ENDOGENOUS NITRIC OXIDE PRODUCTION IN

AIRWAYS OF PATIENTS WITH

CYSTIC FIBROSIS

A thesis submitted to the University of Glasgow

for the degree of Doctor of Medicine

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DECLARATION

I declare that the work has been done and the thesis composed by myself, and that the books and papers cited were all consulted by me personally unless it is otherwise stated.

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SUMMARY

Nitric oxide (NO) is a highly reactive molecule with physiological and pathological roles in the airways. This thesis first investigated the production of NO in the airways of cystic fibrosis patients compared to normal and asthmatic subjects after validating a method to measure this which excluded known confounding factors. The first finding was that exhaled NO, against expectation were not increased in cystic fibrosis patients, even during infective exacerbations. I hypothesised that this could be due to lack of detection of NO (secondary to removal of NO by reaction with other reactive molecules and/or impedance of diffusion of this gaseous molecule into the airway) or lack of production (secondary to decreased expression of nitric oxide synthase II (NOS II), the gene thought to be responsible for NO production within an inflammatory setting). The thesis thus explored these possibilities by investigating a) exhaled NO levels in patients with bronchiectasis, a group of patients with similar suppurative airway disease to cystic fibrosis b) breath condensate levels of hydrogen peroxide, another highly reactive volatile molecule in cystic fibrosis patients compared to normal subjects c) expression of NOS II in cystic fibrosis and normal primary epithelial cells and epithelial cell lines at baseline and in response to proinflammatory stimuli and d) nitrite levels in breath condensate of CF patients. In order to perform the above, methods for measuring hydrogen peroxide and nitrite in breath condensate were developed. The correlation between the levels of

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these molecules and lung function and circulating leucocytes were also determined.

The thesis found that like exhaled gaseous NO, hydrogen peroxide levels were not elevated in exhaled air of patients with CF. However nitrite levels were increased and this correlated with the levels of circulating leucocytes. The expression of NOS II were normal in primary cystic fibrosis epithelial cells and epithelial cell lines in vitro. Therefore, it is concluded that exhaled NO is not helpful as a marker of airways inflammation in chronic suppurative airway diseases of cystic fibrosis and bronchiectasis. This is likely to be due to lack of detection rather than production of NO from the epithelium. Breath condensate nitrite levels may be a more useful tool for measuring airways inflammation in these conditions and further developments in this area are suggested.

ABBREVIATION

AAV	Adeno-associated virus
AP1	activator protein 1
AP2	activator protein 2
ARDS	adult respiratory distress syndrome
BAL	bronchoalveolar lavage
Bcl-2	B cell lymphoma-2
cAMP	adenosine 3', 5' - cyclic monophosphate
CD	cluster of differentiation
CF	cystic fibrosis
CFTE	cystic fibrosis tracheal epithelium
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	guanosine 3', 5' - cyclic monophosphate
COPD	chronic obstructive pulmonary disease
COX	cyclo-oxygenase
CREB/ATF	cAMP response element binding protein/activating transcription factor
cytP450	cytochrome p450
DNA	deoxyribonucleic acid
DEPC	diethyl pyrocarbonate
EDRF	endothelium derived relaxing factor
EGTA	ethyleneglycol-bis (β-aminoethylether)- N, N, N', N'- tetraacetic acid
ERK	extra cellular signal regulated kinase
Emm	emission
Exc	excitation
FAD	flavin adenine dinucleotide
FEV_1	forced expiratory volume in first second
FMN	flavin mononucleotide

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γ-IRE	interferon gamma response element
GTP	guanosine triphosphate
HBE	human bronchial epitelium
HRP	horse radish peroxidase
Hsp	heat shock protein
IFN	interferon
IL	interleukin
JAK	janus kinase
kb	kilo base
kDa	kilo Dalton
LED	lithium emission display
L-NMMA	N^G-monomethyl-L-arginine
LPS	lipopolysaccharide
LTD4	leukotriene D4
MAPK	mitogen activated protein kinase
Mcg	microgram
MEM	minimum essential medium
MMP	matrix metalloproteinase
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide
NANC	non adrenergic non cholinergic
NaOH	sodium hydroxide
NF-1	nuclear factor 1
NF-kβ	nuclear factor kβ
NHA	N-γ-hydroxyl-l-arginine
NO	nitric oxide
NOS	nitric oxide synthase
NOx	nitric oxide species
PCR	polymerase chain reaction
PGF2α	prostaglandin F2α

PHA	phytohaemagglutinin
PHAA	p-hydroxyphenyl acetic acid
RNA	ribonucleic acid
ROS	reactive oxygen species
RS-NO	s-nitrosothiol adducts
RNH-NO	nitrosoamine
rt-PCR	reverse transcription polymerase chain reaction
SAPK/JNK	stress activated protein kinase/c-Jun N-terminal kinase
SD	standard deviation
SH	sulphydryl
Sp1	SV40 virus promoter specific transcription protein 1
STAT	signal transducer and activator of transcription
TB	tuberculosis
TGF-β	transforming growth factor β
Th	T helper
TNF	tumour necrosis factor
TNF-RE	tumour necrosis factor response element
UV	ultraviolet
VEGF	vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1 ENDOGENOUS NITRIC OXIDE AND ITS DISCOVERY

The first indication that the body produces nitrate endogenously came in 1916 when dietary balance studies showed that more nitrate was excreted than was ingested (Mitchell HH, 1916). This excess was thought to be produced by microorganisms and it was not until Tannenbaum and his colleagues proved that nitrate was biosynthesised mainly outside the intestine (Green LC, 1981) that interest in this area was renewed. Urinary nitrate levels in rats could be increased ten fold with injection of bacterial toxins and immunostimulated macrophages were found to produce high amounts of nitrite and nitrate (Ivengar R, 1987).

In 1980, Furchgott and Zawadzki showed that the endothelium was essential for the vasodilator action of acetylcholine in isolated arterial strips (Furchgott RF, 1980). Removal of the endothelium prevented the relaxant effect of acetyl choline and even led to contraction of the artery while stimulation of the endothelial cells caused the release of a substance, which Furchgott named endothelium derived relaxing factor (EDRF). Many other endogenous vasoactive substances including bradykinin, histamine, adenine nucleotides, thrombin, substance P and 5-hydroxytryptamine were found to act through the release of EDRF (Furchgott RF, 1989. Ignarro LJ, 1989. Moncada S, 1993).

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The chemical nature of EDRF was not discovered until 1987 when Furchgott and Ignarro independently pointed out that EDRF and NO were very similar in their biological properties. Soon afterwards, Moncada and colleagues compared the release and bioactivity of NO and EDRF in endothelial cell culture and found that EDRF was indistinguishable from NO (Palmer RMJ, 1987). Since then there has been an explosion of research on NO and its effects. It has been difficult to believe that such a simple molecule should turn out to affect almost every function in the body and have such far reaching consequences. In 1992, *Science* picked NO as the 'Molecule of the Year' and in 1998, Furchgott, Ignarro and Murad (but not Moncada, perhaps unjustly) were awarded the Nobel prize in Physiology and Medicine for their contribution to its discovery.

1.2 THE CHEMISTRY OF NITRIC OXIDE

Nitrogen oxides of biological relevance include elemental nitrogen in five oxidation states (+1 to +5) (table 1). Nitroxyl anion (NO^-) and the product of its protonation, dimerization and dehydration, N_2O, contains nitrogen in the +1 oxidation state. The nitric oxide free radical (NO) contains nitrogen in the +2 oxidation state. There are four important oxides in the +3 states: the weak base, nitrite (NO_2^-) and the corresponding acid, nitrous acid or HNO_2; the nitrosonium cation (NO^+), dinitrogen trioxide (N_2O_3) and the peroxynitrite anion ($ONOO^-$). Nitrogen dioxide radical ($NO_2.$) and its dimer, dinitrogen tetroxide (N_2O_4), with which it is in equilibrium, contain nitrogen in the +4 oxidation state. Finally, in nitrate (NO^{3-}), its corresponding acid (HNO_3, nitric acid), and dinitrogen pentoxide (N_2O_5), nitrogen is in the +5 oxidation state.

In part as a consequence of the multiple oxidation states available for nitrogen, NO does not to persist as the NO moiety for any length of time in biological settings. Rather it is oxidised, reduced or complexed with other biomolecules depending on the conditions that prevail in its microenvironment. These alterations in the molecular form of NO have three distinct effects; firstly they can modify the reactivity of NO so that it can be shuttled from its site of production to its site of effect. Secondly, local reactions may enhance or inhibit the toxicity of NO, and thirdly, as a consequence of the reaction of NO with certain carrier

Redox reactions in the presence of oxygen

$2NO^· + O_2 \rightleftharpoons 2NO_2^·$

$2NO_2^· \rightleftharpoons N_2O_4$

$N_2O_4 + H_2O \rightarrow HNO_2 + HNO_3$

$HNO_3 \rightleftharpoons NO_2^- + H^+$

$HNO_3 \rightleftharpoons NO_3^- + H^-$

$NO^· + NO_2^· \rightleftharpoons N_2O_3$

$N_2O_3 + H_2O \rightarrow 2HNO_2$

$HNO_2 \rightleftharpoons NO_2^- + H^+$
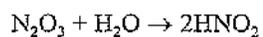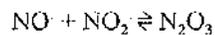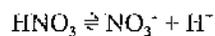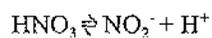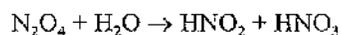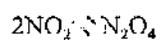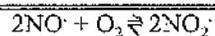
Interconversion among the NO redox array in the presence of transition metals

$NO^· + XFe^x \rightleftharpoons [X\text{-}Fe^x\text{-}NO.] \rightleftharpoons XFe^{x-1}\text{-}NO^+$

$NO^· + YFe^x \rightleftharpoons [Y\text{-}Fe^x\text{-}NO.] \rightleftharpoons YFe^{x+1}\text{-}NO^-$
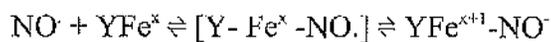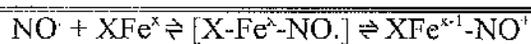
Reaction of NO with superoxide

$NO^· + O_2^{·-} \rightleftharpoons OONO^- \rightleftharpoons OONOH \rightleftharpoons NO_2^·....OH^·$
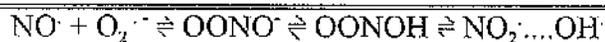
Table 1. Key chemical reactions of nitric oxide.

molecules, the activity of the carrier molecules may be modified so as to change their bioactivity reversibly.

Four chemical reactions are particularly pertinent biologically:

1. Redox reactions in the presence of oxygen.

In oxygenated aqueous and gaseous environments (e.g. air/fluid interface in the airways), rapid interconversion among NO species may occur as shown in table 1 (Marletta MA, 1990. Marletta MA, 1988. Gillespie R, 1989).

Thus, NO· generation in aqueous biological systems and at air-aqueous interfaces is marked by the formation of end products of NO metabolism, NO^{2-} and NO^{3-} (Stamler JS, 1992a). NO^{2-} is widely believed to be the major end product of the reaction between NO· and O_2 in aqueous solutions; however, much of the aqueous chemistry in biological systems remains poorly understood (Gaston B, 1994). Acidic conditions such as those that may be found in the airway are likely to alter the distribution of NO species in aqueous solution, largely as a result of decomposition of NO^{2-} to the reactive NO^+ equivalent, N_2O_3 (Marletta MA, 1988). Transition metal and related redox enzymes may also support the oxidation of NO^{2-} to NO^{3-}, to account for the latter's prevalence in cellular system.

2. Reaction of NO with transition metals.

NO· readily reacts with transition metal ions free in solution and with both haem and non haem metalloproteins (Stamler JS, 1992a. Wan Q, 1991). The interaction

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between NO and iron is biologically important because it forms the basis for the action of NO in at least four physiological systems -

i.   guanylyl cyclase activation, inducing a stereochemical change within the porphyrin ring of the enzyme (Wong SK 1992).

ii. the inhibitory action of haemoglobin in systems in which NO is generated or administered

iii. nitrosylation of iron-sulfur centered proteins in macrophage driven cidal reactions (Hibbs JB, 1988. Hibbs JB, 1987.)

iv. modification of function of metalloproteins like cytochrome c, myoglobin and tyrosinase (Henry Y, 1991).

3. Nitrosation of nitrogen and sulfur containing compounds.

The nitrosation of biologically relevant nucleophilic centres (N,S,C and O) involves the transfer of NO^+ in the form of N_2O_3, N_2O_4, iron nitrosyls, nitrososamines (RNH-NO) and S-nitrosothiol (RS-NO). Under normal physiological conditions, thiols are more reactive than amines and S-nitrosation occurs preferentially over N-nitrosation (Stamler JS, 1992a).

The formation of RS-NO derivatives may be viewed as a means of stabilising NO in the bioactive forms, potentially facilitating its transport in tissue and, importantly in the lungs, mitigating the toxicity arising from the reaction of NO with O_2 and superoxide (Kanner J, 1991).

4. Reactions with superoxide ($O_2.-$)

NO reacts rapidly with superoxide in both gaseous phase and aqueous solution to form peroxynitrite anion ($ONOO^-$). The subsequent protonation and decomposition of $ONOO^-$ to species with NO_2^- and OH^- is an important mechanism by which NO may exert toxic and cidal effects under physiological conditions. Peroxynitrite anion and peroxynitrous acid are strong oxidising agents and their decomposition products, OH^- and NO_2^- facilitate lipid peroxidation (Radi R, 1991a. Radi R, 1991b)

Each of the products of these reactions with oxygen, superoxide and transition metals - NOx species, peroxynitrite and metal-NO adducts support additional nitroactive reactions. S-nitrosothiol formation predominates due to the greater prevalence and reactivity of thiols in biological systems (Stamler JS, 1992a). Accordingly, metal and thiol containing proteins serve as major target sites for NO. The essential components of NO responsive signaling circuitry - signaling proteins, ion channels, receptors, enzymes and transcription factors all contain either transition metals or thiols strategically located at either allosteric or active sites allowing regulation of the protein by nitrosylation of or redox reaction with the metal or thiol component.

1.3 SYNTHESIS OF NITRIC OXIDE IN THE LUNGS

NO is formed in vivo by nitric oxide synthase (NOS), which catalyses the 5 electron oxidation of the guanido nitrogen moiety of L arginine to citrulline via N-hydroxyl-L-arginine (figure 1).

The NOS reaction involves an initial hydroxylation of L arginine to generate N-γ-hydroxy-1-arginine (NHA) (Stuehr D, 1991.). This step is thought to involve the haem moiety of NOS, which directly oxidises NHA. There are at least three isoforms of NOS (described in detail later) designated NOS I-III. The enzyme utilises O_2 and NADPH as cosubstrates and thiol, tetrahydrobiopterin, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) as cofactors (Fostermann U, 1991. Mayer B, 1990.). In the lungs, macrophages, neutrophils, eosinophils, mast cells, iNANC neurons, fibroblasts, vascular smooth muscle cells, pulmonary arterial and venous endothelial cells and pulmonary epithelial cells have all been shown to be capable of expressing NOS and generating NO. (Hibbs JB, 1987. Jorens PG, 1991. Rimele TJ, 1988. Bissonnette EY, 1991. Bult H, 1990. Belvisi MG, 1991. Jorens PG, 1992). The most distinctive phenomenon about NO production by these three isoforms is that NO produced by NOS II (see later) is about 1000 fold greater than that produced by the other two isoforms (Vodovotz Y, 1994). This has direct bearing on the function of NO and is discussed in chapter 1.6.3.
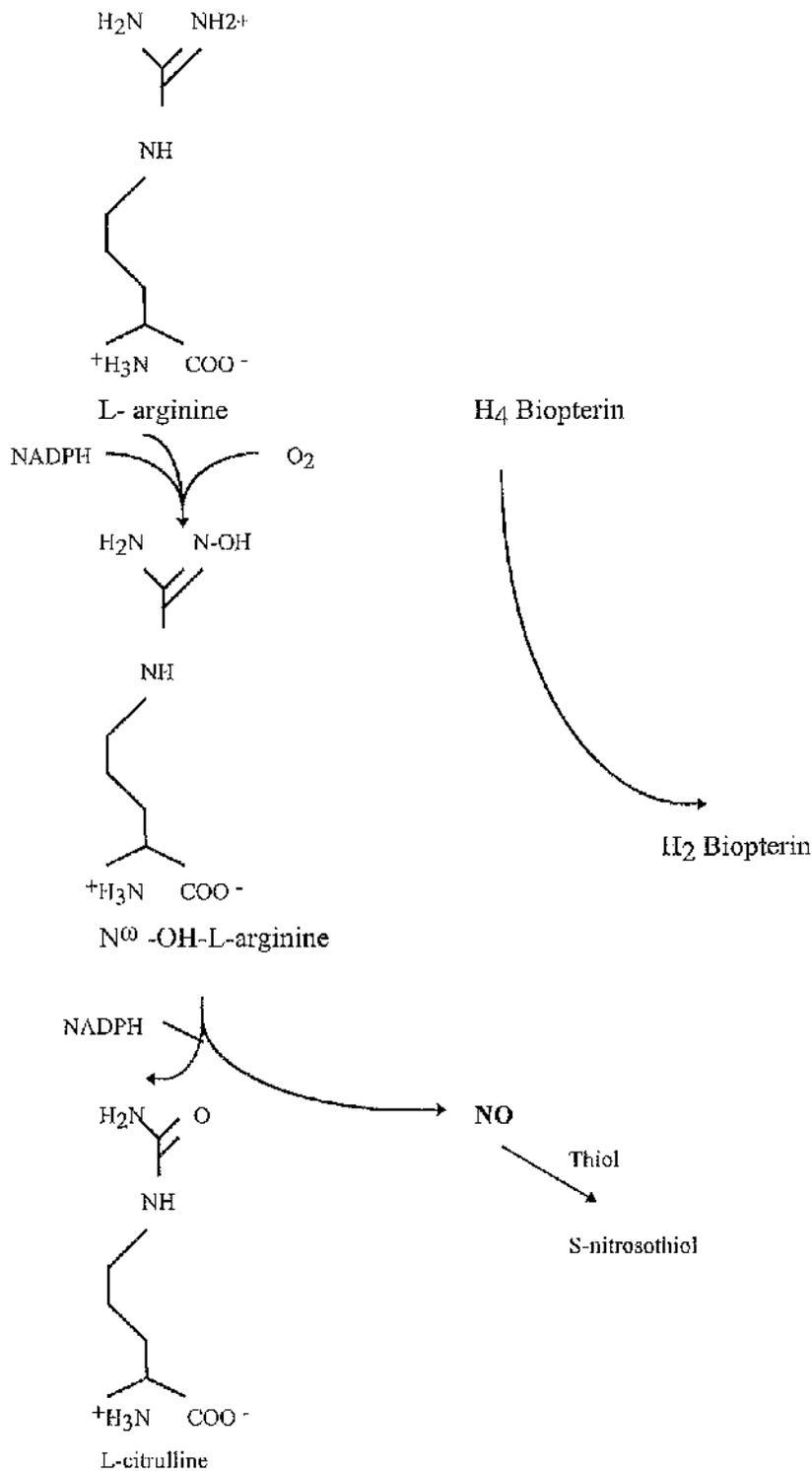
FIGURE 1. Formation of NO by NOS. A guanido nitrogen of L-arginine is oxidised in the presence of cosubstrates NADPH and molecular oxygen to yield NO. Calmodulin, flavoproteins, tetrahydrobiopterin and thiol are enzyme cofactors. NO is converted to more stable RS-NO under physiological conditions, possibly to prevent 'suicidal' inhibition of the NOS haem moiety.

It is widely assumed that NO diffuses passively from the cytosol of its cell of origin to neighbouring target cells to elicit a response (Roberts J, 1992). Its short half life of between 0.1 and 5 seconds in physiological systems (Palmer RMJ, 1987. Kelm M, 1990) has led to the hypothesis that NO is a locally acting substance, perhaps behaving in a paracrine fashion. It is likely that its short half life and reactivity limits it to its site of action but subsequent activity is determined by the amount of NO produced and presence of other molecules in the microenvironment.

1.4 MOLECULAR BIOLOGY OF NITRIC OXIDE SYNTHASES

Two constitutive and one inducible nitric oxide synthases have been described. One of the constitutive enzyme (eNOS) was restricted to endothelium and the other (nNOS) was found in central and peripheral neurons, neuroblastomas, human skeletal muscle, β cells of pancreatic islets and epithelial cells of bronchioli, uterus, and stomach. These constitutive NOS's lie dormant until and so long as a rise in intracellular calcium sustains the binding of calmodulin, leading to NO release over several minutes (Malinski T, 1992). An inducible NOS was found to be expressed in many cell types after challenge with immunological or inflammatory stimuli (Nathan C, 1992) and thereupon generates large amounts of NO. Tonic activation of this isoform is thought to reflect its ability to bind calmodulin even at the trace levels of calcium found in resting cells (Cho IJ, 1992).

Three NOS genes have been identified to date (table 2). These are called types I-III, according to the order of their discovery. Type I (neuronal NOS) is a 29 exon gene found on human chromosome 12q24.2 and extends over 100 kb (Kishimoto J, 1992. Hall AV, 1996). Type II NOS (iNOS) is found on 17cen-q11.2, has 26 exons and is 37 kb in size (Chartrain N, 1997. Marsden PA, Heng HHQ 1994). and type III or the endothelial (eNOS) gene, resides at 7q35-7q36 and comprises 26 exons that span 21 kb (Marsden PA, Heng HHQ, 1993. Robinson LJ, 1994).

human NOS protein	cDNA size	Protein size	Structure	Subcellular site	Primary regulation	NO output	Co-factors
NOS 1	10.0 kb	1433 amino acid 161 kDa	homodimer	Cytosol	Ca^{2+}/ calmodulin	Low (pM)	BH4, NADPH, FAD, FMN, haem
NOS 2	4.1 kb	1153 amino acid 131 kDa	homodimer	Cytosol >> membrane	Gene transcription	High (μm)	BH4, NADPH, FAD, FMN, haem
NOS 3	4.4 kb	1203 amino acid 133 kDa	homodimer	Membrane >> cytosol	Ca^{2+}/ calmodulin	Low (pM)	BH4, NADPH, FAD, FMN, haem

Table 2. Characteristics of the human NOS proteins

Traditionally, types I and III NOS's are thought to be constitutively expressed and intermittently produce small pulses of NO that mediate physiological activities. In contrast, type II or inducible NOS is expressed in response to external stimuli and produces large, sustained amounts of NO. Recent reports, have challenged this paradigm of constitutive and inducible NOS expression. Full type I and III NOS's activity appear to depend upon cytokine induction of GTP cyclohydrolase I, the rate limiting enzyme in the synthesis of tetrahydrobiopterin (Werner-Felmayer G, 1993). Conversely NOS II is also constitutively expressed in some tissues and cell types, such as the large airways of humans and certain mononuclear cell lines (Kobzik L, Bredt DS, 1993. Mannick JB, 1994. Hoffman RA, 1997. Guo FH, 1995). These observations highlight the complexities of NOS gene expression and the fact that NOS expression whether induced or constitutive is not just limited to features of the gene for a specific isoform but also to the cell type and stimulus involved in NOS expression.

1.4.1 REGULATION OF NOS I EXPRESSION

NOS I was the first NOS cDNA ever cloned and this was done from rat cerebellum by Bredt and Snyder in 1990 (Bredt DS, 1990). Since then it has also been cloned in mouse (Ogura T, 1993) and humans (Nakane M, 1993) and appears to be highly conserved across species. The human NOS I cDNA has an open reading frame of 4299 nucleotides encoding for a protein with a molecular weight of 161 kDa. The NOS I gene is approximately 160 kb in size and is

composed of 29 exons and 28 introns (Hall AV, 1996). The major transcription start site is 28 nucleotides downstream from a TATA box. Translation starts in exon 2 and terminates in exon 29. Sequence of the 5' flanking region shows several potential cis acting DNA element including NF-1, AP-2, CREB/ATF and NF-kB like motifs (Hall AV, 1996). Transcriptional control of the human NOS I gene involves two distinct 5' untranslated regions that are encoded through the use of two closely linked but separate promoters for human nNOS gene transcription (Xie J, 1995). This results in alternative splicing of exon 1 in the 5' untranslated region of the mRNA but does not affect actual size of the translated NOS I protein.

Conditions such as cerebral ischaemia (Zhang ZG, 1994) and chronic salt loading (Kadowaki K, 1994) have been shown to upregulate NOS I mRNA in rat brain although the precise molecular mechanisms responsible have not been elucidated. The 3' untranslated region in the 29th exon of the human NOS 1 gene contains a dinucleotide microsatellite repeat and multiple alleles of the repeat exist in normal individuals indicating polymorphism (Hall AV,1996).

Post-transcriptional control of the NOS I gene also exists in the form of alternative mRNA splicing. This structural diversity was first reported in mouse brain where about 5% of neuronal NOS I transcripts lacked two exons encoding 105 amino acids from position 504 to 608 (Ogura T, 1993). Similar structural diversity was also identified in the human cerebellum. This recombinant variant

was unable to convert L-arginine to NO and L citrulline but did retain NADPH diaphorase activity.

With the cloning of NOS I came the recognition that NOS I gene is also highly expressed in skeletal muscle and suggested a possible novel function of NO in the skeletal muscle. An additional alternatively spliced isoform of NOS I is further identified which is expressed in differentiated skeletal muscle (Silvagano F, 1996). Designated neuronal NOS-μ (nNOSμ), this NOS I isoform is slightly larger than NOS I expressed in brain due to an inserted 192 bp alternatively spliced segment between exons 16 and 17. This isoform has similar catalytic activity to cerebellar NOS I and this protein was found only in skeletal muscle, penile corpora cavernosa, urethra , prostate and heart but not spleen, liver, kidney or brain by antibody staining in rat tissues. This demonstrated that alternative mRNA splicing can function as a mechanism for tissue specific regulation of NOS I.

1.4.2 REGULATION OF NOS II EXPRESSION

Human NOS II was first cloned in 1993 from LPS and cytokine stimulated primary human hepatocytes (Geller DA, 1993a). The sequence of hepatocyte NOS II clone reveals a 4145 bp DNA containing 3459 bp open reading frame, MW - 131 kDa. The human NOS II gene itself is about 37 kb long and composed of 26 exons and 25 introns. This genomic structure is similar to that of the human

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NOS I and II genes, suggesting divergence from a common 'ancestral' NOS gene. Compared to the murine macrophage NOS II, the human hepatocyte NOS II sequence is 6 amino acids longer in the amino portion of the protein and 3 amino acids longer at the carboxyl terminus. Similar to other NOS isoforms, NOS II contains consensus recognition sites for the cofactors FMN, FAD and NADPH in the carboxyl half of the protein which have been shown to be important for NOS II enzyme activity. In addition, a consensus calmodulin recognition site is also present. Overall the human hepatocyte NOS II displays 80% sequence identity to its murine macrophage counterpart at both the nucleotide and amino acid levels. It has 51% and 53% amino acid sequence homology with the NOS III and NOS I proteins respectively (Geller DA, 1998).

A number of agents are known to induce NOS II expression. Most of these appear to act in synergy with an interferon (IFN) or IFN inducing agent like bacterial lipopolysaccharide (Nathan C, 1992). The major inducers are the proinflammatory cytokines (IL1β, TNFα and IFNγ), but the list includes 5,6-dimethylxanthenone-4-acetic acid (Thomsen L.L, 1992), picolinic acid (Melilo G, 1993), cAMP elevating agents (Hortelano S, 1993. Koide M, 1993. Imai T, 1994), UV light (Warren JB, 1994) and ozone (Pendino KJ, 1993). However, to put the list in perspective, the most important observation about NOS II expression is that LPS and IFNγ are the strongest inducers of NOS II in monocytes (de Vera ME, 1996). In contrast, most tissue derived cells, including hepatocytes, mesangial cells, vascular smooth muscle cells have NOS II which are more sensitive to IL1β

and TNFα. In nearly all cell types, LPS, IFNγ, ILIβ and TNFα enhance NOS II expression synergistically.

It is worth noting that rodent cells are more easily triggered to express NOS II than human cells, therefore some animal data are unlikely to be applicable to human (Beck KF, 1999).

In terms of speed of expression, studies on human NOS II in a human epithelial cell line (AKN-1) and primary human hepatocytes showed that when stimulated with the cytokine mixture of TNFα, IL1β and IFNγ, NOS II mRNA was observed at 2 hours, peaked at 4 hours and diminished by 24 hours (de Vera ME, 1996). A time dependent rise in NO release was seen that lagged behind the NOS II mRNA expression. Onset of NO production occurred 6-8 hours after induction of NOS II but once induced, this expression persisted for days.

The human NOS II promoter was characterized by de Vera *et al* in 1996 (de Vera ME 1996). It differs significantly from its murine and rodent counterpart. Cytokine responsive elements are found upstream from -3.8 kb in the promoter (de Vera ME, 1996) in contrast to the murine NOS II promoter where only 1 kb of the proximal 5' flanking region is required to confer LPS and cytokine inducibility (Xie QW, 1993). Analysis of the first 400 bp upstream of the human transcriptional initiation site revealed the presence of three interferon gamma response element (γ-IRE) (Pearse RN, 1991), reported to be involved in IFNγ

induced gene expression, an NF-kB site, conserved with the murine promoter, reported to be involved in cytokine and LPS stimulated gene expression and a degenerative TNF-RE site which confers responsiveness to TNFα. NF-kB is the main transcription factor involved in the regulation of NOS II expression. The activation and translocation of this transcription factor to the nucleus leads to a coordinated increase in the expression of many genes including IL1β, TNFα, IL6, IL8 and GM-CSF, and adhesion molecules responsible for recruitment of inflammatory cells. In turn, many stimuli activate NF-kB, including cytokines, protein kinase C activators, viruses and oxidants. Thus induction of NOS II by this pivotal transcription factor involves NO in a coordinated increase in the expression of many genes whose products mediate inflammatory and immune responses (Xie QW, 1994).

There is evidence to suggest that NOS II is expressed in response to factors other than immunological and inflammatory signals. NOS II have been detected constitutively in kidneys of normal rats (Tojo A, 1994) and importantly, in normal airway epithelia (Kobzik L, 1993, Watkins DN, 1997). In the last, it has been argued that this may be a residuum of repeated exposures to airborne stimuli rather than true constitutive expression. If NOS II is constitutively expressed, it suggests that it may serve a physiological role in addition to its pathological ones.

Although NOS II is thought not to be calcium sensitive, the calcium chelating agent ethylene glycol bis (β aminoethyl ether)-*N,N,N',N'*-tetra-acetate (EGTA)

and the calmodulin antagonist, trifluoroperazine decreased NOS activity by 50-60% (Sherman PA, 1993). It is likely that some low level of calcium is required for optimal calmodulin binding to human NOS II.

More recent findings suggest that there are three main signaling pathways responsible for NOS II expression. Induction in murine macrophages is mediated by IFN γ activated sites and IFN γ responsive elements in the promoter (Xie QW, 1993. Martin E, 1994). In rat mesangial cells, binding of NF-kB at the appropriate site on the NOS II promoter is essential for the induction of NOS II by IL1β (Eberhardt W, 1998). In contrast, endothelin 1 inhibits cytokine induced NOS II expression without affecting NF-kB binding suggesting additional mechanisms that are essential for cytokine induced NOS II expression (Beck KF, 1996). For the induction of NOS II by cAMP, enhanced binding of the transcription factors, CAAT enhancer-binding protein (C/EBP) and cAMP responsive element binding protein (CREB) on the NOS II promoter has been reported (Eberhardt W, 1998).

One of the controversies surrounding NOS induction in inflammatory situation is the difficulty encountered in finding NOS II expression in human macrophages (Denis M, 1994). While the debate continues, it is clear that human macrophages have the ability to produce NO under certain inflammatory conditions but at least one group has confirmed a transcriptional basis for hyporesponsiveness of the NOS II gene promoter in human macrophages to LPS and IFNγ compared to murine macrophages (Zhang X, 1996). Two explanations for this

hyporesponsiveness were found - i. multiple inactivating nucleotide substitutions in the human counterpart of the enhancer element that has been shown to regulate LPS and IFNγ induced expression of the murine NOS II gene and ii. an absence of one or more nuclear factors in the human macrophages that are required for maximal expression of NOS II. This may also illustrate the differences between molecular regulation of the rodent and murine NOS II and human NOS II.

Equally important in knowing what upregulates NOS II expression is knowing what turns it off. Glucocorticoids are known to inhibit induced NO synthesis in several cell types (Nathan C,1992. DiRosa M, 1990. Knowles RG, 1990. Radomski MW, 1990). In rat hepatocytes, the effect is a result of decreased NOS II transcription due to the ability of dexamethasone to upregulate I-kB expression and inhibit cytokine stimulated NF-kB activity (Geller DA, 1993b) The latter is thought to be mediated by direct binding of glucocorticoid receptor to NF-kB, preventing it from binding to kB sites on NOS II. Kleiniert found that dexamethasone inhibited cytokine induced NOS II mRNA in human A549 epithelial cells by 70% and that this inhibition was mediated in part by down regulating activation of NF-kB, without an increase in I-kB mRNA levels (Kleinert H, 1996).

TGFβ also partially prevents induced NO synthesis. Suppression of NOS II expression by TGFβ has been shown in macrophages (Ding A, 1990) mesangial cells (Pfeilschiffer J, 1991) and cardiac myocytes (Roberts AB, 1992). However,

this effect is not uniform with different cells and species (Kunz D, 1996). This underscores the complexity of NOS II regulation, lending support to the notion that species, tissue and cell-specific mechanisms are likely to be important in controlling NOS II expression. Induction of heat shock response by hyperthermia blocked subsequent NOS II expression in AKN-1 human liver cells and a similar suppression has been reported in brain astroglial cells (Feinstein DL, 1996). The induction of the heat shock response may be an adaptive defense mechanism that prevents over expression of NOS II during inflammatory conditions.

In terms of post transcriptional control, TGFβ has been found to suppress macrophages NOS II expression by decreasing NOS II mRNA stability and translational efficiency (Vodovotz Y, 1993). There are also reports that dexamethasone can increase NOS II mRNA stability in IL1β stimulated rat mesangial cell and vascular smooth muscle cells (Kunz D, 1996. Perrella MA, 1994.). There are also reports of differential NOS II protein detection in different tissue – thus, although NOS II mRNA can be readily detected in cardiac myocytes and hepatocytes, NOS II protein and NO synthesis are not found in the former (Luss HL, 1997). These data suggest that cell-specific post transcriptional mechanisms may operate; but it is unclear if this is secondary to rapid degeneration of NOS II mRNA or a block in translational machinery.

1.4.3 REGULATION OF NOS III EXPRESSION

The endothelial NOS cDNA was cloned from cultured bovine (Lamas S, 1992) and human endothelial cells (Marsden PA, 1992). The cDNA has an open reading frame of 3609 nucleotides encoding a protein with a molecular weight of 133 kDa. The human NOS III protein has approximately 50% homology with NOS I isoform (Geller DA, 1998). The gene is 21 kb in size and composed of 26 exons and 25 introns (Nadaud S, 1994). A highly polymorphic dinucleotide repeat $(CA)_n$ occurs within intron 13. The human NOS III promoter is 'TATA-less' but does contain a CCAAT box at -286. The 5' flanking region also contains proximal promoter elements Sp1 and GATA which are consistent with constitutively-expressed endothelial cell genes. The 5' flanking region also contains putative sequence motifs for AP-1 and AP-2, NF-1, heavy metal and shear-stress responsive elements (Marsden PA, 1993). Haemodynamic shear stress in vitro (Nishida K, 1992) and chronic exercise in vivo (Sessa WC, Circ Res 1994) have been shown to increase the expression of NOS III mRNA, suggesting possible transcriptional regulation mediated via the shear stress response element in the promoter.

Hypoxia appears to exert different effects on NOS III expression. In rat and porcine arterial endothelial cells, chronic hypoxia up-regulated NOS III expression (Le Cras TD, 1996. Xu X-P, 1995). However, exposure of human or bovine endothelial cells to low oxygen tension resulted in a decrease in the

transcript for endothelial NOS and a corresponding fall in NOS protein (Phelan MW, 1996). Furthermore, the ability of endothelial cells exposed to chronic hypoxia to produce NO in response to bradykinin, a stimulator of NOS III activity, was also impaired. The addition of actinomycin-D abrogated the hypoxic effect of NOS III transcripts, suggesting that new gene transcription is required for NOS III suppression.

In addition to transcriptional control, post transcription regulation has also been identified for the human NOS III gene. TNFα was found to decrease bovine aortic endothelial cell and human umbilical vein endothelial cells (HUVECS) mRNA. In nuclear run on assays TNFα did not change the rate of NOS III gene transcription assays in HUVECs but it did shorten the half life of NOS III mRNA (Yoshizumi M, 1993). Under basal conditions the NOS III mRNA was very stable in HUVECs with half life of 48 hours; exposure to TNFα decreased this to 3 hours. Thus, TNFα decreases NOS III mRNA levels by increasing the degradation. The mRNA destabilization was prevented by cycloheximide, implying that TNFα may induce a protein responsible for message degradation.

1.4.4 POST TRANSLATIONAL CONTROL OF NITRIC OXIDE SYNTHASE PROTEINS

The NOS protein is a complex enzyme involving several tightly bound redox cofactors that are apparently organised into discrete domains that can be

associated with a particular activity (Fostermann U, 1991). The enzyme has significant homology to NADPH cytochrome P450 reductase and has been shown to carry out P450 chemistry in the formation of NO (Bredt DS, 1991). Given that the normal function of P450 reductase is to supply reducing equivalents to cytochrome P450, it has been assumed that this domain in NOS serves the same function.

All three NOS isoforms share features of post translational modification. Mechanisms of post translation control include NOS protein stability, dimerization, phosphorylation, subcellular localisation, co-factor binding and availability of substrates L- arginine and molecular oxygen (Fostermann U, 1995).

Besides suppressing NOS II expression (see chapter 1.4.2), TGFβ also accelerates the degradation of NOS II protein in mouse macrophages (Ding A, 1990. Pfeilschiffer J, 1991). However, it is unknown if similar effect is exerted on other NOS isoforms.

Tetrahydrobiopterin appears to be a rate limiting co-factor in mouse fibroblasts, rat vascular smooth muscle and human endothelial cells (Gross SS, 1993). Full NOS II and NOS III activity appear to be dependent on the induction of GTP cyclohydrolase I, the rate limiting enzyme in biopterin synthesis. The same kinds of immune and inflammatory agents that induce NOS II in endothelium are able to capacitate endothelial NOS III within intact cells by satisfying its

tetrahydrobiopterin requirements (Suschek C, 199). In this way, the activity of a constitutive enzyme may appear partially inducible. However, this has only been shown in vitro, where the naturally occurring tetrahydrobiopterin levels fall rapidly when cells are cultured, raising the possibility that the observation may be a cell culture induced artifact.

One of the most important determinant of protein stability could well be the product of the protein itself, nitric oxide. NO can inhibit NOS (Rogers NE, 1992. Griscavage JM, 1993) and is known in vitro to have a negative feedback ability (Assreuy J, 1993). However, it is not clear whether the relevant reaction of NO is with the thiol-bound haem of NOS, with a critical thiol or other amino acid side chain on the enzyme, or indeed with a redox-sensitive co-factor.

1.5 THE BIOLOGICAL EFFECTS OF NITRIC OXIDE

NO can be viewed as an ubiquitous molecule with three broad remits in its physiological role - as a signaling molecule, a neurotransmitter and a molecule possessing its own specific activities.

1.5.1 NITRIC OXIDE AS A SIGNALING MOLECULE

One of the most basic activities of NO is its role as a signaling molecule in cell responses. This is possibly one the most intense areas of basic NO research in recent years. The most physiologically relevant action of NO in this capacity is the activation of soluble guanylate cyclase by nitrosation of its haem moiety (Ignarro LJ, 1990). The subsequent increase in cGMP level alters the activity of three main target proteins: i. cGMP regulated ion channels, ii. cGMP regulated phosphodiesterases and iii. cGMP dependent protein kinases (Schmidt HHHW, 1993).

NO appears to activate all three parallel mitogen activated protein kinase (MAPK) cascades in Jurkat T cells (Lander HM, 1996) and increase the activity of protein kinase C. However, these effects do not appear consistent and in different cell types may have the opposite effect (Caselli A, 1994. Callscn D, 1998). The current conclusion from a large number of studies is that NO can, in principle deliver signals to all major MAPK cascades including the classical ERK, SAPK/NK and p38 kinase cascades as well as the JAK/signal transducer and

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activator of transcription (STAT) pathway (Beck KF, 1999). An integration of these signals lead to cell type specific responses that critically depend on interactions between individual constituents of a cell and the components of the different signaling cascades. These cascades of events then trigger the phosphorylation of key nuclear proteins, including transcription factors and finally may lead to alteration in gene expression.

Increasing evidence is also accumulating that NO preferentially alters transcription factors that are sensitive to changes in cellular redox status (e.g. NF kB and AP 1) (Sen CK, 1996). Reported mechanisms include scavenging of reactive oxygen intermediates in the cell (and therefore inhibiting the activation of NF-kB) (Zeiher AM, 1994. Peng HB, 1995), direct inhibition of the DNA binding activity of NF-kB family proteins and direct modification of transcriptional activators e.g. AP 1 (Tabuchi A, 1994).

An increasing number of genes have been shown to be under the regulatory control of NO - extracellular matrix proteins (Chatziantoniou C, 1998) and their corresponding proteases (Sasaki K, 1998), growth factors (Tsurumi Y, 1997. Chin K, 1997), hormones (oestrogens) (Wang H, 1998), cytokines (TNFα stimulated IL8 is inhibited by NOS inhibitor in dose dependent manner) (Van Dervort AI, 1994. Villarete LH, 1995. Nuhl H, 1997) and enzymes (Hartsfield CL, 1997).

NO has also been established as a potent inducer of apoptosis in certain cell types (Brune B, 1998. Mebmer UK, 1994) but contradictory effects have been reported

in others, with NO displaying anti-apoptotic effects in lymphocytes, eosinophils, hepatocytes and endothelial cells (Nitsch DD, 1997. Dimmeler S, 1997).

Therefore, most physiological responses are triggered by moderate concentration of NO through the activation of soluble guanylate cyclase. High concentrations of NO operate in part through redox sensitive regulation of transcription factors and gene expression and alter, on a longer term basis, the capacity of the cells to deal with stress conditions.

1.5.2 EFFECTS OF NITRIC OXIDE IN HOST DEFENCE AND IMMUNO-REGULATION

NO formation may have originated as an ancient first line of defence for metazoan cells against intracellular parasites like *L. major*, *T. gondii* and *M. Leprae*. Infection in humans and animals are often associated with significant increase in systemic NO production in plasma and urine (Anstey NM, 1996. Evans TG, 1993. Ochoa JB, 1991.). Microbial products like LPS and lipoteichoic acid stimulate the expression of NOS II in epithelial and macrophages. Production of NO in many animal models can be directly correlated with the ability of the host to contain microbial proliferation. Abrogation of NOS II activity produces dramatic increases in microbial burden (Stenger S, 1996. MacMicking JD, 1997). In vitro studies of phagocytic cells and a variety of microbial targets have demonstrated cytokine inducible microbiostatic or microbicidal activity which is L arginine dependent and inhibitable by NOS inhibitors. Indeed, there is good evidence that

NO release induced in septic shock is a major contributor to cardiovascular collapse. Finally, experiments using the NOS II knockout mouse showed that these animals were less capable of controlling replications of *Listeria monocytogenes* and *Leishmani major* (MacMicking JD, 1995).

Although it is has been shown in vitro that NO has a growth preventing and lethal cidal effect, there is still little information on the biochemical mechanism by which NO blocks the growth of or kills microorganisms. One paper did show that formation of nitrotyrosine around bacteria aids in the ingestion by phagocytes (Evans TJ, 1996). There is also accumulating data to suggest that NO encourages the production of IFNγ which is crucial in the containment of intracellular pathogens like the mycobacteria *spp.* since this cytokine activates macrophages and other effector cells (MacMicking JD, 1997).

The role of NOS II produced -NO in the immune system comprises both effector and regulatory functions. There is considerable evidence that NOS II produced NO can modulate cytokine production from macrophages, T cells, endothelial cells and fibroblasts and lymphocyte proliferation. Low levels of NO have been implicated in lymphocyte activation and proliferation. NO donors like sodium nitroprusside increase lymphocyte uptake of glucose (an early event in lymphocyte activation) and stimulate TNFα production and NF-kB binding activity (Billiar TR, 1995. Farrell AJ, 1996). NO also enhances activity of tyrosine kinase p56 which is implicated in lymphocyte signaling events. NOS inhibitors and L arginine depletion impairs phytohaemagglutinin (PHA) stimulated

proliferation (Efron DT, 1991). Both in vitro and in vivo, L arginine has also been shown to enhance natural killer and lymphokine activated killer activity. In contrast, high concentrations of NO suppress antigen presenting cell activity and T cell proliferation (Mills CD, 1991. Merryman PF, 1993). There is evidence that NO exerts different effects on discrete subpopulations of T cells, for example by inhibiting secretion of IL2 by murine Th1 cells and increasing secretion of IL4 by Th2 cells (Chang RH, 1997). These observations however, differ from those of Bauer who reported that production of Th1 and Th2 associated cytokines are equally impaired by NO donors (Bauer H, 1997).

NO has been shown to promote apoptosis in macrophages, CD4+/CD8+ thymocytes, chondrocytes and pancreatic β islet cells (Blanco FJ, 1995. Kitajima I, 1994. Ankacrona M, 1994. Fehsel K, 1995. Albina JE, 1997). The mechanisms appears to require the activation of poly(ADP-ribose) polymerase and formation of nitrotyrosine and is opposed by Bcl-2 (Melkova Z, 1997). However, in low concentrations (<1 μM), NO has been reported to inhibit apoptosis of hepatocytes, B lymphocytes and eosinophils (Kim YM, 1997).

1.5.3 NITRIC OXIDE IN INFLAMMATION

Endogenous NO is widely hailed as a double edged sword. In high concentrations and under abnormal conditions and environment it has deleterious effect, while in small pulses it mediates physiological processes.

NOS II is induced in response to excessive cytokine production. This is a non-specific event which occurs in a wide variety of cells. Increased production or expression of NOS II has been implicated in sepsis (Hutcheson AR, 1990. Petros A, 1991), ulcerative colitis (Middleton SJ, 1993), psoriasis (Kolb-Bachofen V, 1996), rheumatoid arthritis (McCartney-Francis N, 1993. St Clair EW, 1996), multiple sclerosis (Parkinson JF, 1997), type 1 diabetes (Kolb H, 1992), asthma (Kharitonov SA, 1994. Alving K, 1993) and connective tissue diseases (Belmont HM, 1997). In all of these diseases the pro-inflammatory cytokines, TNFα, IL1β and IFNγ have been implicated. Therefore, the finding of increased NO production is often viewed as a reflection of an immune activated state where inflammatory cytokines and other mediators have upregulated NOS II in diverse tissues.

Several classic signs of inflammation are reversed by NOS inhibitors. Mulligan et al, using a rat model of immune complex lung injury, showed that inhibitors of NO synthesis were protective whereas L arginine exacerbated tissue injury (Mulligan MS, 1991). IL2 infusion in healthy mice results in the expression of NOS II in numerous tissues including the endothelium and muscles of the thoracic wall with accompanying pleural effusion and pulmonary oedema (Orucvic A, 1997). Treatment of these mice with NOS inhibitors reduced IL2 induced pulmonary oedema and pleural effusion.

In keeping with its dual roles, NO also possesses anti inflammatory effects (Kubes PM, 1991). It is able to inhibit leukocyte adhesion and oxidant production and in direct contrast to Mulligans' work on IL2 infusion in mice, Bouchier-Hayes

reported that NO attenuates IL2 induced lung injury (Bouchier-Hayes D, 1997). These seemingly conflicting data underscore the complexity of defining the role of NO in inflammation. While excessive NO production is generally associated with tissue injury, NO constitutively produced by endothelium is believed to play a protective role in the microvasculature (Stamler JS, 1992. Kubes PM, 1991). Here, NO inhibits platelet and neutrophil adhesion to endothelial monolayers as well as inhibiting leukocyte superoxide anion production (Clancy RM, 1992. Utierrez HH, 1996). NO has also been shown to inhibit mast cell degranulation and to block upregulation of p selectin expression (Massini E, 1996). These vascular defensive properties of NO in tissues have been demonstrated by its capacity to protect against tissue injury in vivo in myocardial ischaemia reperfusion injury and in ARDS (Roissant R, 1993. Guidot DM, 1995).

A complex relationship is also emerging between NO and the cyclo-oxygenase (COX) pathways, the latter involved in the production of prostaglandins. Like NOS, COX has constitutive and inducible pathways and COX 2 is the inducible form of the enzyme. A variety of cells e.g. endothelial cells and macrophages produce NO and prostaglandins simultaneously in response to cytokines and other activators. The paracrine effects of these molecules are often similar, including the capacity to relax smooth muscle, inhibit platelet and neutrophil adhesion and neutrophil oxidant production. Small amounts of NO appear to stimulate COX activity, possibly by scavenging superoxide which usually inhibits COX activity (Salvemini D, 1996. Amin AR, 1997. Salvemini D 1993).

Wink and coworkers explain these conflicting roles of NO by its physiological chemistry (Grisham M, 1999). Indeed, it does appear that the chemistry of NO within its microenvironment as described in chapter 1.2 is likely to dictate the type of effect NO has on biological systems. This together with the amount of NO produced are likely to be the most important determinants of the role of NO in any circumstance. Thus NO produced in small fluxes and interacting directly with a biological molecule or target usually results in protective effects. For example, reaction of NO with the haem moiety in guanylate cyclase and subsequent synthesis of cGMP results in anti inflammatory effects such as regulation of vascular tone, inhibition of platelet aggregation and leukocyte-endothelial interactions (Wink DA, 1997). In the NO-COX interactions, small amounts of NO stimulate production of anti inflammatory prostaglandins as described above (Savemini DT, 1993 and 1996, Amin AR, 1997) but sustained over-production of NO actually inhibits COX activity.

Large amounts of NO production appear to be the principal cause of inflammation. The effects of NO become 'indirect' with the formation of reactive nitrogen species, mainly from the interaction of NO and superoxides and oxygen. Peroxynitrite itself is a powerful oxidant, oxidizing thiols, nitrating tyrosine residues, nitrating and oxidising guanosine, degrading carbohydrates, initiating lipid peroxidation and cleaving DNA (Wink DA, 1998).

Another way of promoting inflammation is by removal of NO adducts with anti inflammatory functions. S-nitrosothiol adducts are readily formed during auto-

oxidation of NO (Stamler JS, 1992). In the context of inflammation, s-nitrosothiol can inhibit leukocyte adhesion to microvascular endothelial cells by nitrosation of sulphydryl groups on surface of endothelium and polymorphonuclear neutrophils which are critical for adhesion and infiltration (Kubes PM, 1991). The presence of superoxide in chronic inflammatory situations has been shown to deplete the amount of s-nitrosothiol and therefore promote adhesion and infiltration of inflammatory cells (Marletta MA, 1994).

1.6 THE BIOLOGICAL ROLE OF NITRIC OXIDE IN THE AIRWAYS

In the airways, NO serves three major physiological roles - as a vasodilator, bronchodilator and in mediating host defence. The first two roles are discussed below, its function in host defence has been discussed in chapter 1.5.2. NO is also implicated in the pathophysiology of a number of airways diseases.

1.6.1 ROLE IN PULMONARY VASCULAR TONE

In the airways, NO mediates the vasodilator action of acetylcholine in animal and human pulmonary vessels and acts as a braking mechanism against pulmonary vasoconstriction (Furchgott RF, 1980. Dupuy PM, 1992. Li CG, 1991). Release of NO from endothelial cells in pulmonary circulation counteracts hypoxic vasoconstriction (Liu SF, 1991. Persson MG, 1990) and L-NMMA (a non specific NO inhibitor) injected into pulmonary artery causes an increase in pulmonary vascular resistance (Blitzer M, 1994). Pulmonary arterial and venous endothelial cells release NO in response to a variety of agonists such as acetylcholine and bradykinin that relax pulmonary arterial, venous and lymphatic smooth muscle (Ignarro LJ, 1987. Cherry PD, 1990. Liu SF, 1992). NO mediates both endothelium dependent and endothelium independent iNANC relaxation of guinea pig pulmonary artery in vivo (Liu SF, 1992). It also modulates pulmonary arterial contractile responses to catecholamines (Liu SF, 1992) and PGF2α (Namiki A, 1992), reverses hypoxic pulmonary vasoconstriction (Frostell CG, 1991) and mediates flow-enhanced decreases in vascular resistance (Cook JP, 1990). Its

importance in human physiology is shown by the finding of decreased expression of NOS in pulmonary vascular endothelium of patients with both primary and secondary forms of pulmonary hypertension (Giaid A, 1995). This suggests that sustained attenuation of pulmonary vascular NO production is associated with clinically significant alterations on pulmonary vascular tone.

Under physiological conditions, the pulmonary vasculature exhibits a low degree of resting tone, and operates at pressures approximately 20% of those in the systemic circulation. It has been suggested that this is largely secondary to continuous NO release, because of its vasodilatory effects as described above. This hypothesis is not universally accepted. NOS inhibitors enhance pulmonary vascular tone in some models but have no effect in those that attempt to keep cardiac output constant (Leeman M, 1996). Also, chronic inhibition of NOS in rats causes systemic but not pulmonary hypertension (Hampl V, 1993).

Because NO appears to relax both vascular and airways smooth muscle, it has been proposed to serve as a mediator of regional airflow/ blood flow matching. The use of inhaled NO, however has shed some light on the possible role in these circumstances. There are reports of its successful use in primary pulmonary hypertension (Pepe-Zaba J, 1991), respiratory failure of the newborn (The Neonatal Inhaled NO Study Group, 1997), persistent pulmonary hypertension of the newborn (Roberts JD, 1992. Roberts JD, 1997) and pulmonary hypertension associated with congenital heart disease (Roberts JD, 1993). In ARDS, inhaled NO causes selective pulmonary vasodilatation, reducing pulmonary arterial

pressure and improving oxygenation in a proportion of cases (Rossaint R, 1993). However, the hypoxaemia in ARDS can be completely explained by shunting, hypoxic vasoconstriction being only contributory (Danztker DR, 1979). Under such conditions, inhaled NO preferentially vasodilates arterioles supplying ventilated regions, improving oxygenation and lowering vascular resistance. In other pulmonary conditions where shunt is less significant and the preservation of adequate gas exchange is dependent on the integrity of hypoxic vasoconstriction which diverts blood to better ventilated regions, the use of inhaled NO has been shown to be detrimental. In these conditions, NO appears to worsen ventilation perfusion mismatching and gas exchange. Indeed, this was elegantly demonstrated by Barbera et al who showed that in patients with COPD associated pulmonary hypertension, inhalation of 40 ppb of NO worsened ventilation perfusion mismatching (Barbera JA, 1996).

1.6.2 EFFECTS ON AIRWAY SMOOTH MUSCLE

NO is also known to act as a bronchodilator by increasing the soluble guanylyl cyclase activity, increasing the cGMP and relaxing airways smooth muscle (Gruetter CA, 1989). Certainly in anesthetized rabbits, relatively high concentrations of inhaled NO reduced the bronchoconstrictor effect of nebulised methacholine (Hogman M, 1993a). In vitro, Gruetter *et al* demonstrated that carbachol-contracted proximal airways had greater relaxation to nitroglycerin and nitroprusside (NO donors) than did distal airways (Gruetter CA, 1989). However, dissolved NO is much less potent in mediating the relaxation of bovine trachealis

muscle compared to endothelium denuded bovine pulmonary artery. The potency of NO species in relaxing smooth muscle varies considerably with the oxidation state of the molecule as well as with the type and epithelial integrity of the airway. In both guinea pig and human airways, nitroso-thiols are two logs more potent than nitrite in relaxing smooth muscle. However, these findings in animals have not been reflected in human studies. Inhaled NO has only been shown to be a partial and weak bronchodilator (Hogman M, 1993b), possibly due to the requirement for very high levels of NO locally.

There is also a large amount of evidence to suggest that NO may function as a neurotransmitter of nonadrenergic noncholinergic nerves (Rand MJ, 1992). It is likely that NO is released from nerve endings itself, since the NOS I isoform but not other isoforms was found localised to peripheral nerves (Bredt DS, 1990). NO accounts for approximately half of the inhibitory (bronchodilator) NANC response in guinea pig trachea in vitro, modulates neurobronchoconstriction in vivo and appears to account for most of the bronchodilator NANC response in human airways in vitro (Tucker JF, 1990. Li CG, 1991). NO dependent iNANC response in human bronchi appear to be significantly diminished in previously denervated tissue (Belvisi MG, 1991).

1.6.3 NITRIC OXIDE IN AIRWAY INFLAMMATION

The role of NO in the pathogenesis of airway disease has received less attention than its use as a pulmonary vasodilator. However in the last eight years, since it

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was discovered that exhaled NO levels are elevated in asthma (Kharitonov SA, 1994. Alving K, 1993) , a lot more work has been done in this area. Increased NO production in asthma may mediate the hyperaemia seen in these airways by virtue of it vasodilator properties (Barnes PJ, 1996). It may also increase exudation of plasma by increasing blood flow to leaky post capillary venules, thus increasing airway oedema. Mucus secretion may also be enhanced directly or via an increase in blood flow to the submucosal glands. It has also been suggested that NO may amplify the eosinophilic inflammation in asthma by selective inhibition of T helper 1 (Th1) cells (Barnes PJ, 1995). This causes Th2 polarisation in the T helper cell population, promoting the production of IL5 and IgE.

Some of these suggestions have been supported by correlations between clinical inflammatory parameters and exhaled NO levels in asthmatics. Jatakanon et al found that exhaled NO levels correlated with sputum eosinophils (Jatakanon A, 1998). Bronchial hyperresponsiveness and allergen induced asthmatic responses have been found to be associated with increased NO levels both in animals and steroid naive asthmatics (Mehta S 1997. Nijkamp FP, 1993. Dupont LJ, 1998). Exhaled NO levels are also known to be decreased with steroid administration (Kharitonov SA, 1996a. Kharitonov SA 1996b.Yates DH, 1995).

Taken together, these findings suggest that increased NO production appears to contribute to airways inflammation. However, it is noteworthy that patients with ARDS and COPD do not appear to have elevated levels of exhaled NO (Brett SJ, 1998. Rutgers SR, 1999) while atopic non-asthmatic subjects (Martin U, 1996)

and those with viral upper respiratory tract infections also have very high levels of NO (Kharitonov SA, 1995). This may mean that NO production is not synonymous with airway inflammation *per se*.

1.7 MEASURING NITRIC OXIDE IN THE EXHALED BREATH

In 1991, Gustaffson made the first measurement of NO in exhaled breath of rabbits and humans and observed that administration of L-NMMA intravenously decreased NO levels (Gustaffson LE, 1991). Two independent groups then measured NO in exhaled breath of asthmatics and found this to be elevated (Kharitonov SA, 1994. Alving K, 1993). This discovery has excited many to the possibility of using exhaled NO as a non invasive measure of airway inflammation.

The measurement of nitric oxide levels in the breath is based on the chemiluminescence principle where NO reacts with ozone to produce nitrogen dioxide. The nitrogen dioxide molecule exists at a higher energy level and spontaneously degrades to N and O_2, emitting a photon while doing so. The quantity of photons is directly proportional to the amount of NO and this energy is converted into an electronic signal. NO levels can be measured as volume per volume of exhaled air (ppb) or NO output (V_{NO}) in ml/min.

Not long after this discovery, it became apparent that there were many problems associated with the technique of exhaled NO measurement. The first and most important finding was that NO levels in the nose were at least 100 times higher than that in the lower airways (Lundberg JON, 1994). NOS II was found to be constitutively expressed in the paranasal sinuses of normal subjects (Lundberg JON, 1995) and it was suggested that these high levels may be responsible for the

sterility of these sites. Auto inhalation of nitric oxide may also contribute to the innate host defense mechanisms in the lower airways. However, it meant that levels measured at the mouth may not reflect those produced in the lower airways.

The methods of measuring exhaled NO between 1991 and 1996 were also complicated by different techniques used by different research groups (Massaro AF, 1995. Alving K, 1993. Kharitonov SA, 1994. Borland C, 1993. Robbins RA, 1996. Schedin U, 1993). The main difference involved sampling from mixed expired air (i.e. collection of exhaled air over a defined period into a bag) or from a single exhalation. In addition, some workers asked subjects to inhale NO free air before collecting their expirate while others do not. One other major confounding factor was noted in 1997 when it was found that the rate of exhalation significantly affected NO levels (Silkoff P, 1997).

Exhaled NO levels before 1996 were obtained using methodology that did not exclude contribution from upper airways. Therefore, values in early papers were grossly elevated (compared to papers in subsequent years). This MD research project was started in 1996 and the first remit of the project was to improve and optimise the method of exhaled NO measurement. In 1996, Kharitonov and Barnes developed a method of excluding nasal contamination of the lower airways air by employing a constant mouth pressure during a single exhalation (Kharitonov SA, 1997). This was shown to create a back pressure sufficient to raise the soft palate, sealing off the nasopharygeal cavity during exhalation, thus

preventing mixing of the upper and lower airways air. I adopted the technique and further validated and optimised its use.

1.8 CYSTIC FIBROSIS

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Tsui LC, 1995). It is the commonest inherited disease, affecting 1 in 2500 live Caucasian births. Median survival now approaches 31 years for men and 28.3 years for women (Rosentein BJ, 1998). The identification of the CFTR gene in 1989 combined with extensive physiological, microbiological and biochemical studies have provided a substantially greater understanding of the cellular and molecular biology underlying CF.

In clinical terms, CF is a multisystem disease, characterised by abnormal epithelial secretions. It is manifested in the lungs by obstruction and relentless damage to the airways with recurrent infection culminating in bronchiectasis, fibrosis and respiratory failure. In the gastrointestinal tract, exocrine pancreatic insufficiency leads to impaired digestion, recurrent subclinical pancreatitis and subsequently type 2 diabetes mellitus. Newborns often present with bowel obstruction in the form of meconium ileus and in adult patients, a similar syndrome of obstruction (distal intestinal obstruction syndrome) can occur. A subset of patients develop focal biliary cirrhosis or multilobular cirrhosis which may be further complicated by portal hypertension. Male patients are infertile due to the absence of vas deferens, thought to be secondary to a developmental dysregulation of the Wolfian duct caused by the defective CFTR gene. The major

cause of morbidity arises from complications of lung disease which is the cause of death in more than 95% of CF patients.

1.8.1 PATHOPHYSIOLOGY OF CYSTIC FIBROSIS

The CFTR gene encodes for a protein (CFTR protein) which functions as a cAMP regulated chloride channel in the apical membrane of epithelial cells (Tsui LC, 1995). A normal CFTR gene downregulates the epithelial amiloride sensitive sodium channel (Kunzelmann K, 1997) and upregulates the outwardly rectifying chloride channels (Schwiebert EM, 1995). Therefore, mutations of the CFTR gene not only disrupt the CFTR ion channel but also interfere with its regulation of other ion channels.

Among the greater than 600 mutations now registered by the CF Genetics Consortium, the δF508 mutation is present on about 70% of abnormal CF chromosomes. The δF508 is a biosynthetic trafficking defect in which the mutant protein is retained in the endoplasmic reticulum and degraded by a proteasome dependent pathway (Ward CL, Cell 1995). In vitro studies suggest that the mutant chloride channel has a reduced but measurable open time (Drumm ML, 1991. Welsh MJ, 1993). Reduction or elimination of cAMP mediated chloride transport through the CFTR channel is traditionally thought to reduce the chloride and water secretion into the airway lumen and to cause hyperabsorption of sodium and water from the lumen, producing dehydrated and viscous secretions. However, Smith et al have proposed that airways surface fluid in CF contains increased

concentration of sodium and chloride ions consistent with a decrease in absorption of ions (Smith JJ, 1996). These data were obtained from in vitro studies and at present the salt content of airway surface fluid in CF patients remains an intensely debated subject.

Salt concentration in the airway lining fluid is important because defensins, a group of naturally occurring peptides with antimicrobial activity are known to be salt sensitive and potentially inactivated by higher salt concentration. Several genes in human encoding for these proteins, including β defensin 1 and tracheal antimicrobial peptide have recently been identified (Goldman MJ, 1997). Inactivation of these defensins by higher concentrations of salt might explain why CF airways become chronically colonised with bacterial pathogens such as *P. aeruginosa*.

Other factors also make substantial contribution to the pathogenesis of CF airway disease. Defective acidification of intracellular organelles with secondary alteration of glycoprotein (Barasch J, 1991), changes in normal endocytosis and exocytosis (Bradbury NA, 1992), abnormal functioning of submucosal glands and sulfation of high molecular weight glycoconjugates have all been shown to be significant factors (Engelhardt JF, 1992). The CFTR protein itself may also play an important role in bacterial defences. Epithelial internalisation of *P. aeruginosa*, a process important in defence against this bacterium is thought to be defective in epithelial cells with the mutant CFTR (Pier GB, 1996).
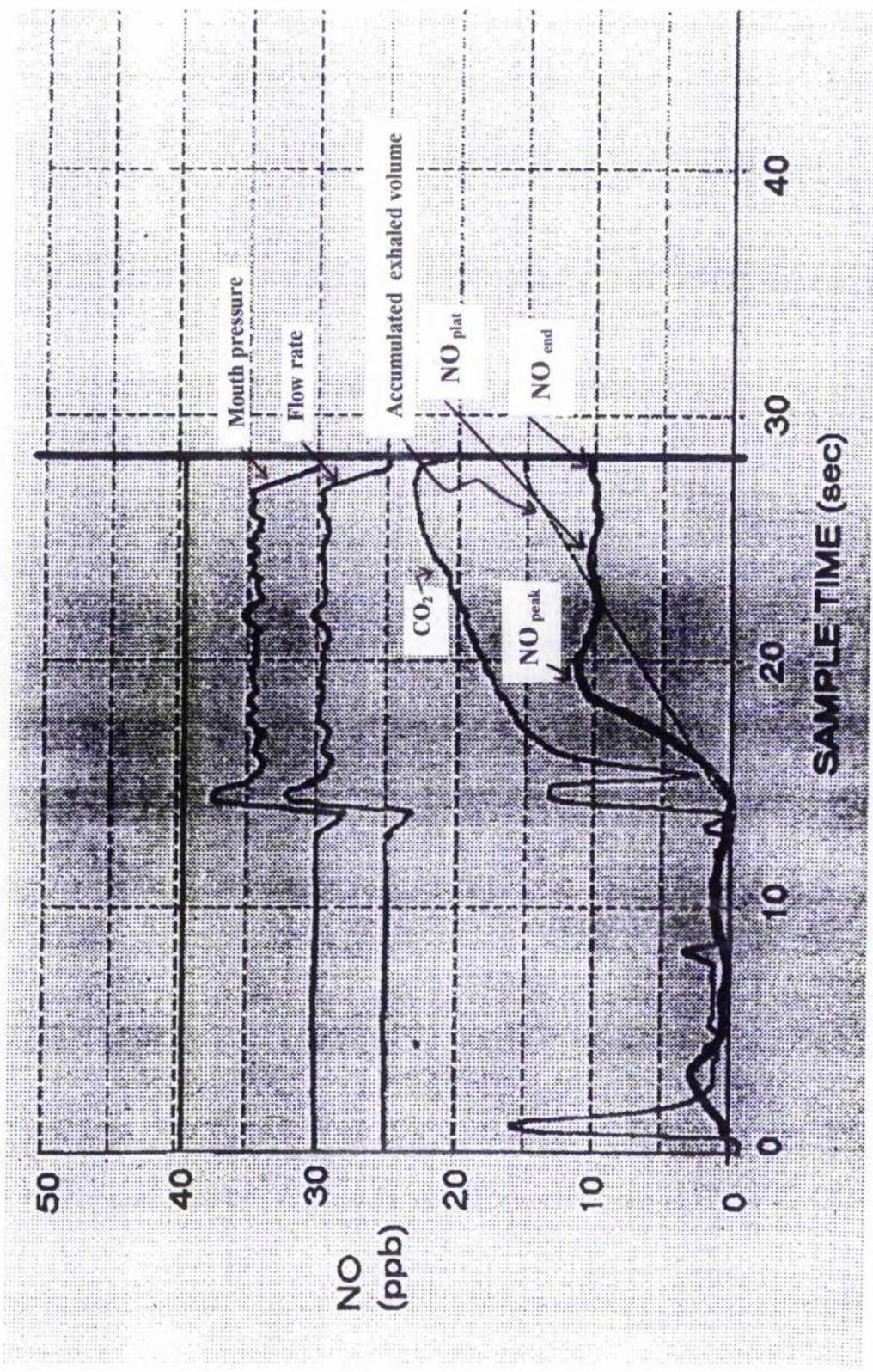
Figure 2. Screen on NO analyser displaying values obtained during a single exhalation. The three points analysed in Study 2.3.2 are illustrated as NO_{peak}, NO_{end} and NO_{plat}.

There is also evidence that inflammation is present in neonatal airways, even before the onset of infection and that the CFTR gene defect may cause upregulation of cytokine gene expression (Khan TZ, 1995). An interesting paper reported the prevention of CF in transgenic mice after in utero gene replacement (Larson JE, 1997). Although the results were preliminary and intriguing questions remain unanswered (e.g. what is the mechanism for this effect), the finding suggests that the CFTR gene is involved in the normal development of the foetus and its defect may result in effects which are predetermined in utero.

1.8.2 DIAGNOSIS OF CYSTIC FIBROSIS

In most cases the diagnosis of CF is considered when one or more typical clinical features are present and then confirmed by a finding of more than 60 mmol/l chloride in sweat by quantitative pilocarpine iontophoresis (Stern RC, 1997). The ability to detect CF mutations and to measure bioelectric properties (Knowles MR, 1995) across the nasal epithelium have greatly expanded the identification of clinical variation in CF. In 2% of patients there is an atypical phenotype which consists of chronic sino-pulmonary disease, pancreatic insufficiency and borderline (40-60 mmol/l) or normal (<40 mmol/l) sweat chloride concentrations. These are thought to be secondary to milder mutations of the CFTR gene which retain a partially functioning and appropriately localised CFTR protein (Rosentein BJ, 1998).

Neonatal diagnosis by prenatal screening programmes are available in most developed and Western countries. In utero diagnosis of CF, based on the detection of two CF mutations in the foetus by chronic villus sampling or amniocentesis is also available.

1.8.3 MANAGEMENT

Most of the morbidity and mortality in CF is attributable to chronic pulmonary disease. Chronic infection of the airways with *Staphylococcus aureus, Haemophilus influenzae* and *Pseudomonas aeruginosa* is typical (Fitzsimmon FC, 1993). Other pathogens such as *Burkholderia cepacia, Stenotrophomonas maltophilia, B gladioli, Aspergillus fumigatus* and non tuberculous mycobacteria are particularly problematic in some patients. Progressive decline in pulmonary function is due to a viscious cycle of airway obstruction, infection and inflammation. Airway inflammation plays a pivotal role as evidenced by raised levels of free DNA, IL8, free neutrophil elastase and neutrophils in the bronchoalveolar lavage fluid (Konstan MW, 1994).

The goals of therapy are to improve nutritional status, prevent or slow decline in pulmonary function and encourage a reasonable quality of life. This is best managed by a multidisciplinary team who can provide continuity of care over an extended period. Patients are encourage to maintain an unrestricted high fat, high caloric diet with liberal salt intake and vitamin and mineral supplements.

Pancreatic enzyme replacement for those requiring this usually takes the form of enteric coated tablets.

The approaches to the prevention of pulmonary deterioration include aggressive antibiotic regimens, airway clearance techniques, anti inflammatory agents, regular exercise, mucoactive agent and treatment of airway reactivity. Bilateral lung or heart lung transplant is the ultimate treatment for end stage lung disease. Recent results indicate 73% survival at one year, 63% at two and 57% at three years post transplant (Trulock EP, 1997). Bilateral lobar transplantation from living donors is offered at some centres as an alternative to cadaveric transplantation, with similar results. This approach is still ethically controversial but is partly driven by the lack of suitable donor organs. Some patients may benefit from non-invasive assisted ventilation while awaiting transplant.

The importance of aggressive antimicrobial treatment is generally agreed but how to do this is not. Potential approaches include short term treatment of acute exacerbations with oral, aerosol or intravenous antibiotics, and long term treatment with oral or aerosol antibiotics and long term treatment with quarterly courses of intravenous antibiotics. The efficacy and safety of high dose aerosolised Tobramycin are well established (Ramsey BW, 1993). It is usually used as a maintenance therapy in patients with chronically infected *P. aeruginosa* to slow the progression of lung disease and to increase the interval between exacerbations. In some centres, it is being used after the initial isolation of *P. aeruginosa* to delay chronic infection. Long term anti staphylococcal therapy early

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in life before the onset of pseudomonas infection is also been employed in some centres (Weaver LT, 1994). Use of dornase-alfa, an agent which depolymerises DNA and decreases the viscosity of airway mucous has been shown to reduce the frequency of pulmonary exacerbations and delay pulmonary decline in a subset of patients (Fuchs HJ, 1994).

Approaches to suppression of airway inflammation include oral alternate day corticosteroids, chronic use of inhaled steroids (Oermann CM 1999) and chronic high dose ibuprofen (Konstan MW, 1995).

1.8.3.1 NEW THERAPIES

Two intensely investigated new therapeutic strategies are gene therapy and more recently, 'protein repair therapy'.

Better understanding of the different types of CFTR mutations has led to new therapeutic approaches. Defects in CFTR production, folding, trafficking, or chloride conduction can be addressed by strategies organised in a genotype-specific way. Class I mutations decrease production of CFTR protein. About 5% of all mutations involve premature stop codons that lead to early termination of CFTR mRNA. These mRNAs are unstable and are never translated into protein. Certain aminoglycoside antibiotics (e.g. gentamicin) may suppress these 'nonsense' mutations and restore full length CFTR mRNA protein to the cell (Howard M, 1996).

Class II trafficking mutations of which δF508 is the most common are defects with respect to structural assembly in the endoplasmic reticulum and are not efficiently glycosylated or transported to the cell surface. This protein retains partial chloride channel activity and is transported to the cell surface after treatment with chemical chaperones like glycerol. These are thought to facilitate protein folding and stabilise protein structure, thereby promoting processing and trafficking to the cell surface (Brown CR, 1997). An alternative to these chaperones is phenylbutyrate, an oral analogue of butyrate that appears to regulate the expression of many genes including those for CFTR, γ globulin and those involved in cell differentiation (Perrine SP, 1993). It has been shown to promote the trafficking of the δF508 protein in vitro and in vivo.

Class III mutations are completely defective with respect to regulation and activation of chloride channel activity whereas class IV mutations (R117H) have partial response to cAMP mediated stimulation. Three agents are being investigated for CFTR activation in these genotypes. Milrinone is a phosphodiesterase inhibitor that improves chloride conductance (Kelley TJ, 1997). Genistein, a tyrosine kinase inhibitor that appears to augments the chloride channel via inhibition of protein phosphatase (Illek B, 1995) and CPX (cyclophenyl-1,3-dipropylxanthine) a xanthine adenosine receptor antagonist that specifically activates the δF508 chloride channel (Guay-Broder C, 1995).

1.8.3.2. GENE THERAPY IN CYSTIC FIBROSIS

The option of gene replacement therapy in CF is attractive since, theoretically, with one therapeutic intervention the myriad downstream effects of a mutant gene can be corrected. This is particularly true for CF since it is a monogenic disease and the target organ, the lung, is readily accessible. Vectors can be applied directly to airway epithelia without the need to harvest the target cells, modify them genetically and return them to the body. Rapid progress towards gene replacement has been made since the identification of the gene resulting in the first Phase 1 clinical trial in the nasal epithelium in 1991 (Zabner J, 1993). The most studied vectors are adenovirus, adeno-associated viruses and liposomes.

Recombinant adenoviral vectors are derived from human serotypes 2 and 5 of the group C adenovirus family. They are not oncogenic and the genome can accommodate 36 kb of double stranded DNA. This large capacity allows removal of selected genes involved in viral replication and replacement with the therapeutic promoter-cDNA construct. Gene delivery by adenovirus vectors in animal models has been limited by low vector entry rates in intact, non inflamed tissue and in CF patients by rapid induction of an inflammatory response to both viral vector and /or modified cells (Ho LP, 1998). Efforts to modify the virus/cell interaction to improve targeting in the lung, to allow repeat dosing, and/or to transiently manipulate the immune system to enhance gene expression are likely to dominate the field in the next few years.

AAV is a very small single stranded DNA defective parvovirus which cannot reproduce itself unless provided with a 'helper' virus like the herpes simplex virus. An AAV vector is formed by replacing the two endogenous viral genes with the promoter and/or cDNA of choice (Curiel DT, 1996). Advantages include the non pathogenic and non tumorigenic character of the wild type virus, the preservation of infectivity after both the viral genes are removed and the potential for site specific integration in the host genome which would tend to increase the persistence of the transduced gene (Flotte TR, 1995). Disadvantages are the lower efficiency when compared to adenoviral vectors and the limited capacity for large exogenous DNA sequences.

Synthetic non viral vectors of which cationic liposomes and cationic polymers are the major examples have advantages over viral vectors with respect to toxicity and immunogenicity (Gao X, 1995). They can deliver large DNA sequences and are easy to produce in large quantities. Gene transfer to the nasal epithelium of CF patients is associated with improvements in chloride transport but the effects are transient and repeat dosing will be necessary for sustained effect.

The march of gene therapy into human trials has been faster and more aggressive than anticipated. At least 11 trials (Ho LP, 1998) have been completed involving both viral and liposomal delivery to the nose and the lungs since the cloning of the gene 9 years ago. A large amount of work is currently being done on improving the vector and understanding the barriers to gene transfer and persistence. The outlook is optimistic but clinical trials have proven that many areas require further

work. One of these is the ability to detect, accurately, correction of the basic genetic defect and its downstream clinical improvement. Most available clinical measurements of efficacy have significant limitations - trans-epithelial potential difference measurements, fluorescence imaging of chloride channels and biopsies for inflammatory cell numbers are limited by information derived from only a small and possibly non representative area. Lung function tests on the other hand are too crude and insensitive to detect small changes in cellular activity and subclinical improvements. It was with this need for a sensitive, reproducible and non invasive method of measuring pulmonary inflammation in mind that this MD project was originally designed.

1.9 AIMS AND OUTLINE OF THESIS.

At the start of this MD project (1996), the aim was to examine the use of exhaled nitric oxide as a possible marker of airway inflammation and clinical severity in patients with cystic fibrosis. It was intended then, that, this measurement may be developed to a standard where it could be used as a clinical measure of efficacy and safety in the CF gene therapy trial in the lungs.

The expression of inducible NOS was known to be increased by proinflammatory cytokines (Robbins RA, 1994) and levels of NO have been reported to be increased in the exhaled air of asthmatics (Alving K, 1993. Kharitonov SA, 1994) and bronchiectasis (Kharitonov SA, 1995). In addition, NOS activity as measured by ability to convert L-arginine to L-citrulline have been found to be increased in CF lung homogenate (Belvisi M, 1995). As discussed, patients with CF have gross pulmonary inflammation. Therefore, I hypothesised that NOS II expression in the persistently inflamed airway epithelial and inflammatory cells in cystic fibrosis patients would be increased. This would be manifested as increased NO levels in the exhaled air which may reflect severity of airways inflammation in these subjects.

However, the first major finding from the project was that exhaled NO was *not* raised in patients with CF in contrast to asthmatics. This was a novel and an unexpected finding at that time and the aims of the study were then redirected

towards examining the possible explanations for this. Broadly, this may be due to two reasons: firstly NO production is increased but not detectable in the exhaled breath. This could be due to removal of gaseous NO by reaction with other free radicals or reactive oxygen species in chronically inflamed airways. Excess secretions could also prevent diffusion of the gaseous molecule into the lumen. Secondly, NO may be produced in lower quantities secondary to a defect in expression of the nitric oxide synthase gene which in turn may be related to the CFTR mutation, the chronic inflammatory state or lack of substrate (l-arginine).

Thus, the thesis first reports the development of exhaled NO as a method for measuring airways NO. Then it reports the finding of exhaled NO in CF patients with comparison to asthmatics and healthy volunteers. In order to explore some of the possible reasons for the observation that exhaled NO is not elevated in cystic fibrosis, the following studies were then conducted:

1) exhaled NO levels were examined in patients with bronchiectasis, a group with suppurative lung disease similar to CF to examine if excess secretion and chronic inflammation may be a cause for the lack of exhaled NO

2) molecular evidence of NOS II expression in CF epithelial cells lines (the purported cells responsible for elevated NO in asthmatics) and primary CF nasal epithelial cell cultures was examined

3) hydrogen peroxide levels in expired breath were measured to examine if levels of another volatile reactive species is increased

4) a different method of NO measurement was developed (nitrite in expired breath) and used in CF patients to explore if NO production may be elevated but not detected as gaseous NO in exhaled breath.

1.10 ETHICAL APPROVAL

There was ethical approval from the Lothian Ethics Committee for all studies involving patients. All subject gave informed consent.

CHAPTER 2

DEVELOPING THE METHODOLOGY OF MEASURING NITRIC OXIDE IN THE EXHALED BREATH

2.1 BACKGROUND

The history of exhaled NO measurement has already been discussed in chapter 1.7. At the start of this MD project (1996), my first aim was to establish a method of measuring exhaled NO levels which was sensitive, reproducible and excluded nasal air contamination. At that time, various methods of measurement were being used and there was wide variation in reported values of exhaled NO in normal and disease states (table 3). The methods available then were single breath collection, mixed exhaled air sampling from a bag and mean values over a few tidal breaths. I chose a new technique which appeared to have the capacity to eliminate nasal air contamination. My first priorities were to determine a representative point from the exhaled NO profile, examine other possible confounding factors and establish the reproducibility of this method in our laboratory.

2.2 THE EQUIPMENT

The nitric oxide analyser was purchased from Logan Research Limited (Rochester, U.K.). It is a rapid and highly sensitive chemiluminescence analyser (model LR 2000, version 2.2) and specified by the company to have a resolution of 0.3 ppb and a 95% response time (time between detecting zero amount of NO

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Author	Method of measurement	NO (ppb)	
		Normal subjects	Untreated asthma
Gustafsson L 1991	VC (gas collected in bag)	8±0.8	
Kharitonov S 1994	VC(over 35–45 sec)	80.2 ± 4.1 (peak)	283 ± 16
Persson MG 1994a	Slow VC (gas collected in bag), 15 sec breath hold	39.0 ± 4	62.6 ± 13.3
	VC, gas collected; end expiratory gas concentration	7.2 ± 1.0	
Persson MG 1994b	Tidal breathing	7.6 ± 0.7	
Schilling	Tidal breathing, gas collected	19 ± 8	
Alving K 1993	Tidal breathing	9 ± 1	22
Borland C 1993	VC, last litre gas collection as minute ventilation	9.4 l/min	
Lundberg 1994	VC	7 ± 2 (plateau)	

Table 3. Mean values of exhaled NO measurements from different investigators. VC=vital capacity, values are presented as mean ± standard deviation, in ppb except for Borland C et al.

and 95% of delivered NO) of 0.4 seconds. It is a single breath analyser connected to a dedicated personal computer and measures NO and CO_2 levels simultaneously in each exhaled breath. These levels are displayed on the screen during each exhalation. There is a short 'lag time' between detection and display on the computer screen of 1.8 seconds for NO and 1.4 seconds for CO_2. The value at any point of the NO exhalation profile is the average of 50 NO levels measured at 0.04 second intervals. The screen also displays the mouth pressure and exhalation flow rate (figure 2).

The amount of NO (vol:vol) in exhaled air is derived from the number of photons that are released from the degradation of NO_2 after NO in the exhaled air reacts with ozone (described in chapter 1.7). The generation of ozone and its reaction with NO is a vital part of the analysis. This takes place in a vacuum which is created by a pump in the analyser. Accurate operation of the analyser is crucially dependent on this vacuum. Carbon dioxide is analysed using a single beam infrared analyser incorporated in the machine.

Schematic illustration of this analyser is shown in figure 3.

An important part of the equipment is the exhalation circuit. This comprises an internal flow resistor which provides a small positive pressure at the mouth (5-20 cm H_2O). There is a visual (LED) feedback panel which tells the subject how much mouth pressure is being generated during the exhalation and the subject is coached during the procedure to keep this mouth pressure constant at 5 cm H_2O.
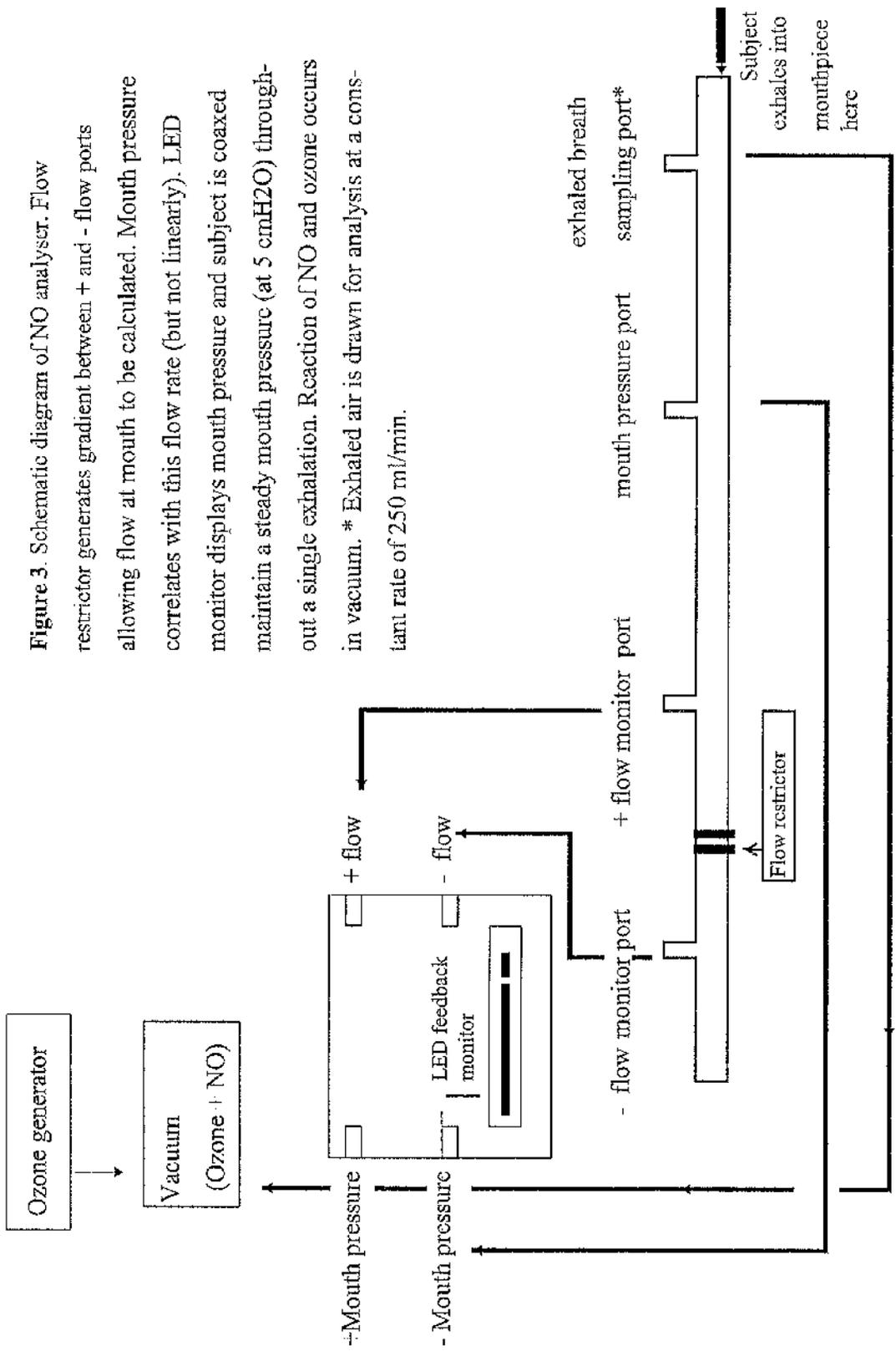
Figure 3. Schematic diagram of NO analyser. Flow restrictor generates gradient between + and − flow ports allowing flow at mouth to be calculated. Mouth pressure correlates with this flow rate (but not linearly). LED monitor displays mouth pressure and subject is coaxed to maintain a steady mouth pressure (at 5 cmH2O) throughout a single exhalation. Reaction of NO and ozone occurs in vacuum. * Exhaled air is drawn for analysis at a constant rate of 250 ml/min.

Theoretically, this creates a back pressure in the oral cavity during exhalation which results in the elevation of the soft palate. This in turn 'seals off' the nasopharyngeal area and eliminates mixing of nasal air with lower airways air. This theory was subsequently confirmed by a study published in 1997 where radioactively tagged argon gas flushed into the nasal cavity was shown to be undetectable in the exhaled air using this method (Kharitonov SA, 1997).

Air from each exhalation is extracted for analysis at 250 ml/min. However, this is affected by how fast the subject exhales, which in turn correlates directly with mouth pressure (although not in a linear fashion). Therefore, during each exhalation, the mouth pressure is the most important part of the maneouvre and constancy of all other parameters depends on keeping the mouth pressure constant.

The analyser is calibrated every third day of use with 103 ppb NO/N_2 gas mixture (BOC, Kent). The mouth pressure and flow rate are calibrated monthly using a hand held pressure manometer and a flow rotameter.

2.3 STUDIES

Three studies were performed to validate the exhaled NO method.

2.3.1 STUDY 1 - POSSIBLE CONFOUNDING FACTORS IN EXHALED NITRIC OXIDE MEASUREMENTS

AIMS

To examine the effect of the following possible confounding factors on exhaled NO levels:

i. ambient NO levels

ii. mouth pressure

iii. breath holding

2.3.1.1. AMBIENT NITRIC OXIDE LEVELS

METHODS

Since ambient NO levels in Edinburgh vary between 0.4 ppb and 50 ppb, it was important to examine if this affected the exhaled NO levels. This was studied in two ways. Firstly, in normal non smoking subjects (n=3), exhaled NO levels were measured after inhaling atmospheric air (with ambient NO level of 0.4 ppb) and air containing 80 ppb of NO. Increased levels of NO in the inspired breath were

generated by adding NO gas to the inspired air. A circuit to provide the correct admixture of NO in air was designed to allow compressed air to pass at 20 l/min through a carbon NO scrubber. This was passed into a T-piece connected to a tubing fed with 50 ppm NO at a flow rate of 0.04 l/min. The net admixture produced air containing about 75-85 ppb at the tube joining the T-piece. The absolute value was checked by the NO analyser and shown to be 80 ppb. Secondly, exhaled NO levels were measured (in 3 normal non smoking subjects) on four days with different levels of ambient NO- 0.2 ppb, 5 ppb, 30.5 ppb and 50 ppb.

RESULTS

During exhalation, the plateau NO level, in contrast to the peak NO level, did not differ whether subjects inhaled 0.4 ppb of ambient NO or inspired air containing 80 ppb NO (table 4).

In keeping with this finding, there was no significant difference in end expiratory (plateau) exhaled NO levels in all three subjects when inhaling different ambient levels (table 5). However it was noteworthy that the peak and early levels in the exhalation profile were markedly affected by the ambient levels of NO. Therefore, if the plateau levels were not attained as in subjects with smaller vital capacity (e.g. in diseased lungs), exhaled NO levels may not be accurate.

Inhaled NO	Subject 1		Subject 2		Subject 3	
	Peak NO	Plateau NO	Peak NO	Plateau NO	Peak NO	Plateau NO
Atmospheric air (0.4 ppb NO)	7.9	7.8	8.6	7.6	3.9	3.5
80 ppb after a single inhalation.	39.0	7.7	36.1	5.8	25.8	3.1
	44.2	7.7	48.0	6.3	26.9	2.7
	40.1	7.4	45.3	6.8	23.6	2.4
80 ppb after 3 tidal breaths		7.8				
80 ppb after 5 tidal breaths		7.3				
80 ppb after 5 minutes of tidal breathing		7.3				

Table 4. Table showing effect of inhaling increased levels of inspired NO on exhaled NO values .

Ambient NO levels	Subject 1		Subject 2		Subject 3	
	NO peak	NO plat	NO peak	NO plat	NO peak	NO plat
0.2	5.4	5.0	6.2	5.1	8.2	7.5
5.0	5.5	5.1	6.7	6.0	8.8	6.1
30.5	31.0	6.0	24.9	5.9	22.0	7.9
50.0	44.0	8.2	39.8	6.3	33.0	8.1

Table 5. Effect of ambient NO levels on highest level of NO on exhalation profile (NO_{peak}) and value at end expiration of exhalation profile (NO_{plat}).

2.3.1.2. MOUTH PRESSURE

METHODS

Although, the manufacturer had recommended a mouth pressure of 5 cmH_2O, I was concerned that some patients may not be able to maintain this specific level. Therefore, I examined if changes in mouth pressure affected exhaled NO levels and if there was a range that could be allowed for patients who experienced difficulties with maintaining the specific value of 5 cmH_2O. Three normal, non-smoking subjects were recruited. In each, mouth pressure and (consequently flow rate) were varied during exhalation (2, 5 and 10 cm H_2O) and exhaled NO levels recorded at the end (plateau) of the exhalation profile.

RESULTS

There was no significant difference between NO levels taken at 5 and 10 cmH_2O but levels at 2 cm H_2O were significantly higher (table 6). In addition, NO levels taken at 2 cmH_2O mouth pressure were highly variable. This suggested that 5 cm H_2O may be the threshold of mouth pressure required to ensure adequate sealing off of the nasopharyngeal area by the soft palate. Pressures lower than that may allow leakage of nasal air and may explain the variability in NO levels.

MP	Subject 1		Subject 2		Subject 3	
(cm H_2O)	NO_{end}		NO_{end}		NO_{end}	
2	5.0	11.2	8.5	13.4	18.8	11.0	5.4	2.4	5.9
5	5.6	5.0	5.8	8.7	8.0	9.1	4.4	4.3	4.0
10	5.0	5.8	6.1	8.1	8.8	8.2	4.1	4.9	4.9

Table 6. Effect of different mouth pressure during a single exhalation on the highest level of NO on exhalation profile (NO_{peak}) and NO value at end expiration of exhalation profile (NO_{plat}). MP = mouth pressure.

2.3.1.3. BREATH HOLDING

METHODS

At this point of the study there were reports using other methods (which did not exclude nasal air contamination) that suggested that breath holding affected NO levels. The same subjects (n=3) were asked to exhale without breath holding and after 10 and 20 seconds of breath hold. This was repeated with subjects exhaling immediately after 2 minutes of continuous conversation.

RESULTS

For breath holding, there was a clear increase of peak levels with increased breath hold time but end expiratory levels were not affected (Table 7). Talking before measurement also affected the peak NO levels. This was likely to be secondary to inadvertent breath holding during conversation.

Breath hold (secs)	Subject 1		Subject 2		Subject 3	
	NO $_{peak}$	NO $_{end}$	NO $_{peak}$	NO $_{end}$	NO $_{peak}$	NO $_{end}$
0	4.1	3.9	7.0	7.1	7.0	5.9
5	5.5	3.8	10.1	6.5	18.7	6.9
10	13.5	3.9	15.1	6.9	22.4	7.8
20	16.1	3.4	19.9	7.1	29.1	7.5
After 2 minutes of conversation	8.5	4.0	12.1	7.0	7.9	7.6

Table 7. Effect of breath holding and conversation on the highest level of NO on exhalation profile (NO $_{peak}$) and NO value at end expiration of exhalation profile (NO $_{plat}$).

2.3.2. STUDY 2 - REPRESENTATIVE NITRIC OXIDE LEVEL ON EXHALATION PROFILE

AIM

To determine the most appropriate point on the NO exhalation profile to record the representative NO level for a subject.

METHODS

The single breath profile is characterised by a small peak and then a longer plateau (figure 2). To determine the agreement at different parts of the profile, NO levels were taken at the:

a) end expiratory point, (NO_{end}, figure 2) defined as the point within the last 3 seconds at the end of expiration (corresponding to the near plateau level of the CO_2 profile)

b) plateau point (NO_{plat}), defined as any point after the first three seconds of achieving a plateau

c) peak level (NO_{peak}) - the highest NO level in a single exhalation

These points were chosen because NO_{end} , No_{plat} are likely to represent the values from the end of expirate and hence the lowest part of the airways. These would be the ideal representative levels. However, the most appropriate point on the plateau phase of the profile was not known and the study examined if there was any significant difference between values near the beginning of the plateau compared to

the end of an expiration. NO_{peak} was chosen as a comparison because this was one of the standard methods of measurement at that stage of exhaled NO research.

Three normal non-smoking subjects with no clinical evidence of upper respiratory tract infections were recruited for these studies. Levels were taken 7 times sequentially on 2 different days. Subjects were seated upright and asked to exhale slowly from total lung capacity following a full inspiration. Subjects wore nose clips and maintained a mouth pressure of 5 cmH_2O. The procedure was repeated to obtain seven technically acceptable manoeuvres (based on manoeuvres performed without breath holding or interruption e.g. from coughing and maintenance of constant mouth pressure of 5 cmH_2O for at least 80% of the exhalation profile). Levels were recorded at points of interest (NO_{peak} , NO_{end} and NO_{plat}) on each exhalation. ANOVA was then performed between these points.

RESULTS

There was no significant difference between levels taken at a point after 3 seconds of plateau had been achieved (NO_{plat}) and at the end of the plateau (NO_{end}), suggesting that levels taken at any point after the first 3 seconds of achieving a plateau are representative of the single exhalation profile. Using pairwise multiple comparison in one way analysis of variance (ANOVA), NO_{peak} was found to be significantly higher than NO_{plat} and No_{end}. NO_{peak} was significantly higher than No_{plat}. The peak value was also more variable within each subject compared to plateau levels and sensitive to even short periods of breath holding.

All values are shown in table 8.

Other observations

During the course of these preliminary experiments, it was noticed that condensation build-up was a frequent occurrence in the exhalation arm of the setup. Since NO is a highly reactive molecule, I examined if the presence of water in the path of the exhaled air affected the NO levels. This was done by measuring NO levels before and after drying the tubing with high flow oxygen flushing. There was no significant difference in the NO levels before and after drying the tubing; there was a drift towards lower levels with the presence of condensate.

FINAL METHODOLOGY FOR EXHALED NO MEASUREMENT

The final method adopted for the measurements in the project incorporated the following features:

1. The NO analyser is set at an extraction flow rate of 250 ml/min.

2. The ambient nitric oxide level is measured by allowing the analyser to sample room air over 30 seconds. If the background levels were high (>10 ppb) and the subject is unable to achieve a substantive plateau (>5 seconds) in the exhalation profile, the procedure is abandoned as an accurate level would not be obtained.

3. All tubing in the collection system is ensured to be free from condensation.

| | Patient 1 | | | | | | Patient 2 | | | | | | Patient 3 | | | | | |
| | Day 1 | | | Day 2 | | | Day 1 | | | Day 2 | | | Day 1 | | | Day 2 | | |
	NO peak	NO plat	NO end	NO peak	NO plat	NO end	NO peak	NO plat	NO end	NO peak	NO plat	NO end	NO peak	NO plat	NO end	NO peak	NO plat	NO end
mean	7.2	3.9	4.0	7.0	4.0	4.7	11	7.1	7.0	10.1	7.5	7.2	4.0	2.4	1.9	4.6	3.2	3.0
S.D.	2.6	0.5	0.3	2.7	0.5	0.3	3.2	0.4	0.6	2.4	0.5	0.7	0.8	0.9	0.4	0.9	0.6	0.5
SEM	0.9	0.2	0.1	1.0	0.2	0.1	1.1	0.2	0.2	0.8	0.2	0.2	0.3	0.3	0.1	0.3	0.2	0.2
COV	36.1	12.8	7.5	39	12.5	6.0	29.0	5.6	8.5	24.0	6.7	9.7	20.0	38.0	21.0	20.0	19.0	16.0

Table 8. Mean values of exhaled NO levels on three patients taken from highest point on exhaled profile (NO peak), within first and last 3 seconds of exhalation plateau (NO plat and NO end respectively), with their respective standard deviation (SD), standard error of means (SEM) and coefficient of variation (COV).

4. The subject is asked to sit upright facing the NO analyser. The procedure is explained to the subject and the subject is asked to breathe normally and discouraged from talking just before the procedure.

5. The subject inspires to total lung capacity and exhales immediately into the mouthpiece while watching the visual biofeedback. Mouth pressure is kept constant at 5 cm H_2O. If there are difficulties in maintaining this pressure, subjects are asked to exhale over the target (> 5 cmH_2O) rather than to allow the pressure to fall below 5 cmH_2O.

6. The subject exhales until the NO profile reaches a plateau and the CO_2 profile a near plateau (it is not physiologically possible to reach a complete plateau for CO_2). A plateau is defined as a horizontal line at an angle of 25° or less, for more than 5 seconds. This is usually visually discernible.

7. This is repeated three times and the representative value from each profile is taken from the end expiratory point. The mean of three levels is taken as a representative value for that subject.

2.3.3. STUDY 3 - REPRODUCIBILITY OF FINAL METHOD

AIM

To examine the reproducibility of the final method.

METHODS

NO plateau levels were obtained from seven subjects on three to six occasions separated by at least a day. All subjects were healthy non-smokers with no respiratory symptoms. To test reproducibility of plateau NO measurements, Bartlett's Test for equality of variance within each patient was first applied. Analysis of variance was then used to determine variance and thus global standard deviation.

RESULTS

Individual results are shown in table 9.

Equality of variance within each patient tested by Bartlett's test showed no significant difference between patients. ANOVA for seven subjects with repeated measurements (between 3-6 times) of exhaled NO gave a pooled standard deviation of 0.84 ppb, and a coefficient of variation = 0.24. The 95% confidence interval for any measurement of NO was thus ±1.72 ppb.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Subject 1	5.5	4.2	6.2	7.1	6.2	5.9
Subject 2	4.9	4.1	5.5	3.5	N/D	N/D
Subject 3	2.4	3.9	1.7	1.9	1.0	0.7
Subject 4	5.1	4.2	4.7	2.9	3.5	4.7
Subject 5	2.2	2.9	2.5	2.9	3.1	3.0
Subject 6	2.6	2.1	4.0	3.1	3.6	N/D
Subject 7	2.2	2.3	3.2	N/D	N/D	N/D

Table 9. Exhaled NO levels (ppb) of 7 normal, non smoking subjects on 6 different days.

2.4 DISCUSSION

The accurate measurement of nitric oxide production in the lower airways is crucial to the examination of its role in the airways. In order to derive correct conclusions it would be important to know what exhaled nitric oxide represents – does it reflect lower airways NO production, if so how much of it does, what is the contribution of different parts of the airways to the exhaled values and what factors affect these measurements?

In 1996, Massaro and co workers demonstrated that exhaled NO levels were indicative of lower airways NO production when they showed that bronchoscopic measurements of NO in the lower airways of asthmatics revealed levels that were elevated and comparable to those measured in the exhaled breath (Massaro AF, 1996). Subsequently, Gabay et al (Gabay E, 1998) using an identical analyser to the one used in this study, showed that there was a good agreement between bronchoscopically measured levels in the lower airways and that at the end expiratory levels in exhaled air. They also confirmed my findings on the repeatability of NO levels at the end expiratory phase of the exhalation profile.

The studies above demonstrated that if all criteria for accurate measurements are followed (as specified under 'Final methodology'), then this method is a reproducible way of measuring NO production from the lower airways. The only caveat was that on days when the ambient NO levels are high (>10 ppb) measurements can only be made in subjects who are able to exhale long enough to obtain a plateau level. This may be a significant limitation in patients with small

vital capacity like those with cystic fibrosis patients and severe bronchiectasis. Fortunately, in Edinburgh, high levels of background NO were very uncommon (< 5% of a month, observed during the preliminary studies). In other cities e.g. London, where levels in excess of 50 ppb are common place, NO free inspired air may be needed.

The finding that high inhaled NO levels did not affect exhaled end expiratory levels of NO was interesting (table 4). Although my aim was to determine whether ambient NO affected expired lower respiratory tract NO, the results also suggested that gaseous NO appears highly labile within the airways. Within the duration of a single inhalation and exhalation (approximately 10-15 seconds) it has either diffused into the pulmonary circulation or metabolised to other products. The most likely explanation is that inhaled NO reaches the alveoli and is immediately absorbed into the pulmonary circulation (Hyde RE, 1997). The first phase of the exhaled breath represents air from anatomical and physiological dead space and reflects high NO levels that are still present this part of the airways. The last phase of the expirate reflects NO levels from the lower parts of the airways. Results from this experiment suggest two points - firstly that NO produced in the vicinity of the alveoli is likely to be reabsorbed very quickly and not reflected in the expirate. Secondly what is detected at the end of an expired breath probably represents high and continuously produced NO from the airways (rather than parenchyma). This method may, therefore, not be able to detect lower (but still elevated) levels of NO generation.

At present, it is unclear where exhaled NO originates. It is likely to represent luminal production since NO in the circulation is tightly bound to the haem component of haemoglobin (Stamler JS, 1988) and does not contributed to the gaseous pool of nitric oxide. However, recent evidence suggest that in disease states the decrease in pH at the air-fluid interface in alveoli encourages the dissociation of NO-haem complexes and under such conditions, luminal NO may be increased by NO from the pulmonary circulation (Gow AJ, 1998). Also, the cellular source of NO production remains unclear. NO is produced by almost all cell types within the airways (Nathan C, 1994). It is likely that airway epithelia by virtue of size and the number of NO producing units may contribute most to an increase in levels. It is also unknown how much each isoform of NOS contributes to the level of exhaled NO. In normal states, nNOS is thought to contribute about 30% of exhaled NO levels in mice. This is derived from studies comparing normal and nNOS knockout mice (De Sanctis GT, 1997). However, in disease states like asthma, it is likely that NOS II driven NO makes up most of the increase in NO levels. NOS II expression in the epithelium of asthmatics have been shown to be increased (Hamid Q, 1993) and NO production by this enzyme is much greater than that from the constitutive enzymes (Vodovotz Y, 1994). No studies have examined the expression of the other isoforms in asthma.

Thus the method used here carries the above unanswered questions and limitations. Exhaled NO levels remain a non specific marker of NO production in the airways.

2.5 CONCLUSION

Exhaled NO can be measured easily and reliably provided *all* criteria for measurements are followed. The main limitation is that it is a non discriminating measure of NO production from the lower airways.

The 95% confidence interval for each individual measurement is ± 1.72 ppb.

CHAPTER 3

EXHALED NITRIC OXIDE LEVELS IN PATIENTS WITH CYSTIC FIBROSIS COMPARED TO NORMALS AND ASTHMATICS.

3.1 INTRODUCTION

As discussed in chapter 1, expression of NOS II is increased by proinflammatory cytokines and LPS and patients with CF have severely inflamed airways. Therefore, it can be hypothesised that NO production is increased in this inflamed environment. The study examines NO levels in the exhaled breath of CF patients and compares this with normal controls and asthmatics.

3.2 HYPOTHESIS

Exhaled NO levels in patients with cystic fibrosis are increased compared to normal subjects and may reflect clinical severity in these patients.

3.3 STUDY DESIGN

Subjects and patients

Thirty six patients with cystic fibrosis were recruited from the Scottish Adult Cystic Fibrosis Service, Edinburgh. All except three had fully defined CF genotypes (the remaining three were $\delta F508/?$). Disease severity varied widely,

with predicted FEV_1 ranging from 18% to 100%. Patients with concomitant asthma and those who were on inhaled steroids were excluded.

As a positive control, 34 patients with asthma were recruited from the respiratory outpatient clinic and from general practices. All patients had documented reversible air flow obstruction and were on inhaled β_2-agonist. 23 were on inhaled steroids (doses ranging from 400µg to 3200µg per day). Eleven asthmatics were steroid naive. The FEV_1 for the whole group ranged from 34% to 100% predicted. All patients were non smokers and clinically stable. Twenty-two healthy volunteers were recruited from hospital staff. These were non- smokers, had no respiratory tract symptoms and did not have active allergic rhinitis at time of sampling. The mean ages ($\pm$ SD) were 32.0 (12.6), 27.1(8.6) and 45.2 (16.8) years for normal subjects, CF and asthma patients respectively.

In 10 CF patients (8 males, mean age 26.5 years), exhaled NO was also examined during infective pulmonary exacerbations. Levels were measured within 24 hours of onset of an infective exacerbation, defined as a worsening of clinical condition requiring intravenous antibiotics accompanied by a fall of 400 mls or 20% of usual FEV_1. All measurements were performed before or within six hours of commencement of IV antibiotics. In eight of these subjects, serial exhaled NO measurements and lung function were recorded over seven days.

Informed consent was obtained from all subjects and the study was approved by Lothian Health Ethics Committee.

Measurement of exhaled nitric oxide

Nitric oxide was measured using a sensitive chemiluminescence analyser (LR 2000, Logan Research Ltd, Kent, U.K.) as described in Chapter 2.

3.4 STATISTICAL ANALYSES

For comparison of NO levels between groups, Mann Whitney rank sum test was used since the asthmatic groups showed deviation from normal Gaussian distribution. Correlation between lung function with exhaled NO in the CF group was measured using Pearson's Product Moment correlation test. For all tests, $p < 0.05$ was assumed to achieve statistical significance.

3.5 RESULTS

Exhaled NO levels in cystic fibrosis, asthma and normal subjects.

NO levels in patients with CF and normal subjects did not differ significantly from each other. The median value in patients with CF was 4.0 ppb (interquartile range, 3.1) and normal subjects had a median value of 4.4 ppb (interquartile range, 3.4) (figure 4). Patients with asthma had a significantly higher median value compared to all the other groups (10.4, interquartile 16.9). There was a positively skewed distribution and a large range of values in the asthmatic population.
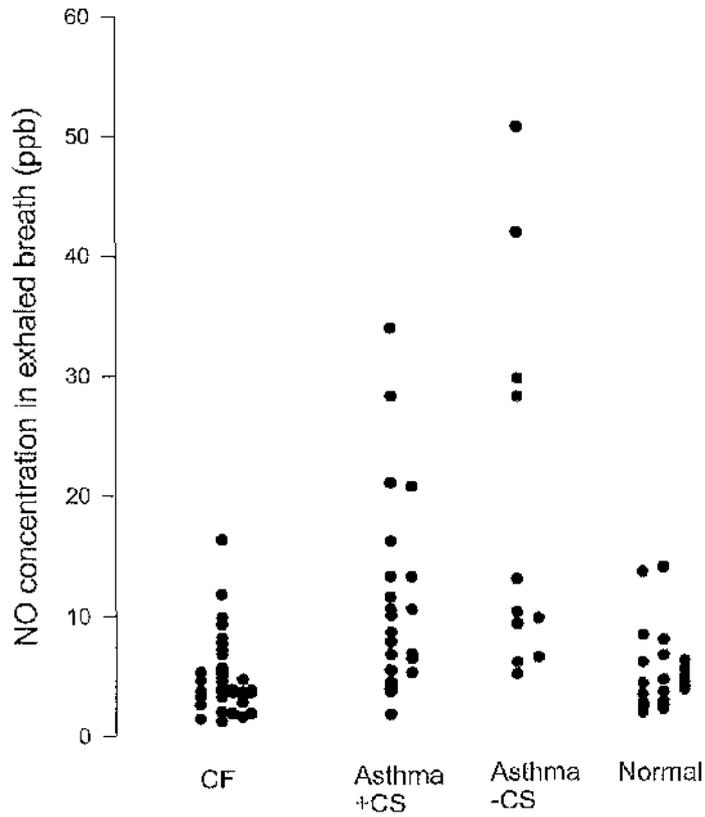
Figure 4. Exhaled NO levels in patients with cystic fibrosis, asthma and in normal subjects.

CS= inhaled corticosteroids. CF= cystic fibrosis.

Correlation between lung function and exhaled NO levels in stable patients.

There was a weak but statistically significant positive correlation between exhaled NO and FEV_1 in patients with CF (r = 0.48, p < 0.05) (figure 5). Thus, patients with better lung function appeared to have higher exhaled NO levels. This was not seen in bronchiectasis or asthma.

Infective pulmonary exacerbations and exhaled NO levels in patients with cystic fibrosis.

During pulmonary exacerbations exhaled NO remained within the normal range (figure 6). In 7 out of 10 patients, the values were the same or lower than when clinically stable. In 3 patients there was a small but insignificant rise. For eight patients serial exhaled NO measurements made over seven days after commencement of IV antibiotics showed no consistent trend with time (figure 7).

3.6 DISCUSSION

In this study, a method which excluded nasal air was used and a single exhalation flow rate for all subjects were employed. With this method , it was demonstrated that exhaled NO levels in CF and bronchiectasis were no different from that in normal subjects. However, in keeping with published work, high levels were detected in asthmatics. These findings in cystic fibrosis were supported by reports,
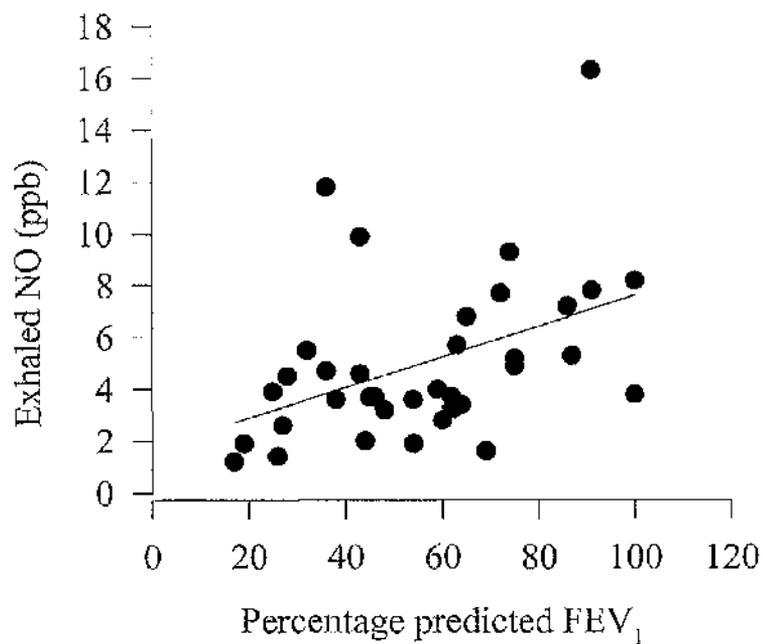
Figure 5. Relationship between exhaled NO levels and lung function in cystic fibrosis patients.
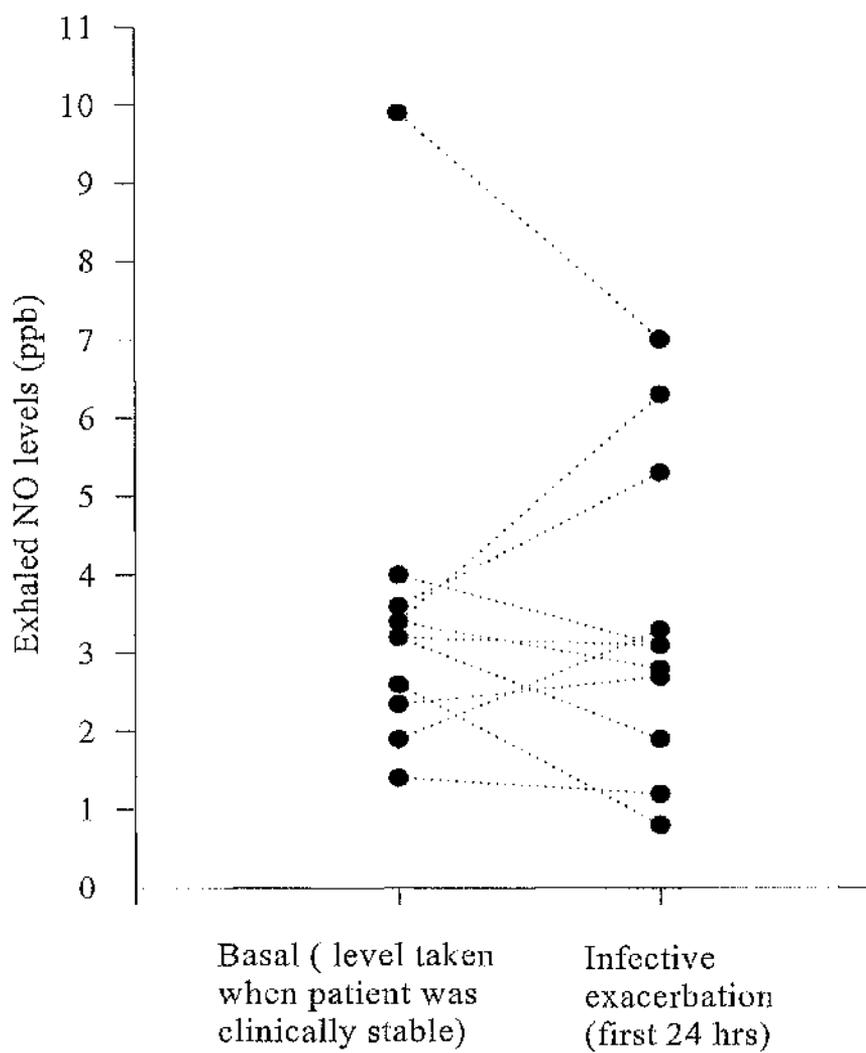
Figure 6. Exhaled NO levels in patients with cystic fibrosis while clinically stable and during the first 24 hours of infective exacerbation.
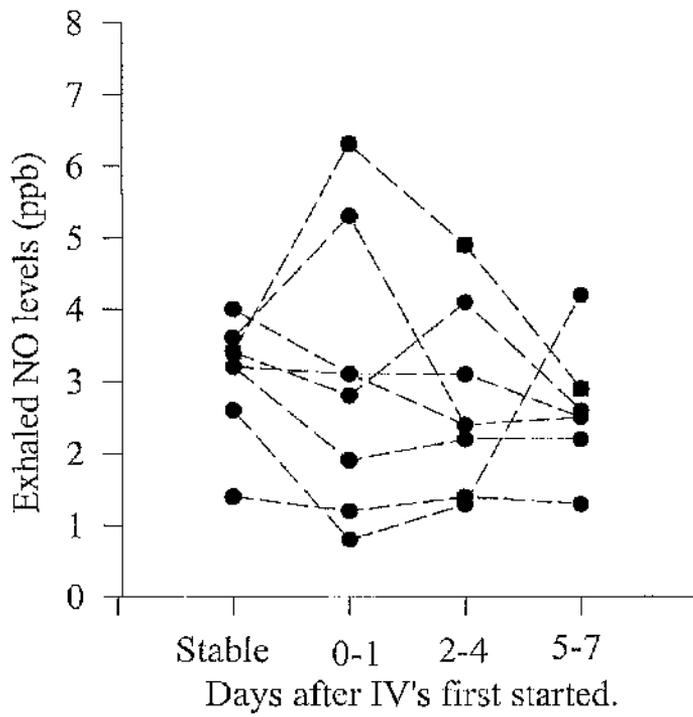
Figure 7. Exhaled NO levels in CF patients over first 7 days of treatment of infective exacerbation .

all in children, which were published around the same time. Methodological differences did not allow absolute comparison but all reported NO values in CF patients that were no different from control subjects (Lundberg JON, 1996. Balfour-Lynn IM, 1996. Dötsch J, 1996). In none of these studies, however, was nasal NO definitely excluded. In one study (Dötsch J, 1996) the response time of the analyser was slow, risking "contamination" from the ambient air, and indeed the study showed good correlation between exhaled NO and ambient NO. Furthermore, levels were obtained from a collection bag containing mixed expired air. In the third study, patient numbers were small.

The absence of elevated levels of NO in the exhaled breath of patients with CF has a number of potential explanations. There may be a decrease in diffusion of NO across secretions of increased viscosity and volume. Alternatively, in a suppurative and inflamed environment, the presence of reactive oxygen species in the vicinity of the NO production may result in chemical interaction with the effective removal of NO by formation, for example, of peroxynitrite (Wink D, 1993). As discussed in chapter 1, NO has been shown to be a potent scavenger of reactive oxidants in the lung. Either explanation would be consistent with our finding that NO levels in the breath of cystic fibrosis patients correlated positively with lung function. Patients with mild disease would have less viscid and fewer secretions, and less production of reactive oxygen species.

Low NO generation in CF and bronchiectasis may have important implications for host defence, since low levels of NO produced by the epithelium may result in

decreased local bacterial killing and thus facilitate chronic bacterial colonisation and repeated infections.

Two other findings from the study were interesting. Firstly, in CF patients with infective exacerbation, levels did not change during the first 24 hours of infection and with follow-up over time. This could mean either that there is a failure to upregulate NOS II in response to infection and increased inflammatory stimuli or that there was an increase in NO production but this was not detected because of the blanket effect of airways secretions. Secondly, patients with better lung function had higher NO levels. This was at odds with the theory that exhaled NO levels reflect airway inflammation. In addition to the suggestion above that detection of gaseous NO may be improved in those with better lung function and hence less viscid secretions, this also supports the possibility that down regulation of NOS II occurred in the airway epithelia of patients with worse lung function and therefore, more severe inflammation.

Although the measurement of exhaled NO in asthmatics was not the principal purpose of this study sufficient numbers were studied in this positive control group to observe wide variation in levels from patients who were steroid naïve. In some patients with more severe asthma but who were on inhaled steroids, levels were noted to be lower. Steroids may well reduce NO levels by downregulation of NOS II thus explaining the observation (Kharitonov SA 1996a&b. Yates DH 1995). This questions whether the elevated exhaled NO levels in asthma are a reflection of the clinical severity of asthma, a guide to the degree of airways

inflammation present or a marker of balance of cytokine activities in the airways and NOS II regulation.

3.7 CONCLUSION

Using a method which excluded nasal air contamination and applying a standard exhalation rate in all our patient groups, exhaled NO levels are not elevated in the inflammatory airways disease of CF, in contrast to asthma. This is the case even during pulmonary infective exacerbations. Exhaled NO is not a helpful clinical marker of airways inflammation in CF.

CHAPTER 4

EXHALED NITRIC OXIDE LEVELS IN PATIENTS WITH BRONCHI-ECTASIS

4.1 INTRODUCTION

The findings from Chapter 3 suggest that the lack of increase in NO levels in CF compared to asthma may be due to lack of detection or lack of production of the gas. To explore the former, levels in patients with bronchiectasis were determined using the same methodology. Bronchiectatic patients were chosen because these patients have very similar airway pathology to CF patients. Both display damaged airways which allowed pooling of excess secretions, bacterial colonisation and recurrent infection. Airways inflammation is driven by chronic bacterial presence.

4.2 HYPOTHESIS

It is hypothesized that if exhaled NO levels in patients with CF are reduced due to poor gaseous diffusion into the lumen, then the same phenomenon would be observed in patients with bronchiectasis.

4.3 STUDY DESIGN

Patients

Sixteen patients with bronchiectasis were recruited from the respiratory outpatient clinic. Bronchiectasis had been diagnosed by classical chest x-ray changes in 6 patients, by classical CT changes in 4 patients and by bronchography in 6 patients. In five patients, bronchiectasis was secondary to childhood bronchopneumonia, in three to tuberculosis, one to whooping cough, two to measles and five had no determined cause for their bronchiectasis. Six patients were on inhaled steroids. None had evidence of asthma or allergic bronchopulmonary aspergillosis. The group had widely varying disease severity (FEV$_1$ 32% to 91% predicted).

Since the difference in mean age between the CF and bronchiectasis group was necessarily large (27.1 years v 57.2), we also tested the relationship of exhaled NO with age. For this, 22 non smoking, normal subjects with no evidence of active atopy or respiratory tract infection were recruited (age range 23 to 66y).

Measurement of exhaled nitric oxide

Nitric oxide was measured using a sensitive chemiluminescence analyser (LR 2000, Logan Research Ltd, Kent, U.K.) as described in Chapter 2.

4.4 STATISTICAL ANALYSES

For comparison of NO levels between the two groups of patients, the Student t-test was used. Correlation between age and exhaled NO was measured using

Pearson's Product Moment correlation test. For all tests, p<0.05 was assumed to achieve statistical significance.

4.5 RESULTS

NO levels in patients with bronchiectasis

The mean value of exhaled NO levels in bronchiectasis was 6.2 ppb (S.D 2.24) compared to 5.0 ppb (S.D 3.2) in CF patients (figure 8). There was no significant difference between the two groups. Similarly when patients with bronchiectasis were divided into those on inhaled steroids and those that were not, the NO levels were not significantly different.

Effect of age on NO levels

There was no correlation between age and exhaled NO; r = 0.28, p = 0.2 (healthy volunteers) (figure 9)
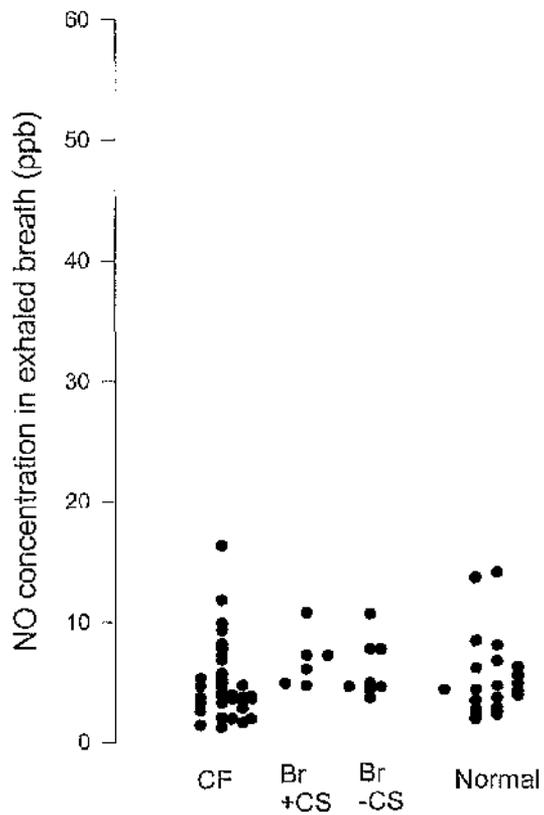
Figure 8. Exhaled NO levels in bronchiectatic patients compared to patients with cystic fibrosis and normal subjects. CS= inhaled corticosteroids, Br= patients with bronchiectasis.
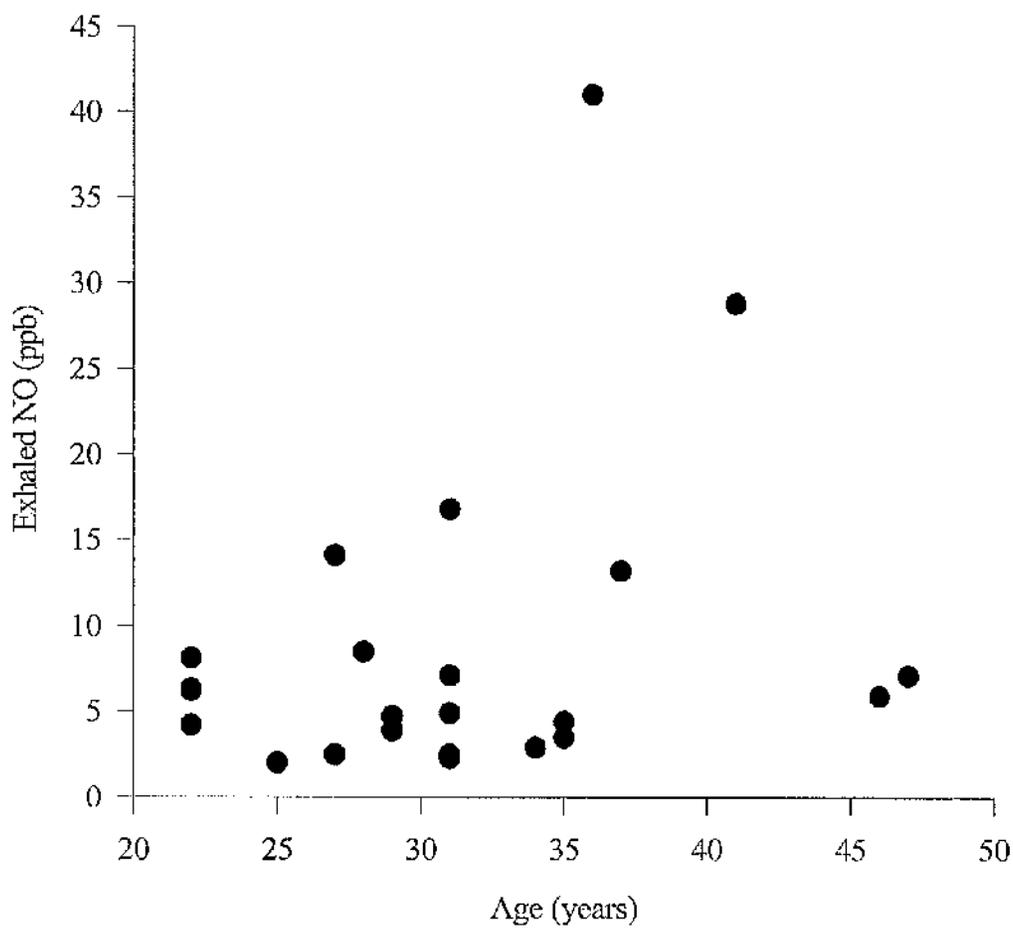
Figure 9. Effect of age on exhaled NO levels.

4.6 DISCUSSION

The findings in bronchiectasis were unexpected, since one report had indicated that exhaled NO was increased in bronchiectasis (Kharitonov SA, 1995). The different methodologies used in measuring exhaled NO may explain the different results in the bronchiectasis patients. Kharitonov and colleagues used the peak level from a long and slow exhalation as the representative NO value. The peak level can be very variable and sensitive to even a short period of breath holding and, as shown in chapter 2, be influenced by ambient NO levels.

This result suggests that NO in patients with CF is low because of inability to detect NO. In both bronchiectasis and CF, gaseous NO could have been prevented from diffusing into the airway lumen, either because of physical impedance or chemical removal. In the latter, reaction with other reactive species in an inflamed environment could effectively remove gaseous NO from the local environment. The inflammatory cell influx in both these conditions produces a large amount of superoxide and hydrogen peroxide (Greening AP, 1983. Shult PA, 1985. Kinnula VL, 1992a&b.). These highly reactive molecules can react with NO to produce peroxynitrite and hydroxonium ions (Gaston B, 1994). This would not only effectively remove gaseous NO from the environment but also enhance the potential for local tissue damage.

Although the finding of low NO in both suppurative conditions suggests the above theory, this cannot be concluded merely from data collected so far. It does not

exclude the possibility that NOS II expression is decreased in chronic inflammation. As discussed in chapter 1, it has been shown that NO has an auto inhibitory effect on NOS expression (Rogers NE, 1992. Griscavage JM, 1993. Assrcuy J, 1993). Also, NOS II protein degradation appears to be enhanced by TGFβ which is produced in large quantities by activated macrophages (Ding A, 1990).

The implications of low NO production in the setting of these diseases are interesting. If production of No is truly decreased, then the protective anti inflammatory effects of NO could be lost and inflammation further enhanced. An even more important loss would be that of the anti microbial effects of NO. This would be particularly pertinent in the setting of chronic bacterial colonisation as seen in CF and bronchiectasis.

Another relevant effect of NO in CF and bronchiectasis is the modulation of chloride channel activity. Kamosinska et al showed that NO can increase chloride currents in human epithelial cell line (Kamosinska B, 1997). There is also evidence that NO is necessary for ciliary motility (Xue C,1996. Jain B, 1995). Curiously, patients with Kartegener's syndrome (ciliary dyskinesia and situs inversus) have almost undetectable levels of nasal NO compared to normal subjects (Lundberg JON, 1994). It is unclear from that study whether this is a secondary or primary phenomenon. NOS expression was not examined.

Finally, if the assumption that exhaled NO or NO production is a reflection of airways inflammation is incorrect, then one may not necessarily expect an increased level of NO in suppurative or infective conditions. Indeed, asthma may be unique in its high production (or high levels in exhaled breath) of NO and this increase may not be related to airways inflammation.

However, it is important to realise that low exhaled NO levels does not necessarily equate to low NO production. It is possible that NO production is normal or high but simply not detected in the exhaled air. Therefore, before the implications of decreased NO production is investigated, firm evidence that NO production is indeed decreased should be obtained.

4.7 CONCLUSION

Exhaled NO is not increased in patients with bronchiectasis. This, together with similar finding in patients with CF suggests that NO in chronically suppurative airways may be impeded from diffusing into the airway lumen and thus detection of gaseous NO is limited. It is also possible that NOS II is downregulated in chronic inflammation.

CHAPTER 5

EVIDENCE FOR MOLECULAR PRESENCE OF NOS II IN CYSTIC FIBROSIS EPITHELIAL CELL LINES AND PRIMARY NASAL EPITHELIAL CULTURES

5.1 INTRODUCTION

In order to examine if lack of increase in exhaled NO levels in patients with cystic fibrosis may be due to the lack of production, I investigated if one of the genes responsible for its production (NOS II or iNOS) is expressed in CF cells. Type II NOS was chosen because it is the enzyme responsible for the largest amount of NO generation. Also, evidence at the time of study design suggested that NOS II expression was increased in airway inflammation (see chapter 1.6.3). In the lungs, type II NOS has been localised in neutrophils, eosinophils, alveolar macrophages and epithelial cells (Gaston B, 1994). Its expression upon stimulation by proinflammatory agents (Robbins RA, 1994) indicates its importance in inflammation while the loss of defence against intracellular pathogens in an NOS II knockout mouse implicates its role in host defence (Wei XQ, 1995). Bronchoalveolar lavage of CF lungs has demonstrated raised IL8 levels and a predominantly neutrophilic inflammatory cell influx (Dean TP, 1993. Dosanjh AK, 1998. Bonfield TL, 1995). However, as seen in chapter 3, exhaled NO levels are low in CF patients, even during infective pulmonary exacerbations. Similarly nasal NO levels which are in the order of 500 ppb in normal subjects have been found to be significantly lower in CF patients (10 ppb), raising the

question of NOS expression in CF epithelial cells. Therefore, one possible explanation for the lack of increase of exhaled NO levels in CF might be the inability to upregulate NOS II in CF epithelium.

5.2 HYPOTHESIS

The lack of increase in exhaled NO levels in the inflammatory setting of CF airways may be secondary to loss of NOS II expression in epithelial cells.

5.3 AIMS OF STUDY

The study investigates the basal and stimulated expression of NOS II in primary human nasal epithelial cells, since these may be representative of cells in the lower airways. Expression of NOS II in the airway epithelial cell line (ΣCFTE) (Gruenert DC, 1995) expressing the most common CFTR mutation, δF508 was also studied to complement the primary culture experiments.

Experiments were performed to address the following questions:

(a) Is NOS II expression in CF epithelial cell lines and CF nasal epithelial cells altered compared to normal controls?

(b) Is this expression increased in vitro on stimulation with proinflammatory cytokines and the bacterial product, lipopolysaccharide (LPS)?

5.4 MATERIALS AND METHODS

5.4.1 CELL CULTURE

Primary nasal epithelial cell culture

Nasal epithelial cells were harvested from the inferior turbinate of subjects using a bronchoscopy cytology brush (BC-5, Olympus Keymed, Essex, England). The brush was advanced and retrieved quickly along the floor of the nose. No local anaesthetic was used. Both nostrils were brushed three times.

Brushings were obtained from 3 CF patients (all δF508 homozygotes), 3 normal subjects and 3 asthmatics. Asthmatics acted as positive controls since they are known to have increased NOS II gene expression and thus acted as positive controls. All subjects were non smokers and did not have active allergic rhinitis or upper respiratory tract infection during, and for 6 weeks before, the procedure. None of the patients were on inhaled steroids at the time of sampling.

After harvesting, cells were re-suspended within 10 minutes in Ham's F12 culture media (GIBCO), supplemented with 2% Ultroser G (IBF Biotechnics), 1% Glutamine, 1% non essential amino acid, 2% penicillin/streptomycin, 0.1% gentamicin and 1% Amphotericin B (all from Life Technologies) and incubated in 5% CO_2 in air at 37°C. For each subject, the total cells were divided into 4
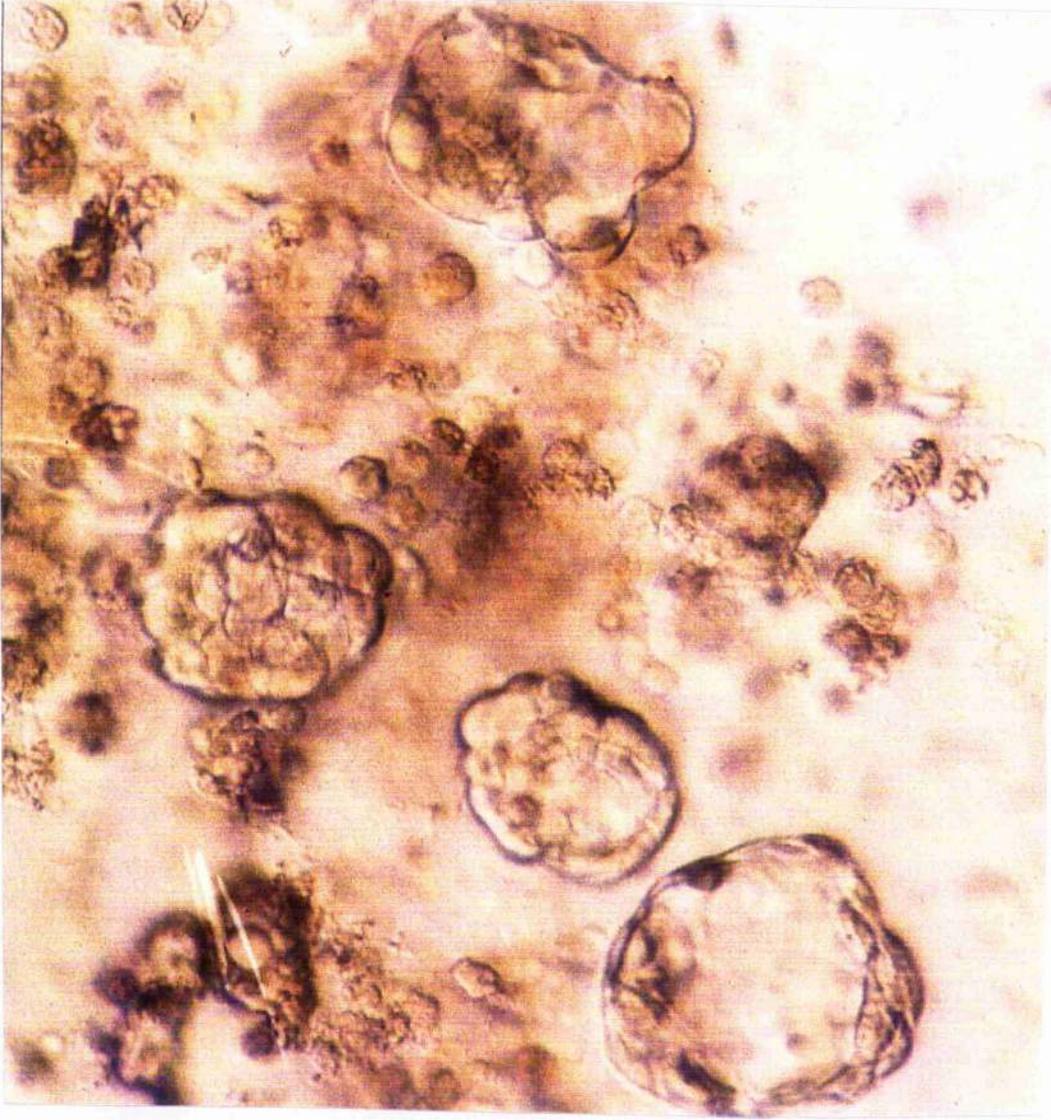
Figure 10. Nasal epithelial cell suspension at 24 hours. Epithelial cells are dislodged from bronchoscopic brush by swirling the brush vigorously in culture medium. Live and intact cells are recognised as strips of epithelial cells with beating cilia. Cells form clumps within a few hours and remain viable for at least a week.
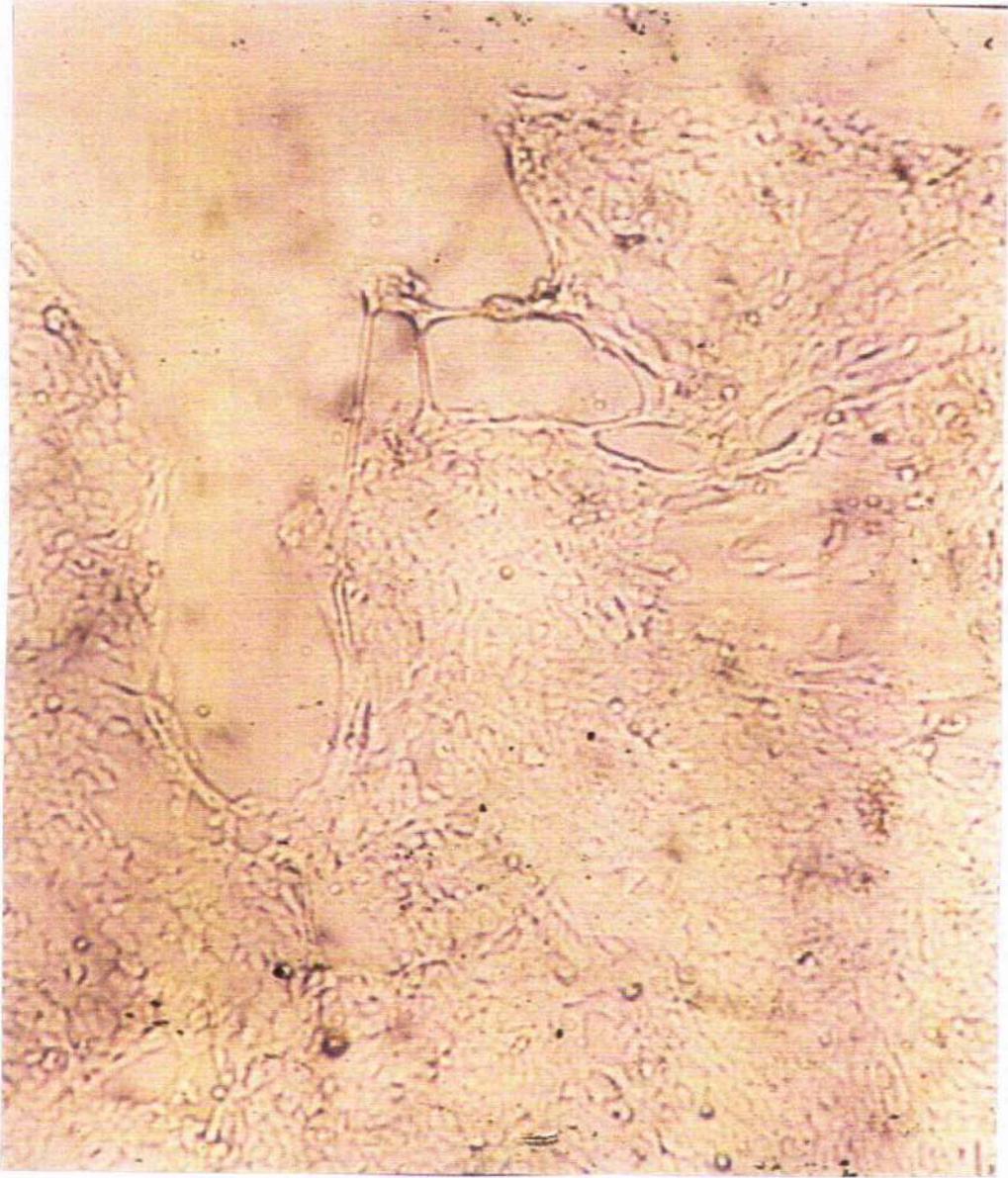
Figure 11. Primary nasal cells after 10 days of culture on plastic, showing morphology characteristic of epithelial cells.

wells. Three wells were used for stimulation studies, while the fourth acted as a control where no stimulants (cytokines and LPS) were added.

Cell viability was assessed by ciliary motility and only wells with at least 90% of cells exhibiting ciliary motion were used. Cells were grown in 96 well plates and all samples contained at least 90% of ciliated epithelial cells (figure 10 and 11). They were incubated for half an hour before stimulation with 200 µl of medium containing 10 ng/ml each of IL1β, TNFα and IFNγ, and 10 µg/ml of LPS for 5 hours.

RNA extraction was performed at the end of stimulation (chapter 5.4.2).

Epithelial cell lines

A human CF tracheal epithelial cell line expressing the δF508 mutation, ΣCFTE, and for control a normal human bronchial epithelial cell line, 16 HBE (both gifts from Professor D Gruenert, UCSF, California) were propagated in MEM medium (GIBCO BRL) with 10% foetal calf serum, supplemented with penicillin/streptomycin, 0.5 mM L-arginine and glutamine in a 5-6% CO_2 incubator at 37°C. Cells were grown to 90% confluence on fibronectin/collagen coated 6 well plates prior to stimulation. As described with the primary cells, these cells were divided into stimulated (with 15 ng each of IL1β, TNFα and IFNγ, and 15 µg of LPS in 1.5 ml of culture medium) and unstimulated samples and incubated for 8 and 24 hours before RNA extraction.

100

5.4.2 MOLECULAR BIOLOGY

RNA extraction. Both primary cultures and cell lines were prepared in the same manner. Cell lines but not primary cells were washed with ice cold phosphate buffered saline (PBS). Total RNA was extracted as follows. Culture media was first removed from cell culture wells. 1 ml RNAzol B (Boehringer Mannheim) was added to each well to lyse cells. Lysed cells are then reacted with 100 μl chloroform, spun down and 450 μl of top layer removed. RNA is precipitated from this layer by addition of 4 μl of glycogen (10 mg/ml) and 450 μl Isopropanolol. This was allowed to react for 15 minutes and then RNA pelleted by centrifuging for 15 minutes. This pellet was washed with 75% ethanol and final pellet resuspended in sterile water.

Reverse transcription. 2 μl of prepared RNA suspended in DEPC treated water was denatured at 65°C for 15 minutes. cDNA is synthesised from RNA according to protocol from Boehringer Mannheim [1st strand cDNA synthesis kit for RT-PCR (AMV)] (Appendix) in a 20 μl reaction containing 2 μl of 10x reaction buffer, 5 mM of $MgCl_2$, 1 mM of deoxynucleotide mix (dNTPs), 0.4 μl of gelatin (at 0.01 mg/ml), 3.2 μg of $p(dN)_6$ primer, 50 units of RNAse inhibitor and 0.8 μl (20 units) AMV-reverse transcriptase at 42°C for 60 minutes. Negative control tubes containing no RNA were also included.

PCR. A semi quantitative PCR was employed, according to methods and primers described by Watkins DN et al (Watkins DN, 1997). The following oligonucleotide primers for reverse transcription PCR were used:

NOS II (+) : 5' ACGTGCGTTACTCCACCAAC-3'

NOS II (-): 5' TCGCAAA GAGGATGGTGACT-3'

This was obtained from Genosys, UK. This produced a 945 bp PCR product.

Primers for β actin, a housekeeping gene was also used in a duplex reaction, using the following oligonucleotide primers:

β actin (+): 5' CGTGACATTAAGGAGAAGCTGTGC-3'

β actin (-): 5' CTCAG GAGGAGCAATGA TC TTGAT-3'

A 25 µl PCR reaction mixture containing 2.5 µl Perkin Elmer 10x buffer, 1.5 µl of 25 mM MgCl$_2$, 50 ng of specific primers, 1 unit Amplitaq DNA polymerase and 0.625 µl of 10 mM dNTPs is prepared with 10 µl of cDNA. Denaturation, annealing and extension temperatures for PCR were 94°C, 55°C and 72°C for 36 cycles.

Products were electrophoresed on a 2% agarose gel. Each sample was compared to a negative control which did not contain reverse transcriptase and samples containing DEPC treated water and no RNA acted as further negative controls. 1

pg samples of plasmid DNA (gift from Dr. Watkins, Queen Elizabeth II Medical Centre, Australia) acted as positive controls.

Southern blotting. RT-PCR products were detected by Southern blotting using P^{32} end labeled oligonucleotides specific for the NOS II and β actin genes. Specific intron spanning 20 mer primers were designed from published cDNA sequences obtained from Geller DA et al (Geller DA, 1993a). PCR products were transferred to a Hybond N $^{+tm}$ nylon membrane by capillary blotting in 10x saline sodium citrate (SSC). Membranes were hybridised at 58°C in 5 X SSC and 0.5% SDS and 10µg heat denatured salmon sperm DNA and 50 ng of heat denatured probe. High stringency wash in 4 x SSC was performed twice before membranes were opposed to a phosphoimager for 24 hours. The images from the phosphor image were processed using the ImageQuant™ programme.

5.5 DATA ANALYSIS

For the cell line experiment, gene expression of NOS II is represented numerically as a percentage of β actin expression. This is performed on the ImageQuant ™ programme.

For primary cell suspension experiments, numerical expression was not calculated because it was not possible to ensure that β actin expression was derived purely from epithelial cells. Thus, the primary culture experiments were regarded as supportive evidence for the cell line experiments.

5.6 RESULTS

NOS II expression in primary epithelial cells from normal, CF and asthma patients

In unstimulated samples, NOS II mRNA was only detected in asthmatic samples (figure 12). However, NOS II mRNA expression was seen in all stimulated samples (at least 2 of three wells for each subject). The housekeeping gene, β-actin mRNA was detected in all samples. All controls (minus reverse transcriptase, water control and minus RNA control) showed no signal, indicating no false positive signals.

NOS II expression in normal and CF epithelial cell lines

Both ΣCFTE and 16 HBE cell lines showed expression of NOS II at baseline with increased expression after 8 and 24 hours stimulation [% NOS II/β actin = 12.7 v 32.2 v 36.7 (16 HBE) and 20.3 v 23.0 v 50.9 (ΣCFTE) respectively]. (Figures 13a&b and 14)

5.7 DISCUSSION

These experiments demonstrated that NOS II expression in CF nasal epithelia, in contrast to asthmatics, is not increased prior to further stimulation with proinflammatory cytokines. Both cell line and primary culture experiments show
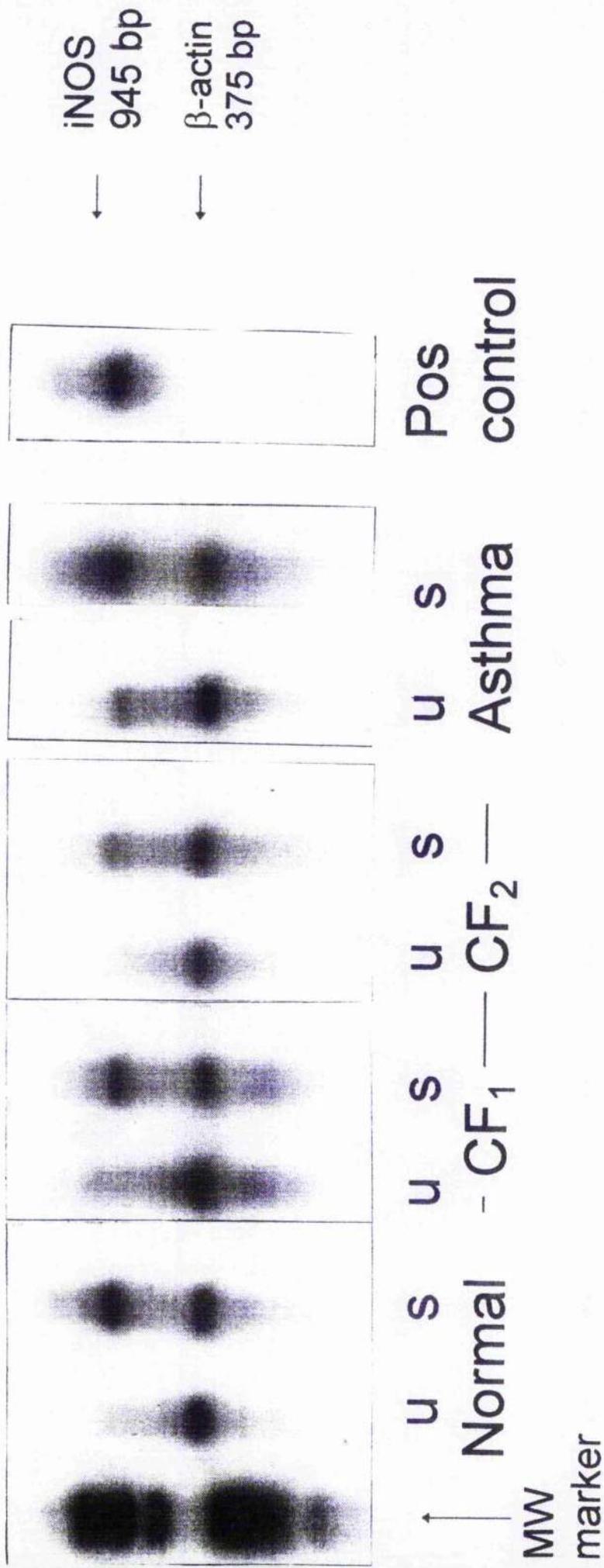
Figure 12. Expression of iNOS in primary airway epithelial cells from CF patients compared to normal subjects and asthmatics. u=unstimulated, s=stimulated for 5 hours with 10 ng/ml of IL1β, IFNγ, TNFα and 10 μg/ml of LPS.
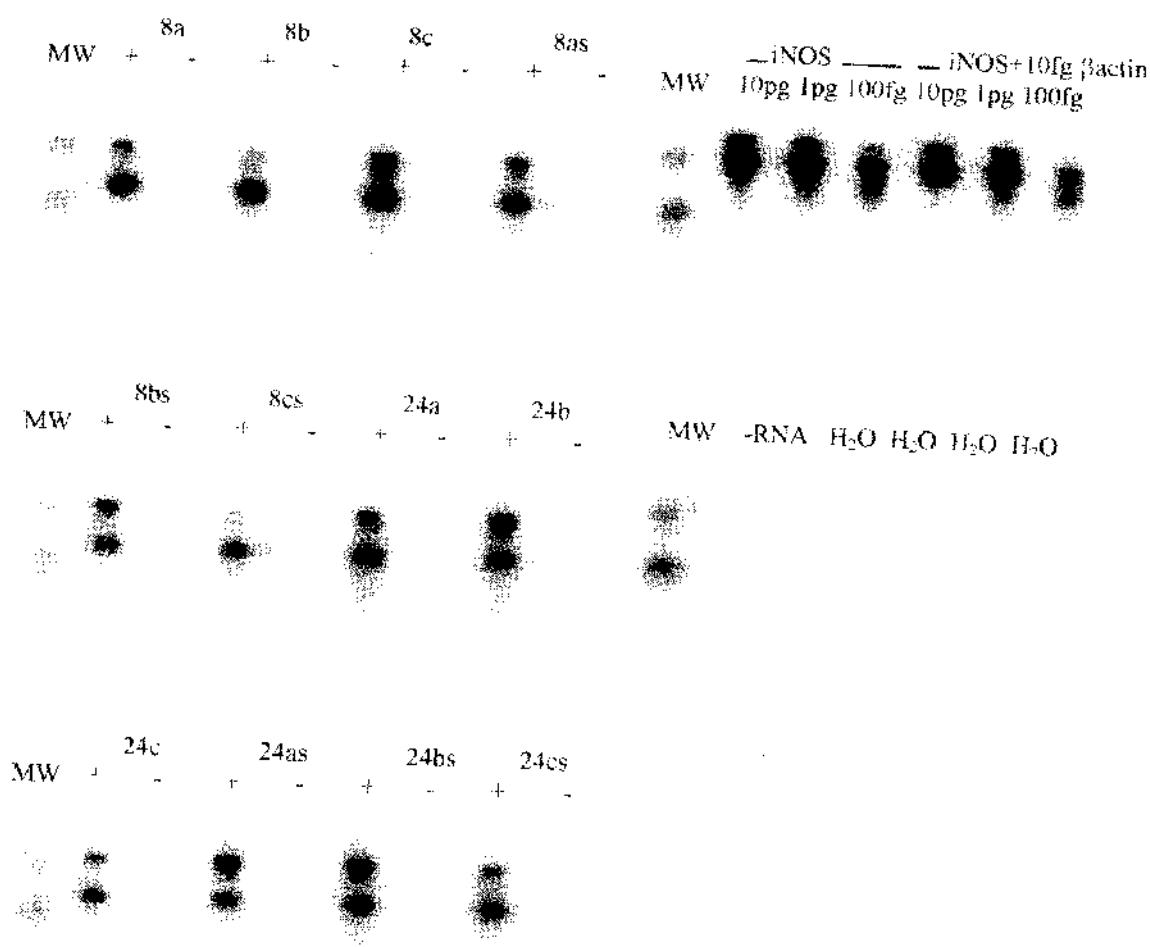
Figure 13a . Phosphorimages of Southern blots showing expression of iNOS in normal cell line (16 HBE) from one experiment.

MW=molecular weight marker. 8a-c and 24a-c= Unstimulated wells at 8 and 24 hours respectively. 8as-cs and 24 as-cs =Wells stimulated with 10 ng/ml of IL1β, IFNγ, TNFα and 10 μg/ml of LPS for 8 and 24 hours respectively. +=with reverse transcriptase; -=no reverse transcriptase control. Controls : (1) -RNA= PCR mix without RNA. (2) H₂0 = PCR mix with sterile water without cDNA.

Figure 13b . Phosphorimages of Southern blots showing expression of iNOS in CF cell line (ΣCFTE) from one experiment.

MW=molecular weight marker. 8a-c and 24a-c= Unstimulated wells at 8 and 24 hours respectively. 8as-cs and 24 as-cs =Wells stimulated with 10 ng/ml of IL1β, IFNγ, TNFα and 10 μg/ml of LPS for 8 and 24 hours respectively. +=with reverse transcriptase; -=no reverse transcriptase control. Controls : (1) -RNA= PCR mix without RNA. (2) H_2O = PCR mix with sterile water without cDNA.
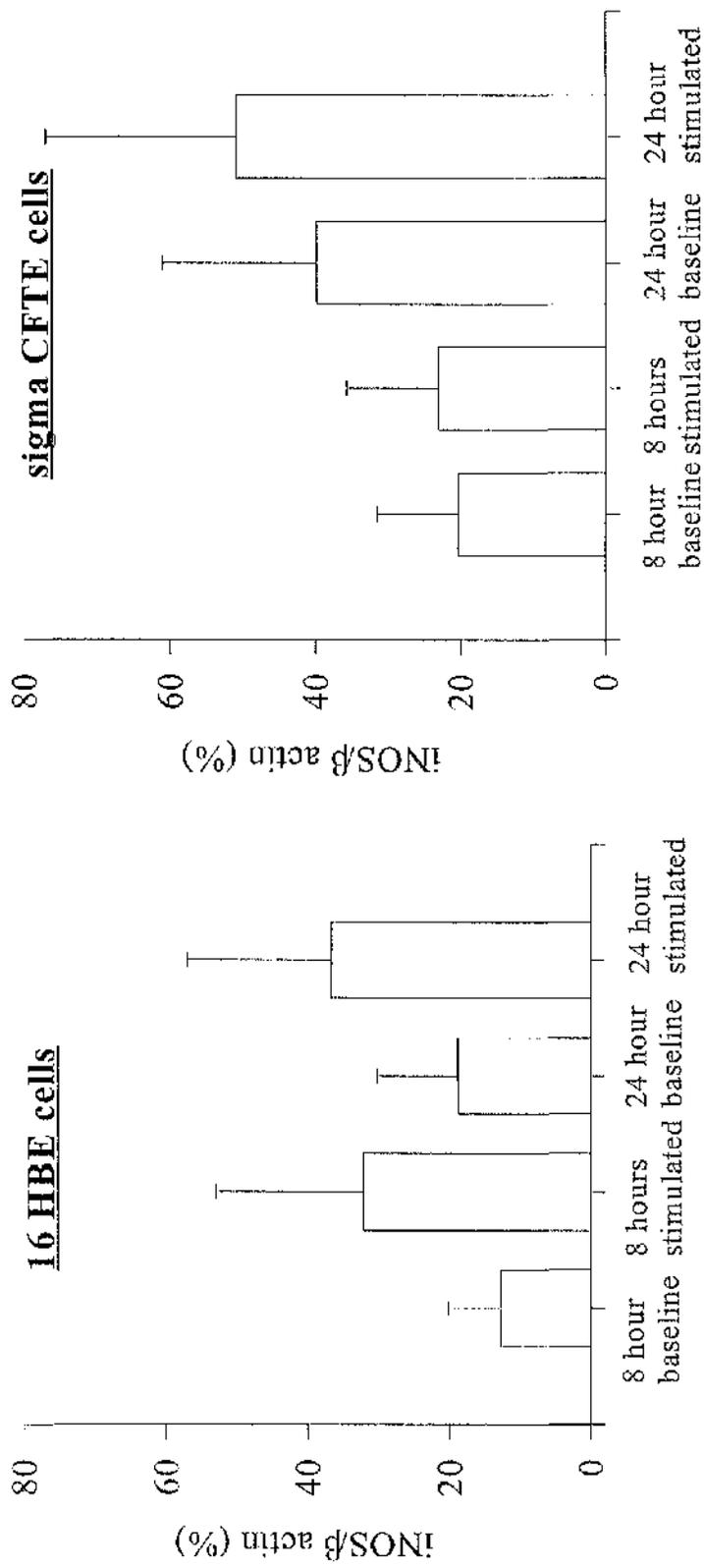
Figure 14. Expression of iNOS in cystic fibrosis (ΣCFTE) and normal (16 HBE) cell lines expressed as percentage of the housekeeping β actin gene. Mean of n=3 each, error bars = S.E.M..

that NOS II expression and response to pro-inflammatory cytokines are similar to normal cells.

From these results it can be concluded that:

1. the presence of CFTR gene mutation do not affect the expression of NOS II in response to inflammatory stimuli. Therefore, low levels of exhaled NO in CF are not secondary to abnormal expression of the NOS II gene to inflammatory stimuli.

2. NOS II expression is increased in asthmatics but not in CF epithelia compared to normal subjects.

The aim of the study was to examine if lack of NOS II expression accounted for the low levels of exhaled NO in CF patients. The definitive study would be to obtain biopsies from the lower airways of patients with CF. However, we did not have ethical permission for this. I examined, instead, the nasal epithelium taking it as a representative of the lower airways. This presented a few limitations. Firstly, bronchial sepsis that occurs in the lower airways is not present to the same degree in the upper airways. So, definitive conclusions about the expression of NOS II in response to suppurative environment cannot be derived. Secondly, the precise nature of the primary cell cultures were a little uncertain. Although visual observation showed almost 90% of cells to be ciliated epithelial cells, I could not exclude the possibility of some inflammatory cells in the culture. The only way of excluding inflammatory or other cells would have been to allow the epithelial cells to adhere and grow to confluence. However, although adherence of the cells

would have allowed washing off of 'contaminant' cells, it would have created the possibility of dedifferentiation of the cells and natural loss of NOS II expression as demonstrated by Guo et al (Guo FH, 1995).

Therefore, from this study it could only be concluded that NOS II expression in the lower airways of CF, like that in the upper respiratory tract (nose) is *likely* to increase in response to inflammatory stimuli in bronchial sepsis.

That aside, a few interesting suggestions arise from these results. In view of increased background inflammation compared to normal subjects, the expectation is for CF epithelial cells to have increased constitutive expression of NOS II, as in asthmatic epithelium. Despite these cells being nasal rather than lower airway cells exposed to bronchial sepsis, previous experiments in our laboratory had shown that nasal biopsies obtained from CF patients demonstrated excess inflammatory cell infiltrate (Porteous DJ, 1997). Hence, it seems that at the cellular level, there appear to be persistent inflammatory stimuli in the nasal epithelium, but these do not appear to result in upregulation of NOS II . It may be that asthmatic epithelia are unique in the upregulation of the NOS II gene in vivo. This may be secondary to a genetic predisposition to epithelial upregulation of NOS II.

At the time of conclusion of these experiments, Polak's group published a study in which the NOS II expression in CF cell lines and bronchial explants from end stage CF patients undergoing heart lung transplant were examined (Meng Q-H,

1998). They found decreased expression of NOS II in these explants and no expression in CF epithelial cell line even after stimulation with proinflammatory cytokines and LPS. They speculated from their findings that the loss of NOS II expression may be secondary to the presence of CFTR mutation. My findings would suggest otherwise, since NOS II is clearly expressed in the CF cell lines used and it appears more likely that in the presence of persistent chronic inflammation, NOS II expression is downregulated and that this effect is unlikely to be unique to CF.

5.8 CONCLUSION

CF epithelial cells, in vitro, are able to express NOS II normally in response to inflammatory stimuli. The studies, however, do not exclude the possibility that NOS II expression may be down regulated by chronic inflammatory stimulation.

CHAPTER 6

DEVELOPMENT OF BREATH CONDENSATE METHODOLOGY

6.1 INTRODUCTION

As discussed in chapter 4.6, one way of exploring NO production in the lower airways is to examine a surrogate marker of this production. This can take the form of an end metabolite like nitrite. Four methods have been developed for sampling of the lower airways for cellular and biochemical activity. They are bronchoalveolar lavage (BAL), induced sputum production, exhaled air and condensed breath. BAL is the oldest of the methods but is also the most invasive and has led to a few asthma deaths (O'Byrne PM, 1994). Breath condensate and exhaled air methods are the least invasive, and arguably sample the whole of the lungs rather than a localised area and provide information on physiological/cellular activity at the time of sampling. They are relative new and the least investigated of the four methods.

The concept of using breath condensate as a medium to measure surrogate markers of airways pathophysiological activity has been suggested since the early 1980's. Then, volatile hydrocarbons were detected in exhaled breath by mass spectrometry and spontaneous chemiluminescence was observed in condensed breath (Barkley J, 1980). More than 200 different volatile components have since been identified in the exhaled air (Manolis A. 1983). The method of detecting

substances of interest in the breath originated from the idea that volatile hydrophilic substances can be concentrated as condensate after passing the warm (36.9°C) exhaled breath through a cold trap. Hydrogen peroxide, a volatile oxygen metabolite can enter the gaseous phase at physiological temperatures (Williams MD, 1982) and has been shown to be detectable in breath condensates. The levels of non volatile but water soluble compounds can also be measured in micro-aerosols produced in condensed exhaled air (Scheideler L, 1993). The use of breath condensate as a research tool is not widespread. This is partly due to the complicated and prolonged collection period to acquire enough fluid for analysis. However, the advantages of this method over induced sputum and bronchoalveolar lavage make it a very attractive option to pursue.

I wanted to develop a collection method that would incorporate the following features:

1) The method should collect enough condensate in a short period of time. This is necessary to ensure that condensate is present in the cold trap for a minimum amount of time before analysis or storage so as to prevent degradation of molecules of interest.

2) The method should be acceptable to patients particularly in CF where the expiration phase could be decreased due to reduction in total lung capacity.

3) There should be no salivary contamination.

4) Minimal or no contribution of air from the nasal passages to the exhaled air.

6.2 METHODOLOGY AND EQUIPMENT

6.2.1 COLLECTION OF BREATH CONDENSATE

METHODS

Teflon perfluoralkoxy was used for the collecting tube. This is an inert material which ensured that no reaction occured between the molecules of interest and collecting tubes. The tubing had a 0.5 cm diameter and was initially coiled in an S shape to collect breath condensate at the most dependent area (Figure 15a). This suffered from salivary contamination and poor volume collection. The setup was thus modified to incorporate a system that allowed increased surface area in order that more condensate can be collected over a shorter period of time. This was achieved by coiling the tube and using a container with a confined space to maintain the coils. Different number of coils were used but the increase in surface area afforded by the increase in the coils was limited by the resistance to exhalation that this created (Figure 15b). Two and a half coils were found to provide the best compromise. Patient acceptability in terms of ability to exhale into the tube over 5-6 minutes duration was tested on three CF subjects with different lung function (FEV1 37% to 85% of predicted). All found the method acceptable .

Approximately one ml of breath condensate could be obtained within 6 minutes using this method. In order to prevent saliva contamination, the collection tubing

Condensate collects in
test tube

Teflon tube

Subject exhales here

Polystyrene ice
bucket

Ice
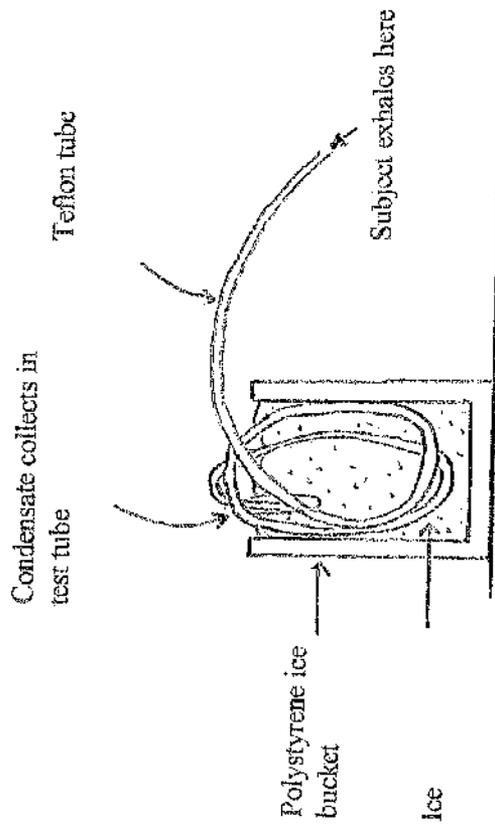
Figure 15b. Final breath condensate collection
method.

Ice

Teflon tube

Subject exhales here

Saliva trap

Polystyrene ice bucket

Condensate
collects
here
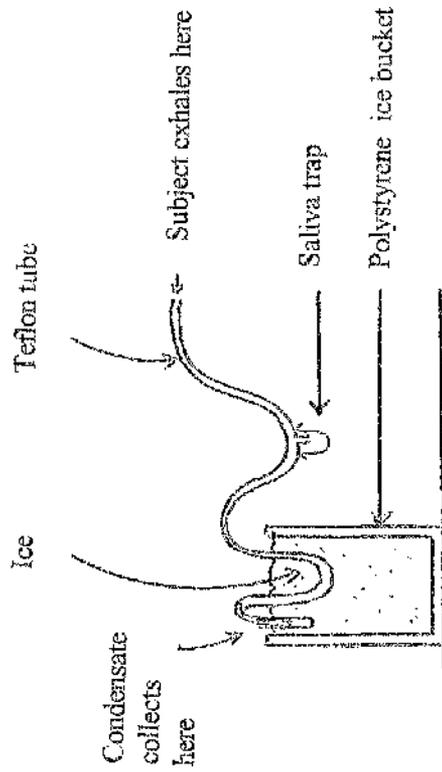
Figure 15a. Earlier set up which suffered
from saliva contamination.

was placed about 5 inches higher than the subjects mouth. The subject was also instructed to swallow his or her saliva before exhalation and to do this at intervals throughout the procedure.

6.2.2 SALIVA CONTAMINATION

METHODS

To test that this method excluded salivary contamination, breath condensate samples were collected in 15 CF subjects over 6 minutes and each tested for salivary amylase using a reflectrometric dry slide method with a Vitros analyser (Ortho Clinical Diagnostics, Strasbourg). Two samples were 'spiked' with 0.5 ml of saliva to ensure that this method of detection worked.

RESULTS

I found no salivary amylase in any of the samples collected. In contrast the spiked samples showed levels in excess of 20,000 IU.

6.2.3. NASAL AIR CONTAMINATION

METHODS

Two subjects were asked to exhale into the Teflon tube in the standard fashion while their noses were being flushed with 100% helium (BOC, Kent) delivered by nasal canulae. (The helium flushing started after inspiration). Expirate emerging from the collecting tube was continuously sampled for helium using a helium analyser (an integral part of a standard body plethysmograph). This was performed during and for 15 seconds after the end of exhalation. In order to establish that this system could detect helium in the expirate, the subjects inhaled to TLC while the nose was flushed with 100% helium and then exhaled into the collecting system where expirate was tested for helium as above.

RESULTS

Both subjects who inhaled to TLC while their noses were being flushed with 100% helium had detectable helium in their expired air within the first 15 seconds (2.8% and 1.5% respectively). However, exhaled air tested when helium was delivered to the nose *after* the start of exhalation showed no helium in the expirate. These data indicate that the method of exhaling against the resistance of the tubing is sufficient to exclude contamination of the expirate by nasal air, presumably by elevating the soft palate and sealing off the posterior nasopharynx.

6.3 FINAL METHOD

In the final method, breath condensate was collected by asking the subject to inspire repeatedly to total lung capacity (TLC) and exhale through 1.5 m Teflon perfluoralkoxy (PFA) tubing with 0.5 cm internal diameter, immersed in ice (figure 15b). Subjects were asked to keep their mouth dry by swallowing their saliva throughout the procedure. This method yielded one ml of breath condensate within 6-8 minutes.

CHAPTER 7

DEVELOPMENT OF NITRITE ASSAY FOR BREATH CONDENSATE

7.1 INTRODUCTION

In an oxygenated aqueous environment, rapid interconversion among NO species may occur as described in chapter 1.2. In the air-fluid interface of the airways, NO generation may be indicated by the formation of stable end products of NO metabolism, nitrite (NO_2^-) and nitrate (NO_3^-).

At this point in time of the MD project, no one had investigated the possibility of detecting nitrite in exhaled breath condensate. In theory, nitrite, being water soluble would be present in the lining fluid of airways. At 37°C, expired breath contains volatile substances and water vapour of which some would have originated from the inspired breath and others derived from the airway lining fluid. It would also contain micro aerosol which may have been generated by the expiration against a mild resistance. Scheideler et al had shown that IL1β, sIL2R and TNFα could be detected in condensed breath (Scheideler L, 1993). Since these molecules were non soluble, it meant that their detection was made possible by condensed micro aerosol generated from the airway lining fluid. It also allowed me to speculate that there was a high probability that a water soluble molecule like nitrite would be detectable.

Nitrite rather than nitrate was selected for the study for three reasons. Firstly it is the main end product of NO metabolism and nitrate is frequently reduced to nitrite in biological systems (Marletta MA, 1988). Secondly, the use of one simple and reliable method of measurement (Griess reaction) decreased the need for increased condensate collection. Thirdly, in a previous (unrelated) study examining NO production in cultured chondrocytes I had already demonstrated that nitrate and nitrite generation correlated strongly with each other (r=0.99) (Ralston S, 1995).

7.2 HYPOTHESIS

Nitrite levels can be measured in condensed expired breath and this may be a surrogate marker of NO production in the lower airways of patients with cystic fibrosis.

7.3 METHODS

The first task was to evaluate if nitrite could be detected in the breath condensate and then to validate the reproducibility and sensitivity of the assay.

Nitrite assay- Griess reaction

I employed a well established method commonly used for measuring nitrite levels in cell culture studies - the Griess reaction (Green LC, 1982). This is a

colorimetric method where the diazotization of the Griess reagent (1% sulfanilamide dihydrochloride and 2.5% H_3PO_4) by nitrite causes a colorimetric change. The intensity of this colour change correlates to the concentration of nitrite and is detected on a spectrophotometrically. The Griess reagent is prepared fresh on the day of use by adding reagent A and B at 1:1 vol:vol. (Reagent A: 2% sulfanilamide in orthophosphoric acid. Reagent B: 0.2% N-1- naphthyl ethylenediamine dihydrochloride (NED) in water). A standard curve is derived on the day of use from a stock of 100 mM of sodium nitrite (0.069 g in 10 ml distilled water) to give a concentration of 0- 10 μM.

Ability to detect nitrite in breath condensate

This protocol was applied to breath condensate collected from four normal subjects and two asthmatics and two CF patients.

Reproducibility

Intrasubject reproducibility on different days (3 - 8 days) was assessed in 7 normal subjects. Bartlett's test was first applied to test for heterogeneity of variance between individuals and then ANOVA was applied to give a pooled standard deviation and 95% confidence interval.

7.4 RESULTS

Nitrite assay

The standard curve showed that the assay was able to detect nitrite levels as low as 0.5 μM and a standard curve from 0 to 10 μM consistently showed a linear relationship with the spectrophotometric reading (r = 0.99) (figure 16). Nitrite was detected in all samples collected from all subjects and was noted to be considerably higher in asthmatics (table 10).

Final methodology

Triplicates of 100 μl of breath condensate were reacted with 25 μl of Griess reagent (0.1% naphthylethylene diamine dihydrochloride, 1% sulfanilamide, 3% H_3PO_4) and measured at absorbance of 570 nm with a microplate reader (MR 710, Dynatech). A fresh standard curve is prepared for each batch of experiments using the same reagents as for the samples. Breath condensate collections were performed as described in chapter 6.2.

Intrasubject variation.

Nitrite levels in individual subjects are shown in table 11.
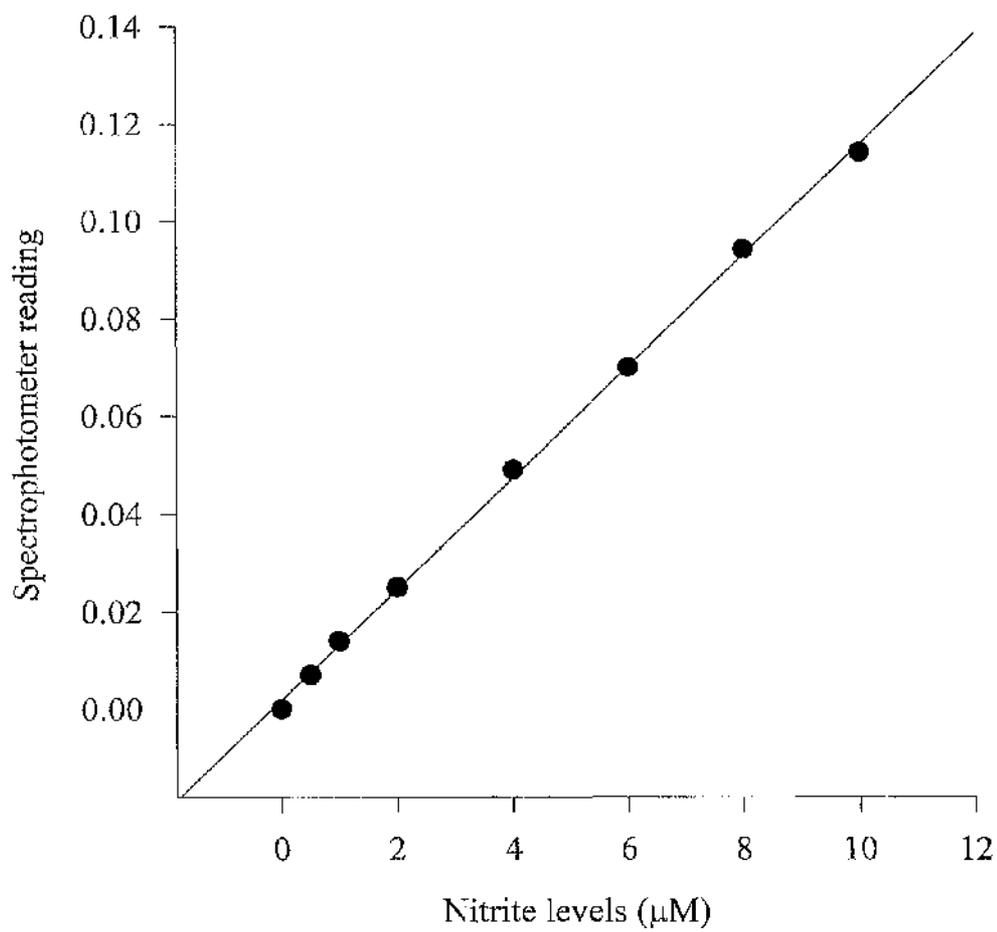
Figure 16. Typical standard curve for known nitrite concentrations derived using Griess reaction.

	Breath condensate nitrite (μM)	
Normal subject 1	0.60	0.64
Normal subject 2	1.20	1.46
Normal subject 3	1.10	1.0
Normal subject 4	0.52	0.54
CF 1	4.30	4.04
CF 2	2.10	2.10
Asthma 1	8.10	8.42
Asthma 2	4.40	4.00

Table 10. Preliminary values of nitrite in asthmatics, CF and normals from one collection on same day.

	Breath condensate nitrite levels (μM)							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Subject 1	1.0	0.0	0.24	0.06				
Subject 2	1.0	1.46						
Subject 3	1.1	0.28	1.2					
Subject 4	0.0	0.52	0.76					
Subject 5	1.1	0.6	1.72	0.67	0.32	0.56	0.98	
Subject 6	0.28	1.29	0.32					
Subject 7	0.88	0.32						

Table 11. Values of breath condensate nitrite (μM) in normal subjects on different days.

Bartlett's test showed no significant difference between the individuals tested ($p=0.91$), thus ANOVA to the whole group could be applied. This gave a pooled S.D. of 0.5 µM, and a 95% confidence interval for any measure of ± 1.12 µM.

CHAPTER 8

NITRITE LEVELS IN EXPIRED BREATH OF CYSTIC FIBROSIS PATIENTS

8.1 INTRODUCTION

Contrary to expectations, exhaled NO levels in cystic fibrosis have been found to be low, even during infective pulmonary exacerbations. As discussed, one explanation is that NO in the gaseous phase may be "trapped" by the viscous secretions in suppurative lung conditions and effectively be removed by its reaction with reactive oxygen species or with water and oxygen. Having demonstrated that nitrite is measurable in breath condensate, I proceeded to examine the levels in CF patients compared to normal subjects. The relationships of breath condensate nitrite with circulating plasma leucocytes, exhaled NO, conventional lung function measurements and the use of inhaled steroids were also analysed.

8.2 HYPOTHESIS

It is hypothesised that in CF airways, excess gaseous NO from inflamed airways is converted to nitrite and nitrate close to the site of production before it is able to move into the airway lumen and be detected as gaseous NO. Thus nitrite levels in breath condensate of CF patients would be elevated compared to normal subjects.

8.3 STUDY DESIGN

Subjects and patients

Twenty one clinically stable CF patients with defined genotypes were recruited. Lung functions were variable, with FEV_1 as percentage of predicted ranging from 20 % to 95 % ; mean ($\pm$ S.D.), 57 (24) %. The mean age for the patients was 26.0 (9.3) years. There were 13 males and 8 females. Twelve healthy normal volunteers acted as controls, with mean age 31.4 (4.9) years (6 males, 6 females). All subjects were non smokers and had no intercurrent respiratory tract infection, active allergic rhinitis or concomitant airways diseases.

All CF patients had spirometry and reversibility to salbutamol. Patients with reversible airways obstruction (increase in FEV_1 of greater than 175 ml after 2.5 mg nebulised Salbutamol) or clinical evidence of asthma were excluded. Within the CF group, 9 were on inhaled steroids, ranging from 200 mcg to 1500 mcg beclomethasone dipropionate per day which was started by their usual physician as anti-inflammatory treatment.

Breath condensate nitrite and exhaled NO of all patients were measured on two separate days. On all occasions, the patients were clinically stable and lung function (FEV_1) was unchanged ($<$ 175 ml). Fourteen subjects consented to venepuncture for measurement of circulating white cell count and differential

white cell count (neutrophils, eosinophils, monocytes and lymphocytes). This was performed on the day of breath collection.

Measurement of exhaled NO and breath condensate were as described in chapters 2.3 and 6.2 respectively.

8.4 STATISTICAL ANALYSES

Comparisons between the two groups were made using the Mann Whitney Rank Sum Test. Correlations between nitrite levels and exhaled NO and between nitrite and lung function (FEV_1) were determined using the Spearman Rank Sum Test.

8.5 RESULTS

Nitrite and exhaled NO levels in CF compared to controls

Nitrite levels were significantly higher in CF patients compared to normal subjects (median 1.93 μM compared to 0.33 μM, $p < 0.001$) (figure 17 and table 12).

In contrast, exhaled NO values were not significantly different compared to the normal range [median of 3.8 (95% confidence interval 3.4-5.2) ppb vs 4.4 (2.9 - 6.3) ppb].
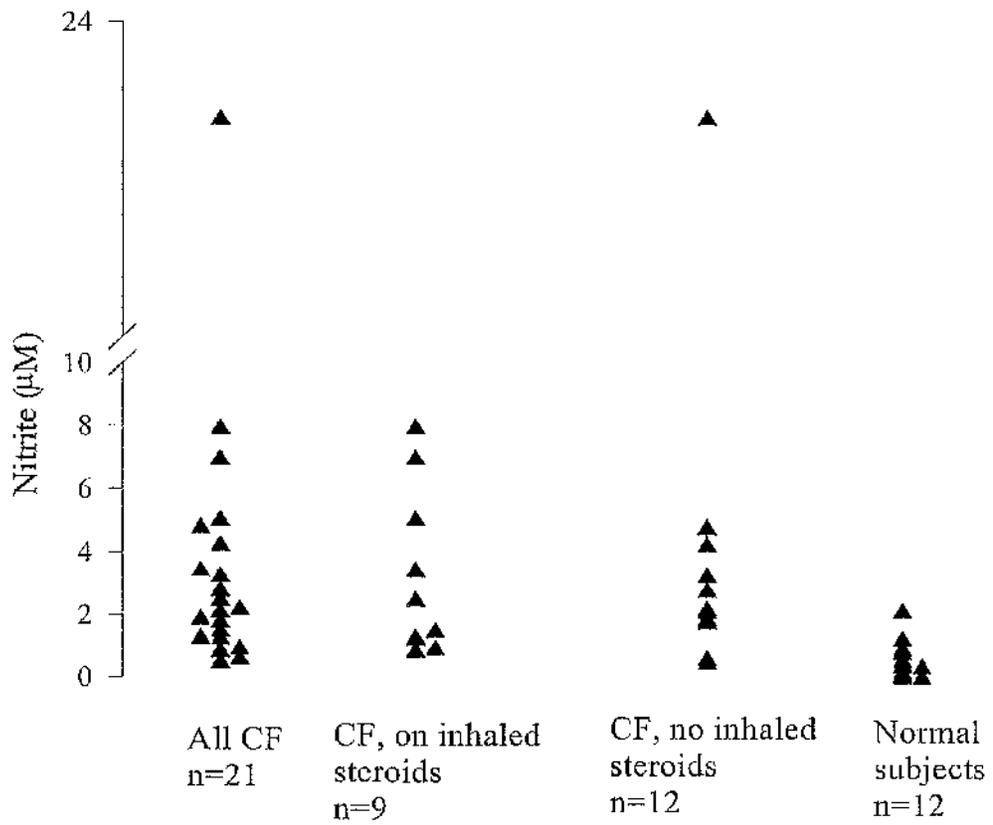
Figure 17. Nitrite levels in breath condensate of patients with cystic fibrosis compared to normal subjects.

	All CF's	Normals	CF (no inhaled steroids)	CF (on inhaled steroids)
n	21	12	12	9
Nitrite (median value)	2.15 μM	0.36 μM	1.94 μM	2.86 μM
Range	0.44–23.69	0–2.10	0.80–7.89	0.56–23.69

Table 12. Breath condensate nitrite levels in normal subjects and patients with cystic fibrosis.

Correlation between nitrite levels and plasma leukocyte levels, lung function and inhaled steroid usage

There was a positive correlation between nitrite levels and circulating leucocytes and neutrophils (r=0.6, p=0.04 and r=0.6, p=0.03 respectively by Spearman Rank Order Correlation) (figure 18). In contrast, there was no correlation between exhaled NO and circulating plasma leukocyte count. Nitrite levels were not significantly different between the patients taking and those not taking inhaled steroids (table 12).

There was no correlation between breath condensate nitrite and exhaled NO or any between breath condensate nitrite and lung function (figures 19 and 20).

8.6 DISCUSSION

This study has demonstrated that, compared to normal subjects, nitrite levels in breath condensate were higher in CF patients but confirmed again that this was not the case with exhaled NO. It also showed that there is no relationship between exhaled NO and nitrite levels.

This suggests that exhaled NO may not reflect total NO production in the airways, particularly if the airways are chronically suppurative. It is likely that NO, as it is being produced, is converted to nitrite and possibly nitrate (although this was not measured here) before it has the chance to diffuse into the lumen as gaseous NO.
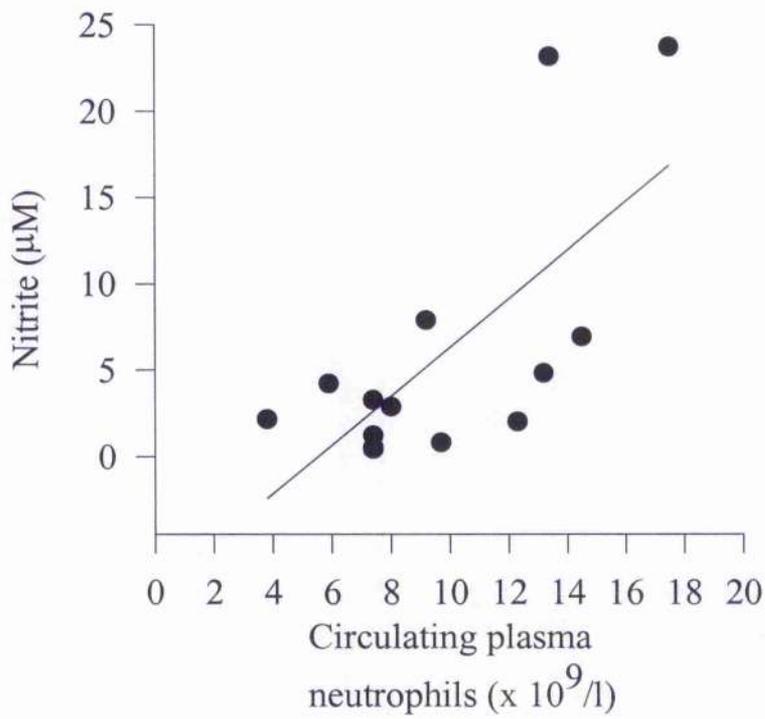
Figure 18. Correlation between breath condensate nitrite levels inpatients with cystic fibrosis and their peripheral neutrophil levels.
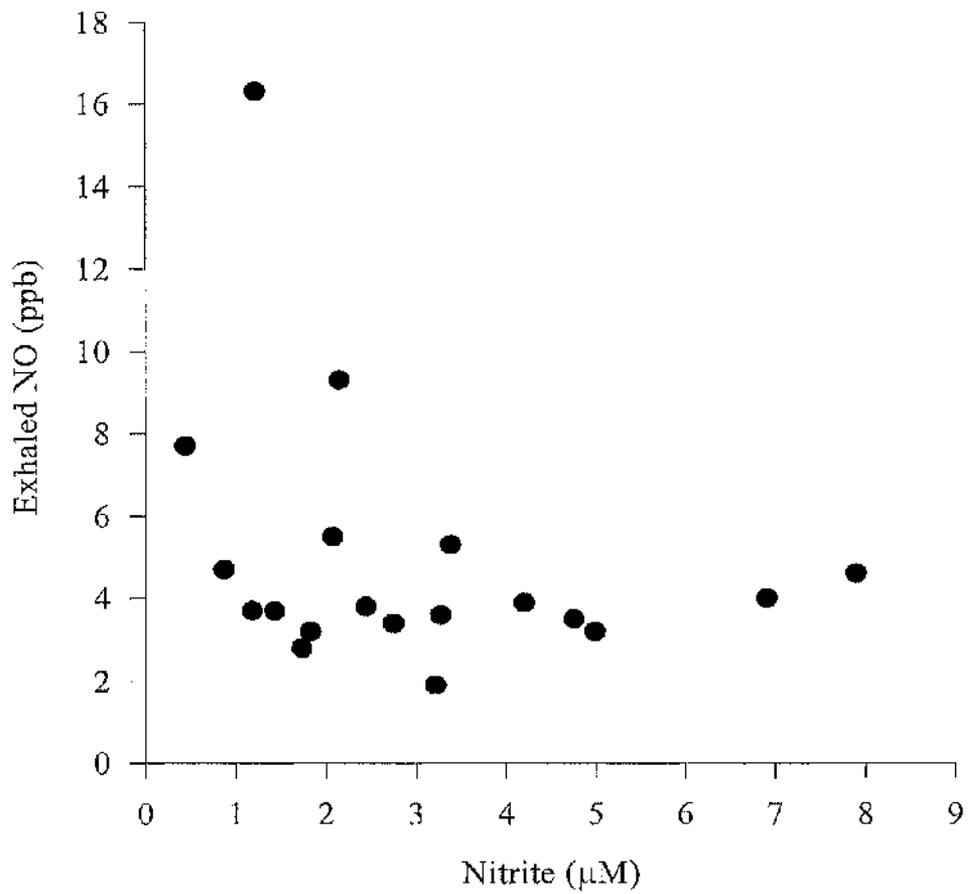
Figure 19. Relationship between breath condensate nitrite levels and exhaled NO levels in patients with CF.
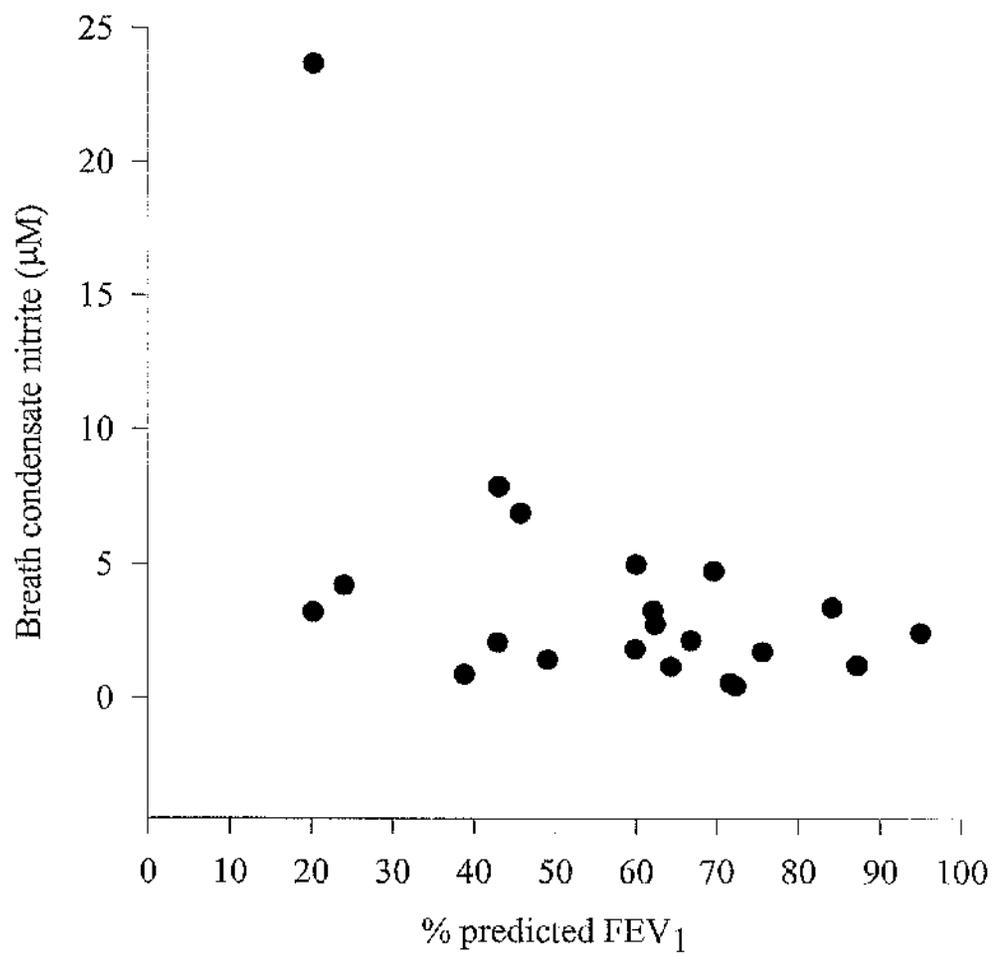
Figure 20. Relationship between breath condensate nitrite levels and lung function in patients with CF.

Excess secretions and mucus in CF airways may inhibit diffusion of gaseous NO into the airways lumen and encourage interaction with the epithelial lining fluid, forming nitrite and nitrate. Other factors may contribute to its removal from detection as gaseous NO, for example reaction with other reactive oxygen species such as superoxide to form peroxynitrite. It is possible that elevated levels of gaseous NO reflect very high levels of NO production which is surplus to that which has metabolised at the site of production.

Breath condensate nitrite also appeared to correlate with peripheral blood total leukocyte counts. It is possible therefore, that nitrite levels in the airways may reflect the burden of inflammation in the airways. It is not surprising to find no correlation between nitrite levels with lung function in spite of its relationship with leukocyte count. This is because FEV_1 in CF patient can be regarded as a 'late' event in the chain of disease processes. It reflects a combination of irreversible end stage airway damage and fibrosis, degree of bacterial colonisation and mucus plugging and may not bear any relationship with ongoing inflammation at the time of sampling. This is particularly pertinent in clinically stable patients as in this study. The finding that inhaled steroids did not affect nitrite levels was more unexpected. It is possible that large parts of the airways in suppurative airways disease like CF are inaccessible to inhaled medication. Lack of compliance with medication in this group of patients could also be a contributory factor to this unexpected result.

Breath condensate nitrite, like exhaled gaseous NO gives no information as to the cellular source of NO production. Polak's group (Meng Q-H, 1998) found that there was significantly decreased or absent epithelial NOS II expression in bronchial explants from end stage CF patients. Our finding is not inconsistent with this, since nitrite levels that we measured may have reflected NO produced by inflammatory cells rather than epithelial cells. This is supported by the relationship with circulating plasma neutrophils and also Polak's finding that NOS II appeared to be normally expressed in neutrophils found in CF airways. Grasseman and colleagues (Grasseman H, 1997. Grasseman H, 1998) have also demonstrated high levels of a related NO metabolite, nitrate, in bronchoalveolar lavage fluid of children with pneumonia and CF, supporting my hypothesis that in inflamed airways, a significant proportion of NO from the lower airways may have been oxidised to nitrite and nitrate.

8.7 CONCLUSION

Breath condensate nitrite levels are elevated in clinically stable CF patients compared to normal controls and is possibly a more sensitive marker of airways NO production compared to exhaled NO. It may be more useful as a marker of airways inflammation compared to exhaled gaseous NO in suppurative airways disease. The findings from the study also suggest that NO generation is high in cystic fibrosis airways in spite of low exhaled NO levels, and that this is likely to be mainly attributable to production by inflammatory cells.

CHAPTER 9

DEVELOPMENT OF HYDROGEN PEROXIDE ASSAY FOR BREATH CONDENSATE

9.1 BACKGROUND

Oxidant induced cellular damage plays a significant role in the pathogenesis of many diseases (Kinnula VL, 1995). The impact of free radicals may be especially important in the lung because of its great surface area and exposure to higher concentrations of oxygen than other tissues. The free radical burden occurs as a byproduct of cellular oxidative metabolism and, in the lung, is further increased by inhaled toxic particles and gases. There is evidence that oxidants play a central role in the pathogenesis of many pulmonary diseases, such as fibrosing alveolitis, emphysema, asthma and ARDS (Barnes PJ, 1990. Crystal RG, 1991. Heffner JE, 1989. Kamp DW, 1992. Sibille Y, 1990). The mechanisms contributing to free radical related tissue injury include reactive oxygen species generation by both normal lung cells and by inflammatory cells.

The primary reactive oxygen metabolites formed in vivo are superoxide and hydrogen peroxide. The latter is also generated through non-enzymatic or enzymatic dismutation of superoxide. These radicals, although toxic themselves, generate an even more reactive and harmful radical - the hydroxyl radical (Halliwell B, 1990). Given the potential deleterious effects of these molecules, the

lungs (and other cells) have efficient antioxidant defence mechanisms and various lung cell types differ in their resistance against oxidants, partly due to differences in these antioxidant defence mechanisms.

Hydrogen peroxide is primarily produced by mechanisms that produce superoxide in the cytoplasm, mitochondria or endoplasmic reticulum (Kinnula VL, 1995). This is generated through enzymatic or non enzymatic dismutation and occurs physiologically as a byproduct of mitochondrial respiration or via cytoplasmic reactions catalysed by enzymes like xanthine oxidase. Such H_2O_2 production is contained within the cell by rapid scavenging locally. However, H_2O_2 is more stable than superoxide and diffuses easily through the plasma membrane. Therefore, in circumstances when intracellular H_2O_2 generation exceeds the capacity of the antioxidant scavenging systems of the cell (catalase and glutathione), it diffuses extracellularly (Kinnula VL, 1992c). Extracellular release also occurs from activated inflammatory cells and cells like alveolar epithelial and vascular endothelial cells, partly from superoxide generation at the cell membrane (Dupuy C, 1991. Kinnula VL, 1992a). Production by this method is usually much greater and is catalysed by membrane associated NADPH oxidase. This forms the respiratory burst in these cells which are stimulated by various proinflammatory agents.

Measurement of H_2O_2 therefore may be seen to reflect the oxidant burden in biological systems. In conditions like cystic fibrosis, it may be a reflection of the inflammatory cell activity or a measure of phagocyte response to stimulation. The

finding that nitrite, but not gaseous NO, levels are increased in exhaled air of CF patients suggests that NO, being a highly reactive molecule, may have reacted close to its site of production (e.g. to an end metabolite like nitrite) and is prevented from being detected in the exhaled air. I was interested in investigating the levels of H_2O_2 in the exhaled air for two reasons:

1. If the hypothesis that NO production in CF is high but simply not detected because it has reacted close to its site of production or because its diffusion into the gaseous phase is impeded by secretions is correct then H_2O_2, being a highly reactive molecule, would suffer the same fate.

2. If, the above hypothesis is not true and the levels of H_2O_2 in breath condensate are high then this may be a useful method for monitoring response of airways inflammation to novel treatment in CF (e.g. gene therapy, chapter 1.8.3.2).

The first task was to validate a method for measuring H_2O_2 produced in the airways.

9.2 METHODS

Several methods have been used for the determination of H_2O_2 (Root RK, 1975. Ruch W, 1983 Roberts IM, 1991. Cathcart R, 1983. Giulivi C 1994. Pick E, 1980). Almost all utilised the principle of fluorometry where hydrogen peroxidase catalyses the oxidation of a compound ('substrate') by hydrogen peroxide to form a fluorogenic molecule. Most of the substrates are phenolic compounds which act

as hydrogen donors. The choice of substrate may be determined by the system to be tested; e.g. in cell culture experiments, p-hydroxyphenyl acetic acid may be cytotoxic in certain concentrations and therefore other substrates like homovanillic acid may be preferable.

Other methods use CO_2 liberation from catalase dependent oxidation of radiolabelled formate (Iyer GYN, 1961). The main disadvantage of this method is the need of incubation vessels suitable for the trapping of gaseous CO_2. Inhibition of cellular catalase by aminotriazole, catalase being susceptible to this only when complexed with H_2O_2, has also been used as an indirect assay. However, it is not suitable for phagocytic studies as these cells only have low levels of catalase (Gee JBL, 1970). The most commonly used assay is probably one based on the quenching of fluorescence mediated by horseradish peroxidase and H_2O_2 dependent oxidation of scopoletin (Root RK, 1975). It has high sensitivity but unfortunately suffers from a few disadvantages. A non-linear loss of fluorescence occurs as scopoletin concentration decreases and H_2O_2 production can only be followed for a short time interval due to exhaustion of scopoletin. In addition, since direct fluorometry is required, the assay cannot be performed easily on cell monolayers. Some of these problems are eliminated by using phenol red as the H-donor with production of an oxidation product (Pick E, 1980). However, this method is relatively insensitive.

In terms of breath condensate H_2O_2 levels, different assays and indeed different collection methods have been employed. This has inevitably resulted in a variety

of H_2O_2 levels which appear to be laboratory specific (table 13). However, in general, all levels are very low and clearly a sensitive method is important. At the start of this MD project, no publication had studied the intrasubject reproducibility of the assay.

Breath collection

Breath condensate was obtained as described in chapter 7.

H_2O_2 assay

P-hydroxyphenyl acetic acid was chosen as the substrate since this method has been used for breath condensate measurements in asthmatics (Dohlman AW, 1993) and also been shown by other investigators to be the most sensitive method for use in cell culture systems (Zaitsu K, 1980). With this method, the concentration of H_2O_2 is quantified by using a fluorometric assay based on the formation of an intermediate compound (compound I) from H_2O_2 by hydrogen peroxidase and the oxidation of p-hydroxyphenyl acetic acid (PHAA) by this compound to a fluorogenic compound.

H_2O_2 $\rightarrow$ compound I (transient oxidised intermediate)
$\downarrow$
PHAA $\rightarrow$ oxidised compound (fluorogenic)

The method was first described by Hyslop and Sklar (Hyslop PA, 1984). Fluorescence from the oxidised product was read from the excitation and emission

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Author / year	Airways disorder	H2O2 levels (µM)
Baldwin SR, 1986	Ventilated ARDS	1.68 (0.35)
	Ventilated, no ARDS	0.34 (0.08)
Sznajder JI, 1989	ARDS and pulmonary infiltrates	2.34 (0.2 - 6.1)
	ARDS and respiratory failure	0.99 (0.05-3.0)
	Respiratory failure but no ARDS	2.45 (0.1-6.2)
Kietzmann D, 1993	Respiratory failure & ventilated	0.1 (0.15-0.89)
	ARDS	0.55 (0.15-0.89)
	Post operation ventilation	0.04 (0.02-0.60)
Dekhuijzen PNR , 1996	Stable COPD	0.21 (0.12-0.55)
	COPD exacerbation	0.5 (0.2-0.15)
	Normal subjects	0.03 (0.0-0.1)

Table 13. Breath condensate hydrogen peroxide levels in different patient groups. Figures in parenthesis = SD or range. ARDS = adult respiratory distress syndrome, COPD = chronic obstructive pulmonary disease.

scans at 310 nm and 414 nm wavelengths. Ultra violet range cuvettes were used in a model LS-5B, Perkin Elmer fluorometer.

H_2O_2 assay protocol

An assay used by Greening AP et al (1983) for cell culture work was adapted for breath condensate measurements of H_2O_2 levels. 7.4 mg/ml p-hydroxyphenyl acetic (PHAA) was prepared (0.074 g in 10ml distilled water) on the day of use. Horse radish peroxidase type II (HRP) (Sigma Aldrich) at 0.1 mg/ml was prepared by making 10mg/ml concentration (0.05g in 5 ml) in distilled water and diluted 1:100 with in 2.5 % Hanks Hepes to obtain 0.1 mg/ml. HRP was then added to PHAA in a 3:1 (vol:vol) ratio. 1 ml of breath condensate was added to 1 ml of the final PHAA and HRP mixture. 600 μl of borate buffer (12.37g borate buffer in 1 l distilled water, adjusted to pH 10.4 with NaOH) was then added. The mixture was allowed to react for 10 minutes at room temperature in dark.

For the standard curve, H_2O_2 stock solution was prepared from 3% H_2O_2 solution (Sigma) - 0.01M solution was first prepared by dilution with distilled water (1.13 ml 3% H_2O_2 and 98.87 ml distilled water), then diluted to 2 μM stock solution. The 2 μM solution was made fresh on the day of use. A standard curve was prepared from varying proportion of 2 μM H_2O_2 and distilled water, and 600 μl borate buffer to give final concentrations of 0 to 0.73 μM.

Breath condensate was obtained from three CF patients (mean FEV_1, 56.8%, mean age 19 years) and three normal subjects (mean age 28 years) for preliminary testing of this protocol.

9.3 RESULTS

The standard curve showed a good correlation coefficient (r=0.99) for concentrations between 0 and 0.77 uM (figure 21).

Results on three CF patients and two normal subjects suggested discrimination at low levels (table 14).

It was decided that this method would be adopted for patient studies.

9.4 OTHER OBSERVATIONS

Background fluorescence. At the start of the studies it was noted that there was a significant amount of fluorescence in the final mixture, when no H_2O_2 was present (zero of standard curve). The cause of this was explored by changing the starting concentration, cuvettes, change in concentration of HRP, age of HRP and PHAA, ratio of volume of HRP to PHAA, change in diluent for H_2O_2 stock solution, HRP and PHAA and changes in pH of final concentration.
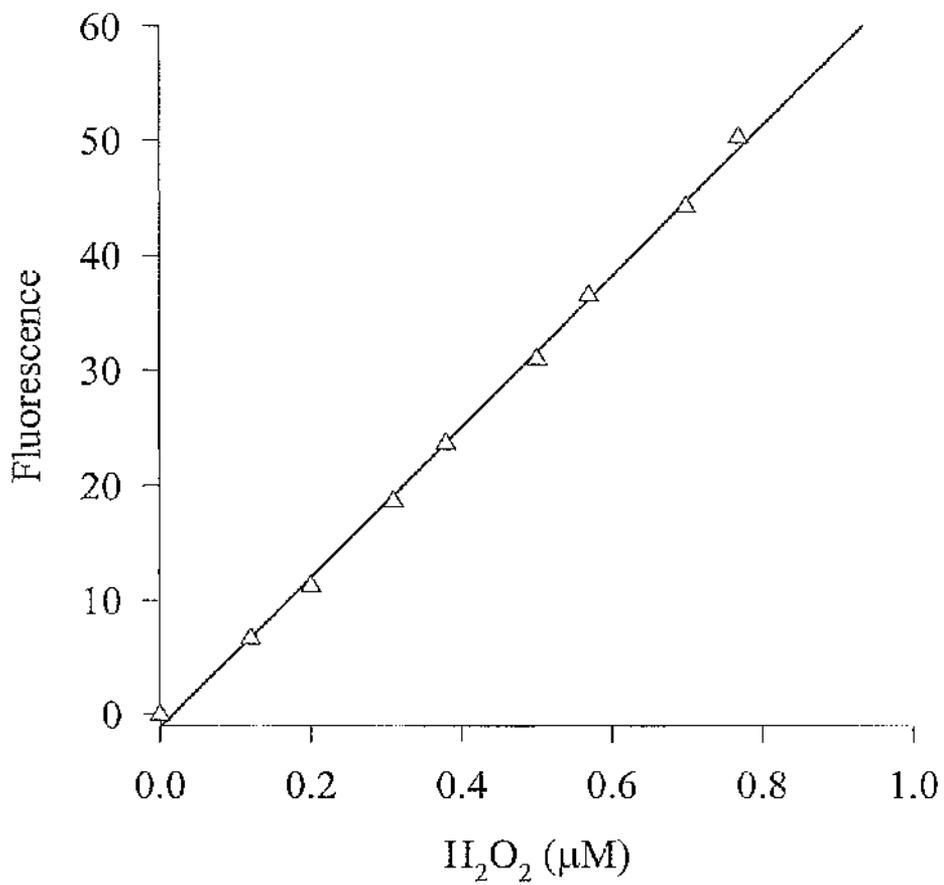
Figure 21. Standard curve showing fluorescence for known incremental concentrations of hydrogen peroxide.

Subject	H$_2$O$_2$ levels in breath condensate (μM)	
	Day 1	Day 2
CF1	0.55	0.61
CF2	0.30	0.63
CF3	0.50	0.83
Normal subject 1	0.20	0.44
Normal subject 2	0.06	0.0

Table 14. Breath condensate hydrogen peroxide values for patients with CF and normal subject on two different days.

It was discovered in the end that the unexplained fluorescence was secondary to the age of the PHAA solution. As PHAA was made up and left in the laboratory it is slowly oxidised in the atmosphere and this contributed to the high background fluorescence even without the addition of H_2O_2. This was an important finding as a high background fluorescence contributed to the loss of sensitivity of the assay since the substrate (PHAA) was already partially consumed before the assay was used.

9.5 EFFECT OF STORAGE AT -20°C ON SAMPLES

Methods

Using the final method, two sets of standard dilutions were made by diluting 2 μM H_2O_2 stock solution to obtain concentrations from 0-0.77 μM. Fluorescence from these samples were measured immediately (fresh) after addition of reactants and after being kept in the dark for 15 and 30 minutes. On a different day, another three sets of standard dilutions were prepared. Fluorescence was then measured immediately, and after 24 hours, 72 hours and 7 days storage at -20°C. In addition 5 breath condensate samples were divided into two, one measured immediately after collection for H_2O_2 levels and the other stored without added reactant at -20°C for 5 days before being thawed and assayed.

Results

Reacted samples are stable at room temperature in the dark for up to 30 minutes after addition of reactants (PHAA and HRP) (figure 22). However with storage at -20°C, samples showed increasing variability and loss of fluorescence with increase in storage time (figure 23). Samples kept without having had reactant added first showed variably decreased amounts of fluorescence compared to freshly assayed samples (table 15).
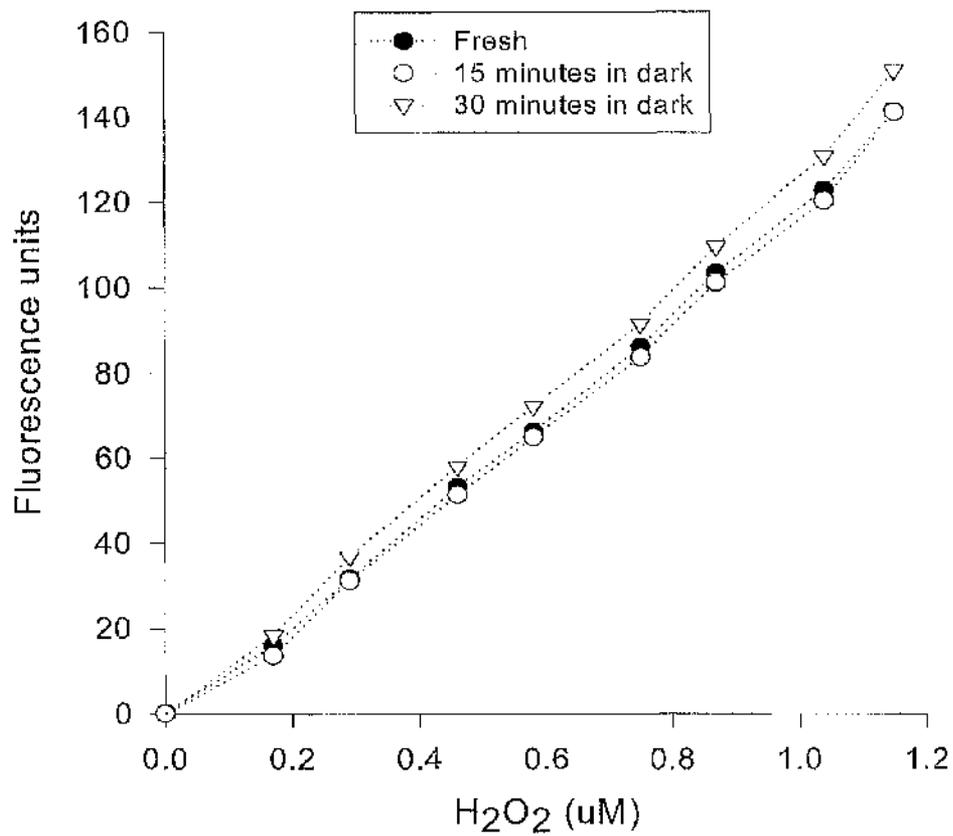
Figure 22. Fluorescence of known concentration of H_2O_2 are maintained after 15 and 30 minutes storage in the dark.
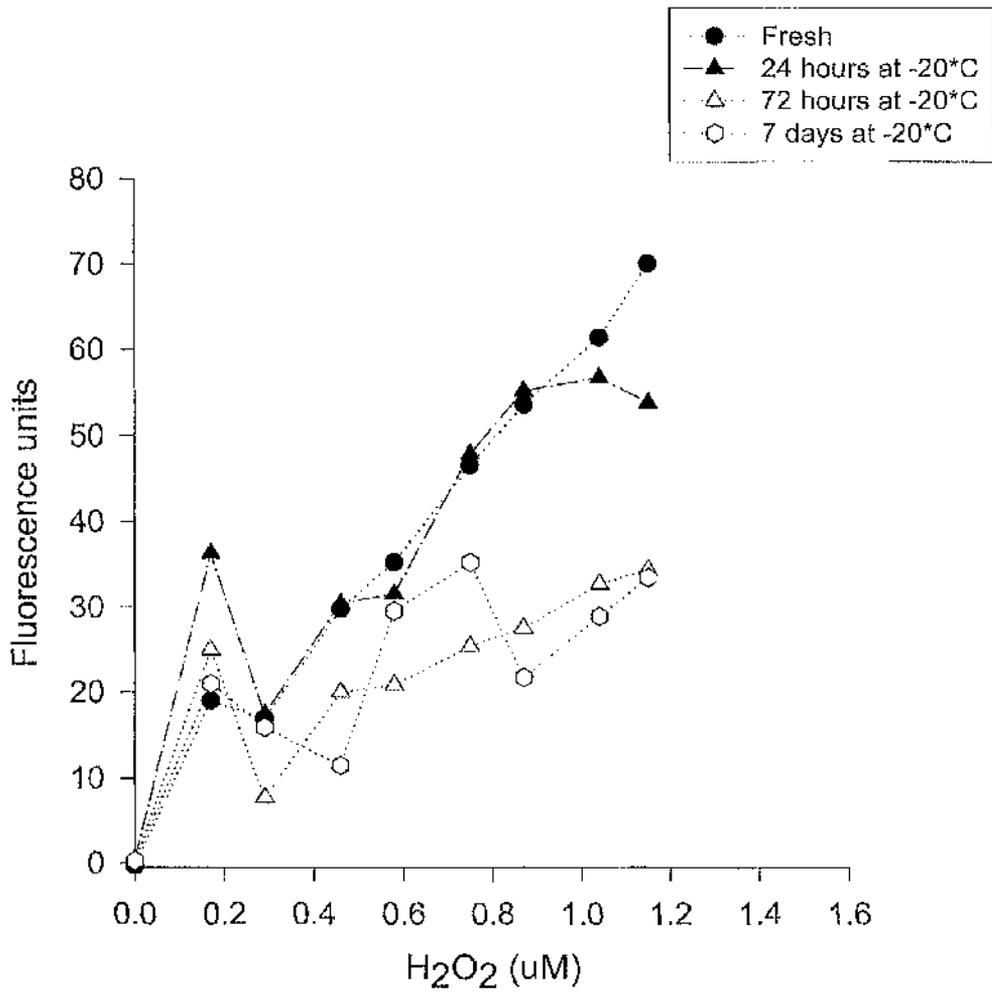
Figure 23. Graph showing changes in fluorescence of known concentration of H_2O_2 after storage at 20°C for 24 hours, 72 hours and 7 days.

Sample	Freshly assayed	Stored for 5 days and then assayed
1	15	-5
2	21	8
3	15	-12
4	19	10
5	35	2

Table 15. Effect of storage in -20°C on breath condensate hydrogen peroxide levels from 5 normal subjects . Values in fluorescence signals.

9.6 FINAL H$_2$O$_2$ ASSAY PROTOCOL

A solution comprising 50 mM PHAA (Sigma-Aldrich,U.K.), 0.1 mg/ml Type II borseradish peroxidase (Sigma-Aldrich,U.K) in 1:3 volume was freshly made on the study day. One ml of breath condensate is added to 1 ml of this solution within 5 minutes of collection and a pH of 9-10 was then achieved by addition of 1 ml of borate buffer (made to pH 10.4). The fluorescence from the oxidised product was then read from the excitation and emission scans at 310 nm and 414 nm wavelengths performed in UV range cuvettes using model LS-5B, Perkin Elmer fluorometer. A fresh standard curve is performed each day using 3% hydrogen peroxide stock solution diluted in ultra heat purified distilled water.

9.7 REPRODUCIBILITY OF FINAL ASSAY

Methods

Breath condensate was collected from five normal subjects on two different days (1 week apart) and H$_2$O$_2$ assay was performed on the samples in the same fashion as described. Reproducibility was measured using the Bland-Altman test. Statistical significance was assumed at $p < 0.05$.

Results

Individual results are shown in Table 16.

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Subject	H$_2$O$_2$ levels (μM)	
	Day 1	Day 2
1	0.14	0.15
2	1.60	0.94
3	0.08	0.03
4	0.10	0.16
5	0.11	0.10

Table 16. Breath condensate hydrogen peroxide levels in 5 different normal subjects on 2 different days.

Bland-Altman test showed a mean difference between 2 days of 0.023 mM with a

95% confidence interval of -0.04 to 0.08 mM

9.8 DISCUSSION

The storage of samples resulted in variable loss of fluorescence activity. Therefore, all samples were assayed within 10 minutes of collection. It is unclear why this occurred and the finding is at variance with other investigators. However, samples with reactants added can be kept in the dark at room temperature for up to 30 minutes.

9.9 CONCLUSION

H_2O_2 can be measured reproducibly in breath condensate with an assay that detects H_2O_2 down to 0.1 μM.

CHAPTER 10

HYDROGEN PEROXIDE LEVELS IN BREATH CONDENSATE OF CYSTIC FIBROSIS PATIENTS

10.1 INTRODUCTION

Lung injury in CF patients has been attributed to various factors, although basic mechanisms have not been fully elucidated. As discussed in chapter 1.8.1, abnormal airways secretions, decreased mucociliary clearance and loss of activity of innate antibacterial molecules like β-defensins generate a permissive environment for bacterial colonisation and infection, driving chronic inflammation. The major contribution to cell damage is thought to be secondary to proteolytic and oxidative products released by these inflammatory cells (Doring G, 1996). The primary reactive oxygen metabolites formed in vivo are superoxide and hydrogen peroxide, released during oxidative bursts associated with sequestration of invading organisms. Hydrogen peroxide is also generated through nonenzymatic and enzymatic dismutation of superoxide. Because of their lability, direct detection of superoxides is difficult. H_2O_2 on the other hand is volatile, more stable and enters the gaseous phase at physiological temperatures.

10.2 HYPOTHESIS

It was hypothesised that H_2O_2 levels in the lungs are elevated in patients with CF due to the increased neutrophilic inflammation and activated macrophages. This

may be reflected in increased levels of H_2O_2 in the condensed breath which may be used to follow airways inflammation in these patients.

10.3 STUDY DESIGN

In this study the levels of H_2O_2 in breath condensate of CF patients and normal healthy controls were measured and examined for correlation with lung function and circulating plasma leucocytes.

Expired breath condensate was collected from all subjects and assayed immediately for H_2O_2. All samples were fresh and processed immediately after collection. Each patient contributed one (duplicate) sample. Within 2 hours of breath collection, FEV_1 was performed and venous blood was collected for measurement of total circulating leukocyte count and differential analysis of leucocytes for neutrophils, monocytes and eosinophils.

Subjects and patients

Sixteen clinically stable CF patients (mean age 25.3 years, 9 males) and 14 normal subjects (mean age 29.9 years, 6 males) were recruited. Lung function in the CF subjects ranged from 14.8 % to 77.8% of predicted FEV_1 (mean FEV_1 : 50.2 %) but all were clinically stable at time of sampling (table 17). Eight of these patients also had asthma, diagnosed by clinical history and reversible airways obstruction (increase in FEV_1 of greater than 175 ml after 2.5 mg nebulised salbutamol) and

	Cystic fibrosis patients (n=16)	Normal subjects (n=14)
Mean age (S.D.) (years)	25.3 (6.5)	29.9 (6.2)
Gender	9 males/7 females	6 males/8 females
Mean FEV_1 (S.D.)	50.2% (20.1%)	Not done

Table 17. Demographic data of the study groups.

were on inhaled steroids. The dose of inhaled steroids ranged from 400 µg beclomethasone dipropionate per day to 2000 µg fluticasone propionate per day. Normal subjects were healthy volunteers, with no evidence of active allergic rhinitis, concomitant respiratory tract infection or respiratory symptoms. For both groups, smokers were excluded.

Collection of breath condensate

Breath condensate was collected using the method described in Chapter 6.3.

H_2O_2 assay

H_2O_2 was assayed using the method described in Chapter 9.6.

10.4 RESULTS

H_2O_2 levels in breath condensate

The median level of H_2O_2 concentration in breath condensate of CF patients was lower than that in normal subjects (63.5 nM v 89.5 nM) but this did not reach statistical significance (p=0.198, Mann Whitney Rank Sum Test). There was a bigger range within the normal group (figure 24). Further analysis between CF patients on inhaled steroids and those not, showed no difference in mean levels
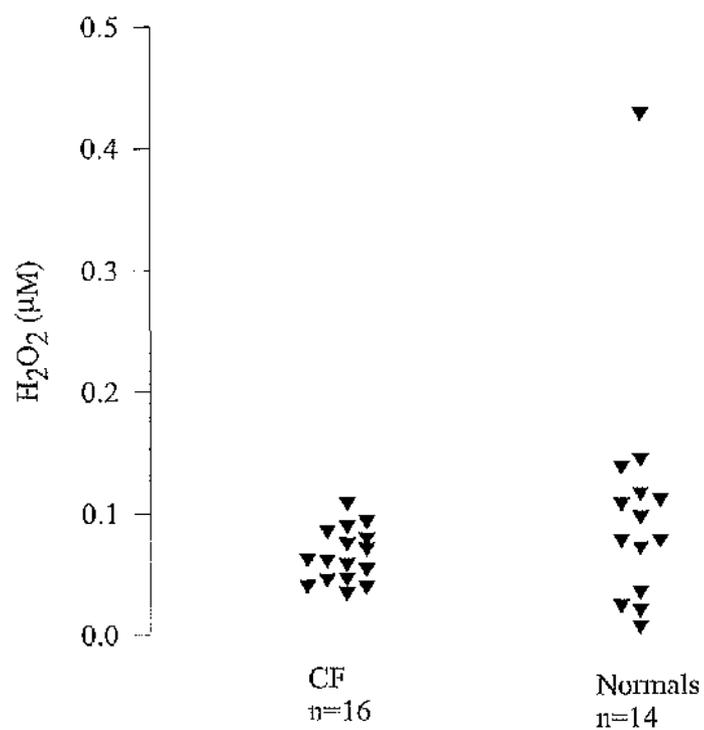
Figure 24. Breath condensate levels of hydrogen peroxide in patients with CF compared to normal subjects.

between the two groups (patients on inhaled steroid, n=8; 65.5 nM and not on inhaled steroids, n=8; 68.4 nM)

Relationship of H_2O_2 levels and lung function

Within the CF group, there was no significant correlation between H_2O_2 level and lung function, although the trend is for higher levels of H_2O_2 in better patients (r= -0.20, p=0.55) (figure 25).

Relationship of H_2O_2 levels and circulating inflammatory cells

There was a modest but insignificant positive correlation between H_2O_2 and circulating leucocytes and neutrophils (figure 26).

10.5 DISCUSSION

It has been shown previously that activated human macrophages release a large amounts of H_2O_2 extracellularly (Greening AP, 1983). Even larger amounts of H_2O_2 are released from neutrophils (Shult PA, 1985) and H_2O_2 release has been measured from apical surfaces of tracheal epithelial cells, monocytes, rat alveolar type II cells and eosinophils (Kinnula VL, 1992a&b). The levels that we have observed in the CF patients (range of 0.04 to 0.11 μM) were below the range reported previously by other groups working in stable asthmatic children (mean of 0.54 μM and median of 0.6 μM from two different authors) (Jobsis Q, 1997,
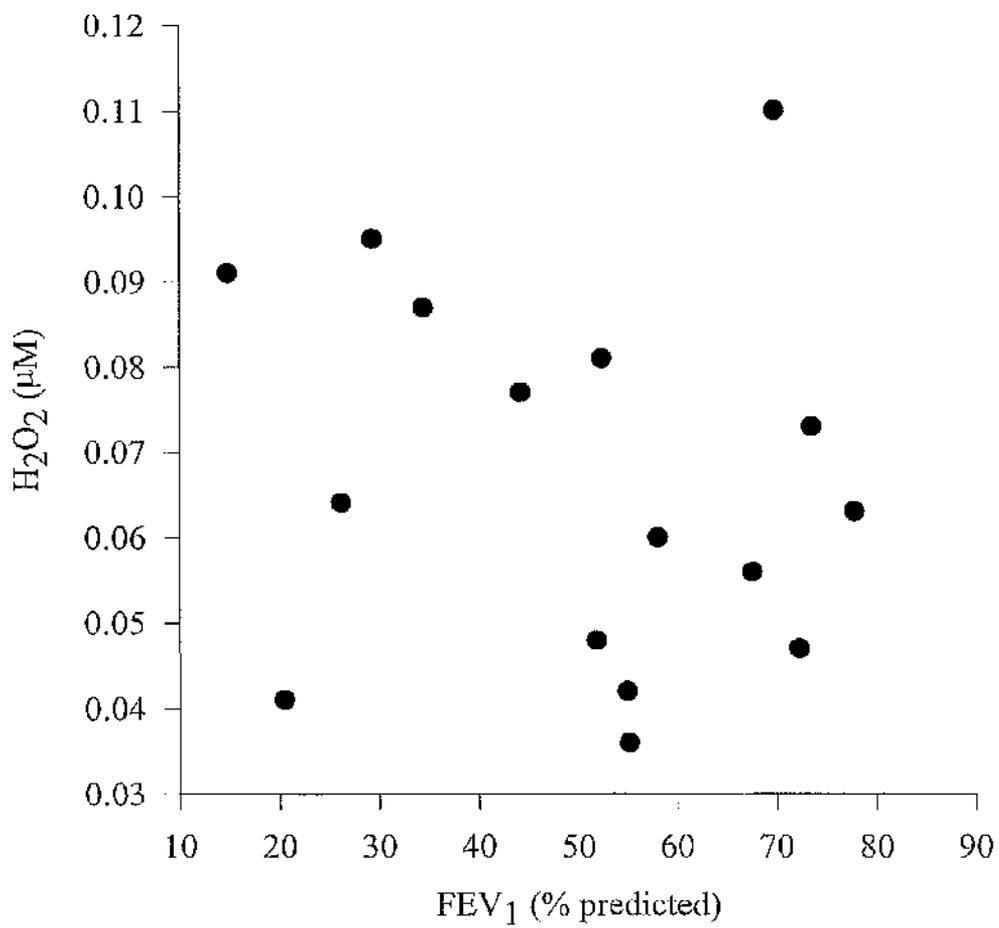
Figure 25. Relationship between breath condensate H_2O_2 levels and lung function in patients with CF.
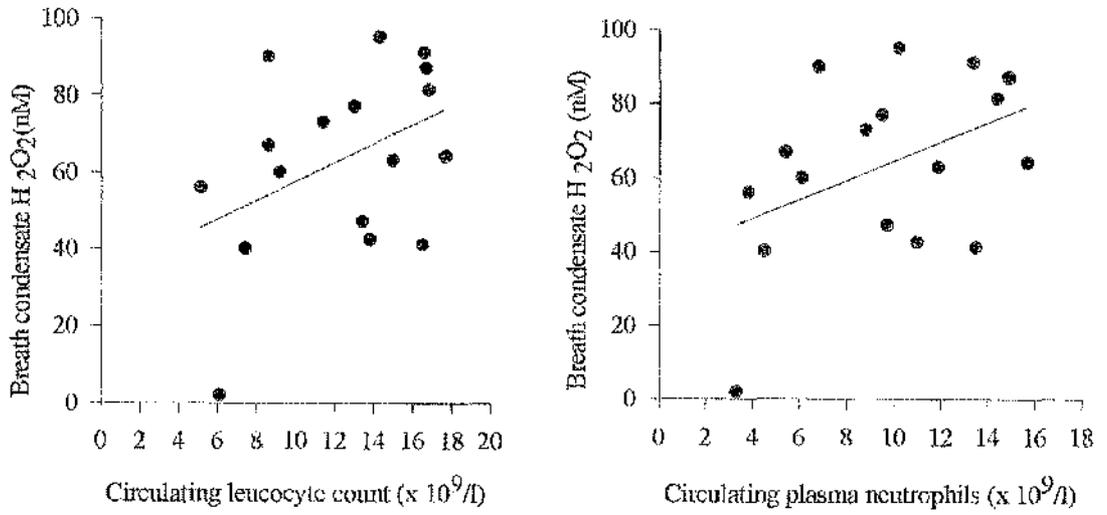
Figure 26. Relationship between breath condensate H_2O_2 and peripheral total leucocyte and neutrophil count.

Dohlman AW, 1993) and stable COPD (0.12-0.55 µM) (Dekhuijzen PNR, 1996). All the normal ranges, however, were similar to ours : 0-0.8, 0-0.1 and 0.08-0.23 µM compared to our levels of 0.008-0.15 µM.

In experimental animals, lung injury caused by complement activation can be attenuated by superoxide dismutase, catalase and desferrioxamine (Seekamp A, 1993. Ward PA, 1983). All are antioxidants which scavenges the free radicals released by these cells. In ARDS, where neutrophil accumulation and stimulation is thought to amplify lung damage, glutathione levels, a major antioxidant in the airway epithelial fluid lining has been found to be decreased (Pacht ER, 1991) and H_2O_2 in the exhaled air of intubated patients elevated (Baldwin SR, 1986. Kietzmann D, 1993). Thus in CF, where neutrophilic inflammation predominates and alveolar macrophages are thought to be constantly stimulated by bacterial colonisation, it was expected that hydrogen peroxide generation would be high and that this might be detected in expired breath. In this study I have found no elevation of H_2O_2 in very inflamed airways. This is the case, even in the patients with concomitant asthma, although inhaled steroids may have been a confounding factor.

There are several possible explanations for this. In the context of the general investigation of this MD project, it is possible that hydrogen peroxide is generated but not detectable due to rapid reaction with other reactive species - superoxide, a source of extracellular H_2O_2 is known to react rapidly with the free radical NO to form peroxynitrite. The other possibility is that with large amount of viscous

secretions within the airways, volatile substances like H_2O_2 are prevented from diffusing into the airway lumen. This is supported by the small range of H_2O_2 levels within our CF group - suggesting that whether the levels were high or low, only a certain amount was able to escape into the lumen. Another supporting factor comes from the modest correlation between H_2O_2 in breath condensate and circulating leukocyte count. This suggests that there may be a correlation with inflammatory burden but that this was not detectable in the expired breath.

An alternative explanation may be that there is an increase in the release, by neutrophils and monocytes within the CF environment, of myeloperoxidase or an antioxidant like catalase (Worlitzsch D, 1998). The former generates hypohalous acids in the presence of H_2O_2 and halides, thus effectively removing H_2O_2. Both catalase and MPO are positively charged while the airway secretions are negatively charged (due to excess DNA from degraded inflammatory cells). They may therefore be "trapped" for a longer time in this environment. If the residence time of catalase is indeed increased, then it could be suggested that airways secretions in CF may be protective against further inflammatory cell injury. There is some evidence suggesting the protective nature of airways secretions in CF- preliminary in vitro studies have shown increase in levels of free IL8, a peptide with affinity for the anionic DNA, with the addition of recombinant DNA-ase to sputum *in* vitro (Perks B, 1997).

10.6 CONCLUSION

These results, taken together with the findings on exhaled NO and nitrite suggest that the lack of elevation of NO in the exhaled air is likely to be secondary to decreased detection of gaseous molecules in cystic fibrosis rather than decreased in production. It is unlikely that decreased levels of two reactive gaseous products are attributed to lack of production in the face of overwhelming in vitro evidence for increase in its production.

CHAPTER 11

SUMMARY OF THESIS FINDINGS

The thesis concludes that

1. Gaseous NO can be measured reproducibly in the exhaled air of normal subjects. With my method, the 95% confidence interval for any measurement of NO is ± 1.72 ppb.

2. Using the method of detection as in this project, increased levels of ambient NO does not affect measurement of endogenous NO from the airways.

3. Exhaled nitric oxide is not increased in patients with cystic fibrosis in contrast to asthmatics, in spite of airways inflammation and acute infective exacerbation.

4. There is a significant positive correlation between exhaled NO levels and FEV_1 in CF patients.

5. Exhaled NO is not elevated in bronchiectatic subjects compared to normal subjects and in contrast to asthmatics.

6. Exhaled hydrogen peroxide can be measured in the breath condensate of CF and normal subjects.

7. Hydrogen peroxide levels are not increased in CF patients compared to normal subjects.

8. Nitrite, a stable end product of nitric oxide can be assayed reliably in breath condensate.

9. Breath condensate nitrite levels in CF subjects are increased compared to normals, in contrast to exhaled NO levels. This suggests that NO production in airways of CF subjects is increased. The cellular source of this NO is unknown.

10. In these patients, there is a significant positive correlation between condensate nitrite levels and circulating leucocytes and neutrophil counts but not with FEV_1.

11. NOS II gene is expressed in both primary nasal epithelial cells and epithelial cell line ($\Sigma CFTE$) of CF patients and respond normally to proinflammatory stimuli. The presence of CFTR mutation does not affect NOS II expression in epithelial cells.

CHAPTER 12

OVERALL DISCUSSION AND CONCLUSION OF THESIS

This thesis has explored issues pertaining to a newly available methodology for measuring exhaled NO levels, reported new findings on NO production in the airways of patients with CF and bronchiectasis and developed new methods for detecting NO production in the lower airways. It has also raised questions about the interpretation of exhaled NO levels and our ability to measure endogenous NO production in chronically inflamed airways.

The first part of the thesis validated a new method for measuring NO levels in the exhaled breath. This was an important part of the thesis as subsequent studies and conclusions depended on a reproducible and accurate method of measuring airway NO. I determined a consistent point of measurement on the exhalation profile and found that this was not affected by inspired NO levels. Also, it was found that mouth pressure and therefore, exhalation rate affected the end expiratory NO levels. An important factor for accurate measurement was the ability to exclude contribution of nasal or paranasal NO to lower airway NO. I was able to do this with an analyser which had a novel built-in mechanism that prevented mixing of nasal and lower airway air.

The finding of low levels of exhaled NO in cystic fibrosis patients stimulated me to examine the reasons for this. This was important because if, indeed, NO

production was low, then this could potentially contribute to decreased bacterial resistance in CF airways. It was also possible that the presence of the CFTR mutation may have contributed to a defect in expression of the NOS II gene.

Putting all my findings together, it appears most likely that exhaled NO levels in CF are reduced because of a defect in detection rather than total production of NO in the airways. This is suggested by the finding of elevated levels of breath condensate nitrite in contrast to NO in gaseous form. The anomaly in detection may occur because although exhaled NO appears an easy method of measuring NO production in the airways, it suffers from a few limitations. Firstly it requires NO to be in the gaseous phase and in the presence of excess secretions, the gas is impeded from diffusing into the exhaled air. Secondly within severely inflamed environments, gaseous NO reacts quickly with other reactive molecules (not usually present in normal airways) to form other products. These factors are most relevant to chronic suppurative states like CF and bronchiectasis.

It can be argued that similar reactions could occur in asthmatics because these patients also have increased levels of reactive molecules in their airways. However, two points ought to be borne in mind with exhaled NO levels in asthmatics in this regard. Firstly, not all asthmatics have increased levels of exhaled NO and the reason for this is still unclear. My study in chapter 3 showed that exhaled NO levels in asthmatics ranged between 2.2 and 52 ppb. The large range in NO levels may be secondary to varying amounts of reactive species in the airways. Secondly, the cause of increased NO levels in asthmatics is still

uncertain. There is evidence to suggest that exhaled NO levels do not bear any relationship to clinical severity or lung function in asthmatics (Ho LP, 1998. ten Hacken NHT, 1997). Its levels may bear a closer relationship to eosinophilic inflammation rather than neutrophil influx as seen in CF (Jatakanon A, 1998). Therefore, comparison of inflammation in general between these two disease processes may be inappropriate. Indeed, airways inflammation is a heterogeneous entity. Hence in cystic fibrosis this is characterised by an infection driven process and the cytokine pattern is dominated by IL6, IL8 and TNFα with neutrophils being the predominant inflammatory cells. In contrast, asthmatic inflammation is mediated in main by leukotriene (especially LTD4), IL4, IL5 and IL13, with major inflammatory cells being the eosinophils, mast cells and CD4+ T cells (Tak HL, 1998).

At present, the relationship of *exhaled* NO (as opposed to NO production) with inflamed airways is still under debate. NO levels are not elevated in conditions like ARDS which is characterised by overwhelming sequestration of neutrophils in pulmonary extravascular tissues. My studies on CF have contributed to the debate on the interpretation of increased *exhaled* levels of NO in inflammatory airway conditions and suggested that elevated NO levels do not necessarily equate to inflammatory activity in the airways. This is partly because the tool we have for measuring NO production is imperfect, contributed in part by the highly reactive nature of gaseous NO.

It is also possible to speculate that increased exhaled NO levels are unrelated to neutrophil or eosinophilic inflammation; instead, they may be an allergen-induced phenomenon and secondary to upregulation of NOS II expression in epithelial cells, mast cells or antigen presenting cells (e.g. macrophages), on contact with airborne allergen. This will be in keeping with data showing a positive correlation between exhaled NO levels and bronchial hyperresponsiveness in animals and humans (Dupont LJ, 1998. Persson MG, 1993). Further, exhaled NO levels are also increased in asthmatics who are atopic, some normal atopic subjects and some with allergic rhinitis (Martin U, 1996) The determination of enzymatic and cellular sources of NO detected in the exhaled breath is a current challenge in NO research. The answer to this question is unlikely to be derived solely from human studies and work on NOS knockout animals are likely to be more productive and conclusive.

The thesis highlighted another potentially important finding. Taken together with Polak's study (Meng Q-H, 1998) where NOS II expression was found to be absent in bronchial epithelia of end stage CF patients, my finding of low exhaled NO levels but preserved NOS II expression in the epithelial cells of CF may mean that epithelial NO production in chronically inflamed lower airways may be decreased. However, NOS II expression in inflammatory cells in CF is normal and this is the likely reason for the increased nitrite levels that I found in breath condensate of these patients. This implies that chronic inflammatory stimuli or persistent NO production may downregulate the expression of NOS II in epithelia. NO production is persistently provided by 'freshly' infiltrated inflammatory cells. This

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makes sense physiologically as very high levels of NO would be detrimental to a biological system and a mechanism to switch this off should be in place to prevent injurious effect. A few studies have reported autoinhibitory effects of high NO on its production (see chapter 1.6.3). Therefore, if there was persistent migration of new neutrophils to the airways with accompanying high NO generation from these cells, then it can be envisaged that epithelial NO production might be switched off. This may be inappropriate if the there remains a need for NOS II activity.

The contribution of epithelial NO production to host defence is unknown. Traditionally, the airway epithelium has been regarded as a relatively passive physical barrier to invasion of foreign organisms. However, its role in host defence has become more prominent in the recent years. It is able to exert active effects by producing chemicals with anti-microbial activity like β defensins and the CFTR protein itself may aid ingestion of bacteria. It can be envisaged that epithelial NO may contribute actively to the function of the epithelium in host defence. NO is also likely to have an effect on ciliary activity and therefore, low or absent epithelial NO production may affect mucociliary clearance. Recent studies have also demonstrated that there is 'constitutive' expression of NOS II in airway epithelia (Guo HF, 1995). Whether this is truly 'constitutive' and unprovoked or whether the samples are representative of atopic, mildly stimulated epithelium (e.g. by airborne allergen) is still unknown. However, if it is true that normal airway epithelia express NOS II, then it means that there is constant background requirement for high level NO production and the loss is inappropriate, especially during chronic infective states. It is also known that NO

is involved in the modulation of T helper (Th) lymphocyte differentiation, causing polarisation to a Th2 subset (Huang FP, 1998). This T cell subset is characterised by production of IL4, IL5 and IL13, cytokines which promote humoral and allergic response in contrast to IFNγ, IL2 and TNFα produced by Th1 subset which promote cellular immunity. Conceivably, different levels of NO may influence the Th cell differentiation and hence the associated type of inflammation.

My studies of nitrite and hydrogen peroxide levels in condensed breath had a dual purpose of aiding the understanding of why exhaled NO levels were not elevated in CF and examining two other possible methods of non invasively monitoring airway inflammation. In terms of the latter, breath condensate hydrogen peroxide was not found to be useful as the levels were very low in both normal and cystic fibrosis patients. Realistically the discriminatory power of such low levels is poor and although longitudinal studies are needed to determine that they remain low during infective episodes, it appears unlikely that this method will prove useful.

Nitrite levels however, showed widely differing levels within the patient group and also between patients and normals. Encouragingly, the levels also correlated with peripheral circulating leukocytes. The assay is easily and quickly performed on microplates, making the method viable in the context of a clinical trial. This method offers an alternative to the use of induced sputum as a non invasive method of sampling airway inflammatory activity. Induced sputum, although non invasive, has a few drawbacks - it is not possible to gauge the relative

contribution of different parts of the airways to the final sputum collection, nor how long the sputum has been present in the airways. In addition, there is a small risk of inducing severe bronchospasm with hypertonic saline. Breath condensate measurements, on the other hand, allow instantaneous sampling of inflammatory events at the time of measurement. They also sample the airways 'globally' thus representing net inflammation within the lungs.

The studies on breath condensate studies have also highlighted areas that need further development. The present system of breath collection involves condensation of exhaled breath within a coiled Teflon tube immersed in ice. The method poses two major limitations: a) the requirement for constant supply of wet ice, thus limiting its 'portability' and practicality in large studies and clinics and b) inability to store collected samples. This may be secondary to presence of other inhibitors or molecules within the breath sample/condensate which react with the molecule of interest. Two important improvements to the assay would be the ability to collect samples quickly and to store these indefinitely. Recent reports have suggested that freeze drying breath condensate samples may provide a reliable method of storage and this may be a way forward for this assay.

LESSONS FROM THE PROJECT

With the benefit of hindsight, there are a few things that I would have done differently if I were to do the project again. It would have been much more interesting and profitable to have concentrated on two strands of finding - that of

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high nitrite production in breath condensate and that of low NOS II expression in epithelial CF cells. The studies I have performed have, in large, been cross sectional studies. Although these were necessary to detect differences from the normal population, I would have followed the lead from these studies and examined reasons for the abnormalities detected. Hence, in the exhaled nitrite studies, I would have selected a group of patients who were not on inhaled steroids and followed the course of breath condensate nitrite levels longitudinally. Knowledge of day to day baseline variation in NO levels within CF patients and the effect of exacerbation and treatment would have provided important information on its potential use in clinical practice.

With regards to the studies on NOS II gene expression, I would have included patients with bronchiectasis in the study. This would have given me more information on the effect of chronic inflammation on NOS II expression. The use of nasal epithelium was necessary because of ethical considerations but this clearly limited my conclusions on activities of the NOS II gene in the lower airway. In retrospect, I would have obtained bronchial epithelial cells from CF explanted lungs and concentrated on culturing these cells and examining NOS II expression at baseline, to proinflammatory cytokines and to prolonged inflammatory stimulus. This would be compared to cells obtained by bronchoscopic brushings of normal bronchi from subjects undergoing diagnostic bronchoscopy who subsequently turn out not to have active disease.

One other experiment I would have done in the nasal epithelial cell studies would have been to stimulate the cells with cytokines and LPS for longer than 24 hours. It was originally thought that since cells removed from the bronchus show a natural loss in NOS II expression after 8 hours, experiments performed longer than that would be confounded by this factor. Therefore, experiments were limited to 24 hours. However, with hindsight, a control well (with no stimulus), to show change of gene expression over time could have been included. I would also have looked at the effect of excess NO, by incubating the cells with NO donors, and the effect of IL8, on NOS II expression.

Further, I would have measured the levels of nasal nitric oxide simultaneously with obtaining nasal cells. This would have helped determine if the levels of NO produced in the nose correlated with epithelial NOS II expression.

FUTURE DIRECTIONS

The project has highlighted the question of the source of NO detected in the exhaled breath. It is not clear whether NO levels originated mainly from NOS II or other NOS's or indeed, if levels are NOS II derived, whether this is NOS II from the inflamed epithelia or inflammatory cell influx. The answer to this question is unlikely to be derived fully from human studies and work on animal models are likely to be more productive and conclusive. Recently NOS I, II and III knockout transgenic mice have been generated. Direct comparisons of NO levels in the

exhaled air of these animals and the consequent effect of these levels on bronchial hyperresponsiveness would add substantially to available human data.

The relationship between NO and other inflammatory mediators and transcription factors such as NF-KB and the effect of oxidative stress on transcription in chronic inflammation may provide some answers to the cause of down regulation of NOS II expression. However, it will be important first to substantiate the finding that epithelial NOS II is indeed decreased in chronic inflammatory states and second to examine if this decrease affects host defence, specifically if bacterial colonisation, infection and adherence to cilia is decreased. The CF transgenic mouse with chronic pulmonary bacterial infection is likely to provide a good model for examination of the effects of chronic inflammation on NO production and recurrent bacterial infection *in vivo*. Until these studies are performed, the implication of low NO levels on host defence remains speculative.

Still linked with host defence but from a different perspective, is the effect of NO on ciliary motility. It has been demonstrated that nasal NO is low in CF patients and also in Kartegener's disease. There are preliminary reports which showed that patients with primary ciliary dyskinesia also have very low levels of nasal NO. These data suggest that NO may be involved in the signaling pathway for ciliary activity. Apart from the possibility of using nasal NO levels as a diagnostic test for primary ciliary dyskinesia, exploration of this area could also lead to mechanisms of restoring appropriate NO levels and improvement of ciliary motility in these patients.

Outwith the area of cystic fibrosis, exhaled NO research is moving into studies on the utility of exhaled NO levels in clinical practice, particularly in relatively more common conditions like COPD. Potentially, exhaled NO levels could be used as a sensitive marker of disease deterioration and predictor of corticosteroids requirement. This would greatly enhance clinical practice and management of respiratory diseases like COPD and asthma. In this sense, long term longitudinal studies will be needed together with studies on the effect of natural history of the disease on NO levels. This thesis has shown that exhaled NO levels vary widely in asthmatic patients and a large proportion of steroid-free the patients have normal levels. The reason for this is unclear. There is still some way to go in understanding the biological role of NO in asthma and the implications of increased levels of exhaled NO before its use in clinical practice becomes a reliable possibility.

In recent years, large community studies on the effect of atopy on NO have also emerged, showing that atopic children have higher levels of NO compared to normal children. The logical next step in these studies will be to examine if increased levels of NO in these children are a predictor of development of asthma. In terms of understanding basic mechanism, it will be interesting to determine whether this increase is actually 'protective' - i.e. is the increase secondary to upregulation of the NOS1 gene in bronchial epithelium and iNANC pathways to overcome bronchoconstriction? This is pertinent since there are studies which

suggest that low levels of NO (in the form of s-nitrosothiols) may be responsible for a more severe outcome in asthmatic exacerbation.

CONCLUSION

In summary, this thesis has reported that exhaled NO measurement is reproducible but levels in the exhaled air may not necessarily reflect total NO production. I have shown that exhaled NO levels in CF and bronchiectasis are not increased but breath condensate nitrite levels are (in CF). These data suggest that 'low' levels are likely to reflect lack of detection rather than decreased production from the lower airways. The thesis highlights the point that there remains no clear information as to the major enzymatic, cellular and anatomical sources of NO detected in the exhaled breath. We are also uncertain about what proportion of total NO production is reflected by exhaled gaseous NO since a large proportion could theoretically have reacted close to its site of production. All this information is important to the basic understanding of the role of NO in pathological processes and development of strategies to address the abnormalities in production. The possibility of downregulation of *epithelial* NOS II due to chronic inflammation rather than abnormal expression secondary to CFTR mutation is suggested by normal response in cell lines and primary cell culture to proinflammatory stimuli together with findings from Polak's group that NOS II expression is markedly reduced in end stage CF bronchial epithelium.

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