CHAPTER 14

Involvement of the Endothelins in Airway Reactivity and Disease

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A. Introduction

In 1988, a previously uncharacterized endothelium-derived contractile factor was isolated, purified and identified as a novel 21 amino acid sequence and named endothelin-1 (ET-1) (YANAGISAWA et al. 1988; INOUE et al. 1989). Some of the pharmacological activity of ET-1 was also reported in these studies, although there soon followed an avalanche of published research data from other laboratories describing the biology of this peptide in great detail in several mammalian systems, with particular emphasis on its spasmogenic actions in vascular tissues. In addition however, the potent contractile effects of ET-1 in airway smooth muscle were also reported in 1988 and again in 1989 (TURNER et al. 1989; UCHIDA et al. 1988), predictably, followed rapidly by evidence for high densities of ET receptors in airway smooth muscle (TURNER et al. 1989; POWER et al. 1989). Autoradiographic analyses in human and animal airway tissues established the presence of significant numbers of such receptors in several airway wall cell types in addition to airway and vascular smooth muscle (HENRY et al. 1990; GOLDIE et al. 1995). Taken together, this information constituted a reasonable basis for speculating that the actions of ET-1 might be associated with obstructive airway diseases such as asthma (HAY et al. 1993a, 1996; GOLDIE et al. 1996c), as well as with pulmonary hypertension (ALLEN et al. 1993; STELZNER et al. 1992; STEWART et al. 1991; FOLKERTS et al. 1998). (The involvement of ETs in pulmonary hypertension will be dealt with in detail in Chap. 15.) Since then, there has been a constant stream of published reports demonstrating that ET-1 can mimic many of the features of asthma in addition to its powerful spasmogenic activity in airway smooth muscle, all of which add weight to the concept of a mediator role for ET-1 in this disease (GOLDIE et al. 1996c). Evidence continues to emerge implicating the ETs in this and other lung pathologies, including degenerative fibrotic diseases such as fibrosing alveolitis (SALEH et al. 1995; SEINO et al. 1995; MUTSAERS et al. 1998).

In this review, we will evaluate much of the evidence implicating the ETs in respiratory diseases, with particular emphasis on asthma for which a strong, but still circumstantial case can be made.

T. D. Warner (ed.), *Endothelin and Its Inhibitors*

B. The Endothelin System

I. Is There a Link to Asthma?

Over the years, many substances have been proposed as mediators in asthma. However, only some of these, such as the cysteinyl leukotrienes, have been confirmed as significant players in this disease after years of rigorous evaluation. Similarly, the theory that ET-1 (and/or related endogenous peptides) is a significant mediator in asthma will only receive universal acceptance after various standard criteria are fulfilled. First, ET-1 must induce actions in the respiratory tract that mimic most if not all of the features and symptoms of this disease. Second, relevant receptors must be present and actively involved in mediating relevant cellular responses to these peptides in the airways. A true asthma mediator must be an endogenous substance, synthesized, released and degraded at appropriate sites in the lung. Furthermore, the levels of the mediator must be elevated in asthma, with a positive correlation existing between these levels and disease symptom severity. Finally, ET receptor antagonists or inhibitors of ET synthesis should relieve asthma symptoms and thus be of at least potential therapeutic benefit. Before exploring these aspects further, it is important to outline briefly some of the fundamental features of the endothelin system in the airways as far as they are presently understood.

II. ET Structure, Synthesis and Degradation

1. Structure

ET-1 is one of a family of 21 amino acid endogenous mammalian peptides (ET-1, ET-2 and ET-3), each of which have similar sequences. In each sequence, two disulfide bridges spanning positions 1, 15 and 3, 11 constrain their structures as seen in Fig. 1. The sarafotoxins, which are spasmogenic components of the venom of the Middle Eastern burrowing asp, *Atractaspis engaddensis* (MASAKI et al. 1992) are also 21 amino acid sequences with similar structural characteristics and sequences to the ETs (see Chap. 2). It is perhaps not surprising then that the sarafotoxins also evoke contraction of vascular smooth muscle. Recently, a 31-amino acid ET-1 sequence was also identified which was derived from prepro-ET-1 via the action of mast cell chymase (NAKANO et al. 1997) (Fig. 1). This peptide is also a directly acting spasmogen in both vascular and airway smooth muscle, i.e. biological activity is not dependent upon cleavage to the 21 amino acid sequence, although this conversion can occur (YOSHIZUMI et al. 1998a, b; KISHI et al. 1998).

2. Synthesis

The formation of the ETs is preceded by the synthesis of 212 amino acid precursors known as prepro-ETs, e.g. prepro-ET-1 (YANAGISAWA et al. 1988; see Chaps. 3, 7). These precursors for ET-1, ET-2 and ET-3 are encoded by genes found on chromosomes 6, 1 and 20, respectively (INOUE et al. 1989; BLOCH et

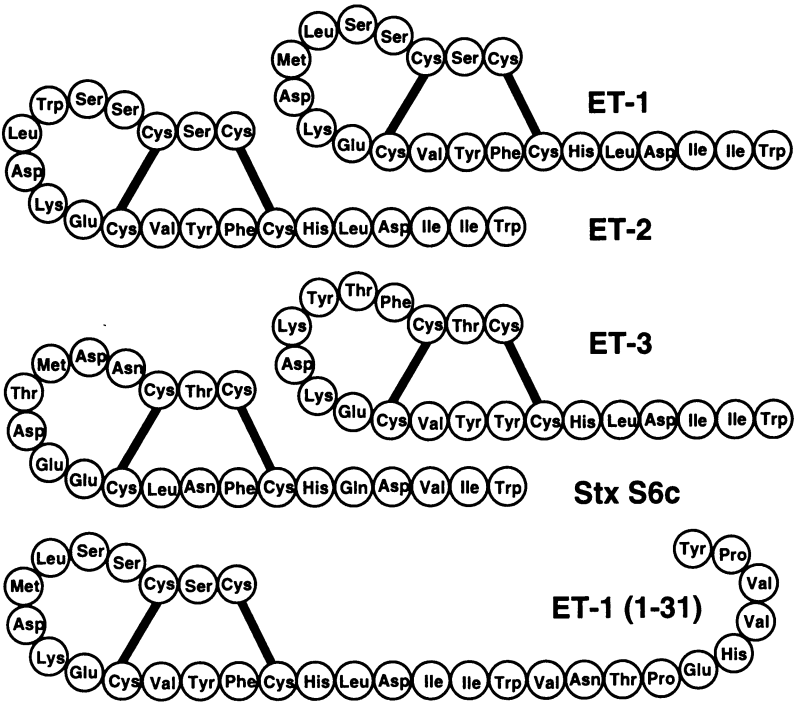

Fig. 1. Diagrammatic representation of the chemical structures of ET-1 and related peptides

al. 1989a, b, 1991) and are subsequently cleaved via dibasic amino acid residue-specific endopeptidases or by the mammalian convertase known as furin (DENAULT et al. 1995), to provide 38 amino acid residues called big ETs (INOUE et al. 1989; ITOH et al. 1988). The big ETs are not ET receptor agonists and must be cleaved to the receptor-activating 21 amino acid sequences (YANAGISAWA and MASAKI 1989; MASAKI et al. 1992; OPGENORTH et al. 1992) via ET converting enzyme (ECE) (XU et al. 1994). This enzyme is a phosphoramidon-sensitive, membrane-bound, neutral metalloprotease (EMOTO and YANAGISAWA 1995; OPGENORTH et al. 1992; VEMULAPALLI et al. 1992).

3. Degradation

Neutral endopeptidase (NEP) is found in abundance in the airways (JOHNSON et al. 1985) and the ETs have high affinity for and are actively metabolized by this enzyme (VIJAYARAGHAVAN et al. 1990; FAGNY et al. 1991). Interestingly, in activated human polymorphonuclear neutrophils, cathepsin G, rather than NEP, may be the enzyme responsible for eliminating ET-1 (FAGNY et al. 1992).

C. ET Receptors

The detection by molecular cloning of just two ET receptor subtypes in mammalian cells, designated ET_A and ET_B (ARAI et al. 1990; SAKURAI et al. 1990; SAKAMOTO et al. 1991; ADACHI et al. 1991; see Chap. 4) is entirely consistent with data derived using other approaches (SAKURAI et al. 1992; MASUDA et al. 1989; TAKAYANAGI et al. 1991) in mammalian systems including the lung (HICK et al. 1995; KONDOH et al. 1991; KOZUKA et al. 1991; HAGIWARA et al. 1992). However, in amphibian cells, an ET_C receptor has been cloned and may be functional (KARNE et al. 1993). In recent years, several studies involving vascular tissues have provided evidence for the existence of distinct subtypes within both the ET_A and ET_B receptor families (SOKOLOVSKY et al. 1992; WARNER et al. 1993). This possibility has also been raised for ET_B receptors in human bronchus, with the reporting of apparently anomalous contractile responsiveness to ET_B receptor agonists (HAY et al. 1998). Despite such functional evidence, the existence of genetic codes for such receptors has not been confirmed. Accordingly, the existence of these novel receptor subtypes must for the moment remain uncertain (BAX and SAXENA 1994). Indeed, some of the functional data for novel ET receptor subtypes may be explained in terms of differences in the kinetics of ligand interactions with ET receptors (DEVADASON and HENRY 1997).

D. ET and the Major Pathologies in Respiratory Diseases

I. Asthma

Asthma is recognized as a chronic inflammatory lung disease (BARNES et al. 1988), involving several pathologies (KAY 1991) including airway hyperreactivity (BOUSHEY et al. 1980). It is also clear that asthma is an obstructive airway disease and that a significant component of the obstruction is caused by increased airway smooth muscle tone (JAMES et al. 1989). However, occlusion of the bronchi is also a result of the hypersecretion of mucus together with reduced clearance of mucus from the airways (BEASLEY et al. 1989). Epithelial cell damage and desquamation and the addition of epithelial debris and inflammatory cells to luminal mucus further reduces the patency of the airways (NAYLOR 1962; LAITINEN et al. 1985; BEASLEY et al. 1989). Finally, airway wall restructuring as evidenced by submucosal oedema, airway smooth muscle and mucous gland hyperplasia (HEARD and HOSSAIN 1973; CARROLL et al. 1993; KNOX 1994; ROCHE 1998) and sub-epithelial fibrosis (BREWSTER et al. 1990; ROCHE et al. 1989) accompanying chronic airway inflammation further reduce lumen diameter and elevate bronchial resistance to airflow. Importantly, ET-1 has actions within the bronchial wall which mimic and potentially reproduce many of these pathologies and symptoms and these issues will be discussed in detail in later sections. It is also important to realize that treatment with anti-inflammatory glucocorticoids (TRIGG et al. 1994) and the removal of

provoking stimuli, e.g. diisocyanates (Saetta et al. 1995) has been linked to reversal of such structural changes to the bronchial wall.

II. Allergic Rhinitis

The human nasal mucosa contains the mRNA for prepro-ET-1 and expresses immunoreactive ET-1 (ir-ET-1) (Mullol et al. 1993; Casasco et al. 1993). Specific binding sites for ET-1 that are presumably ET receptors were found in nasal submucosal glands, venous sinusoids and small muscular arterioles and ET-1-induced stimulation of these sites in vitro caused serous and mucous cell secretions (Mullol et al. 1993) and induced prostanoid release Wu et al. 1992). Riccio and co-workers conducted the first study describing the effects of intranasal ET-1 in human rhinitics and healthy volunteers (Riccio et al. 1995). This study demonstrated mucosal hyperresponsiveness to ET-1 in rhinitics, since sneezing frequency and the amounts of nasal secretions were increased. It has subsequently been shown that the levels of mRNAs for prepro-ET-1 and ECE were significantly increased in chronic rhinitis (Furukawa et al. 1996). The possibility of a mediator role for ET-1 in rhinitis requires further investigation.

III. Adult Respiratory Distress Syndrome (ARDS)

Lung injury resulting in compromised pulmonary gas exchange in ARDS is most often the result of sepsis, but may have other causes. Elevated circulating ir-ET levels have been linked to deterioration in ARDS and clinical improvement was associated with significant falls in these levels (Druml et al. 1993; Langleben et al. 1993). Animal studies suggest that the release of ir-ET in this condition may reflect the influence of endotoxin released in sepsis (Weitzberg et al. 1991). In rat models of respiratory distress, abnormal blood gas levels and pulmonary oedema are seen, together with increases in ir-ET levels in bronchoalveolar lavage (BAL) fluid (Herbst et al. 1995; Simmet et al. 1992). Importantly, the abnormal blood gases and oedema were partly corrected in the presence of ET_A receptor antagonist BQ-123, suggesting the involvement of ET-1 in this model (Herbst et al. 1995).

IV. Cryptogenic Fibrosing Alveolitis (CFA)

As the name suggests, CFA is characterized by peripheral lung fibrosis involving fibroblast proliferation and collagen deposition. However, CFA is also linked to lung inflammation and type II pneumocyte proliferation. The production of ir-ET was up-regulated in these cells, and in airway epithelium (Giaid et al. 1993). Importantly, the extent of type II cell proliferation was closely correlated with the levels of ir-ET. Morphological changes were also detected in pulmonary vessels in which the endothelium over-expressed ir-ET (Giaid et al. 1993).

V. Pulmonary Fibrosis

Pulmonary fibrosis can be induced in the rat (MUTSAERS et al. 1998) and hamster by pretreatment with bleomycin (SEINO et al. 1995). In the rat, intratracheal bleomycin caused a significant elevation in ir-ET which preceded the increase in lung collagen (MUTSAERS et al. 1998). In the hamster, the ET_A receptor-selective antagonist BQ-123, attenuated bleomycin-induced alveolar fibrosis and the accompanying right ventricular hypertrophy. These data suggest that endogenous ET-1 released in this condition was mitogenic in pulmonary fibroblasts, indicating a significant link to the disease process in these models and raising the possibility of a role in the pathogenesis of human pulmonary fibrosis. Consistent with this, increased levels of ir-ET and ir-ECE-1 have been co-localized in several cell types, including proliferating type II pneumocytes, in patients with idiopathic pulmonary fibrosis (SALEH et al. 1995). Pulmonary fibrosis also occurs in systemic sclerosis where increased ir-ET levels in BAL fluid were also detected (CAMBREY et al. 1994). The levels of ir-ET in BAL fluid were high enough to induce an ET_A receptor-dependent proliferation of fibroblasts in vitro. In this disease, a significant amount of ir-ET probably comes from alveolar macrophages which have been shown to produce excessive amounts of this peptide in response to endotoxin (ODOUX et al. 1997). In other fibrotic lung conditions in the human, including that associated with scleroderma (ABRAHAM et al. 1997) and in a rat model of bronchiolitis obliterans (TAKEDA et al. 1998), significant increases in ir-ET were detected in both alveolar and peripheral bronchial epithelia. Interestingly, in scleroderma-associated lung fibrosis, total ET receptor number was increased two- to threefold. The ratio of ET_A:ET_B sites also changed, with ET_B receptor numbers increased and ET_A receptor numbers reduced (ABRAHAM et al. 1997).

VI. Pulmonary Tumours

ET receptors and prepro-ET-1 mRNA have been detected in HeLa and HEp-2 human tumour cell lines and ir-ET was also released from these cells (SHICHIRI et al. 1991). ET-1 evoked increased cytosolic free Ca^{2+} and proliferation of these cells (SHICHIRI et al. 1991). In addition, ir-ET and mRNA for prepro-ET has been detected predominantly in pulmonary squamous cell carcinomas and adenocarcinomas (GIAID et al. 1990). The precise role of ET-1 in these tissues is not established, although activity as an autocrine/paracrine growth factor cannot be dismissed.

E. ET-1 and the Standard Criteria for Mediator Status in Asthma

The following is an outline of the extent to which the standard criteria for confirming the identity of an endogenous chemical mediator of disease, as described in Sect. B.I., have been fulfilled for ET-1 in asthma.

I. Synthesis, Release and Degradation of the ETs in the Lung

A true endogenous asthma mediator must be synthesized, released and degraded at appropriate sites in the lung.

1. Synthesis

The first reports describing the synthesis of the ETs established the endothelium as the primary vascular source of the peptides (YANAGISAWA et al. 1988; INOUE et al. 1989). The endothelium of small blood vessels within the airway wall is also a significant site of ET synthesis in the lung (GIAID et al. 1991; SPRINGALL et al. 1991; MACCUMBER et al. 1989). However, in relation to the status of ET-1 as a possible asthma mediator, arguably more important support for the case came in the form of several studies establishing that this peptide was also synthesized and released by the airway epithelium (ROZENGURT et al. 1990; GIAID et al. 1991; SPRINGALL et al. 1991; RENNICK et al. 1992; MACCUMBER et al. 1989). This is significant because this tissue represents a relatively large proportion of the airway mucosal volume and can potentially produce relatively large amounts of peptide which might diffuse to critical submucosal target tissues including airway smooth muscle and nerves.

As previously mentioned, ECE is a phosphoramidon-sensitive metalloprotease responsible for the conversion of Big ETs to the "mature" 21 amino acid ET peptides. This critical enzyme and has been found in guinea-pig, rabbit and human airways (BIHOVSKY et al. 1995; ISHIKAWA et al. 1992; PONS et al. 1992b). The lung contains high levels of the ETs relative to most other organs (PERNOW et al. 1989; YOSHIMI et al. 1989) and it is the airway epithelium in animal and human lung that is the richest source of these peptides (ROZENGURT et al. 1990; RENNICK et al. 1992; MACCUMBER et al. 1989; SPRINGALL et al. 1991).

2. Release

Under basal conditions, ET-1 is released abluminally from human (MATTOLI et al. 1990), porcine and canine (BLACK et al. 1989) cultured bronchial epithelial cells and from cultured tracheal epithelium from the guinea-pig (ENDO et al. 1992) and rabbit (RENNICK et al. 1993). Despite this, ETs released from vascular endothelium and some inflammatory cells (MACCUMBER et al. 1989; GIAID et al. 1991) including mast cells (EHRENREICH et al. 1992) and macrophages (EHRENREICH et al. 1990) may also play important functional roles within the airway wall in asthma.

3. Degradation

The ETs are catabolized primarily by neutral endopeptidase (NEP), a phosphoramidon-sensitive enzyme found in abundance in the airway epithelium (JOHNSON et al. 1985). Not surprisingly, both removal of the epithelium or pretreatment with phosphoramidon causes marked potentiation of the contrac-

tile actions of ET-1 in guinea-pig tracheal (HAY 1989) and human bronchial airway preparations (CANDENAS et al. 1992; YAMAGUCHI et al. 1992). These data are consistent with the notion that epithelial NEP is the major degradative enzyme for the ETs in the lung and, as such, acts as a significant modulator of the sensitivity of airway smooth muscle to this peptide (DI MARIA et al. 1992; BOICHOT et al. 1991b).

II. ET Receptor Distribution

ET receptors must be present in the lung at sites relevant to the expression of asthma symptoms.

1. Airway Smooth Muscle

Autoradiographic studies have been valuable in mapping the distribution and localization of both ET receptor subtypes in the lung. Such studies have verified the presence of high densities of ET receptors in many tissue types in the respiratory tract in the human (HEMSEN et al. 1990; HENRY et al. 1990; BRINK et al. 1991; MCKAY et al. 1991) and in animals (TURNER et al. 1989; POWER et al. 1989; TSCHIRHART et al. 1991). Consistent with the potent spasmogenic activity of the ETs in the airways, ET receptors are found in greatest density in airway smooth muscle, with many studies showing that both ET_A and ET_B receptor subtypes are expressed. There is a wide spectrum of subtype ratios detected in airway smooth muscle from different species. For example, approximately equal numbers of these subtypes are detected in mouse and rat tracheal smooth muscle (HENRY and GOLDIE 1994; HENRY 1993). However, there are some notable exceptions to this general pattern, with ET_B receptors constituting approximately 90% of the total population in human bronchial airway smooth muscle (GOLDIE et al. 1995). This proportion falls to about 70% when non-airway components of the human bronchial wall are included in the assessment (FUKURODA et al. 1996). ET_B receptors also greatly outweigh the numbers of ET_A receptors in rabbit bronchus (MCKAY et al. 1996) and also predominate in guinea-pig bronchus (GOLDIE et al. 1996a) and pig trachea (KOSEKI et al. 1989). At the other end of the spectrum, ET_A receptors exist as a homogeneous population in sheep tracheal airway smooth muscle (GOLDIE et al. 1994), strongly predominate in canine airway smooth muscle (MCKAY et al. 1996) and constitute approximately 70% of the total in pig bronchus (GOLDIE et al. 1996a).

2. Other Sites

Many cell types within the airway wall, other than airway smooth muscle, also express either or both ET_A and ET_B receptors. For example, specific [^{125}I]-ET-1 binding was also detected in epithelium and submucosal tissues, as well as in blood vessels in human bronchus (POWER et al. 1989; HENRY et al. 1990; GOLDIE et al. 1995) and in mouse, rat, guinea-pig and pig tracheal tissue

(Tschirhart et al. 1991; Henry et al. 1990; Goldie et al. 1996a; Koseki et al. 1989). In sheep trachea, submucosal glands and sub-epithelial tissues expressed relatively high levels of ET_B receptors (Goldie et al. 1994). Peripheral lung alveoli also express very high levels of both ET_A and ET_B receptors. This has been verified in human lung (Knott et al. 1995) and in lung from the rat (Turner et al. 1989; Power et al. 1989), guinea-pig and pig (Goldie et al. 1996b). Neuronal tissue also contains ET receptors of both subtypes. Specific [^{125}I]-ET-1 binding has been detected in airway parasympathetic ganglia and with paravascular nerves and other neuronal pathways (McKay et al. 1991; Kobayashi et al. 1993). ET receptors mostly of the ET_B subtype, have been localized to guinea-pig tracheal adrenergic and cholinergic nerve cell bodies, processes and varicosities in primary culture (Takimoto et al. 1993). Recently, we used an immunofluorescence approach with confocal microscopy to show that both receptor subtypes existed in rat tracheal nerves grown in cultures (Fernandes et al. 1998; Goldie et al. 1998). The actions of the ETs at these various sites may also be relevant to the airway obstruction in asthma. This will be discussed below.

In the context of assessing the role of the ETs in asthma, it is clearly important to determine whether this disease is associated with significant changes in ET receptor distribution or subtype densities in the lung. We have assessed these parameters in central (Goldie et al. 1995, 1996c; Hay et al. 1993a) and peripheral airways (Knott et al. 1995) from both asthmatic and non-asthmatic subjects. Interestingly, in asthma, no significant differences were detected in ET_A/ET_B receptor ratio in either central bronchial airway smooth muscle (non-asthmatic = 12%:88%; asthmatic = 18%:82%) (Goldie et al. 1995), or in alveolar wall tissue (non-asthmatic = 32%:68%; asthmatic = 29%:71%) (Knott et al. 1995). However, issues such as altered levels of ET synthesis and release in asthmatics compared with healthy individuals may be of importance in asthma.

III. ET Receptor-Mediated Responses Relevant to Asthma

1. Altered Bronchial Tone

ET receptors in the lung must mediate responses relevant to asthma symptoms and pathologies.

The autoradiographic detection of specific ET binding sites does not establish these sites as functional ET receptors. However, ET-1 has been shown to induce a wide range of acute and chronic effects within the bronchial wall and in peripheral lung in tissue known to contain specific binding sites for [^{125}I]-ET-1, that may be highly relevant to a mediator role in asthma. Of all the effects that an asthma mediator might be expected to induce, airway smooth muscle contraction is arguably one of the most important, since episodic and chronically elevated airway tone are cardinal features of this disease.

a) Direct Airway Smooth Muscle Contraction

The airway spasmogenic effects of ET-1 and related peptides was reported very soon after the identification of these substances in 1988 (UCHIDA et al. 1988). Subsequently, airway smooth muscle contraction has become the most widely studied action of ET-1 in the airways. ET-1 is one of the most powerful and potent spasmogens in human isolated bronchial smooth muscle preparations (UCHIDA et al. 1988; HAY et al. 1993a; TSCHIRHART et al. 1991; HEMSEN et al. 1990; MAGGI et al. 1989; ADVENIER et al. 1992; HAY 1990). Contraction to ET-1 is relatively slow to develop, but is persistent and resistant to reversal by washout. This may be explained in part by the pseudo-irreversible nature of ET-1 binding to ET receptors (NAMBI et al. 1994; WAGGONER et al. 1992; WU-WONG et al. 1994; WATAKABE et al. 1992; IHARA et al. 1995), which causes sustained receptor activation and signal transduction, consistent with the persistence of elevated bronchial tone often seen in asthma.

Although ET_B receptors greatly predominate in number in human bronchial smooth muscle, functional studies indicate that the smaller ET_A receptor population can also mediate ET-1-induced contraction (GOLDIE et al. 1995; FUKURODA et al. 1996). This is also the case in animal airway smooth muscle in which both receptors are expressed (HENRY and GOLDIE 1994; HENRY 1993; HAY et al. 1993d; GOLDIE et al. 1996a; KOSEKI et al. 1989) and in sheep tracheal smooth muscle where only ET_A receptors were detected (GOLDIE et al. 1994; ABRAHAM et al. 1993). Interestingly, in asthma, a decrease in sensitivity to the contractile effects of the ET_B receptor-selective agonist sarafotoxin S6c was demonstrated in bronchial tissue, suggesting that ET_B receptor desensitization may have occurred. It is possible that such desensitization was the result of increased synthesis and release of ET-1, with subsequent over-exposure of ET receptors to this ligand. Thus, a mediator role for ET-1 in asthma cannot be attributed in any degree to up-regulation of ET receptor function (GOLDIE et al. 1995) or density (GOLDIE et al. 1995; KNOTT et al. 1995).

b) Modulation of Neurotransmission

Neuronal pathways play an important role in bronchial wall homeostasis, including the regulation of airway tone. In asthma, the function of such systems may be perturbed, resulting in altered airway resistance or the activity of gland secretory processes. For example, airway obstruction resulting from increased airway smooth muscle tone could be induced by hyper-activity of bronchial cholinergic nerves (WARD et al. 1994; SHEPPARD et al. 1982; BEAKES 1997), or of the excitatory non-adrenergic, non-cholinergic neuronal system (BARNES et al. 1991) innervating this target tissue. Airway calibre might be similarly influenced by hypofunction of inhibitory sympathetic pathways (GOLDIE et al. 1986; GOLDIE 1990) or of inhibitory non-adrenergic, non-cholinergic nerves (ELLIS and UNDEM 1992). Thus, the balance of activities between these systems may be important in the etiology and progression of obstructive airway diseases

including asthma (GOLDIE 1990; BARNES 1992). Mediator-induced modulation of such pathways is one mechanism through which this balance might be disturbed.

Recent evidence that the ETs induced potentiation of cholinergic neurotransmission in the guinea-pig ileum (WIKLUND et al. 1989) provided a rationale for assessing the influence of these peptides on airway neuronal pathways. The first evidence for such an effect in the respiratory tract came with a report that ET-3 potentiated cholinergic nerve-evoked bronchial contraction in the rabbit (MCKAY et al. 1993). This was soon followed by similar findings with ET-1 and the ET_B receptor-selective agonist sarafotoxin S6c in mouse (HENRY and GOLDIE 1995) and rat (KNOTT et al. 1996) trachea. With regard to a mediator role for ET peptides in asthma, it is particularly exciting to find that sarafotoxin S6c also caused powerful and potent potentiation of cholinergic nerve-mediated contraction in human isolated bronchial preparations (FERNANDES et al. 1996). Importantly, very recent, but as unpublished data from our laboratory indicate that ET-1 also potentiated cholinergic contraction in human bronchial ring preparations, an effect involving activation of both ET_A and ET_B receptors located on post-ganglionic nerves. These findings are consistent with results in rat tracheal tissue, where both receptor subtypes were linked to increased release of acetylcholine (KNOTT et al. 1996) and provide another mechanism through which ET peptides might influence airway tone in asthma. Ovine tracheal muscle tissue represents an interesting anomaly, in that prejunctional ET_B receptor stimulation caused inhibition of contraction due to suppression of acetylcholine release (HENRY et al. 1996). Thus, sheep airways do not provide a model which mimics the neuronal activities of ET-1 in human bronchus. We are presently evaluating the effects of exogenously applied ET-1 and similar peptides on non-cholinergic neuronal pathways in the airways.

c) Bronchoconstriction In Vivo

ET-1 caused increased airway resistance, presumably primarily as a result of bronchospasm, in several animal species, including the rat (MATSUSE et al. 1990), dog (UCHIDA et al. 1992a), guinea-pig (NOGUCHI et al. 1993; NAGASE et al. 1995; TOUVAY et al. 1990) and sheep (NOGUCHI et al. 1995). Both ET_A and ET_B receptors were involved in this effect in the guinea-pig (NOGUCHI et al. 1993; NAGASE et al. 1995) where sustained bronchoconstriction to either intravenous or aerosolized ET-1 involved the production of the secondary mediators thromboxane and platelet-activating factor (PAF) (MACQUIN-MAVIER et al. 1989; PAYNE and WHITTLE 1988; LAGENTE et al. 1989). Importantly, allergic sensitization in this model was accompanied by hyperresponsiveness to ET-1, perhaps related to reduced epithelial NEP activity (BOICHOT et al. 1990, 1991b). In addition, the early phase response to ovalbumin in this species, due largely to histamine and leukotriene release (BARNES et al. 1988), may also involve ET release early in the cascade of events, since ET_B receptor block-

ade inhibited allergic bronchoconstriction (UCHIDA et al. 1995). The late phase reaction to allergen may also involve ET production and the activation of ET_A receptors, since inhibition at these sites attenuated this response, as well as the usual hyperresponsiveness to inhaled carbachol (NOGUCHI et al. 1995).

ET-1 may also induce bronchial hyperresponsiveness in some models. For example, ET-1 challenge in rabbits has recently been reported to enhance bronchoconstriction to inhaled histamine, an action that involved the activation of capsaicin-sensitive airway sensory nerves (DAGOSTINO et al. 1998). Hyperresponsiveness to spasmogens such as histamine and methacholine is also commonly observed in asthmatics (BOUSHEY et al. 1980). Inhaled ET-1 has been shown to induce potentiated responsiveness to such agonists in the sheep (NOGUCHI et al. 1995), although this could only be demonstrated in one (KANAZAWA et al. 1992) of five (MACQUIN-MAVIER et al. 1989; BOICHOT et al. 1991a; PONS et al. 1992a; LAGENTE et al. 1990) studies in the guinea-pig. In heterozygous ET-1 knockout mice in which ET-1 levels were abnormally low, airway responsiveness to methacholine was increased rather than reduced as might be predicted (NAGASE et al. 1998). This suggests that underproduction of this peptide might also be accompanied by anomalous production of another factor(s) such as a bronchodilator like nitric oxide (NO) (NAGASE et al. 1998).

Studies involving the actions of the ETs in human subjects, particularly asthmatic individuals, have long been awaited, since asthma is a peculiarly human condition for which there are no completely adequate animal models. The first report of the actions of inhaled ET-1 in human asthmatic and non-asthmatic subjects showed that inhaled aerosolized ET-1 had little influence on lung function in non-asthmatics, but induced severe bronchoconstriction in asthmatic patients who were also hyperresponsive to inhaled methacholine (CHALMERS et al. 1997). The lack of potency in normals may have been the result of protection of the submucosa via the degradative barrier actions of epithelial NEP. In contrast, in asthmatics, this protection might have been compromised by damaged epithelium reducing the amounts of available NEP, or by the greater permeability of the epithelium in asthmatics, allowing the penetration of ET-1 to sub-epithelial targets including airway smooth muscle and cholinergic nerves.

2. Mitogenesis

a) Fibroblasts

The pathologies which accompany chronic asthma include restructuring of various tissue elements within the bronchial wall, a phenomenon often described as bronchial remodelling. Amongst the most prominent of these changes is an increase in airway smooth muscle volume (CARROLL et al. 1993; KNOX 1994) and increased thickness of the sub-epithelial matrix caused by increased deposition of extracellular matrix protein (BREWSTER et al. 1990; ROCHE et al. 1989). The question is whether the action of ET peptides can con-

tribute to these events. If so, then further circumstantial evidence is provided in support of a mediator role for the ETs in asthma. Indeed, the evidence from animal models clearly demonstrates the mitogenic potential of ET-1 in fibroblasts and airway smooth muscle cells. For example, ET-1 induced the proliferation of Swiss 3T3 fibroblasts in culture (Takuwa 1993). In addition, ET-1 and ET-3 have been shown to be chemoattractants for fibroblasts and also to promote the replication of rat pulmonary artery-derived fibroblasts (Peacock et al. 1992).

Importantly, evidence from human cells is consistent with data from these animal studies. Fibronectin released from human bronchial epithelial cells is both an important extracellular matrix component and itself a chemotactic factor for fibroblasts. ET-1 enhanced fibronectin gene expression and fibronectin release from these cells, actions that were mediated via ET_A receptors (Marini et al. 1996). Furthermore, in human asthmatic bronchial epithelial cells pretreated with allergen, the cytokine granulocyte/macrophage colony-stimulating factor (GM-CSF) stimulated ET-1 production, which in turn was associated with transformation of epithelial cells into myofibroblasts, cells which play a critical role in extracellular matrix deposition (Sun et al. 1997). Thus, ET-1 and epithelial cells from which it is derived, are pivotal to the promotion of sub-epithelial fibrosis. Interestingly, the mitogenic action of interleukin-1 beta (IL-1β) in porcine epithelial cells appear to be mediated in part by ET-1-induced ET_A receptor activation (Murlas et al. 1997).

b) Airway Smooth Muscle

ET-1 has also been shown to be a potent but relatively weak mitogen in cultured tracheal airway smooth muscle cells from the guinea-pig, rabbit (Noveral et al. 1992) and sheep (Glassberg et al. 1994). Similar results were obtained in human cultured bronchial smooth muscle cells, adding considerable weight to the proposition that ET-1 might be an asthma mediator. Interestingly, although the mitogenic activity of ET-1 was modest, this action was not blunted by pretreatment with β-adrenoceptor bronchodilators such as salbutamol which had marked inhibitory effects against the proliferative effects of other mitogens including thrombin (Tomlinson et al. 1994). Thus, ET-1 is unusual amongst mitogens in this regard and this characteristic enhances its profile as a putative asthma mediator. Perhaps more importantly, ET-1 is a potent co-mitogen in human bronchial airway smooth muscle cells, i.e. although alone, ET-1 was only weakly active, this peptide dramatically potentiated (three- to fourfold) the already powerful mitogenic effects of epidermal growth factor (EGF), an action mediated exclusively via ET_A receptors (Panettieri et al. 1996).

c) Mucous Glands

Bronchial mucous gland hyperplasia is also observed in chronic asthma, a phenomenon which presumably is linked to excessive production of mucus in this

disease (BEASLEY et al. 1989; HEGELE and HOGG 1996). Although not tested, given the mitogenic effects of ET-1 in bronchial smooth muscle and fibroblasts, it seems likely that mucous gland cells might also proliferate in response to ET-1.

3. Secretion of Mucus

Receptors for ET-1 have been detected in submucosal glands associated with their venous sinusoids and small muscular arterioles (MULLOL et al. 1993). Studies in animal models suggest that ET-1 has effects in mucous glands consistent with the production of mucus-obstructed airways, as often reported in asthma. Namely, ET-1 increased mucous glycoprotein secretion in feline isolated tracheal submucosal glands (SHIMURA et al. 1992) and in ovine tracheal tissue, and reduced tracheal mucus velocity as a result of ET_A receptor activation (SABATER et al. 1996). ET-1-induced increased mucus production coupled with reduced mucus clearance are effects consistent with the promotion of airway mucus plugging as seen in asthma. Importantly, stimulation of ET receptors in human cultured nasal mucosal tissue also results in increased serous and mucous secretions (MULLOL et al. 1993) and increased production of prostanoids (WU et al. 1992). Furthermore, the vascular endothelium and venous sinusoidal tissue in human nasal mucosal tissue produced ET-1 (CASASCO et al. 1993).

4. Altered Microvascular Permeability

Asthma is an inflammatory lung disease. Accordingly, the permeability of the airway microvasculature may be increased, resulting in bronchial submucosal oedema, since increased microvascular permeability is an obligatory accompaniment to airway inflammation (PERSSON 1991; GOLDIE and PEDERSEN 1995). In addition, oedema-associated bronchial wall swelling is a potentially important component of airway obstruction in asthma (PERSSON 1991). Another potentially important action of ET-1 in relation to asthma is the fact that it has been shown to increase airway microvascular permeability. This has been reported in perfused rat lung where the response was leukocyte- and plasma-dependent (RODMAN et al. 1992). The formation of secondary mediator prostanoids may also be involved, although this remains uncertain (RAFFESTIN et al. 1991; HORGAN et al. 1991; PONS et al. 1991; ERCAN et al. 1993). However, there is some consensus that ET-1-induced oedema formation in this model involves increased microvascular pressure (RAFFESTIN et al. 1991; RODMAN et al. 1992), mediated in part by ET_A receptor activation (FILEP et al. 1993a). ET_A receptors also mediated permeability increases in the guinea-pig (FILEP et al. 1995).

5. ET-1 as a Pro-Inflammatory Mediator

Given the inflammatory nature of bronchial asthma, a link between inflammatory processes in the lung and the actions of ET-1 would certainly support

the case that this peptide is an asthma mediator. A large body of evidence suggests that ET-1 has significant pro-inflammatory activities in vitro, although some data suggest otherwise.

a) ET-1-Induced Pro-Inflammatory Mediator Release

The concept of ET-1 as a pro-inflammatory mediator in the airways, is largely dependent upon data from animal studies, including the description of an indomethacin-sensitive increase in the release of the prostanoid precursor arachidonic acid from feline cultured tracheal epithelial cells (Wu et al. 1993) and from epithelial membrane phospholipids (Plews et al. 1991). Other studies have also demonstrated ET-1-induced release of various pro-inflammatory mediators from various airway cells types. For example, ET-1 activated the release of the pro-inflammatory cytokines tumour necrosis factor alpha (TNF-α), IL-1β and IL-6 from human monocytes (Helset et al. 1993) and of thromboxane A_2 from an unidentified source(s) in perfused guinea-pig lung (de Nucci et al. 1988). In addition, thromboxane and PGD_2 were detected following stimulation with ET-1 of cells from canine bronchial lavage lavage fluid (Ninomiya et al. 1992). Furthermore, the levels of 15-HETE and of oxygen radicals in BAL fluid in the rat were raised in response to intravenous ET-1 (Nagase et al. 1990), as was the case for oxygen radical levels in BAL fluid from the guinea-pig (Filep et al. 1995). Guinea-pig alveolar macrophages also released arachidonic acid and thromboxane in response to ET-1 (Millul et al. 1991) and superoxide production was raised by ET-1 in human alveolar macrophages (Haller et al. 1991). ET-1 also potentiated superoxide production from alveolar macrophage in response to FMLP and PAF, via an ET_A receptor-mediated mechanism (Filep et al. 1995). ET-1 caused the release of histamine from guinea-pig pulmonary mast cells (Uchida et al. 1992b) and of histamine, 5-HT and LTC_4 from IL-4-treated murine bone marrow-derived mast cells (Egger et al. 1995).

b) ET-1-Induced Inflammatory Cell Chemotaxis

ET-1 may also be a chemotactic factor under some conditions, since it caused adhesion of leukocytes to pulmonary vascular endothelium and induced the sequestration of leukocytes from pulmonary capillaries (Helset et al. 1994). Antigen challenge in the sensitized mouse also caused the influx of eosinphils into the respiratory tract, an effect attenuated by selective ET_A receptor blockade, or pretreatment with the dual ET_A/ET_B receptor antagonist SB 209670, but not by selective ET_B receptor blockade (Fujitani et al. 1997).

c) Some Evidence Against a Major Pro-Inflammatory Role for ET-1 in the Airways

Despite these apparently positive indications of pro-inflammatory activity and reports that ET-1 stimulated the release of prostanoids from guinea-pig

trachea (HAY et al. 1993c), human bronchus (HAY et al. 1993b) and human cultured nasal mucosal tissue (WU et al. 1992), ET-1 failed to activate histamine or leukotriene release from intact airways from these sources (HAY et al. 1993b, c). Some evidence from studies in vivo also do not indicate ET-1-associated inflammatory activity. For example, in the guinea-pig, ET-1 was not a chemoattractant for inflammatory cells as assessed by histological examination of lung alveolar or vascular walls (MACQUIN-MAVIER et al. 1989; BOICHOT et al. 1991a). In addition, in this model, exposure to ET-1 was not associated with airway epithelial damage or elevated microvascular permeability (MACQUIN-MAVIER et al. 1989; PONS et al. 1992a). The chemotactic influence of ET-1 on human blood monocytes is also disputed, with one study reporting chemotaxis (ACHMAD and RAO 1992) and another failing to demonstrate this phenomenon (BATH et al. 1990).

IV. Airway Inflammation, Increased ET Levels and Asthma

Levels of ETs should be elevated in asthma and should positively correlate with disease severity.

1. Evidence from Models In Vitro

If ET-1 is involved in the chronic generation of asthma symptoms and pathologies, levels of this peptide might be expected to be significantly increased as a result of the actions of other inflammatory mediators. Indeed, it has been demonstrated that endotoxin, thrombin and other inflammatory stimuli including various cytokines, stimulate the release of ET-1 from tracheal epithelial cells in culture (NINOMIYA et al. 1991; ENDO et al. 1992; RENNICK et al. 1993; FRANCO-CERECEDA et al. 1991). In addition, in human bronchial epithelial cells in culture, the cytokines IL-1α, IL-1β and TNFα enhanced the expression of prepro-ET-1 mRNA and increased ET-1 release (NAKANO et al. 1994), providing further strong support for the proposition that inflammation and elevated ET levels in the airways were causally linked.

2. Evidence from Animal Models Ex Vivo or In Vitro

If disease-associated airway inflammation (e.g. in asthma) involves elevated ET-1 levels in the airways, it should be possible to mimic this in animal models of airway inflammation. This is indeed the case in several systems. Some investigators have used sephadex administered intra-tracheally or intravenously to model the airway inflammation of asthma, since this stimulus causes an acute airway eosinophilic inflammatory response (BJERMER et al. 1994; KUBIN et al. 1992). Results clearly demonstrate that immunoreactive ET (ir-ET) was significantly increased in BAL fluid and this response was markedly attenuated by the glucocorticoid budesonide (ANDERSSON et al. 1992, 1996). Recent work has established that the increase in lung ir-ET after intra-tracheal sephadex in the rat occurred in bronchial epithelium and macrophages and importantly,

that this response preceded the airway eosinophilia, suggesting a role for ET-1 in the initiation of airway inflammation (FINSNES et al. 1997). This study also demonstrated that the ET_A/ET_B receptor antagonist bosentan blocked this inflammatory reaction (FINSNES et al. 1997). Tissue levels of ir-ET were also significantly increased in mice with airway inflammation associated with an Influenza-A respiratory tract viral infection (CARR et al. 1998).

Similarly, increased levels of ir-ET have been detected in plasma, BAL fluid and tissue in actively and passively ovalbumin-sensitized guinea-pigs which have an accompanying airway inflammatory cell infiltrate (FILEP et al. 1993b; XU and ZHONG 1997). BAL fluid contained enough ir-ET to induce significant proliferation of bronchial airway smooth muscle cells in culture. It was concluded that increases in TNFα, in response to allergic sensitization, induced the increased production of ET-1 which promoted airway smooth muscle cell proliferation (XU and ZHONG 1997). This is consistent with evidence that TNFα induced elevated ET-1 levels in human airway epithelial cells lines (AUBERT et al. 1997). In ovalbumin-sensitized mice, the accompanying airway eosinophilia and neutrophilia were attenuated by about 50% following ET_A receptor blockade, but not by selective antagonism of ET_B receptors (FUJITANI et al. 1997). Taken together, these data clearly indicate that airway inflammation results in the enhanced production and release of ETs which then has the potential to activate responses within the airway wall relevant to obstructive diseases such as asthma. However, it is important that such a link be established in human asthma.

3. Asthma-Associated Airway Inflammation and ET Levels

As previously mentioned, epithelial cells are a major potential source of ETs in the respiratory tract, although in healthy individuals, the expression of these peptides under basal conditions is very low (VITTORI et al. 1992; SPRINGALL et al. 1991). It might be expected that these epithelial levels would be significantly elevated in inflamed airways in asthma. Several studies have now established that this is so. Thus, the expression of both mRNA for prepro-ET-1 and of ET-1 protein in bronchial epithelial cells from asthmatics was significantly greater than in similar tissue from healthy volunteers or from chronic bronchitics (VITTORI et al. 1992). Cells derived from asthmatics have also been shown to produce increased amounts of ET-1 in response to pro-inflammatory stimuli compared with amounts produced in tissue from nonasthmatic subjects. This has been demonstrated for epithelial cells in response to IL-1, histamine (ACKERMAN et al. 1995) and GM-CSF (SUN et al. 1997). Peripheral blood mononuclear cells from asthmatics also released greater amounts of ET-1 than similar cells from non-asthmatics and allergen immunotherapy suppressed this response (CHEN et al. 1995).

Importantly, various studies have now shown that active asthma is associated with increases in the production of ETs in the lung. In the first of these, Nomura and co-workers reported tantalizing preliminary data of a sixfold

elevation in the levels of ir-ET in BAL fluid from an individual in status asthmaticus (NOMURA et al. 1989). It was subsequently shown that asthmatics have increased circulating blood (CHEN et al. 1995; AOKI et al. 1994) and BAL fluid levels of ir-ET (MATTOLI et al. 1991; SOFIA et al. 1993; BATTISTINI et al. 1991), suggesting that epithelial ET-1 levels should also be raised in asthma. Studies assessing ir-ET levels in bronchial biopsies confirmed this (SPRINGALL et al. 1991; REDINGTON et al. 1997). It is also significant that ir-ET-1 levels in lung tissue were not significantly raised in asthmatics receiving anti-inflammatory glucocorticoid therapy (REDINGTON et al. 1997). In such asthmatics demonstrating reduced ir-ET levels, the symptoms of asthma should be reduced in severity if ET-1 is a significant mediator.

4. Respiratory Tract Viral Infection, Asthma and ETs

Respiratory tract infections with viruses such as respiratory syncytial virus (RSV) have long been associated with exacerbations of asthma and bronchial hyperresponsiveness to inhaled spasmogens (BEASLEY et al. 1989; NICHOLSON et al. 1993; TEICHTAHL et al. 1997; SCHWARZE et al. 1997). Although many mechanisms for this have been proposed (BUSSE 1990; FOLKERTS et al. 1998), the phenomenon remains poorly understood. However, it is known that such infections involve airway inflammation and in the case of RSV this response involves an eosinophilia (FOLKERTS et al. 1998; SCHWARZE et al. 1997). This raises the possibility that up-regulated epithelial production of ET-1 makes a significant contribution to the induction of asthma symptoms. Consistent with this, we have recently shown that influenza-A virus infection in the mouse was linked to markedly elevated levels of ir-ET in both central and peripheral airways (CARR et al. 1998). Behera and colleagues have now established that RSV infection in human airway epithelial cell lines caused markedly increased expression of ET-1 mRNA, ET-1 protein, 5-lipoxygenase activity and cysteinyl leukotrienes (BEHERA et al. 1998). Taken together, these data suggest that ET-1 could be a significant mediator of virally-triggered asthma.

5. ET Levels and Asthma Symptoms

It has now been established that glucocorticosteroids, which are an important anti-inflammatory therapy in asthma, reduced the amount of epithelial ET-1 released in asthmatics (VITTORI et al. 1992; MATTOLI et al. 1991; REDINGTON et al. 1995). Most importantly, treatment with inhaled β-agonists and oral glucocorticoids for 15 days reduced the previously elevated BAL ET levels in asthmatics to levels approaching those detected in healthy control subjects and this was accompanied by improvements in lung function (MATTOLI et al. 1991). In a separate study, the suppressant influence of steroids on BAL ir-ET levels in asthma was confirmed (REDINGTON et al. 1995). Significantly, it was also noted that the percentage of predicted FEV_1 was lower in patients not receiving this treatment and that this correlated with the higher levels of BAL ir-ET in these patients. In contrast, in patients with nocturnal asthma, the fall in FEV_1

overnight was inversely related to the levels of ir-ET-1 in BAL fluid (KRAFT et al. 1994).

F. Conclusions

There are now many hundreds of published research papers describing the actions of the ETs in the respiratory tract, many of which support a case for ET-1 as a significant contributor to the pathologies of asthma. However, to our knowledge, there have been no studies assessing the clinical effects of ET receptor antagonists in asthmatic subjects. An alternative therapeutic approach would be to use agents that inhibit the synthesis or release of the ETs within the airway wall. Once again, studies evaluating such inhibitors have not been conducted. Until studies of this type are done and their therapeutic efficacy established, the last of the standard criteria for confirmation of ET-1 as an asthma mediator must remain unfulfilled.

Despite the fact that the case in support of this contention is circumstantial, the weight of evidence is impressive. In particular, the facts that the actions of ET-1 in human respiratory tissues mimic so many of the signal features of asthma, and that asthma/airway inflammation has been linked to increases in epithelial ET levels, provide powerful evidence suggesting that ET-1 is indeed a mediator in this disease. However, most of the data also suggest that the ETs are not initiators of this disease. Rather, their production seems to be upregulated following the establishment of airway inflammation, promoting the expression of disease symptoms. This view might have to be modified in the light of data from the study by Finsnes and co-workers, who reported a rise in ir-ET in the airways which preceded the eosinophilia in response to a pro-inflammatory stimulus (FINSNES et al. 1997).

Acknowledgements. The authors acknowledge the research funding support of the National Health and Medical Research Council (Australia).

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