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The effects of eosinophil cationic granule proteins on cholinergic gene expression in nerve cells.

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The Effects of Eosinophil Cationic Granule Proteins on Cholinergic Gene Expression in Nerve Cells

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I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of Doctor of Medicine is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed 

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List of Abbreviations:

Ach	Acetylcholine
AChE	Acetylcholinesterase
AD	Atopic dermatitis
AP-1	Activator protein 1
cDNA	copy DNA
CCR	C-C chemokine receptor
CGL	Cholinergic gene locus
ChAT	Choline acetyltransferase
COPD	Chronic obstructive pulmonary disease
DAPI	4'-6-Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EBP	Enhancer binding protein
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
EDN	Eosinophil derived neurotoxin
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
EPO	Eosinophil peroxidase
Ets	E-twenty six
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
GM-CSF	Granulocyte Macrophage colony stimulating factor
h	Hour(s)
HEP1	Heparinase 1
ICAM1	Intracellular adhesion molecule 1
IFN	Interferon
IL	Interleukin
IkB	Inhibitory factor kB
LT	Leukotriene
MBP	Major basic protein
MCP	Monocyte chemoattractant protein
min	Minute(s)
MIP	Macrophage inflammatory protein
mRNA	messenger RNA
NF	Nuclear factor
NRSE	Neuron-restrictive silencer element
NSF	N-ethylmaleimide sensitive fusion protein
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PLA	Poly l Arginine
PLG	Poly l Glutamate
PMSF	Phenylmethylsulfonyl fluoride
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted

REST	Repressor element 1 silencing transcription factor
ROS	Reactive oxygen species
RT	Room temperature
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SDW	Sterile distilled water
SNAP	Soluble NSF attachment protein
SNARE	Soluble NSF attachment protein receptor
TAK	Transforming growth factor activating kinase
TGF	Transforming growth factor
TH2	T-Helper 2
TNF	Tumour necrosis factor
VAChT	Vesicular acetylcholine transporter
VCAM1	Vascular adhesion molecule 1
VEGF	Vascular endothelial growth factor
VLA4	Very late antigen 4

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Abstract

Interaction between eosinophils and nerve cells is known to occur in conditions such as asthma. Within nerve cells this interaction increases intracellular signaling via mitogen activated protein (MAP) kinase phosphorylation and nuclear factor (NF) κB activation resulting in increased anti-apoptotic and cholinergic gene expression. Activated eosinophils release potentially toxic cationic granular proteins, including major basic protein (MBP), eosinophil derived neurotoxin (EDN) and eosinophil peroxidase (EPO) in an adhesion-dependent manner. MBP has been shown to induce dysfunction of nerve cell M_2 receptors inhibiting cholinergic negative feedback and, like whole eosinophils, protect nerve cells from apoptosis by up-regulating Bfl-1 expression in an (NF)κB dependent manner.

This study was designed to examine the effects of eosinophil granule proteins on cholinergic gene expression in IMR32 nerve cells and assess the interactive and intracellular mechanisms leading to these changes.

EPO caused up-regulation of both choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) gene expression. ChAT mRNA up-regulation occurred early and transiently with levels returning to baseline at 4 hours. VAChT up-regulation however was first observed at 4 hours and sustained up to 24 hours of co-incubation. ChAT and VAChT protein levels were also increased between 18 and 24 hours of co-incubation with VAChT levels remaining elevated up to 48 hours. MBP caused an up-regulation of VAChT at 24 hours but had no effect on ChAT expression. EDN failed to up-regulate either gene.

The mechanism of ECP interaction with the cell membrane was assessed using the cationic charge mimetic poly-L-arginine (PLA), charge counteraction with the anionic

poly-L-glutamate (PLG) and cleavage of heparan sulphate moieties of glycosaminoglycans (GAG's) with heparinase 1. PLA had no effect on either ChAT or VAChT expression at any time point. However, both PLG and heparinase 1 reversed the up regulatory effect of EPO on ChAT and VAChT RNA and protein suggesting that ECP charge is essential but in itself insufficient to instigate intracellular cascasdes and gene up-regulation.

EPO was demonstrated to cause ERK activation by phosphorylation. Electrophoretic mobility shift assay (EMSA) showed activation of NF-κB in IMR-32 cells within 10 min of treatment with EPO. ERK inhibition led to complete reversal of all up regulatory effects of EPO on ChAT and VAChT RNA and protein at relevant time points.

Using confocal microscopy, cellular localization of fluorescently labeled EPO to IMR32 cells is seen very clearly as early as 1 hour. There is evidence of preferential interaction with certain areas of membrane likely representing GAG rich areas and evidence of internalization at later time points. PLG and heparinase 1 block this interaction suggesting that charge may play a synergistic role to ECP adhesion at GAG rich sites on the cell membrane.

CHAPTER 1

INTRODUCTION

1.1 THE EOSINOPHIL

Eosinophil granulocytes are white blood cells that comprise an important component of the immune system of vertebrates. Along with mast cells, they also control mechanisms associated with allergy and asthma and play an important role in a number of other diseases outlined below.

In normal individuals eosinophils constitute about 1-5% of white blood cells, and are about 12-17 μm in size and can easily be identified because of their strong affinity to the acidic dye eosin in blood and tissues (Simon and Simon 2009). Eosinophils are released from the bone marrow into the peripheral blood, where they circulate only a few h before they enter the tissues. Tissue survival time exceeds 2 weeks (Simon *et al.* 1997, 2007; Blanchard and Rotenberg 2009). Eosinophils, under physiological conditions are found mainly in the lymphoid tissues, thymus, and gastrointestinal tract with the exception of the oesophagus (Kato *et al.* 1998).

1.2 EOSINOPHIL PRODUCTION

Eosinophils are produced in the bone marrow from multipotent hematopoietic stem cells. Subsequent commitment of CD34 haemopoetic progenitor cells to the myeloid lineage then yields myeloblasts with shared eosinophil and basophil properties. A separate eosinophil lineage then ultimately leads to the production of mature eosinophils. This process is under the tight control of a number of transcription factors and soluble mediators (Blanchard *et al.* 2009).

1.2.1 Transcription factors

- CCAAT/enhancer-binding protein family of transcription factors (C/EBP's): This family of transcription factors exerts a biphasic influence on the production of eosinophils. These are vital initially for the induction of myeloid lineage commitment from progenitor cells. Subsequently, sustained activation is required for the development of mature eosinophils (Nerlov *et al.* 1998).

- PU.1: This member of the E-twenty six (Ets) transcription factor family member is involved in the switching between lymphoid and myeloid lineage and plays an important role in monocyte, macrophage and dendritic cell lineage commitment and for neutrophil differentiation (McNagny *et al.* 2002).

- GATA-1: This transcription factor is so named due to its affinity for GATA promoter sequences. It is the most important transcription factor in eosinophil lineage commitment. This has been demonstrated *in vivo* whereby GATA-1 mutant mice lose eosinophil lineage (Yu *et al.* 2002). GATA-1 also plays a pivotal role in the expression of numerous eosinophil related genes including eosinophil granule protein genes and the GATA-1 gene itself which is located on chromosome X in humans (Blanchard and Rothenberg, 2009)

1.2.2 Soluble mediators

Interleukin (IL)-3, IL-5, and granulocyte macrophage colony stimulating factor (GM-CSF) play a vital, synergistic role in influencing eosinophil differentiation (Rothenberg *et al.* 1988, Takatsu *et al.* 1994). IL-5 is the most specific to the

eosinophil lineage, and its influence on selective eosinophil development has been clearly demonstrated in animal models (Sanderson 1992). IL-5 deficient mice show a marked reduction in eosinophil levels in the blood and tissues in allergic models (Kopf *et al.* 1996, Mishra and Rothenberg 2003). Conversely, over expression of IL-5 in mice has been shown to cause marked rise in eosinophil numbers in the blood, bone marrow and spleen (Tominaga *et al.* 1991, Lee *et al.* 1997). In humans, anti-IL-5 therapy of asthma patients has been shown to inhibit eosinophil maturation in the bone marrow and migration into the bronchial mucosa (Blanchard and Rothenberg 2009).

1.3 EOSINOPHIL TRAFFICKING AND TISSUE MIGRATION

The three cardinal sequential steps in eosinophil migration from the blood stream to the tissues comprise rolling, adhesion and vascular endothelial transmigration. Each of these steps is intricately regulated by a series of soluble mediators and adhesion molecules (Blanchard and Rothenberg 2009).

Vascular endothelial adhesion, necessary for eosinophil tissue migration, is mediated by interaction of the P-selectin-glycoprotein ligand (PSGL)-1 on eosinophils with P-selectin on endothelial cells. The inflammatory cytokines IL-1 and tumour necrosis factor (TNF)-α up-regulate P and E-selectins on endothelial cells (Lampinen *et al.* 2004). This initial endothelial interaction is followed by firm cellular attachment mediated by eosinophil surface very late activation antigen (VLA)-4 (integrin α4 β1) and CD11b/CD18 binding to vascular adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 on the vascular endothelium respectively. IL-4 and interferon (IFN)-γ up-regulate VCAM-1 and

ICAM-1 expression (Spoelstra *et al.* 1999). The expression of VLA-4 expression on eosinophils is up-regulated by eotaxin, which also plays a key role in morphological changes necessary for eosinophil "cell rolling" and tissue migration (Rosenberg *et al.* 2007).

After entering the extracellular matrix, eosinophil integrins serially engage and disengage extracellular matrix (ECM) proteins such as fibronectin along chemokine gradients to reach target tissues. Chemokines involved in this process include eotaxin, RANTES and monocyte chemoattractant protein (MCP)-1, 2, 3, and 4 (Simon and Simon 2007, Elsner and Kapp 1999). The eotaxin and RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted) receptors include C-C chemokine receptor (CCR)-3, a seven-transmembrane spanning, G-protein coupled receptor which is up-regulated during allergic inflammation (Sehmi *et al.* 2003).

IL-5 plays an important role in eosinophil migration mainly by augmentation of eotaxin-CCR-3 interaction and amplification of CCR-3 mediated responses. IL-5 is a key stimulant for eosinophil clonal expansion and mobilization from the bone marrow to the lung following allergen exposure (Hogan *et al.* 1997). Antigen-induced tissue migration of eosinophils can also occur independently of IL-5. This has been demonstrated in animal and human *in vivo* studies using IL-5 knockout mice and in asthmatic patients undergoing anti IL-5 therapy respectively (Blanchard and Rothenberg 2009).

IL-4 and IL-13 play a role in the up-regulation of eotaxin expression and hence play an accessory role in increasing eosinophil tissue migration especially in T-helper (TH)-2 mediated immunological responses (Blanchard and Rothenberg 2009).

Other mediators such as complement factor C5a, platelet activating factor (PAF), leukotriene B4, and prostaglandin D2 play important roles in eosinophil trafficking and have been shown to recruit eosinophils to the lung and peritoneal cavity in helminth infections (Simon and Simon 2007, Bandeira-Melo 2000).

1.4 EOSINOPHIL SURVIVAL

Delayed eosinophil apoptosis is a central pathological mechanism in allergic conditions such as asthma where it serves to maintain tissue eosinophilia.

IL-5, IL-3 and GMCSF have demonstrated anti-apoptotic activity. Intracellular tyrosine kinase phosphorylation and activation of transcription STAT proteins result in up-regulation of anti apoptotic genes such as Bcl-2 and inhibition of pro apoptotic Bax protein translocation. IL-5 has also been shown to up-regulate anti apoptotic proteins such as survivin and cIAP-2 *in vitro* (Vassina *et al.* 2006).

1.5 EOSINOPHIL DEGRANULATION PRODUCTS

Four microscopically distinct groups of eosinophil cytoplasmic granules have been identified, each of which contain a repertoire of protein and lipid contents. These are the primary, secondary, small and lipid body granules (Blanchard and Rothenberg 2009).

The primary granules contain galectin-10, the Charcot-Leyden crystal protein, which binds lysophospholipases (Ackerman *et al.* 2002). The secondary granules contain the cationic granule proteins major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) (Hamann *et al.* 1991). These secondary granules are composed of a crystalloid core

of MBP-1 (and MBP-2), and a matrix of ECP, EDN, and EPO (Gleich and Adolphson 1986).

Small granules mainly store proteins, such as arylsulphatase B and acid phosphatase. Lipid bodies contain arachidonic acid, which is essential for eicosanoid production (Bandeira-Melo *et al.* 2000). Cytokines, chemokines and growth factors are mainly stored in secondary and lipid body granules (Simon and Simon 2007).

1.6 EOSINOPHIL CATIONIC GRANULE PROTEINS

1.6.1 Eosinophil cationic protein (ECP)

ECP is a small, basic protein found in the matrix of the eosinophil secondary granules. It exhibits cytotoxic, helminthotoxic, and ribonuclease activity. The molecular weight of ECP ranges between 16–21 kDa. This heterogeneity in size is due to differential glycosylation. Two isoforms, ECP-1 and ECP-2, have been identified (Blanchard and Rothenberg 2009).

Extensive 65% aminoacid sequence homology exists between ECP and the related EDN protein with an 89% cDNA sequence overlap between the two proteins. Indeed ECP exhibits ribonuclease activity which is about 100 times less potent than EDN (Slifman *et al.* 1986). ECP has also demonstrated antiviral activity. This cytotoxicity is likely mediated by voltage-insensitive, ion-selective toxic pores in the membranes of target cells which facilitates cytotoxic molecules entry into target cells (Rosenberg and Domachowske 2001, Young *et al.* 1986).

ECP also exhibits numerous cellular modulatory effects including the suppression of T cell proliferation, the augmentation of immunoglobulin synthesis by B cells, mast cell degranulation, and stimulation mucus secretion in the airways (Blanchard and

Rothenberg 2009).

Eosinophils store abundant amounts of ECP which may be released upon repetitive stimulation with the same agonist. Mature eosinophils thus do not require significant *de novo* ECP synthesis (Simon *et al.* 2000).

1.6.2 Major basic protein (MBP)

MBP exists as two isoforms (MBP-1 and MBP-2) which are derived from two separate genes. MBP-1, a small protein of 117 amino acids, has a molecular weight of 13.8 kDa, and a high isoelectric point (>11) (Hamann *et al.* 1991). MBP-1 is synthesized early in eosinophil development and stored exclusively in crystalloid granules. Mature eosinophils lose the ability to synthesize MBP1 (Voehringer *et al.* 2007, Blanchard and Rothenberg 2009).

MBP2 is specific to eosinophils, and are more specific marker than MBP1 for tissue eosinophilia (Plager *et al.* 2006). The two MBP isoforms share 63% amino acid sequence homology. In fact MBP2 has biological activity which is similar but less potent than MBP1. This is explained by differences in charge density whereby MBP2 carries a negative charge of half the potency of MBP1 (Blanchard and Rothenberg 2009).

MBP is highly cytotoxic (Gleich *et al.* 1979). Its cationicity affects the charge of surface membranes resulting in disturbed permeability, disruption, and injury of cell membranes and perturbation of the cell surface lipid bilayer (Wasmoen *et al.* 1988). MBP is at least partly responsible for tissue damage associated with eosinophil infiltration in bronchial mucosa in asthma. At low concentrations, MBP stimulates mediator production by other inflammatory cells (Furuta *et al.* 2005, Hisamatsu *et*

al. 1990).

1.6.3 Eosinophil-derived neurotoxin (EDN)

EDN is an eosinophil granule-derived secretory protein with ribonuclease and antiviral activity. EDN is instrumental in the recruitment, migration, maturation and cytokine release by dendritic cells (Yang *et al.* 2003). Dendritic cell activation is mediated by intracellular signaling pathways triggered by engagement of TLR2 toll like receptor with EDN and activation of the TLR2-myeloid differentiation factor 88 (Myd88) signaling pathway.

EDN also plays an important role in selective enhancement of antigen-specific Th2 immune responses mediated by IL-5, IL-6, IL-10, IL-13 (Blanchard and Rothenberg 2009).

1.6.4 Eosinophil peroxidase (EPO)

EPO is localized in the matrix of secondary eosinophil cytoplasmic granules where it accounts for approximately 25% of granular protein mass. EPO is composed of two subunits, a heavy chain (50–57 kDa) and a light chain (11–15 kDa). The peroxidase activity of EPO is known to catalyze the oxidation of halides and nitric oxide to form highly reactive oxygen species (hypohalous acids), and reactive nitrogen metabolites (nitric dioxide). This action promotes oxidative stress and target cell apoptosis (MacPherson *et al.* 2001).

Eosinophils contain nitrotyrosine-positive proteins in specific granules. EPO plays a central role in post translational modification of eosinophil granule contents by tyrosine residue nitration. The process occurs during eosinophil maturation, is

accentuated by acute and chronic inflammation and is dependent on EPO. Nitration sites occur at Tyr-349 in EPO and Tyr-33 in both ECP and EDN (Blanchard and Rothenberg 2009).

1.7 EOSINOPHIL DEGRANULATION

Upon activation, eosinophils specifically release their granule proteins by exocytosis. A prerequisite for exocytosis is the docking of the vesicles/granules to the cell membrane mediated by membrane-associated proteins forming soluble N-ethylmaleimide sensitive fusion (NSF) attachment protein (SNAP) receptors (SNAREs) (Fasshauer *et al.* 1998). Thus, regulated exocytosis occurs by the formation of a docking complex composed of soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) located on the vesicle (v-SNAREs) and the target membrane (t-SNAREs). Two broad types of SNARE molecules are classified based upon the presence of a conserved amino acid arginine (R) or glutamine (Q). The plasma membranes of human eosinophils express the Q-SNAREs SNAP-23 which interacts with the R-SNARE VAMP-2 on cytoplasmic secretory vesicles (Blanchard and Rothenberg 2009).

Intracellular mechanisms governing granular exocytosis in eosinophils have been proposed based on *in vitro* observations with mast cells where degranulation is induced by an IkB kinase dependent phosphorylation of SNAP-23 (Suzuki and Verma 2008). Whether the same mechanism occurs in eosinophils is not known.

1.8 PHYSIOLOGICAL FUNCTIONS OF EOSINOPHILS

1.8.1 The "Respiratory burst" and extracellular traps

Eosinophils primed with IL-5 or IFN-γ and stimulated with eotaxin, C5a or CCR3 ligands release mitochondrial DNA and the granule proteins MBP and ECP to form extracellular bactericidal traps. This process requires free radical production via NADPH oxidase. This ejection of mitochondrial DNA is extremely rapid, occurring in less than one second after cell stimulation in a catapult like fashion. The process does not lead to eosinophil apoptosis as nuclear disruption does not occur (Yousefi *et al.*2008). A recent study by Dworski and colleagues has demonstrated eosinophil extracellular traps composed of mitochondrial DNA and MBP are almost universally the airways of atopic asthmatic patients. Both eosinophil and neutrophil traps were observed in airway biopsy samples. Notably, no apoptotic features were found in relation to the releasing cells indicating exclusive mitochondrial DNA release (Dworski *et al.* 2011).

The NADPH oxidase complex in Eosinophils is especially robust. It represents a prominent mechanism whereby eosinophils exert their antimicrobial and antiparasitic actions. Activated NADPH oxidase catalyzes O_2 to O_2^-, which enters further redox pathways to produce hydrogen peroxide (H_2O_2) in the presence of superoxide dismutase, or hydroxyl and nitrogen dioxide radicals, after combining with nitric oxide. EPO subsequently plays a pivotal role by oxidizing bromide, nitrite, and thiocyanate in the presence of H_2O_2. These products are sufficiently potent to disrupt cell membranes and thereby increase intracellular stress. (Wang *et al.* 2006; Simon and Simon 2007). Eosinophils and neutrophils assemble NADPH oxidase complexes in similar fashions at the molecular complex level. However

differences exist between the intracellular localization of these complexes between the two cell types. In eosinophils, the complexes translocate (in association with the GTP-binding protein Rac) to the plasma membrane, while in neutrophils the distribution is predominantly intracellular. Thus, eosinophils, in contrast to neutrophils, generate most of their O_2^- extracellularly. This accounts for the tissue damaging and remodulatory capacity of eosinophils in conditions such as asthma (Lacey *et al.* 2003).

1.8.2 Cytokine production and release

Eosinophils synthesize numerous cytokines and growth factors which illustrate in part their role in both homeostasis and inflammation. Eosinophils from normal individuals express IL-4 and IL-10 constitutively with further up-regulation occurring in response to inflammatory signals (Nakajima *et al.* 1996). Both these cytokines along with IL-2, IL-5, and IL-13 are stored in the crystalloid core (Simon and Simon 2007). Cytokine up-regulation and release by eosinophils is under the influence of mediators such as IL-3, IL-5, GM-CSF, IFN-γ, TNF-α, and complement factor C5a (Lampinen *et al.* 2004, Kariyawasam *et al.* 2006, Simon and Simon 2007).

Eosinophil activation by surface receptor engagement leads to the secretion of a large variety of pro inflammatory cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16, IL-18, and TGF-a/b), chemokines (RANTES and eotaxin-1), and lipid mediators (PAF and LT-C4), as well as GMCSF which has autocrine antiapoptotic function (Blanchard and Rothenberg 2009).

1.8.3 Mast cell regulation

Eosinophils regulate mast cell functions through the release of granule protein and cytokines. Animal studies utilizing rat mast cells have confirmed concentration-dependent histamine release in response to MBP, ECP and EPO (Zheutlin *et al.* 1984). MBP has been demonstrated to induce the release of histamine, PGD-2, GM-CSF, TNFα, and IL-8 from human cord mast cells *in vitro* (Piliponsky *et al.* 2002). Eosinophils also play a key role in mast cell survival whereby nerve growth factor (NGF) release from eosinophils confers an activating and anti apoptotic effect on mast cells. NGF is notably induced by autocrine eosinophil stimulation by EPO (Solomon *et al.* 1998, Blanchard and Rothenberg 2009).

1.8.4 Leukotriene generation

Leukotrienes possess pro inflammatory activities. They have been shown to act as inflammatory cascade amplifiers by chemotaxis as well as potentiators of cytotoxic protein release (Simon and Simon 2009). They may also act as signaling molecules in the regulation of IL-4 release (Bandeira-Melo *et al.* 2002).

The synthesis of leukotriens by eosinophils requires priming by IL-3, IL-5 and GM-CSF and activation by C5a, PAF (Thomet *et al.* 2001, Takafuji *et al.* 1991). Stimulation of G-protein-coupled and cytokine receptors results in an activation of the MAP kinase cascade resulting in phosphorylation cytosolic phospholipase A2 (PLA2). Final leukotriene generation is subsequently achieved by calcium dependent activation of phosphorylated PLA2 (Thomet *et al.* 2001).

1.8.5 *Antigen presentation and T-cell proliferation*

In vitro experiments have demonstrated that eosinophils are capable of antigen processing and MHC class II molecule expression. This suggests that they may function as antigen-presenting cells in stimulating T-cell responses (Shi 2004). Eosinophils from normal individuals do not express MHC class II molecules; however, HLA synthesis occurs in response to stimulation with IL-3, IL-4, GM-CSF or IFN-γ. HLA expression is further increased during tissue migration (Yamamoto *et al.* 2000).

Eosinophils are capable of promoting T cell proliferation by processing and presenting a variety of antigens. These include viral and parasitic antigens (Handzel *et al.* 1998), superantigens and allergens (MacKenzie *et al.* 2001).

Eosinophils also promote T cell proliferation and activation via a variety of cytokines including IL-2, IL-4, IL-6, IL-12, IL-10 (Lacy and Moqbel 2000, Shi *et al.* 2000, Blanchard and Rothenburg 2009). Eosinophils regulate TH1 and TH2 imbalance via indoleamine 2,3-dioxygenase (IDO). IDO catalyzes the conversion of tryptophan to kynurenines which promotes TH1 apoptosis (Odemuyiwa *et al.* 2004).

Eosinophils have been microscopically demonstrated to traffic into the draining lymph nodes and localize to the T cell rich paracortical regions. Here Eosinophils activate effector T cells and promote their proliferation (Blanchard and Rothenberg 2009, MacKenzie *et al.* 2001).

1.8.6 *Uterine and mammary gland development*

Eosinophils play an important role in uterine maturation. Under the influence if IL-5, eotaxin-1, RANTES, and macrophage inflammatory protein (MIP)-1α, eosinophils

localize to the endometrial stroma and at the endometrial–myometrial junction of the uterus (Sferruzzi-Perri *et al.* 2003). *In vivo* experiments have in fact shown that eotaxin-1 deficient mice have a deficiency of eosinophils in the uterus, and a delay in onset of estrus (Gouon-Evans *et al.* 2002).

Eosinophils also localize to the mammary glands under similar chemotactic stimulation. Here they promote duct branching and end bud formation; a process thought to be mediated by eosinophil secretion of transforming growth factor (TGF)-β (Gouon-Evans *et al.* 2000).

1.8.7 Eosinophils and the thymus

Eotaxin-1 is constitutively expressed in the thymus where it is the dominant chemotactic factor in recruiting eosinophils (Matthews *et al.* 1998). IL-4 and IL-13 are also linked to eosinophil recruitment and activation in the thymus. Animal studies have demonstrated eosinophil recruitment to the thymus glands of neonatal mice with maximal numbers noted at 14 days returning to basal levels by 28 days of age. At this stage, eosinophils are preferentially localized to the corticomedullary region of the thymus. A secondary increase in thymic eosinophil levels in mice has been demonstrated at 16 weeks; at this stage localizing to the medullary region. Notably, this period also coincides with the onset of thymic involution and eosinophils may promote developing-thymocyte apoptosis since thymic eosinophils have a high level of free radicals due to high levels of NADPH oxidase activity (Blanchard and Rothenberg 2009).

Thymic eosinophils also act as antigen presenting cells (APC) expressing class II molecules and intermediate levels of class I molecules. Activated thymic eosinophils

also express pro-inflammatory cytokines, IL-6 and TNF-α. Eosinophils within inflammatory foci are protected by GM-CSF and IL-5 which act as autocrine anti apoptotic factors (Blanchard and Rothenberg 2009, Throsby *et al.* 2000).

1.9 EOSINOPHILS IN DISEASE

1.9.1 Chronic obstructive pulmonary disease

Airway eosinophilia has been reported in COPD during exacerbations as well as in stable disease. Higher numbers of eosinophils have been found in bronchial biopsies from patients with COPD compared to healthy controls (Balzabo *et al.* 1999). Eosinophilia in COPD appears to be linked to active smoking as well as to an asthmatic component to the disease (Lams 1998). Airway obstruction has been shown to correlate with an increase in the number of activated eosinophils in the airways (Lams 2000). Sputum eosinophilia in patients with COPD relates to an increased steroid responsiveness and may serve as a predictor for therapeutic steroid responsiveness (Pizzichini 1998).

ECP and EPO levels are also raised in the sputum and bronchoalveolar lavage fluid in patients with COPD during exacerbations (Keatings and Barnes 1997, Rutgers *et al.* 2000).

Eosinophils in COPD airways are postulated to have a role in antiviral host defense which is thought to be related to the ribonuclease effect of degranulation products such as EDN (Tetley 2005).

Eosinophilia in COPD has been shown to be independent of IL-5. IL-6 and IL-8 (known for its actions on neutrophil chemotaxis) have been suggested to recruit and activate eosinophils in COPD (Yamamoto *et al.* 1997). Expression of RANTES,

eotaxin and CCR3 is up-regulated during acute COPD exacerbations (Simon and Simon 2009).

1.9.2 Infection

- Parasitic infection

Eosinophils have been shown to play a pivotal role in the defense against helminthic infection. They have been demonstrated to mediate antibody and complement host responses as well as to aggregate and degranulate in areas of infection in the vicinity of damaged parasites. *In vivo* studies in parasite infected IL-5 knockout mice have suggested a role for IL-5 in conferring immunity following helminthic infection infection with Strongyloides venezuelensis and others (Korenaga *et al.* 1991). In studies involving CCR3 and eotaxin-1-deficient mice a role for eosinophils has been postulated in combating parasitic infection by larval encasement (Gurish *et al.* 2002).

- Viral infection

Eosinophil granule protein ribonuclease activity, namely on the part of EDN and ECP has been shown to degrade single stranded RNA containing viruses (Rosenberg and Domachowske 2001). Co incubation of Parainfluenza and RSV viruses with eosinophils in the presence of antigen-presenting cells has been shown to induce EPO release by eosinophils

(Davoine *et al.* 2008).

- Fungal infection

The antifungal effect of eosinophils is mediated by direct, contact dependent killing by cytotoxic granule proteins. Eosinophils localization to fungal cells and subsequent

degranulation is mediated by the I-domain of eosinophil CD11b integrin which adheres to fungal β-glucan cell wall components (Blanchard and Rothenberg 2009).

- Bacterial infection

Eosinophil release extracellular traps consisting of mitochondrial DNA and cytotoxic cationic proteins ECP and MBP in response to exposure to bacteria, C5a or CCR3 ligands. Trap structures composed of mitochondrial DNA and the granule proteins have been shown to bind and kill bacteria both *in vitro* and under inflammatory conditions *in vivo* (Yousefi *et al.* 2008).

1.9.3 Atopic dermatitis

Cutaneous tissue and blood eosinophilia are prominent features in atopic dermatitis (AD). Eosinophil and ECP levels in the blood have been shown to correlate with disease activity. Skin biopsies from patients with AD have consistently shown high levels of MBP staining.

In AD, IL-5 plays a prominent role in eosinophils clonal proliferation in the marrow, chemotaxis, activation, and survival. IL-4 has been shown to mediate an associated TH2 response as well as an augmentation of eosinophil cytokine production (Blanchard and Rothenberg 2009).

1.9.4 Gastrointestinal diseases

In health eosinophils are present in the lamina propria throughout the GI tract from the stomach to the colon with the exception of peyers patches (DeBrosse *et al.* 2006, Kato *et al.* 1998). Under normal conditions, the paired immunoglobulin-like receptor B (PIR-B), expressed by eosinophils, provides an inhibitory signal that limits eosinophil accumulation into the GI tract. The pathological accumulation of eosinophils in the GI tract is a common feature of numerous disorders such as

hypereosinophilic syndrome, allergic colitis and inflammatory bowel disease (Rothenberg 2004) as well as primary eosinophilic GI diseases such as eosinophilic oesophagitis and gasrtoenteritis. Eosinophilic infiltration of the gastrointestinal tract in disease states usually occurs in the absence of peripheral blood eosinophilia. This highlights the prominence of GI chemotaxis and site specific regulation of eosinophil numbers in the pathogenesis of these diseases (Blanchard and Rothenberg 2009).

Eotaxin-1, Eotaxin-3 and CCR-3 are the dominant eosinophil chemotacic factors to the GI tract. Animal studies on eotaxin-1 and CCR-3 deficient mice have yielded GI specimens almost completely free of eosinophils (Matthews *et al.* 1998, Mishra *et al.* 1999). IL-13 has been shown to up-regulate eotaxin-3 expression in primary esophageal epithelial cells in patients with eosinophilic oesophagitis (Blanchard *et al.* 2007).

Eosinophils show substantial accumulation in histological oesophageal samples of patients with eosinophilic oesophagitis. Animal models have suggested a central role for IL-5 in the maintenance of systemic eosinophil levels required for oesophageal eosinophilic accumulation (Mishra *et al.* 2002). In humans, IL-5 mRNA expression is up-regulated in the biopsies of eosinophilic oesophagitis patients compared to healthy controls (Straumann *et al.* 2005).

The eosinophil granule proteins MBP, EPO and EDN are present in the esophageal eosinophils, and MBP deposition has been detected by immunohistochemistry in esophageal biopsies of patients with eosinophilic oesophagitis suggesting a cytotoxic component to the pathophysiology of this condition mediated by ECP's (Blanchard and Rothenberg 2009).

1.10 THE ROLE OF EOSINOPHILS IN ASTHMA

The most common driver of eosinophilia in asthma is the increased generation of IL-5-producing T-cells. There is extensive literary evidence implicating blood and pulmonary tissue eosinophilia in the development of asthma. Levels of eosinophils and their products in blood, sputum, bronchoalveolar lavage fluid, or bronchial biopsies (Simon and Simon 2007, Wardlaw *et al.* 2000) have been found to correlate with disease severity. During asthmatic inflammation, eosinophils interact with lymphocytes, neutrophils, mast cells, and macrophages. Additionally interaction with resident tissue cells, such as epithelial cells, endothelial cells, smooth muscle, fibroblasts, and nerve cells has been documented (Jacobsen 2001). Disappointing results from clinical trials investigating the efficacy of anti-IL-5 therapy in asthma has cast doubt on the role of eosinophils in the pathogenesis of asthma in recent years. Studies in genetically targeted mice have demonstrated that eosinophils are critical for airway hyper responsiveness and remodeling (Humbles 2004). Expired NO is elevated in patients with asthma (Simon and Simon 2007, Alving 1993). It is used as a diagnostic tool in monitoring asthma and is thought to reflect the extent of eosinophilic inflammation of the lower airways (Turner 2007).

The cytotoxic effects of the eosinophil cationic granule proteins MBP, EPO, ECP, and EDN result in extensive epithelial desquamation and destruction leads to airway dysfunction (Jacobsen 2007). Histological samples from the airways of severe asthmatics, as well as post mortem samples following fatal asthma attacks have immunohistochemically localized MBP to the sites of epithelial damage. High levels

of MBP have also been detected in induced sputum samples from asthmatic (Gleich 2000). Eosinophils from asthmatic patients contain and release larger amounts of EDN compared to healthy controls in response to inflammatory stimuli (Sedgwick 2004). ECP levels in blood, sputum, and bronchoalveolar lavage fluid of patients with asthma have been found to correlate with the airflow obstruction clinically (Venge 1999). EPO also plays a key role in mucosal damage in the airways, and excessive levels of reactive oxygen species have been produced *in vitro* from eosinophils obtained from the airways of asthmatic patients (Kariyawasam 2006). In addition to functioning in an anti viral capacity, the RNAse effects of EDN and ECP have been shown to cause bronchial hyperreactivity in patients with asthma (Simon and Simon 2007, Gleich 2000, Rosenberg and Domachowske 2001).

MBP and EPO have been shown to augment the cholinergic phenotype of airway nerves by binding to the muscarinic M2 receptors and causing loss of negative feedback to Ach release at nerve termina resulting in vagal overstimulation and bronchoconstriction (Jacoby *et al.* 1993). MBP is also known to induce histamine release from mast cells and basophils (O'Donnell *et al.* 1983) further potentiating bronchoconstriction.

Airway remodeling in asthma is characterized by structural changes in the lungs including epithelial hypertrophy, subepithelial deposition of extracellular matrix proteins, mucus gland hypertrophy, smooth muscle hypertrophy, and vascular changes. Many of the known airway remodulatory factors are expressed by eosinophils. These include fibroblast growth factor (FGF)-2, IL-4, IL-11, IL-13, IL-17, nerve growth factor (NGF), and vascular endothelial growth factor (VEGF) (Simon and Simon 2007).

Clinical studies have demonstrated that patients receiving anti-IL-5 antibody therapy had a reduced deposition of extracellular matrix proteins beneath the bronchial basement membrane. This suggests a central role played by eosinophils in the remodulatory process.

Eosinophil granule proteins also play a role in tissue remodeling. For example, MPB interacts with IL-1 and TGF-β and stimulates lung fibroblasts (Simon and Simon 2007). MBP has also been shown to inhibit neurite outgrowth in airway nerve cells in dose dependent fashion in a manner similar to *in vitro* studies with whole eosinophils (Kingham *et al.* 2003).

Eosinophils are known to secrete metalloproteinases, especially matrix metalloproteinase (MMP)-9 which has been found to be increased in severe persistent asthma and following allergen challenge (Mattos *et al.* 2002). Eosinophils also potentiate the actions of pro angiogenic mediators such as VEGF and FGF-2 hence promoting angiogenesis, which presents an additional important mechanism whereby eosinophils promote chronic inflammation and tissue remodeling in the aiways (Puxeddu *et al.* 2005). Activation of smooth muscle cells is another mechanism of airway remodeling mediated, at least in part, by eosinophils. IL-25, generated by eosinophils, is known to have stimulatory action on airway smooth muscle cells by induction and promotion of extracellular matrix components synthesis. IL-25 has been found in endobronchial histological samples of asthmatic patients (Foley *et al.* 2007).

The immunoregulatory role of eosinophils in asthma is multifactorial. On the one hand, the wide range of cytokines produced by eosinophils including IL-4, IL-5, IL-13 may sustain TH2 mediated responses in asthma which are well documented

(Schmid *et al.* 2002). HLA-DR up-regulation and surface expression by antigen exposed airway eosinophils renders them capable of functioning as antigen-presenting cells in stimulating T-cell responses (Sedgwick *et al.* 1992). Eosinophil produced TNF-α has been shown to enhance the inflammation in asthma, and leukotriene production by eosinophils are known to increase mucus production and bronchoconstriction. LTC4 derivatives are also known to reduce eosinophil apoptosis (Lee *et al.* 2000).

1.11 TARGETTED ANTI-EOSINOPHILIC THERAPY IN ATHSMA

The multifaceted approaches targeting eosinophil mediated processes in asthma and their clinical efficacy corroborate the integral role played by the eosinophil in the pathogenesis of this condition. These therapeutic targets are geared towards reducing eosinophil production and migration into inflamed tissues, increasing eosinophil apoptosis, preventing eosinophil activation, and blocking of eosinophil mediators.

Humanized anti-IL-5 decreases eosinophil load in humans, dramatically lowering eosinophil levels in the blood, decreases eosinophil activation and to a lesser extent eosinophil levels in the inflamed lung (Blanchard and Rothenberg 2009).

Three initial studies assessing the efficacy of mepolizumab in the treatment of allergic asthma have yielded disappointing results regarding sustained spirometric benefits and quality of life. The reasons for this seem to be centered on the selectivity of action on mature eosinophils in the peripheral blood. Less effect in reducing tissue eosinophilia in the airways was noted implying that the multiplicity

of chemotactic factors involved in tissue migration limited the efficacy of this treatment in the cohorts of asthmatics studied. A more recent study (Nair *et al.* 2009) has shown promising results in patients with asthma in the presence of sputum eosinophilia despite glucocorticoid therapy. In this randomized, placebo controlled trial involving 20 patients with steroid resistant asthma and sputum eosinophilia, 9 patients received mepolizumab in 5 monthly 750 mg infusions versus 11 assigned to the placebo group.

There were 12 asthma exacerbations in 10 patients who received placebo, 9 of whom had sputum eosinophilia at the time of exacerbation. In comparison, only one patient who received mepolizumab had an asthma exacerbation, and this episode was not associated with sputum eosinophilia (P=0.002). Patients who received mepolizumab were able to reduce their prednisone dose by a mean (+/-SD) of 83.8 +/- 33.4% of their maximum possible dose, as compared with 47.7 +/- 40.5% in the placebo group (P=0.04). The use of mepolizumab was associated with a significant decrease in the number of sputum and blood eosinophils. Improvements in eosinophil numbers, asthma control, and forced expiratory volume in 1 second were maintained for 8 weeks after the last infusion.

Also, encouraging results were obtained in a recent study in patients with eosinophilic bronchitis with or without asthma (Nair *et al.* 2008). A randomized placebo controlled trial was conducted and found that patients who received mepolizumab were able to reduce their prednisone dose by 90% of their maximum possible compared to 55% in the placebo arm ($p<0.05$). Mepolizumab treatment was accompanied by a significant decrease in sputum and blood eosinophils and improvements in asthma control, FEV1 and asthma quality of life that were

maintained for 8 weeks after the last infusion, suggesting that mepolizumab is an effective prednisone sparing therapy in patients with eosinophilic bronchitis with or without asthma

1.12 CHOLINERGIC INNERVATION OF THE AIRWAYS

Cholinergic airway innervation originates in the nucleus ambiguous of the brain stem. Fibers travel along the vagus nerve and synapse in parasympathetic ganglia within the airway wall. Post-ganglionic fibers travel to airway smooth muscle and submucosal glands. Ach is released from cholinergic nerve termini in response to nerve activation. This activation of muscarinic cholinergic receptors in smooth muscle and gland cells by Acetylcholine (Ach) mediates bronchoconstriction and mucous secretion. Bronchoconstriction is mediated by M3 receptors and mucous secretion appears to be mediated by M1 and M3 receptors.

Inhibitory autoreceptors have been demonstrated on cholinergic nerves of airways in animals *in vivo*, and in human bronchi *in vitro* (Fryer *et al.* 1999). These autoreceptors act to inhibit Ach release limiting cholinergic effects on the airways. Autoreceptors belong to the M2 receptor subtype in airway nerves, whereas those on airway smooth muscle and glands belong to the M3 receptor subtype. *In vivo* activation of M2 receptors has been shown to inhibit reflex bronchoconstriction (Barnes and Thomson 2002). This feedback mechanism seems to be defective in asthmatics suggesting M2 receptor dysfunction and eosinophilic involvement in the pathogenesis and disease progression. The mechanisms of pre-junctional M2 receptor dysfunction may involve M2-receptors oxidative damage as well as allosteric inhibition by eosinophil degranulation products (Sawatzky *et al.* 2003).

Cholinergic bronchoconstriction mainly involves larger airways, with relative diminution of cholinergic control in small airways. Hence, anticholinergic drugs are likely to be less useful than β-agonists when bronchoconstriction involves small airways (Barnes and Thomson 2002).

Inflammatory mediators such as thromboxane and prostaglandin D2 may influence cholinergic neurotransmission via pre-junctional receptors facilitating ACh release from post-ganglionic nerves in the airways (Barnes 1992).

The role of cholinergic nerves in asthma

Cholinergic tone is increased in the airways of asthmatics. This is mediated by stimulation of sensory nerves in the airway walls by inflammatory mediators such as histamine and prostaglandins. The increased release of ACh from cholinergic nerves is also a critical component of inferring a cholinergic phenotype on airway nerves. As discussed later, this is at least in part mediated by augmentation of cholinergic gene expression. Reduction in M2 – receptor efficacy by allosteric inhibition is also a central pathological process. There is no evidence for increased M1 or M3 - receptor expression in asthmatic lungs. Decrease in the neuromodulators (VIP, NO) which tend to inhibit neurotransmission may also play a part (Barnes and Thomson 2002).

The clinical consequences of the accentuated cholinergicity of airway nerves in response to whole eosinophils and ECP's are readily apparent. Release of acetylcholine by airway nerve cells results stimulation of muscarinic receptors and subsequent airway smooth muscle contraction and release of secretions from submucosal mucous glands. Acetylcholine actions at M3 receptors results in a

reduction in intracellular cAMP levels and smooth muscle contraction. Contraction and relaxation of airway smooth muscle accounts for much of the rapid changes in airflow limitation characterizing asthma and is the basis for beta-agonist action.

Large airway obstruction leads to airflow limitation and decreased flow rates, while obstruction in small airways leads to air trapping with increase in residual volume and dynamic hyperinflation (Mitchell *et al.* 2004). Studies have demonstrated heterogeneous narrowing of large airways on high resolution CT scanning (King *et al.* 2004). The relative prominence of large airway contribution to airflow obstruction in response to acetylcholine release is illustrated by the efficacy demonstrated to date of bronchial thermoplasty in improving asthma control (Cox *et al.* 2007).

Acetylcholine has been proposed to accentuate airway hyperresponsiveness to stimuli. (Chiba *et al.* 1999). Bronchial hyperresponsiveness is a manifestation of reversible airflow obstruction due to smooth muscle contraction. It represents an exaggerated response to a variety of chemical and environmental stimuli. Patients with asthma are hyperresponsive to a wide variety of inhaled stimuli including histamine, bradykinin and cold air. This heightened reactivity is markedly reduced or abolished by anticholinergics such as ipratropium bromide or atropine (Canning 2006), suggesting that these stimuli cause an exaggerated parasympathetic nerve response.

Increasing evidence suggests that a subset of patients with asthma have irreversible airflow obstruction, which is believed to be caused by airway remodeling (Limb *et al.* 2005) which refers to structural changes in the airways that may cause

irreversible airflow limitation, superimposed on the effects of smooth muscle contraction.

Hallmarks of airway remodelling include an increase in the number mucous-producing goblet cells, fibrotic thickening of the sub-epithelial reticular basement membrane, increased numbers of myofibroblasts and vascularity within airway walls, increased airway smooth muscle mass, and increased extracellular matrix. These structural changes contribute to bronchial wall thickening, and account for the changes in physiologic consequences of smooth muscle contraction in the airways of patients with asthma.

1.13 THE SYNTHESIS OF ACETYLCHOLINE

Parasympathetic nerve stimulation in the airways induces Ach release which leads to mucous secretion and contraction of bronchial smooth muscle. Mucous production and bronchoconstriction are central features of airway diseases such as asthma. Parasympathetic nerves may hence play a pivotal role in the pathogenesis. There are several key steps in the synthesis, storage and release of Ach (Figure 1).

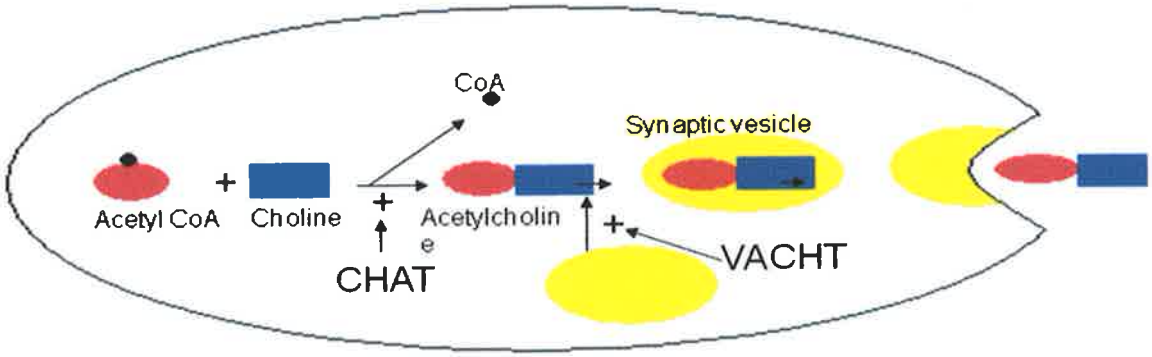


FIGURE 1: Synthesis and release of Acetylcholine

Ach is synthesized from choline and acetyl CoA under the enzymatic action of choline acetyltransferase (ChAT). ACh is transported into synaptic vesicles by way of the vesicular Ach transporter (VAChT).

Once released, ACh stimulates muscarinic receptors on both target organs as well as the nerves themselves by stimulating M_2 muscarinic receptors. M_2 muscarinic receptors function as autoreceptors to limit further ACh release. ACh is metabolized into choline and acetate by the action of Acetylcholinesterase (AChE). Hence, a core group of enzymes and receptors are responsible for controlling the synthesis, activity and turnover of ACh in cholinergic nerves.

The genes involved in the synthesis, control of release and metabolism of ACh are under the control of a variety of intracellular signaling pathways, including several protein kinases and MAP kinases culminating in the activation of nuclear factor KB which subsequently regulates the expression of cholinergic genes such as ChAT and VAChT as well as genes that effect cell survival such as the antiapoptotic gene Bfl-1 (Durcan *et al.* 2006, Morgan *et al.* 2004). Key to the understanding of the role played by cholinergic nerve phenotype and remodeling in the asthmatic airways is to elucidate the factors effecting expression of this "cholinergic gene locus" and the

regulatory factors caused by muscarinic receptor modification (Durcan *et al.* 2006).

1.14 THE CHOLINERGIC GENE LOCUS

The human cholinergic gene locus (CGL) was first identified in 1994 on the long arm of chromosome 10. The cholinergic gene locus contains the genes for both ChAT and VAChT (Kitamoto *et al.* 1998, Berse *et al.* 1995). The ChAT gene contains three 5' noncoding exons: exon (exon R); exon 2 (exon N); and exon 3 (exon M). The three exons result in multiple 5' mRNA species, which are produced from different promoters and by alternative mRNA splicing. The VAChT gene lies within the first intron of the ChAT gene, between the R and N exons, and has the same transcriptional orientation. Multiple VAChT mRNA species with different 5' noncoding exons have also been reported, and some of these share the R exon with ChAT mRNA species (Kitamoto *et al.* 1998). This arrangement permits coordinated regulation of expression. In vitro studies have illustrated the role of protein kinase A on regulation of expression of the cholinergic gene locus in PC12 cells. This is mediated by a 21-bp DNA sequence known as the repressor element (RE-1)/neuron-restrictive silencer element (NRSE) (Shimojo *et al.* 2004). This nucleotide series are bound by repressor element-I silencing transcription factor (REST)/ neuron-restrictive silencer factor (NRSF) which blocks the expression of many neuronal RE-I/NRSE containing genes in neurons (where they regulate neuronal gene expression) and non neuronal cells. REST4, one of the REST/NRSF isoforms has been shown to regulate transcription of the cholinergic gene locus by blocking the repressor activity of REST/NRSF. Protein kinase A up-regulates the synthesis of REST4, which in turn depresses the repressor activity of REST/NRSF.

1.15 EOSINOPHILS AND AIRWAY NERVES

1.15.1 Eosinophils localize to airway nerves

The presence of eosinophils and their degranulation products in tissues is the hallmark of a variety of diseases, including the allergic conditions asthma and rhinitis, as well as inflammatory bowel disease and eosinophilic gastroenteritis (Walsh *et al.* 2003, Rothenberg *et al.* 2001). Increased numbers of degranulated eosinophils are found in the airways in postmortem studies of patients with acute fatal asthma attacks (Jacoby *et al.* 2000). Eosinophils localize to specific tissue structures under the coordinated influence of specific chemoattractants and through interactions with adhesion molecules at sites of inflammation (Frenette *et al.* 1996, Walsh *et al.* 2003). Direct cell-to-cell contact between eosinophils and airway nerves has been demonstrated microscopically in patients with asthma (Kingham *et al.* 2003). Adhesion leads to degranulation of the eosinophils and release of mediators, including MBP, EPO, EDN, ECP and leukotriene C_4, and to the generation of reactive oxygen species (ROS) within the nerve cells through an NADPH oxidase-dependent mechanism (Sawatzky *et al.* 2001, Kingham *et al.* 2001). In animal models, eosinophil MBP is associated with the development of vagally mediated hyperreactivity of cholinergic nerves; this is due to inhibition by MBP of protective M_2 muscarinic autoreceptor activity (Costello *et al.* 2000).

Eosinophils express the integrin adhesion molecules CD11/18 complex and very late antigen-4 (VLA-4), which interact with intercellular adhesion molecule-1 (ICAM-1)

and vascular cell adhesion molecule-1 (VCAM-1), respectively on nerve cells (Sawatzky *et al.* 2002)

In vitro, airway parasympathetic nerves expressed the adhesion molecule VCAM-1, whereas ICAM-1 is expressed only after pre treatment of the cells with TNF-α and IFN-γ which also stimulate eosinophil adhesion to these cells via a CD11/18-dependent mechanism (Sawatzky *et al.* 2002). The human cholinergic cell line IMR-32, which has been used as an experimental model for airway nerves, constitutively express VCAM-1 and ICAM-1. Human eosinophils adhered to IMR32 cells via these adhesion molecules in a time-dependent manner, and this adhesion leads to eosinophil activation and degranulation. This adhesion is prevented by co-incubation with Ethylenediaminetetraacetic acid (EDTA) and experimentation at low temperatures. This suggests a metabolically active process. The interaction is also inhibited by antibodies to CD11/18, ICAM-1 and VCAM-1 as well as the VLA peptide inhibitor ZD-7349. Hence, eosinophil adhesion involves both ICAM-1 and VCAM-1. The VLA4-VCAM-1 and CD11/18-ICAM-1 interactions have also been shown to be synergistic. This synergy is mediated by cytohesin-1, released by VLA-4 integrins upon interaction with VCAM-1. Cytohesin-1 subsequently promotes B2 integrin clustering thus facilitating CD11/18-ICAM-1 interactions (Sawatzky *et al.* 2002, Dwarakanath *et al.* 1995).

Eosinophils induce neurite retraction in parasympathetic nerves and in the IMR32 nerve cell line. This is accompanied by only a modest increase in the number of apoptotic nerves with no effect on MTT metabolism. This suggests that neurite

retraction is a physiological remodelling process induced by eosinophils (Kingham *et al.* 2003).

Blocking adhesion of eosinophils to nerves with an anti-CD18 MAb and a VLA-4 peptide inhibitor attenuates eosinophil-induced neurite retraction as does inhibition of NADPH oxidase with diphenyliodonium. This suggests that ROS, generated through cell adhesion plays an important role in the remodulatory process (Kingham *et al.* 2003). ROS have been demonstrated to induce microtubular reconfiguration, and this has been suggested as a mechanism for the above neurite retraction (He *et al.* 2002).

Intracellular protein tyrosine phosphorylation has been shown to occur in response to eosinophil-nerve interaction (Tilghman *et al.* 2002). With regards to morphological alteration such as neurite retraction, the involved proteins are in the range 30-45 kDa suggesting p38 MAP kinase activation. The p38 MAP kinase inhibitor SB-239063, inhibits neurite retraction but not ROS production. Equally, inhibiting ROS production had no influence on p38 MAP kinase activity. In contrast, the tyrosine kinase inhibitor genistein blocks both ROS production and neurite retraction. This suggests two separate pathways involving ROS and p38 activation control neurite retraction and remodelling (Kingham *et al.* 2003).

Incubation of IMR32 cells with eosinophil conditioned media fails to duplicate the effect of whole eosinophils with regards to neurite retraction. Degranulation products do however reduce the ability of the nerve cells to extend neuritis during differentiation. This indicates a partial role for ECP's in the remodulatory process.

Eosinophil MBP has been shown to inhibit neurite outgrowth in a dose-dependent manner. The mechanism of this effect is unknown, but may be related to the cationic charge MBP as poly-L-lysine has a similar effect (Kingham *et al.* 2003).

1.15.2 M2 receptor dysfunction

The dominant innervation of airway smooth muscle is by the vagal parasympathetic nerves. Nerve stimulation causes release of Ach onto M_3 muscarinic receptors to mediate bronchoconstriction, production of mucus, and dilation of the bronchiolar vasculature (Nadel *et al.* 1984). This mechanism is subject to feedback control by M_2 muscarinic receptors present on the parasympathetic cholinergic terminals. Ach acting on these M_2 receptors leads to a reduction in the further release of Ach (Fryer *et al.* 1984). The M_2 muscarinic receptor is a G protein-coupled receptor linked to inhibition of adenylate cyclase and to the Egr family of transcription factors (Von Den Kammer *et al.* 1984).

Airway hyperreactivity has been shown to result from M2 receptor dysfunction which is prevented by inhibiting eosinophil localization to the airways (Elbon *et al.* 1995). The eosinophil protein major basic protein (MBP) is an allosteric antagonist at M_2 muscarinic receptors in vitro. The neutralization of MBP with heparin (Fryer *et al.* 1992) or an anti-MBP antibody (Evans *et al.* 1997) restores M_2 receptor function and prevents antigen-induced hyperreactivity *in vivo*. Hence, eosinophils increase Ach release by preventing negative feedback. Eosinophil granule proteins, particularly MBP are critical to this process (Walsh *et al.* 2003).

In addition to the acute effect of eosinophil-mediated loss of M_2 receptor function,

prolonged exposure leads to an initial up-regulation of the M_2 receptor itself (Sawatzky *et al.* 2003). M_2 receptor protein levels increase by 225% following 24 h of eosinophil co-culture (Durcan *et al.* 2006). Mechanistic studies showed that eosinophil adhesion to nerves via the adhesion molecules ICAM-1 and VCAM-1 is necessary but in itself insufficient to up-regulate M_2 receptor expression. No alteration in gene expression was observed in the presence of adhesion inhibitors. However, incubation of nerve cells with eosinophil membrane preparations failed to induce these changes. This indicates a prominent role for eosinophil degranulation products in this process. Indeed, subsequent studies have shown that MBP and EPO increase M_2 receptor *expression by* 170% and 300% respectively after 24 h of co-incubation (Durcan *et al.* 2006).

Sequence analysis studies of the M2 gene have identified the presence of six different mRNA transcripts with five exons. Alternative splicing of exons 2 and 5 has been shown (Krejci *et al.* 2004). Three regions of transcription initiation in the human muscarinic M_2 receptor gene are known. Each has multiple transcription start sites (TSSs) in close proximity to each other. TSS1 region appears to be the most commonly used transcription start site. The major regulatory region lies immediately upstream of TSS3 with repressor elements operating upstream of TSS1 (Durcan *et al.* 2006). The M_2 receptor promoter, as with all muscarinic receptor promoters is TATA-less. Sp1, AP1, AP2 and GATA transcription factors have been identified with the highest incidence of TF sites within the region of maximum transcriptional regulatory activity, immediately upstream of TSS3 (Zhou *et al.* 2001, Saffen *et al.* 1999, Durcan *et al.* 2006). It remains to be determined which of these transcription factors are involved in eosinophil induced up-regulation.

1.15.3 Eosinophils increase Ach release from nerve cells

IMR32 cells take up radiolabelled choline and release the radiolabel in response to electrical stimulation or a solution of high K^+. This release is abolished in Ca^{2+}-free medium and hence not the result of cellular damage (Sawatzky *et al.* 2003).

Inhibition of Ach release by approximately 20% is achieved using M1/M2 receptor agonist arecaidine. Conversely, the selective M_2 receptor antagonist AF-DX 116 potentiates Ach release by approximately 75% (Re 1999, Sawatzky *et al.* 2003).

Whole eosinophils potentiate Ach release from IMR32 cells. The maximum enhancement is somewhat less than that produced by the M_2 receptor antagonist AF-DX 116 and is, therefore, within the range that could be explained by an effect on M_2 receptors. Also, carbachol, which causes internalisation of the M2 receptor prevents eosinophil mediated increase in Ach release and prevents the Ach receptor antagonist AF-DX 116 from enhancing Ach release. Inhibition of eosinophil adhesion to the nerve cells by interruption of CD11/18-ICAM-1 or VLA-4-VCAM-1 interactions reverses the enhanced Ach release (Sawatzky *et al.* 2003).

Eosinophil conditioned medium also increases Ach release from IMR32 cells. The magnitude of the increase is similar to that achieved with whole eosinophils. This indicates that eosinophil products contribute to an increased release of Ach from nerves. The most likely candidates for this effect are EPO and MBP, both of which are antagonists at M_2 muscarinic receptors (Jacoby *et al.* 1993).

The enhancement of Ach release caused by contact between eosinophils and IMR32 cells is completely abolished by treatment of eosinophils with either antibody to CD11/18 or the VLA-4 inhibitor ZD-7349 confirming the central role played by integrin interaction on eosinophil degranulation (Sawatzky *et al.* 2003).

1.15.4 Eosinophils cause cell signalling

Co-culture of eosinophils with IMR32 nerve cells leads to activation of neuronal transcription factors by adhesion-dependent mechanisms. Adhesion of eosinophils to IMR-32 cells induces activation of the transcription factors NF-κB and activator protein (AP)-1 both within 5 min of co-incubation with a return to baseline by 2 h. This is then followed by a second surge in activity between 3 to 24 h. Inhibition of eosinophil binding via ICAM-1 inhibited NF-κB activation while inhibition of eosinophil binding via VCAM-1 had no significant effect on NF-κB. In contrast, eosinophil adhesion to VCAM-1 leads to activation of AP-1. These up-regulatory effects on NF-κB and AP-1 are duplicated by incubating IMR32 cells with eosinophil membranes alone, and inhibited by specific inhibitors of adhesion. Thus, the observed activation of MAP kinases and nuclear transcription factors is due to membrane–membrane interactions between nerves and eosinophils (Walsh *et al.* 2003).

The intracellular intermediates involved in NFκB and AP-1 activation involve the MAP kinases ERK1/2 and p38, both of which are rapidly and transiently activated in IMR32 cells in response to eosinophil adhesion. Activation of p38 is dependent on VCAM-1, and inhibition of VLA-4/VCAM-1 interaction delays the onset of p38

activation from 5 min to 1 h and inhibition AP-1 activation. Inhibition of ERK1/2 has little effect on AP-1 activation. ERK1/2 activation and phosphorylation with subsequent NFκB activation is primarily dependent on ICAM-1–mediated adhesion. Thus, adhesion of eosinophils via CD11/18/ICAM-1 and VLA-4/VCAM-1 induced the activation of a network of intracellular pathways in IMR32 cells (Walsh *et al.* 2003).

A number of signal transduction pathways that result in activation of NF-κB have been described. These include a pathway involving the serine-threonine kinase, interleukin-1 receptor-associated kinase-1 (IRAK-1). This is part of the common downstream signaling pathway that is triggered by the Toll/interleukin-1 receptor (TIR) family of transmembrane receptors which include the IL-1, IL-18, and the Toll-like receptors. These receptors share a common cytoplasmic motif, the TIR domain (Martin *et al.* 2002).

Upon ligand binding, the TIR domain recruits the cytoplasmic adapter molecule, myeloid differentiation protein (MyD88) which complexes with IRAK-1, which undergoes hyperphosphorylation, dissociates from the membrane-bound complex, and binds to tumor necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 then ubiquitinises and triggers the phosphorylation and activation of transforming growth factor-β-activated kinase-1 (TAK-1). TAK-1 then activates the inhibitory factor κB (IκB) kinase complex, which results in the phosphorylation and degradation of the NF-κB inhibitory protein IκB, thus allowing the transcriptionally active NF-κB dimer to translocate from the cytoplasm to the nucleus, whereupon it regulates gene transcription (Curran *et al.* 2005, Martin *et al.* 2002, Schmidt *et al.* 1995). IRAK-1 activation occurs within 10 min of coculture with eosinophil

membranes. IRAK-1 activation is dependent on the adapter protein MyD88 and inhibited by preventing eosinophil adhesion via ICAM-1. ERK1/2 inhibition prevents adhesion-induced IRAK-1 activation (Curran *et al.* 2005).

Eosinophil adhesion results in the up-regulation of the ICAM-1 gene. ICAM-1 protein expression is also dependent on the IRAK-1 pathway, and is inhibited by transfection of IMR-32 cells with the dominant-negative mutant of MyD88 before coculture with eosinophil membranes (Curran *et al.* 2005).

1.15.5 Eosinophils protect nerve cells from apoptosis

Eosinophils and eosinophil membranes have been shown to protect cholinergic IMR-32 nerve cells against apoptosis induced by serum deprivation or by the cytokines IL-1β, TNF-α, and IFN-γ (Morgan *et al.* 2004).

This protection against apoptosis is mediated by direct cell-cell contact and is reversed by neutralizing antibodies to the adhesion molecules ICAM-1 and VCAM-1, alone or in combination (Morgan *et al.* 2004). MAP kinase inhibitor studies have demonstrated that the antiapoptotic effect of eosinophils on nerve cells is entirely mediated by ERK1/2 and not p38. This is consistent published work implicating the MEK/ERK pathway in cell survival in different cell types (Koulich *et al.* 2001, Jin *et al.* 2002, Buckley *et al.* 1999).

Bfl-1 anti apoptotic gene is known to be a key regulator of apoptosis in endothelial cells (Noble *et al.* 1999) and is thought to mediate its actions by preventing mitochondrial membrane depolarization and sequestering the pro apoptotic BH3

domain-only members of the Bcl-2 family (Werner *et al.* 2002). The Bfl-1 gene is regulated in neurons by MEK/ERK dependent pathways and subsequently by NF-κB activation (Zong *et al.* 2001).

Incubation of IMR-32 cells with eosinophil membranes causes up-regulation of the anti- apoptotic Bfl-1 gene in the IMR-32 cells. This is dependent on adhesion through ICAM-1 and VCAM-1 on the nerve surface and phosphorylation of ERK1/2. Up-regulation of Bfl-1 expression occurs as early as 2 h after of co incubation with eosinophil membranes and is sustained for over 24 h. Bid and Bad, pro apoptotic Bcl-2 family members, are constitutively expressed by IMR-32 cells, but their transcription was not influenced by eosinophil adhesion (Morgan *et al.* 2004).

In vivo models demonstrate no evidence of nerve cell death in conditions such as asthma and inflammatory bowel disease where eosinophils are found in close association with nerves. This is despite the presence of inflammation which might be expected to lead to an increase in nerve cell death in these tissues (Morgan *et al.* 2004).

1.15.6 Eosinophils increase cholinergic gene expression

Eosinophils actively up-regulate the expression of cholinergic genes in IMR32 cells. ChAT and VAChT mRNA and protein production are significantly up-regulated following 24h of co-culture. Eosinophils induce a five-fold increase in ChAT gene expression following 24 h of co-incubation with IMR32 cells with a corresponding two fold increase in ChAT protein expression by 24 h. VAChT gene expression is increased by 132% following 1 h of eosinophil co-incubation with IMR32 cells with a

two fold increase in VAChT protein expression after 24 h. Additionally, eosinophils induce a modest increase (140% at 12 h) in AChE mRNA with a minor associated increase in enzyme protein level between 1 and 12 h of co-incubation (Durcan *et al.* 2006).

1.15.7 ECP's cause intracellular signaling and protect nerve cells from apoptosis

The cationic eosinophil granule protein MBP has been shown to protect IMR-32 cholinergic nerves from an apoptosis induced by serum deprivation. This finding is not duplicated with EDN (Morgan *et al.* 2005). Differences in intracellular signalling induced by the two proteins explains this discrepancy. While both MBP1 and EDN activate NFκB, they do so over different time courses. MBP1 induces a prolonged activation of NF-κB, extending from 10 min to 12 h and declining by 24 h. This activation is dependent on both ERK 1/2 and Akt pathways in the early phases, and relies solely on ERK 1/2 in later phases. EDN-induced NF-κB activation is short-lived, lasting less than 1 h, and independent of both ERK 1/2 and Akt.

The sustained activation of NFκB by MBP leads to up-regulation of the anti apoptotic gene Bfl-1, EDN induced no such up-regulation. Interestingly, ERK inhibition reverses only 70% of the MBP induced anti apoptotic activity rather than completely abolishing it, further highlighting the multiplicity of intracellular pathways involved in Bfl-1 gene up-regulation (Morgan *et al.* 2005).

In summary, cholinergic remodeling of airway nerves is known to occur in response to eosinophil co-localization in conditions such as asthma. Eosinophils protect nerve cells from apoptosis and induce M2 receptor dysfunction, preventing cholinergic negative feedback. Adhesion dependent intracellular cascades culminate in ERK and NFκB activation and result in up-regulation cholinergic gene locus expression and production of acetylcholine. Early studies with eosinophil cationic granule proteins have shown that MBP and EDN both activate NFκB but via different mechanism and over different time courses. MBP has been shown to protect nerve cells from apoptosis in vitro while EDN has not. These findings are discussed in detail in sections 1.15.1 – 1.15.7 and have led to the formulation of the hypothesis below.

1.16 HYPOTHESIS

Eosinophil granule proteins lead to an increase in cholinergic gene expression accounting, at least in part, for the cholinergic remodelling effects of eosinophils in conditions such as asthma.

Study goals:

The purpose of this study is to examine:

1) If the Eosinophil granule proteins EPO, MBP, EDN induce cholinergic gene expression in IMR32 cells
2) What intracellular signaling mechanisms could be involved.
3) The mechanism of interaction of EPO with the cells to induce the changes as a representative of ECP's.

CHAPTER 2

MATERIALS AND METHODS

All chemicals and reagents used in this study were supplied by Sigma Aldrich, Ireland unless otherwise specified.

2.1 IMR32 CELL CULTURE

2.1.1 Proliferation Medium

For 500 ml Dulbecco's Modified Eagles Medium with Glutamax add:

5% (v/v) foetal calf serum (FCS) (25 ml)

1 % (w/v) penicillin-streptomycin (5 ml)

1µl/ml gentamicin (0.5 ml)

2.1.2 Differentiation Medium

For 500 ml Dulbecco's Modified Eagles Medium with Glutamax add

2% (v/v) FCS (10 ml)

2 mM sodium butyrate (0.11g)

1 % (w/v) penicillin-streptomycin (5 ml)

1 µl/ml gentamicin (0.5 ml)

2.1.3 Thawing

Cells thawed quickly by placing cryovial in 37°C incubator.

Contents of cryovial transferred into 5 ml medium containing 10% (v/v) FCS (twice as much FCS is needed to initially boost cells; when first passage needed use ordinary 5% (v/v) FCS Proliferation Medium) in sterile 15 ml conical tube. Cells spun at room temperature (RT), 1000 x g, 10 min.

Supernatant discarded and cells resuspended in 1 ml 10% (v/v) FCS medium.

Cells transferred to culture flask (T75) containing 15 ml 10% (v/v) FCS medium (prewarmed to 37°C).

Labelled and incubated at 37°C, 5% CO_2.

2.1.4 Passaging

Old media from culture flask poured down to approximately 0.5 ml.

Flask tapped until cells sheet off from bottom of flask.

5 ml fresh Proliferation Medium (prewarmed to 37°C) added and remaining cells rinsed off bottom of flask.

Medium with suspended cells transferred into sterile 50 ml conical tube.

Approximately 10 µl of cells removed into sterile eppendorf tube for counting.

Cells spun at 1000 x g, RT, 10 min.

Based on cell count, cells resuspended in appropriate volume of proliferation media and cells passed through syringe with 21G needle (green) 3-4 times to break up clumps of cells.

Cells transferred to flasks and plates as appropriate.

2.1.5 Differentiation

Cells plated to appropriate number on cell culture plates. 24-48 h after plating, proliferation medium replaced with differentiation medium. Cells allowed to grow for 7- 8 days, changing medium every 1-2 days.

2.1.6 Freezing

Cells removed from bottom of flask and spun down. Based on cell count, cells re-suspended in Freezing Mix [90% (v/v) FCS; 10% (v/v) DMSO] at approximately $5x10^6$ per ml and 1 ml placed in each cryotube. Labelled and placed at -80°C overnight before transferring to liquid nitrogen.

2.1.7 Cell Culture

IMR32 cells were seeded into 6 well plates at a density of 2 X 10^5 cells/well in proliferation media. After 3-4 days cells were fed with differentiation media. Cells were maintained in differentiation media for approx 4 days prior to experimentation.

2.2 CELL TREATMENTS

2.2.1 Eosinophil Granule protein EPO time points

To assess the effects of EPO on cholinergic gene expression, differentiated IMR32 cells were incubated with 1µg of purified EPO per 1ml of medium. Incubation times of 1, 4, 16 and 24 h were used to establish the regulatory time course of EPO on ChAT and VAChT gene expression.

Two negative controls were utilized for all experiments with IMR32 cells incubated in differentiation medium alone. All experiments were conducted in duplicate.

After co-incubation, IMR32 cell monolayers were rinsed 3 times with chilled PBS and harvested from the wells with cell lysis in TRI-Reagent and RNA isolated as

described below.

EPO acquired from laboratory of Dr. Gerald Gleich, University of Utah.

2.2.2 Poly-L-Arginine charge mimetic

The first of the mechanistic studies undertaken was the establishment of the role of the cationic charge of EPO on ChAT and VAChT gene expression. For this purpose, the synthetic cationic polypeptide Poly-L-Arginine was used as a cationic charge mimetic.

IMR32 cells were incubated with 1µg/ml Poly-L-Arginine (PLA) per 1ml of medium for 1,4,16 and 24 h as previously undertaken with purified EPO. Two negative controls were used as previously and all experiments were conducted in duplicate.

The IMR32 cell monolayer was again harvested and lysed in TRI-Reagent and RNA isolated as described below.

2.2.3 Inhibition of Adhesion PLG/heparinise/PD95098 at specific time points

The dependence of EPO gene regulatory effects on direct interaction with the cell membrane was studied using the cell adhesion inhibitors Poly-L-Glutamate and Heparinase 1.

Poly-L-Glutamate (PLG) is a polyanionic polypeptide used to inhibit the interaction EPO with the IMR32 cell surface by charge neutralization. Heparinase 1 was used to inhibit EPO interaction with Glycosaminoglycan moieties in the IMR32 cell membrane by disulfide bond cleavage.

From the previous experiments, the time point at which EPO exerts maximal effects on ChAT and VAChT gene expression were selected for all subsequent mechanistic

inhibitor studies. At selected time points, IMR32 cells were incubated with EPO (1µg/ml) in the presence and absence of PLG (1µg/ml) or Heparinase 1 (4U/ml). Two negative controls were used and all experiments were conducted in duplicate.

The intracellular pathway resulting in genetic effects in the nucleus were studied by inhibiting the MAP kinase ERK1 using PD98059.

IMR32 cells were incubated with EPO (1µg/ml) in the presence and absence of PD98059 at 50µM concentration.

2.3 RNA ISOLATION

TRI REAGENT was used in the simultaneous isolation of RNA, DNA and protein. A single-step liquid phase separation resulted in the simultaneous isolation of RNA, DNA and protein.

After adding chloroform and centrifuging at 12000 x g for 15 mins, the mixture separated into 3 phases: an aqueous phase containing the RNA, the interphase containing DNA and an organic phase containing proteins. Each component was then isolated after separating the phases. One ml of TRI REAGENT was sufficient to isolate RNA, DNA and protein from 5-10 X 10^6 cells or 10 cm^2 of culture dish surface for cells grown in monolayer.

This is one of the most effective method for isolating total RNA and can be completed in only 1 h starting with cells. The procedure is very effective for isolating RNA molecules of all types from 0.1 to 15 kb in length. The resulting RNA is intact with little or no contaminating DNA and protein.

Monolayer cells:

1. Cells lysed directly on the culture dish. 1 ml of the TRI REAGENT per 10 cm^2 of glass culture plate surface area was used. After addition of the reagent, the cell lysate were passed several times through a pipette to form a homogenous lysate.

2. Samples allowed to stand at RT for 5 min. 200ul of chloroform was added per ml of TRI REAGENT used in step 1.

Samples then covered and shaken for ~15 seconds and allowed to stand for 2-15 min at RT.

Centrifuged at 12,000g for 15 min at 4^0 C.

3. The aqueous phase was transferred to a fresh tube and 0.5 ml of isopropanol added per ml of TRI REAGENT used in sample preparation, step 1, and mixed.

Sample allowed to stand for 5-10 min at RT then centrifuged at 12,000 g for 10 min at 4°C. The RNA precipitate formed a pellet on the side and bottom of the tube.

4. The supernatant was removed and the RNA pellet washed by adding 1 ml (minimum) of 75% (v/v) ethanol per 1 ml of TRI REAGENT used in Sample Preparation, Step 1. The sample was then centrifuged at 7,500 x g for 5 min at 4°C.

5. The RNA pellet was dried for 5-10 min by air- drying or under a vacuum. The RNA pellet was not allowed to dry completely, as this greatly decreases its solubility. An appropriate volume of formamide, water or a 0.5% (w/v) SDS solution was added to the RNA pellet. To facilitate dissolution, it was mixed by repeated pipetting with a micropipette at 55-60°C for 10-15 min.

Final preparation of RNA should had a 260/280 ratio of ~1. 7.

2.4 cDNA SYNTHESIS

Using the 1st Strand cDNA Synthesis Kit, RNA was reverse transcribed into single-stranded cDNA. In this method, AMV Reverse Transcriptase synthesizes the new cDNA strand at a site(s) determined by the type of primer used: at the 3'-end of the poly(A)-mRNA when Oligo-p(dT)15 is used as a primer. The resulting first strand cDNA can then be used as a template for PCR. AMV Reverse Transcriptase is well suited to the preparation of cDNA for use as a PCR template. Fully active in PCR buffer, AMV Reverse Transcriptase features a high specific activity, ensuring large amounts of template. At 42°C, this highly processive enzyme is more efficient than M-MuLV Reverse Transcriptase

The following components were mixed in a sterile microfuge tube (table 1):

TABLE 1: cDNA synthesis reagents

Reagent	Volume/ 1 sample	Final Concentration
10X Reaction Buffer	2µl	1X
25 mM $MgCl_2$	4µl	5mM
Deoxynucleotide Mix	2µl	1mM
Oligo-p(dT)15 Primer	2µl	0.04 A_{260} Units
RNase Inhibitor	1µl	50 units
AMV Reverse Transcriptase	0.8µl	>20 units
Sterile water	Variable	
RNA sample*	Variable	
TOTAL	20µl	

* 1µg total RNA used

2. Briefly vortexed and mixture centrifuged to collect the sample at the bottom of the microfuge tube.

3. Reaction incubated at 25°C for 10 min and then at 42°C for 60 min. During the first incubation the primer annealed to the RNA template. The RNA was subsequently reverse transcribed resulting in cDNA synthesis during the second incubation.

4. Following the 42°C incubation, the AMV Reverse Transcriptase was denatured by incubating the reaction at 99°C for 5 min and then cooling to 4°C for 5 min - If not denatured, AMV Reverse Transcriptase may interfere with subsequent applications. Reaction tube was stored at -20°C pending use for quantitative studies.

2.5 QUANTITATIVE PCR USING THE LIGHTCYCLER

Amplification of cDNA was carried out by quantitative PCR using the Light Cycler using the double-stranded DNA binding dye SYBR Green 1 (Roche Molecular Biochemicals). The samples were continuously monitored during the PCR, and fluorescence was acquired every 0.1 °C. PCR mixtures contained 0.5 µM of either β-Actin (Forward 5' GGA CTT CGA GCA AGA GAT GG 3', Reverse 5' AGG AAG GAA GGC TGG AAG AG 3') or primers specific to gene of interest (Synthesised by MWG Biotech) (table 2). The samples were denatured at 95 °C for 10 min followed by 45 cycles of annealing and extension at 95 °C for 12 s, 55 °C for 5 s, and 72 °C for 10 s. The melting curves were obtained at the end of amplification by cooling the samples to 65 °C for 15 s followed by further cooling to 40 °C for 30 s. Serial 10-fold dilutions were prepared from known quantities of β-Actin and gene of interest PCR products, which were then used as standards to plot against the unknown samples.

Quantification of data was analyzed using the LightCycler™ analysis software, and values were normalized to the level of β-Actin expression for each sample on the same template cDNA.

TABLE 2: Primer sequences

PRIMER	SEQUENCE	Tm(°C)
β-Actin F	5′ – GGA CTT CGA GCA AGA GAT GG – 3′	59.4
β-Actin R	5′ – AGG AAG GAA GGC TGG AAG AG – 3′	59.4
ChAT F	5′ – CTA CAG GCT CCA CCG AAG AC – 3′	61.4
ChAT R	5′ – GTC AGT CAC GGC TCT CAC AA – 3′	59.4
VAChT F	5′ – ACT ATG CGG CCT CTG TTT TG – 3′	59.4
VAChT	5′ – CGC TGC CAT AGA CTG AGA CA – 3′	59.4

Semi-Quantitative RT-PCR

In semi quantitative RT-PCR, the integrity of RNA extraction and cDNA synthesis was verified by PCR by measuring the amounts of β-actin cDNA in each sample using β-actin-specific primers. PCR mixtures contained 10X reaction buffer (Promega, Madison, WI), 2.5 mM $MgCl_2$, 1.25 units of *Taq* polymerase, and 0.2 mM of each dNTP (Promega). Thermocycling conditions for M_2 cDNA were 95 °C for 5 min, 42 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Twenty nine cycles were used to amplify the more abundant β-Actin cDNA. A final extension step of 72 °C for 10 min was followed by resolution of the ChAT, VAChT and β-Actin products on a 1.5% (w/v) Tris borate-EDTA agarose gel containing 0.5 µg/ml ethidium bromide. M_2 PCR products were captured and quantified by densitometry using the Image Master VDS-Cl and software Total Lab v1.00 (Amersham).

2.6 PROTEIN PREPARATION AND WESTERN BLOTTING

2.6.1 Experiment layout for protein studies

IMR32 cells (5 x 10^5) were differentiated for 5-7 days with sodium butyrate as described above and then incubated with or without 1µg/ml EPO for varying time periods from 12 to 24 h. In some experiments, IMR32 cells were pre-treated with inhibitors of EPO cell surface interaction inhibitors [PLG (1 µg/ml) and heparinase 1 (4U/ml)] as well as MAP kinase inhibitor [PD98059 (50µM)]. Experiments were carried out in the presence or absence of these inhibitors. All experiments were conducted in duplicate with two negative controls. Cytoplasmic/nuclear and membrane protein extracts were prepared following the protocols outlined below. ChAT was assayed from the cytoplasmic fraction and VAChT from the membrane fraction. Relative quantification was assessed absorbimetrically with western blotting using the protocols outlined.

2.6.2 Nuclear and Cytoplasmic protein preparation

Nuclear and cytoplasmic extracts were isolated from IMR32 cells, essentially as in Greene *et al.* (2000) with some modifications. Briefly, cells were harvested in 1 ml ice-cold PBS and pelleted by centrifugation at 3800 x *g* for 5 min at 4^0C. Cells were resuspended in 1 ml hypotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 0.5 mM DTT] and pelleted by centrifugation at 13,000 x *g* for 10 min at 4^0C before lysis for 10 min on ice in 20 µl hypotonic buffer containing 0.1 % (v/v) Igepal CA-630. Lysates were centrifuged as before and the supernatant cytoplasmic extract removed to fresh tubes. Protein concentration was established

by the Bradford method (Bradford, 1976) and cytoplasmic extract stored at –80^{0}C The nuclear pellet was lysed in 15 µl lysis buffer [20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25 % (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] for 15 min on ice. After centrifugation, as before, supernatant nuclear extracts were removed into 35 µl storage buffer [10 mM HEPES (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 20 % (v/v) glycerol, 0.5 mM PMSF, 0.5 mM dithiothreitol (DTT)]. Protein concentration was determined as before and nuclear extracts stored at –80^{0}C.

2.6.3 Membrane protein preparation

Solutions

1. PBS
2. 0.5M EDTA in PBS (37^{0}C)
3. **Buffer A** (4^{0}C)

   5mM Tris-HCL pH 6.8

   2mM EDTA

   Protease Inhibitors (to be added fresh)

   5ug/ml Leupeptin

   0.7ug/ml Pepstatin

   5ug/ml Benzamidine

   1mM PMSF

4. **Buffer B** (4^0C)

   20mM Tris-HCL (pH 6.8)

   150mM NaCl

   10mM EDTA

   10mM EGTA

   1% (w/v) Triton X 100

   Protease Inhibitors (As for buffer A)

Protocol

1. Cells washed with PBS
2. Cells detached with 0.5mM EDTA in PBS
3. Cells peletted at 1,500 x g for 5 min and resuspend in buffer A.
4. Cells forced through a 22 gauge needle 6-8 times.
5. Spun at 55,000 x g for 20 min at 4^0C using polycarbonate ultracentrifuge tubes.
6. The resulting pellet contained the membrane fraction.
7. Pellet resuspended in approx 100ul buffer B.
8. Supernatant retained in buffer A.

2.6.4 Gel Electrophoresis Stage

2.6.4.1 Materials

10% Separating Gel

Acrylamide - 3.3 ml

Sterile distilled water (SDW) -3.9 ml

1.5 M Tris (pH 8.8) -2.5 ml

200ul

10% (w/v) SDS 200 µl

10 % (w/v) APS 0.1% (w/v) – 67 µl

TEMED - 6.7 µl

SDS Sample Treatment Buffer

1M Tris (pH 6.8) - 1ml

10% (w/v) SDS – 2ml

0.1% (v/v) Bromophenol Blue –

dH_2O – 5ml

Glycerol – 2ml

6 – mercaptoethanol – 1ml

4% Stacking Gel

Acrylamide - 500 µl

dH20 -2.25 ml

0.5 M Tris (pH 6.8) -950 µl

10% (w/v) SDS -38 µl

10% (w/v) APS -25 µl

TEMED -3.8u1

Running Buffer 5X

Tris - 7.5g

Glycine – 36g

SDS – 25g

Make up to 500ml in SDW

Transfer Buffer 1X

Tris – 2.42g

Glycine – 11.26g

10% (w/v) SDS – 1ml

Make up to 200 ml in methanol,

Add 800ml SDW.

Blocking Buffer

0.2g I-Block

PBS – 100ml

0.1% (v/v) Tween 20 – 100 µl

Washing Buffer

1 sachet PBS

1 L SDW

Tween 20 – 1ml

Assay Buffer

SDW – 18ml

10X Assay Buffer – 2ml

Substrate/Blot

CDP-Star – 1.9ml

Nitro Block – 100 µl

10X Assay Buffer

200mM Tris-HCL pH 9.8

10mM $MgCl_2$

2.6.4.2 SDS-PAGE

Proteins were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel overlayed with 4% stacking gel.

Separating gel was made up and added to 1 cm of top of gel caster set-up, layered with ethanol and allowed to set for 5-10 min. Meanwhile, stacking gel was made up. When separating gel was set EtOH was removed and stacking gel was poured on. Comb was inserted making sure no bubbles had formed, then gel was left to set for 30 min in gel casette.

The amount of protein in samples was measured according to the Bradford method.

The hot plate was pre-heated to 94^{0}C. All samples were made up to the same amt (~10 µl) using SDW. The same amount of SDS sample treatment buffer was added (in fume hood) and proteins were denatured for 5 min. Side supports and elastic were removed from gel which was then added to chamber. Backing support was

added to other side.

350 ml IX running buffer (70 ml 5X buffer and 280 ml dH2O) was made up and gradually added to electrophoresis chamber making sure no bubbles formed. The comb was then carefully removed.

10 µl of molecular weight marker was added and -20 µl of sample applied to wells and electrophoresis was run at 30mAmps/gel > 500 V > 20W for approx 1 hr or until dye had run to the bottom of the gel.

2.6.4.3 Western blotting

The proteins were transferred to nitrocellulose membranes at 80 mA overnight using a wet electrophoretic blotting system.

4 blotting papers and one nitrocellulose membrane were cut. 1200 ml transfer buffer was added to chamber.

Blot sandwich was made up as follows:

White grid > Two foams > Pre-wetted blotting paper > Pre-wetted nitrocellulose > Gel cut to size > Pre-wetted blotting paper > Two foams>Black grid

The blot sandwich was clipped together and placed in transfer chamber with white side facing forward to + side. Transfer was run at 80 mA, 500 V, 30 W overnight.

2.6.4.4 Blocking Stage

Blot was removed from sandwich, Ponceau S solution added and left for a few min to show up proteins. Ponceau S solution was then washed off with water. Blot was then immersed in blocking buffer for 1 hr. Buffer was tipped out and sheet placed on tissue paper. If looking at more than one protein, sheet cut in appropriate place.

2.6.4.5 Washing and primary/secondary antibody stage

Primary antibody (table 3) was made up in blocking buffer and 5 ~ 10 ml added to 50 ml tubes. Blot was immersed in primary antibody with light shaking at 50 x g overnight at 4ºC. Blot was then removed and washed with 50 ml washing buffer 6 times x 5min on shaker. Secondary antibody (table 3) then made up in blocking buffer. Blot was immersed in antibody and shaken for 1 hr. Washing procedure was repeated to remove secondary antibody.

TABLE 3 Primary and secondary antibodies used in Western blotting

Primary antibody	Supplier	Dilution	Cat no#
ERK2 (C-14)	Santa Cruz	1:1000	*sc-154*
ChAT (H-95)	Santa Cruz	1:1000	*sc-20672*
VAChT (H-160)	Santa Cruz	1:1000	*sc-15315*
NFκB (C20)	Santa Cruz	1:10,000	*sc-372X*
IκB-α	Santa Cruz	1:1000	*sc-1643*

Secondary antibody	Supplier	Dilution	Cat no#
Anti-rabbit IgG-HRP	Santa Cruz	1:10,000	Sc-2370

2.6.4.6 Assay & Substrate stage

Assay buffer was made up and blot washed with 10ml twice for 2 min to prepare for substrate. Substrate was made up just prior to use and pipetted onto blot placed on plastic sheet and left for 5 min. Excess substrate was removed from blot and sandwich between plastic folders. Blot was then exposed to x-ray film for 30 sec -10 min. Exposed films were digitally photographed and subjected to denditometric analysis using GeneSnap software (Synoptics).

2.7 INTERACTION AND CO-LOCALISATION STUDIES

IMR32 cells were incubated with fluorescently labelled EPO in order to visually assess the interaction of EPO with nerve cells.

2.7.1 Fluorescent labelling of EPO

The following procedure was employed to label EPO with fluorescein isothicyanate (FITC)

1. 2 mg/ml EPO solution was prepared in 0.1 M sodium carbonate buffer, pH=9.
2. FITC was dissolved in anhydrous dimethyl sulfoxide (DMSO) to a concentration of 1 mg/ml.
3. 50 ml of FITC for every 1ml of EPO solution was added in 5 ml aliquots while continuously stirring the protein solution.
4. EPO-FITC reaction mixture incubated in the dark for 8 hours at 4 °C.
5. NH4Cl was added to a final concentration of 50 mM and incubated for 2 hours at 4 °C.
6. Xylene cyanol then added to 0.1% and glycerol to 5% concentrations.

7. Unbound FITC separated from the conjugate by gel filtration using a fine-sized gel matrix with an exclusion limit of 20,000. With the column flow stopped, the reaction mixture was layered onto the top of the column.
8. The reaction mixture was then allowed to flow into the column.
9. PBS then added to the top of the column and connected to column buffer supply
10. Of the two bands formed on the column, the faster moving (conjugated protein) was eluted and stored in the column buffer in a light-proof container at 4°C. The slower moving band, representing unbound FITC and Xylene cyanol, was discarded.
11. Fluorescein to Protein ratio was calculated as per manufacturer's instructions by measuring the absorbance at 495 nm and 280 nm. F/P approx 0.5.

2.7.2 Primary experiment (incubation time and protein concentration optimization)

1. IMR32 cells were split and plated onto Lab-Tek II Chamber slides (Nalge Nunc International Corp). In order to prevent cell detachment during washes, glass slide surfaces were pre-coated with cell culture grade Gelatin 1% (w/v).

   1ml of sterile gelatin solution was placed in each of the two wells of each slide. This was left to sit for 10 min. The gelatin solution was then aspirated and the slides were left to dry at room temperature under cell culture conditions.

   The dry slides were then washed once with proliferation medium.
2. IMR32 cells were then plated at 10,000 cells per well in 1.5 ml of proliferation medium for 48 h. Cells were then differentiated for 5 days prior to co-

2.8 STATISTICAL ANALYSIS

Values are expressed as mean +/- SEM. The statistical significance of differences between treated samples and the appropriate time point control was evaluated by ANOVA; *$p<0.05$, **$P<0.005$

CHAPTER 3

RESULTS PART 1

The up-regulatory effect of Eosinophil Peroxidase on ChAT and VAChT cholinergic genes in IMR32 cells

3.1 VERIFICATION OF DETECTABLE PRESENCE OF PCR PRODUCT

3.2 THE EFFECTS OF EPO, MBP and EDN ON CHOLINERGIC GENE LOCUS EXPRESSION
- 3.2.1 EPO up-regulates ChAT RNA and protein
- 3.2.2 EPO up-regulates VAChT RNA and protein
- 3.2.3 MBP up-regulates VAChT but not ChAT RNA
- 3.2.4 EDN does not up-regulate ChAT or VAChT RNA

3.3 MECHANISTIC STUDIES
- 3.3.1 Poly-L-Arginine fails to mimic EPO effects of up-regulation of ChAT and VAChT RNA
- 3.3.2 Heparinase 1 and Poly-L-Glutamate reverse EPO effects on ChAT and VAChT RNA
- 3.3.3 ERK inhibition reverses the effects of EPO on ChAT and VAChT RNA
- 3.3.4 Heparinase 1, Poly-L-Glutamate and ERK inhibition reverse EPO up-regulatory effects on ChAT and VAChT protein
- 3.3.5 EPO leads to nuclear factor κB activation

3.1 VERIFICATION OF DETECTABLE PRESENCE OF PCR PRODUCT

Studies were first carried out to confirm the presence of ChAT and VAChT cDNA in the cell samples by verifying that PCR primers yielded measurable levels of PCR product utilizing primers outlined in Table 2.

Optimization of magnesium concentrations for all subsequent qualitative PCR studies was conducted at the outset using the "magnesium curve" whereby PCR reactions were carried out in the presence of varying concentrations of MgCl2. Magnesium concentration of 1mM was found to be optimal for both ChAT and VAChT primers (Figure 2A).

Initial qualitative PCR was then performed to confirm the presence of CHAT and VAChT cDNA amplification products in the presence of MBP, EDN and EPO. The purpose of the second standardization was to identify any difficulty in the detection of ChAT or VAChT with respect to treatment with any of the proteins from the outset as well as to the concentration of protein used. Results shown (Figures 2B, C) clearly show good yield of both ChAT and VAChT cDNA amplification products at predetermined optimal Magnesium concentration (1mM) after incubation of differentiated IMR32 cells with MBP, EDN and EPO using primers designed to yield 100-200 bp amplification products. The dose of 1µg/ml of protein showed good product signal throughout for all three proteins. This concentration of EPO was chosen as previous studies have shown it to be non toxic to nerve and epithelial cells (Morgan *et al.* 2005, Pegorier *at al.* 2006).

Thus, it was confirmed that it was possible to amplify ChAT and VAChT PCR product following cell treatment with MBP, EDN or EPO. Although MBP, EDN and EPO all amplified ChAT and VAChT PCR product, EPO was the most efficient

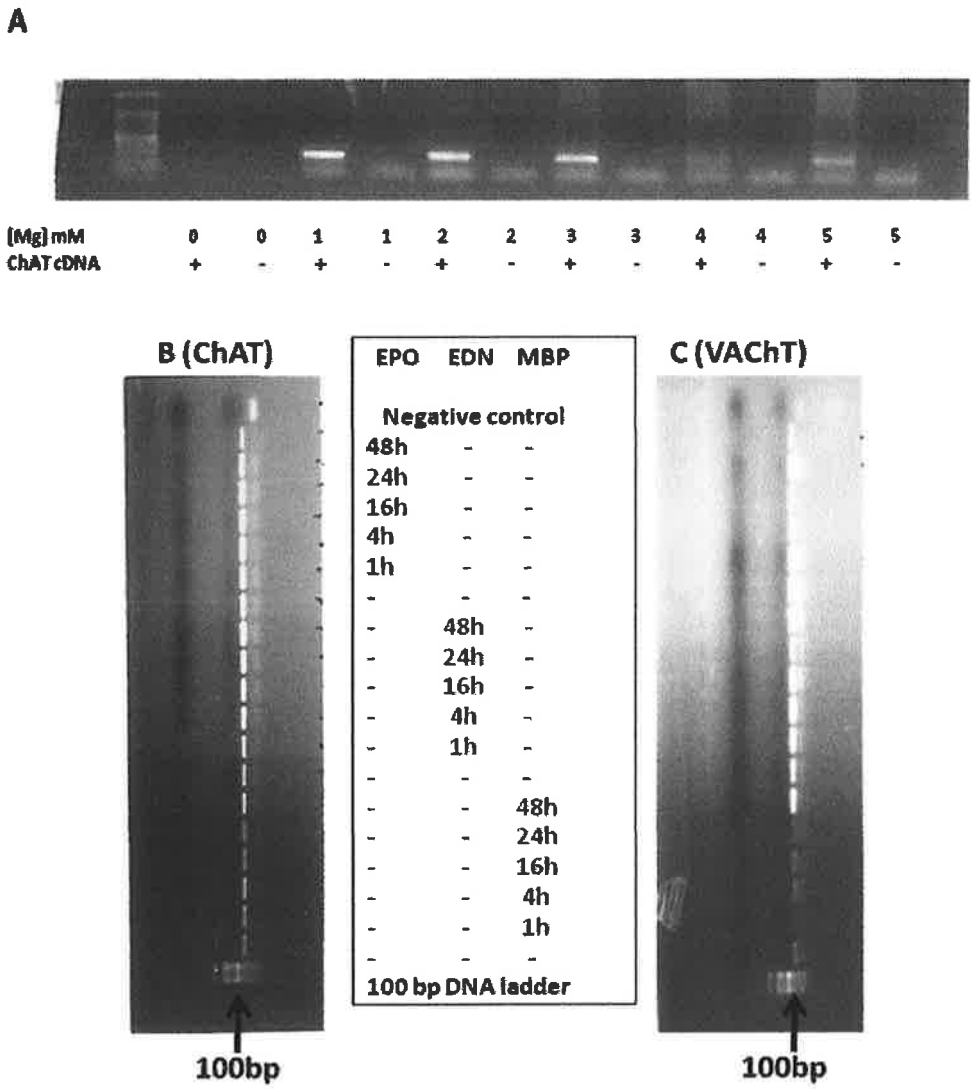


FIGURE 2: Verification of detectable PCR product

A: Magnesium curve optimization for ChAT PCR. Agarose gel electrophoresis of PCR products from cDNA obtained from IMR32 cells incubated with EPO (1µg/ml). The PCR was performed with varying concentrations of Magnesium ions (0, 1, 2, 3, 4, 5 mM). Every Mg^{2+} concentration point is performed in the presence and absence of ChAT cDNA. Magnesium concentration of 1mM was optimal for PCR with ChAT primer. The image shown is of ChAT PCR. In cDNA (-) wells RCR reactions were conducted in the absence of cDNA. Bands seen in the cDNA (-) wells are due to primer dimers.

B,C: IMR32 cells that had differentiated for 7 days were incubated with Eosinophil Granule proteins MBP, EDN, EPO (1µM) for 1, 4, 16 and 24 h. PCR was then performed with ChAT (B) or VAChT (C) primers at predetermined optimal Magnesium concentrations. Agarose gel electrophoresis was used to confirm the presence of product. The negative control well represents the product of PCR reaction conducted with all mastermix constituents and primers but in the absence of cDNA.

3.2 THE EFFECTS OF EPO MBP AND EDN ON CHOLINERGIC GENE EXPRESSION

3.2.1 EPO up-regulates ChAT RNA and protein

In order to test the hypothesis that eosinophil cationic granule proteins independently contribute to plasticity of cholinergic nerves in vitro, IMR32 cells were used as models of airway nerves as described (Walsh *et al.*, 2004). The expression of these genes by IMR32 cells in response to co-incubation with non cytotoxic concentrations of Eosinophil Peroxidase (EPO 1 μg /ml) for specific time points was assessed. Prior work in the Costello laboratory and by others that this concentration is not toxic when there is no substrate for the peroxidase enzyme (Morgan *et al.* 2005). IMR32 nerve cells were maintained in differentiation medium for 7 days prior to experimentation and then exposed to EPO for varying periods of time. Incubation of IMR32 cells with EPO (1μg/ml) caused an up-regulation in the expression of ChAT mRNA at 1 h of co-incubation ($p=0.027$ $n=7$) (Figure 3A). Three further experiments confirmed that this up regulation is not detectable at time points earlier than 1 h co-incubation (Figure 3B). ChAT mRNA levels returned to baseline within four h. In fact, at four h statistically significant down-regulation of RNA is observed prior to return to baseline ($p=0.001$, $n = 3$).

The suggested explanations for these changes in expression are explored within the DISCUSSION section.

EPO also significantly up-regulated ChAT protein expression within the nuclear protein fractions of IMR32 cells (Figure 4A, 4B). As discussed in the introduction ChAT is most strongly localized to the nuclear subfraction of these cells. A total of 3 separate experiments showed a statistically significant increase in the up-regulation

of ChAT protein expression at time points between 18 and 24 h of co-incubation ($p<0.01$). The protein levels return to baseline levels by 48 h (Figure 4). Uniform gel loading was assured by reprobing the blot with anti-ERK2 antibody hence utilizing ERK2 as a nuclear loading control. This control has been previously utilized in quantitative analyses of ERK activation dependent pathways (Morgan et al 2005).

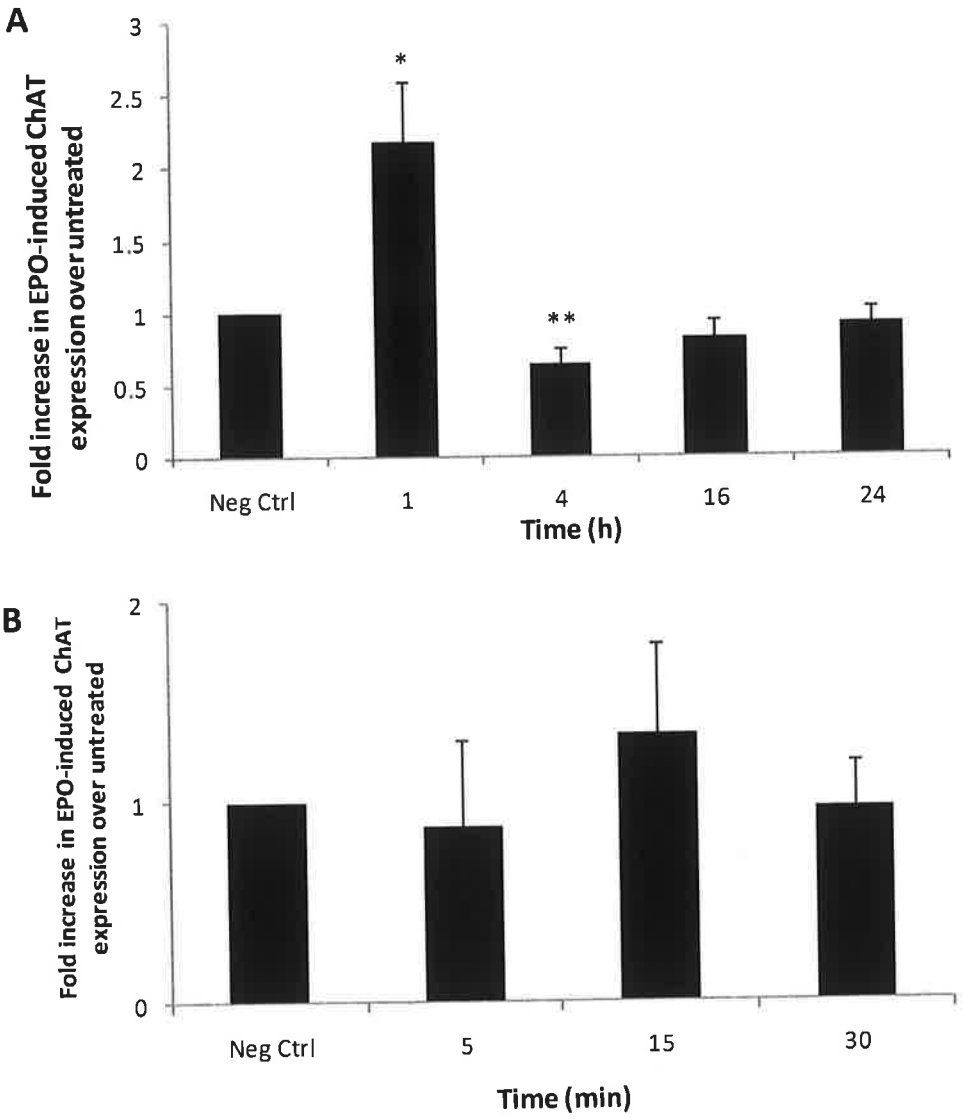


FIGURE 3: EPO causes up regulation of ChAT RNA.

IMR32 cells that had been differentiated for 7 days were incubated with Eosinophil Peroxidase (1 µg/ml) for 1, 4, 16 and 24 h. RNA was isolated, reverse transcribed to cDNA and subjected to quantitative PCR in a Lightcycler (Roche) with primers for ChAT or β-Actin. Quantification for each gene was achieved by relating fluorimetric data for each time point to that of serial dilutions of pre-synthesized genetic standards. The ratios of raw absorbance data for ChAT and β-Actin at each time point were then calculated. ChAT RNA was up regulated by EPO at 1 h (A). This effect had subsided by 4 h of co-incubation. (n=7; mean +/- SEM; *p=0.027 versus untreated IMR32 cells). Paradoxical, statistically significant down-regulation of ChAT mRNA is observed at 4 h (p=0.001). This effect is transient and mRNA levels return to normal by the 16 h time point. Analyses performed in duplicate. Appendix 1 shows raw fluorimetric data for all 7 experiments.
In 3 separate experiments, no statistically significant increase in ChAT expression in response to EPO was noted earlier than 1 h (B).

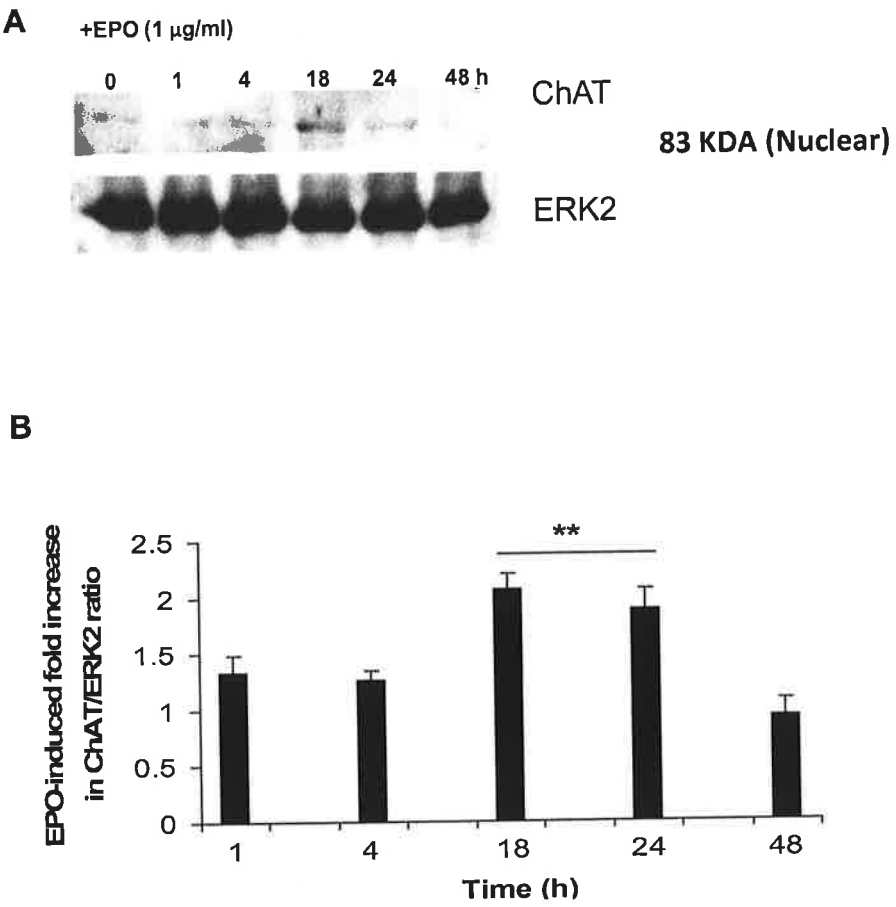


FIGURE 4: EPO increase the expression of ChAT protein in the nucleus of IMR32 cells in a time-dependent manner.

(A) Representative Western blots of IMR32 nuclear protein (10 µg) from IMR32 cells treated with EPO (1 µg/ml) for the indicated times, probed with anti-ChAT antibody (top panel) then stripped and re-probed with anti-ERK2 antibody are shown. (B) Graph shows the quantitative fold increase in the ratio of ChAT versus ERK2 expression in nuclear extracts of IMR32 cells exposed to EPO (1 µg/ml) for the indicated times (n=3; mean±SEM; $^{**}p<0.01$ versus untreated IMR32 cells).
Nuclear protein isolation techniques were performed as previously outlined in METHODS and Western Blotting was used to quantify ChAT protein expression.

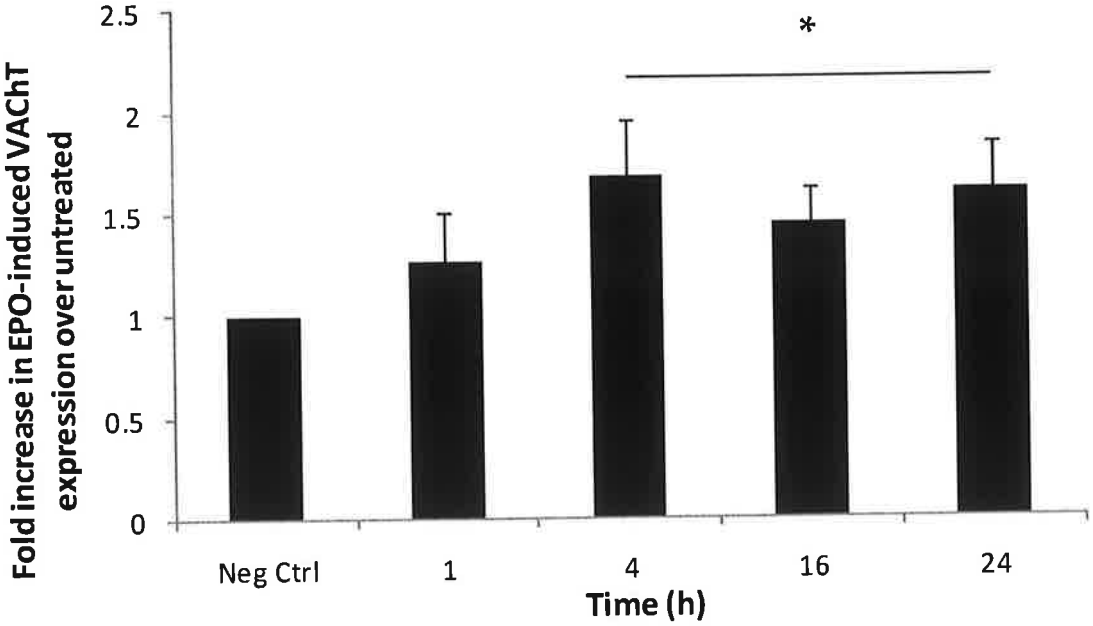


FIGURE 5: EPO causes modest but sustained up-regulation of VAChT RNA.

IMR32 cells that had been differentiated for 7 days were incubated with EPO (1µg/ml) for 1, 4, 16 and 24 h. RNA was isolated, reverse transcribed to cDNA and subjected to quantitative PCR in a Lightcycler 1.0 (Roche) with primers for VAChT and β-Actin. Quantification for each gene was achieved by relating fluorimetric data for each time point to that of serial dilutions of pre-synthesized genetic standards. The ratios of raw absorbance data for VAChT and β-actin at each time point were then calculated. VAChT RNA was up regulated by EPO between 4 h up to 24 h (n=9; mean +/- SEM; $p<0.05$ versus untreated IMR32 cells). Data from 9 separate experiments showed sustained up regulation of VAChT by EPO. All analyses were performed in duplicate.
Appendix 2 shows raw fluorimetric data for all 9 experiments.

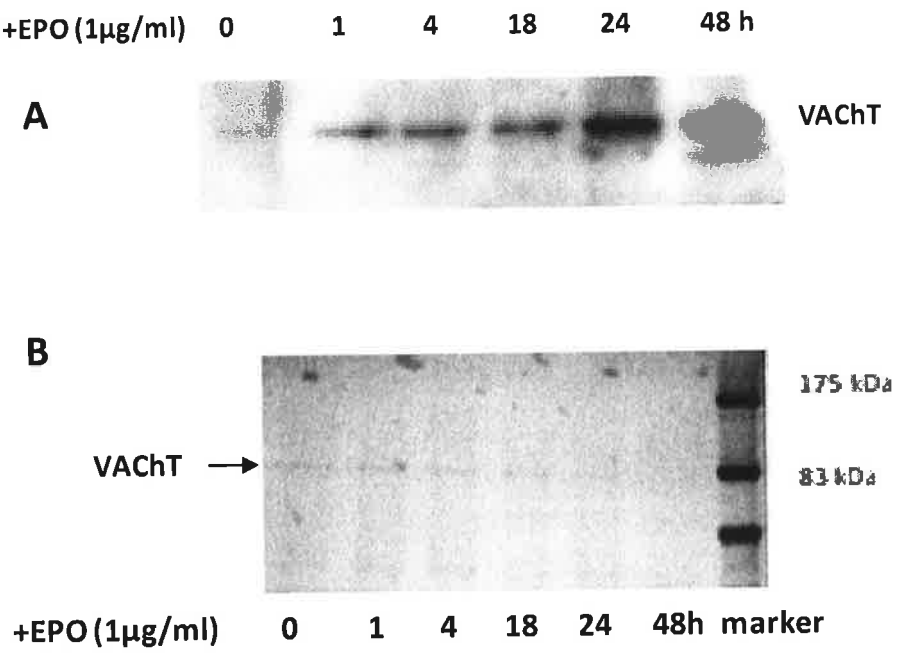


FIGURE 6: EPO increases expression of VAChT protein in the membrane of IMR32 cells in a time-dependent manner.

Representative Western blots of IMR32 membrane protein (10 µg) from cells stimulated with EPO (1 µg/ml) for the indicated times, probed with anti-VAChT antibody showing upregulation of VAChT protein in response to co-incubation of IMR32 cells with EPO with maximal up-regulation at 24 hours sustained at 48 hours (A). Representative Western blot of IMR32 membrane protein probed with anti-VAChT antibody confirms the presence of product of expected 80KDa molecular weight (B).

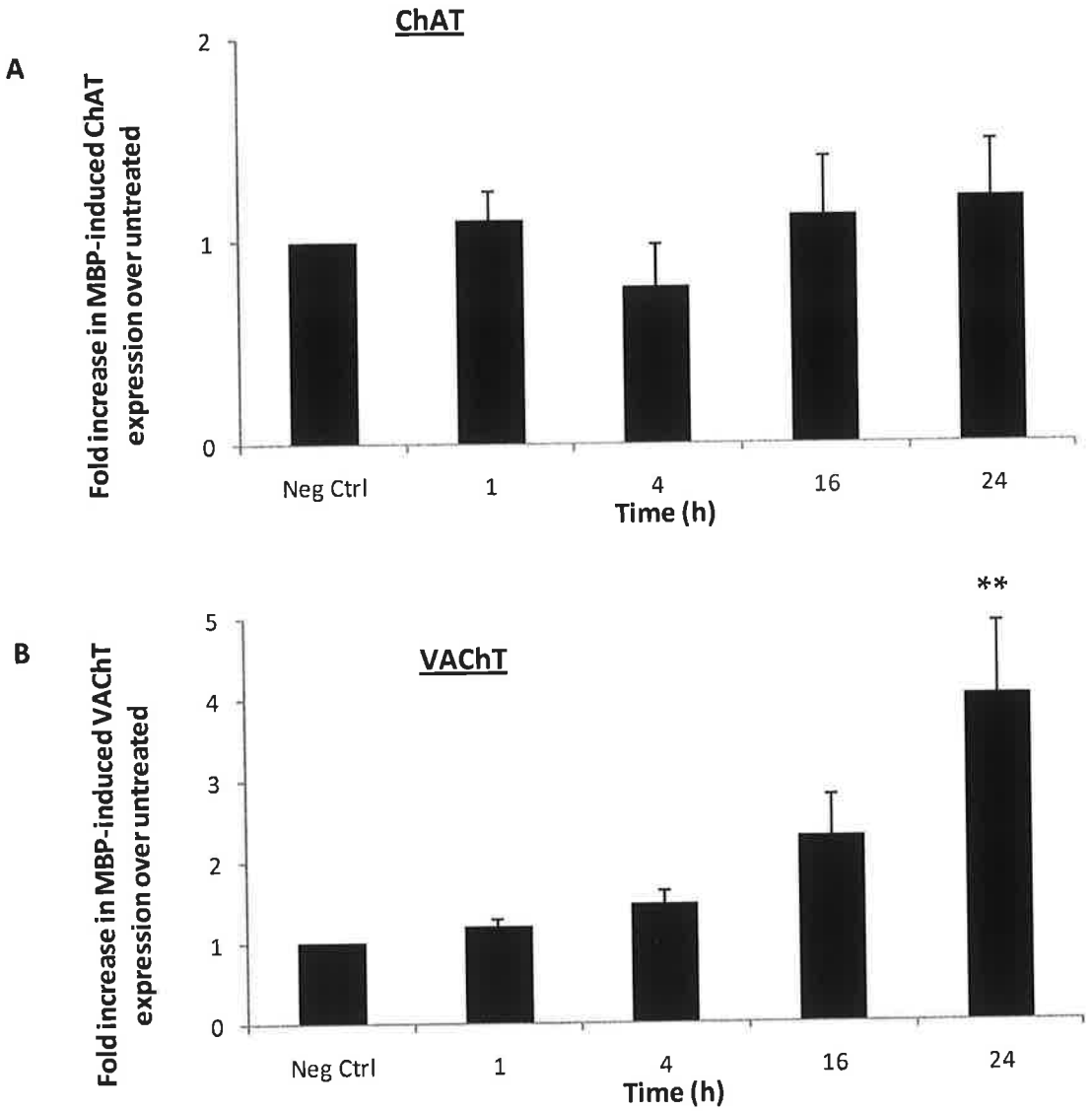


FIGURE 7: MBP up-regulates VAChT but not ChAT RNA.

IMR32 cells that had been differentiated for 7 days were incubated with MBP (1 μg/ml) for 1, 4, 16 and 24 h. RNA was isolated, reverse transcribed to cDNA and subjected to quantitative PCR in a Lightcycler 1.0 (Roche) with primers for ChAT or VAChT or β-Actin. Quantification for each gene was achieved as in previous experiments. All analyses were performed in duplicate.
MBP induced no statistically significant change in ChAT expression at any time point from 1 to 24 hours (A). VAChT RNA was up-regulated by MBP in an incremental, time dependent manner which was statistically significant at 24 h (B) (n=4; mean +/- SEM; *p=0.0022 versus untreated IMR32 cells).

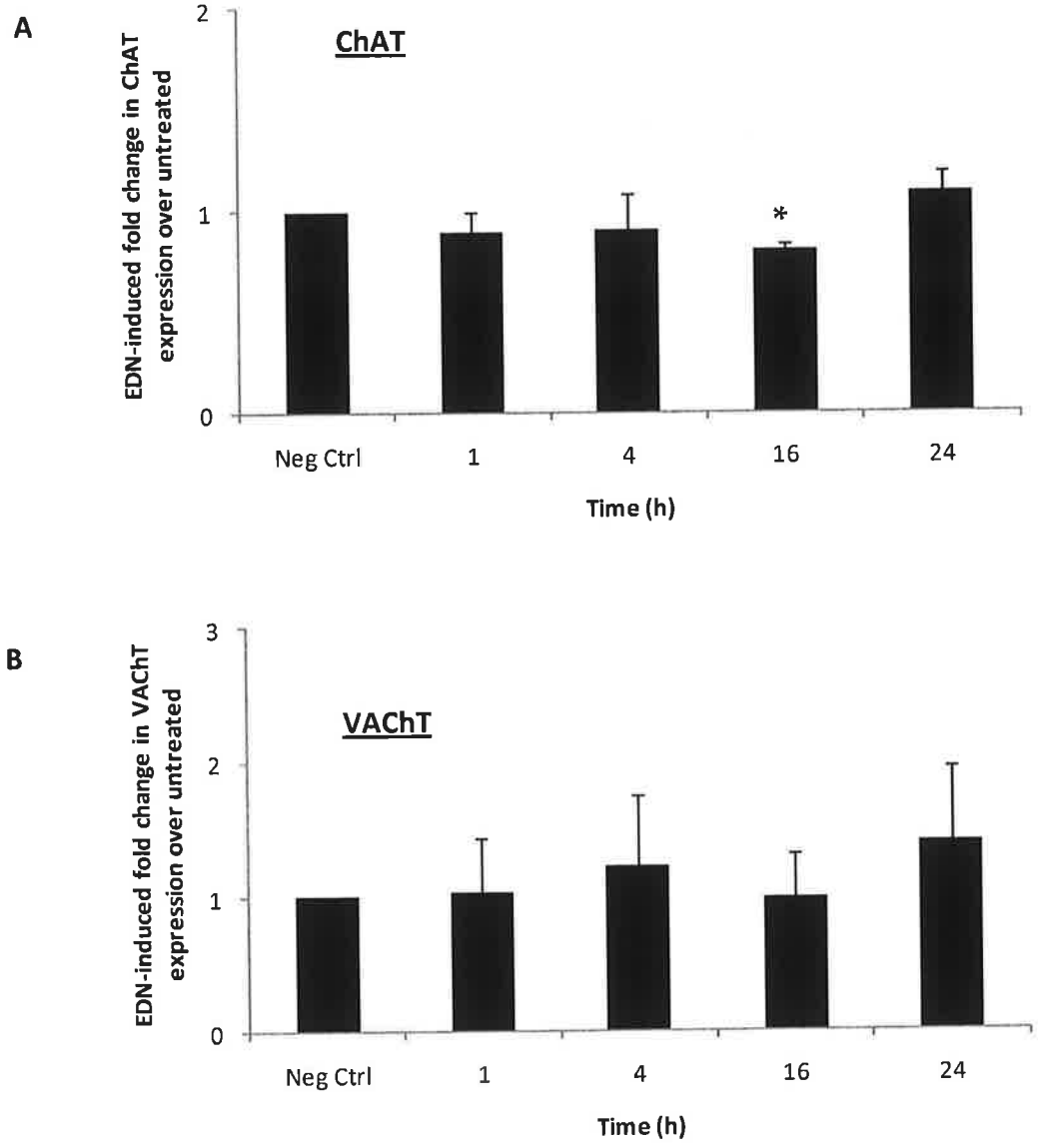


FIGURE 8: EDN does not up-regulate ChAT or VAChT.

IMR32 cells that had been differentiated for 7 days were incubated with Eosinophil Derived Neurotoxin (1 µg/ml) for 1, 4, 16 and 24 h. RNA was isolated, reverse transcribed to cDNA and subjected to quantitative PCR in a Lightcycler 1.0 (Roche) with primers for ChAT or VAChT or β-Actin. Quantification for each gene was achieved as in previous experiments.
Each experiment was performed in duplicate.
EDN induced no up-regulation of ChAT RNA at any time point. However, statistically significant down-regulation of ChAT RNA was seen at 16 h (n=4; mean +/- SEM; *p=0.035 versus untreated IMR32 cells) (A). No statistically significant up or downregulation of VAChT RNA was noted at any time point from 1 to 24 hours in response to EDN (B).

3.3 MECHANISTIC STUDIES

In order to investigate the mechanisms involved in EPO-induced up-regulation of ChAT and VAChT RNA and protein expression, mechanistic studies were carried out to assess both the direct effects of cell surface interaction of the protein molecule as well as the intracellular signalling mediators involved in gene up-regulation. As previously discussed (chapter 1), studies investigating the effects of eosinophil granule proteins on nerves showed that the MBP and EDN activate several intracellular signal pathways within the cell including the phosphorylation of the MAP kinases including ERK 1/2, p 38 and AKT (Morgan *et al.* 2005). In particular they also lead to activation of the nuclear transcription factor NFκB with resultant gene transcription up regulation (Morgan *et al.* 2005). What is not certain is which pathways EPO induces and what receptor is activated by EPO. Prior studies with EPO have suggested an important role played by its cationic charge in instigating intracellular cascades and remodelling factor synthesis in epithelial cells (Pegorier *et al.* 2006). So, experiments were performed to study how EPO may up-regulate cholinergic genes in IMR32 cells. In particular, cell surface interaction mechanisms and the role of MAP kinase and NFκB pathways in increasing ChAT and VAChT expression were assessed.

The time points selected in these mechanistic studies correspond to the time when the maximal effects occurred in the previous gene and protein expression studies (Figures 3-6). All experiments were conducted in triplicate with untreated controls.

3.3.1 *Poly-L-Arginine fails to mimic EPO effects on ChAT and VAChT RNA*

All eosinophil granule proteins are known to be strongly cationic in charge with dense charge concentration (Morgan *et al.* 2005). However, EPO, MBP and EDN all had different effects on ChAT and VAChT expression with only EPO inducing up-regulation. This implies that it is not the simple cationicity of EPO which explains its effects. Hence, the first experiments investigating the mechanism of regulation of gene expression investigated the efficacy of a synthetic cationic molecule on the regulation of cholinergic gene mRNA. To this goal, the cationic PLA, which is of similar charge concentration as EPO, was used to treat IMR32 cells. Real time PCR experiments were used to assess the effects of PLA on ChAT and VAChT gene expression. The concentration of PLA utilized was also identical to that of EPO or MBP or EDN used above (1µg/ml) (Figures 3-8). This concentration of PLA has been utilised in published work (Pegorier *et al.* 2006).

In both experiments IMR32 cells were incubated with PLA (1µg/ml) for 1, 4, 16 or 24 h. In three separate experiments, PLA had no effect on either ChAT (Figure 9A) or VAChT (Figure 9B) expression at any time point. In fact, a paradoxical statistically significant down regulation of both genes was noted at 1 h of co-incubation with PLA ($p=0.002$ for ChAT and 0.05 for VAChT). This down regulatory effect is transient; the effect has returned to normal by 4 h (raw fluorimetric data in appendices 3a, 3b). Hence, cationic charge alone is not sufficient to induce the up-regulation of ChAT and VAChT observed in the presence of EPO or of VAChT by MBP.

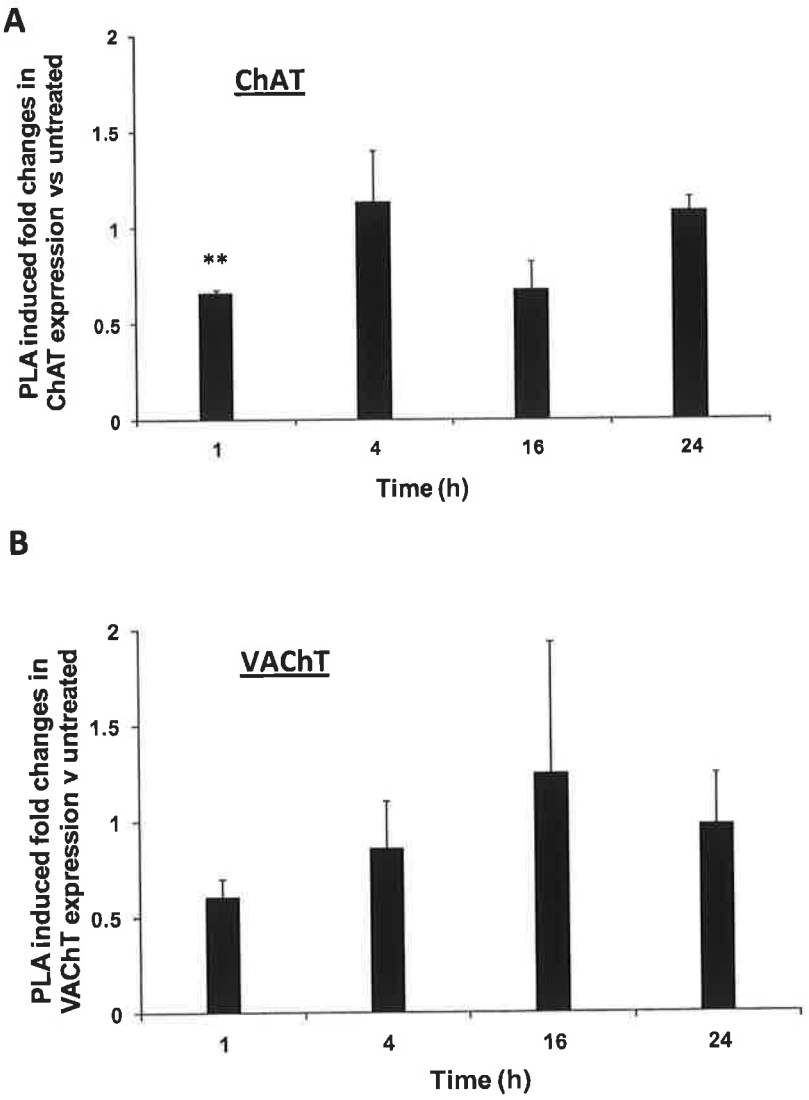


FIGURE 9: PLA fails to mimic the up-regulatory effects of EPO on ChAT or VAChT RNA.

The cationic molecule PLA (1µg/ml) was used to attempt to mimic the effects of EPO on expression of cholinergic genes ChAT (A) and VAChT (B). IMR32 cells were differentiated for 7 days then incubated either alone (negative control) or in the presence of PLA (1µg/ml) for 1, 4, 16, or 24 h. A total of 3 experiments were performed in duplicate. No statistically significant up regulatory effects on ChAT or VAChT RNA were seen at any of the indicated experimental time points. This indicates that charge alone is insufficient to trigger the intracellular pathways necessary for gene up regulation. There was statistically significant down-regulation of ChAT mRNA by PLA at 1 h.
Raw fluorimetric data presented in appendix 3, Mean +/- SEM, n=3, **p<0.005.

3.3.2 / 3.3.3 Heparinase 1, PLG, and ERK inhibition reverse EPO effects on ChAT and VAChT RNA

In order to further assess the impact on role played by the cationic charge of ECP's on their cholinergic up-regulatory effects, an inhibitory study was conducted using PLG. This polyanionic molecule has a molecular weight of 77 KDa which is similar to EPO and carries a charge of -514e at neutral pH with a polymerization rate of 515 amino acids per molecule (Barker *et al.* 1991). PLG was utilized at concentrations of 1µg/ml as optimized in published studies (Pegorier *et al.*, 2006). We hypothesized that if the cationic charge of eosinophil cationic proteins played some role in the interaction of ECP's with nerve cells, this should be counteracted by PLG.

PLG demonstrated partial reversal of the up regulatory effect of EPO on ChAT RNA at 1 h (Figure 10 and appendix 4) ($p=0.002$, $n=5$) and complete reversal of VAChT RNA up regulation at 4 h (Figure 11 and appendix 5) ($p=0.018$, $n=6$).

Extracellular proteins are also known to interact with cell surfaces by direct interaction with highly sulfated anionic glycosaminoglycan moieties of the cell membrane. Heparinase 1, which enzymatically cleaves highly sulfated regions of heparan sulfate-like glycosaminoglycans at 2-O-sulfated uronic acids, was used to further investigate the interactive mechanisms of action of ECP's with nerve cells.

Heparinase 1 demonstrated complete reversal of EPO up regulatory effects on ChAT (Figure 10 and appendix 4) ($p=0.011$, $n=6$) and VACHT (Figure 11 and appendix 5) ($p=0.016$, $n=6$).

These results suggest that EPO may act by interacting with cell membrane GAG's in a charge related mechanism to affect cholinergic transformation. This is further elaborated on in the DISCUSSION section.

The role of EPO-induced MAP kinase activation was investigated. PD98059 is a potent, selective and cell-permeable inhibitor of extracellular signal regulated protein kinases (ERK1/2). It inhibits phosphorylation of MAP kinase by MAP kinase kinase. Our previous results indicate that ERK was essential for MBP-induced effects on nerve cell survival (Morgan *et al.* 2006).

IMR32 cells were incubated with EPO (1μg/ml) with and without pre-treatment with PD98059 (50 μM). ERK inhibition leads to complete reversal of all up regulatory effects of EPO on ChAT (Figure 12 A) and VAChT (Figure 12 B) at relevant time points.

EPO was also demonstrated to cause ERK activation by phosphorylation (Figure 12 C). Western blots of cytoplasmic protein from IMR-32 cells treated with EPO (1 μg/ml) for various times from 10 min to 24 h were probed with an antibody specific to the dual phosphorylated form of ERK 1/2 and then re-probed with anti-ERK 2 antibody to quantify phospho-ERK. The intensity of the signal for the dual phosphorylated form of ERK 1/2 was quantified against that for ERK 2 at each time point. EPO induced a sustained increase in phosphorylation of ERK 1/2 over untreated levels in IMR-32 cells. This was evident from 10 min and lasted up to 12 hours of treatment. Previous work has demonstrated Sustained ERK activation in response to MBP detectable as early as 10 minutes and sustained at 24 hours (Morgan *et al* 2005). Thus, it was undertaken to assess for early 10 minute ERK activation in response to EPO. Additionally this early time point was studied given the early NF-KB activation also demonstrated in this work.

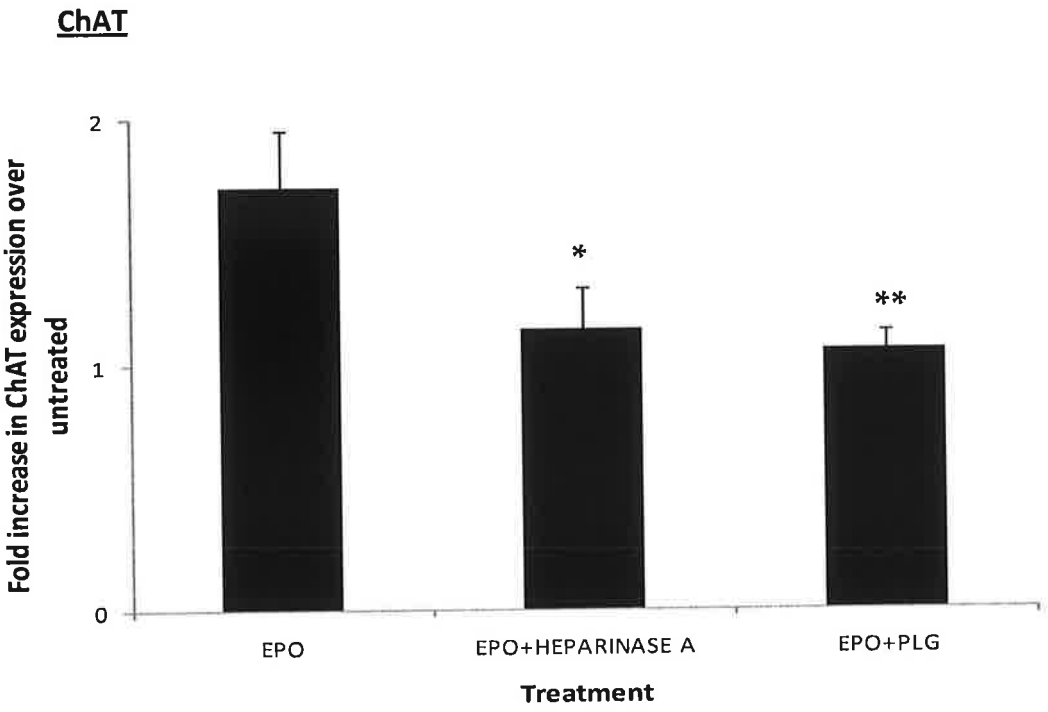


FIGURE 10: Heparinase I or PLG reverse the up regulatory effects of EPO on ChAT gene expression.

IMR32 cells were differentiated for 7 days then incubated with EPO (1µg/ml) for 1 h in the presence and absence of heparinase 1 (4U/ml) or PLG (1µg/ml). ChAT gene expression was then quantified by quantitative PCR on the Lightcycler 1.0 (Roche) as previously described. Both Heparinase I and Poly-L-Glutamate (PLG) demonstrated almost complete reversal of the up regulatory effect of EPO on ChAT gene expression (n=5; mean +/- SEM; *$p<0.05$, **$p<0.005$ versus IMR32 cells incubated with EPO alone).
All analyses performed in duplicate. Fluorimetric raw data shown in appendix 4.

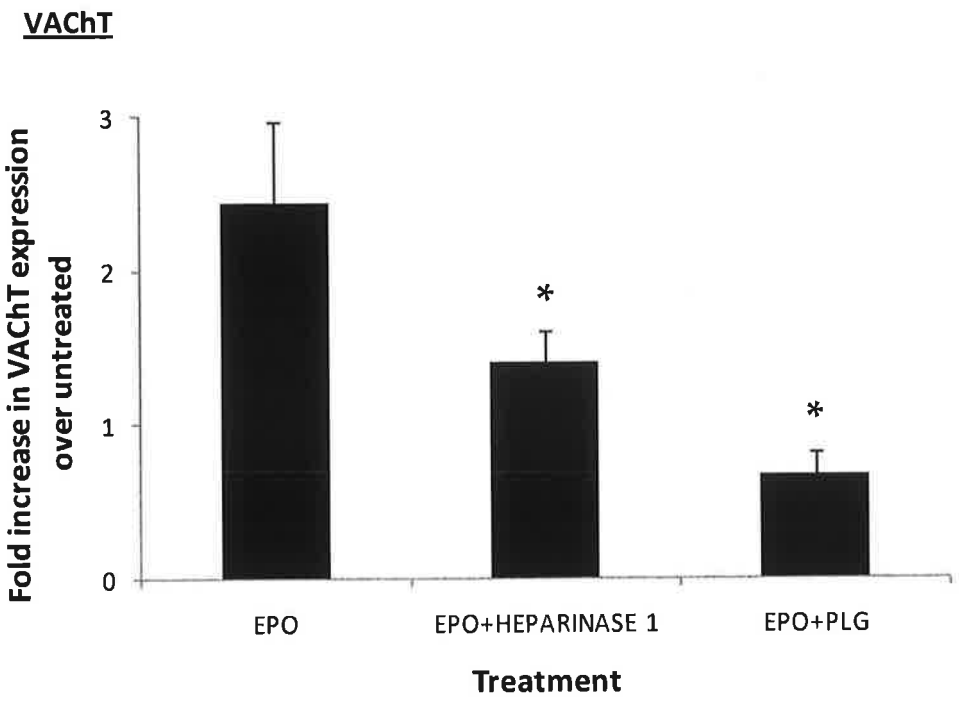


FIGURE 11: Heparinase I and PLG reverse the up regulatory effects of EPO on VACHT RNA.

IMR 32 cells were incubated with EPO (1µg/ml) for 4 h in the presence or absence of heparinase I (4U/ml) or PLG (1µg/ml). The results of 6 experiments showed either complete (PLG, n=6 +/- SEM, $p<0.05$) or partial (Hep 1, n=6, mean +/- SEM, $p<0.05$) reduction in EPO effects on VAChT up-regulation. All analyses performed in duplicate. Raw fluorimetric data shown in appendix 5.

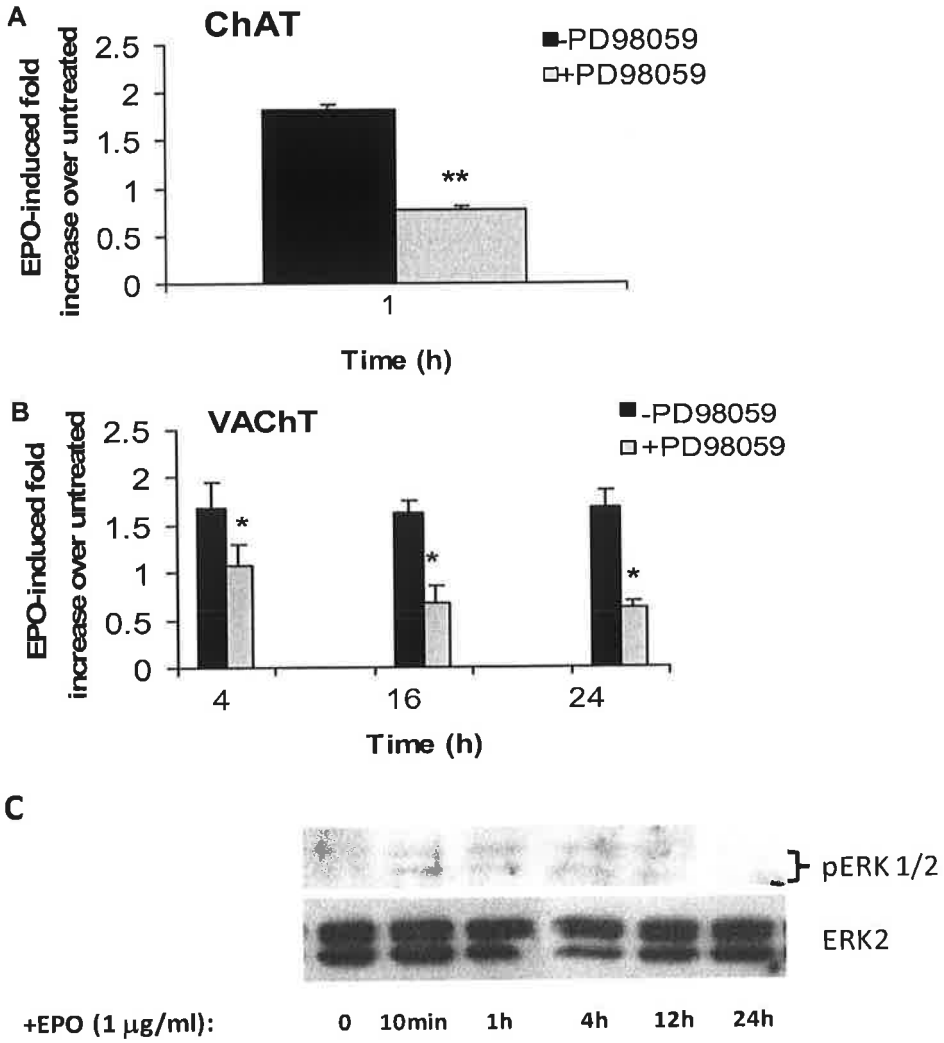


FIGURE 12: MEK/ERK inhibition reverses the effects of EPO on ChAT and VACHT up-regulation.

IMR32 cells were differentiated for 7 days then incubated in the presence or absence of EPO for indicated time periods. Pre-treatment with the MEK/ERK inhibitor PD98059 (50μM) yielded:

A: Complete reversal of the up regulatory effects of EPO on ChAT expression at the 1 h time point. (n=3; mean +/- SEM; **p<0.01 compared with cells incubated with EPO alone).

B: Complete reversal of the up regulatory effects of EPO on VAChT expression at 4, 16 and 24 h (n=3; mean +/- SEM; *p<0.05 for all three time points compared to cells incubated with EPO alone).

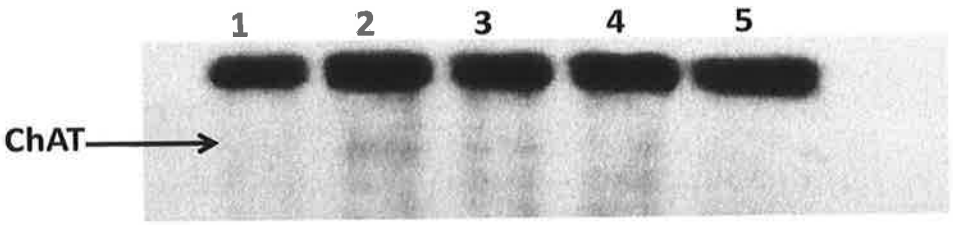
C: Western Blot demonstrating the early activation of ERK by phosphorylation as early as after 10 min of incubation of IMR32 cells with EPO (1μg/ml). The effect is sustained to 12 h of co-incubation

3.3.4 Heparinase 1, PLG and ERK inhibition reverse EPO up-regulatory effects on ChAT and VAChT protein

Western blotting was used to quantify the effects of charge counteraction, GAG heparan sulphate moiety cleavage or ERK inhibition in a time dependent manner (Figure 4 and 6).

Inhibitory studies using heparinase 1, PLG or PD98059 were performed at the 18 h time point for ChAT and both 24 and 48 time points for VAChT.

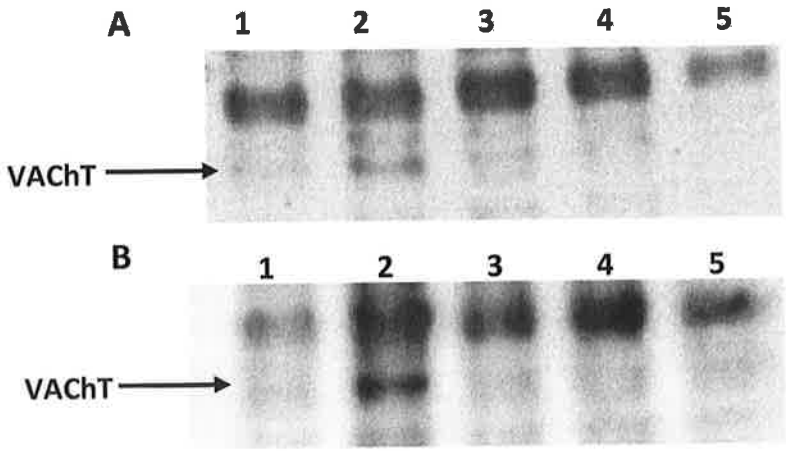
Results show that either heparinase 1 or PLG inhibited the up-regulatory effects of EPO on ChAT protein at 18 h (Figure 13) and VAChT protein at 24 h (Figure 14 A) and 48 h (Figure 14 B). Similarly, protein up regulation was also reversed by ERK inhibition for both ChAT (Figure 13) and VAChT (Figure 14 A and B).



EPO	–	+	+	+	+
Hep 1	–	–	+	–	–
PLG	–	–	–	+	–
PD98059	–	–	–	–	+

FIGURE 13: EPO induced ChAT protein up-regulation is inhibited by heparinase 1, PLG or ERK inhibition.

IMR32 cells were differentiated for 7 days then co-incubated with EPO (1μg/ml) for 18 h in the presence or absence of heparinase 1 (4u/ml), PLG (1μg/ml) or PD98059 (50μM)
Representative Western blots of IMR32 nuclear protein (10 μg) probed with anti-ChAT antibody. Heparinase 1, PLG and PD98059 all reverse the up-regulatory effects of EPO on ChAT protein. Three independent replicate experiments were conducted.



EPO	–	+	+	+	+
HEP 1	–	–	+	–	–
PLG	–	–	–	+	–
PD98059	–	–	–	–	+

FIGURE 14: EPO induced VAChT protein up-regulation is inhibited by heparinase 1, PLG or ERK inhibition.

IMR32 cells were differentiated for 7 days then co-incubated with EPO (1µg/ml) for 18 h in the presence or absence of heparinase 1 (4u/ml), PLG (1µg/ml) or PD98059 (50µM)
Representative Western blots of IMR32 nuclear protein (10 mg) probed with anti-VAChT antibody. Incubation time for the experiments is 24 h (A) and 48 h (B). Heparinase 1, PLG and PD98059 all reverse the up-regulatory effects of EPO on VAChT protein. Three independent replicate experiments were conducted.

3.3.5 EPO leads to nuclear factor κB activation

Previous published data have demonstrated MAP kinase and NFκB activation after incubation with whole eosinophils and eosinophil membrane preparations (Curran *et al.*, 2004, Walsh *et al.* 2004). The following experiments aimed to test the hypothesis that EPO as a representative ECP could independently up regulate NFκB expression in nerve cells as a prerequisite for up regulation of cholinergic gene expression. NFκB has been shown to be important for cholinergic gene expression (Durcan *et al.*, 2006).

IMR32 cells were incubated with EPO for 4, 12 and 24 h with an early time point of 10 min also included given that NFκB activation must precede that of ChAT temporally. Electrophoretic mobility shift assay (EMSA) showed that EPO induced activation of NF-κB in IMR-32 cells within 10 min of treatment (Figure 15).

This data of early unsustained ChAT up regulation and late sustained up regulation of VAChT allows for temporally coordinated production, packaging and exocytosis of Ach and the resulting *in vivo* consequences.

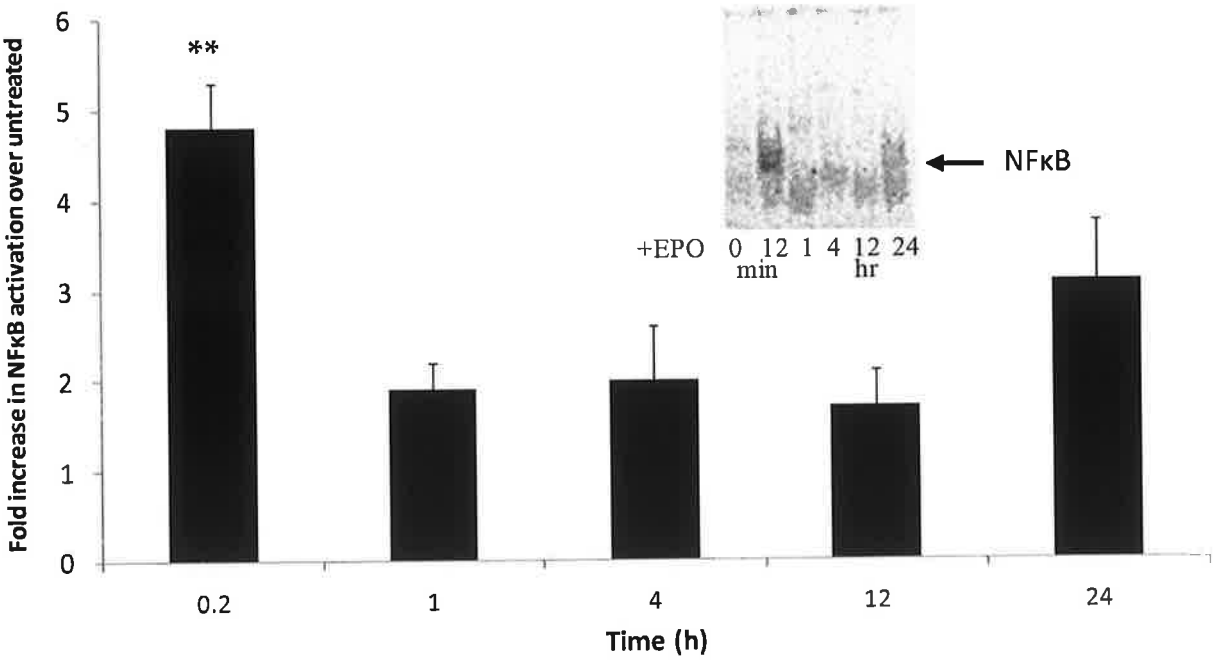


FIGURE 15: EPO induces NFκB activation in IMR32 cells

Representative EMSA and quantification of NF-κB in nuclear extract (10 μg) from IMR32 cells left untreated (0) or treated with 1 μg/ml of EPO for the indicated times. Fold increase shown in graph is based on a comparison of band intensities from EMSA gels of nuclear extract, calculated from the area under the curve of plots of pixel intensities generated using ImageQuant on the Storm 820 phosphoimaging system. Data are mean +/- SD from three separate experiments.
NFκB activation in response to incubation of IMR32 cells with EPO (1μg/ml) is shown in this figure to occur as early as 12 min with the transient effect subsiding by 1 h. A second rise in NFκB activation is noted at 24 hours. (n=3; mean +/- SEM; **p<0.01).

In summary, these quantitative and mechanistic studies clearly illustrate that, like whole eosinophils, EPO leads to up regulation of cholinergic genes. This is one of the causes of airway remodeling in asthma. The effects of EPO start with cell surface interaction at GAG rich moieties of the nerve cell membrane in a charge dependent manner. It seems that the charge of ECP's is necessary but not sufficient to instigate intracellular pathways leading to up regulation of gene expression. The intracellular events following surface interaction depend on MAP kinases cascade phosphorylation. This culminates in NFκB activation and cholinergic locus up-regulation.

CHAPTER 4

RESULTS PART 2

Imaging of EPO - IMR32 membrane interaction

4.1 INTERACTION BETWEEN FLUORESCENTLY LABELLED EPO AND IMR32 CELLS
4.2 MECHANISTIC STUDIES

Previous experiments have clearly implicated a role for interaction between eosinophil peroxidase and the IMR32 cell membrane in a charge dependent manner at GAG rich moieties, resulting in MAP kinase activation and hence cholinergic gene up regulation. Also whole Eosinophils tagged with MBP specific markers have been shown in close association with airway nerve cells in patients with fatal asthma attacks (Jacoby *et al.* 2001). The mechanisms of these interactions are discussed in the INTRODUCTION.

Given our experimental results, microscopic evidence was sought for the interaction of ECP's directly with the cell membrane. The time course of cell surface adhesion was hypothesized to occur early and a sustained manner given the time pattern of cholinergic gene up-regulation previously determined. Another purpose of these confocal experiments was to visualize the pattern of membrane-EPO interaction and look for evidence of internalization in to the nerve cells.

4.1 INTERACTION BETWEEN FLUORESCENTLY LABELLED EPO AND IMR32 CELLS

IMR32 cells were grown on gelatin coated chambered slides for 48 h then differentiated for 7 days. Cells were then incubated with the labeled EPO at the same concentration previously shown to up-regulated cholinergic genes and proteins (1µg/ml) (Figures 3-6). Identical time points to the genetic studies were also used (1, 4, 16 and 24 h). The slides were then prepared for confocal microscopic visualization.

The images obtained (Figure 16 A-D) showed that EPO interacts with the IMR32 cell surface as early as 1 h (A). The maximal interaction occurred at 4 h (B) with persistence up until 16 h (C), following which disengagement seems to occur in conjunction with evidence of cell damage and apoptosis (lifting of cells from the slides and cytoplasmic "blebbing") (D). This is likely the result of the high concentrations of EPO used, the toxic effect of FITC, as well as the length of time needed to grow, differentiate and expose the cells to EPO. The pattern of cell surface interaction is aggregated to specific areas of the cell surface surmised to coincide with GAG rich areas. Time dependent progressive background homogeny is seen to occur maximally at 16 h on a foreground of speckled surface interaction. This may indicate a degree of cellular internalization to the cytosoplasm (C).

4.2 MECHANISTIC STUDIES

As in previous experiments, inhibitor studies were undertaken using Heparinase 1 and Poly-L-Glutamate to corroborate the charge-GAG dependence of EPO interaction with IMR32 cells. The 16 h time point was chosen for the inhibitor studies with concurrent images confirming the 4 and 16 h optimal interaction (Figure 17 A, B respectively).

At 16 h, both Heparinase 1 and PLG each inhibit IMR32 cell membrane and EPO interaction. This is further evidence that charge is necessary to the physiological effects of ECP's on nerve cells, although insufficient in itself to instigate genetic up regulatory effects.

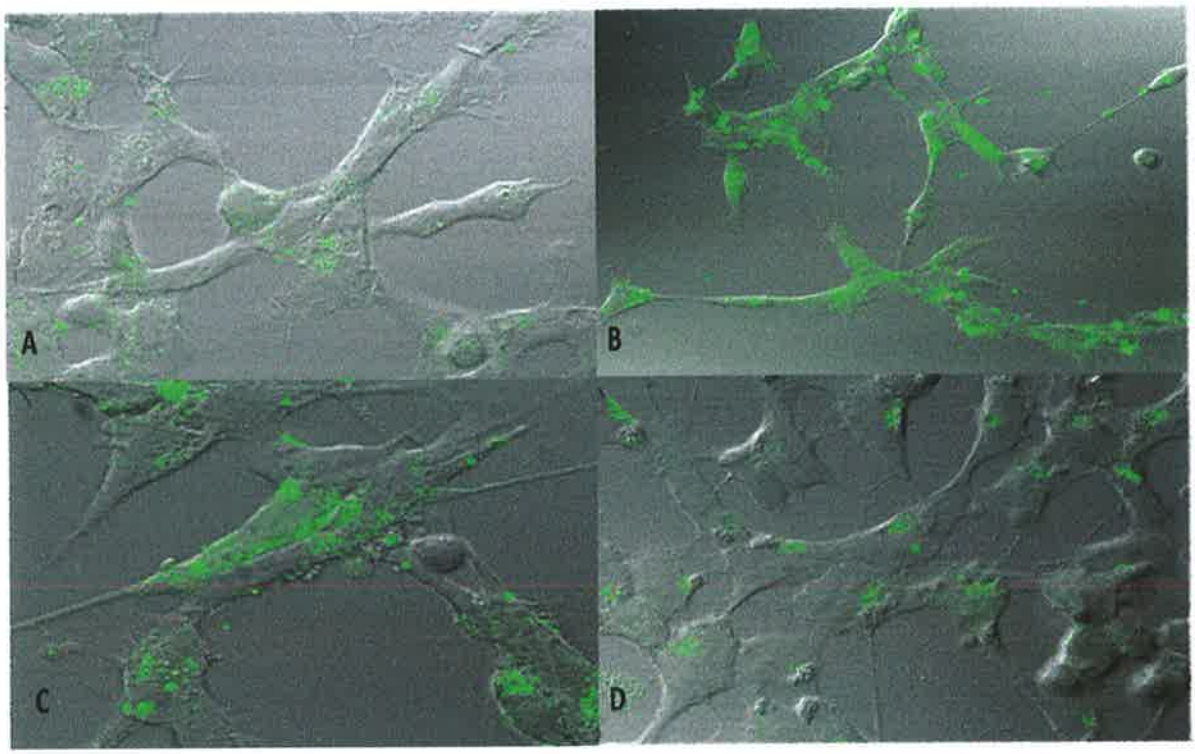


FIGURE 16: FITC labeled EPO interacts with IMR32 cells

IMR32 cells incubated with FITC labeled EPO (1µg/ml). Incubation times of 1h(A), 4h (B), 16h (C) and 24h (D). There is clear evidence of early interaction with the cell membrane as early as 1 h. Maximal association is seen to occur at 4 h and 16 h. At 24 h there is less cell surface signal with microscopic evidence of cellular stress and apoptosis.

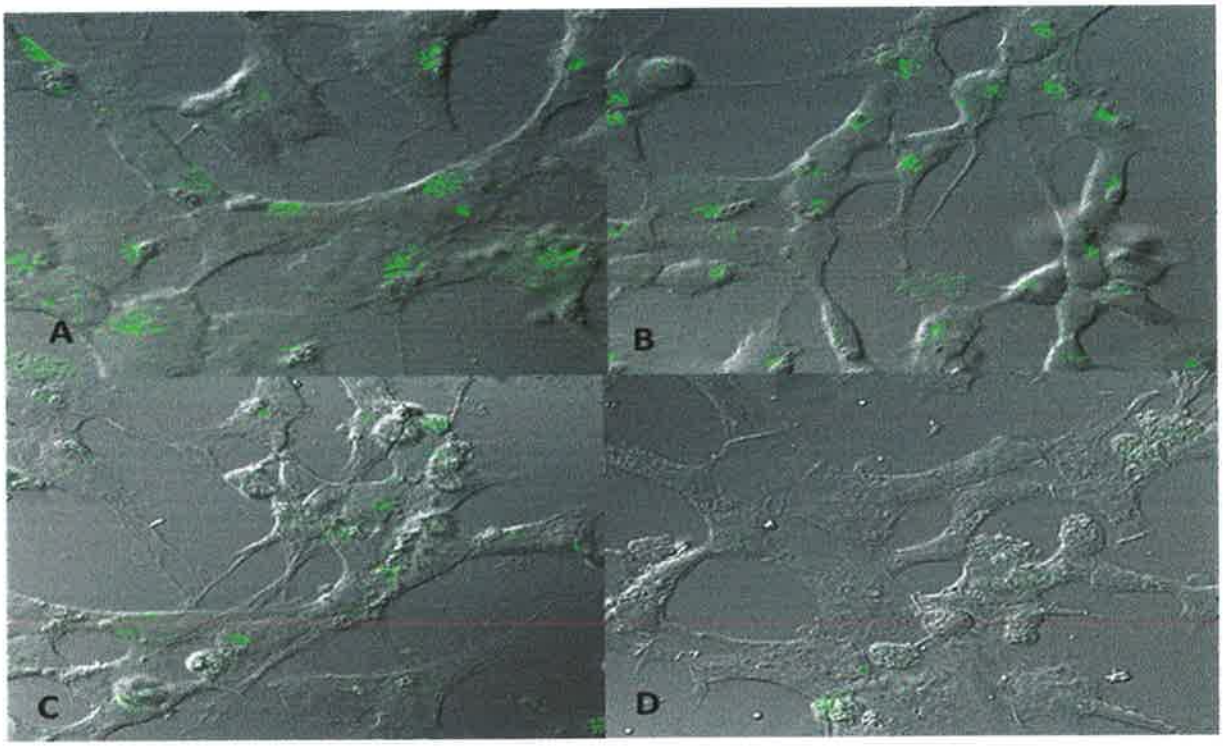


FIGURE 17: Heparinase 1 and PLG prevent EPO interaction with IMR32 cells

Optimum time points for incubation of IMR32 cells with EPO alone are established at of 4 and 16 h and are shown in (A) and (B) respectively to standardize the subsequent inhibitor images which were both performed at the 4 h time point.
IMR32 cells were incubated with EPO in the presence of heparinase 1 (4U/ml) (C) and in the presence of Poly-L-Glutamate (1µg/ml) (D) both for 4 h. Clear evidence of the interaction inhibitory effects of both GAG heparan sulphate moiety cleavage (heparinase 1) and charge counteraction (PLG) are seen.

CHAPTER 5

GENERAL DISCUSSION

5.1 OVERVIEW

5.2 EOSINOPHIL PEROXIDASE UPREGULATES CHOLINERGIC GENES

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5.6 METHODOLOGICAL PROBLEMS ENCOUNTERED

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5.1 OVERVIEW

This research has focused on the cholinergic phenotype conferred on airway nerves in response to the cationic granule proteins released from eosinophils upon activation. The results show that EPO up-regulates the expression of ChAT and VAChT; which may in turn increase the expression of Ach by airway nerves. Furthermore, the results indicate that the mechanism of induction of these proteins is mediated by adhesion of EPO to the nerve cell membrane via a cationic interaction with an as yet unidentified glycosaminoglycan and that this in turn leads to intracellular signaling via the ERK1/2 pathway. Finally, the studies presented in this thesis indicate that the activation of ERK1/2 mediates the regulation of ChAT and VAChT. These studies indicate a novel action of EPO and identify critical aspects of the mechanism of action of EPO.

5.2 EOSINOPHIL PEROXIDASE UPREGULATES CHOLINERGIC GENES

This project investigated the effects of EPO, MBP and EDN at a concentration of 1µg/ml. This concentration was previously shown to be non toxic to nerve and epithelial cells (Morgan *et al.* 2005, Pegorier *at al.* 2006). The effects on the expression of cholinergic gene locus components ChAT and VAChT in IMR32 neuroblastoma cells was examined. IMR32 cells were chosen as experimental models because they synthesize, store and release Ach in response to the normal physiological signals that regulate nerve function (Kingham *et al.* 2003) meaning that they are useful models of parasympathetic nerves in vitro.

The results of this study have demonstrated that co-incubation of IMR32 cells with

EPO lead to a time dependent up-regulation of ChAT and VAChT mRNA and protein. In contrast to the clear effects of EPO MBP only up-regulated VAChT expression at a late time point and EDN showed no up-regulatory effects on either gene.

EPO caused an up-regulation in the expression of ChAT mRNA by 1 h of co-incubation, an effect not detectable any earlier time point. Also, the effect was transient with a return of mRNA levels to normal by 4 h of co-incubation and remained at baseline. It must be noted that a statistically significant paradoxical down-regulation of ChAT RNA is actually seen at 4 h. This is quite significant given that the statistics were based on 7 separate experiments. The cause for this has yet to be determined but is likely related to the way both cholinergic genes are oriented spacially within the cholinergic gene locus on chromosome 10. The *VAChT* and *ChAT* genes form a phylogenetically conserved "cholinergic locus" in which the former gene is nested within the first intron of the latter (Kitamoto *et al.* 1998). This unusual genomic arrangement of these two separate but related genetic functions may contribute to the coordinated regulation of both genes in cholinergic neurons. Work conducted by Blusztajn and Berse using cell culture systems have indicated coordinate regulatory properties for *VAChT* and *ChAT* expression (Berse *et al.* 1995). Alfonso, Rand and others proposed alternative splicing of a common precursor to be responsible for producing two distinct mRNAs from the cholinergic locus in *C. elegans* (Rand *et al.* 2000, Alfonso *et al.* 1993). In vertebrates, however, it seems that ChAT and VAChT specific mRNAs result from a combination of differential promoter usage and/or alternative RNA processing. Blusztajn and others, using Northern analysis, studied the modulation of ChAT and VAChT expression in a murine septal cell line (SN56) by cAMP, retinoids and other

compounds. All these agents increased the ACh level in the cells up to 2.5-fold. Dibutyryl cAMP had a greater effect on the level of VAChT mRNA (4-fold induction) than on the level of ChAT mRNA (2-fold induction), suggesting a quantitatively differential transcriptional regulation of the two genes by the cAMP pathway (Berse *et al.* 1995). The effects of the three groups of agents studied on ChAT and VAChT mRNA levels were additive, pointing to several independent mechanisms by which the cholinergic properties of septal neurons can be modulated (Berse *et al.* 1995).

EPO is the only one of the cationic protein to show up-regulatory effects on both cholinergic genes. MBP induced a late increase in VAChT mRNA production and there was no effect of MBP on ChAT and, as mentioned above, EDN had no effect on either gene. These differences in the time points at which the different proteins exert an effect is interesting because none of them individually correspond to the effects that whole eosinophils have on the cholinergic gene locus. Durcan and colleagues in 2006 demonstrated that whole eosinophils up-regulate ChAT RNA to five fold baseline levels at 24 h, while VAChT levels increase as early as 1 h after co-incubation (Durcan *et al.* 2006). These differences may be due to the fact that whole eosinophils were also seen to secrete other factors including NGF and BNF. This emphasizes that the effects *in vivo* of eosinophils are likely to be complicated with multiple interactions occurring between the various factors produced by these cells.

It is noted that EDN, instead of up-regulating cholinergic genes tended to cause the opposite effect at numerous time points. This actually reached statistical significance at the ChAT 16 h time point. This down regulation of cholinergic genes may be explained by the ribonuclease effect of EDN on mRNA.

H. Rosenberg and colleagues have described the unusual manner in which this ribonuclease lineage has evolved, with diversification observed in primate as well as in rodent EDNs and ECPs. The studies demonstrated the direct, ribonuclease-dependent, reduction exerted by eosinophils on the infectivity of RSV in vitro. EDN was shown to function alone as an independent antiviral agent (Rosenburg *et al.* 1999).

Earlier studies by Gleich, Slifman and others demonstrated the RNase effects of EDN and to a lesser extent ECP by spectrophotometric assays of acid soluble nucleotides formed from yeast RNA. Purified EDN showed RNase activity similar to bovine pancreatic RNase, whereas ECP was 50 to 100 times less active (Slifman *et al.* 1986). Howard, Yang and colleagues have also shown that EDN, and to a lesser extent human pancreatic RNase (hPR), activates human dendritic cells (DCs), leading to the production of a variety of inflammatory cytokines, chemokines, growth factors, and soluble receptors. EDN production by human macrophages was also demonstrated to be induced by pro-inflammatory stimuli, indicating that EDN is a likely participant of inflammatory and immune responses (Yang *et al.* 2004).

The *in vivo* cumulative effect of all three proteins remains to be studied. MBP acts by surface charge alteration in cell permeability and cell membrane injury and EPO exerts its effects via oxidative stress induced apoptotic stimuli. Studies involving cumulative eosinophil granule protein incubations would shed more light on this issue to assess approximation to the effects of whole eosinophils.

Given the dual effects of EPO on the up-regulation of both cholinergic genes at different time point profiles, it was chosen for subsequent analysis from the

mechanistic point of view as a representative eosinophil protein. Obviously some variation in the mechanisms of initiating intracellular cascades may well exist between the different proteins. This is particularly true for MBP which is smaller and more densely cationically charged than EPO.

5.3 INTRACELLULAR CASCADES

Prior studies in our laboratory have shown the ECP's cause activation of nuclear transcription factors namely NFκB and AP-1 which directly lead to the up-regulation of genes including receptor genes (Durcan *et al.* 2006), anti apoptotic genes (Morgan *et al.* 2005), and cholinergic genes. The events leading up to nuclear factor activation is via MAP kinases such as ERK1/2 and p38 (Walsh *et al.* 2003). MAP Kinases are dual-specificity protein kinases that function in a mitogen activated protein kinase cascade controlling cell growth and differentiation. Activation of MEK1 and MEK2 occurs through phosphorylation of two serine residues at positions 217 and 221 (in the activation loop of subdomain VIII) by Raf-like molecules. MEK1/2 is activated by a wide variety of growth factors and cytokines and also by membrane depolarization and calcium influx. MEK activates p44 and p42 MAP kinase by phosphorylating both threonine and tyrosine residues at sites located within the activation loop of kinase subdomain VIII (Crews *et al.* 1992). All of these effects are adhesion dependent. Eosinophils express the integrin adhesion molecules CD11/18 complex and very late antigen-4 (VLA-4), which interact with intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), respectively on nerve cells (Sawatzky *et al.* 2002). Inhibition of eosinophil binding via ICAM-1 inhibited NF-κB activation while inhibition of

eosinophil binding via VCAM-1 had no significant effect on NF-κB. In contrast, eosinophil adhesion to VCAM-1 led to activation of AP-1. These effects on NF-κB and AP-1 in IMR32 cells can be induced by incubating IMR32 cells with eosinophil membranes alone, and inhibited by specific chemical inhibitors of adhesion (Walsh *et al.* 2003). Hence adhesion to nerves is one mechanism of activation of the MEK/ERK pathway.

Similar cell signalling was hypothesized to yield phenotypic effects in response to eosinophil granule proteins. Morgan and colleagues have indeed demonstrated both MBP1 and EDN activate NFκB (Morgan *et al.* 2005). However there are differences in time course and intracellular pathways patterns activated by the two proteins. MBP1 induced a prolonged activation of NF-κB up to over 12 h, a process dependent on both MAP kinases ERK and Akt in the early phases and ERK alone later. EDN on the other hand induced only a very short lived NF-κB activation lasting less than 1 h independent of either ERK 1/2 or Akt (Morgan *et al.* 2005). EPO had not been studied previously and so the events following EPO incubation were assessed in this work.

Our results showed firstly that EPO caused statistically significant activation of NFκB. This effect was detectable as early as 10 min and sustained up to 24 h. This talies with the up-regulatory effects of EPO on both cholinergic genes over different time frames. EPO has also been shown to concomitantly cause degradation of IK-B in a manner similar to whole eosinophils and eosinophil membranes previously demonstrated (Durcan *et al.* 2006). With regards to the aforementioned intracellular cascades leading up to NFκB activation, we employed ERK inhibition

using PD98059 which has been shown to act *in vivo* as a highly selective inhibitor of MEK1 activation and the MAP kinase cascade. PD98059 binds to the inactive forms of MEK1 and prevents activation by upstream activators such as c-Raf.

ERK inhibition was found to cause complete reversal of the up-regulatory effects of EPO on both ChAT and VAChT indicating an ERK dependent pathway at all time points. Western blots of ChAT and VAChT proteins also showed reversal of the EPO effects in response to ERK inhibition. These results illustrate the role of ERK dependent pathways culminating in NFκB activation in the up-regulation of cholinergic genes. We next investigated how ERK may be activated by EPO.

5.4 CELL SURFACE INTERACTION

While the cell surface interaction mechanisms of eosinophils and eosinophil membranes with nerve cells are well established and outlined above, how the eosinophil granule proteins initiate their intracellular cascades at the cell surface necessitated further studies. There are no known receptors for these proteins on the cell surface. It was hypothesised that this interaction either resulted from cationic charge interaction or at highly sulphated moieties of glycosaminoglycans. Pegorier, Pretolani and colleagues demonstrated the remodulatory effects of eosinophil granule proteins MBP and EPO on airway epithelial cells. They determined that these proteins augmented transcription of endothelin-1, TGF-α, TGF-β, PDGF, MMP-9, fibronectin and tenascin while down-regulating transcription of MMP-1. The effects of these proteins were only partially mimicked by the

membrane regions but does alter their morphology. Heparan sulphate GAG's are similar in structure to heparin, however the disaccharide units are organized into distinct sulfated and non-sulfated domains. Heparan sulfate occurs as a proteoglycan (PG) in which two or three HS chains are attached in close proximity to cell surface or extracellular matrix proteins. It is in this form that HS is known to bind to a variety of protein ligands and regulates a wide variety of biological activities, including developmental processes, angiogenesis, blood coagulation and tumour metastasis (Hacker *et al.* 2005).

Thus, it seems that eosinophil granule proteins interact with GAG's in a biphasic way, initially by charge then at a structural level to instigate MAP kinase activation and subsequent gene up-regulation in an NFκB dependent manner. To test this hypothesis further, imaging experiments were performed to demonstrate fluorescently labeled granule protein interaction with cell membranes.

5.4.4 Confocal microscopy

Direct visualization of the above described interaction between EPO and nerve cells was achieved by confocal microscopy. As described in the METHODS section, IMR32 cells were incubated with fluorescently labeled EPO for time points ranging between 1 and 24 h. Cellular localization of EPO is seen very clearly as early as 1 h, with maximal interaction at 4 h. By 24 h, a degree of disengagement seems to occur associated with residual apoptotic features left behind on the IMR32 cells. There is clear evidence of fluorescent clustering on the cells at all time points. This suggests preferential interaction with certain areas of membrane. It is likely that these represent GAG rich areas. Evidence of internalization of fluorescence is evident at

later time points with an acquired background homogeneity of fluorescence underlying the speckled cell surface protein. Interestingly charge counteraction with PLG almost completely reversed the interaction corroborating the hypothesis of essential charge related "priming" preceding biologically active morphological association between EPO and GAG's on the cell surface. Heparinase 1 partially blocks the interaction but not completely. This suggests that the initial charge interaction is further augmented by a secondary GAG interaction leading to structural membrane changes with initiation of intracellular events. It is possible that intracellular cytoskeletal elements play a role in this process.

In conclusion, EPO, chosen as a representative ECP has been demonstrated to up-regulate the expression of the cholinergic gene locus in vitro. Like whole eosinophils, EPO achieves its genetic effects by instigating intracellular ERK dependent pathways leading to early activation of NFκB and subsequently ChAT and VAChT. MBP also up-regulates VAChT while EDN seems to play a modulatory role likely related to its ribonuclease properties which merits further study in cumulative ECP effects on nerve cells. EPO exerts its effects by initially interacting with the cell membrane at GAG rich areas. The interaction seems to be biphasic with an initial charge related potentiation followed by a structure altering allosteric effect leading to a translation into intracellular signalling. Charge is hence essential but insufficient to account for the effects of EPO on cholinergic gene up-regulation.

It seems that with regards to airway cholinergicity and remodeling, ECP's play a propagatory role to the whole eosinophil, the interactive mechanisms of which are well established.

5.5 CLINICAL IMPLICATIONS

This work highlights the importance of eosinophil granule proteins in up-regulating the expression of the cholinergic gene locus of airway nerve cells. This process is known to occur in response to whole eosinophils (Sawatzky *et al.* 2003, Durcan *et al.* 2006).

The clinical implications of this cholinergic up-regulation are notable in both the acute and chronic phases of asthma. From an airway pathohysiological perspective, the increased actions of acetycholine on M3 receptors increase smooth muscle contraction, mucus secretion, airway hyperresponsiveness and airway remodelling. Actions ECP's on M2 receptor dysfunction also blunt cholinergic negative feedback responses (Fryer *et al.* 1992, Evans *et al.* 1997, Walsh *et al.* 2003).

Inhaled anticholinergics have been shown in clinical trials to reduce hospital admission rates and improve pulmonary function to a greater extent than a short-acting beta agonist alone. The positive effects of ipratropium are seen in both adults and children with moderate and severe airflow obstruction during exacerbations (Rodrigo *et al.* 2000).

Corticosteroids are the most common agents used in clinical practice to treat exacerbations of asthma and COPD. They markedly reduce eosinophilia by increasing apoptosis rates and inhibit cytokine mediated survival pathways (Rothenberg 1998, Schleimer and Bochner 1994). Glucocorticoids also inhibit cytokine and chemokine production by leukocytes, such as T-cells by down-regulating expression of inflammatory mediator genes such as IL-3, IL-4, IL-5, GM-CSF and the expression of chemokines such eotaxin. They have also been shown to

destabilize eosinophil cytokine mRNA thereby reducing their half-life (Stellato *et al.* 1999).

Glucocorticoids exert their effects by influencing target cell transcriptional regulation and protein synthesis. Upon entering the cell, they bind to a cytoplasmic glucocorticoid receptor. The hormone-receptor complex then translocates to the nucleus, where it binds to glucocorticoid-response elements and modulates transcription of target genes.

A study by Auphan and colleagues has clearly demonstrated inhibition of NFκB activation in mice and cultured cells by corticosteroids. This inhibition was mediated by induction of the IκB inhibitory protein (Auphan *et al.* 1995). This is of particular interest as it implies downregulation of cholinergic gene locus expression in response to systemic steroid therapy. Further studies assessing the effects of corticosteroids on ChAT and VAChT expression and Ach release in nerve cells would be of value.

5.6 METHODOLOGICAL PROBLEMS ENCOUNTERED

Methodological difficulty associated with the use of IMR32 cell line was encountered during the preparation of slides for confocal microscopy. Cells were poorly adherent to glass slide surfaces and cells detached from slide surfaces during PBS washing phases of experiments. To overcome this problem, slide surfaces were coated with 1% sterile gelatine and allowed to dry. This greatly improved the cellular adherence and facilitated the multiple washes necessary to assure minimisation of background fluorescence.

5.7 AVENUES FOR FUTURE RESEARCH

Areas of interest for future research based on the current work may include the following:

- The effect of EPO on nerve cell survival and its regulatory effect on Bfl-1 gene expression.
- The cumulative effect of all three eosinophil granule proteins (MBP, EDN and EPO) on cholinergic gene expression and the quantification of acetylcholine production.
- Co-localization studies using confocal fluorescent microscopy with DAPI nuclear staining to further clarify the degree of fluorescently labelled eosinophil granule protein cellular internalization and nuclear interactions.

Appendix 1

EXP	ChAT untx	Bactin untx	ratio untx	ChAT 1h	Bactin 1h	ratio 1h	FOLD 1h	ChAT 4h	Bactin 4h	ratio 4h	FOLD 4h	ChAT 16h	Bactin 16h	ratio 16h	FOLD 16h	ChAT 24h	Bactin 24h	ratio 24h	FOLD 24h
1	4082	262500	0.015	5361	223400	0.024	1.600	799	99900	0.008	0.530	1478	100600	0.015	0.980	3411	340800	0.010	0.660
2	2622	175600	0.015	10843	314300	0.035	2.300	597	180900	0.003	0.650	1674	167200	0.010	0.660	4489	213800	0.021	1.400
3	6555	278800	0.024	8597	223900	0.038	1.600	2580	122300	0.021	0.880	4917	200700	0.025	1.020	4926	311800	0.016	0.660
4	4543	234500	0.019	11890	223500	0.053	2.800	2363	200300	0.012	0.620	4950	232400	0.021	1.120	3733	200700	0.019	0.980
5	1338	198700	0.007	1644	167800	0.010	1.400	493	123300	0.004	0.620	1977	290800	0.007	0.970	1398	199800	0.007	1.020
6	4554	365800	0.013	5318	98850	0.054	4.300	552	78850	0.007	0.580	876	230500	0.004	0.300	917	145500	0.006	0.500
7	2322	189600	0.012	2650	166700	0.016	1.300	3433	254300	0.014	1.360	1980	210700	0.009	0.770	2917	199800	0.015	1.200
AVG							2.186				0.647				0.831				0.917
SEM							0.410				0.110				0.110				0.110
p							0.027				0.001				0.164				0.525

Appendix 2

Exp	VAChT untx	Bactin untx	ratio	VAChT 1h	Bactin 1h	Ratio 1h	FOLD 1h	VAChT 4h	Bactin 4h	ratio 4h	FOLD 4h	VAChT 16h	Bactin 16h	ratio 16h	FOLD 16h	VAChT 24h	Bactin 24h	ratio 24h	FOLD 24h
1	113.8	200800	0.0006	123.8	209800	0.0006	**1.030**	212.8	213000	0.0010	**2.000**	77.1	88950	0.0009	**1.500**	323.3	230900	0.0014	**2.540**
2	70.8	77750	0.0009	399.1	231600	0.0017	**1.880**	144.2	110900	0.0013	**1.440**	294.1	245100	0.0012	**1.230**	179.7	112300	0.0016	**1.720**
3	321.5	176900	0.0018	589.6	112300	0.0052	**2.880**	1885.1	299500	0.0063	**3.430**	938.5	312900	0.0030	**1.670**	206.5	129000	0.0016	**0.880**
4	211.6	234000	0.0009	99.1	98950	0.0010	**1.120**	376.1	313200	0.0012	**1.320**	277.3	298200	0.0009	**1.032**	173.8	289700	0.0006	**0.650**
5	234.5	289800	0.0008	59.8	77650	0.0008	**0.970**	234.1	329600	0.0007	**0.890**	191.3	233300	0.0008	**1.044**	453.4	266700	0.0017	**2.130**
6	115.6	145400	0.0008	288.2	315800	0.0009	**1.140**	493.1	394500	0.0013	**1.550**	187.2	98550	0.0019	**2.430**	86.9	87650	0.0010	**1.320**
7	288.0	330900	0.0009	320.8	299800	0.0011	**1.230**	319.2	414500	0.0008	**0.880**	310.7	172000	0.0018	**2.130**	114.8	67550	0.0017	**1.990**
8	235.2	314200	0.0008	75.9	190800	0.0004	**0.520**	241.8	219800	0.0011	**1.450**	139.2	188100	0.0007	**0.990**	178.6	231900	0.0008	**1.040**
9	97.7	180500	0.0005	24.9	658250	0.0004	**0.660**	141.2	117700	0.0012	**2.210**	105.5	188700	0.0006	**1.044**	135.5	112900	0.0012	**2.200**
AVG							**1.270**				**1.686**				**1.452**				**1.608**
SEM							**0.230**				**0.270**				**0.170**				**0.230**
p							**0.274**				**0.017**				**0.021**				**0.014**

Appendix 3a

EXP	Chat untx	Bactin untx	Ratio	ChAT 1h	Bactin 1h	Ratio 1h	Fold 1h	ChAT 4h	Bactin 4h	Ratio 4h	Fold 4h	ChAT 16h	Bactin 16h	Ratio 16h	Fold 16h	ChAT 24h	Bactin 24h	Ratio 24h	Fold 24h
1	2380	180500	0.013	1589	175600	0.009	0.687	2290	250600	0.009	0.694	1816	275600	0.007	0.500	1748	140500	0.012	0.944
2	3776	245500	0.015	3204	320500	0.010	0.650	4504	180900	0.025	1.620	3662	245500	0.015	0.970	3622	210300	0.017	1.120
3	1455	145000	0.010	1359	210400	0.006	0.644	1911	176400	0.011	1.080	2396	330200	0.007	0.558	2746	230100	0.012	1.190
AVG							0.660				1.131				0.676				1.085
SEM							0.016				0.332				0.183				0.091
p							0.002				0.673				0.160				0.367

Appendix 3b

EXP	VAChT untx	Bactin untx	Ratio	VAChT 1h	Bactin 1h	Ratio 1h	Fold 1h	VAChT 4h	Bactin 4h	Ratio 4h	Fold 4h	VAChT 16h	Bactin 16h	Ratio 16h	Fold 16h	VAChT 24h	Bactin 24h	Ratio 24h	Fold 24h
1	100.6	142500	0.00071	128.5	321300	0.00040	0.57	90.3	180600	0.00050	0.706	49.7	110500	0.00045	0.627	86.5	120200	0.00072	1.01
2	210.4	120300	0.00175	303.6	221600	0.00137	0.785	232.7	98600	0.00236	1.348	1296.8	280700	0.00462	2.64	128.1	145600	0.00088	0.5
3	252.7	245100	0.00103	97.7	207900	0.00047	0.457	59.7	110500	0.00054	0.525	113.6	231900	0.00049	0.479	198.5	135000	0.00147	1.428
AVG							0.604				0.860				1.249				0.979
SEM							0.096				0.250				0.697				0.268
p							0.054				0.631				0.755				0.946

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