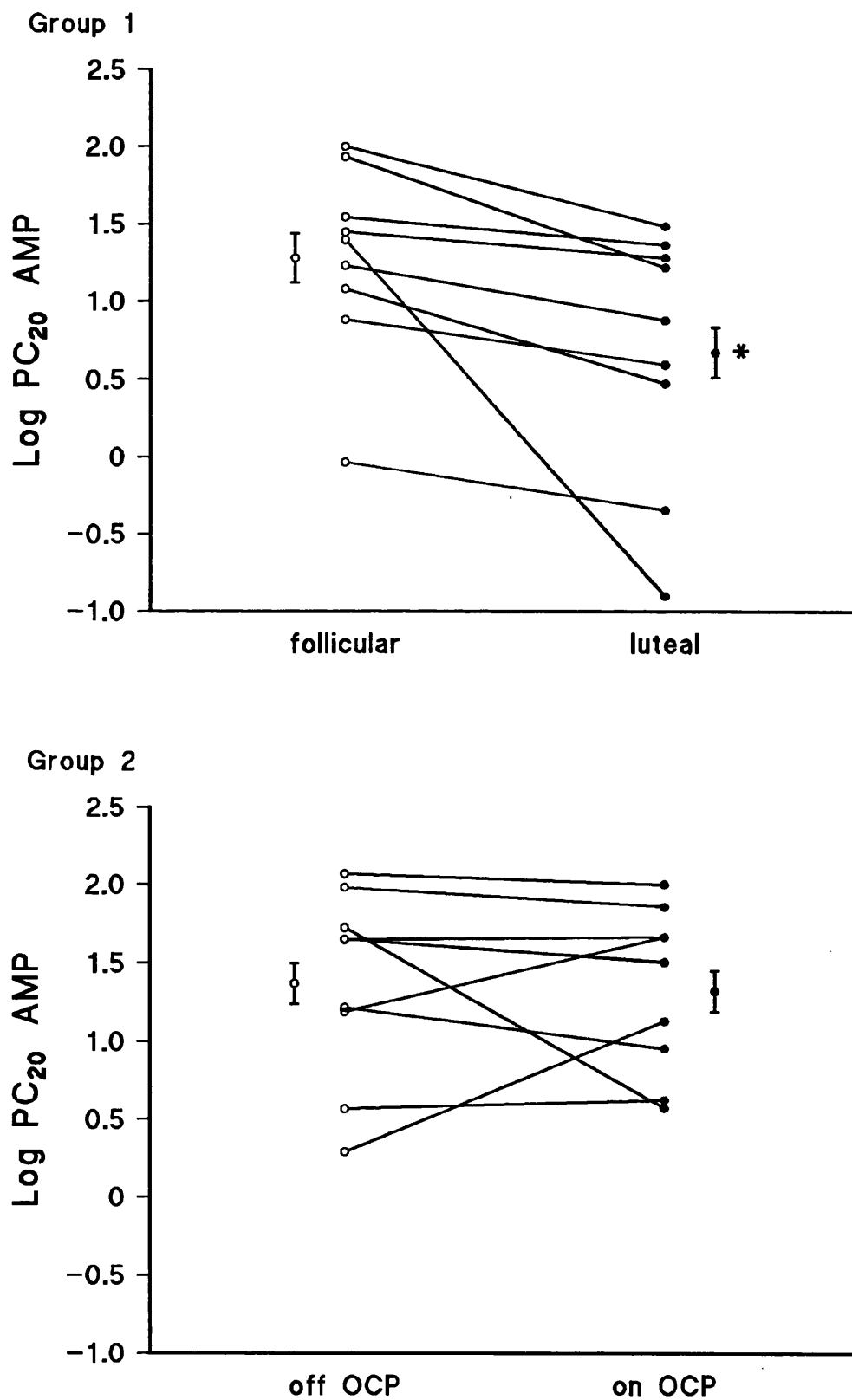


Figure 13.2 Airway reactivity to AMP during the menstrual cycle in group 1 (with natural cycles) and group 2 (on the OCP). * indicates $p < 0.05$ for visit 1 versus 2.

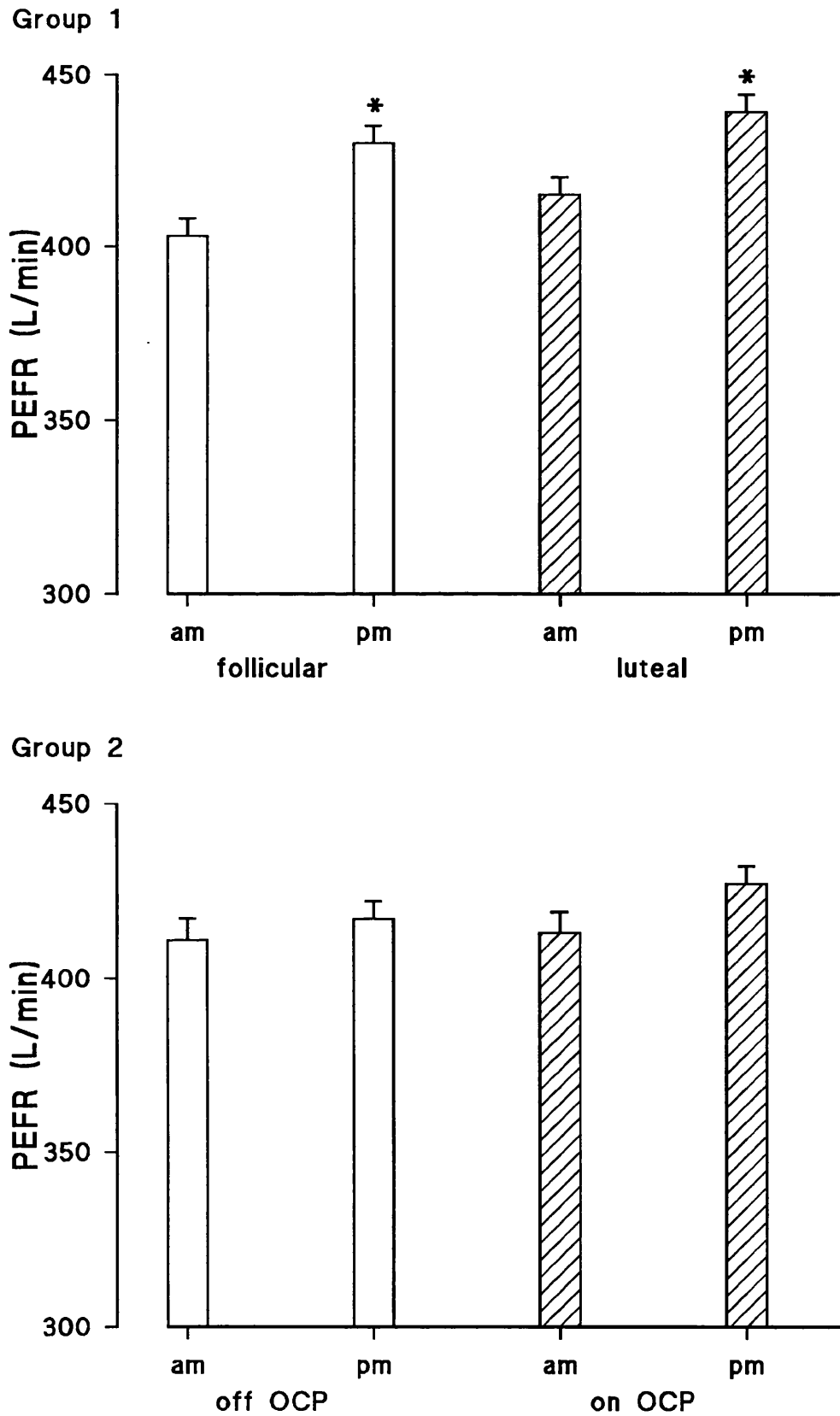


Figure 13.3 Morning and evening domiciliary PEFR during the menstrual cycle in group 1 (with natural cycles) and group 2 (on the OCP). * indicates $p < 0.05$ for morning PEFR versus evening PEFR.

13.4 DISCUSSION

This study has demonstrated that airway reactivity to AMP shows cyclical change in female asthmatics during the menstrual cycle, with greater reactivity at the luteal phase, as shown previously in Chapter 10. This was accompanied by a significant and appropriate increase in female sex-steroid hormones at this phase of the menstrual cycle. In contrast, endogenous female sex-steroid hormones were suppressed and showed no increase during visit 2 in the group of asthmatics who were taking the oral contraceptive, as would be expected. Airway reactivity in this latter group did not show any variation during the cycle, in contrast to those with natural cycles. It is also interesting to note that this cyclical change in airway reactivity occurred in the group of asthmatics with natural cycles even though they were on a higher median dose of inhaled corticosteroid. Our patients had mild disease and so it may not be possible to extrapolate the present results to more severe asthmatics and particularly those with symptomatic premenstrual deterioration. It is interesting to note that in a recent study by Skobeloff *et al* (1996), almost half of all female admissions for acute asthma occurred during the perimenstrual phase.

Previous studies using histamine or methacholine, which act directly on airway smooth muscle have not shown any change in airway reactivity during the menstrual cycle. In the studies by Pauli *et al* (1989) ($n=11$) and Weinmann *et al* (1987) ($n=9$), the subjects studied had natural cycles and none were receiving the oral contraceptive. Juniper *et al* (1987) studied ten subjects with natural cycles and seven subjects receiving the oral contraceptive. The observation that 'direct' bronchoconstrictors like histamine and methacholine show no cyclical change together with the results in this present study would suggest that the mechanism of hormonal influence on airway reactivity is on mediator release. Since AMP mediates bronchoconstriction via mediator release from mast cells (Cushley, 1985), it is possible that the normal post-ovulatory rise in sex-hormones may sensitise mast cells, and so, lower the threshold

for mediator release. This in turn suggests that in the luteal phase during the premenstrual period, deterioration in asthma control may be due to augmented inflammatory mediator release. Thus, suppression of endogenous hormone levels by the contraceptive pill may offer an explanation for the attenuation of airway reactivity seen in this group of subjects.

When evaluating changes in airway reactivity it is important to consider the intrinsic variability of this measurement. This is such that PC₂₀ values can vary by up to two fold ie \pm one doubling dose - even in stable asthmatics (Juniper, 1978). In our study, we have shown that, in those with natural cycles, airway reactivity to AMP increased by four fold during the luteal phase, equivalent to 2 doubling doses of AMP. Thus, as well as being statistically significant, this increase is also likely to have clinical significance as it exceeds the limit of biological variability. It is also important to note that baseline airway calibre as assessed by FEV₁ was not significantly different between the two visits for the two groups. This is relevant to the evaluation of PC₂₀ as airway geometry has an important influence on assessing airway responsiveness to a bronchoconstrictor agent (Chung, 1982).

The group receiving the contraceptive pill were also found to have attenuated diurnal PEF variability compared with those with natural cycles. Clearly, it is important to know that the two groups were similarly matched for age and disease severity. In this respect, there were no significant differences in FEV₁ and PC₂₀ at visit 1. This makes it likely that the difference in PC₂₀ and PEF variability between the two groups was due to the effect of the contraceptive pill rather than any other confounding variables due to their asthma. Ideally, we would like to have evaluated the group of asthmatics while on and off the oral contraceptive. However, this was not possible as the subjects concerned were reluctant to consent to this because of the increased risk of pregnancy while off the OCP. Likewise the Tayside Committee for Medical Research Ethics felt that this was ethically unacceptable.

Cyclical menstrual changes are not confined to the airways but are also evident in skin-prick test reactions to histamine and allergen, with greater weal-and-flame reactions during the early luteal phase (Kalogeromitros, 1995). There are other possible mechanisms by which sex-steroids may influence the airways. In rabbit lung, oestrogen and progesterone have been shown to modify β -adrenergic receptor density with oestrogen increasing and progesterone decreasing the number of sites (Moawad, 1982). Prostaglandins are known to be modulators of airway tone (Hyman, 1978) and monthly cyclical variations in circulating $\text{PGF}_{2\alpha}$ metabolite have been reported (Koullapis, 1980). However, treatment with meclofenamate, a potent prostaglandin synthase inhibitor, did not prevent exacerbations of premenstrual asthma (Eliasson, 1987).

What then is the clinical relevance of our findings? It is possible that exogenous female sex-steroid hormones may be used therapeutically in females with unstable asthma or those with premenstrual asthma, not controlled by conventional therapy, by smoothing out cyclical changes in airway reactivity. However, the effects of exogenous female sex-steroid hormones are not entirely clear. In a recent questionnaire-based prospective cohort study (Troisi, 1995), there was an increased risk of developing adult-onset asthma in those women who had used hormone-replacement therapy or oral contraceptives, and this risk was related to duration of use and dose of oestrogen. Nevertheless, the modulatory effect of exogenous female sex-steroids may be beneficial rather than deleterious in those with established asthma, by attenuating the cyclical changes in airway responsiveness. Clearly, further studies are needed to identify which hormone is responsible for these airway changes, perhaps by administering the hormones individually by aerosol to the lung and assessing changes in airway responsiveness.

CHAPTER 14

CONCLUSIONS

CONCLUSIONS

This series of studies has examined the ways in which corticosteroids and β -agonists interact on the β_2 -AR and the role which abnormal hormone regulation of β -AR plays in females with premenstrual asthma. The results, which we believe are novel and exciting, add to the existing body of knowledge on these important subjects.

The initial study, (Chapter 3), showed that asthmatic patients developed bronchodilator subsensitivity after regular use of the long-acting β -agonist, formoterol. A bolus of systemic corticosteroid rapidly (within 1h) restored bronchodilator responsiveness. Two recent studies have investigated the effect of a bolus of inhaled corticosteroid on the reversal of loss of functional antagonism of β -agonists. Although the actual mechanism underlying the loss of bronchoprotection *in vivo* is unknown, it probably relates to β_2 -AR down-regulation (Svedmyr, 1990). Yates *et al* (1998) treated 15 steroid-naïve asthmatic patients with terbutaline 500 μ g four times daily for 12 days in a double-blind crossover study. Bronchoprotection of terbutaline was evaluated with methacholine and AMP challenges after a seven day run-in, at days 7 and 8 of dosing and on days 11 and 12. On day 10, they received either inhaled budesonide 800 μ g or placebo 12h before challenge. The bronchial challenges were performed on separate days to avoid possible interaction. After regular terbutaline treatment, there was significant loss of bronchoprotection to AMP challenge (1.7 doubling dilutions), while there was no such tolerance to methacholine (0.1 doubling dilutions). Although this was in keeping with the hypothesis that mast cell β_2 -AR are more susceptible to tolerance than smooth muscle β_2 -AR, it is surprising that tolerance was not demonstrated against methacholine challenge after terbutaline (Chapter 1). Inhaled

budesonide did not have any significant effect on methacholine or AMP challenge compared with placebo. Aziz *et al* (1997, abstract) studied nine asthmatic subjects on inhaled steroid treatment in double-blind crossover fashion. Patients received inhaled formoterol 24 µg twice daily for two separate one week treatment periods with one week washout between periods. A single dose of inhaled budesonide 1600µg or placebo was administered at the end of each treatment period at the same time with the last dose of formoterol having withheld usual inhaled corticosteroid treatment for 24h. AMP challenge was performed 2h after the first dose of formoterol and after one week of dosing. Following formoterol treatment, there was significant loss of bronchoprotection against AMP, amounting to 5.55 fold difference between the first and last dose of formoterol. However, after budesonide inhalation, there was significant reversal of the loss of bronchoprotection which amounted to a 5.66 fold difference compared with placebo, such that there was no difference from baseline. There are several possible explanations for the different results between the two studies. Firstly, the choice of β -agonist might be important. Formoterol, being a long-acting and full agonist at the β_2 -AR, is a more potent inducer of β_2 -AR tolerance and down-regulation. Corticosteroids are more likely to demonstrate an effect on β_2 -AR function in desensitised tissue. Chapter 3 describes how *in vivo* β_2 -AR responses (except for heart rate) were not augmented by a bolus of systemic corticosteroid after placebo treatment, compared to the effect seen after dosing of regular formoterol. In other words, the 'up-regulating' effects of corticosteroid are heightened as tissue desensitisation increases. Secondly, there may be a dose-response effect of inhaled corticosteroids in that the higher dose of budesonide (1600µg) used by Aziz *et al* may have had a greater facilitatory effect on β_2 -AR. Finally, duration of action may be significant. Aziz *et al* carried out AMP challenge 2h after inhaled budesonide, while

Yates *et al* performed the first challenge 12h after budesonide dosing, with the second challenge up to 36h afterwards. Corticosteroid effect may be more apparent at the peak, rather than at the end of the dose. We similarly demonstrated in Chapter 3 that systemic corticosteroids had an effect on β_2 -AR function within 1h of dosing. The studies by Yates and Aziz question whether high-dose inhaled corticosteroids could be effective in acute asthma, given that repeated use of β -agonists could lead to β_2 -AR tolerance in this situation. The avoidance of oral steroids, where possible is attractive to both doctors and patients. In a general practice study of mild asthma exacerbations, Levy *et al* (1996) examined 413 patients randomised to receive two weeks of either inhaled fluticasone propionate 1mg twice daily or a reducing course of prednisolone (starting at 40mg daily). No significant difference was found between the two treatments, with the primary outcome measure being 'treatment failure' (peak flow < 60% of best/predicted, no improvement in symptom score or withdrawal from study). However, with a power of only 57% in detecting a difference between treatments, there was increased likelihood of a type 2 error in missing an actual difference. Despite this, these findings should prompt further work on investigating high-dose inhaled corticosteroids in more severe asthma exacerbations and in children and adolescents who may be more susceptible to the effects of systemic steroids. We have shown that systemic corticosteroids could reverse formoterol-induced bronchodilator subsensitivity (Chapter 3). It is not yet known whether this facilitatory effect can be demonstrated with high dose inhaled corticosteroid.

In Chapters 5 and 6, we demonstrated the loss of bronchoprotection against methacholine, a direct-acting stimulus, and against AMP, an indirect-acting stimulus with regular treatment with short and long-acting β -agonists. These findings in themselves are not novel. What is interesting is that in Chapter 5, the residual level of

protection against methacholine was the same, irrespective of differing formoterol dose and dosing regimen, and when compared with a short-acting β -agonist, terbutaline. In Chapter 6, once daily and twice daily formoterol produced a similar degree of tolerance to the bronchoprotection against AMP. This could be explained by the prolonged receptor occupancy formoterol and bronchoprotective effect (up to 24h) which formoterol has been shown to have against methacholine (Rabe, 1993). Tolerance to bronchoprotective effects of β -agonists can be reversed by a single dose of inhaled corticosteroids (Aziz, 1997, abstract). The same group showed that this bronchoprotective subsensitivity cannot be overcome by a high dose of β -agonist (Lipworth, 1998, abstract). This involved treatment of ten asthmatic patients in cross-over fashion with placebo, inhaled salmeterol 50 μ g twice daily and formoterol 12 μ g twice daily for nine days each, with a one week washout between treatment periods. Methacholine challenge was performed 1h after the first dose and after seven days. After nine days' treatment, a third methacholine challenge was performed 1h after inhalation of a single 1600 μ g dose of salbutamol. After regular treatment with salmeterol and formoterol, there was loss of bronchoprotection against methacholine of 0.9 doubling doses and 0.5 doubling doses respectively. After nine days the bronchoprotective effect of salbutamol after placebo was 2.7 fold greater than after salmeterol treatment and 3.6 fold greater than after formoterol treatment. This inability of β -agonists to overcome prior subsensitisation to long-acting β -agonists has important implications. In acute asthma, patients may conceivably be desensitised due to repeated doses of β -agonists, or the use of a regular long-acting β -agonist. Following an encounter with a bronchoconstricting stimulus such as allergen or exercise, further administration of β -agonists may therefore be of limited protection. Asthmatic subjects in both these studies were receiving inhaled corticosteroid, again highlighting

the fact that these drugs do not protect against the development of tolerance. This is further confirmed by their lack of facilitating effect on β_2 -AR parameters on lymphocytes in asthmatic patients (Chapters 7 and 8). Single and repeated doses of high-dose fluticasone propionate (up to 2mg) failed to have any effect on β_2 -AR density and cAMP responsiveness. This is in contrast with a single dose of oral prednisolone (50mg) which up-regulated β_2 -AR density and increased cAMP responsiveness to isoprenaline. However, high-dose fluticasone propionate may have demonstrated facilitatory action if β_2 -AR were initially down-regulated. To conclude the discussion on corticosteroids, low dose prednisolone was shown to protect against the development of *in vivo* β_2 -AR desensitisation to formoterol (Chapter 9). Prednisolone 15 mg a day attenuated the fall of *in vitro* cAMP response to isoprenaline, while receptor number down-regulation was not prevented, in keeping with data from Davies and Lefkowitz (1984). Further research on this subject would include a dose-ranging study of oral prednisolone on the up-regulating effect on lymphocyte β_2 -AR. It would also be of interest to discover whether high-dose inhaled corticosteroid protects against *in vivo* β_2 -AR desensitisation to β -agonists and whether high dose corticosteroids are able to reverse bronchodilator subsensitivity induced by long-acting β -agonists. Recent preliminary data from Rahman *et al* (1997), on the contrary, suggests that inhaled corticosteroid might exhibit differential protection against β_2 -AR tachyphylaxis in normal and asthmatic subjects. They studied eight subjects (four asthmatic) who were treated with inhaled salbutamol and inhaled fluticasone 1500 μ g twice daily for two weeks. There was no significant tachyphylaxis in airway and systemic β_2 -AR responses, whilst pulmonary β_2 -AR density as assessed by positron emission tomography (PET) was unchanged in the asthmatic subjects. The normal

subjects on the other hand, developed significant airway and systemic β_2 -AR tachyphylaxis together with a 23% decrease in pulmonary β_2 -AR.

In a number of the studies, we have used lymphocyte β_2 -AR parameters as a surrogate for pulmonary β_2 -AR. Lung material for β_2 -AR can rarely be obtained while peripheral leukocytes are easily available and exclusively yield the β_2 -AR subtype (Brodde, 1981; Aarons, 1979; Davis, 1980). Therefore, peripheral leukocyte β_2 -AR have been claimed to be a reliable model for examining properties of β_2 -AR in human lung of healthy and asthmatic subjects. In several studies (Newnham, 1994a; Newnham, 1995; Grove, 1995), lymphocyte β_2 -AR down-regulation has occurred to a similar degree as bronchodilator subsensitivity, making it a useful surrogate for airway β_2 -AR. However, we recognise there is controversy as to whether it is valid to make this extrapolation. Tashkin *et al* (1982) demonstrated lymphocyte β_2 -AR down-regulation in asthmatic patients following treatment with oral terbutaline while bronchodilator responses to inhaled isoprenaline and subcutaneous terbutaline were maintained. Hauck *et al* (1990) examined the relationship between lymphocyte and pulmonary β_2 -AR directly. They evaluated 18 patients undergoing lung resection for bronchial carcinoma. Ten received subcutaneous terbutaline 0.5 mg twice daily for 24-72h preoperatively while eight received no treatment. Leukocyte β_2 -AR density was found to be reduced by 43% whilst there was no significant change in lung β_2 -AR density. While this may represent a constitutional difference in susceptibility of the two receptor populations to down-regulate, it is also possible that inadequate tissue penetration was achieved with the dose and the route of administration used, or that the pulmonary β_2 -AR were already down-regulated as a result of prior use of β_2 -agonists (five of the treated group were already on regular β_2 -agonists). In a recent

study, seven healthy subjects were treated with oral (up to 8 mg twice daily) and inhaled (up to 400 μ g four times daily) salbutamol for two weeks. They found that the down-regulation in mononuclear leukocyte β_2 -AR density correlated closely with that of *in vivo* pulmonary β_2 -AR changes as assessed by PET (Hayes, 1996). The authors conclude that it may be possible to predict changes in pulmonary β_2 -AR by measuring changes in peripheral blood mononuclear leukocyte β_2 -AR. Thus, the validity of this technique as an index of pulmonary β_2 -AR function remains unclear.

We have performed some preliminary work on β_2 -AR polymorphism in asthma. As detailed in Chapter 4, homozygous Gly 16 asthmatic patients were more susceptible to bronchodilator desensitisation after regular formoterol than homozygous Arg 16 patients. This is in keeping with *in vitro* studies with cultured human airway smooth muscle cells which have shown that the Gly 16 form of the β_2 -AR predisposes to agonist-induced down-regulation and desensitisation to a greater degree than the Arg 16 form (Green, 1995a). Recent preliminary data support the role of β_2 -AR polymorphism in the development of tolerance (Rahman, 1996). 12 male subjects (six with mild asthma not requiring treatment) were treated with inhaled salbutamol up to 1.2 mg four times daily for two weeks. Eight subjects (homozygous Arg 16, Gly 16 and heterozygous Arg 16/Gly 16) developed significant bronchodilator and systemic β_2 -AR tachyphylaxis associated with pulmonary β_2 -AR down-regulation on PET. The four subjects who were homozygous Glu 27 were resistant to β_2 -AR desensitisation. Our study was a retrospective analysis of three studies (Chapter 3; Newnham, 1994a; Newnham, 1995) and it would be important to confirm this finding by performing a prospective study. Subjects evaluated in Chapter 3 were genotyped for β_2 -AR

polymorphism at codon 16 and 27. It would appear that reversal of desensitisation to systemic steroids is not related to β_2 -AR polymorphism, although the small number did not allow statistical analysis. *In vitro* data suggest that β_2 -AR polymorphisms affect agonist-induced down-regulation by altering susceptibility to protein degradation of the receptor (Green, 1995b). As receptor synthesis is not influenced by β_2 -AR polymorphism, we may therefore not expect that corticosteroid reversal of β_2 -AR down-regulation is also unaffected. β_2 -AR polymorphism does not appear to influence development of tolerance to the bronchoprotective effect of formoterol against AMP challenge (Chapter 6). Here, again, numbers were too small for formal analysis. Tolerance to the bronchoprotective effect of β -agonists is readily demonstrable, in contrast to bronchodilator tolerance and thus β_2 -AR polymorphism is unlikely to be an important influence. However, this can only be confirmed by repeating the study using adequate patient numbers with known β_2 -AR polymorphisms. Future researchers will be required to know the genotypes of asthmatic patients, before examining both β_2 -AR function and the role of β -agonists.

The development of tolerance to the beneficial effects of inhaled β -agonists has been suggested as one possible explanation for the deleterious effects of frequent use of these agents in asthma. This has come about with the association of increased β -agonist use and asthma mortality and morbidity (Pearce, 1990; Grainger, 1991; Spitzer, 1992). With *in vitro* and now *in vivo* data showing that homozygous Gly 16 β_2 -AR polymorphism is predisposed to β_2 -AR down-regulation and tolerance, one would expect that this particular polymorphism would expose the affected individual to increased risk of asthma-related death. Contrary to this hypothesis, recent abstracted data (Weir, 1997) showed a reduced prevalence of the Gly 16 allele in patients with

fatal or near fatal asthma compared with those with mild asthma. The Glu 27 allele showed equal prevalence. Although there may be several explanations for these findings they illustrate that a single gene polymorphism is unlikely to account for the complex process of asthma exacerbation. The regular use of a short-acting β -agonist, fenoterol, in asthma patients, of whom the majority were taking an inhaled corticosteroid, has been associated with increased exacerbations, decline in baseline lung function and an increase in airway hyperresponsiveness to methacholine (Sears, 1990; Taylor, 1993). However, three studies with long-acting β -agonists produced different results (Greening, 1994; Woolcock, 1996; Pauwels, 1997). Greening *et al* and Woolcock *et al*, in large multicentre studies, found that the addition of salmeterol to inhaled corticosteroid was more effective than using a higher dose of corticosteroid in those with persistent asthma symptoms, resulting in improved peak expiratory flow rates, symptom control and the use of rescue bronchodilator over a period of six months. There was no effect on exacerbation rates as these studies were not powered to detect this. More recently, Pauwels *et al* studied 852 patients with asthma in a parallel group design. They were assigned to one of four treatments for one year: budesonide 100 μ g twice daily plus placebo, budesonide 100 μ g twice daily plus formoterol 12 μ g twice daily, budesonide 400 μ g twice daily plus placebo or budesonide 400 μ g twice daily plus formoterol 12 μ g twice daily. Exacerbation rates were significantly reduced when formoterol was added to budesonide. The higher dose of budesonide alone was found to significantly reduce exacerbation rates. Likewise, peak expiratory flow rates and asthma symptoms were improved with the addition of formoterol. We should be cautious, however, in extrapolating results from stable, mild asthmatic patients to those with severe asthma, in whom bronchodilator

and antibronchoconstrictor tolerance may be significant, particularly in the face of worsening disease.

Our studies with female sex steroid hormones indicate that there may be abnormal hormonal regulation of β_2 -AR in female asthma patients. In healthy women, lymphocyte β_2 -AR are normally influenced by ovarian sex steroid hormones, in that the post-ovulatory rise in progesterone and oestradiol is accompanied by an increase in receptor density and cAMP responsiveness to isoprenaline (Wheeldon, 1994). In Chapter 11, healthy women with natural menstrual cycles were given single doses of ethinyloestradiol or medroxyprogesterone during the pre-ovulatory follicular phase when endogenous levels of sex hormones are low, to mimic luteal phase events when the hormone levels are elevated. Exogenous progesterone, but not oestradiol, resulted in a significant increase in lymphocyte β_2 -AR density, suggesting that the normal post-ovulatory response is mediated by a rise in progesterone levels. However, as described in Chapter 12, the same study design produced paradoxical results in mild stable female asthmatic patients, when medroxyprogesterone produced a decrease in lymphocyte β_2 -AR numbers and cAMP responsiveness to isoprenaline. This suggests an abnormal regulation of β_2 -AR in female asthmatics, and might be a possible mechanism for premenstrual asthma, as progesterone levels are high during the luteal phase. In Chapter 10, this hypothesis is supported by the finding that β_2 -AR regulation shows a loss of the normal cyclical pattern in stable asthmatic women with natural cycles. This occurs despite an appropriate post-ovulatory rise in sex hormones during the luteal phase. In addition, there are cyclical changes in airway hyperresponsiveness to AMP, the latter being highest during the premenstrual period (Chapters 10 and 13). We had postulated that this may be due to regulation of

adenosine receptors on airway mast cells by circulating sex hormones. However, there was no correlation between hormone levels and changes in PC₂₀ AMP. Alterations in asthmatic inflammation within female airways during the menstrual cycle could account for this. AMP challenge may be a more discriminatory method in detecting this airway inflammation than the direct smooth muscle stimuli methacholine or histamine, as adenosine acts indirectly on both airway mast cells and airway sensory nerves (Polosa, 1991). Our findings, which may be the result of increased inflammation could be confirmed by bronchoalveolar lavage and biopsies during different phases of the menstrual cycle in female asthmatic patients. However, there are now several non-invasive ways to evaluate airway inflammation in asthma that may be applicable here. Exhaled nitric oxide is known to be elevated in asthma (Kharitonov, 1994b), and is thought to be a marker of airway inflammation, as the abnormally elevated levels in symptomatic asthma are reduced by inhaled corticosteroid treatment (Kharitonov, 1996). A recent study showed that exhaled nitric oxide is elevated during the luteal phase of the menstrual cycle in healthy women (Kharitonov, 1994a), and this could aid further understanding of the possible changes in airway inflammation in premenstrual asthma. Exhaled nitric oxide measurements should therefore be performed in female asthmatic patients and in those with premenstrual exacerbations. Likewise, induced sputum has also been used to show changes in asthmatic airway inflammation, particularly in absolute eosinophil numbers and sputum ECP levels (Pizzichini, 1996). This technique could also be used to assess airway inflammation throughout both phases of the menstrual cycle in female asthmatic patients. Our results are further supported by recent preliminary results of 107 female asthma patients, which found that those with premenstrual asthma had an increase in airway responsiveness to methacholine of almost 2 doubling doses during the late luteal phase compared to the follicular phase (Dauletbaev, 1996 abstract).

In Chapter 13, we found that these cyclical changes in airway responsiveness to AMP appear to be abolished in asthmatic women taking the combined oral contraceptive pill, with suppression of the normal post-ovulatory luteal phase rise in sex steroid hormones. However, it is not possible to draw firm conclusions from our studies as all the patients had stable asthma with no premenstrual symptoms. Further research is therefore needed in patients with premenstrual asthma to evaluate whether altered β_2 -AR function and airway hyperresponsiveness to AMP provide an explanation for the underlying mechanisms in this condition. Studies are also required to investigate whether the combined oral contraceptive pill and adenosine antagonists such as theophylline and the cromones might benefit patients with premenstrual asthma, given the paucity of data on treatment options. By studying pregnant and menopausal asthma patients, as well as those on hormone replacement therapy, more information could be obtained on the ways in which progesterone and oestradiol might influence β_2 -AR function and airway hyperresponsiveness. In a prospective, questionnaire-based study in a large number of post-menopausal nurses over the course of 10 years, the risk of developing new-onset adult asthma was associated with the use of exogenous oestrogen therapy (Troisi, 1995). The age-adjusted relative-risk of developing asthma for women who reported ever using postmenopausal hormones was 1.49 compared to women who reported never using these. This association was also related to the duration of use and dose of oestrogen. There was no difference in whether the hormone therapy contained progesterone or not.

Our studies have provided some insight into the facilitatory effects of corticosteroids and female sex steroid hormones on β_2 -AR function and regulation in asthma. There

are still many unanswered questions and the studies we hope have opened up several possible avenues for further research in this area of asthma.

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