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# The role of cytokines in regulating platelet-activating factor (PAF) synthesis in endothelial cells.

by

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## ABSTRACT

Asthma is a disease of the airways in which an inappropriate inflammatory response occurs. It is characterized by the expression of Th2 type cytokines such as IL-4. PAF is a potent phospholipid autocoid that participates in leukocyte recruitment during inflammation. Human umbilical vein endothelial cells (HUVEC) synthesize and express PAF in response to several agonists including histamine and thrombin. In this study we examined the role of cytokines in regulating PAF synthesis. HUVEC were first treated for 24 hr with IL-4 and then stimulated for 5 min with histamine or thrombin. IL-4 stimulation primed HUVEC for increased PAF production in response to either histamine or thrombin. IL-4 also increased the sensitivity of HUVEC to histamine. We conclude that the increase in histamine sensitivity seen in IL-4 treated HUVEC is due, in part, to an increase in histamine receptors. We were unable to define the mechanism behind increased histamine or thrombin-induced PAF synthesis in IL-4 treated HUVEC.

**Acknowledgements**

I would like to thank Dr. Kamala Patel for her guidance and support throughout this endeavor. To the 'Patel Lab,' especially Evelyn, Shahina, and Bao, thank you for enduring my presence and making my stay an incredible experience! I have learned so much from all of you that you have each become a part of me. I leave now with a greater understanding of what kind of person I would like to be, and for that I am eternally grateful.

To my parents and brother for always being there for me. You have always taught me to be more than I am, see the potential within myself, realize its beginnings and encouraged me to explore the endless possibilities that it holds.

Finally, to the Medical Research Council of Canada whose funding made this research possible.

## Dedication

To my parents, my everything.

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## Abbreviations

| | |
|---|---|
| AHR | airway hyperresponsiveness |
| AM | acetoxymethyl ester |
| BAL | bronchoalveolar lavage |
| COX | cyclooxygenase |
| $cPLA_2$ | cytoplasmic phospholipase $A_2$ |
| DAG | diacylglycerol |
| ECM | endothelial cell media |
| ECP | eosinophil cationic protein |
| EDN | eosinophil derived neurotoxin |
| ELISA | enzyme-linked immunosorbent assay |
| EPO | eosinophil peroxidase |
| ERK | extracellular signal-responsive kinase |
| ESL-1 | E-selectin ligand-1 |
| FEV | forced expiratory volume |
| HBSS | Hank's balanced salt solution |
| HBSS/A | Hank's balanced salt solution/albumin |
| HPLC | high pressure liquid chromatography |
| HRP | horseradish peroxidase |
| HUVEC | human umbilical vein endothelial cells |
| $IC_{50}$ | inhibition of $control_{50}$ |
| ICAM-1 | intercellular cell adhesion molecule-1 |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| $IP_3$ | inositol triphosphate |
| IRS | insulin receptor substrate |
| LT | leukotriene |
| M199 | medium 199 |
| MAPK | mitogen activated protein kinase |

| | |
|---|---|
| MBP | major basic protein |
| MCP | monocyte chemoattractant protein |
| MIP-1α | macrophage inflammatory protein-1α |
| PAF | platelet-activating factor |
| PAF-AH | platelet-activating factor- acetyl hydrolase |
| PAF-AT | platelet-activating factor- acyltransferase |
| PAGE | polyacrylamide gel electrophoresis |
| PG | prostaglandin |
| $PIP_2$ | phosphotidylinositol bisphosphate |
| PKC | protein kinase C |
| PLC | phospholipase C |
| PSGL-1 | P-selectin glycoprotein ligand-1 |
| RANTES | regulated on activation of normal T cell expressed and secreted |
| RIA | radioimmunoassay |
| RT-PCR | reverse transcription–polymerase chain reaction |
| SEM | standard error of the mean |
| SH2 | src homology domain |
| STAT-6 | signal transducers and activators of transcription |
| TLC | thin layer chromatography |
| TNF | tumor necrosis factor |
| VLA-4 | very late antigen-4 |
| VCAM-1 | vascular cell adhesion molecule-1 |

## Epigraph

*Se eye ndzeye pa enum yi a, na eye barima... enyimnyam, awerehyemu, akokodur, ehumbobor, gyedzi.*

- Fante tribal proverb

# CHAPTER ONE: INTRODUCTION & BACKGROUND

Leukocyte adhesion is a pivotal process by which cells migrate from the blood stream to the tissue during an inflammatory process. In asthma, eosinophils are selectively recruited to airway tissue by chemoattraction and the adhesion process. Asthmatic lungs have up to 200 fold increases in eosinophils when compared to neutrophil influx into airway tissue [1]. This increase is due to a number of factors including the enhanced production of eosinophils, recruitment of eosinophils to the airways and finally, a prolonged survival in the tissue. The adhesion events leading to eosinophil recruitment in allergic asthma and related chemotactic aspects are currently a topic of intense investigation.

Adhesion of circulating leukocytes occurs through a defined set of events [2]. Tissue injury results in the activation of endothelial cells, capture of leukocytes and their subsequent emigration. Upon activation, endothelial cells lining the blood vessel walls of a particular tissue will express selectins, which engage specific ligands on passing leukocytes (reviewed in [3]). This process is known as tethering and is responsible for the capture and rolling of leukocytes at the site of recruitment. In addition, receptor-ligand interactions lead to activation of the leukocytes, causing upregulation and activation of integrins [4]. Subsequent engagement of integrins induces a halt in the rolling of leukocytes and causes firm adherence [2]. Finally, the adherent leukocytes emigrate through the endothelial cell barrier via a combination of integrin engagement and chemotaxis to specific attractants [2]. In order for recruitment to occur, endothelial cells must be activated. Specific agonists such as histamine from mast cells or basophils are capable of inducing effects in endothelial cells within minutes. The result is the surface expression of P-selectin in endothelial cells. At the same time, platelet-activating factor (PAF) is rapidly synthesized and expressed on the surface of endothelial cells [5]. Together, PAF and P-selectin engage receptors on the circulating leukocyte and activate these cells [6].

Along with these adhesion molecules, a number of other molecular messengers play a role in the recruitment of leukocytes. Cytokines are small molecular weight compounds that are involved in a number of immune processes including leukocyte

adhesion and emigration [7]. Cytokine-stimulated endothelial cells have been shown to upregulate adhesion molecules that mediate tethering and rolling [8]. In addition, they induce cell expression of chemokines such as MCP-1 [9], RANTES [10] or IL-8 [11], which participate in leukocyte activation and recruitment.

Cytokines are a major contributor to the development of chronic inflammation in the airways of asthmatics. A number of immune cells are involved in the production, secretion and response to cytokines [12]. These include T cells, basophils, mast cells, B cells and eosinophils, all of which play a role in the pathogenesis of asthma (reviewed in [13]). The chronic expression of Th2 type cytokines such as IL-4 and IL-5 play a role in the selective recruitment of eosinophils to the lung (reviewed in [1]). Studies by this lab and others have demonstrated that eosinophils are selectively recruited on IL-4-treated endothelial cells [14-16]. Further, by obstructing PAF receptor engagement using WEB 2086, a blocking antibody, this recruitment can be significantly inhibited (unpublished observations).

The purpose of this study was to determine what effect chronic exposure of IL-4 on endothelial cells had on the production of PAF when the cells were exposed to a secondary agonist such as histamine. We found that IL-4 had a priming effect on histamine-induced-PAF synthesis. Priming occurred in two ways: 1) IL-4-treatment increased the total amount of PAF produced and 2) IL-4 increased endothelial cell sensitivity to histamine. We investigated the mechanism behind these priming effects of IL-4 and determined that in part, they were due to an upregulation of the number of histamine receptors on the surface of the endothelial cells.

## 1. Asthma.

### 1.1 What is Asthma?

Asthma is a disease of the airways resulting from the dysregulation of the immune system [17]. Currently, in the United States alone, there are approximately 12-14 million people afflicted with this condition, of which, approximately 2 million are seen at emergency rooms annually [18]. The mortality rate is 2 per 100 000 people in the United States [19]. The financial burden placed on the health care system is over 6 billion dollars

as a result of treatment for acute asthma attacks alone [18]. In the past two decades the prevalence of this disease has increased at an alarming rate despite great advances in treatment [20].

Although the exact cause(s) of asthma remain unclear, the past decade of research has revealed much of the pathobiology of the disease. Asthma is a chronic inflammatory disorder of the airways resulting from the actions of many immune system cells. In particular, T cells, mast cells, eosinophils and B cells [13]. A challenge or trigger will result in a characteristic narrowing of the airways, secretion of excess mucous and eventual suffocation if not treated. Symptoms include recurrent episodes of wheezing, breathlessness, cough and tightening of the chest [21]. While some of the symptoms and underlying chronic inflammation is constant between asthmatics, the known causes are diverse. As such, sub-classification of this disease remains difficult. A basic division between atopic asthma (allergy driven) and non-atopic asthma has been drawn; the majority of asthmatics are atopic [17]. Though both conditions show very little differences in pathology or response to treatment by corticosteroids, atopic individuals have a characteristic elevated IgE serum level and are prone to allergy related attacks. A more detailed classification system of asthma is based on the frequency of symptoms, inhaled steroid use, baseline lung function and airway responsiveness to challenge. These patients are categorized as persistent, obstructed, episodic, or in remission asthmatics (reviewed in [21]).

## 1.2 Asthma: the pathogenesis of the disease.

### 1.2.1 Pathology

The pathobiology of asthma has been studied using a variety of techniques including pathology studies of fatal asthma attacks, and bronchoscopy to obtain biopsy and BAL samples (reviewed in [21]). When compared to a normal lung, asthmatics exhibit a reduction in airway diameter due to contraction of airway smooth muscle, edema of the bronchial wall and shedding of the epithelium/mucosal exudates. The result of these alterations is an increase in airway resistance and a decrease in forced expiratory volumes (FEV) and flow rates as well as alterations in arterial blood gasses [22]. The hallmark

features of a patient who has asphyxiated from an asthma attack includes gross over distension of the lungs, mucosal plugging of bronchial branches to the terminal bronchiole, hypertrophy of bronchial smooth muscle, epithelial desquamation, thickening of the basement membrane and eosinophils in the bronchial wall [13;22]. In chronic asthma, these acute symptoms may not be visible but underlying abnormalities in airway function as well as inflammation are constantly involved [13].

### 1.2.2 T cells and their role in the asthmatic lung.

A number of immune cells as well as molecular mediators are involved in the pathogenesis of asthma (see figure 1.1). Of prominent importance, T cells play a role in both the establishment of the disease and the recruitment of other cell types to the airways. T cells are generally classified into two groups; CD4+ (helper) cells, which recognize foreign antigens presented on MHC class II antigen presenting cells and are involved in humoral response. The other group is CD8+ (cytotoxic) cells, which recognize antigens presented on MHC class I and are involved in cell-mediated killing. CD4+ helper cells have been implicated in the pathogenesis of the asthma through a number of studies. They are upregulated both in the peripheral blood [23] and in the airways of asthmatics [24]. BAL studies show a correlation between the number of CD4+ T cells and airway hyperresponsiveness [24]. Further, activated CD4+ cell numbers correlate with the number of eosinophils and severity of disease [25]. Finally, allergen challenge of atopic asthmatics has been shown to induce IL-4, IL-5 and GM-CSF mRNA expression in CD4+ T cells [26]. Their importance in the disease process is underscored by the finding that glucocorticoid therapy decreases the number of CD4+ cells in peripheral blood and BAL samples which correlates with an improvement in clinical symptoms of asthma [27].

T helper cells have been divided into two categories, either Th1 or Th2 cells depending upon their expression of cytokines. In a chronic disease such as asthma, CD4+ cells differentiate and follow a Th2 type profile. Th2 cells express cytokines such as IL-4, IL-5, IL-9 and IL-13 as shown by mRNA expression in samples obtained by BAL [24;28-31]. These cytokines play a vital role in the development of the inflammatory response in the airways by setting up a complex set of immune cell players. IL-4 and IL-13 are involved in the isotype switching of immunoglobulin expression of B cells to IgE

[32], recruitment of eosinophils [1:33] and the induction of Th2 type in CD4+ cells [34]. IL-5 is involved in the differentiation, activation and prolonged survival of eosinophils in the airway tissue [35]. Finally, IL-9 is involved in the proliferation and differentiation of mast cells [29]. Each of these cells plays an important role in the subsequent development of inflammation in the lung.

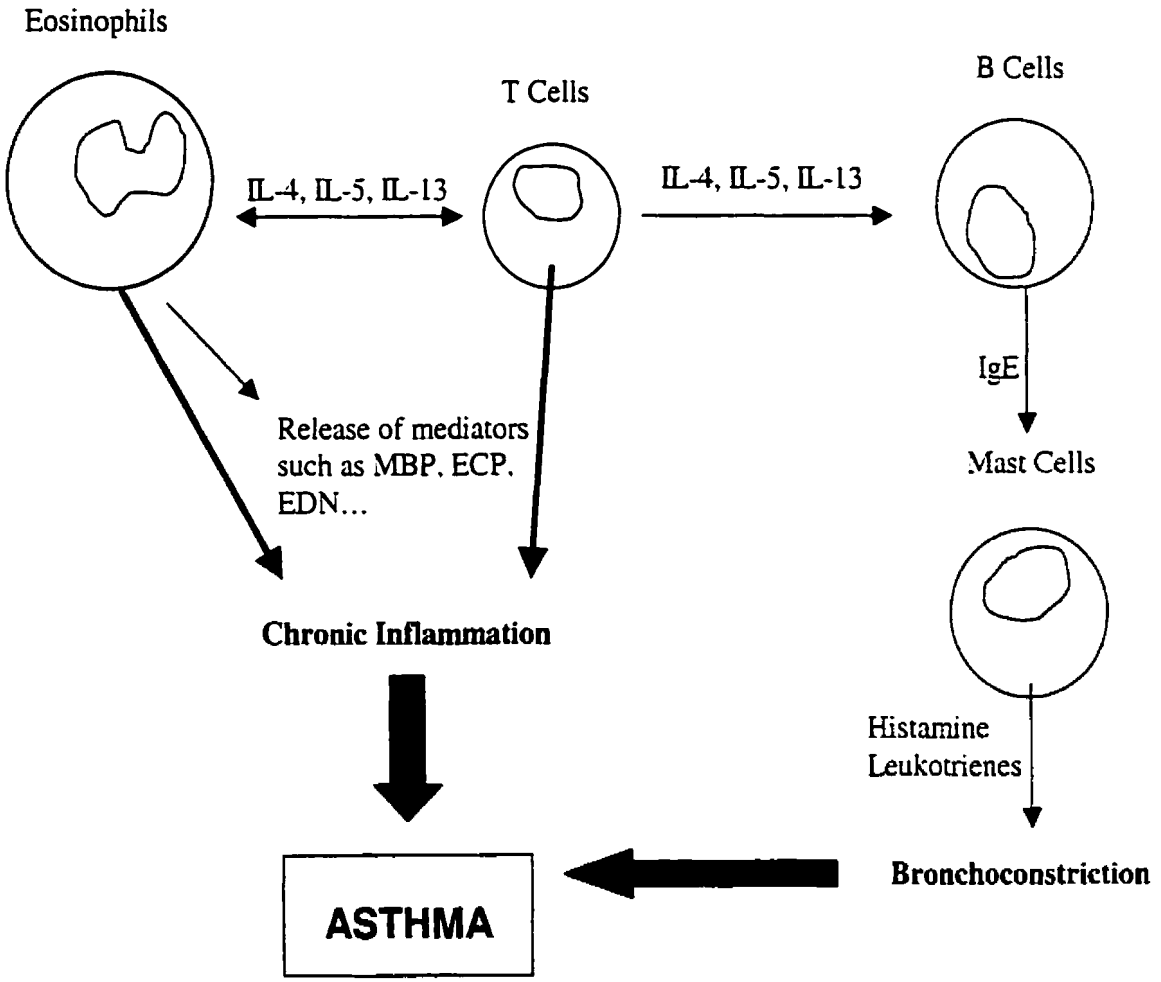



**Figure 1.1** Immune cells involved in the inflammatory response in asthma.

### 1.2.3 Of Mice and Men: The Th1 / Th2 paradigm in asthma.

While Th2 expressed cytokines are predominant in human samples, studying this profile in animals has been difficult to replicate accurately. In murine models the Th1/Th2 cytokine profile of CD4+ T cells is well defined. Th1 cells secrete TNFα, IL-2 and INFγ. In contrast Th2 cells secrete IL-4, IL-5, IL-13 and IL-10 [36]. These two profiles determine the immune response where Th1 cells mediate a delayed type hypersensitivity reaction and are involved in phagocyte-mediated defense mechanisms. In contrast, Th2 cells are thought to mediate chronic events such as allergic reactions in which serum IgE levels are increased. While these two subsets are clearly distinguished in mice, in humans the distinction is not as clear (reviewed in [12]). Although the cytokines secreted are generally the same in allergic conditions, there are some inconsistencies that have been documented. For example, unlike in the mouse models of allergic conditions, IL-2, a Th1 cytokine, is produced both in control and allergic human subjects [37;38].

Generally in humans, IL-4 and IL-5 levels are increased in the asthmatic lung as demonstrated by increased mRNA for these cytokines in biopsy samples as well as direct measurement in BAL fluid [13;39]. In addition, IFNγ levels are low when compared to IL-4 in asthmatic allergic conditions [39]. Because of the wide variability in production of Th2 cytokines, it has been suggested that the amounts of these cytokines is not as important as the ratio between the Th1 and Th2 profiles [12]. The results of these increases in IL-4 and IL-5 correlate with the elevated numbers of activated T cells and eosinophils in BAL samples from asthmatics [25;26].

Recently, the role of IL-13 in asthma has come under investigation [40;41]. IL-13 shares a common IL-4Rα chain in signaling through its receptor and has been shown to perform the same function as IL-4 [42]. Both are able to selectively recruit eosinophils by causing an upregulation of VCAM-1 and P-selectin on the surface of endothelial cells [33;43]. In addition, IL-13 mRNA has been found to be elevated in asthmatics BAL samples when taken from allergen challenged humans [44]. When studied in an allergen challenged mouse model, IL-13 was found to play an important role in the ability of the mouse to develop AHR [40;41]. By blocking IL-13 with a soluble fusion protein, challenged mice showed no AHR, little eosinophil infiltration and decreased mucous production [40]. Interestingly, neutralization of IL-13 was able to reverse AHR when established while

blocking IL-4 cannot. This suggests that IL-4 is more important for establishing a Th2 immune response and not contributing to effector functions [40]. Taken together, though in humans the Th2 profile is not as defined as in mice, there is still a trend toward the expression of Th2-type cytokines.

### 1.2.4 The role of eosinophils in asthma.

Eosinophils are an important cell type found in the airways and are characteristic of asthma sufferers. Eosinophils constitute approximately 1-5% of leukocytes in BAL samples [25] and upon activation produce cytotoxic proteins, lipid mediators and cytokines [13]. In biopsy studies, eosinophils show an average 60-fold increase when compared to the number of infiltrating neutrophils, demonstrating a selective recruitment for eosinophils in the asthmatic lung [1]. After an allergen challenge, eosinophils influx in 4-6 hours and persist for at least 24 hours in these samples [45;46]. Eosinophils are capable of producing and releasing a number of mediators such as major basic protein (MBP), eosinophilic cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN) [47]. Mediators, such as MBP, are found in sputum samples and are suspected to play a role in epithelial shedding as well as bronchoconstriction of the airway [48;49]. Eosinophils are also capable of synthesizing lipid mediators such as LTC4 and PAF. In particular, LTC4 plays a role in bronchoconstriction, mucous secretion and changes in vasopermeability in airway tissue [50]. Eosinophils capacity to cause tissue damage and alter airway function is furthered by their expression of a number of cytokines and chemokines (see figure 1.2).

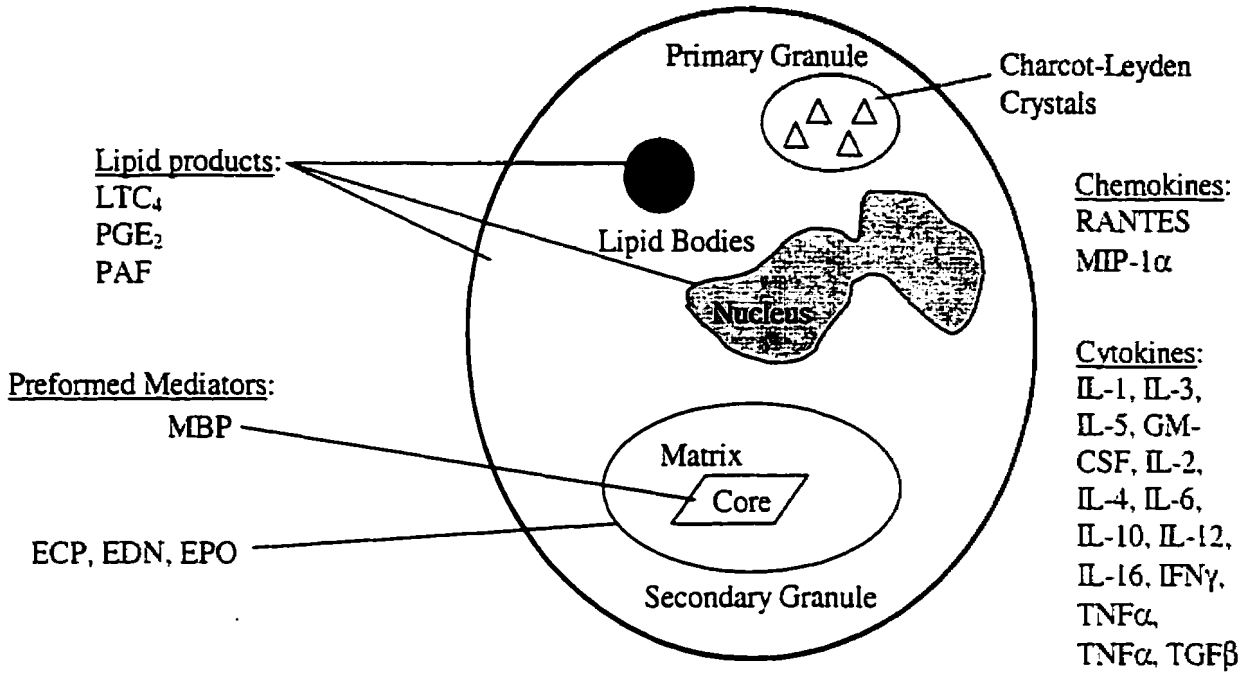



**Figure 1.2** Mediators of inflammation released by eosinophils.

The importance of eosinophils in the development of AHR in asthma is demonstrated by a number of animal models. For example, IL-5 is required for the recruitment, activation and prolonged survival of these cells in the airway tissue. Animal models in which IL-5 was ablated with antibodies or by gene knockout show an inability to mount an eosinophilic allergic response and AHR [51:52]. IL-13 knockout studies have demonstrated an important role for eosinophils and development of AHR in a murine system [40:41]. Finally, studies of P-selectin-deficient mice demonstrated a reduction in the recruitment of eosinophils (and other lymphocytes) as well as reduced AHR in a challenged model [53]. Together, current evidence suggests a strong correlation between the presence of eosinophils and the development of AHR though this is not always the case. A recent study by Tournoy *et al.* demonstrated that eosinophils were not required for allergen-induced AHR when low concentrations of allergen were used [54].

Bronchial biopsies of asthmatic lungs have been performed and are representative of the actual site of the disease. Inflammatory cells such as eosinophils are seen adhering to vessel walls, the epithelial cells are shed and there is airway edema. Eosinophils have been found in the sputum and epithelial layer of the airway, which requires that they travel across the basement membrane [55;56]. There is also an increase of eosinophils in the bronchial mucosa, which is related to an increase in severity of asthma symptoms. The thickening of the lamina reticularis seen in asthmatic lungs is due to an increase in collagen, fibronectin and tenascin deposition [21;57]. The presence of laminin allows for the attachment of monocytes, T cells, mast cells and eosinophils to the basement membrane. Eosinophils can adhere to the extracellular matrix via integrin $\alpha 6\beta 1$ for laminin [58] and $\alpha 4\beta 7$ for fibronectin [59]. The latter interaction has been shown to promote eosinophil survival in the extracellular matrix and would enable eosinophils to cross the basement membrane and enter the airway lumen.

### 1.3 Models of Asthma.

A number of models for asthma have been used including those in primates, rabbits, horses, dogs and guinea pigs, however the recent trend is to use rats and mice to understand the role of the immune system in airway abnormality. Mice have been particularly useful because their genes can be either knocked out or over expressed and the effects on AHR assessed. However, some variations in AHR seen in murine models are due to genetic differences between strains of inbred mice. Because of this phenomena, these models have been instrumental in the study of gene candidates for AHR (reviewed in [60]). While murine models have been very useful, they still have not been accurately descriptive of the human disease. Models have not been able to mimic the chronic inflammation seen in human airways. Instead, they have been more acute and do not show significant airway remodeling or other characteristic signs of human asthma such as epithelial shedding or eosinophil degranulation in the epithelium and edema.

For example, one model used to study inflammation in the lungs chronically exposes rats to $SO_2$ gas. These rats develop chronic bronchitis and exhibit airway inflammation, hyperresponsiveness and mucosal hypersecretion [60]. However, analysis of lavage fluid and airway tissue for mononuclear cells and neutrophils show differences in

the number of infiltrating cells into the lumen between humans and rats. This is most likely due to differences in chemotactic and adhesion molecules expressed by epithelial and sub-epithelial cells in the airways of rats and humans (reviewed in[60]).

The understanding of the role of Th2 cytokines and eosinophilic inflammation has been furthered due to studies in transgenic mice. For example, IL-5 deficient mice showed normal IgE production and T cell responses to allergen challenge demonstrating that IL-5 is not required for B and T cell responses [52:61]. However, IL-5 is required for eosinophil accumulation in the lungs and these deficient mice failed to develop airway hyperresponsiveness suggesting that eosinophil infiltration is a prerequisite for developing AHR [52]. Also, studies of IL-4 deficient mice did not develop IgE production in response to airway challenge and demonstrated very little eosinophil infiltration of the airways. Again, this is associated with failure to develop AHR to methacholine challenge in this model [62]. The relative importance of either IL-4 or IL-5 in the development of AHR remains controversial with conflicting data demonstrating either of the two cytokines being responsible for recruitment of eosinophils [40:41:51:63]. However, these studies have led to greater insight as to the mechanism of action of cytokines seen in human asthmatics.

We have chosen to study endothelial cells using HUVEC as our model system. These cells are cultured and used after one passage in order to retain their differentiated endothelial cell characteristics such as the ability to produce PAF. A number of studies have used HUVEC as a model for analyzing the molecular interactions of adhesion, including the role of PAF in this process [64-66].

## 2. The Endothelium and Adhesion.

### 2.1 The adhesion pathway.

During an inflammatory response leukocytes are recruited from the vasculature to a specific site in the tissue. In asthma, this inflammatory process occurs inappropriately,

resulting in the accumulation of leukocytes such as eosinophils in the airways of the lungs.

The recruitment of leukocytes to tissue follows a sequentially organized cascade of events beginning with the tethering and rolling of leukocytes on the endothelial cells lining the blood vessel wall (see figure 1.3) [2]. This initial step is mediated predominately by a family of molecules known as selectins [3]. Selectins are a group of transmembrane glycoproteins that bind to sialylated and fucosylated oligosaccharides in a calcium-dependent manner [3;4]. There are three members of this family: P- and E-selectin, which are expressed on the surface of endothelial cells, and L-selectin, which is expressed on the surface of leukocytes [4]. P-selectin is stored pre-formed in Weibel-Palade bodies in endothelial cells and upon activation with agonists such as histamine or thrombin, P-selectin is expressed on the cell surface [67]. Expression is fast (within minutes) and is followed by rapid internalization and recycling of the P-selectin molecule to intracellular stores [68]. It's ligand, PSGL-1, is found on the surface of most bone marrow derived cells including eosinophils [4]. In addition, E-selectin has been shown to mediate leukocyte tethering under physiological shear stress in a L-selectin-dependent manner [69]. There are a number of ligands for E-selectin including PSGL-1, ESL-1 and L-selectin itself [4].

Following leukocyte rolling, the cells firmly adhere to the endothelial cell layer. This process is mediated by a second family of adhesion molecules known as integrins that bind to immunoglobulin superfamily adhesion molecules on endothelial cells [2]. Integrins are upregulated in response to specific signaling molecules such as chemokines, cytokines or other mediators including PAF [70]. There are four major subfamilies of integrins; the β1 integrins, β2 (or leukocyte) integrins, β3 (or cytoadhesins) and finally the β7 integrins. All integrins are heterodimers composed of both an α and β chain that undergo conformational changes upon activation (reviewed in [4]).

Integrins are generally thought to play a primary role in the firm adhesion process of leukocyte attachment. In particular, two major families involved in leukocyte adhesion include the α4's, such as VLA-4 or very late antigen-4 (α4β1;CD49d/CD29) and the β2's, such as LFA-1 or lymphocyte function-associated antigen-1 (αLβ2; CD11a/CD18). Their ligands vary and include members of the immunoglobulin superfamily such as VCAM-1 and ICAM-1 as well as extracellular matrix proteins such

as fibronectin, laminin and collagen [4]. In addition to their role in adhesion, integrins have been shown to mediate tethering and rolling interactions; I will discuss this in detail in regards to eosinophil recruitment in latter sections. The result of the activation of the adhesion pathway is the diapedesis of leukocytes to specific tissue sites in response to a chemotactic signal.

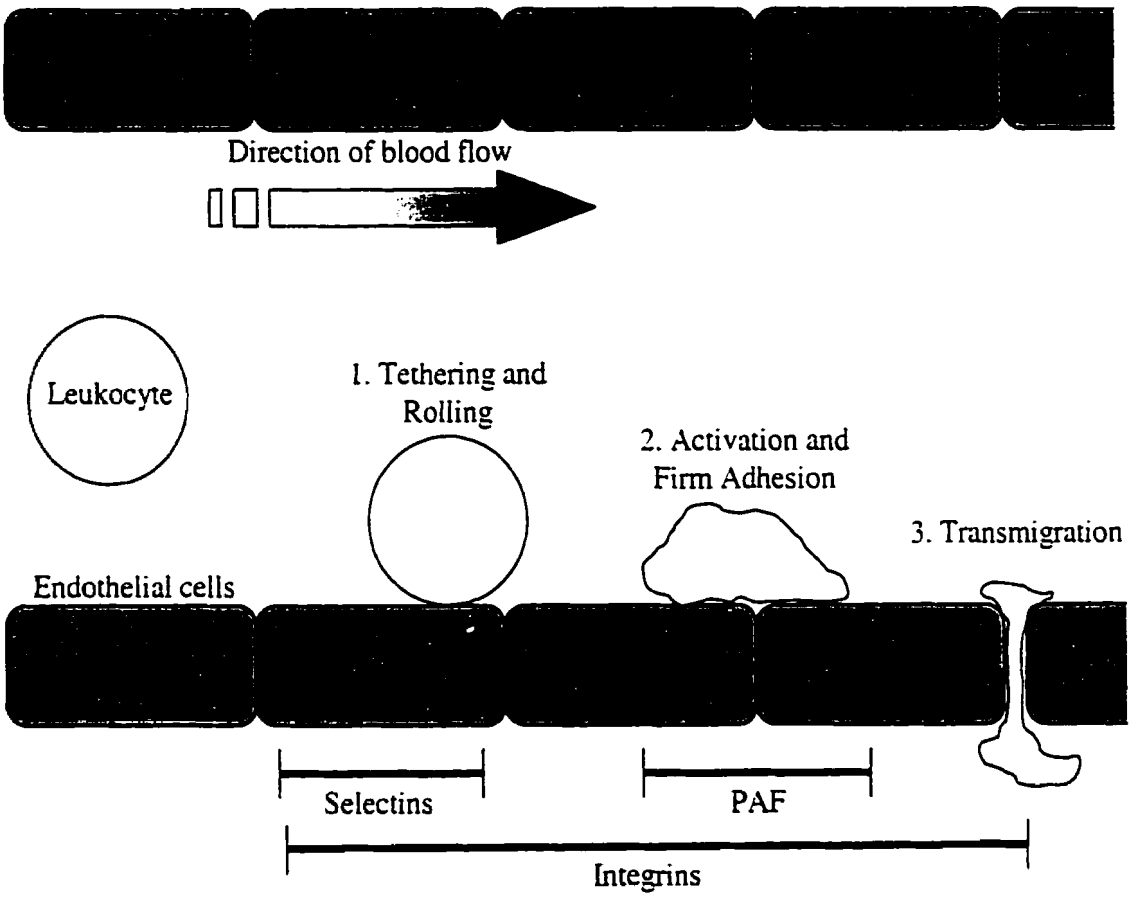



**Figure 1.3** The Adhesion Pathway.

## 2.2 The recruitment of eosinophils.

As previously mentioned, eosinophils are selectively recruited from the circulation into the airway tissue in asthma. Endothelial cells lining the blood vessel walls mediate this selectivity, in part. Upon stimulation with specific agonists, the endothelial cells will upregulate expression of certain adhesion molecules [71;72]. Specifically, activation of endothelial cells results in the increased surface expression of P-selectin, E-selectin and VCAM-1 [4]. Exposure to IL-4, oncostatin M or IL-13 will induce P-selectin expression on the surface of HUVEC [33;73]. Eosinophils bind with greater affinity to P-selectin compared to neutrophils, which binds with a greater affinity to E-selectin [68;74]. The importance of P-selectin expression is demonstrated in animal studies where eosinophil accumulation was lower in antigen challenged P-selectin deficient mice [53]. Further, when P-selectin and E-selectin were blocked using antibodies, only the P-selectin-blocked mice showed reduced eosinophil accumulation, suggesting a greater role for P-selectin in eosinophil recruitment [75].

Interestingly, the tethering and rolling step of eosinophils on endothelium seems to work best when P-selectin and the integrin VLA-4 are working in conjunction [33;76]. It should be mentioned that VLA-4 has been demonstrated to mediate tethering of eosinophils under flow conditions [77]. While VLA-4 is expressed on the surface of eosinophils, it is not expressed by neutrophils and is therefore suspected to be involved in the selective recruitment of eosinophils and T-cells [1]. IL-4 and IL-13 have been shown to be required for airway hyperactivity to inhaled antigen in murine models [40;41]. Both IL-4 and IL-13 are able to induce upregulation of VCAM-1, the ligand for VLA-4. Animal studies in which VLA-4 or VCAM-1 were blocked showed that eosinophil migration and subsequent airway hyperresponsiveness could be inhibited in the lungs [78](reviewed in [1]).

Finally, the afore mentioned immunoglobulin superfamily molecules VCAM-1 and ICAM-1 act as ligands for integrin engagement, thus facilitating firm adhesion of leukocytes to the apical surface of the endothelial cell. The final step in the adhesion pathway is diapedesis, in which the VLA-4/VCAM-1 and CD18/ICAM-1 interactions play a crucial role (reviewed in [4]).

In addition to upregulation of adhesion molecules, endothelial cells produce a host of leukocyte stimulating factors. These include chemokines such as monocytes

chemotactic protein-1 (MCP-1) and eotaxin [79] as well as lipid mediators such as PAF. Each of these pro-inflammatory molecules is able to activate leukocytes and in some cases, cause deleterious effects on surrounding tissue. Activation of tethered eosinophils is an essential step in the adhesion process. Exposure to PAF, which is synthesized and expressed by endothelial cells in response to specific stimuli such as histamine or thrombin, has been shown to upregulate integrins in neutrophils [6] and activate eosinophils [80] (see figure 2.2). In addition, eosinophils are able to respond to exposure to chemokines such as eotaxin, MCP-3 and -4 and MIP-1α, all of which are upregulated in asthmatics [1]. These chemokines act through the CCR-3 receptor (except for MIP-1α which acts through CCR-1) and are chemotactic for eosinophils. Interestingly, the cytokines IL-4 and IL-13 are able to induce expression of eotaxin in fibroblasts and epithelial cells. thereby contributing to the recruitment of eosinophils to the airways.

In summary, eosinophils are recruited to the airways through a number of events. A variety of factors influence the selective recruitment of eosinophils to the airway tissue including cytokines, chemokines and adhesion molecules. In particular, the effect of Th2 cytokines such as IL-4 and IL-13 demonstrate an ability to recruit eosinophils over neutrophils. These cytokines are implicated in the upregulation of both P-selectin and VCAM-1/VLA-4 interactions as well as the production of chemoattractants such as eotaxin. IL-4 stimulated endothelial cells have been shown to selectively recruit eosinophils over neutrophils from whole blood [14]. Thus, a major contributor to the selective recruitment of eosinophils seems to lie in the actions of the cytokine IL-4.

## 3. The major players in this study: PAF, IL-4 and Histamine.

### 3.1 Platelet-activating factor.

PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) or platelet-activating factor is a relatively simple phospholipid molecule whose function gives way to a complex array of effects. These include such biological activities as platelet activation, airway constriction, development of bronchial hyperresponsiveness and induction of microvascular leakage and edema [81]. PAF is synthesized by a number of cell types

including endothelial cells, neutrophils, eosinophils and lymphocytes [82]; interestingly, only endothelial cells express PAF on their surface, whereas other cell types secrete PAF [83].

PAF has been extensively studied in endothelial cells. It is derived from processing of membrane phospholipids which contain a phosphocholine group. Two pathways within cells synthesize PAF: the remodeling pathway or the *de novo* pathway. The latter is generally thought to be involved in the maintenance of normal physiological concentrations of PAF rather than in inflammatory responses [84]. An illustration of the remodeling pathway is shown in figure 1.4. The basic synthetic pathway begins with the cleavage of membrane phospholipids by phospholipase $A_2$ ($PLA_2$) to form the intermediate molecule lyso-PAF. Subsequently, PAF-acetyltransferase (PAF-AT) transfers an acetyl group from acetyl-CoA to the *sn*-2 position of lyso-PAF to produce the final product, PAF. While activation of $PLA_2$ is necessary for initiating synthesis of PAF, the rate determining step of the reaction is hypothesized to be the activity of PAF-AT. Studies by Holland *et al.* have demonstrated that thrombin increased the activity of PAF-AT in endothelial cells, which resulted in an increase in PAF production [85].

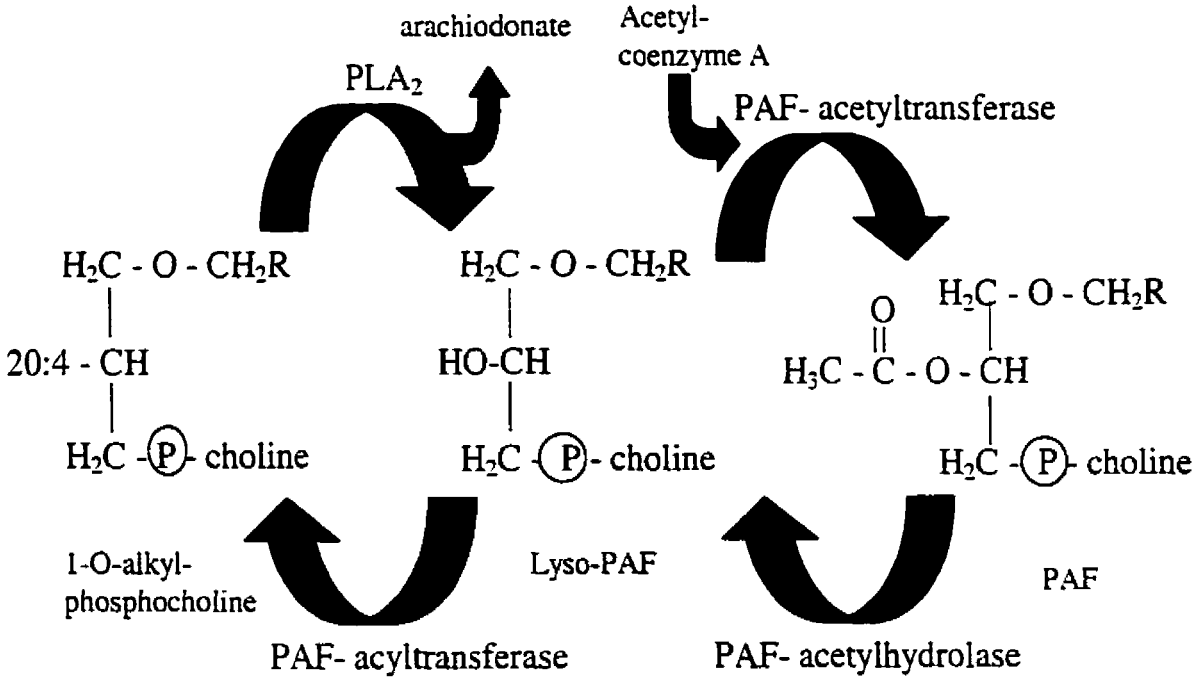



**Figure 1.4** The remodeling pathway for PAF synthesis.

The molecular structure of PAF is important for its ability to act as an effector molecule (see figure 1.5). At the *sn*-2 position, a short acyl chain is esterified to the glycerol backbone structure of the phospholipid. This acetate group is critical for PAF's biological activity as increasing the chain length will reduce its ability to bind to its receptor. In addition, the *sn*-1 ether linkage of a fatty alcohol chain (usually 16-18 carbon atoms) is essential for PAF activity (reviewed in [86]). A study by Whateley *et al.* demonstrated that two isoforms of PAF are non-selectively produced when endothelial cells are stimulated [87]. The *sn*-1-alkyl as well as the *sn*-1-acyl analog of PAF is synthesized simultaneously with the latter being the predominant form [87]. However, the potency of this acyl-PAF analog is approximately 1% that of PAF [86]. Further studies by Bussolino *et al.* have contradicted these findings using HPLC-tandem mass spectroscopy showing that endothelial cells stimulated with either TNFα, IL-1 or elastase were capable of synthesizing alkyl-PAF over acyl-PAF in 3-5 fold greater quantities [88]. The types of bonds and structural changes described above illustrate the importance of stereochemistry for biological activity of PAF.

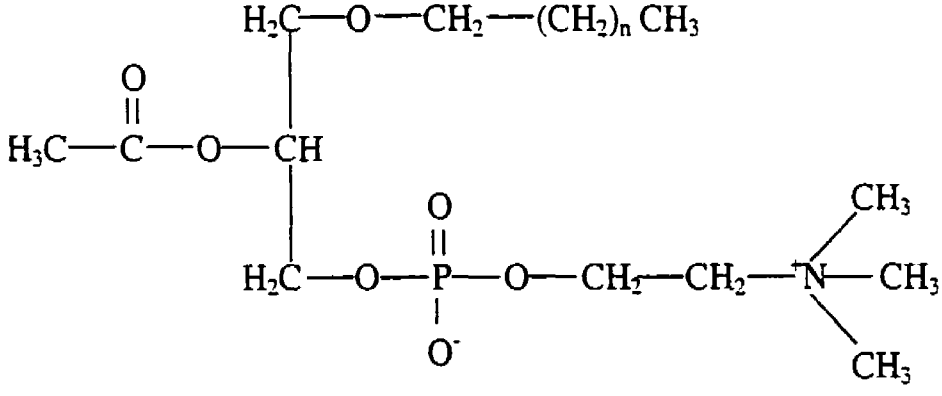


**Figure 1.5** Chemical Structure of PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); n= 14-16

Synthesis of PAF is tightly regulated and requires an influx of intracellular calcium [85:89]. For PAF to be synthesized, precursor lipids must be available for processing and conversion by $PLA_2$ and acetyltransferase enzymes [84]. This process begins with the activation of G protein coupled receptors such as histamine or thrombin. The result is an activation of the enzyme PLC which couples to phosphatidylinositol 4,5-bisphosphate ($PIP_2$) in membrane phospholipids and acts to convert $PIP_2$ into inositol 1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG) [90]. This is achieved by a $Ca^{2+}$ dependent coupling of $PIP_2$ to the PH domain of either PLC-β or γ and the subsequent hydrolysis into $IP_3$ and DAG products [90]. $IP_3$ acts to release intracellular stores of calcium within the cell cytosol by binding directly to its receptor on $Ca^{2+}$ storage vessicles. In addition, the binding of DAG to protein kinase C (PKC) reduces the enzymes requirement for $Ca^{2+}$ and increases its activity [91]. The $PLA_2$ enzyme is then activated by an influx of calcium from the extracellular milieu and the phosphorylation mediated by active PKC [92]. PKC reduces $PLA_2$'s sensitivity to calcium which is required for binding of $PLA_2$ to membrane phospholipid [92]. PKC can also play a role in $PLA_2$ activation by triggering a kinase cascade leading to MAPK activation [93]. Activation of the p42 or p44 MAPKs increases the activity of $PLA_2$ and results in a characteristic gel shift in *in vitro* experiments [94:95]. Interestingly, PKC is also involved in the inactivation of the PAF synthesis pathway by inhibiting the calcium mobilization necessary for $PLA_2$ activation. A negative feedback pathway has been hypothesized to exist from studies by Heller *et al.* in which a brief stimulation with PMA inhibited thrombin-induced PAF production in endothelial cells [96]. However, this PKC mediated process has not been characterized. Finally, activation of PKC and $PLA_2$ generates other inflammatory mediators such as arachiodonate metabolites including prostaglandins ($PGI_2$ and $PGE_2$) [97:98]. (see figure 1.6)

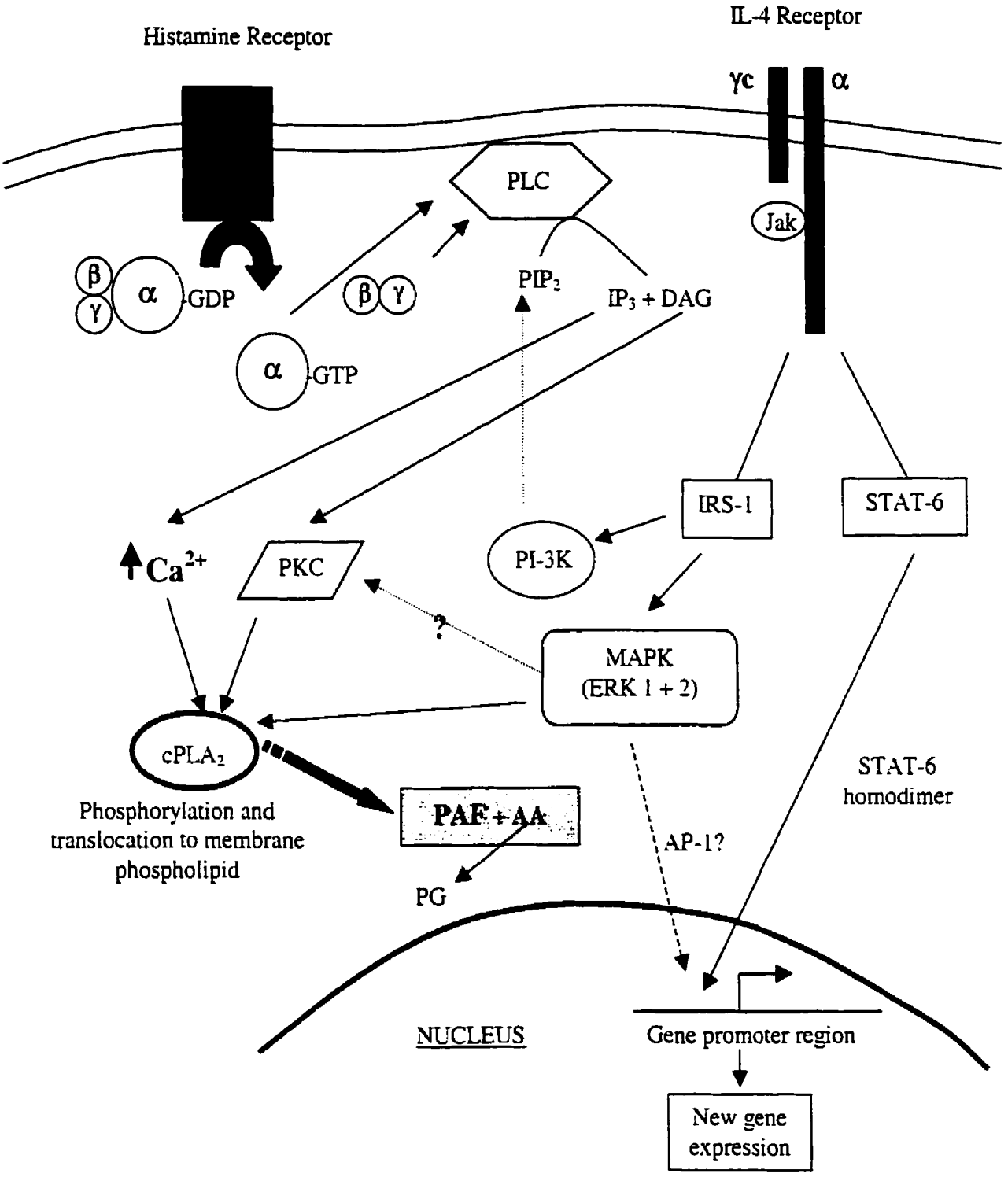



**Figure 1.6** Summary of the intracellular signaling events of histamine and IL-4 receptor engagement.

PAF binds to a specific receptor, which was originally isolated and cloned from the lungs of a guinea pig [99]. It is a seven transmembrane, G-protein coupled receptor whose engagement results in activation of the receptor mediated by multiple G proteins. These receptors are found on the surface membranes of neutrophils and eosinophils and activate P-selectin tethered leukocytes, causing them to upregulate the expression of integrins on their surface. Lorant *et al.* have shown that a rise in intracellular $Ca^{2+}$ and the functional upregulation of β2-integrins are the result of PAF receptor engagement on neutrophils [100]. The result is increased binding between the endothelium and the leukocyte causing firm adhesion and eventually leading to diapedesis. Current evidence shows that PAF plays an important role in the activation of leukocytes, and its upregulation increases the transmigration rate of leukocytes into tissue [72].

PAF has been suggested to be a potent mediator in asthma. When given by inhalation, PAF causes bronchoconstriction in normal and asthmatic lungs as well as increases AHR (reviewed in [81]). It is also involved in the recruitment and activation of eosinophils, release of $LTC_4$ from eosinophils and increase in mucous secretion [81;101]. Despite it's significant involvement in the pathogenesis of asthma, clinical trials using PAF receptor antagonists such as WEB2086, UK74505 and most recently, SR27417A have been unable to show a significant effect in asthmatic patients [102].

Finally, PAF is broken down through the action of PAF-acetylhydrolase (PAF-AH), which removes an acetyl group from the *sn*-2 position of PAF leaving lyso-PAF [103]. Next, PAF-acyltransferase converts lyso-PAF to a phosphocholine containing phospholipid molecule [86]. PAF-AH is found both in plasma and in tissue with a number of isoforms identified including intra- and extracellular forms and is constitutively active at basal levels [103]. PAF-AH deficiency has been shown to be a modulator in asthma and correlates to increased severity of the disease [104]. A recent study in a murine model suggested the potential therapeutic value of using recombinant PAF-AH as a means of down regulating the effects of PAF in airway hyperresponsiveness [105]. While the study indicated a reduction in eosinophil infiltration in the airway tissue as well as reduced sensitivity to methacholine challenge, results from clinical human trials have shown no significant benefit to asthmatic patients [106].

The localization of PAF production has not been characterized conclusively in cells. $PLA_2$ has been found to couple to a number of sites within cells including the nuclear membrane, endoplasmic reticular membrane and plasma membrane of the cell [107](reviewed in [86]). Besides the membrane phospholipids, another possible source of substrate for PAF are lipid body structures. Studies have shown that lipid bodies can be sites of eicosanoid production as the enzymes involved in their production such as cyclooxygenase, 5-lipoxygenase and $LTC_4$ synthase have been localized to these structures [108:109]. This suggests that lipid bodies may act as a possible source for substrate used in PAF synthesis although no clear evidence for this exists. These lipid bodies have been shown to be upregulated in leukocytes in response to certain inflammatory mediators such as PAF and other arachidonic acid containing lipids. Though the formation and function of these bodies is not understood, the induction of these bodies is regulated by both PLC and PKC activity [110:111].

### 3.2 Interleukin-4.

IL-4 is a pleiotropic cytokine produced primarily by T cells, but also by mast cells and basophils in response to IgE cross-linking of its receptor, and finally, eosinophils. Its main functions are to induce IgE production in B cells and promote the shift to a Th2 cytokine profile in CD4+ cells (reviewed in [112]). The IL-4 receptor is found on endothelial cells and consists of a 140-kDa IL-4Rα chain that binds to both IL-4 and IL-13 [42]. Physiological signaling is then mediated through the heterodimerization of the IL-4Rα chain to a γc chain [112]. The IL-4Rα chain is essential for signaling as demonstrated by IL-4 knockout mice, which show no IgE synthesis, AHR or Th2 mediated immune responses [63:113]. Upon binding of IL-4 to its receptor, the IL-4 receptor is phosphorylated and Jak kinases are activated. Jak1 associates with the IL-4Rα chain while Jak3 associates with the γc chain [42:112].

Signaling by the IL-4 receptor can then follow three alternative routes (see figure 1.6) (reviewed in [112]). The first is through the signal transducers and activators of transcription-6 (STAT6) pathway, which is responsible for activation or expression of IL-4 responsive genes. Here, STAT6 binds to the phosphorylated receptor through a SH2 domain. The phosphorylated STAT6 molecule is released from the receptor chain and

binds with another STAT6 molecule to form a homodimer. This active homodimer then translocates to the nucleus and binds to the promoter regions of specific IL-4 responsive genes. The importance of STAT6 signaling is illustrated through knockout studies that show a lack of Th2 mediated inflammation as well as IgE production [114]. In addition, these STAT6-/- mice had no eosinophilia and reduced AHR to allergen challenge [115]. The second route of IL-4 receptor signaling is through the insulin receptor substrate-1 and -2 (IRS-1 and -2) which are involved in IL-4 mediated cell proliferation. Finally, the third alternative signaling pathway in IL-4 receptor engagement is the activation of PI3-kinase. The PI3-K enzyme is heterodimer containing a regulatory (p85) and a catalytic (p110) subunit. The regulatory subunit engages a phosphorylated IRS-1/2 molecule, which is attached to the IL-4Rα chain. This engagement leads to a conformational change in the PI3-K complex and allows the catalytic subunit to phosphorylate membrane lipids and Ser/Thr residues of proteins (reviewed in [116]). Signaling through this pathway can potentially contribute to the increase in $PIP_2$ levels, used in the synthesis of PAF [112].

### 3.3 Histamine and its receptors.

B cells are responsible for the production of antigen specific immunoglobulins in mounting a humoral response to antigen. The exposure of IL-4 and IL-13 to B cells induces a switch to IgE synthesis by these cells [32]. IgE molecules are then able to bind to FcεRI receptors on the surface of mast cells. Interestingly, IgE is found in BAL fluid from atopic asthmatics [117], however, an increase in B cell numbers has not been seen in biopsy studies of asthmatics [118]. Upon allergen exposure, binding of foreign particles cross-links the IgE bound to Fc receptors and activates mast cells. Activation of mast cells by exposure to an allergen or other stimulus will cause degranulation and the release of a number of mediators such as histamine or lipid mediators from these cells. Histamine has been found to be elevated in the BAL fluid of asthmatics [119]. It has a variety of actions on different tissue including the mediation of bronchoconstriction of smooth muscle [120] and the increase of mucosal secretion by pulmonary tissue [121], both of which are primary afflictions in asthmatics.

Histamine is primarily stored in mast cells and basophils in humans; mast cells are found in the alveolar walls of lung tissue [122;123]. It is produced by the degradation of

histidine by the enzyme histamine decarboxylase [124]. Upon activation, mast cells or basophils are able to secrete histamine along with other inflammatory mediators, which results in bronchoconstriction. In fact, studies have shown a correlation between increased histamine concentration in bronchoalveolar lavage samples and increased bronchoconstriction [125]. As well, increased histamine concentrations have been associated with hyperresponsiveness induced by methacholine challenge as measured by BAL samples [119]. However, of most importance is the observation that BAL fluid from asthmatics contains higher concentrations of histamine than BAL fluid from normal subjects[119:125], leading to the hypothesis that histamine plays an important role in the pathogenesis of allergic asthma.

One function of histamine in the inflammatory cascade is the induction of PAF synthesis within endothelial cells [83]. Histamine has also been demonstrated to induce prostaglandin synthesis in HUVEC [83] and lung tissue [126]. While there are three major receptors for histamine found on the surface of cells, the majority of effects seen in endothelial cells have been demonstrated to be mediated by the H1 receptor [127-130]. Studies on histamine-induced $IP_3$ accumulation and $PGI_2$ synthesis in HUVEC have shown these responses are mediated exclusively through the H1 receptor [127:128]. Though HUVEC histamine receptors have never been fully characterized, studies to date have only shown a role for the H1 receptor; presence of the H2 or H3 receptors on HUVEC cannot be proven until these receptors have been cloned [127]. The H1 receptor is a seven transmembrane-G protein coupled receptor with phosphorylation sites for protein kinase C [131]. It has been detected in airway smooth muscle and is responsible for a variety of intracellular responses such as the production of inositol phosphates, influx of calcium and the release of arachidonic acid [127:128:130]. H1 receptors signal through the $G\alpha_q$ family of G proteins which activate PLCβ and mitogen-activated protein kinase (MAPK) pathways in cells [132]. This has been shown by Ab mediated blocking of the $G\alpha_q$-like proteins in histamine-induced synthesis of inositol phosphates [133]. (see figure 1.6)

## 4. New alternatives for treatment of asthma.

While most current therapy is designed to treat symptoms of asthma such as reversing bronchoconstriction, the most effective therapy is to prevent chronic airway inflammation. New avenues of reversing and preventing airway inflammation are currently being explored. Two general approaches are being taken, the first is to prevent adhesion of specific cells such as eosinophils and their recruitment to airway tissue. For example, by inhibiting VLA-4 interactions with VCAM-1, eosinophils cannot successfully roll and adhere to the endothelium and would prevent their emigration into airway tissue. Results of clinical trials testing the effectiveness of low molecular weight VLA-4 antagonists are expected in the near future.

The second approach to altering the asthmatic lung is to manipulate the cytokine profile of Th2 producing cells. Blockade of IL-5 responses in animal models using antibodies have demonstrated an inhibition of eosinophilia as well as AHR [61]. Studies involving IL-5 neutralizing antibodies have been tested in humans as well and have shown a significant decrease in eosinophil accumulation in blood and airways however, upon allergen exposure, no significant reduction in AHR is seen [134]. IL-4 and its receptor are also potential targets of therapeutic intervention as suggested by IL-4 knockout studies in mice as well as limited trials in humans. A recent study by Henderson *et al.* demonstrated the use of soluble IL-4 receptor in a murine models of asthma. Results showed reduced eosinophil airway infiltration, decreased VCAM-1 expression, and mucous secretion and finally, a reduction in late phase pulmonary inflammation [135]. Clinical trials of sIL-4R treatment have shown a reduced need for corticosteroids in moderate asthmatics during Phase I/II trials [136]. Finally, an induction of a Th1 profile in subjects remains a potential route of alleviating the Th2 induced disease. This is achieved through the administration of IL-10, IL-12 or IFNγ, which have been shown to be upregulated upon corticosteroid therapy and play a role in the active suppression of Th2 cells [137].

Other novel potential therapies have looked at leukotriene inhibitors which have proven to be effective in some forms of asthma such as aspirin-induced asthma. While no clear protocol has been established for anti-leukotriene therapy, current studies

suggest it may be useful in supplementing current treatment in severe asthmatics [138]. In addition, studies in blocking IgE have shown possible benefits by inhibiting mast cell degranulation which triggers histamine and leukotriene release. Clinical trials on IgE neutralizing antibodies such as rhuMAb-E25 have shown significant reduction in AHR to allergen challenge, interestingly however, their total amount of circulating IgE increased with treatment [134].

## 5. Enhancing the model of leukocyte adhesion; can endothelial cells be primed?

The leukocyte adhesion pathway is complex and has been studied extensively; this includes the role of PAF during the leukocyte recruitment process. While it has been demonstrated that inhibition of PAF will significantly reduce emigration of leukocytes [139], its ability to activate rolling leukocytes is not completely understood. Histamine is able to induce the production and expression of PAF on the surface of endothelial cells. However, the exposure of cytokines such as IL-4 to endothelial cells and their subsequent ability to recruit leukocytes has not been completely characterized. We have studied the effects of chronic exposure of cytokines normally found in asthmatic lungs on endothelial cells. Specifically, the role of IL-4 and how it affects PAF production within endothelial cells in response to histamine induction. As well, other cytokines such as TNFα were used as a comparison to determine if IL-4 effects on HUVEC are specific to this cytokine.

## 6. Hypothesis and specific aims:

**Hypothesis:** ***We hypothesize that the cytokine IL-4 is able to prime endothelial cells to produce platelet-activating factor (PAF).*** *This priming event would enhance the leukocyte adhesion process allowing for higher emigration of cells such as eosinophils into the lung tissue as seen in asthma.*

**Specific Aims and objectives:**

1. *Are cytokines such as IL-4 and TNFα able to prime HUVEC for histamine induced PAF synthesis?*
    - Do cytokines alone induce PAF synthesis in HUVEC?
    - To determine what effect cytokine treatment has on the production of PAF in histamine-induced HUVEC?
    - Is the priming effect specific for the cytokine IL-4?
    - Is the priming effect observed specific for histamine induction of PAF, or are other secondary agonists capable of inducing the same effect?
    - Is new protein synthesis required for priming?

Priming is defined as a reduction in the threshold required for a stimulus to exact its effects as compared to basal levels. In IL-4 priming of histamine-induced PAF synthesis, two components are seen. The first is an increase in the total amount of histamine-induced PAF synthesis following IL-4-treatment. The second is an increase in sensitivity to histamine in the IL-4-treated HUVEC. Specific Aims 2 and 3 will address the mechanisms behind each of these phenomena separately.

2. *Characterize the mechanism responsible for increased PAF production in IL-4 treated HUVEC focusing on the PAF synthesis pathway and substrate availability.*
    - Determine the effect of IL-4 on $PLA_2$ protein levels and activity as a possible mechanism of increased PAF synthesis.
    - Is the accumulation of PAF the result of reduced breakdown of PAF by PAF-acetylhydrolase?
    - Is the increased amount of PAF in IL-4-treated cells due to an increase in the amount of substrate (lipid bodies)?
    - Does IL-4 affect processing (PLC) of membrane phospholipids in the treated HUVEC?

3. *Characterize the role of the histamine receptor in IL-4-mediated priming of HUVEC.*
    - Does IL-4 prime HUVEC for other responses induced by histamine? These include changes in sensitivity for histamine-induced calcium flux and prostaglandin synthesis.
    - Is there an increase in H1 receptors on IL-4-treated HUVEC?

# CHAPTER TWO: METHODS & MATERIALS

## 2.1 Materials

Human recombinant IL-4, TNFα and IL-1β were purchased from R & D Systems Inc (Minneapolis, MN). Histamine and thrombin were from Sigma (St Louis, MO). $PGE_2$ and 6 keto- $PGF_{1\alpha}$ kits were purchased from PerSeptive Biosystems (Framingham, MA). The PAF acetylhydrolase assay kit was purchased from Cayman Chemical Inc (Ann Arbor, MI). [$^3$H]-acetic acid and [$^3$H]-pyrilamine were from NEN Life Science Products, Inc. (Boston, MA). Human cytoplasmic $PLA_2$ antibody as well as human PLC β1 antibody were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). PLC γ1 antibody was from Upstate Biotechnology (Lake Placid, NY). Hanks' balanced salt solution with $Ca^{2+}$ and $Mg^{2+}$ (HBSS) and Media 199 (M199) were from Gibco BRL, Life Technologies (Grand Island, NY). Thirty-five-millimeter dishes were from Corning (Corning, NY) and all other plasticware was from Becton Dickinson (Franklin Lakes, NJ).

## 2.2 Experimental Methods

### 2.2.1 Human Umbilical Vein Endothelial Cells (HUVEC)

To study the behavior of endothelial cells and their production of PAF, primary human endothelial cells were cultured as previously described [140]. This model allowed for analysis of cytokine treatments on PAF synthesis in response to secondary agonists such as histamine and thrombin. Human placentas were obtained from recently delivered babies (<12 hours) at the Foothills Hospital. The umbilical cord was cut from the placenta and rinsed with cord buffer to remove any formed blood clots. The cord was then cannulated and clamped. Using a syringe, collagenase (1 mg/mL) was added to the vein and incubated for 10 minutes. Following incubation, the cord was gently massaged to loosen the endothelial cells and poured into a 50 mL disposable tube containing endothelial cell media (ECM) (with 20% human serum). The cord was washed once with cord buffer and its contents poured into the tube. The cells were then centrifuged at 1200

rpm for 10 minutes and resuspended in endothelial cell media. Cells were grown in gelatinized (0.2%) 75 $mm^2$ tissue culture flasks at $37^0C$ in 5% $CO_2$ until confluent. The HUVEC were split into the appropriate culture dishes for experiments by removal with trypsin-EDTA and resuspension in ECM.

### 2.2.2 Measurement of Platelet-activating Factor synthesis.

The quantification of newly synthesized PAF was carried out as previously described by McIntyre *et al.* [83]. Following cytokine treatment of HUVEC, PAF assays were carried out in response to stimulation with a secondary agonist (figure 2.1). Briefly, the media on the HUVEC monolayers was removed and the cells were washed once with HBSS. The media was replaced with HBSS/A. Cells were loaded with [$^3$H]-acetic acid (50 μCi), for 10 minutes at $37^0C$ (figure 2.2) then stimulated with either histamine or thrombin for the specified times. The reaction was stopped by the addition of acidified methanol to each well. Phospholipids were isolated by the method of Bligh and Dyer [141]. Briefly, the cells were scraped from the wells and added to glass culture tubes containing acidified methanol/chloroform/water (2.5:1.25:1) and excess cold PAF (5 ng/reaction) forming a monophase. The monophase was broken with the addition of chloroform and water. The lower phase was washed once by removing the upper phase and adding 2 volumes of pre-equilibriated upper phase buffer. Finally, the lower phase was transferred to glass culture tubes and dried at $37^0C$ under $N_2$ gas. The dried phospholipids were resuspended in 100 uL of 9:1 chloroform/methanol, of which 25 uL was immediately transferred to scintillation vials for direct counts (vial A). The remaining 75 uL of resuspended phospholipids were separated by thin layer chromatography on silica gel plates. This method of separation of PAF was employed because it allowed us to process many samples at once as opposed to separation by HPLC which can analyze only one sample at a time. PAF was identified by co-migration with cold standard. The material in the PAF spot has been previously shown to be authentic PAF in two ways: 1) the material was further characterized by HPLC and shown to be both alkyl and acyl PAF and 2) the material was scraped from the TLC plate and used to stimulate PMNs. The increased PMN adhesion was inhibitable with a PAF receptor antagonist [142]. The sample lanes are scraped into two vials; the PAF only spot in vial B and the rest of the lane (all

other phospholipids) in vial C. The scintillation vials were counted using a β-counter (Tri-carb 2100TR Liquid Scintillation Analyzer, Mississauga, ON) and the DPMs for 5 minutes were recorded. PAF synthesis was calculated using the formula:

$$\textbf{PAF Counts} = \frac{\text{Vial B}}{\text{Vial B + Vial C}} \times 4(\text{Vial A})$$

Vial A= direct count PAF

Vial B= PAF spot

Vial C= rest of phospholipds

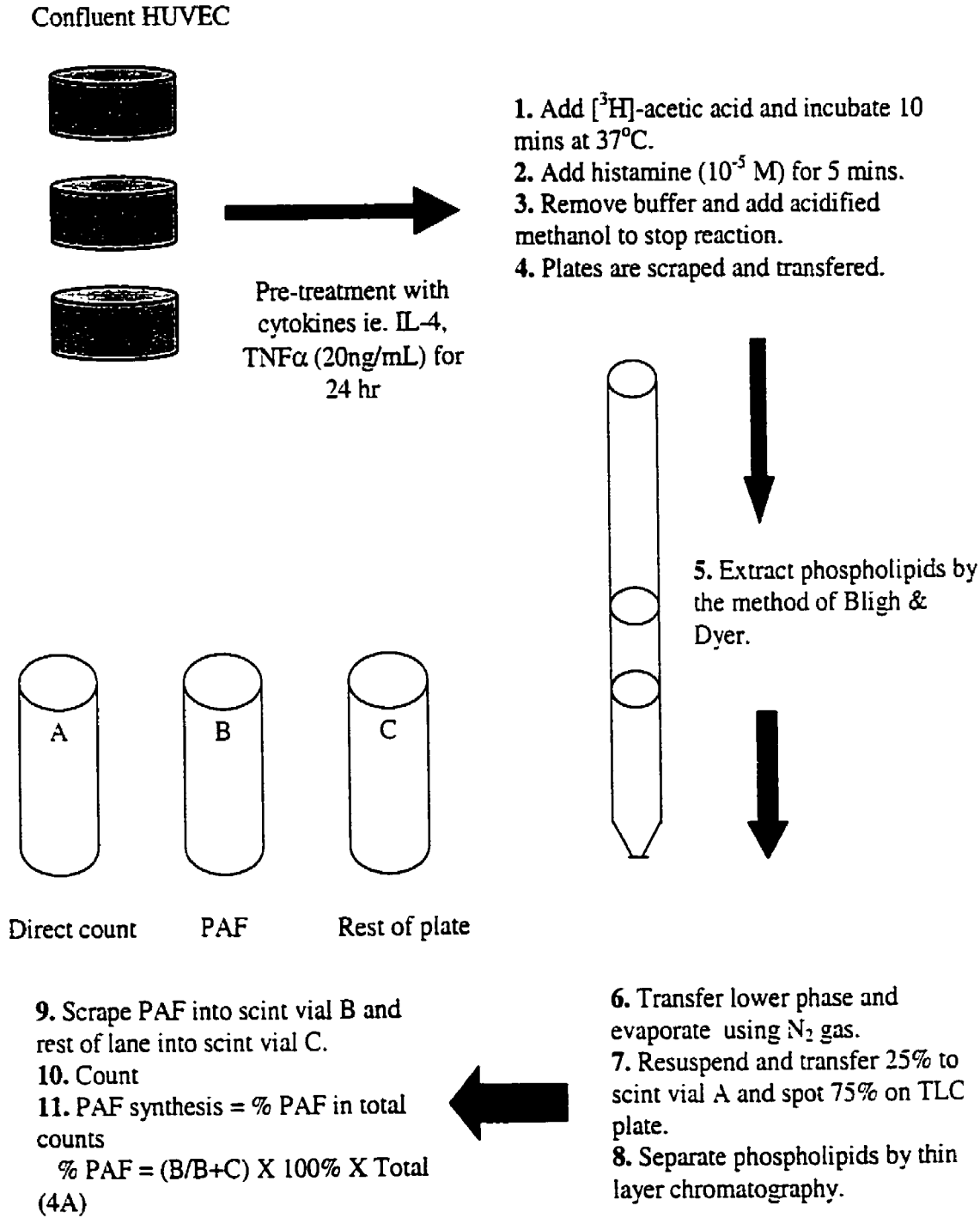



**Figure 2.1** A brief summary of the measurement of newly synthesized PAF in endothelial cells.

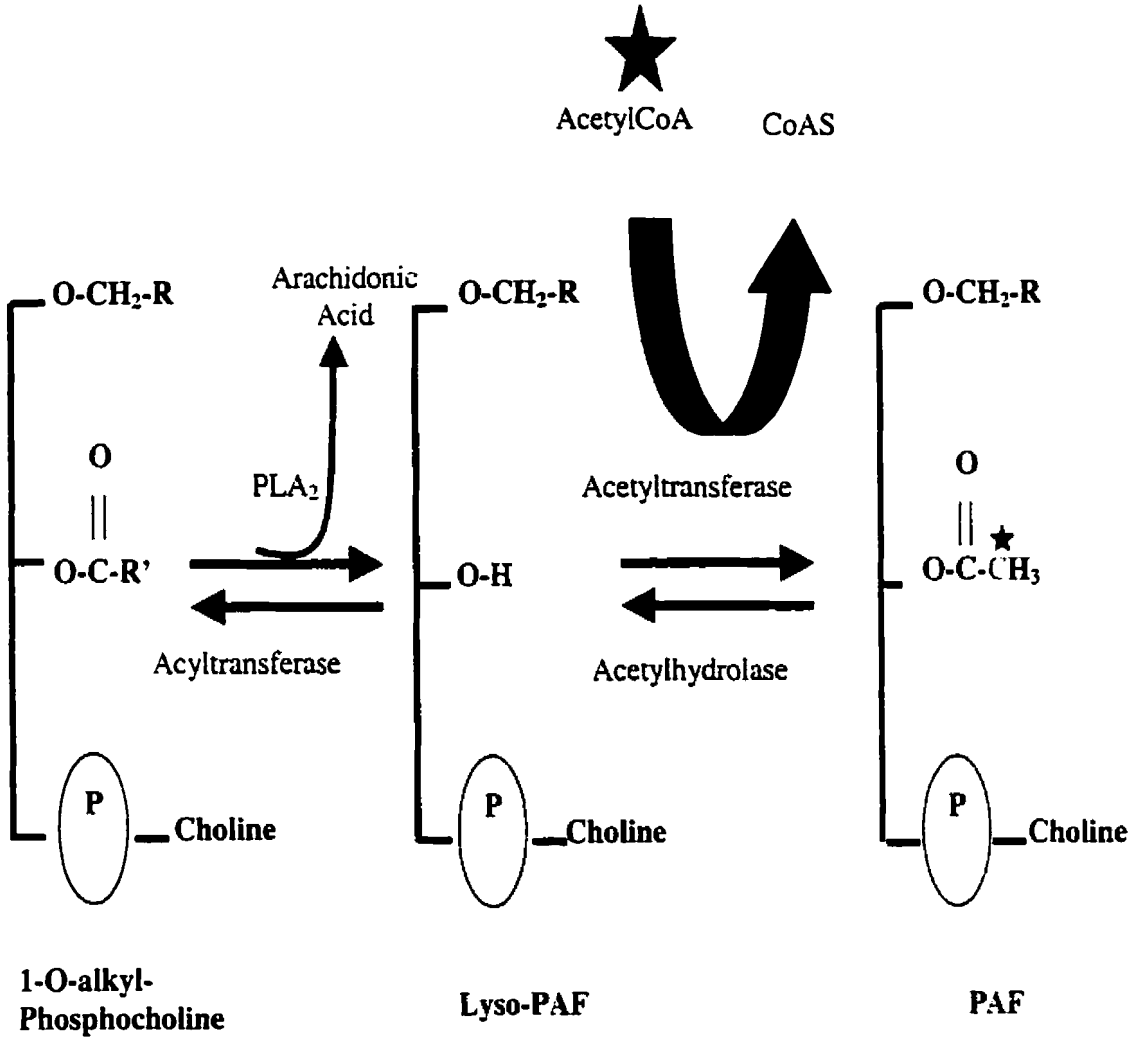



**Figure 2.2** The incorporation of [$^3$H]-acetic acid into newly synthesized PAF.

### 2.2.3 Cytokine treatment of HUVEC and stimulation.

Cells were stimulated when monolayers reached confluence. The monolayer was washed once with warm HBSS then M199/A was added with the appropriate amount of cytokine added to each well. For histamine induction, the media was removed and the monolayer was washed once with HBSS. HBSS/A was added to the cells and allowed to incubate for 5 minutes at $37^0$C. Histamine was then added directly to each well in the concentrations and times indicated. HUVEC used in all experiments were first passage cells and tightly confluent when stimulated.

### 2.2.4 Western blots and Immunoprecipitations for enzyme levels.

To assess protein levels and tyrosine phosphorylation of PLC γ as well as $cPLA_2$, we used PAGE and western blotting for separation and detection of these enzymes. HUVEC were stimulated as described above and then lysed in a buffer containing 1% triton X-100, 5 mM EDTA, 1 mM PMSF, 12 μg/mL leupeptin, 42 μM pepstatin and 2.5 μM sodium vanadate. Samples were prepared by scraping each well and transferring its contents to a microfuge tube allowing them to incubate on ice for 10 minutes. The tubes were centrifuged at 14 000 rpm for 10 minutes at $4^0$C. Supernatants were separated from the pellets and both the triton-soluble and -insoluble fractions were resuspended in 1X laemeli's sample buffer. Samples were separated by PAGE in 10% SDS-gels.

For PLC β1, immunoprecipitation experiments were performed to ensure specificity. Samples were lysed and prepared as above. Lysates were cleared with Protein A-Sepharose CL-4B beads for 4 hours, spun down and supernatants transferred to clean microfuge tubes. PLC β1 Ab was added and rotated at $4^0$C overnight, then Protein A-Sepharose CL-4B was added to each sample and rotated for 2 hours. The tubes were spun briefly and the supernatants discarded. The pellets were washed 4 times then laemeli's was added to each sample. Samples were separated using 10% SDS-PAGE.

Proteins were transferred to PVDF membranes using a semi-dry transfer apparatus at 0.2 Amps for 1 hour. The membranes were blocked using the specified blotto (either 5% milk or 5% BSA in TBS) as recommended by the manufacturer for each antibody. Blots were probed using anti-human cytoplasmic $PLA_2$, anti-phospholipase C γ1 and anti-phospholipase C β1 antibodies. Using the appropriate IgG-HRP conjugated

secondary antibody (Amersham Life Science, Buckinghamshire, UK), the membranes were developed using either ECL reagent (Amersham Life Science, Buckinghamshire, UK) or SuperSignal (Pierce, Rockford, IL). The resulting image was captured on either Hyperfilm (Amersham Life Science, Buckinghamshire, UK) or using the Fluor-S Max imager (Bio-Rad, Hercules, CA).

### 2.2.5 $PGE_2$, 6-keto $PGF_{1\alpha}$ and PAF acetylhydrolase kits.

Generation of $PGE_2$ and 6-keto $PGF_{1\alpha}$ by cytokine-activated endothelial cells was measured using RIA according to manufacturer's instructions. PAF acetylhydrolase activity in cytokine-stimulated endothelial cells was determined by monitoring conversion of a colorometric substrate. The media was removed from the stimulated endothelial cells and the plates were placed on ice. The cells were scraped into 0.1 M HEPES (pH 7.5), disrupted by sonication (4 pulses at 100 Watts; 3-5 seconds each) and enzyme activity was determined according to manufacturer's instructions.

### 2.2.6 Lipid body staining of HUVEC.

HUVEC were cultured on gelatin coated glass coverslips in 4 well dishes and treated with M199/A alone or M199/A containing 20 ng/mL of IL-4 for 24 hours. Lipid bodies were stained using two methods: procedures were kindly provided by Dr. Peter Weller. For nile red staining of lipid bodies, cells were washed with HBSS and fixed with 3.7% formaldehyde for 10 minutes. Cells were washed twice then incubated with 1 mg/mL of nile red for 10 minutes. The coverslips were washed, mounted on slides and observed under fluorescent microscopy. Photographs were taken of random fields using tungsten film (Kodak, Toronto, ONT) and a Leitz Diavert Inverted Microscope (Midland, ONT) under 100X objective.

Osmium staining for lipid bodies was performed by fixing the treated cells with 3.7% formalin for 10 minutes. Cells were washed with water then stained with 0.1 M cacodylate buffer (pH 7.4) and 1.5% osmium tetraoxide (pH 7.4) for 30 minutes. Coverslips were washed, stained with 1% thiocarbohydrazide for 5 minutes, washed, stained again with cacodylate buffer and osmium tetraoxide. The coverslips were washed, mounted on slides and observed using light microscopy under oil immersion.

Random fields were photographed using a Leitz Diavert Inverted Microscope (Midland, ONT) under 100X objective.

### 2.2.7 Calcium imaging.

HUVEC were cultured on gelatin coated glass coverslips and treated with M199/A alone or M199/A containing 20 ng/mL of IL-4 for 24 hours. Cells were washed with HBSS/A then loaded with 5 μM fura-2-AM for 30 minutes at room temperature. The fura-2- loaded cells were placed in a perfusion chamber on a Zeiss Axiovert microscope and fluorescent images were captured using a 20X objective as previously described [143]. The fura-2-loaded cells were excited at both 340 and 380 nm and emission was monitored at 510 nm. The experiment was started and control images were recorded every 5 seconds for 1 minute. Histamine was then added to the endothelial cells at increasing concentrations as indicated in the figure legend and images were collected. Changes in intracellular calcium are expressed as a ratio of the emission at 510 nm. Data were analyzed using ImageMaster software (Photon Technology International. Monmouth, NJ).

### 2.2.8 Histamine receptor quantification.

HUVEC were treated with M199/A alone or with M199/A containing 20 ng/mL IL-4 for 24 hours. Cells were washed with HBSS/A and then increasing concentrations of pyrilamine were added. After 5 minutes, $10^{-5}$M histamine was added and PAF synthesis was determined. Alternatively, a homologous competition binding assay was performed. 2.5 nM [$^3$H]-pyrilamine was added to control or IL-4-treated HUVEC in the presence of increasing concentrations of cold pyrilamine and receptor affinity was determined by comparing the slopes of the curves using Prism software from Graphpad. A single scatchard analysis experiment was performed to confirm the high receptor binding affinity of pyrilamine. Cells were washed with HBSS/A then increasing concentrations of hot [$^3$H]-pyrilamine with 2.5 nM cold pyrilamine were added to either control or IL-4-treated HUVEC. Both the bound and unbound [$^3$H]-pyrilamine were

measured using a β–counter and the results were analyzed as bound/free versus bound [$^3$H]-pyrilamine.

Another method employed to quantify histamine receptors in HUVEC was to use RT-PCR to give a semi-quantitative indication of the histamine receptor mRNA. Primers were designed against the histamine receptor type 1 DNA sequence [144] as shown in Table 1. Histamine receptor DNA was amplified along with a β-actin control and a positive control, MCP-4, which is upregulated in response to IL-4-treatment (unpublished observation).

Finally, flow cytometry was used to quantitate surface expressed histamine receptors. Cells were grown in 6 well plates and removed using trypsin-EDTA then placed in FACS tubes. The cells were resuspended in 1X PBS at $1x10^6$ cells/tube then incubated with the indicated concentrations of pyrilamine for 30 minutes on ice. Endothelial cells were washed and a histamine-fluorescein compound (Molecular Probes, Eugene, OR) was then added at $10^{-4}$ M for 30 minutes on ice. The unbound histamine was removed from the cells with 2 washes and resuspended in 1 mL of 1X PBS. Flow cytometry was performed on the histamine labeled endothelial cells using $10^4$ events per assay.

### 2.2.9 ELISA for thrombin receptors

To quantify the surface expressed thrombin receptor levels on IL-4-treated HUVEC, cells were cultured in 24-well plates and an ELISA was performed. HUVEC were treated with M199/A alone or M199/A containing either IL-4 (20 ng/mL) or TNFα (20 ng/mL) for 24 hours. Cells were washed with HBSS/A and kept on ice. The thrombin receptor Ab WEDE 15 (2 ug/mL) (Coulter-Immunotech, Burlington, ONT) was used to detect thrombin receptors. An IgG control was used as well as a positive control, CD 106 (VCAM-1) which is upregulated with IL-4-treatment in HUVEC [145]. The primary Ab was incubated for 30 minutes at 37$^0$C, wells were washed and secondary Ab IgG-HRP was added and incubated for 30 minutes at 37$^0$C. Cells were washed and the Ab binding was determined using a trimethyl-benzamidine one-step substrate (DAKO, Carpinteria, CA). Plates were read at 450 nm using a Spectra-Max Plus plate reader (Molecular Devices, Sunnyvale, CA).

### 2.3 Statistics.

All experiments were performed at least three times (unless otherwise specified) and the data are presented as the mean ± SEM. Alternatively, the data from a single representative experiment are presented as the mean ± range of duplicate determinations. Two-tailed Mann-Whitney *U*-test (paired or unpaired) was performed and $p<0.05$ was considered significant.

**Table 1.** Primers used to amplify the histamine type 1 receptor in an RT-PCR reaction.

| DIRECTION | SEQUENCE |
|---|---|
| **Forward 5'→3'** | **AGATCTACAAGGCCGTACGA** |
| **Reverse 3'→5'** | **TGGTATCTGAGTCCGTTCGA** |

# CHAPTER THREE: SPECIFIC AIM 1

**Hypothesis:** Cytokines such as IL-4 and TNFα are able to prime HUVEC for histamine-induced PAF synthesis.

*Objectives:*

1) Do cytokines alone induce PAF synthesis in HUVEC?
2) To determine what effect cytokine treatment has on the production of PAF in histamine-induced HUVEC?
3) Is the priming effect specific for the cytokine IL-4?
4) Is the priming effect observed specific for histamine induction of PAF, or are other secondary agonists capable of inducing the same effect?
5) Is new protein synthesis required for priming?

## 3.1 RESULTS:

*Histamine induces PAF production in human endothelial cells.* McIntyre *et al.* have previously described the effects of histamine on the induction of PAF synthesis in human endothelial cells [83]. To establish whether we could reproduce the effects of histamine-induced PAF production in HUVEC, we performed a time course for histamine stimulation of HUVEC and measured PAF synthesis. HUVEC were cultured and grown to confluence in 6-well plates. The monolayers were washed once and then stimulated with histamine in the presence of [$^3$H]-acetate. At various times the reaction was stopped by the addition of acidified methanol and total PAF synthesis was measured. As shown in figure 3.1, the greatest amount of PAF produced occurred within 5 minutes of stimulation. This level continued with 10 minutes of histamine exposure but eventually began to fall by 15 minutes. These results confirmed previously described data in the literature that 5 minutes of histamine treatment is sufficient to stimulate PAF synthesis in HUVEC.

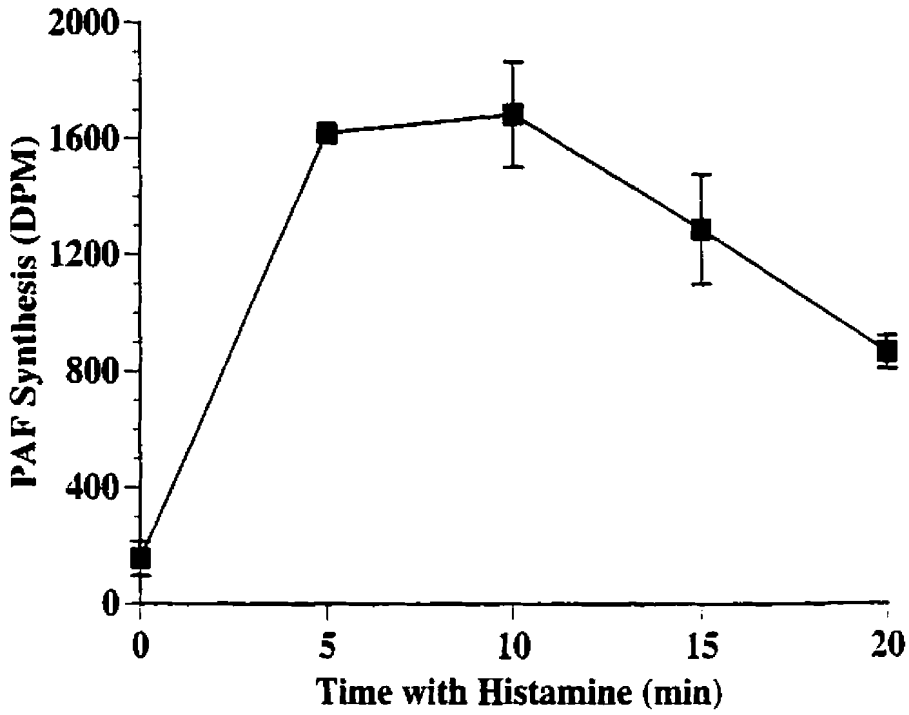



**Figure 3.1.** *Histamine time course for PAF induction in HUVEC.* Confluent HUVEC monolayers were washed once with HBSS and then treated with HBSS/A alone or HBSS/A containing $10^{-5}$M histamine for the specified times. PAF synthesis was measured by [$^{3}$H]-acetic acid incorporation as described in the methods. This was a single experiment with duplicate points performed to reproduce previously described data in the literature.

We next examined the concentration dependence of histamine-mediated PAF synthesis. Figure 3.2A shows the results of 4 experiments where PAF synthesis was measured after treatment with increasing concentrations of histamine. While the results show a maximal amount of total PAF with as little as $10^{-5}$ M histamine, the error bars are large. This is due to the variability between donor cords whose individual ability to produce PAF is dependent upon the genetic makeup of the person. Such individual variability in the magnitude of the response would account for the differences in PAF synthesis between repeats of experiments and thus, large error bars. Therefore, in subsequent experiments we performed all assays in duplicate points. As shown in figure 3.2B, an individual experiment with duplicate points shows a maximum amount of histamine-induced PAF synthesis at $10^{-5}$ M histamine. These results confirm the existing literature and demonstrate our ability to replicate these values.

*Cytokine treatment alone does not induce PAF synthesis in HUVEC.* The ability of cytokine stimulation alone to induce PAF synthesis in HUVEC was evaluated. Previous experiments by Bussolino *et al.* showed that TNFα or IL-1 alone are capable of synthesizing PAF [146;147]. As seen in figure 3.3, histamine ($10^{-5}$ M) was able to induce PAF synthesis, as expected. However, neither IL-4, TNFα, IL-1 nor, oncostatin M were able to induce PAF synthesis at the times indicated. The TNFα and IL-1 data, interestingly, does not concur with the existing literature, which has previously described these two cytokines ability to produce PAF in HUVEC [147].

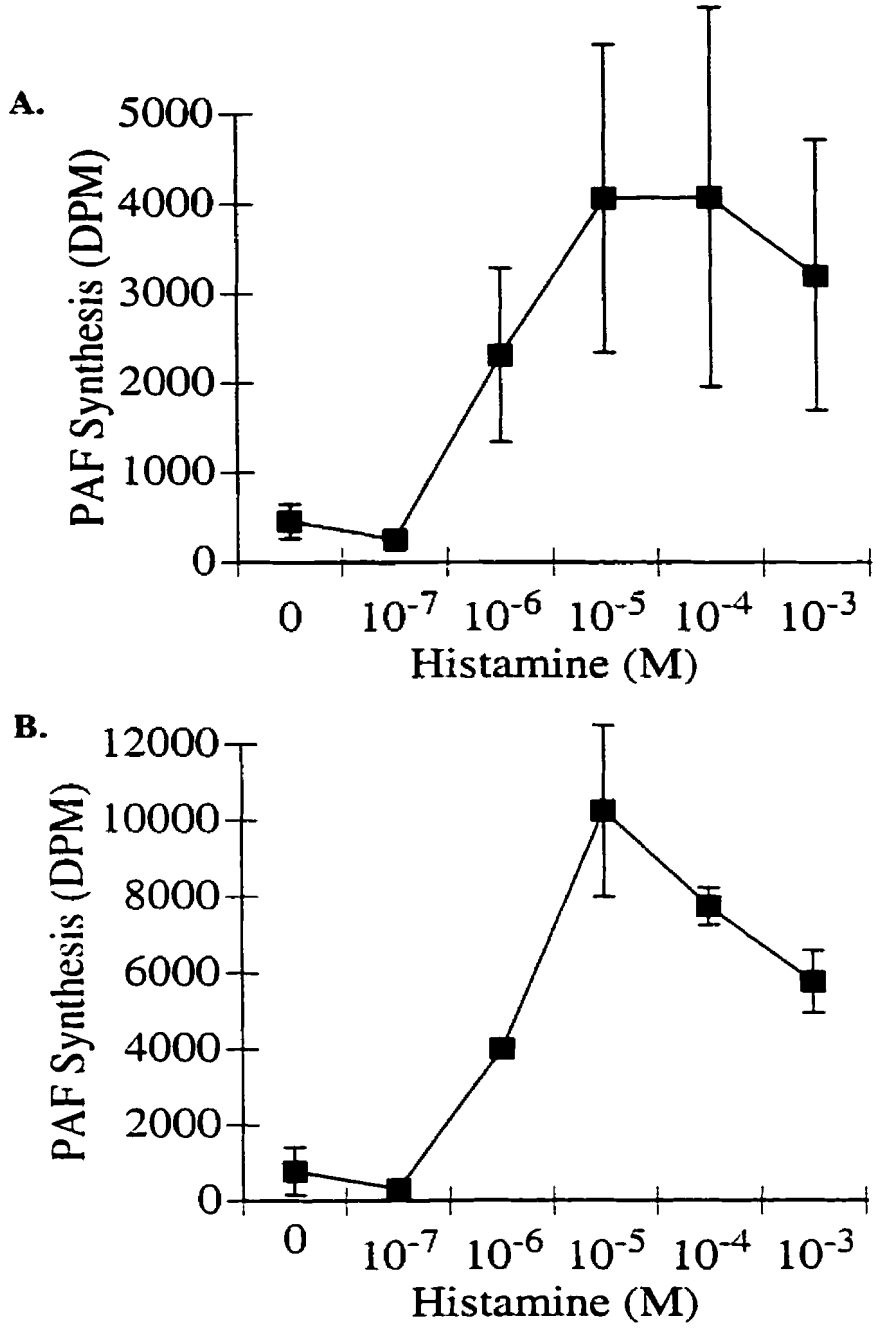



**Figure 3.2.** *Histamine concentration curve for PAF induction in HUVEC.* Confluent HUVEC were washed once with HBSS then treated with HBSS/A alone or with the specified concentrations of histamine for 5 minutes. PAF synthesis was measured by [$^3$H]-acetic acid incorporation as described in the methods. A) The mean ± SEM of 4 experiments show large error bars due in part to the variability in donor cords while B) a representative experiment is shown (mean ± range of duplicate points).

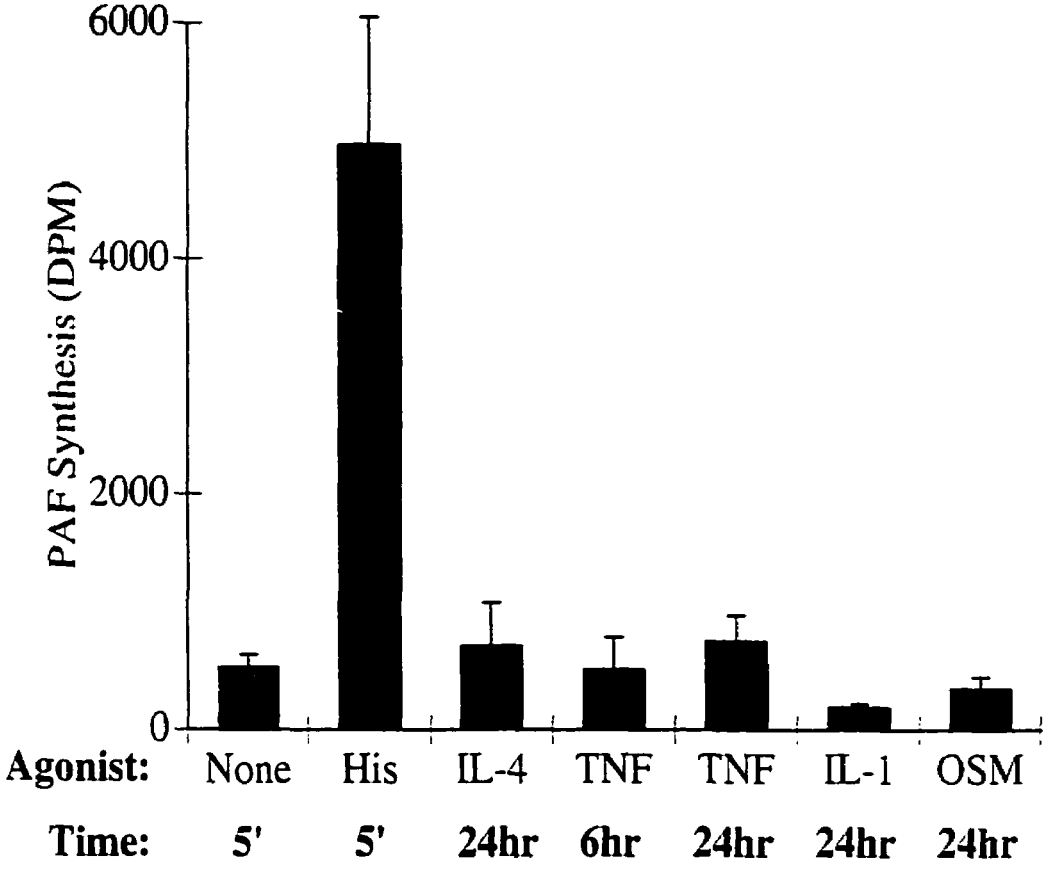



**Figure 3.3.** *Cytokines alone do not increase PAF synthesis in HUVEC.* Confluent HUVEC monolayers were treated with M199/A alone or M199/A containing IL-4 (20 ng/ml), TNFα (20 ng/ml), IL-1β (20 ng/ml), oncostatin M (20 ng/ml), or histamine ($10^{-5}$ M) for the specified times. Following cytokine treatment, the monolayers were washed once and pulsed with [$^3$H]-acetic acid. PAF synthesis was measured by [$^3$H]-acetic acid incorporation as described in the methods. The data represent the mean ± SEM of at least three experiments.

*IL-4, not other cytokines, is able to enhance histamine-induced PAF synthesis in HUVEC.* While cytokines alone were unable to induce PAF synthesis, the combination of IL-4-treatment for 24 hours and subsequent stimulation with 5 minutes of histamine increased PAF production in HUVEC 2.25 fold over control cells (figure 3.4). This effect was not seen with either TNFα at 6 hours, IL-1 at 24 hours or oncostatin M for 24 hours. HUVEC pretreated with TNFα for 24 hours showed a significant decrease in PAF synthesis when compared to the control.

We asked if the increase in overall PAF synthesis was specific for histamine by using thrombin as a secondary agonist. Thrombin has previously been shown to induce PAF production in HUVEC [140]. IL-4-treated HUVEC stimulated with thrombin showed a 1.7 fold increase in PAF synthesis over control (figure 3.4). This indicates that the increased amount of total PAF produced in IL-4-treated HUVEC is not restricted to histamine stimulation.

While IL-4-treated HUVEC showed an increased amount of PAF synthesis at 24 hours, it also showed an increase in sensitivity to histamine (figure 3.5). Here, cells were treated with IL-4 for 24 hours then subjected to the indicated concentrations of histamine. A significant increase in the total amount of PAF synthesized at $10^{-5}$ M histamine is seen in the IL-4-treated HUVEC compared to control. There is also a shift in the concentration curve to the left. This shift is significant at $10^{-7}$ M histamine. A shift in the dose response curve indicates increased sensitivity to the secondary agonist histamine.

This increase in sensitivity to the secondary agonist is not seen when thrombin is used as a secondary agonist. Cells were treated with IL-4 for 24 hours and then stimulated with the indicated concentrations of thrombin (figure 3.6). A representative experiment using duplicate points shows a significant increase in total PAF synthesis in IL-4-treated HUVEC at 0.1, 0.2 and 0.5 units/mL of thrombin compared to control (figure 3.6B). However, a shift in the dose response curve was not significant.

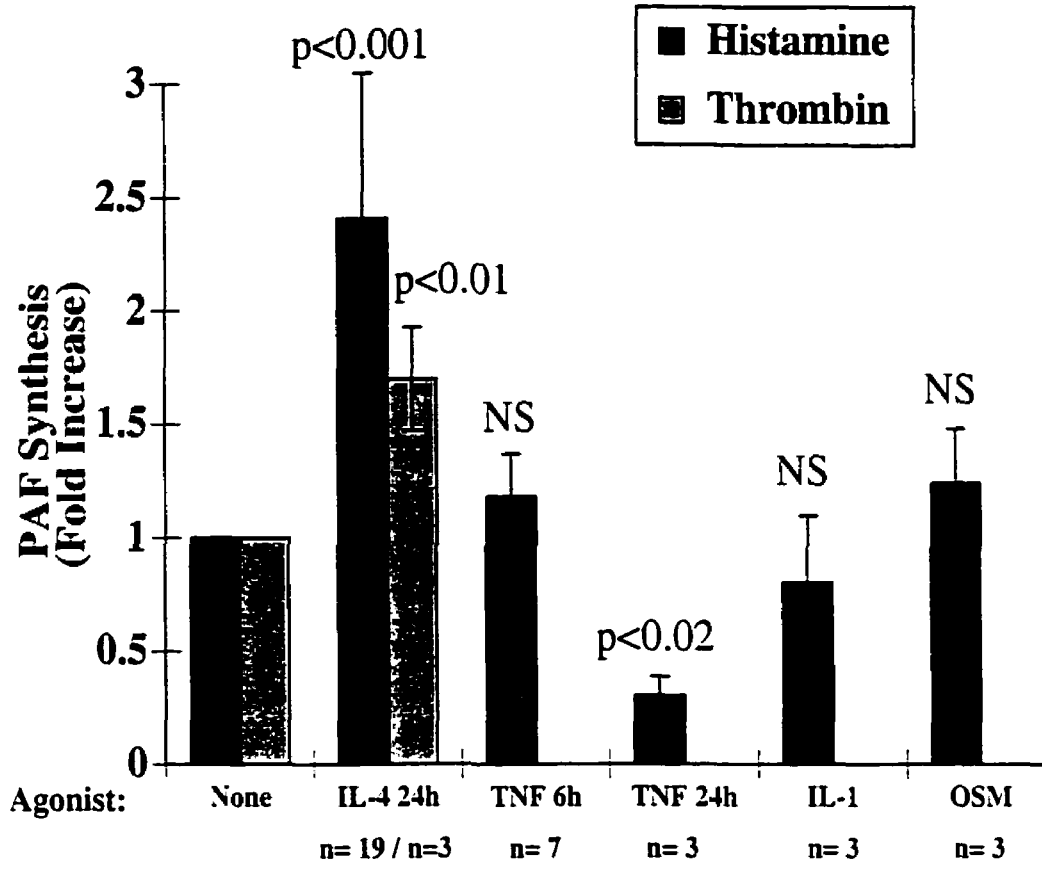



**Figure 3.4.** *IL-4 primes HUVEC for histamine- or thrombin-induced PAF synthesis.* Confluent HUVEC were treated with M199/A alone or M199/A containing TNFα (20 ng/ml) for 6 or 24 hours, IL-4 (20 ng/ml), IL-1β (20 ng/ml) or oncostatin M (20 ng/ml) for 24 hours. Following cytokine treatment, the monolayers were washed once, pulsed with [$^3$H]-acetic acid and then stimulated with either $10^{-5}$ M histamine or 2 units/mL thrombin for 5 minutes. PAF synthesis was measured by [$^3$H]-acetic acid incorporation as described in the methods. The histamine data shown represents 19 paired comparisons of PAF synthesis in control versus IL-4 treated HUVEC. The thrombin data shown represents 3 experiments.

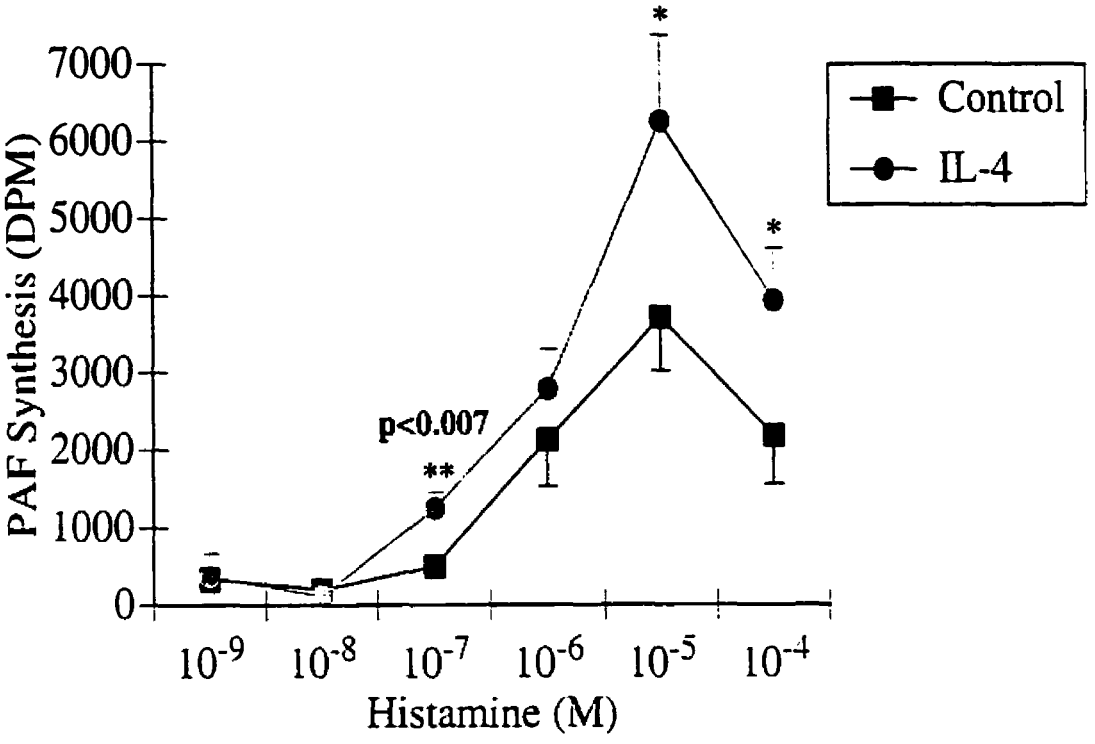



**Figure 3.5.** *IL-4 increases HUVEC sensitivity to histamine.* HUVEC were stimulated with M199/A alone or M199/A containing IL-4 (20 ng/mL) for 24 hours. Following stimulation, cells were washed with HBSS/A pulsed with [$^3$H]-acetic acid and then treated with increasing concentrations of histamine for 5 minutes. PAF synthesis was measured by [$^3$H]-acetic acid incorporation as described in the methods. A comparison of the IL-4 treated HUVEC to control shows a shift in the concentration curve for histamine-induced PAF synthesis. Data shown are the mean ± SEM of between 3 and 6 experiments. *$p<0.05$.

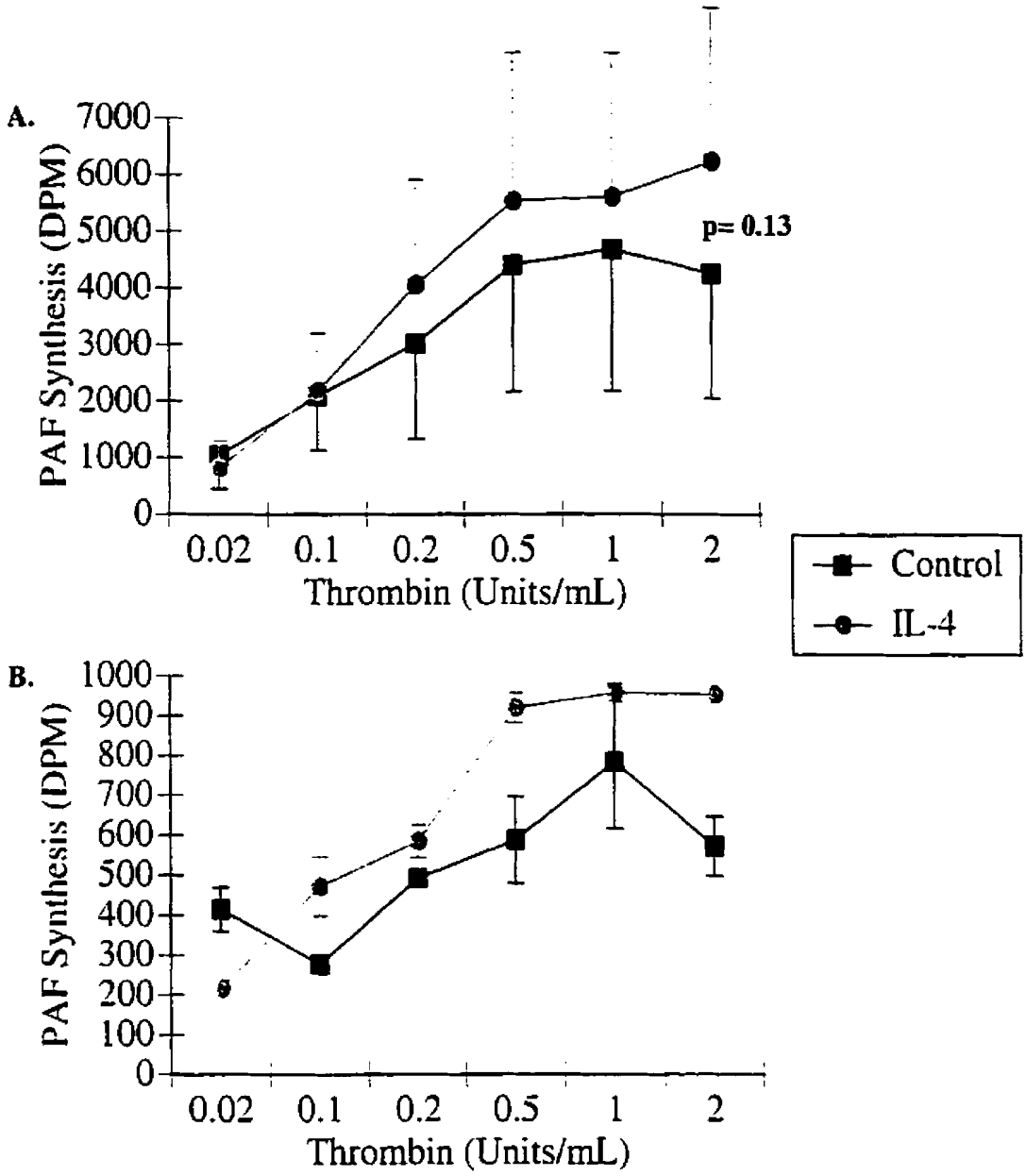



**Figure 3.6.** *IL-4 does not significantly increase HUVEC sensitivity to thrombin.* HUVEC were stimulated with M199/A alone or M199/A containing IL-4 (20 ng/mL) for 24 hours. Following stimulation, cells were washed once with HBSS/A, pulsed with [$^3$H]-acetic acid and then treated with increasing concentrations of thrombin. PAF synthesis was measured by [$^3$H]-acetic acid incorporation as described in the methods. A) A summary of 3 experiments with the mean ± SEM is not considered significant; B) a representative experiment is shown with the mean ± range of duplicate points.

*IL-4 time course requires 24 hours of stimulation for increase in histamine-induced PAF synthesis in HUVEC.* A time course was performed to determine the minimum amount of time required for IL-4-treated HUVEC to exhibit an increase in histamine-induced PAF synthesis over control. Figure 3.7 shows that HUVEC treated for either 2 or 6 hours with IL-4 then stimulated for 5 minutes of histamine had no significant increase in total PAF synthesis. At 12 and 24 hours of IL-4-treatment there was a significant difference between IL-4-treated HUVEC and control ($p<0.01$ and $p<0.003$ respectively). In addition, we looked at 5 minute stimulations of IL-4 for HUVEC to determine if IL-4 had an immediate effect on histamine-induced PAF synthesis. Three experiments of 5 minute IL-4-treatment with subsequent histamine stimulation was performed with no significant difference found between the IL-4 and control treated cells (data not shown). Finally, it is interesting to note that the amount of PAF produced stays constant for the first 6 hours in both IL-4 and control cells. However, as time progresses we see a decrease in the amount of PAF produced in control cells, where as, in the IL-4-treated cells the amount of PAF produced does not decrease as dramatically.

*A potential role for new protein synthesis.* The results of the time course indicated that at 24 hours of IL-4 stimulation there was a significant increase in histamine-induced-PAF synthesis, suggesting that new protein synthesis may be required. We used cyclohexamide, a translational inhibitor, to determine if new protein synthesis was required for IL-4 mediated priming. 24 hour treatment of cyclohexamide caused a significant increase in both the control and IL-4-treated HUVEC for histamine-induced PAF synthesis (figure 3.8). These PAF synthesis values for both control and IL-4-treated HUVEC were consistently higher than those generally observed in non-cyclohexamide treated cells (refer to figure 3.5). Other protein or RNA synthesis inhibitors were subsequently employed, including emetine and actinomycin D, as a means of avoiding the effects of cyclohexamide treatment on increasing total PAF synthesis in histamine-induced HUVEC. However, optimization experiments to establish the ideal concentration of each inhibitor to use for 24 hour treatment proved unfruitful (data not shown). As the current results stand, no valuable conclusions can be drawn from the above data.

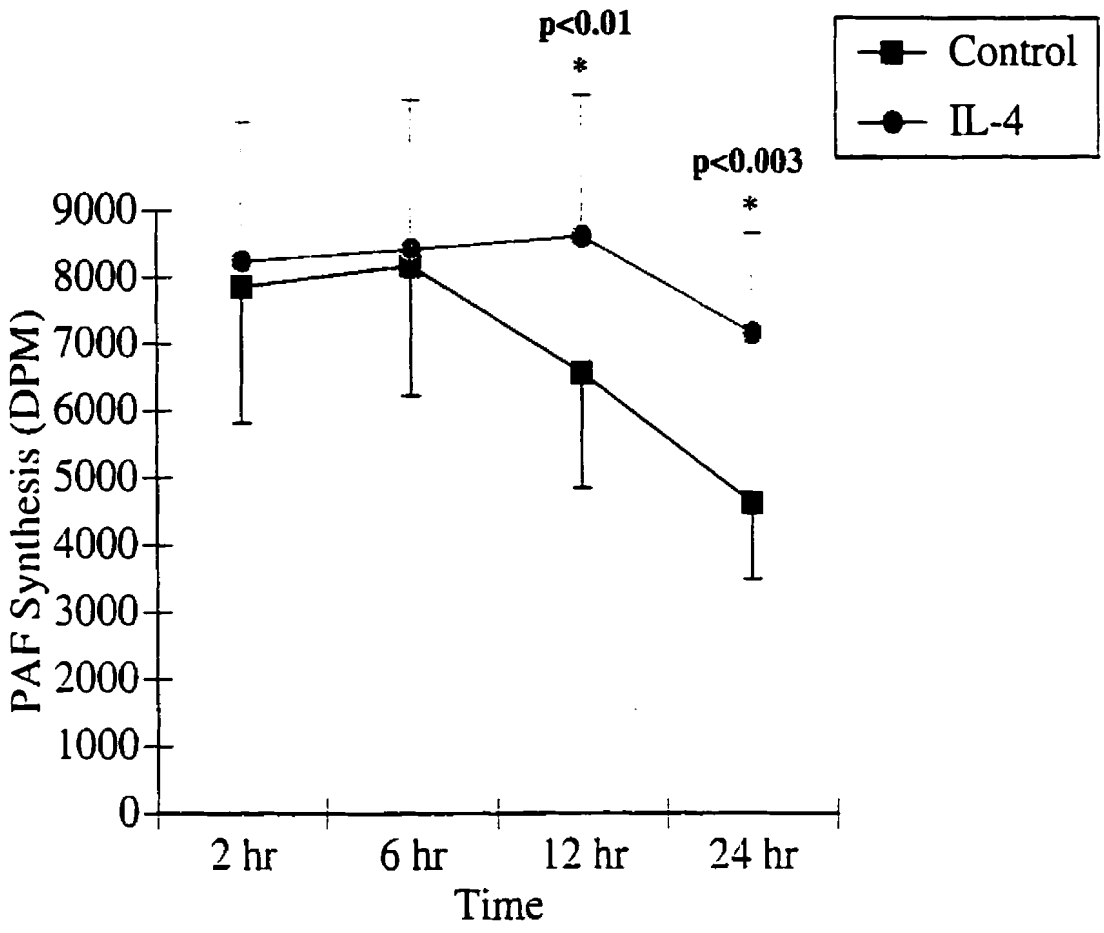



**Figure 3.7.** *Treatment of HUVEC with IL-4 for less than 12 hours has no effect on histamine induced PAF synthesis.* HUVEC were stimulated with M199/A alone or M199/A containing IL-4 (20 ng/mL) for the indicated time points. Following stimulation, cells were washed once with HBSS/A, pulsed with [$^3$H]-acetic acid then treated with histamine ($10^{-5}$ M) for 5 minutes. PAF synthesis was measured by [$^3$H]-acetic acid incorporation as described in the methods. Data shown are the mean of duplicate points ± SEM and are representative of between 3 and 7 experiments.

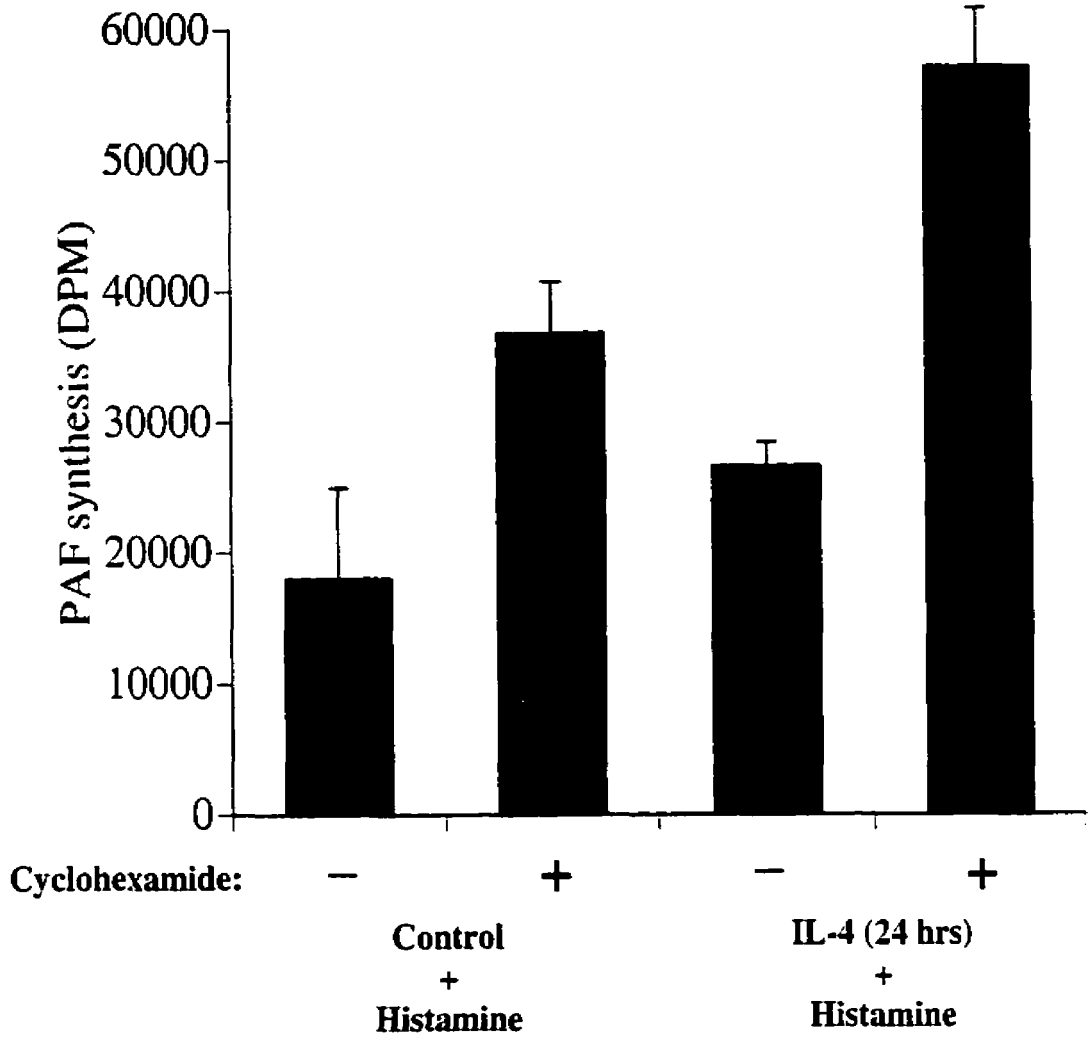



**Figure 3.8.** *Cyclohexamide treatment of HUVEC enhances histamine-mediated PAF production in both control and IL-4-treated cells.* HUVEC were stimulated with either M199/A alone or M199/A containing IL-4 (20 ng/mL) for 24 hours. Cells were treated with histamine as previously described. In some wells, HUVEC were also treated with cyclohexamide (5 μg/mL) for 24 hours. Following stimulation, cells were washed once with HBSS/A, pulsed with [$^3$H]-acetic acid then treated with histamine ($10^{-5}$ M). PAF synthesis was measured by [$^3$H]-acetic acid incorporation as described in the methods. Data shown is the mean ± SD of duplicate points and is representative of at least three experiments.

## 3.2 DISCUSSION:

The recruitment of leukocytes is an essential step in the inflammatory response. Asthma is a disease in which an inappropriate inflammatory response occurs in the airway tissue of the lungs. The selective recruitment of eosinophils into the airway tissue is a characteristic of asthma where these cells play an important role in the pathogenesis of the disease. Asthma, particularly allergy-mediated asthma, is also characterized by Th2 cytokine expression within the airway tissue. In particular, cytokines such as IL-5, IL-4 and IL-13 are elevated as shown by BAL samples and biopsy studies in humans [31;39]. Animal models in which these cytokines have been knocked out show decreased eosinophilia as well as reduced AHR, clearly illustrating the importance of these cytokines in asthma pathogenesis [40;41;51;52;63]. In addition, *in vitro* studies have demonstrated that IL-4 and IL-13 increased VCAM-1 and P-selectin expression on endothelial cells. Both of these adhesion molecules are important in the recruitment of eosinophils under flow conditions [1,33]. TNFα-stimulated HUVEC have also been shown to mediate neutrophil recruitment through E-selectin expression [148]. TNFα-treated HUVEC also supports eosinophil recruitment; however they do not show a selective recruitment over neutrophils as seen in IL-4-treated HUVEC under flow conditions [14].

In order for eosinophils to adhere to endothelial cells and eventually emigrate into tissue, a number of events must occur. As described above, the role of cytokines such as IL-4 in upregulating specific adhesion molecules for eosinophil recruitment has been demonstrated both *in vitro* and in animal model systems. PAF is also an important mediator of the adhesion process and is involved in the activation and priming of eosinophils [149]. Endothelial cells stimulated with either histamine or thrombin have both been shown to induce PAF synthesis in endothelial cells [83]. Newly synthesized PAF is expressed on the surface of endothelial cells where it is able to engage a specific receptor on passing leukocytes. PAF functions in concordance with P-selectin expression in the tethering and activation of circulating leukocytes including eosinophils. Its importance in the adhesion pathway is illustrated by *in vitro* blocking experiments, which show a reduction in the number of transmigrating leukocytes on endothelial cells [139]. In this study, we address the effects of chronic exposure to Th2 type cytokines on endothelial

cells and their ability to produce PAF. Specifically, does exposure to cytokines such as IL-4 prime endothelial cells for production of PAF in response to physiologically relevant mediators of asthma such as histamine.

Previous reports by Bussolino *et al.* have shown that HUVEC treated with TNFα or IL-1β are able to synthesize PAF [147]. We investigated whether IL-4 or other cytokines would induce PAF synthesis in HUVEC by assaying for their PAF producing activity. Results showed that cytokine treatment of HUVEC was unable to induce PAF synthesis on its own while histamine stimulation can induce PAF synthesis as previously reported (figure 3.3) [83]. It is important to note that in experiments carried out by Bussolino's group, cells were stimulated for only 30 minutes with either TNFα or IL-1β, a much shorter exposure to cytokines than what we used. Our data does not support Bussolino's observations, although a possible explanation for this discrepancy could also lie in the method by which we measured PAF synthesis. There are a number of methods available for the measurement of PAF synthesis. These include rabbit platelet aggregation assays, HPLC, and RIA. In our experiments we measured PAF synthesis by monitoring incorporation of [$^3$H]-acetate into the *sn*-2 position of PAF. PAF is purified from the total phospholipids and identified by co-migration with excess cold PAF when separated on silica gel plates by TLC. This method has been previously used by a number of other labs as a means of quantifying total PAF production in cells and is beneficial for analysis of multiple samples [82;83;87;150]. The method used by Bussolino *et al.* purifies PAF again by TLC however, the PAF spot is identified by a standard R*f* value and recovery measured by addition of [$^3$H]-PAF. Differences in PAF measurement techniques have previously been shown to yield different quantifications when compared directly and may explain the discrepancy between results [151].

IL-4-treated HUVEC showed an over two-fold increase in histamine-induced PAF synthesis. This increase was not seen with any other cytokine treatment, which demonstrates the specificity of this response for IL-4. We chose to look at TNFα and IL-1β treatment because they had previously been described as being able to induce PAF synthesis. Oncostain M was investigated as a possible PAF producer since it has been shown to induce new synthesis of P-selectin in HUVEC, similar to the effects of IL-4 [73].

Therefore, it was suspected that oncostatin M may demonstrate the same priming effects as IL-4, however, our results did not support this (figure 3.4).

The results of the cytokine studies suggested that only IL-4 was able to prime HUVEC for histamine-induced PAF synthesis. However, a number of other agonists such as phorbol esters, calcium ionophore as well as physiological mediators such as leukotrienes and thrombin are all able to induce PAF production in endothelial cells [64]. We investigated the specificity of priming seen with IL-4-treatment for the secondary agonist by stimulating HUVEC with thrombin instead of histamine. While both the thrombin and histamine receptors are G protein coupled receptors, they differ in their structure and activation. Thrombin receptors are G protein coupled receptors that are activated by proteolysis, specifically the PAR-1 and PAR-3 receptors found on endothelial cells [152]. Histamine receptors on the other hand are not cleaved when activated and couple to the $G\alpha_q$ family of G proteins upon ligand engagement [132]. Our results showed that the increase in PAF synthesis observed in IL-4-treated HUVEC was not limited to the secondary agonist histamine. Thrombin showed a similar increase in PAF synthesis, albeit, not as high (figure 3.4).

Priming of these endothelial cells by IL-4 for either histamine- or thrombin-induced PAF synthesis was broken down into two components. An increase in total PAF produced in response to these agonists was seen, however, a second component illustrated a difference in response to histamine versus thrombin. Dose response curves of IL-4-treated HUVEC with histamine showed at least a fold increase in sensitivity to the agonist in PAF production (figure 3.5) while response to thrombin showed no statistically significant change in the sensitivity curves (figure 3.6).

IL-4-treated HUVEC showed an increase in histamine-induced PAF synthesis as well as an increase in sensitivity to histamine. In contrast, the increase in priming of thrombin-induced PAF synthesis in IL-4-treated cells was not as great when compared to histamine. In addition, there was no increase in sensitivity to thrombin in the IL-4-treated HUVEC. Together, these observations suggest two different mechanisms behind the IL-4 priming of endothelial cells due to the differing responses to the secondary agonists. Differences in the downstream signaling effects of thrombin and histamine receptors may

account for this variation. However, these signaling pathways have not been well characterized and further investigation is warranted.

Time course studies were performed to determine the minimum amount of time for IL-4 stimulation of HUVEC to exhibit an increase in histamine-induced PAF production. Results showed a significant difference in PAF synthesis as early as 12 hours and a large change by 24 hours (figure 3.7). This suggests that new protein synthesis may be required for the increased production of total PAF seen in IL-4-treated cells. An interesting observation in the time course curves shows that PAF production at early time points remains constant. However, as treatment times increase, the amount of PAF produced does not increase but remains elevated when compared to control cells, which falls more than in the IL-4 cells. This suggests that IL-4 may in fact confer a protective effect on the protein machinery involved in the production of PAF. So in fact, the elevated PAF seen in IL-4-treated HUVEC may not be due to enhanced production but instead, retained capabilities of synthesis over time. Possible explanations for this include the upregulation of substrate available for PAF synthesis, or the prevention of negative regulation of the PAF synthesis production pathway.

Inhibitor studies using cyclohexamide, a translational inhibitor, were performed to determine if new protein synthesis was required for IL-4 mediated priming for histamine-induced PAF synthesis (figure 3.8). However, PAF production in both control and IL-4-treated cells was increased dramatically when treated for 24 hours with cyclohexamide. This unexpected result could be explained by cyclohexamide's potential ability to super-induce gene transcription as previously described by Gajdusek *et al.* [153] in which IGF-1 mRNA was over-expressed in bovine endothelial cells. A possible explanation for the increased PAF production observed in HUVEC could be the activation of the p38 MAPK pathway by cyclohexamide [153]. Though the characterization of the MAPK pathways involved in production of histamine-induced PAF synthesis is not yet characterized, their potential involvement has been previously hypothesized [93-95]. This is suggested due to the involvement of PKC, which is required for PAF synthesis, and has been shown to be activated by the MAPK pathway. The synergistic effects of activation of MAP kinases between cyclohexamide and histamine may contribute to the 'super-induction' of PAF seen in the cyclohexamide treated HUVEC. Continuing studies using a variety of

inhibitors are ongoing, however, as the results stand thus far, no significant conclusions can be drawn as to the requirement of new protein synthesis for PAF production in IL-4-treated HUVEC.

The implications of these findings suggest that chronic exposure to the Th2 cytokine IL-4 contributes to the recruitment of leukocytes such as eosinophils by enhancing endothelial cells ability to produce PAF. This prolonged expression of PAF in response to a lower amount of histamine than what is normally required to induce PAF expression could play a significant role in the pathogenesis of asthma. Since PAF receptors are found on many leukocytes such as neutrophils, its role in adhesion is not limited to selective recruitment of eosinophils. However, when taken in conjunction with the upregulation of P-selectin and VCAM-1, as well as the subsequent activation of integrins on eosinophils upon PAF receptor engagement, PAF can play an important role in the selective recruitment of eosinophils to airway tissue.

## 3.3 FUTURE DIRECTIONS:

Continued studies using protein synthesis inhibitors would be of great interest in dissecting the mechanisms behind IL-4 priming of HUVEC and their retained ability to produce PAF as compared to control cells. Emetine as well as actinomycin D are alternative inhibitors that can prevent translation and transcription respectively. Implications of the cyclohexamide results suggest a role for the activation of MAPK pathway, which has not been previously characterized in histamine-induced PAF synthesis and would be of great interest for future inhibitor therapies. As previously stated, both the signaling pathways of histamine and thrombin-induced PAF synthesis are sparsely characterized and need to be fully elucidated before the effects of IL-4 on these pathways can be completely understood.

The role of IL-4 in priming HUVEC was investigated in this study, however, another important Th2 cytokine which is overexpressed in asthma is IL-13. The effect of this cytokine alone or in conjunction with IL-4 PAF production in HUVEC would be of interest, as both these cytokines are found in the tissue and may have combinatorial

effects. In addition, IL-13 shares a similar signaling pathway with IL-4 through the IL-4Rα chain and subsequent activation of the STAT6 homodimer. It would be interesting to see if IL-13 exhibits the same priming effect on histamine-induced PAF synthesis as IL-4 does. Recent studies in knockout mice have demonstrated an important role for IL-13 in airway hyperresponsiveness and eosinophil recruitment to airway tissue. The authors in this study suggest that IL-4 may play a greater role in the establishment of a Th2 response in asthmatic airways while IL-13 mediates eosinophil recruitment and AHR once the inflammation has occurred. Perhaps IL-13 may be a more potent mediator of eosinophil recruitment than IL-4 in asthma.

## CHAPTER FOUR: SPECIFIC AIM 2

**Purpose:** IL-4 priming of HUVEC involved both an increase in total PAF synthesis as well as an increase in sensitivity to histamine. ***To ascertain the mechanism behind the increased PAF production (refer to figure 3.4 and figure 4.1, component A) the effects of IL-4 on the PAF synthesis pathway, as well as the availability of substrate for the production of PAF was examined.***

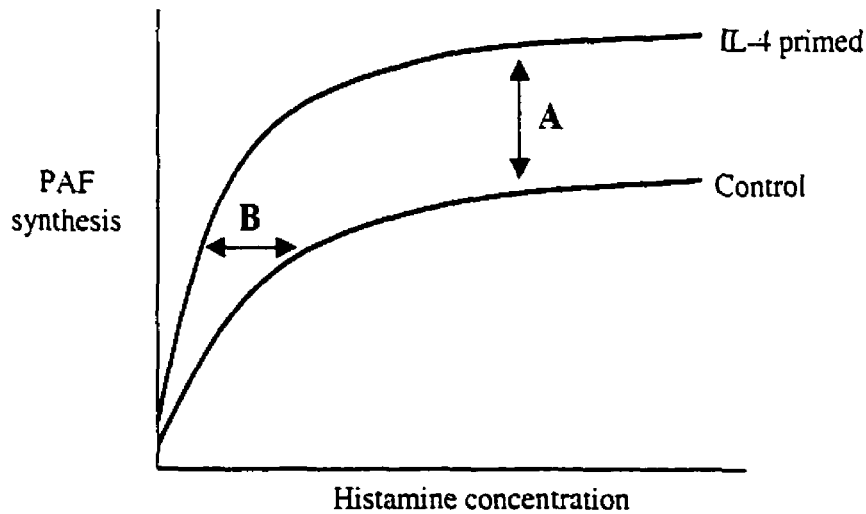



**Figure 4.1** A breakdown of the priming observed with IL-4-treated HUVEC. Both an increase in total PAF synthesis (A) and increase in sensitivity (B) is seen.

*Objectives:*

1) Determine the effect of IL-4 on $PLA_2$ protein levels and activity as a possible mechanism of increased PAF synthesis.

2) Is the accumulation of PAF the result of reduced breakdown of PAF by PAF-acetylhydrolase?
3) Is the increased amount of PAF in IL-4-treated cells due to an increase in the amount of substrate (lipid bodies)?
4) Does IL-4 affect processing (PLC) of membrane phospholipids in the treated HUVEC?

## 4.1 RESULTS:

*Cytoplasmic $PLA_2$ levels and activity are not altered by IL-4 treatment of HUVEC.* PAF synthesis occurs through the sequential action of phospholipase $A_2$ ($PLA_2$) and PAF-acetyltransferase (PAF-AT); refer to figure 1.4. An increase in $PLA_2$ amount or activity would offer an explanation for the increase in PAF production seen in IL-4 primed cells. To address this we first looked at the effect of both IL-4 and TNFα on the levels of $PLA_2$ in HUVEC. No significant difference was observed in $cPLA_2$ protein levels between the control, TNFα or IL-4-treated cells (figure 4.2).

Although IL-4 did not alter $cPLA_2$ protein levels, western blots do not address the issue of $cPLA_2$ enzymatic activity. Since $PLA_2$ generates arachidonic acid, we looked at $PLA_2$ activity by quantifying the breakdown products of arachidonic acid, which include prostaglandins ($PGE_2$) and prostacyclins ($PGI_2$) in HUVEC. Endothelial cells were treated with either TNFα or IL-4 for 24 hours and then $PGE_2$ and $PGI_2$ (which is detected by its breakdown product 6-keto-$PGF_{1\alpha}$) were measured after subsequent stimulation with histamine. Figure 4.3A shows that histamine stimulation increased $PGE_2$ levels in control cells. This indicates that $cPLA_2$ is active. The TNFα pretreated cells showed a significant increase in $PGE_2$ production over control when stimulated with histamine. This is expected since TNFα upregulates COX-2, also known as prostaglandin H synthase 2. However, the IL-4-treated HUVEC did not demonstrate a significant increase in $PGE_2$ levels. This suggests that neither $PLA_2$ nor COX-1 or COX-2 show increased activity in IL-4-stimulated HUVEC. In figure 4.3B, the levels of 6-keto $PGF_{1\alpha}$ were measured in cytokine treated HUVEC similar to the above assay. As expected, the

histamine-induced cells demonstrated an increase in 6-keto-$PGF_{1\alpha}$ levels indicating that $cPLA_2$ is active. As with $PGE_2$, the TNFα treated HUVEC showed a significant increase in $PGF_{1\alpha}$ levels over control; however, the IL-4-treated cells did not.

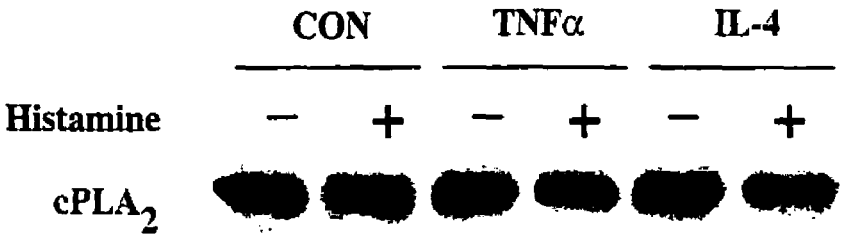



**Figure 4.2.** *IL-4 does not increase $cPLA_2$ levels in histamine-induced HUVEC.* HUVEC were treated with M199/A alone, or M199/A containing TNFα (20 ng/ml) or IL-4 (20 ng/ml) for 24 hours. Following stimulation, the monolayers were washed with HBSS/A and then treated with $10^{-5}$ M histamine for 5 minutes. Cells were lysed in triton X-100 containing lysis buffer and the soluble proteins were separated by SDS-PAGE. Proteins were transferred to PVDF membranes and probed with anti-$cPLA_2$ mAb as described in the methods. Data shown is representative of at least three experiments.

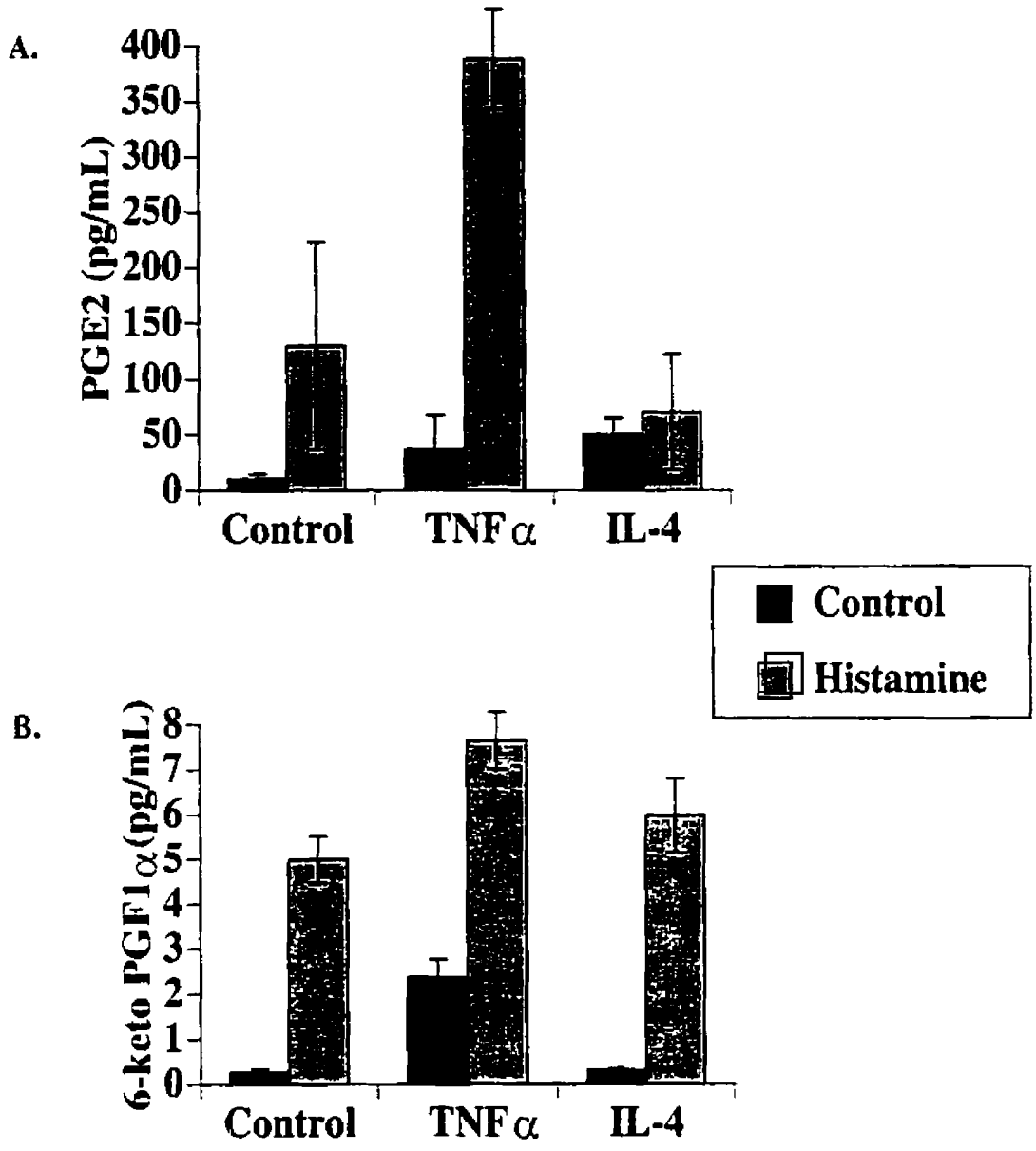



**Figure 4.3.** *IL-4 does not prime HUVEC for histamine-induced synthesis of prostaglandin or prostacyclin.* HUVEC were treated with M199/A alone, or M199/A containing TNFα (20 ng/ml) or IL-4 (20 ng/ml) for 24 hours. Following stimulation, the monolayers were washed with HBSS/A and then treated with $10^{-5}$ M histamine for 5 minutes. (A) $PGE_2$ and (B) $PGI_2$ (as detected by the breakdown product 6-keto-$PGF_{1}\alpha$) were measured as described in the methods. The data in (A) and (B) represent the mean ± SEM of three experiments.

To summarize, the TNFα treated HUVEC showed a significant increase in both prostaglandin and prostacyclin production. This increase is expected and has previously been described [154]. The production of prostaglandin or prostacyclin in response to IL-4-treatment did not show any significant changes over control. These data suggest that IL-4 did not affect the activity of $cPLA_2$ in HUVEC.

*The breakdown of PAF by PAF-acetylhydrolase remains unaltered by IL-4 or TNFα treatment.* An alternative explanation for the increase in PAF synthesis seen in IL-4-treated HUVEC is the accumulation of PAF by delayed degradation, which is normally carried out through PAF-acetylhydrolase activity. We used a substrate assay kit to evaluate the effect of TNFα and IL-4 on HUVEC PAF-acetylhydrolase levels. Cell lysates were incubated with 2-thio PAF substrate and PAH-AH activity was detected by colometric change resulting from the detection of free thiols. A positive control and negative control supplied by the manufacturer were run to ensure the assay was working properly (figure 4.4). The results demonstrate there is no significant difference between control, TNFα or IL-4-treated cells with or without histamine induction. When compared to the negative control, each assay showed a slightly elevated activity level of PAF-AH, which indicates a constitutive level of PAF-acetylhydrolase activity is present in all HUVEC.

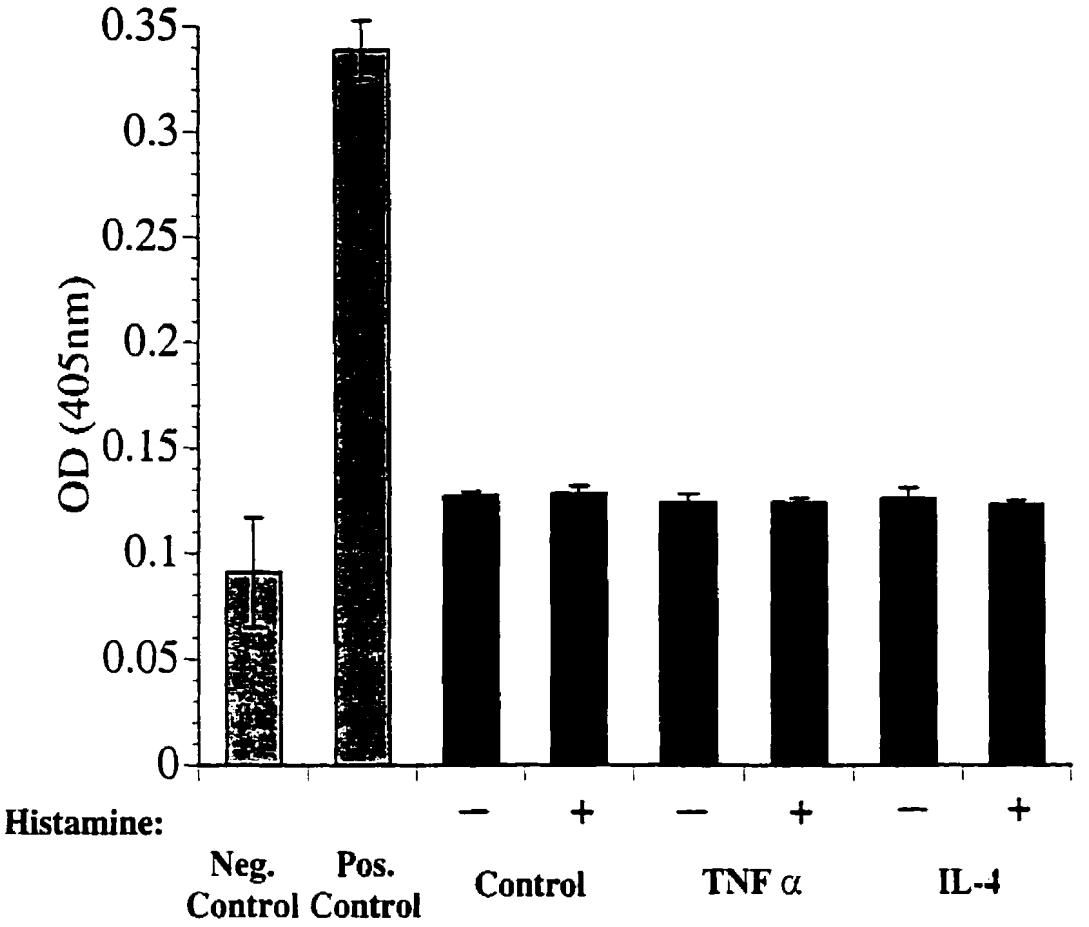



**Figure 4.4.** *PAF-acetylhydrolase activity is unaltered by either TNFα or IL-4 treatment of HUVEC.* Cells were cultured in 6-well plates and confluent monolayers were treated with either M199/A alone or M199/A containing IL-4 (20 ng/mL) or TNFα (20 ng/mL) for 24 hours. Cells were washed with HBSS then scraped and lysed in 0.5 M HEPES solution by sonication as described in the methods. PAF-acetylhydrolase activity levels were assessed as described in the methods. Data shown is the mean ± SD of duplicate points and is representative of two experiments.

*PLCγ and $\beta_1$ levels do not change with TNFα or IL-4 pre-treatment.* The phospholipase C enzyme is involved in the breakdown of $PIP_2$ to $IP_3$ and DAG. DAG in turn is responsible for the activation of protein kinase C (PKC) whose activity is required for $cPLA_2$ activity. In addition, $IP_3$ is responsible for rises in intracellular $Ca^{2+}$, which is also a requirement for $cPLA_2$ activity. In order to determine if PLC levels are altered by cytokine treatment in HUVEC, we performed western blots to look at the different PLC isoforms. Western blots of the whole cell lysate showed no difference in PLCγ levels for either 24 hour treatments of TNFα or IL-4 when compared to the control (figure 4.5).

There are 3 major isoforms of PLC. Another isoform that could be altered in levels or activity that could affect PAF synthesis is PLCβ. Histamine receptors are G protein coupled receptor whose $\alpha_q$ subunit could activate the PLCβ isoform. Thus, we looked at the levels of $PLC\beta_1$ by immunoprecipitation for the enzyme and western blot as shown in figure 4.6. No difference was seen between the IL-4-treated and control HUVEC.

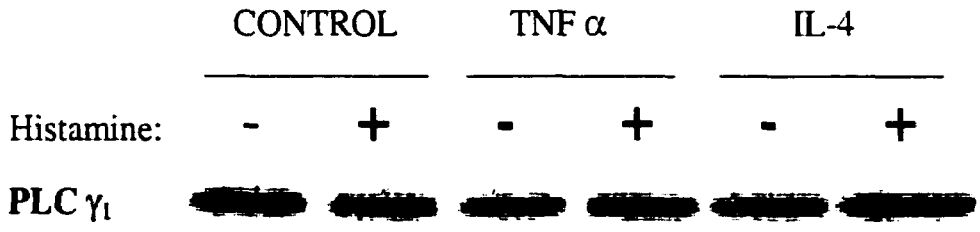



**Figure 4.5.** *Phospholipase Cγ1 levels are unaltered in cytokine treated HUVEC.* Cells were grown to confluence in 6-well plates and treated with either M199/A alone or M199/A containing TNFα (20 ng/mL) or IL-4 (20 ng/mL). HUVEC were then induced with buffer or $10^{-5}$ M histamine then washed once and lysed in a triton X-100 based lysis buffer as described in the methods. Protein was separated by SDS-PAGE and transferred to PVDF membranes, then blotted using an anti-human PLC-γ1 primary Ab and an anti-mouse IgG-HRP secondary Ab. Data shown is representative of at least three experiments.

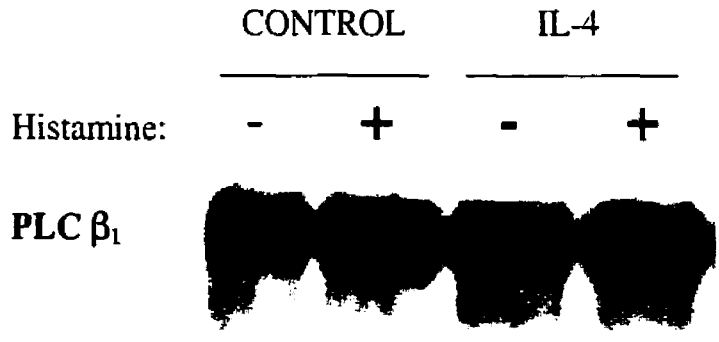



**Figure 4.6.** *Phospholipase $\beta_1$ levels are unaltered by IL-4 treatment of HUVEC.* Cells were cultured as previously described and stimulated with either M199/A alone or M199/A containing IL-4 (20 ng/mL). HUVEC were then induced with buffer alone or $10^{-5}$ M histamine then lysed as described in the methods. Lysates were immunoprecipitated for PLC $\beta_1$ then run on SDS-PAGE and transferred to PVDF membrane. Immunoblots were performed using a PLC $\beta_1$ primary Ab and an $\alpha$-rabbit IgG-HRP secondary Ab. Data shown is representative of at least three experiments.

*Lipid bodies do not increase in IL-4 treated HUVEC.* Lipid bodies have been described as a structure within cells that act as a source for arachiodonate-containing lipids as well as a site for phospholipid metabolism [108:155]. As such, we decided to investigate the existence of lipid bodies in HUVEC and see if they are altered in number as a result of IL-4-treatment. Two methods were employed to visualize the lipid bodies within confluent HUVEC monolayers. Figure 4.7 shows the results of nile red fluorescent staining for lipid bodies which are seen as bright green spots when visualized under blue light. A comparison between control and IL-4 stimulated cells shows an increase in lipid bodies in the IL-4-treated cells (figure 4.7A). However, this difference was not always consistent between different fields of view within one experiment or between individual cords (figure 4.7B). A second method, staining cells with osmium tetraoxide, was used to visualize the lipid bodies, which appear as black spots (figure 4.8). IL-4-treated HUVEC appears to show an increase in number of lipid bodies when compared to control (figure 4.8A). However, the difference was not significant when random counts were performed on multiple fields between three experiments (figure 4.8B). A summary of the counts performed on the osmium stained slides is shown in Table 4.1.

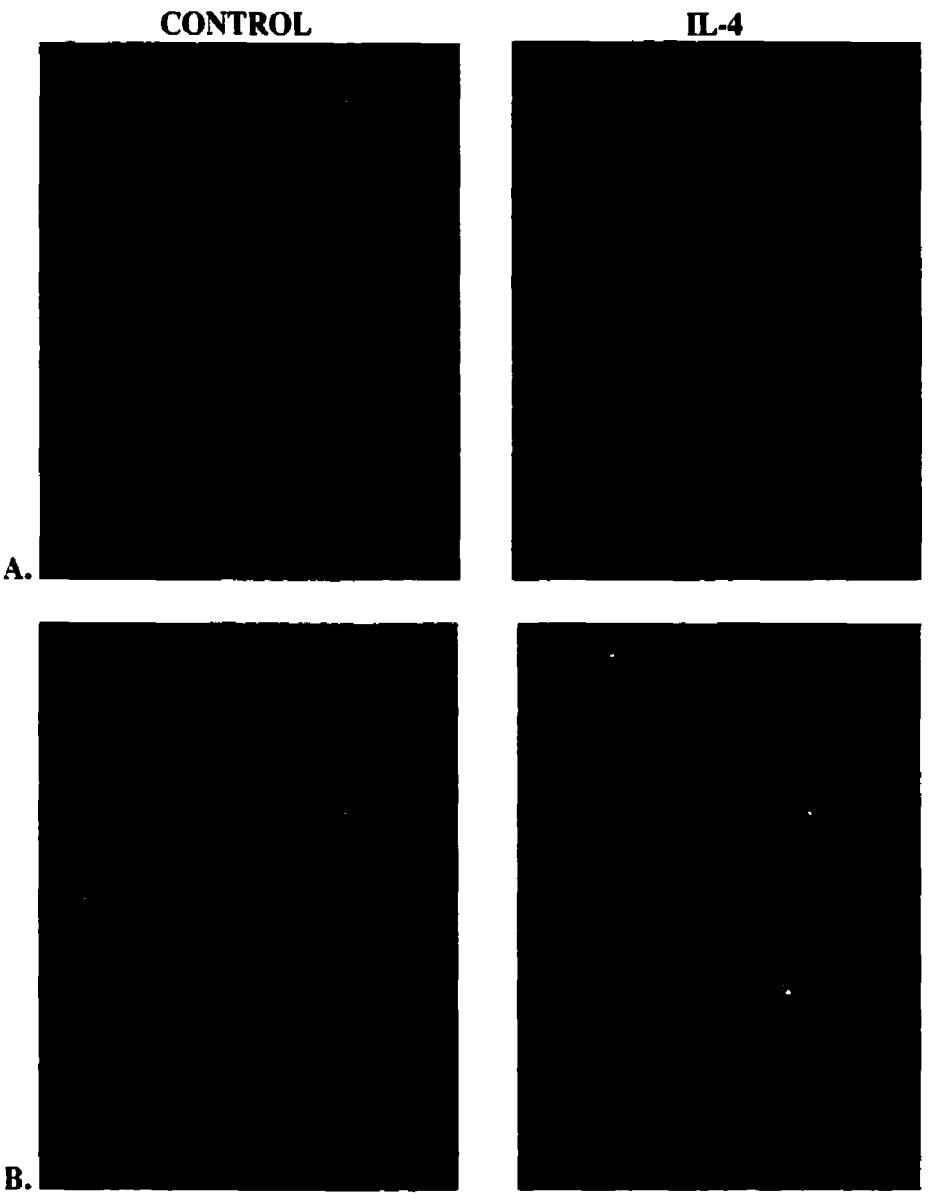



**Figure 4.7.** *Nile red staining for lipid bodies in IL-4-treated HUVEC.* HUVEC were cultured on 12mm glass coverslips in 4-well plates until confluent. HUVEC were treated with M199/A alone or M199/A containing IL-4 (20 ng/mL) for 24 hours. Cells were stained for lipid bodies using a fluorescent nile red dye as described in the methods. The coverslips were visualized using a fluorescent light microscope under 100X objective and random fields were photographed. (A) A comparison between control and IL-4 HUVEC shows a distinct increase in the number of lipid bodies contained in IL-4 treated cells however, as illustrated in panel (B) the difference is not always consistent. This experiment was repeated at least three times.

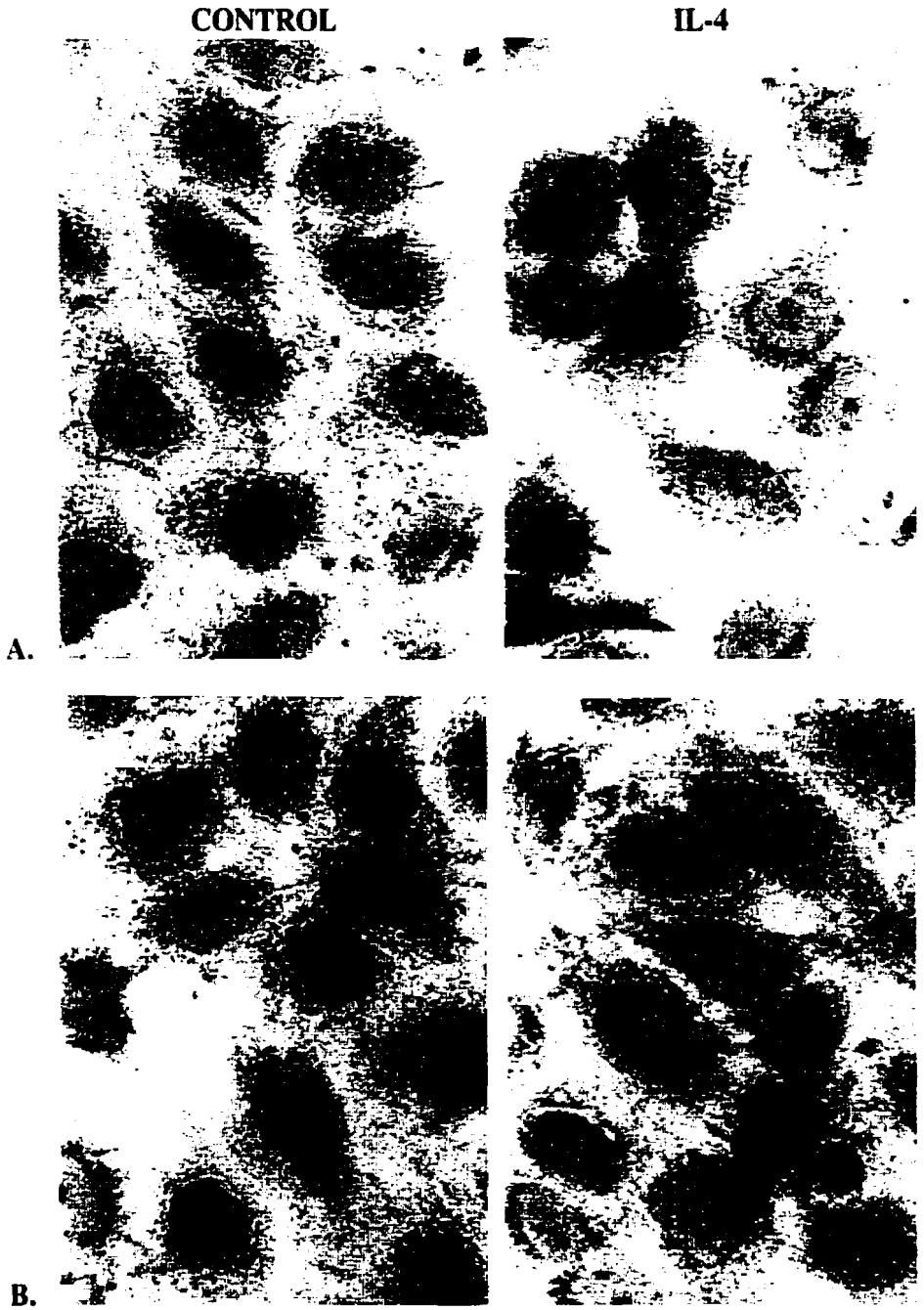



**Figure 4.8.** *Osmium tetraoxide staining for lipid bodies in IL-4-treated HUVEC show an inconsistent difference.* As described in figure 4.7, HUVEC were treated with M199/A alone or M199/A containing IL-4 (20 ng/mL) for 24 hours. Cells were stained for lipid bodies using osmium tetraoxide as described in the methods. (A) Lipid bodies are dramatically increased in the IL-4 treated HUVEC compared to the control cells however, (B) the difference is not consistent (see Table 1). This experiment was repeated at least three times.

**Table 4.1.** Lipid body counts for untreated or IL-4 (20 ng/mL) treated HUVEC show no significant difference when stained with osmium tetraoxide.

| EXPERIMENT: | TREATMENT: | LIPID BODIES / CELL: |
|---|---|---|
| Cord 1 | Control | 14.6 ± 2.5 |
| | IL-4 (24 hours) | 14.7 ± 7.5 |
| Cord 2 | Control | 16.5 ± 9.4 |
| | IL-4 (24 hours) | 9.8 ± 1.0 |
| Cord 3 | Control | 32.3 ± 9.5 |
| | IL-4 (24 hours) | 23.1 ± 1.4 |

## 4.2 DISCUSSION:

The effects of IL-4 priming on HUVEC were broken down into two components; an increase in total PAF produced and secondly, an increase in sensitivity to histamine (refer to figure 4.1). Our studies showed that HUVEC primed with IL-4-treatment for 24 hours demonstrated both an increase in histamine-induced PAF production as well as an increase in thrombin-induced PAF production. This increase when compared in a time course was in fact a retention of PAF produced when compared to control cells which were unable to synthesize elevated PAF levels as seen in earlier time points (figure 3.7). We first investigated the possible mechanisms behind the increase in total PAF synthesis seen in the IL-4-treated cells.

A number of possible explanations could account for the elevated PAF production observed in IL-4-treated cells. However, the response was seen in both histamine- and thrombin-induced cells suggesting that IL-4 mediated changes downstream of the receptors, but common to both signaling pathways. We therefore began by investigating the enzymes directly involved in production of PAF. Synthesis of PAF is dependent on the sequential action of $PLA_2$ and PAF-AT and degradation requires PAF-AH. A previous study by Wu *et al.* [156] demonstrated that TNF$\alpha$ is able to upregulate $PLA_2$ protein levels in human bronchial epithelial cells. We investigated the possibility of increased $PLA_2$ levels by western blots and found that the IL-4-treatment did not alter protein levels of $PLA_2$ (figure 4.2). During PAF synthesis, $PLA_2$ activity results in the cleavage of an arachidonic acid chain from membrane phospholipids. This arachidonic acid is used as a substrate by the cyclooxygenase enzymes (COX-1 and COX-2) in the production of prostaglandins and prostacyclins. While COX-1 is a constitutively active enzyme, COX-2 is an inducible form of the cyclooxygenase enzymes and acts to convert arachidonic acid to $PGG_2$ or $PGH_2$ [157]. These prostaglandins are rapidly converted into $PGI_2$ and $PGE_2$ in endothelial cells. The inducible form of cyclooxygenase is expressed when cells are exposed to inflammatory stimuli and phorbol esters activating PKC [158]. In addition, glucocorticoids will suppress the activity of COX-2 and not COX-1 suggesting a role for COX-2 expression in inflammation [159;160]. Previous studies have shown that both histamine and thrombin are able to induce prostacyclin production in endothelial

cells [83;154]. Studies by Watanabe *et al.* have shown that TNFα is able to potentiate $PGI_2$ synthesis in response to the secondary agonist thrombin in endothelial cells [154]. Our results confirmed this event as seen by the increased $PGI_2$ and $PGE_2$ expression in histamine-induced TNFα treated HUVEC. However, IL-4-treatment did not alter levels of $PGE_2$ and $PGI_2$ indicating that unlike TNFα, IL-4 did not affect the cyclooxygenase enzymes directly or alter $PLA_2$ activity (figure 4.3).

It is interesting to note that TNFα treatment had profoundly different effects on processing of phospholipids than IL-4 on HUVEC. Within 24 hours of TNFα treatment we observed a significant decrease in histamine-induced PAF synthesis (refer to figure 3.4) and yet, we saw no difference in $PLA_2$ levels. Further, we see an increase in metabolism of arachidonic acid, a downstream event of $PLA_2$ activity, in TNFα treated cells but not in IL-4-treated cells. Studies have shown that TNFα is able to induce COX-2 expression in endothelial cells [161]. Our results demonstrate that TNFα is able to induce $PGI_2$ production alone but not PGE2 production. Together, the observations suggest that differential regulation of signaling by TNFα and IL-4 occurs at either the regulation of $PLA_2$ or the COX-2 enzymes.

While the elevated levels of PAF found in histamine or thrombin-induced IL-4-treated HUVEC could not be explained by an increase in $PLA_2$ activity, it remained a possibility that the PAF was not being degraded and was simply accumulating. There are a number of forms of PAF-AH currently described in the literature, both a plasma form and an intracellular form. Unlike the $PLA_2$ or PAF-AT enzymes, PAF-AH does not require $Ca^{2+}$ for activation and thus is constitutively active at basal levels [103]. The importance of this enzyme in asthma is demonstrated by a study in which a Japanese population of asthmatics was analyzed and found that a homozygous mutation in the PAF-AH gene was associated with an increased severity of the disease [104]. In addition, recent studies by Henderson *et al.* have shown that administration of PAF-AH to a murine model of asthma inhibited eosinophil infiltration and decreased AHR in the lungs [105]. Assays to measure PAF-AH activity showed no change in control or IL-4-treated cells with or without histamine induction (figure 4.4). Consistent with the literature, all assays showed a slightly elevated level of PAF-AH activity over the negative control

suggesting that the enzyme was present and active at a basal level, which was unaltered by agonists.

PAF-AH mediated degradation of PAF was not shown by our experiments however, an alternative explanation may be that IL-4-treatment could increase histamine-induced PAF-AT activity. PAF-AT is the second enzyme in the PAF synthesis pathway involved in converting the intermediate molecule Lyso-PAF into PAF by transferring an acetate group from Acetyl-CoA into the *sn*-2 position of the fatty acid. Unfortunately, PAF-AT has yet to be isolated and cloned in mammalian systems. While $PLA_2$ activity is essential for PAF synthesis to occur, the PAF-AT enzyme is considered the rate-determining step of the synthetic pathway. Previous studies measuring PAF-AT activity in response to thrombin have shown that this enzymes activity increases to a maximal state of activation within 60 seconds [85]. Ongoing studies in our lab are investigating the effects of IL-4 on histamine-induced PAF-AT activity to determine if the increase in PAF synthesis seen in these cells is a result of increased PAF-AT activity.

Upstream of the PAF synthetic pathway, IL-4-treatment of HUVEC could potentially alter the enzyme mediated events that are required for $PLA_2$ activation. As mentioned previously, $PLA_2$ requires both a rise in intracellular $Ca^{2+}$ as well as PKC activation. In fact, some isozymes of PKC require $Ca^{2+}$ for activation, however, in histamine-induced endothelial cells, the particular PKC isoforms activated are unknown. Therefore, we investigated the enzymes responsible for the rise in intracellular $Ca^{2+}$ in response to either histamine or thrombin. Upon receptor engagement, phospholipase C couples to membrane phospholipids and hydrolyzes $PIP_2$ into $IP_3$ and DAG. $IP_3$ binds to a specific receptor on intracellular $Ca^{2+}$ storage vesicles and induces a release of these $Ca^{2+}$ stores into the cytoplasmic milieu. This brief rise in intracellular $Ca^{2+}$ leads to the opening of $Ca^{2+}$ channels in the cell membrane, allowing extracellular $Ca^{2+}$ to enter the cell. The resulting sustained elevation of intracellular $Ca^{2+}$ levels is required for $PLA_2$ activity. In addition, the DAG binds to PKC reducing its threshold for $Ca^{2+}$ and leads to PKC activation. There are three major families of PLC; of interest to us is the PLCγ and PLCβ. Each of these families contain a growing list of isoforms. PLCγ has been shown to be involved in the production of prostaglandins previously [162]. We therefore looked at PLCγ levels in IL-4-treated HUVEC and did not find any difference in protein levels

(figure 4.5). Further, PLCγ anti-phosphotyrosine blots did not show an alteration in phosphorylation of the enzyme in IL-4-treated HUVEC (data not shown). PLCβ is the only family shown to be directly activated by G proteins [90]. Upon G protein activation, the Gα subunit dissociates from the βγ complex. Both these subunits are capable of directly docking with PLCβ and activating the enzyme [163]. Our results again showed no significant difference in PLCβ levels of IL-4-treated cells. Unfortunately, PLCβ is not tyrosine phosphorylated so we were unable to assess activity of this enzyme by measuring changes in phosphorylation. In addition, no broad spectrum antibody for PLC is available. There are at least 4 different PLCβ and 2 different PLCγ isozymes currently identified and it remains a significant possibility that we did not assess the protein levels of the correct isozyme.

Finally, we investigated the possibility that substrate levels for PAF synthesis may be elevated in IL-4-treated HUVEC and may potentially be the source of the elevated levels of PAF observed in the histamine-induced cells. Although the exact location of membrane phospholipids used for PAF synthesis is unknown, $PLA_2$ has been shown to directly associate with a variety of lipid sources. In neutrophils, $PLA_2$ and PAF synthesis have been localized to the cell membrane surface. Other studies have shown that $PLA_2$ localizes to either the nuclear or endoplasmic reticulum membranes [86]. In fact, one study by Sierra-Honigmann *et al.* demonstrated that $PLA_2$ localized to either the nuclear membrane or the cell surface membrane depending upon the confluence of HUVEC used [107]. Another possible source of membrane phospholipids in endothelial cells are lipid bodies. Although the majority of studies conducted in the current literature have focused on lipid bodies within eosinophils, they have been described in a number of different cell types. Evidence suggests that lipid bodies are rich sites of esterified arachiodonate where eicosanoid enzymes can aggregate. Lipid bodies are inducible as previously described in eosinophils which show an upregulation in the number of lipid bodies following stimulation with PAF [108]. Further, evidence in the literature suggests $cPLA_2$ and the ERK 1 and 2 MAPKs co-localize to these lipid body sites [155]. We stained for lipid bodies in HUVEC using two separate methods, however, IL-4-treated cells did not show a significant increase in the number of lipid bodies (figures 4.7 and 4.8). Variability in lipid body numbers between random fields of view in the stained slides resulted in

insignificant results. A possible reason for this variability within each assay could be due to the imprecise nature of the staining protocol as well as the activation state of the HUVEC prior to staining.

To summarize, the investigations into the mechanism of elevated levels of histamine-induced PAF synthesis in IL-4-treated HUVEC have lead to no significant conclusions thus far. However, the results of our studies have eliminated a number of potential avenues behind the mechanism of IL-4 priming. A significant difficulty remains in that the signaling pathways for histamine- and thrombin-induced PAF synthesis is incomplete. The involvement of MAPKs in the activation of both PKC and $PLA_2$ as well as the lack of isozyme characterization for PLC and PKC remain a hindrance in our studies. Further characterization of these proteins that are involved in the PAF synthesis pathway needs to be completed. In addition, the localization and source of membrane phospholipids involved in PAF production would be an asset.

## 4.3 FUTURE DIRECTIONS:

Suggested studies in regards to investigating the mechanism behind the elevated levels of histamine- and thrombin-induced PAF synthesis in IL-4-treated cells include further characterization of the PLCβ isoforms involved in PAF synthesis. As well, specific inhibitor studies to determine the precise isoforms of $G\alpha_q$ proteins involved in histamine receptor engagement would be of great value. These could be done using either specific anti-sense mRNA inhibitors or antibodies directed against these isoforms as previously described by Gutowski *et al.* [133]. Finally, characterization of the PAF-AT activity in histamine-induced PAF synthesis for both control and IL-4-treated cells could potentially lead to an explanation as to the prolonged elevated levels of PAF seen in IL-4-treated HUVEC. Perhaps IL-4 is able to sustain increased PAF-AT activity over that of untreated HUVEC.

# CHAPTER FIVE: SPECIFIC AIM 3

**Purpose:** The priming effect of IL-4 on HUVEC not only increased PAF production but, increased the sensitivity of these cells to histamine (refer to figure 3.5 and figure 4.1, component B). ***We investigated the mechanism of increased histamine sensitivity by focusing on the histamine receptor and downstream effects of receptor engagement.***

*Objectives:*

1) Does IL-4 prime HUVEC for other responses induced by histamine? These include changes in sensitivity for histamine-induced calcium flux and prostaglandin synthesis.
2) Is there an increase in H1 receptors on IL-4-treated HUVEC?

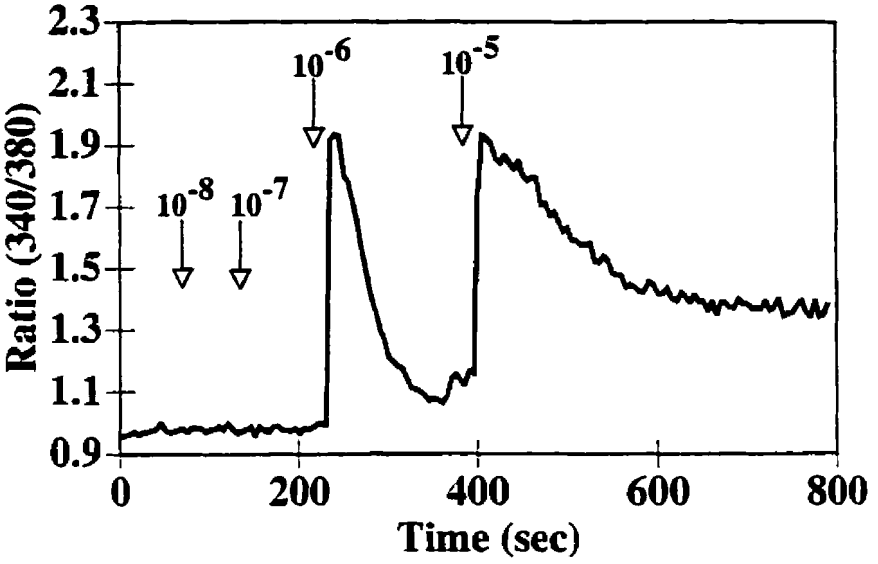
## 5.1 RESULTS:

*IL-4 treated HUVEC have a 100-fold increased sensitivity to histamine-induced calcium flux.* PAF synthesis is a calcium dependent phenomenon, which requires an increase in intracellular $Ca^{2+}$ levels in order for synthesis to occur [89]. Therefore, we measured changes in intracellular $Ca^{2+}$ within HUVEC to determine what effect IL-4-treatment had on these cells. Untreated HUVEC (figure 5.1A) were measured for intracellular $Ca^{2+}$ changes in response to increasing concentrations of histamine. The first dose of histamine that induced a $Ca^{2+}$ flux in the HUVEC occurred at $10^{-6}$ M. However, when compared to the IL-4-pretreated HUVEC, an induction of $Ca^{2+}$ flux occurs at 100X lower concentrations of histamine. Figure 5.1B shows that IL-4-treated HUVEC experience a $Ca^{2+}$ flux when induced with $10^{-8}$ M of histamine. Both the untreated and IL-4-treated cells are able to flux again after returning to basal levels as demonstrated by the successive induction with either the same concentration of histamine (data not shown) or with an increase in histamine concentration. To summarize, a shift in the dose response curve for $Ca^{2+}$ flux is seen in IL-4-treated HUVEC compared to

control. Thus less histamine is required to elicit a response for $Ca^{2+}$ flux in IL-4-treated cells.

The shape of the curves for the initial influx of $Ca^{2+}$ in the control ($10^{-6}$ M) and in the IL-4-treated HUVEC ($10^{-8}$ M) indicate a rapid increase and subsequent decrease in intracellular $Ca^{2+}$. However, in the second spike brought about by a log increase in histamine concentration, both control and IL-4-treated cells experience a longer recovery time. A difference in the control and IL-4 cells is seen during the recovery period at $10^{-5}$ M histamine for the control HUVEC which returns the level of intracellular $Ca^{2+}$ to elevated levels (1.4 units). The IL-4-treated cells return to basal levels (1.0 units) after the second dose of histamine ($10^{-7}$ M).

**A. Control**




**B. IL-4**

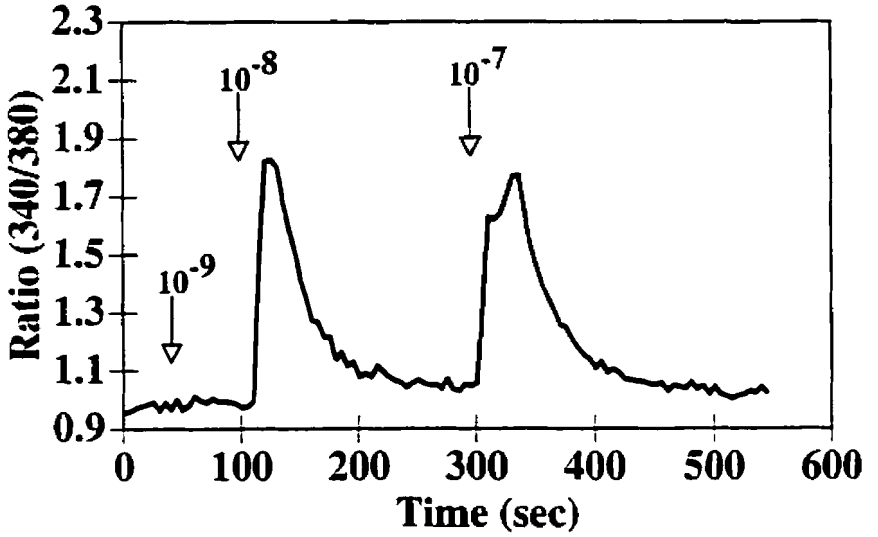



**Figure 5.1.** *IL-4 primes HUVEC for histamine-induced mobilization of intracellular calcium.* HUVEC were cultured on gelatin-coated glass coverslips in 4-well plates. Once confluent, HUVEC were treated with (A) M199/A alone or (B) M199/A containing IL-4 (20 ng/mL) for 24 hours. Following stimulation, cells were washed and loaded with 5μM fura-2-AM for 30 minutes and then placed in a $Ca^{2-}$ imaging chamber as described in the methods. Increasing concentrations of histamine were added to the cells and changes in intracellular $Ca^{2-}$ measured. The data shown are representative of three experiments with equivalent results.

*An increase in histamine sensitivity is reflected in a shift in the dose response curve in prostacyclin production.* An increase in histamine receptors on the surface of IL-4-treated HUVEC should not only increase the sensitivity to histamine for PAF production but for other downstream effects of receptor engagement as well. Since we already observed a shift in $Ca^{2+}$ flux, we next looked at the dose response curve of prostacyclin production in response to increasing concentrations of histamine. A shift in the dose response curve for IL-4-treated HUVEC is observed; a significant increase in $PGI_2$ production occurred at $10^{-6}$ and $10^{-7}$ M histamine in the IL-4-treated cells (figure 5.2A). A dose response curve was performed using thrombin as a secondary agonist. Results showed a slight shift in the IL-4 $PGI_2$ production curve as compared to control (figure 5.2B).

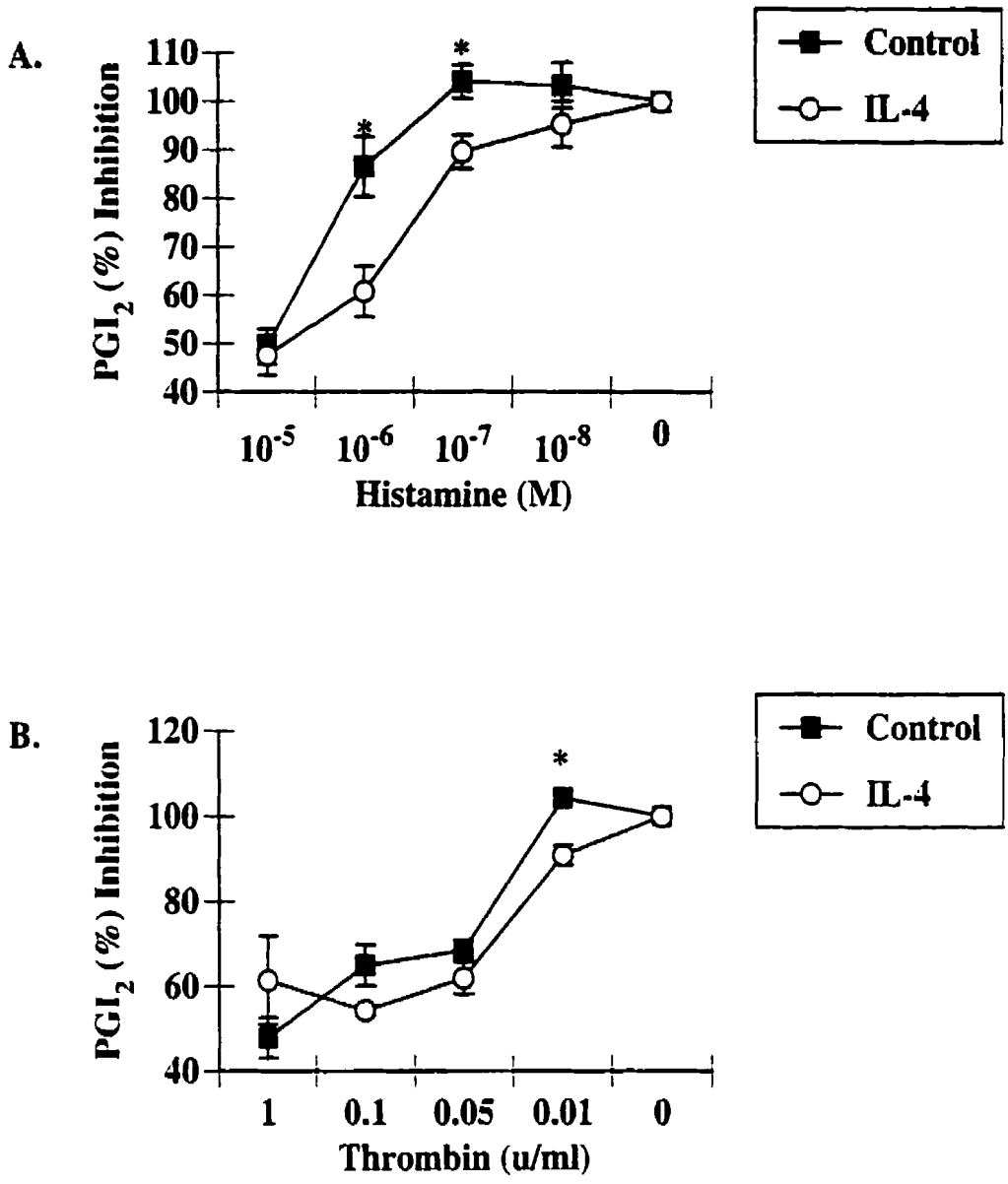



**Figure 5.2.** *IL-4 primes HUVEC for a shift in histamine- but not thrombin-induced prostacyclin production.* HUVEC were treated with M199/A alone or M199/A containing IL-4 (20 ng/mL) for 24 hours. Following stimulation, the monolayers were washed with HBSS/A and then treated with A) histamine or B) thrombin, at the indicated concentrations for 5 minutes. $PGI_2$ (as determined by the breakdown product 6-keto PGF $1\alpha$) were measured as described in the methods. The data in A) and B) represent the mean ± SEM as compared to control of at least 3 experiments. * $p<0.05$

*IL-4 treated HUVEC show a possible increase in the number of histamine receptors on their surface.* We investigated the increase in sensitivity of histamine in IL-4-treated HUVEC seen both in the $Ca^{2+}$ results and $PGI_2$ dose response curves. A possible explanation for the increased sensitivity is an upregulation in the number of histamine receptors on the surface of HUVEC. To address this we first investigated the histamine receptor expression using a heterologous competitive binding assay. There are three types of histamine receptors on the surface of endothelial cells. A specific antagonist for the H1 receptor, pyrilamine, was used to completely block the PAF response. Both control and IL-4-treated HUVEC did not produce PAF in response to histamine stimulation when treated with 100 nM pyrilamine (see figure 5.3A). This indicates that histamine-induced PAF synthesis is mediated through engagement of the H1 receptor. We performed RT-PCR using primers against the H1 gene sequence (see Table 2.1). The H1 receptor mRNA was amplified as a method of indicating the amount of H1 receptors in HUVEC. A positive control was run in each experiment for MCP-4, which is upregulated upon IL-4-treatment (unpublished data). The results demonstrated a slight increase in the H1 mRNA when compared to control cells (see figure 5.4) however, this method is not considered accurate for quantification. We continued to investigate this increase in H1 mRNA by measuring surface expressed histamine receptors.

A competitive binding assay was performed using increasing concentrations of pyrilamine to out compete histamine (see figure 5.3B). The $IC_{50}$ for both control and 24 hour IL-4-treated HUVEC was determined. Results showed an $IC_{50}$ of 11 nM for the IL-4-treated cells and an $IC_{50}$ of 2.2 nM for the control cells indicating that it took approximately one log fold increase in pyrilamine to inhibit 50% of the histamine receptor function in PAF synthesis. This result suggested one of two possibilities, firstly that there are more histamine receptors on the surface of IL-4-treated HUVEC or second, that the IL-4-treated HUVEC have an altered binding affinity for histamine when compared to control cells.

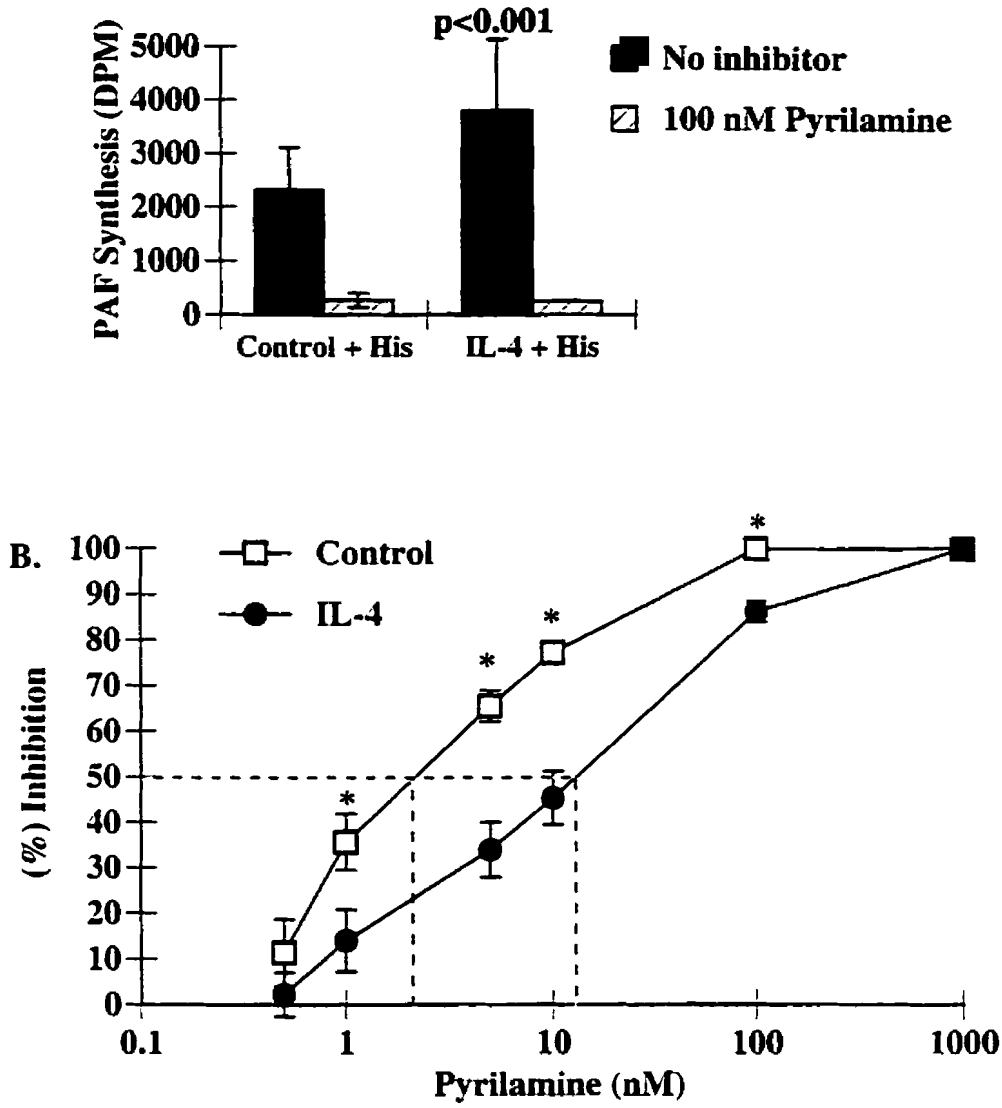



**Figure 5.3.** *IL-4 increases the $IC_{50}$ for pyrilamine on HUVEC.* HUVEC were stimulated with M199/A alone or M199/A containing IL-4 (20 ng/mL) for 24 hours. (A) Following stimulation, cells were washed with HBSS/A pulsed with [$^3$H]-acetate and then treated with $10^{-5}$ M histamine in the presence or absence of $10^{-6}$ M pyrilamine. After 5 minutes PAF synthesis was measured. (B) Following cytokine stimulation, cells were washed with HBSS/A pulsed with [$^3$H]-acetic acid and then treated with $10^{-5}$ M histamine in the presence of increasing concentrations of the H1 antagonist pyrilamine. After 5 minutes PAF synthesis was measured. Data shown are the mean ± SEM of between 4 and 6 experiments. *p<0.05.

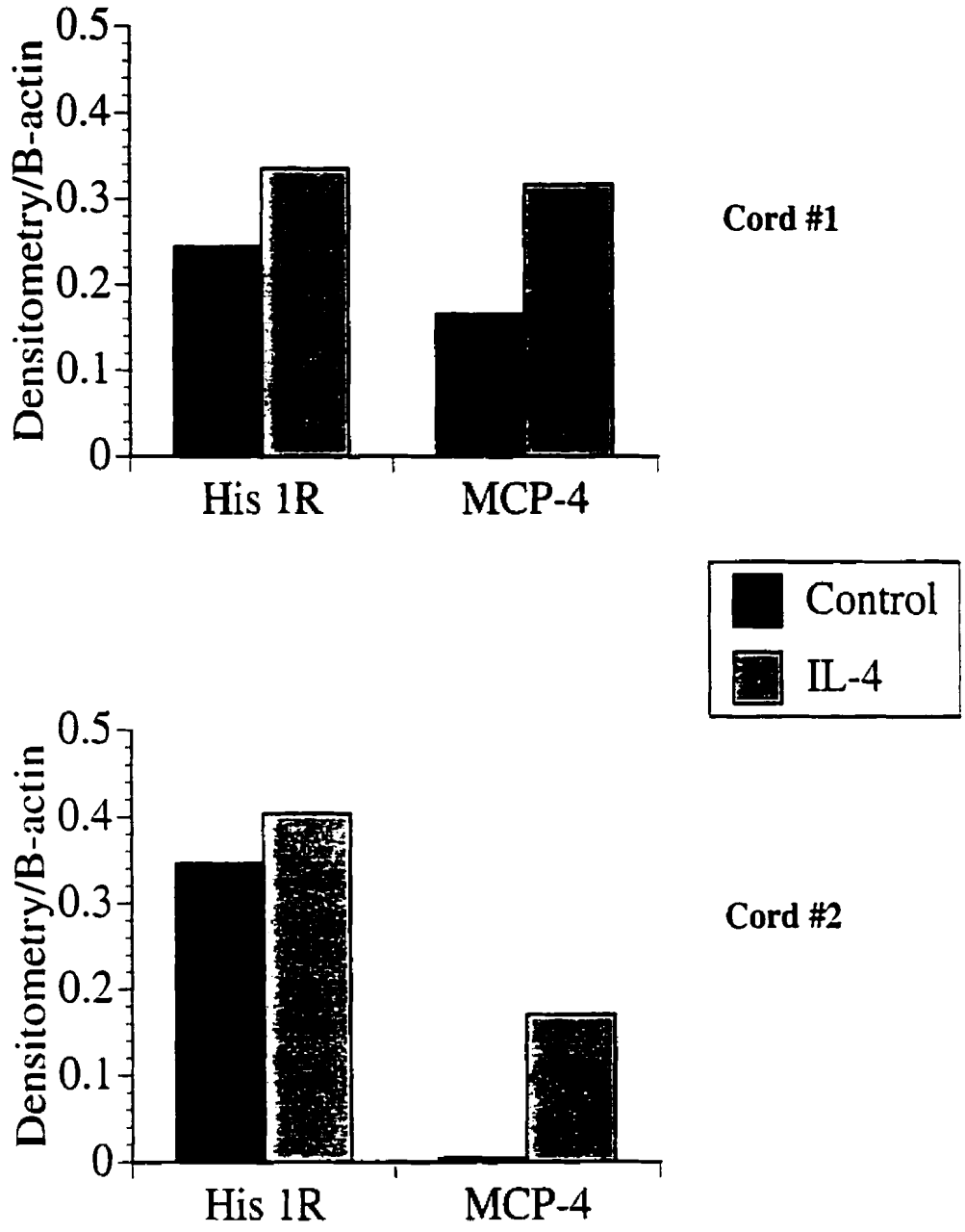



**Figure 5.4.** *Histamine-induced mRNA expression in IL-4-treated HUVEC.* HUVEC were stimulated with M199/A alone or M199/A containing IL-4 (20 ng/mL) for 24 hours. Following stimulation, RNA was extracted using TRIzol reagent according to manufacturer's instructions (Gibco BRL) and amplified by RT-PCR. RNA was resolved using a 2% agarose gel containing ethidium bromide and expression quantified using Fluor-S Max software (BioRad). Data shown are for 2 experiments.

A second method was then employed to quantify the histamine type 1 receptors using homologous competitive binding assays. Here, increasing amounts of cold pyrilamine were added to a constant amount of hot pyrilamine to out compete the surface binding of the antagonist molecule. The excess [$^3$H]-pyrilamine was then washed off and the remaining amount of bound [$^3$H]-pyrilamine was quantified. Results show there was no difference between the control and IL-4-treated cells as illustrated by the line of best fit for both control and IL-4-treated cells (see figure 5.5A). A possible explanation for these results may be that the binding affinity of pyrilamine to the histamine receptor is extremely high, in which case the determination of receptor number by homologous competitive binding assays may be impossible. To confirm this hypothesis a scatchard analysis experiment was performed (see figure 5.5B). Results show a steep slope indicating a very high binding affinity of pyrilamine for the histamine receptor in both the control and IL-4-treated HUVEC. Both conditions showed very similar binding curves indicating that the binding affinity had not changed due to IL-4-treatment. Therefore, the shift in the $IC_{50}$ curve was most likely due to an increase in histamine receptor numbers on the surface of the cells.

Finally, we attempted to quantify the number of H1 receptors on the surface of HUVEC using flow cytometry. A histamine-fluorescein conjugated compound was used to bind to histamine receptors then out competed with increasing concentrations of pyrilamine. Results demonstrated that the cells had a heterogeneous amount of histamine receptors on their surface which could not be competed out with pyrilamine to a quantifiable amount (see figure 5.6). Therefore the results could not be interpreted for H1 receptor numbers.

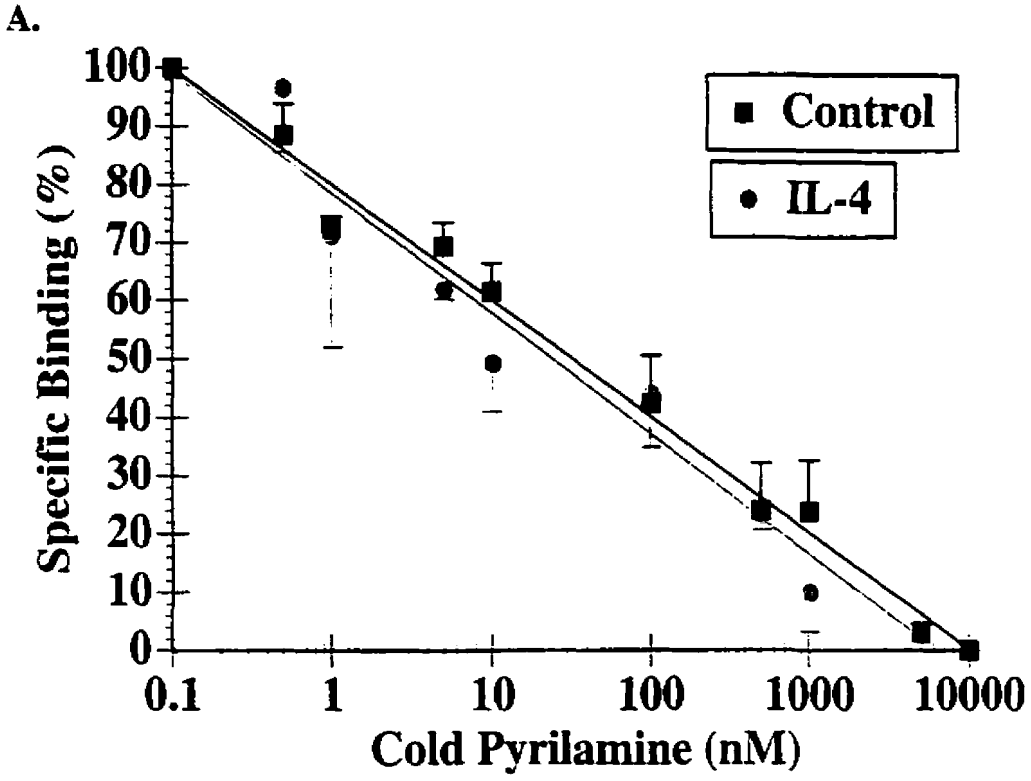
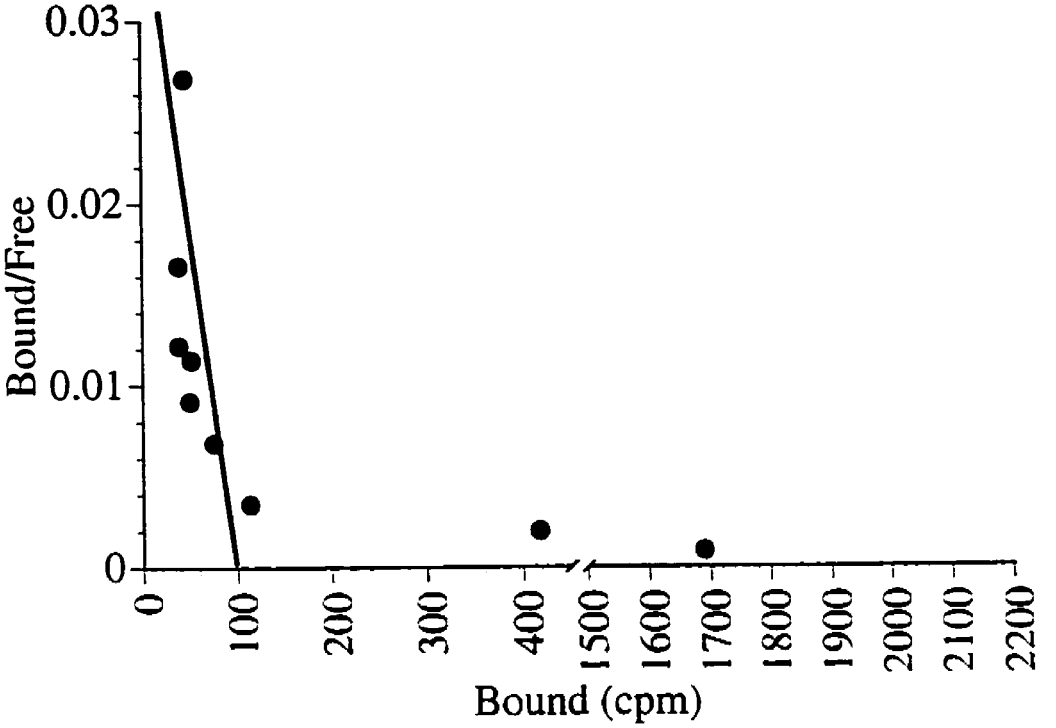


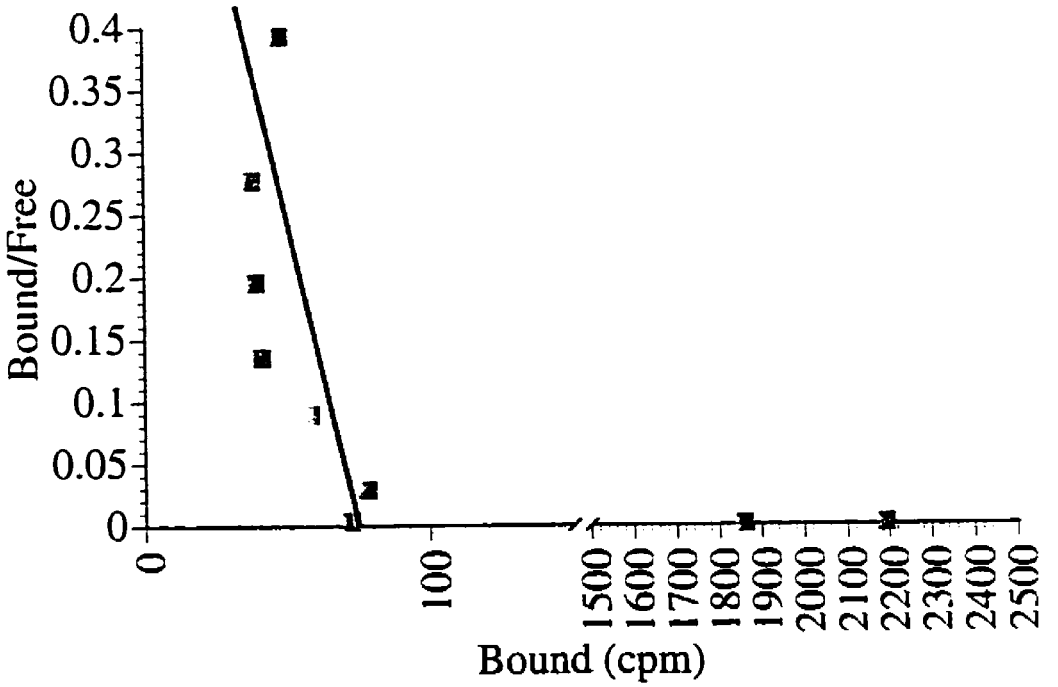

**Figure 5.5.** *IL-4 does not change the affinity of H1 receptors on HUVEC.* HUVEC were treated as in figure 5.3. A) Following stimulation cells were washed twice and increasing amounts of cold pyrilamine were added in the presence of 2.5 nM [$^3$H]-pyrilamine. After 30 mins at 37$^0$C, the cells were washed three times, [$^3$H]-pyrilamine was counted and the competetive binding curves were plotted. Receptor affinity is a function of the slope of the inhibition curve. Data shown are the mean ± SEM of 4 experiments. B) A second method, Scatchard analysis, was employed to determine the receptor affinity (n of 1). The results confirmed the high binding affinity seen in panel A.

B.

Control HUVEC




IL4 HUVEC



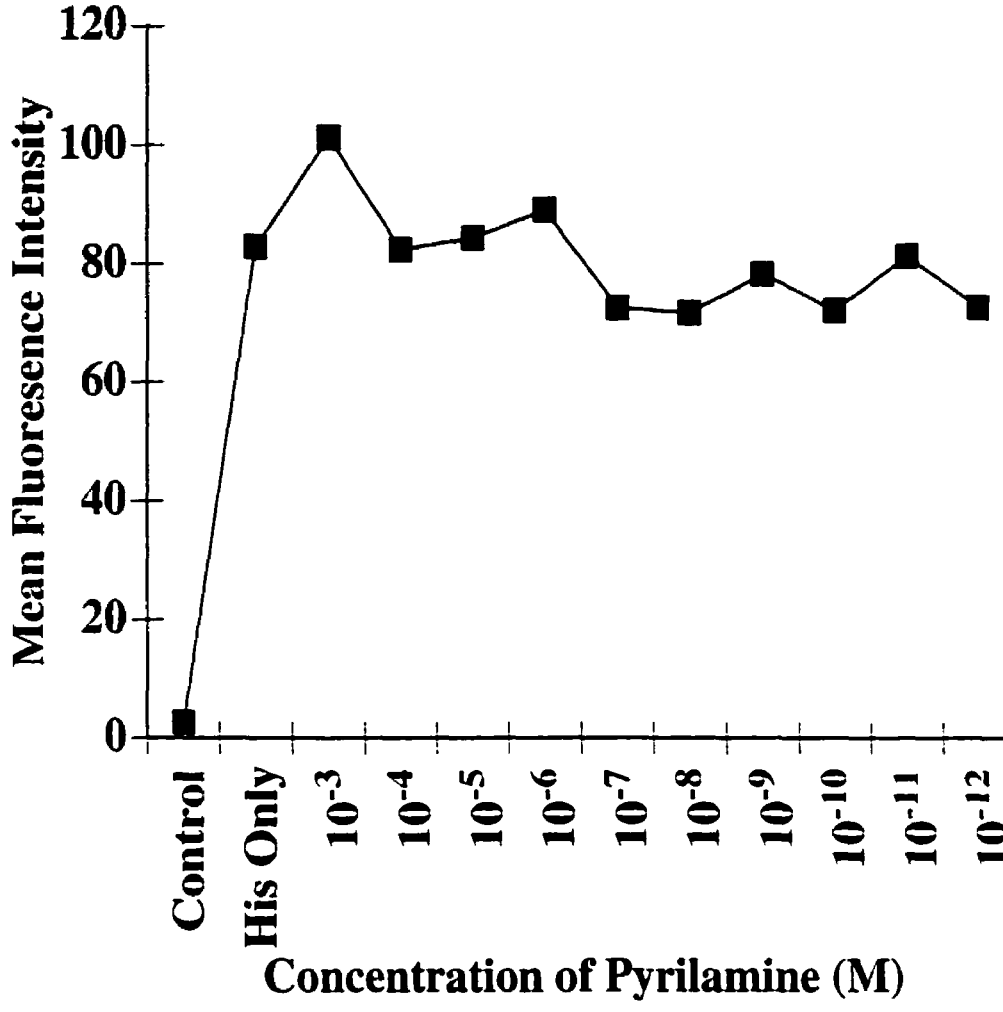



**Figure 5.6.** *Histamine receptor number determination by inhibition with pyrilamine using flow cytometry was unquantifiable.* HUVEC were grown to confluence in 6 well plates, washed with PBS and detached with trypsin. $1X10^6$ cells per assay were incubated with fluor-conjugated histamine ($10^{-4}$ M) for 30 minutes and the indicated concentration of pyrilamine. Fluorescence levels were assessed by flow cytometery. No detectable change in the histamine receptor level could be detected by shift in the pyrilamine inhibition curve. Data is representative of at least three experiments.

*IL-4 treatment does not alter thrombin receptor levels.* Thrombin dose response curves for PAF synthesis (refer to figure 3.6) or $PGI_2$ production (refer to figure 5.2B) showed no or very little shift in IL-4-treated HUVEC compared to control. Experiments demonstrated that an increase in histamine receptors in IL-4-treated HUVEC correlated with the increase in histamine sensitivity. Though we did not see a considerable shift in sensitivity for the thrombin dose curve we performed ELISA experiments to measure thrombin receptor levels in response to cytokine treatment. Results demonstrate that neither 24 hour treatment of TNFα or IL-4 significantly altered the number of thrombin receptors on the surface of HUVEC when compared to control (figure 5.7).

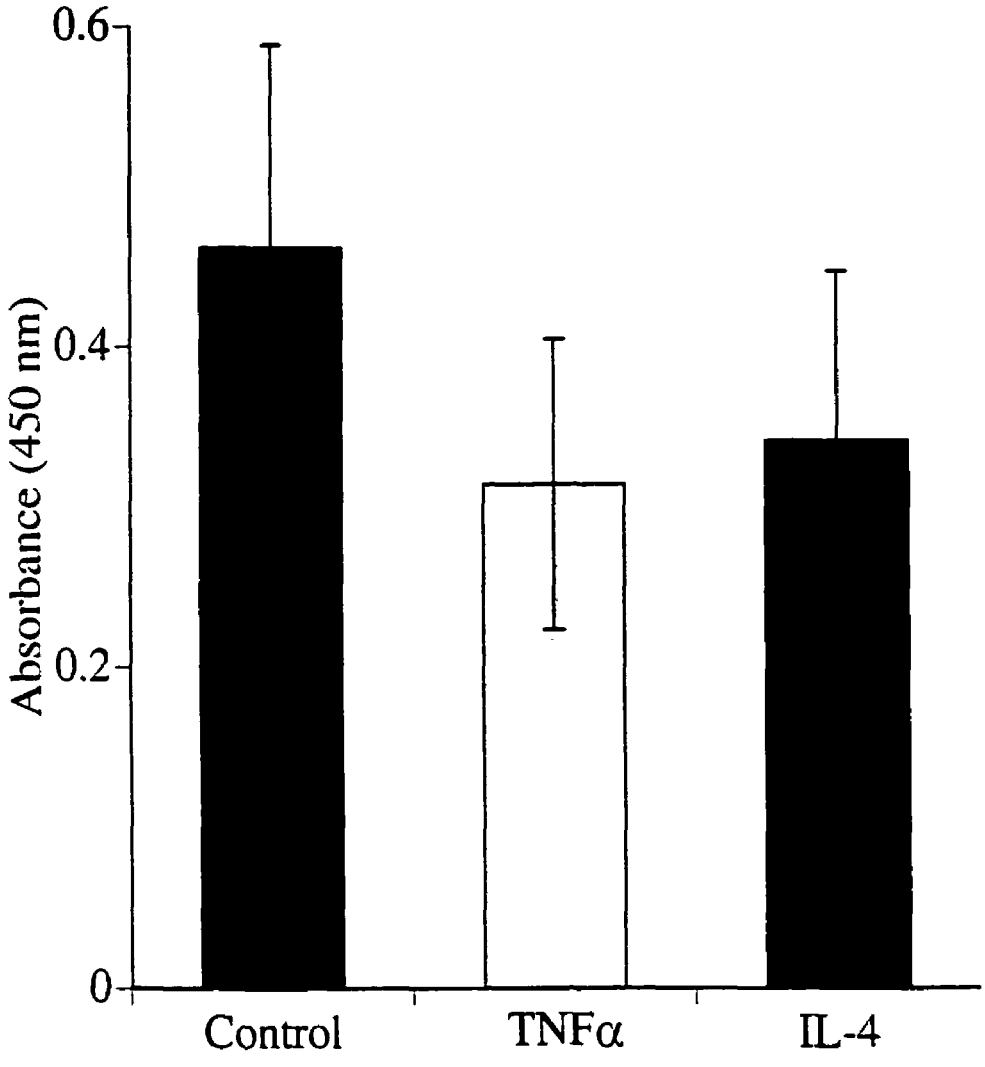



**Figure 5.7.** *Neither IL-4 nor TNFα treatment of HUVEC altered thrombin receptor levels on the surface of HUVEC.* HUVEC were cultured to confluence in 24-well plates and treated as previously described with either M199/A alone or with M199/A containing 20 ng/mL of either IL-4 or TNFα. An ELISA was performed using the primary Ab WEDE 15 (2ug/mL) against the thrombin receptor and an IgG-HRP conjugated secondary Ab. A positive control, VCAM-1, was run with each experiment. Data shown are the mean ± SD of 3 experiments.

## 5.2 DISCUSSION:

The priming of HUVEC with IL-4 showed an increase in histamine- and thrombin-induced PAF synthesis. In addition, a second component of this priming was demonstrated by an increase in sensitivity to histamine but not thrombin in PAF production (refer to figures 3.5 and 3.6). We investigated the mechanism of this increased sensitivity to histamine by first looking at the downstream effects of histamine receptor engagement. An immediate effect upon histamine receptor engagement is the coupling of G proteins to PLCβ which induces formation of $IP_3$. This results in $IP_3$ mediated rises in intracellular $Ca^{2+}$ levels, a requisite for enzyme activation in the PAF synthesis pathway [89]. To determine if an increase in histamine sensitivity altered the upstream effects on the PAF synthesis machinery, we examined the effect of IL-4-treatment on histamine-induced rises in intracellular $Ca^{2+}$ levels. IL-4-treated HUVEC demonstrated a 2-fold log increase in sensitivity for histamine for changes in intracellular calcium levels (figure 5.1). Our data shows that IL-4-treated HUVEC are able to induce a change in intracellular calcium levels, and subsequently synthesize PAF to what is otherwise sub-optimal concentrations of histamine. It is interesting to note that a calcium flux occurred at $10^{-8}$ M histamine in the IL-4-treated cells, however, this concentration of histamine did not induce PAF synthesis in HUVEC (refer to figure 3.5). A significant difference in PAF synthesis first occurred at $10^{-7}$ M histamine suggesting that the $10^{-8}$ M histamine-induced calcium entry was too brief to activate calcium dependent enzymes such as $PLA_2$. While previous studies have shown that a calcium ionophore such as A23187 is sufficient for inducing PAF synthesis [64], it is important to note that these agonists are used in excess concentrations and are capable of sustaining a prolonged intracellular flux of calcium. However, with the initial rise in intracellular calcium seen in the $10^{-8}$ M histamine stimulated cells, the calcium level is not maintained and is rapidly restored to baseline. It is likely that this brief exposure of calcium to the enzymes involved in PAF synthesis is insufficient for sustained activation of $PLA_2$. In addition, other events such as the activation of PKC and MAPK mediated phosphorylation of $PLA_2$ are required for its activation and at $10^{-8}$ M histamine stimulation, these events potentially may not have occurred.

Engagement of either histamine or thrombin receptors will induce prostacyclin synthesis in endothelial cells. The activation of $PLA_2$ results in cleavage of an arachidonic acid chain from membrane phospholipids. Subsequent processing by COX-1 and other prostaglandin synthases results in the production of $PGI_2$. To confirm the increased sensitivity to histamine for $PLA_2$ activation, we demonstrated that downstream events such as prostacyclin production also responded to sub-optimal concentrations of histamine in IL-4-treated HUVEC (figure 5.2). Taken together, the data generated thus far suggested that the increase in sensitivity to histamine in IL-4-treated cells was due to an alteration of function at the receptor level.

A number of possibilities exist for the increased activation of histamine receptors on the surface of IL-4-treated HUVEC. IL-4 may cause a conformational change in the receptor itself, such that it may bind to histamine more rapidly. In addition, some G protein receptors have been shown to overlap in function, acting synergistically with a related receptor to increase the number of G protein mediated events associated with receptor engagement. For example, activation of thrombin receptors enhances the thromboxane A2 affinity for it's ligand and increases coupling of $G\alpha_q$ subunits to the receptor [164]. An increase in receptor coupling of $G\alpha_q$ proteins to the histamine receptor mediated by IL-4-treatment remains a possible explanation for the increase in sensitivity to histamine by these receptors. However, no previous studies have demonstrated that cytokines could exert this effect on G proteins and this mechanism is not a likely event. In addition, increased binding affinity for histamine through conformational changes in the receptor is not very likely as the binding affinity for histamine is high already. The dissociation constant (Kd) for pyrilamine engagement in HUVEC has been measured at 0.74 nM, indicating a tight association of pyrilamine to the histamine receptor [129].

There are three types of histamine receptors known, however, only the H1 receptor has been shown to mediate prostacyclin production [127] and rises in intracellular $IP_3$ levels in human endothelial cells [128]. In addition, a previous study of histamine binding to endothelial cells found that histamine bound to only one receptor as demonstrated by scatchard analysis [129]. As well, a previous study of IL-4-treatment of rheumatoid synovial fibroblasts demonstrated that IL-4- induced production of H1 mRNA [165]. These studies lead us to hypothesize that the increase in sensitivity to

histamine in IL-4-treated HUVEC was due to an upregulation of the number of H1 type histamine receptors on the surface of the cells. An increased number of receptors on the IL-4-treated cells could mediate the effects seen in rises in intracellular $Ca^{2+}$, PAF synthesis and $PGI_2$ production to lower concentrations of histamine than in control cells.

However, no changes were seen in the sensitivity for thrombin-induced PAF synthesis or prostacyclin synthesis in IL-4-treated cells. We investigated the difference between thrombin and histamine sensitivity in IL-4-treated HUVEC by comparing the cytokine treated and untreated cells for changes in receptor numbers. An increase in histamine receptor numbers but not thrombin receptors in IL-4-treated HUVEC could explain the differences in sensitivity to the respective agonists.

While there is no antibody for histamine receptors commercially available, we first quantified histamine receptor expression through competitive inhibitory studies as well as mRNA amplification. Confirming the current literature, we showed that PAF synthesis was induced through the H1 type receptor as PAF synthesis was completely inhibited by use of a specific competitive inhibitor, pyrilamine (figure 5.3). In addition, we measured IL-4-induced H1 mRNA levels using a semi-quantitative method of RT-PCR amplification. A slight increase in H1 mRNA levels was seen in IL-4-treated HUVEC suggesting that H1 expression could be elevated in these cells (figure 5.4). We further investigated this using competitive inhibitor studies which demonstrated a shift in the $IC_{50}$ for pyrilamine (figure 5.3). This observed shift means an increased amount of inhibitor was required to prevent IL-4-treated HUVEC's production of histamine-induced PAF. This suggests that an increase in the number of receptors or a change in receptor affinity for histamine has occurred, however as shown through scatchard analysis, the binding affinity of histamine receptors is extremely high in both control and IL-4-treated cells (figure 5.5). Therefore, our data strongly suggests that IL-4-treatment of HUVEC causes an increase in the number of H1 receptors on the surface of the cells and is the cause for the increased sensitivity to histamine observed.

While IL-4 did induce a change in histamine sensitivity in HUVEC, we did not observe a change in thrombin sensitivity. This was shown for both thrombin-induced PAF synthesis (figure 3.6) and prostacyclin synthesis (figure 5.2). Thrombin receptors, like histamine receptors, are G protein coupled receptors. However, thrombin receptors

belong to the proteinase-activated receptor (PAR) family which are cleaved upon engagement by serine proteases such as thrombin. Endothelial cells express both PAR-1 and PAR-3 on their surface, however, only PAR-1 has been characterized for thrombin mediated activation. Unlike histamine receptors, when engaged, PAR receptors are cleaved and then internalized and degraded [152]. We investigated the effect of IL-4 on thrombin receptor levels and found no change in the thrombin receptor expression (figure 5.7). This supports our findings that no increase in sensitivity to thrombin is seen in IL-4-treated, thrombin-induced PAF synthesis. In addition, the upregulation of histamine receptors but not thrombin receptors could explain the larger increase in amounts of PAF produced between the histamine and thrombin stimulated IL-4-treated cells (figure 4.1).

The implications of this increase in sensitivity to histamine *in vivo* are enormous. In an asthmatic lung where chronic exposure of airway tissue to Th2 cytokines such as IL-4 occurs, an increased sensitivity to histamine could have profound effects during an allergen challenge. Potentially, in a normal lung, when an allergen challenge occurs and histamine is released into the tissue, no significant effects on endothelial cells occur and the person quickly recovers. However, in an asthmatic lung, an allergen challenge that causes release of histamine would cause activation of endothelial cells to produce PAF and may lead to an increase in eosinophil recruitment to the tissue.

## 5.3 FUTURE DIRECTIONS:

Further studies into the direct measurement of histamine receptor numbers on the surface of endothelial cells would confirm this work. Though antibodies to this receptor are not currently available, alternative methods such as flow cytometry using a fluorescent conjugated histamine compound could potentially yield valuable results. We attempted to quantify the H1 receptor using this compound and competitively inhibiting the receptor with pyrilamine, however, the results thus far have shown a variable expression of histamine receptors on the surface of individual endothelial cells (see figure 5.6). Because histamine binds to its receptor with extremely high affinity, conjugating the flourescein-histamine compound to beads and measuring the histamine receptor

numbers may work. Potential problems arise however due to the low number of histamine receptors on the surface of HUVEC as well as the fact that histamine can bind to three different receptors, of which, we are only interested in one. Though scatchard analysis suggests that only one receptor is bound by histamine in endothelial cells, this method is considered inaccurate for high binding affinity compounds and low receptor expression. Until the H2 and H3 receptors are cloned, we cannot confirm their absence in endothelial cells.

Other follow-up experiments include using an antibody to block the IL-4 receptor and determine if there is still a change in PAF synthesis and histamine receptor numbers on the surface of IL-4-treated cells. This would confirm the specificity of IL-4 for inducing the observed changes in PAF synthesis in HUVEC. Further, it would be interesting to characterize the signaling pathway of IL-4 which leads to an increase of histamine receptor gene expression. The histamine receptor promoter contains a number of transcription factor sites such as AP-1 and NF-*k*B. These transcription factors may be activated by the IL-4Rα chains instigation of the ERK 1 and 2 pathway [166]. Finally, studies using IL-13 may also show an increased sensitivity to histamine for PAF synthesis; histamine receptor expression may be upregulated by this cytokine as well.

# CHAPTER SIX: SUMMARY

In this study we examined the role of cytokines in regulating PAF production in endothelial cells. IL-4 is chronically expressed in the asthmatic airway and is known to upregulate adhesion molecules such as P-selectin and VCAM-1 which mediate the selective adhesion of eosinophils to endothelial cells *in vitro*. During an asthma attack, a number of acute mediators are released into the airway tissue including histamine. Histamine is able to induce rapid synthesis and expression of PAF on the surface of endothelial cells. PAF then plays a role in the recruitment of leukocytes by activating tethered leukocytes, causing them to firmly adhere to the endothelial cell lining of blood vessel walls. Although the effect of either IL-4 or histamine alone have been investigated, there have been no studies that have examined the effects of histamine on endothelial cells that have been exposed to cytokines.

Our study focused on the effects of IL-4 exposure on PAF-production by histamine-induced HUVEC. Although IL-4 alone had no effect on PAF-synthesis, we found that IL-4 was able to prime endothelial cells for PAF production in response to histamine. This effect was specific and not reproducible with other cytokines such as TNFα, IL-1β or oncostatin M. This priming effect was broken into two components. First, an increase in the total amount of PAF synthesized was observed. The elevated level of PAF seen in the IL-4 treat HUVEC appeared to be the result of enhanced PAF synthesis over time when compared to control cells. This indicated that IL-4 may be conferring a protective effect on the PAF synthesis machinery within the treated cells. The second aspect of the priming phenomenon was an increase in sensitivity to histamine such that IL-4-treated HUVEC were able to synthesize PAF in response to sub-optimal amounts of histamine. The increased sensitivity to histamine was not restricted to PAF-synthesis since both $Ca^{2+}$ flux and prostaglandin synthesis were also affected. This suggested that an early step in the pathway was being affected. Indeed our results suggested that this increase in sensitivity is due to an increase in the amount of surface expressed H1 receptors in the IL-4-treated HUVEC.

Though we were unable to determine the mechanism for the increase in total PAF synthesis following histamine stimulation in IL-4-treated HUVEC, we were able to

eliminate a number of possibilities. Further investigation is warranted, however, because of incomplete knowledge in the signaling pathways of histamine receptor engagement this will be difficult.

Currently, a number of new treatments for asthma are being explored. These include the inhibition of cytokines such as IL-4, IL-5 and IL-13 as well as PAF. It is important to understand the roles of these mediators in the inflammation of tissue in asthma in order to design effective treatments. Current therapy aims to treat the symptoms of asthma attacks and control the inflammation of the airways. However, future therapy will be aimed at the reversing of airway remodeling and reduction or complete alleviation of inflammation in the airway tissue. Should this goal be reached, asthma may one day be a curable condition instead of just a treatable one.

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