ACTIVATION OF THE PGE_2 RECEPTOR EP_4 PROTECTS AGAINST OXIDANT-INDUCED APOPTOSIS IN AIRWAY EPITHELIAL CELLS

By

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ABSTRACT

Heme oxygenase (HO)-1 expression is induced in pulmonary diseases characterized by oxidative stress, including CF, and is cytoprotective in nature. Prostaglanin (PG) E_2 can protect against oxidant-induced apoptosis via upregulaton of HO-1 mediated via one of its receptor subtypes, EP_2. Using model human airway epithelial cells, I have investigated whether pharmacological activation of PGE_2 receptors (EP_2 and EP_4), induces HO-1 expression, and if so, can this protect the cells against oxidant-induced damage. EP_2 and EP_4 receptor activation had no significant effect on HO-1 expression, either at the gene or protein level; however, PGE_2 itself and EP_4 receptor activation protected against oxidant-induced apoptosis. EP_2 receptor activation had no effect. Activation of neither receptor, nor PGE_2, was effective at reducing H_2O_2-induced lipid peroxidation. Thus, while EP_4 receptor activation is protective against oxidant-induced apoptosis in airway epithelial cells, this mechanism seems to be independent of HO-1 induction, and may reflect PGE_2 production.

LIST OF ABBREVIATIONS AND SYMBOLS USED

INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium (salt)
5-LO	5-Lipoxygenase
T-75	75 cm^2 polystyrene tissue culture flasks
Ac-DEVD-pNa	acetyl-Asp-Glu-Val-Asp p-nitroanilide
AP-1	activator protein-1
ADO-R	adenosine receptors
ATP	adenosine triphosphate
AC	adenylate cyclase
ASL	airway surface liquid
ANOVA	analysis of variance
AA	arachidonic acid
bZip	basic leucine zipper
bp	base pair(s)
Bcl-2	B-cell lymphoma 2
BAD	Bcl-2 associated death promoter
β	beta
HCO^{3-}	bicarbonate ion
BSA	bovine serum albumen
BALF	bronchoalveolar lavage fluid
Ca^{2+}	calcium ion
CaCC	calcium activated chloride channel
CO_2	carbon dioxide
CO	carbon monoxide
Ac-DEVD-CHO	caspase-3 inhibitor
c	centi, 10^{-2}
COPD	chronic obstructive pulmonary disease
Cl^-	chloride ion
CoPP	colbalt protoporphyrin IX
cDNA	complementary deoxyribonucleic acid
Cu	copper
cAMP	cyclic adenosine monophosphate
CF	cystic fibrosis
CFBE41o- (CFBE)	cystic fibrosis bronchial epithelial
CFTR	cystic fibrosis transmembrane conductance regulator
COX	cyclooxygenase
cPGES-1	cytosolic prostaglandin E_2 synthase-1
°C	degrees Celsius
Δ	delta
DNA	deoxyribonucleic acid
dNTP(s)	deoxyribonucleic triphosphate(s)
DMSO	dimethyl sulfoxide

Egr-1	early growth response-1 factor
ELISA	enzyme-linked immunosorbent assay
ENaC	epithelial Na^+ channel
EDTA	ethylenediaminetetraacetic acid
Epac	exchange protein activated by caMP
ERK	extracellular signal-related kinase(s)
Fe^{2+}	ferric ion (iron)
Fe^{3+}	ferrous ion
FBS	fetal bovine serum
GPCR(s)	g-protein coupled receptor(s)
GSH	glutathione
GSSH	glutathione (oxidized)
GSR	glutathione reductase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
g	gram
HO	heme oxygenase
HPLC	high-performance liquid chromatography
HAT(s)	histone acetyltransferase(s)
HDAC(s)	histone deacetylase(s)
hr	hour
16HBE14o- (HBE)	human bronchial epithelial cells
H^+	hydrogen ion
H_2O_2	hydrogen peroxide
$^{\bullet}OH$	hydroxyl radical
HClO	hypochlorous acid
HPRT	hypoxanthine guanine phosphoribosyl transferase
IL	interleukin
Keap1	kelch-like ECH-associated protein 1
kb	kilobase(s)
kDa	kiloDalton
LDH	lactate dehydrogenase
λ	lambda
LPS	lipopolysaccharides
LPO	lipid hydroperoxide
l, L	litres
MARE(s)	maf recognition element(s)
$MgCl_2$	magnesium chloride
MDA(s)	malondialdehyde(s)
Mn	manganese
MAPEG	membrane-associated proteins involved in eicosanoid and glutathione metabolism
m	meter (s)
μ	micro, 10^{-6}
mPGES-1	microsomal prostaglandin E synthase- 1
m	milli, 10^{-3}

MEM	minimum essential media
min	minute(s)
M	molar
mol	mole
O_2	molecular oxygen
M-MLV	Moloney Murine Leukemia Virus
MCC	mucociliary clearance
n	nano, 10^{-9}
Nrf_2	NF-E2 related factor
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydride
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NO	nitric oxide
NF-κB	nuclear factor-kappa B
#	number
oligoDT	oligodeoxythymidylic acid
%	percent
PCL	periciliary liquid layer
PMSF	phenylmethanesulfonyl fluoride
PI	phosphatidyl inositol
PI3K	phosphoinositide-3 kinase
PBS GE (PBS)	phosphate buffered saline with glucose and EDTA
K^+	potassium ion
PCR	polymerase chain reaction
PUFA(s)	polyunsaturated fatty acid(s)
1°	primary
HAE	primary human airway epithelial cells
PCD	programmed cell death
PG	prostaglandin
PAR(s)	protease-activated receptors
PKA	protein kinase A
PKB (Akt)	protein kinase B
RIPA buffer	radioimmunoprecipitation assay buffer
ROS	reactive oxygen species
RNS	reactive nitrogen species
RANKL	receptor activator for NF-κB ligand
RFC (x g)	relative centrifugal force
RT	reverse transcription
rpm	revolutions per minute
RNA	ribonucleic acid
2°	secondary
s	second(s)
STAT3	signal transducer and activator of transcription protein-3

siRNA	small inference RNA
SDS	sodium dodecyl sulphate
Na^+	sodium ion
SEM	standard error of the mean
StRE	stress response elements
$O_2^{\bullet}$	superoxide
SOD(s)	superoxide dismutase(s)
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
t	time
X	times
TBARS	thiobarbituric acid reactive substances
$5SCN^-$	thiocyanate ion
TBE	tris/borate/EDTA
TBS	tris-base saline
TBS-T	tris-base saline with Tween
C_T	threshold cycle
TxA_2	thromboxane A_2
TNF-α	tumor necrosis factor- α
U	units
$^v/_v$	volume to volume
V	volts
H_2O	water
$^w/_v$	weight to volume
Zn	zinc

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CHAPTER 1: INTRODUCTION

1.1 Airway Epithelial Physiology

The proximal conducting airways, stemming from the nasal passage and trachea, lead to branching bronchi and bronchioles that serve the non-respiratory functions of host defense and air conduction. The distal respiratory region includes respiratory bronchioles, alveolar ducts and sacs, which are the primary structures where gas exchange takes place (Harkema *et al.,* 1991). Epithelial cells line the lumen of the extensive branching network that makes up the airways, and are critical for maintaining respiratory health. The epithelium preserves a clear path for air to reach the alveoli and defends the lung against a multitude of endogenous and environmental insults (Crystal *et al.*, 2008; Tam *et al.*, 2011). Like other epithelial cells, those found in the airways have two important characteristic properties; firstly, they are polarized and secondly, they have tight junctions. Polarization of the epithelial cells results in transport proteins being asymmetrically distributed between the apical and basolateral membranes, leading to the ability to regulate fluid and ion movement through cells via transepithelial transport (Muth and Caplan, 2003; Figure 1). Tight junctions control the movement of fluid and ions between cells, a process termed paracellular transport (Steed *et al.*, 2009; Tam *et al.*, 2011; Figure 1). The epithelial cell population lining the luminal surface of the airways is complex, consisting of at least twelve different cell types that possess a great range of physiological functions (Crystal *et al.*, 2008; Tam *et al.*, 2011).

The major epithelial cell types in the bronchi, columnar ciliated cells as well as goblet and serous cells, which both secrete mucus work in collaboration in order to carry out a critical defense process, mucociliary clearance (MCC). The lungs are constantly exposed to pathogens and particulates from the external environment, and efficient MCC ensures the airways are kept clean and clear from these potential threats. The airway surface liquid (ASL) that blankets the airway epithelium consists of two layers; a viscous mucus layer that traps unwanted inhaled particulates sits on top of a more fluid periciliary layer (PCL) that bathes cilia, and whole volume is tightly regulated for optimal ciliary beating (Widdicombe, 2002; Boucher, 2003; Tarran, 2004). Each ciliated epithelial cell possess approximately 200-300 apically located motile cilia that beat in a coordinated

fashion in order to move the mucus towards the pharynx, where it is either swallowed or coughed out (Sleigh *et al.*, 1988; Chilvers and O'Callaghan, 2000).

Along with its physical MCC functions, the ASL also contains compounds that defend the airways in a chemical nature. Anti-oxidants, namely glutathione (GSH), anti-microbial agents such as lysozyme and lactoferrin, as well as inflammatory mediators such as interleukin (IL)-8 (Singh *et al.*, 2000; Travis *et al.*, 1999) are secreted by airway epithelial cells to help prevent injury and infection (Chilvers and O'Callaghan, 2000; Tam *et al.*, 2011).

The maintenance of an optimal PCL depth and composition is important for efficient MCC and airway functioning and is primarily regulated by ion channels (Widdicomb, 2002; Boucher, 2003; Tarran, 2004; Figure 1). The ASL is about 5-7 μm in depth, which is approximately the same length as the cilia (Boucher, 2003). If the fluid is too deep, cilia are unable to contact the mucus in order to transport it, whereas if it is too low, motility of the cilia is severely diminished (Gudis and Cohen, 2010). Additionally, the activity of many cytoprotective agents is disrupted when the osmolarity of the ASL deviates from normal (Chilvers and O'Callaghan, 2000). The osmolarity of the ASL is usually maintained at an isotonic level or slightly higher than that of the interstitium, at approximately 285 mOsM (Boucher, 2003). Changes to the PCL can arise in pathologies such as cystic fibrosis (Widdicomb, 2002), and confirms the importance of proper airway epithelial cell functioning in the overall health of the lungs.

1.2 The CFTR Cl^- Channel and Cystic Fibrosis

Cystic fibrosis (CF) is currently the most common fatal genetic disease in the Caucasian population, affecting one out of every 3600 live births in Canada (Cystic Fibrosis Canada, 2012). This autosomal recessive disease is caused by mutations of the cystic fibrosis transmembrane conductance regulator gene (CFTR), which codes for a membrane bound chloride (Cl^-) channel of the same name (Kerem *et al.*, 1989; Riordan *et al*., 1989; Rommens *et al*., 1989). Since gene discovery and characterization by Kerem and colleagues in 1989 (Kerem *et al.*, 1989; Riordan *et al*., 1989; Rommens *et al*., 1989), a great deal of work has been done to increase understanding of the molecular and physiological aspects of the disease.

CFTR is a large 250 kilobase (kb) gene that is found on chromosome 7 (Boucher, 2004). According to the Cystic Fibrosis Mutation Database (www.genet.sickkids.on.ca, 2011), to date, 1910 different mutations of the CFTR gene have been identified, although the most common mutation, ΔF508, accounts for nearly 90% of disease cases (Cystic Fibrosis Canada, 2012). This mutation is a 3 base pair deletion that results in a missing phenylalanine amino acid at position 508 (Riordan *et al.*, 1989). ΔF508-CFTR results in decreased channel function due to improper protein folding and trafficking (Ward *et al.*, 1995).

In the airways, lack of functional CFTR channels leads to a variety of physiological problems, primarily related to defective airway defense mechanisms (Döring and Gulbins, 2009). Mucocilary clearance (MCC), is severely defective in CF. Modified cellular transport of Cl^- and sodium (Na^+) ions as well as water leads to a dehydrated, viscous mucus layer that is not easily cleared from the epithelial lining of the lungs. This mucus buildup creates an optimal environment for inhaled bacteria such as *Pseudomonas aeruginosa* to proliferate and cause chronic bacterial infections (Matsui *et al.*, 1998). These infections lead to severe chronic inflammation, airway remodeling, tissue destruction and ultimately death from respiratory insufficiency (Cohen and Prince, 2012).

While the median life expectancy of CF has greatly increased from childhood to greater than 40 years of age over the past few decades, there still remains no cure for this fatal disease. Currently, treatments are mainly based on controlling infection and inflammation through antibiotic and anti-inflammatory drugs, nutrition and physiotherapy (reviewed in Wilschanski and Kerem, 2011), rather than taking care of the defective defense mechanisms or original genetic flaw. Progressive studies looking into airway hydration via hypertonic saline or mannitol (reviewed in Thelin and Boucher, 2007), gene therapy (reviewed in Griesenbach and Alton, 2011) or protein modification (reviewed in Powell and Zeitlin, 2002 and Elborn, 2012) have been proposed, but have had limited success. To date, only one drug has been approved in the United States that treats the root cause of the disease. Ivacaftor or Kalydeco (VX-770), a CFTR potentiator (which restores gating defects), is suitable to treat patients with at least one G551D-CFTR mutation (Ramsey *et al.,* 2011). Although such advances are significant, the

G551D-CFTR mutation only accounts for 4% of CF patients (Hadjiliadis, 2012). Therefore, investigating the basic molecular and physiological defects in CF remains an important task. More work must be done in order to find alternative therapies to help treat the underlying cause or increase the functioning of the innate defense mechanisms that are disrupted by the disease.

1.3 Oxidant Stress in the Airways

Oxygen is essential for life; however, at concentrations that surpass physiological limits, or in forms that have become unstable, oxygen can become toxic to cells. The lung are directly exposed to the external environment that contains high levels of atmospheric oxygen and air pollutants such as exhaust fumes and cigarette smoke, which leads to the lung having the highest susceptibility to exposure of exogenous oxidants (Rahman *et al.*, 2006). Oxidants or reactive oxygen species (ROS), is a collective term that includes oxygen radicals that are unstable due to unpaired electrons, as well as other non-radical oxygen derivatives such as hydrogen peroxide (H_2O_2) that are all capable of causing severe tissue injury by degrading and/or modifying deoxyribonucleic acid (DNA), proteins and lipids via oxidation reactions (Halliwell and Cross, 1994; Rahman *et al.*, 2006). Oxidation of these macromolecules can lead to the induction of apoptosis (Chandra *et al.*, 2000) and interfere with a host of important cellular and physiological processes such as cell proliferation and inflammation. ROS also have a significant role in mediating important signaling pathways (Rahman *et al.*, 2006). The most physiologically relevant oxidants include H_2O_2, superoxide ($O_2^{\bullet-}$) and the hydroxyl radical ($^{\bullet}OH$). These oxidants are not only produced exogenously by environmental pollutants and aerobic bacteria, but can also be produced endogenously via 'leaky' metabolic processes including the mitochondrial electron transport chain and deliberately by inflammatory action. *In vivo,* $O_2^{\bullet-}$ and H_2O_2 are produced by activated inflammatory cells as a defense mechanism to prevent infection because the oxidants are highly toxic to bacteria. H_2O_2 is especially useful because of its bacterial killing abilities but is a less potent oxidant and thus results in less damage to the surrounding tissue. In fact, H_2O_2 is generated by reactions of $O_2^{\bullet-}$ and H_2O via superoxide dismutases to rid the system of the more dangerous $O_2^{\bullet-}$ and is used by inflammatory cells to oxidize Cl^- via myeloperoxidase to

make hypochlorous acid (HClO) that is also a potent anti-bacterial agent (Wright *et al.,* 1994; Rahman *et al.*, 2006).

As a result of the presence of these oxidants, it is essential for the airways to possess innate defense mechanisms, other than MCC, in order to combat these potential threats. Fortunately, a healthy lung possesses equally high levels of anti-oxidants that protect the lung from the deleterious effects of oxidants before substantial damage can occur (Rahman *et al.*, 2006). Anti-oxidants can be either enzymatic or non-enzymatic. Anti-oxidant enzymes include superoxide dismutases (SODs), reductases, peroxidases and catalases. These enzymes act by catalyzing the transition of oxidants to safer derivative forms. The main non-enzymatic anti-oxidants of the lung include water and lipid soluble compounds such as GSH and vitamins A, C and E (Ryter *et al.,* 2007). GSH is considered the most critical non-enzymatic anti-oxidant, as it is able to scavenge ROS and neutralize them by becoming oxidized itself. Oxidized glutathione (GSSH) is quickly regenerated into its reduced form via glutathione reductase (GSR) and the electron acceptor nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) (Kohen and Nyska, 2002). Enzymatic and non-enzymatic anti-oxidants work at a large-scale level and are the first line of defense against oxidants. More recent research has highlighted the role of other, more subtle anti-oxidants. These molecules, such as perioxiredoxins, thioredoxins, glutaredoxins and heme oxygenases, are induced by a number of stressors, including ROS, to serve as endogenous protective proteins and defend against damage caused by their initial stimulus (Rahman *et al*, 2006; Ryter *et al*, 2007).

When the equilibrium between oxidants and anti-oxidants is disrupted, a state of imbalance, referred to as oxidative stress occurs (Rahman *et al.*, 2006; Figure 2). Oxidative stress is thought to be a potential common factor in the pathogenesis and progression of all inflammatory airway diseases including asthma (Dworski, 2000), chronic obstructive pulmonary disease (COPD; Repine *et al.*, 1997) and CF (van der Vliet *et al.*, 1997; McGrath *et al.*, 1999). In CF lung disease, both oxidants and anti-oxidants deviate from their normal levels. An increased oxidant burden is caused by the recruitment of neutrophils that produce $O^{\bullet -}$, H_2O_2 and HClO to fight off the common bacterial infections that plague CF patients (Gao *et al.,* 1999; Rahman *et al.*, 2006; Döring and Gulbins, 2009). Decreased anti-oxidant levels seen in CF can be attributed to

the reduced absorption of lipid soluble vitamins from the diet (Farrell *et al.*, 1977) as well as decreased GSH transport via defective CFTR Cl^- channels (Linsdell and Hanrahan, 1998). Indeed, it has been suggested that CFTR itself may play a key role in the anti-oxidant defense system (Gao *et al.,* 1999; Rottner *et al.,* 2011). Other than its role in GSH transport, it has been shown that CFTR can be regulated via oxidant stress (Nguyen and Canada, 1994; Cowley and Linsdell, 2002; Cantin *et al.,* 2005). Also, several studies have shown that inflammation and oxidative stress may occur in CF in the absence of infection (Weber *et al.,* 2001; Zaman *et al.,* 2004; Verhaeghe *et al.,* 2007; Vij *et al.,* 2009), suggesting that although bacterial infections are responsible for the large scale inflammatory and oxidant state, the absence of efficient CFTR could be responsible for constitutive defects in the innate defense system, leading to an early and excessive inflammatory and oxidant load (Gao *et al.,* 1999; Rottner *et al.,* 2011). Several studies using CF cell lines have shown excessive pro-inflammatory cytokine expression (Bonfield *et al.,* 1999; Zaman *et al.,* 2004) as well as increased susceptibility to apoptosis due to high levels of oxidative stress (Rottner *et al.* 2011) in environments where infection is not an issue. Therefore, new therapeutics targeting the dysfunctional defense mechanisms involving CFTR, in order to reduce oxidative stress and consequential damage, has potential to limit the pro-inflammatory status seen in CF.

1.4 Heme Oxygenase

Heme oxygenase (HO) is a member of the heat shock family of proteins and was previously known as heat shock protein 32. HO catalyzes the rate-limiting step of heme oxidation into equal amounts of the bile pigment biliverdin, which is quickly converted to bilirubin by biliverdin reductase, carbon monoxide (CO) and iron (Tenhunen *et al.,* 1968; Yoshida and Kikuchi, 1978). There are three isoforms of heme oxygenase; HO-1, HO-2 and HO-3, all products of separate genes (Donnelly and Barnes, 2001). HO-1 is a 32 kDa inducible form, while HO-2 and HO-3 are reportedly expressed constitutively. The role of HO-2 is not very well understood; it is expressed in high levels in the testes and the brain (Yachie *et al.,* 1999); however, HO-2 is also expressed in respiratory neural tissue (Prabhakar, 1998), therefore further investigation of the role of HO-2 in the airways is needed. HO-3 has only been detected in rat neurons (McCoubrey *et al.*, 1997) and its

existence has been questioned, with some evidence that HO-3 is in fact a pseudogene of HO-2 (Hayashi *et al.*, 2004). Therefore HO-3 will not be considered in this study.

Although HO-1 is primarily expressed at high levels in the spleen, liver and bone marrow in order to break down hemoglobin from aged erythrocytes, HO-1 has also been found in most other tissues at low levels during basal conditions. The HO-1 system in these tissues reportedly responds to a variety of harmful chemical and physical stimuli in order to help manage the deleterious effects of its stimuli at a cellular level (Ryter *et al.*, 2006). The mechanism through which HO-1 produces its cytoprotective effects is not yet fully elucidated and seems to differ between tissue types; however, it is now clear that it has significant anti-oxidant and anti-inflammatory roles. HO-1 knockout mice have been reported to be substantially more sensitive to oxidant stress compared to wild-type control mice (Poss and Tonegawa, 1997). Furthermore, the only reported case of HO-1 deficiency in humans displayed impeded growth, anemia, increased iron deposition, low bilirubin levels and an overall sensitivity to oxidant stress which lead to premature death (Yachie *et al.*, 1999; Kawashima *et al.,* 2002).

Work investigating heme metabolites, biliverdin, bilirubin, CO and iron demonstrated that HO-1 primarily exerts its cytoprotective effects through ridding the system of excess free heme (which is well characterized as pro-inflammatory and pro-oxidant) and these heme degradation products (Raval and Lee, 2010). Excessive accumulation of the bile pigment bilirubin, as occurs in jaundice, can cause harm, especially to newborns. However, Stocker *et al.* (1987) demonstrated that biliverdin and bilirubin have powerful anti-oxidant properties in many *in vitro* models. CO is a gas primarily known as a toxic asphyxiant, as it competes with molecular oxygen (O_2) for binding sites on hemoglobin, with an affinity 200-250 times that of O_2 (Ryter *et al.*, 2006). However, a significant amount of CO is created endogenously through oxidation of heme proteins (primarily heme, but also myoglobin, cytochrome p-450 and mitochondrial cytochromes) and it is now thought that low levels of CO may exert beneficial effects. Interestingly, elevated levels of CO in exhaled air have been reported in patients with many inflammatory lung diseases (reviewed in Gajdócsy and Horváth, 2010), including CF (Paredi *et al.*, 1999). It is thought that HO-1 derived CO exerts its anti-oxidant effects by substituting for nitric oxide (NO) in its signaling without the

deleterious affects of NO, such as generating reactive nitrogen species (RNS) (Carter *et al.*, 2004). CO also reportedly plays anti-inflammatory, anti-apoptotic and anti-proliferative effects (reviewed in Ryter *et al.*, 2007). Although iron is generally thought to be pro-oxidant, HO-derived iron has been shown to promote the synthesis of an iron-sequestering molecule, ferritin, which is inherently cytoprotective (Vile *et al.*, 1993). There is also evidence that HO-1 couples with an iron pump in order to rid the cell of the iron created by heme oxidation (Baranano *et al.*, 2000; Ferris *et al.,* 1999), thus promoting its recycling and limiting its pro-oxidant effects.

HO-1 induction and regulation has been a main focus of HO research, as heme metabolites have great therapeutic potential (Ryter and Choi, 2009; 2010). HO-1 has been shown to be induced by not only its substrate heme, but also by a number of stimuli related to oxidative stress and inflammation including hyperoxia or hypoxia, pro-inflammatory cytokines, bacterial endotoxins, nitric oxide, heavy metals, ultraviolent radiation, heat shock, shear stress, airborne particulates and potent oxidants such as thiol-reactive substances and H_2O_2 (Carter *et al.*, 2004; Rahman *et al.*, 2006; Ryter *et al.*, 2007). The regulation of HO-1 is complex, as HO-1 has many inducing agents and its regulation is species, tissue and cell specific (Ryter *et al.,* 2002). Upregulation of the HO-1 gene in mice is modulated by two enhancer sequences located -4kb (E1) and -10kb (E2) from the transcriptional start site. These enhancer sequences have repeated stress response elements (StRE), which are also the recognition sequences for the small Maf family of proteins and are therefore also known as Maf recognition elements (MAREs). These sequences have the binding sites for oxidant sensitive transcription factors such as NF-E2 related factor 2 (Nrf_2; Kim *et al.*, 2008). In humans, HO-1 regulation is further complicated as the human HO-1 gene contains multiple additional promoter elements with binding sites for numerous transcription factors including early growth response-1 factor (Egr-1), nuclear factor-κB (NF-κB), signal transducer and activator of transcription protein-3 (STAT3), activator protein-1 (AP-1), repressor Bach1, among others. The function of this complexity is not yet fully understood; however, it likely involves a coordinated interaction of multiple transcription factors (Ryter *et al.,* 2002).

HO-1 is expressed in inflammatory cells and airway epithelial cells, including those of the bronchi (Carraway *et al.*, 2000). HO-1 induction has been linked to a number

of lung diseases associated with oxidant stress, such as COPD, asthma and CF (Carter *et al.*, 2004). In fact, lung samples from patients with CF showed increased HO-1 expression (Zhou *et al.*, 2004). This study also used a CF epithelial cell line to show that over-expression of HO-1 resulted in protection against *Pseudomonas aeruginosa* infection, which typically results in severe injury or apoptosis. These results suggest that induction of HO-1 in CF patients is cytoprotective in nature and that increasing its expression could potentially be therapeutic against bacterial injury and the resulting inflammation and oxidant stress that overburdens the CF lung (Zhou *et al.*, 2004).

1.5 Prostaglandin E_2 and its Receptors

Arachidonic acid (AA) metabolites, termed prostanoids, play a multitude of important roles throughout the body (Legler *et al.,* 2010). The five major AA products include the prostaglandins (PG) $PGF_{2\alpha}$, PGD_2, PGI_2, PGE_2 as well as thromboxane A_2 (TxA_2). These AA products are synthesized by the action of cyclooxygenases (COX-1 or COX-2) and various PG synthases (Figure 3) and exert their effects through several prostanoid receptors (Regan, 2003; Furuyashiki and Narumiya, 2009; Scher and Pillinger, 2009). Prostanoid receptors are G-protein coupled receptors (GPCRs), termed FP, DP, IP, EP and TP, to refer to their endogenous prostanoid ligand. These receptors have considerable heterogeneity due to separate genes encoding subtypes and alternative messenger ribonucleic acid (mRNA) slicing (Coleman *et al.,* 1994).

Among the prostanoids, PGE_2 is the most widely produced in the body and also demonstrates the most versatile actions (Sugimoto and Narumiya, 2007). The versatility of PGE_2 may be explained by the fact that PGE_2 exerts its effects through four EP receptor subtypes, each the products of different genes (EP_1, EP_2, EP_3, EP_4). There are also two alternative splice variants of EP_1 and eight variants of EP_3 (Regan, 2003). These receptor subtypes have similar ligand binding properties to PGE_2, but show their major differences in their mechanisms of signal transduction (Figure 4). Since the discovery of nonsteroidal anti-inflammatory drugs (reviewed in Vane and Botting, 2003), which block prostanoid synthesis by COX inhibition, prostanoids have been tightly linked to promoting a pro-inflammatory state. Indeed, there is irrefutable evidence that prostanoids, especially PGE_2, are involved in promoting pain and inflammation;

however, recent investigations display increasing evidence that PGE_2 can also exhibit anti-inflammatory characteristics (reviewed in Scher and Pillinger, 2009). Therefore, it has been hypothesized that prostanoids, including PGE_2, may regulate both the onset and the termination of the inflammatory response. This hypothesis is plausible, as the action of PGE_2 may be context (inflammatory status) and receptor specific.

1.6 Does Prostaglandin E_2 Signaling Play a Role During Oxidative Stress?

It has been reported that exposure to acute oxidant stress, in the form of H_2O_2, can stimulate CFTR anion efflux (Nguyen and Canada, 1994; Cowley and Linsdell, 2002), hypothesized to increase additional water movement, aiding MCC and potentially providing a way to help clear the oxidant stress source.

One mechanism by which H_2O_2 can reportedly acutely stimulate CFTR is via PGE_2, which was originally studied using kidney cells (Soodvilai *et al.,* 2007). Initial work investigating the relationship between prostanoids and CFTR in the airways focused on the actions of isoprostanes, prostanoid-like compounds generated when oxidants react with polyunsaturated fatty acids (PUFAs; prostanoid precursors) and act through activation of prostanoid receptors (reviewed in Janssen, 2001). Indeed, acute treatment with the isoprostane 8-iso-PGE_2 was found to stimulate anion efflux via CFTR by activating the EP_4 receptor in Calu-3 airway epithelial cells (Joy and Cowley, 2008), demonstrating a role for prostanoid receptors in mediating oxidant stress in airway epithelial cells. Using the same model, Jones *et al.,* (2012) recently demonstrated that H_2O_2 rapidly stimulates PGE_2 production, which stimulates CFTR via activation of the EP_4 receptor. Therefore, PGE_2 EP_4 signaling may indeed be a critical part of the CFTR dependant host defense system.

When looking at the longer-term (24 hrs) effects of activation of the EP_4 receptor in another airway epithelial cell model, Calu-3, via the EP_4 agonist PGE_1-OH, Li *et al.,* (2011) found increased levels of PGE_2 as well as several other mediators of inflammation, thus demonstrating that prolonged activation of the EP_4 receptor produces a PGE_2 positive feedback loop that leads to a potentially pro-inflammatory epithelial environment. Interestingly, studies have demonstrated excessive production of prostanoids, including PGE_2, in the bronchoalveolar lavage fluid (BALF), saliva and

urine of patients with CF (Strandvik *et al.,* 1996; Corvol *et al.,* 2003). Furthermore, Chen *et al.* (2012) recently showed evidence to suggest that CFTR negatively regulates the PGE_2 mediated inflammatory response, demonstrating a CFTR dependant defense process that would be dysfunctional in CF and promote excessive inflammation seen in CF tissues.

Studies outside airway models have linked PGE_2 and other prostanoid metabolites to oxidative stress and subsequent cytoprotection (George *et al.,* 2007, Aoudjit *et al.,* 2006). Interestingly, a role for HO-1 has been implicated in this process. 15-Deoxy-$\Delta^{12.14}$-prostaglandin J_2, a terminal dehydration product of PGD_2, thought to have pro-apoptotic activity, was found to exert anti-apoptotic effects after H_2O_2 oxidant stress in rat pheochromocytoma (PC12) cells, through Nrf_2 mediated up-regulation of HO-1 (Kim *et al.,* 2008). Park *et al.,* (2009) demonstrated that PGE_2 treatment reduced oxidant-induced apoptosis by up-regulating HO-1 via the EP_2 receptor in rat C6 brain cells. Conversely, using a IL-1β-induced osteoarthritic chondrocyte model to induce PGE_2, inflammation and apoptosis, Megías *et al.,* (2009) showed that induction of HO-1 via colbalt protoporphyrin IX (CoPP) exerted its beneficial effects by down-regulating the PGE synthases COX-2 and microsomal (m)PGES-1. Megías *et al.,* (2009) hypothesized this downregualtion would thereby reduce subsequent PGE_2 production and PGE_2-mediated apoptosis. mPGES-1 is a perinuclear membrane bound protein that is part of the MAPEG family (for membrane-associated proteins involved in eicosanoid and glutathione metabolism) and involved in the final step of PGE_2 formation (Murakam *et al.*, 2003). As stated previously, it is likely that PGE_2 exerts its effects differently, depending on the tissue or cell type, receptor activated or inflammatory status. Therefore, more work must be done in order to define the role of PGE_2 and its receptors in the airways during oxidative stress. Also, it may be of therapeutic benefit to delineate a potential role of HO-1 in PGE_2 signaling.

1.7 16HBE14o- Cell Line

The 16HBE14o- (hereafter referred to as HBE) cell line was generated by the transformation of normal human bronchial epithelial cells by Cozens *et al.,* (1994). Since its creation, it has been a widely used model for studying airway epithelial cell

physiology as the cells are easily maintained and reproduced at a fraction of the cost of maintaining primary cultures. It has been shown that HBE cells remain well differentiated as epithelial cells (Ehrhardt *et al.,* 2002) and the cell line contains both the mRNA and protein for CFTR, which is key for performing a study relating to CF (Ehrhardt *et al.,* 2002).

1.8 Study Objectives

HO-1 is expressed in airway epithelial cells (Carraway *et al.*, 2000), and its expression induced in pulmonary diseases characterized by oxidant stress, including CF (Carter *et al.*, 2004). HO-1 over-expression protects against *Pseudomonas aeruginosa* infection (Zhou *et al.*, 2004), suggesting induction of HO-1 in CF is cytoprotective in nature and increasing its expression could potentially be therapeutic against bacterial infection, oxidative stress and inflammation. Our laboratory is interested in the role of prostanoid receptors in mediating oxidant stress in airway epithelia. It is well documented that HO-1 is upregulated by several different stimuli (Carter *et al.*, 2004; Rahman *et al.*, 2006; Ryter *et al.*, 2007) and recently it was demonstrated that PGE_2 could protect against oxidant-induced apoptosis via upregulation of HO-1 mediated by the EP_2 receptor (Park *et al.,* 2009). Interestingly, our laboratory has established a body of evidence to suggest that the EP_4 receptor plays an important role in mediating the effects of oxidant stress in the airways (Jones *et al.,* 2012). Therefore, using the model human airway epithelial cells 16HBE14o-, I hypothesize that upon pharmacological activation of the EP_2 and/or EP_4 receptors, induction of HO-1 expression will occur and that, if so, this induction will protect the cells against oxidant-induced damage.

However, it has also been suggested that PGE_2 is harmful and it has been demonstrated that induction of HO-1 reduces available PGE_2 by inhibiting the expression of substrates necessary for PGE_2 formation COX-2 and mPGES-1 (Megías *et al.,* 2009). Thus, using CoPP to induce HO-1, I wish to delineate if upregulation of HO-1 inhibits the gene expression of these substrates in airway epithelial cells.

Understanding the role of HO-1 in the airways, as well as its relationship with PGE_2 and its receptors is useful, as it has the potential to lead to therapies targeting the reduction of damage caused by oxidative stress that plagues inflammatory lung diseases.

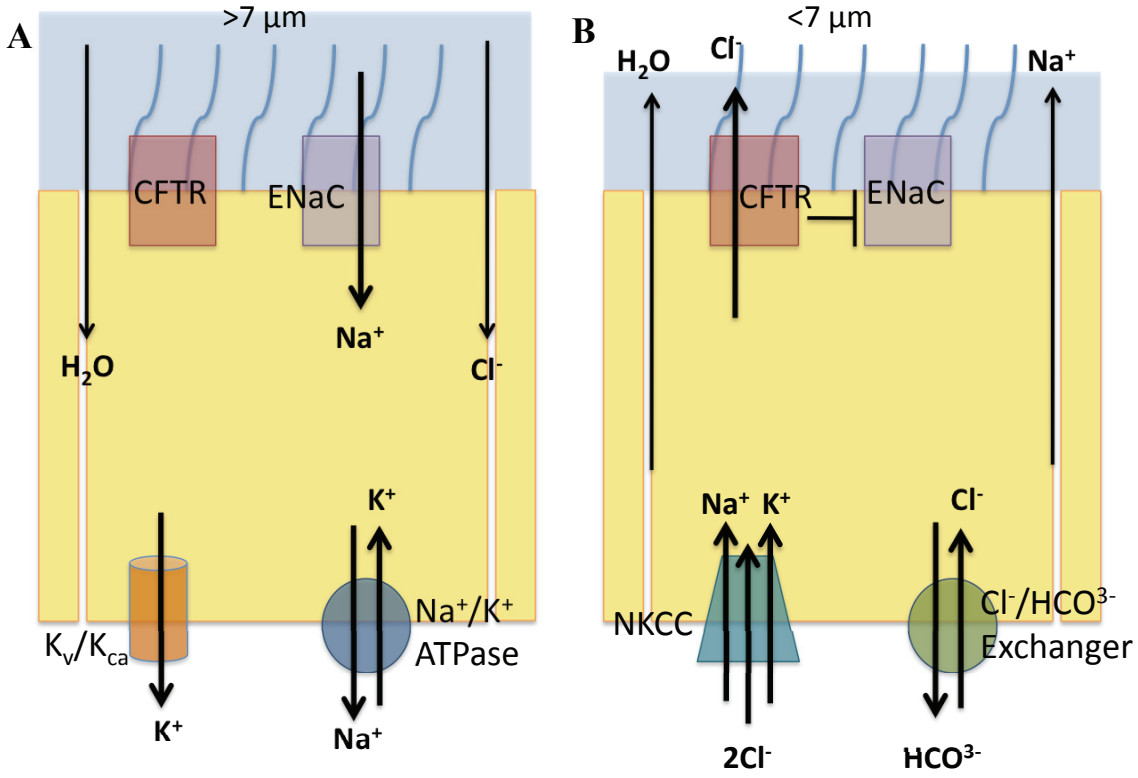

Figure 1. Model of Ion and Fluid Transport in Airway Epithelial Cells. Maintenance of the periciliary layer (PCL) is tightly regulated by airway epithelial cells via paracellular and transepithelial ion and fluid transport. A delicate balance between Na^+ mediated absorption and Cl^- mediated secretion is essential for optimal PCL height and efficient mucociliary clearance (MCC). **A)** Na^+ absorption involves Na^+ entry via apical Na^+ channels, mainly the epithelial Na^+ channel (ENaC), and exit via the Na^+/K^+-ATPase pump. K^+ ions are recycled through basolateral and apical (not shown) K^+ channels. **B)** Cl^- secretion involves entry of Cl^- through the $Na^+/2Cl^-/K^+$ co-transporter and the Cl^-/HCO^{3-} exchanger on the basolateral membrane, followed by its secretion via apical Cl^- channels, including CFTR, which is known to regulate other ion channels including ENaC. Na^+ and K^+ ions are recycled via the Na^+/K^+-ATPase pump and basolateral and apical K^+ channels (not shown). When Na^+ or Cl^- ions undergo transepithelial transport via channels and transporters, H_2O and other ions osmotically follow via paracellular transport (Bardou *et al.,* 2008). Dysregulation of these transport mechanisms as a result of dysfunctional or absent CFTR leads to cystic fibrosis disease (Widdicombe, 2002; Tarran, 2004; Boucher, 2003).

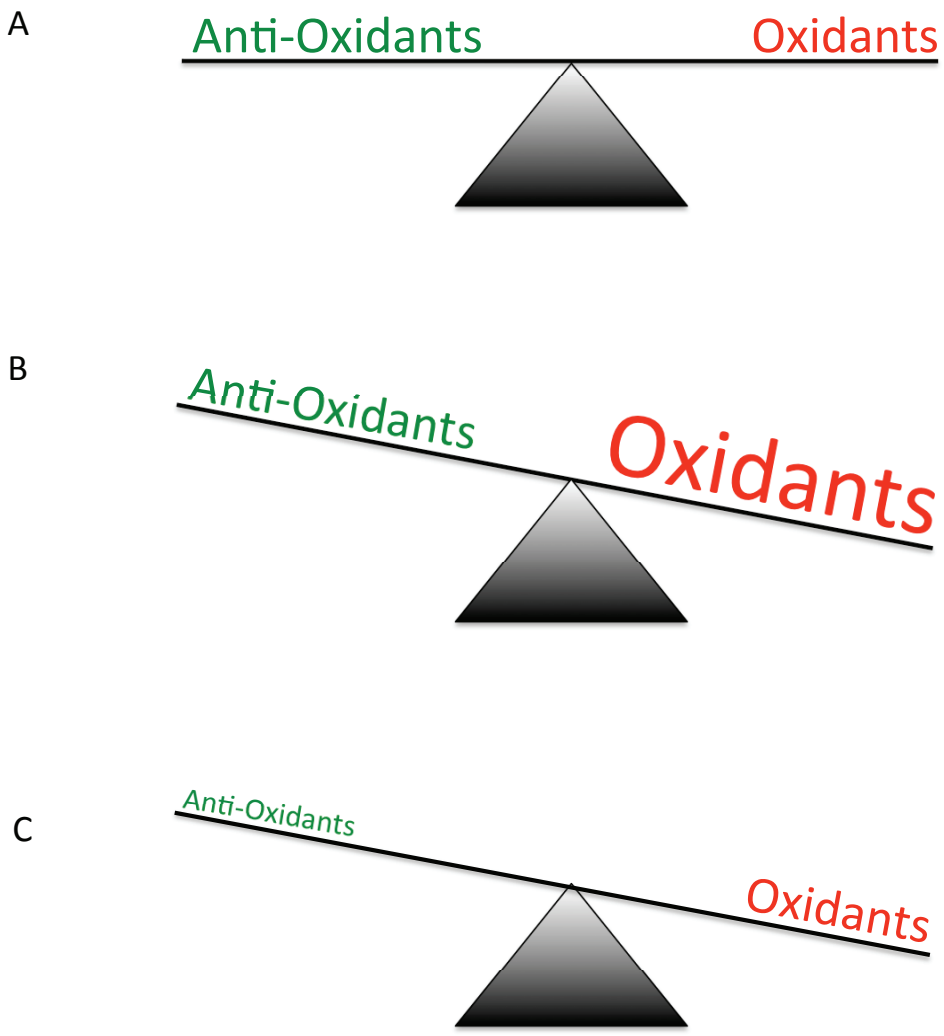

Figure 2. Unbalanced Levels of Oxidants and Anti-Oxidants Leads to Oxidative Stress. In healthy tissue (A) there are equal amounts of oxidants and anti-oxidants, providing a neutral environment for cells. In many disease states, increased generation of oxidants (B), decreased anti-oxidant intake and/or production (C), or both cause an imbalance that leads to oxidative stress (Rahman *et al.*, 2006).

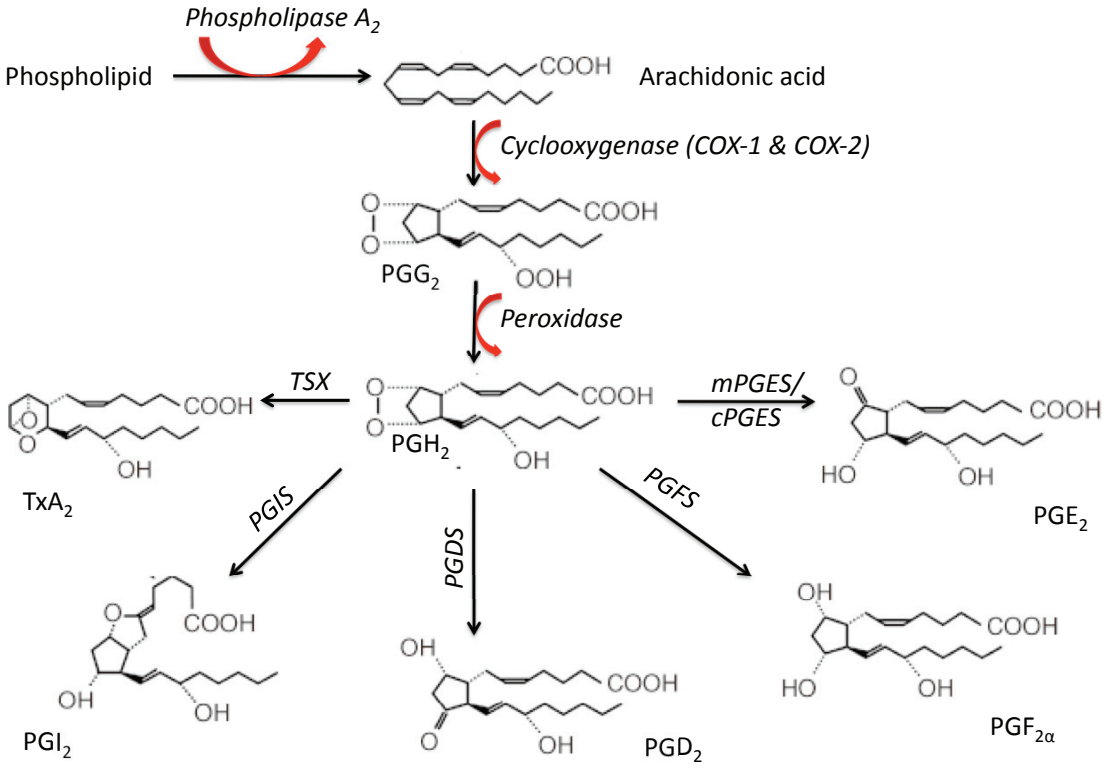

Figure 3. Prostanoid Synthesis and Structure. Prostanoids are not stored within cells, but are synthesized as required in response to stimuli. First, their substrate polyunsaturated fatty acid (PUFA), arachidonic acid (AA), is released from the cellular phospholipids by the action of the enzyme phospholipase A_2. Next, the free acids are acted upon by either cyclooxygenase-1 or cyclooxygenase-2 (COX-1 and COX-2). Both enzymes catalyze the same two reactions form prostoglandin (PG) PGG_2. PGG_2 is then reduced by a peroxidase reaction to form PGH_2. PGH_2 is converted to PGE_2 by prostaglandin E synthases. The cytosolic enzyme cPGES, which is expressed constitutively, is linked functionally to COX-1 to promote immediate PGE_2 production. A second microsomal enzyme (mPGES) is induced by inflammatory stimuli and functions with the inducible COX-2. Similarly; $PGF_{2\alpha}$, PGD_2, prostacyclin PGI_2 and thromboxane TXA_2 are formed by the action of prostaglandin F synthase, prostaglandin D synthase, prostacyclin synthase and thromboxane A synthase respectively. Before they can function, prostanoids that have been newly synthesised must be transported from the cytosol and cross various membranes via active transporter systems (Regan, 2003).

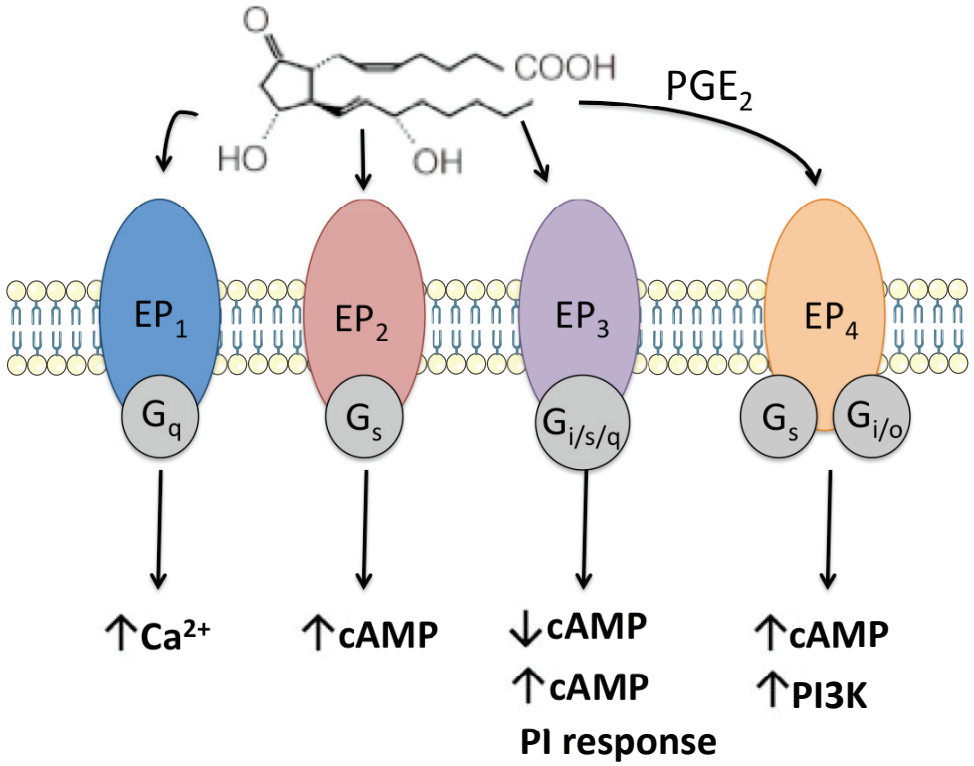

Figure 4. Prostaglandin E_2 Receptor Signaling. PGE_2 has a wide variety of functions, achieved by the signaling of four different G-Protein Coupled Receptors (GPCRs; EP_1-EP_4) with cell specific responses. EP_1 signals primarily through G_q, which produces a transient rise in intracellular calcium (Ca^{2+}). The three remaining EP receptors, $EP_{2\text{-}4}$, are primarily involved in cAMP production. EP_2 and EP_4 are coupled to G_s, which increases cAMP synthesis through adenylate cyclase (AC), which converts ATP to cAMP. However, activation of the EP_4 receptor can also cause an increase in phosphoinositide-3 kinase (PI3K), hypothesized to be mediated by $G_{i/o}$. There are several isoforms of the EP_3 receptor that are linked to $G_{i/s/q}$; therefore, the EP_3 receptor can result in both increases and decreases in cAMP as well as cause a phosphatidyl inositol (PI) response (Sugimoto and Narumiya, 2007).

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Culture

16HBE14o- (HBE) human bronchial epithelial cells were obtained from Dr. Dieter Gruenert (California Pacific Medical Center, San Francisco, CA, USA) and grown in a humidified incubator at 37°C with 5% CO_2. Stock cells were grown in minimum essential media (MEM) containing phenol red, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin (all from Invitrogen, Burlington, ON, Canada), which was replaced every 2-3 days. Stock cells were grown in 75 cm^2 polystyrene tissue culture flasks (T-75; Corning, Lowell, MA, USA) and passaged once per week. All experiments were performed on cells between passages 8-20, when cells became 90-100% confluent.

To passage the stock cells, the media was removed when the cells were approximately 80-90% confluent, and washed with 10 ml 1XPBS GE with glucose and ethylenediaminetetraacetic acid (EDTA; PBS; Appendix B) for 30 s. In order to detach the cells from the surface of the flask, 1 ml of 0.25% trypsin (Invitogen) was applied and the cells incubated at 37°C for 20-30 min. Following detachment, the cells were re-suspended in 6 ml of media and from this solution, approximately 500,000 HBE were put into T-75 flasks in order to continue the cell line. In order to perform several different experiments, culturing and treatment protocols varied and are described below as well as summarized in Appendix A.

2.2 RNA Extraction

In order to detect and quantify changes in mRNA expression of genes of interest in HBE cells, total RNA was extracted after various drug treatments (Table 1) by the following procedure. First, 200,000 HBE cells were seeded in 6-well cell culture plates coated with approximately 100 μM fibronectin coating solution for 3 hr prior to use (Invitrogen; Sigma-Aldrich, Oakville, ON, Canada; BD Biosciences, Franklin Lakes, NJ, USA) (Appendix B) as described by Ehrhardt *et al*, 2002 and grown for 4 days. Following specific drug treatments (Table 1), media was removed and the cells were washed with 2 ml of PBS for 30 s, and 1 ml of Trizol Reagent (Invitrogen) was added to

each well. The cells were incubated at room temperature for 5 min then homogenized by pipetting. The samples were then transferred into 1.5 ml micro-centrifuge tubes, and 200 µl of chloroform (Fisher Scientific, Ottawa, ON) added. The tubes were vortexed for 15 s, incubated at room temperature for 3 min, and then centrifuged at 4°C for 15 min at 13,000 revolutions per minute (rpm). The 400 µl top colourless aqueous phase containing RNA was carefully removed and transferred into new 1.5 ml micro-centrifuge tubes, and 500 µl of isopropanol (Sigma-Aldrich) added. The tubes were then vortexed, incubated at room temperature for 10 min, and centrifuged at 4°C for 10 min at 13,000 rpm. The supernatant was discarded, and the tubes were briefly centrifuged at room temperature to bring down any residual RNA. The RNA pellet was then washed with ice-cold 75% ethanol. The tubes were vortexed and then centrifuged at 4°C for 5 min at 13,000 rpm, and this wash step repeated. The final supernatant was discarded, and the lids were left open to allow the remaining ethanol to evaporate. Once the ethanol was completely evaporated, 20 µl of sterile water was added to each tube, the pellet was dissolved by pipetting, and the samples were stored in at -80°C.

The concentration and purity of the extracted RNA was measured using a GeneQuant Pro Spectrophotometer (Biochrom, Cambridge, U.K.). 1 µl of each sample was added to 69 µl of sterile water in 0.5 ml mini-centrifuge tubes and gently vortexed. The spectrophotometer was calibrated with 70 µl of sterile water in the cuvette as a reference. After calibration, 70 µl of each of the RNA samples were added to the cuvette and the RNA concentration (in µg/µl) was recorded. The purity of the RNA was also measured and recorded as the 260/280 ratio, with a value between 1.6-1.8 indicating a sample of nucleic acid with minimal protein contamination.

2.3 Reverse Transcription of RNA to Single Stranded cDNA

Complimentary DNA (cDNA) was formed by reverse transcription (RT) of all the RNA samples. 2 µg of RNA was added to 1 µl of 500 µg/ml oligoDT (Promega, Madison, WI) and enough sterile water was added to reach a final volume of 15 µl. The samples were heated to 70°C for 3 min, and cooled to 4°C for 2 min. 2 µl of 1st strand cDNA buffer, 2 µl of 5 mM deoxynucleotide triphosphate (dNTP) mix, and 1 µl of 200 U/µl M-MLV RT enzyme (all Invitrogen) were added to each sample and mixed by

pipetting. To verify the lack of genomic DNA contamination, a negative reverse transcription control was performed, which included 1 µl of sterile water instead of 1 µl of Moloney Murine Leukemia Virus (M-MLV) RT enzyme. The samples were incubated at 42°C for 1 hr, and then heated to 80°C for 5 min to inactivate the reverse transcription enzyme. When the reaction was complete, 80 µl of sterile water was added to bring the final volume to 100 µl and the samples were stored at -20°C.

2.4 Polymerase Chain Reaction (PCR)

The target gene fragments were amplified using PCR. For this to occur, each sample contained 2 µl of cDNA, 15.5 µl sterile water, 1.5 µl of 25 mM $MgCl_2$ (Fermentas, Burlington, ON), 2.5 µl of 10X Taq Buffer (Fermentas), 1 µl of 5 mM dNTP mix (Invitrogen), 1 µl of 10 µM specific sense primer, 1 µl of 10 µM specific antisense primer (Invitrogen), and 0.5 µl of Taq DNA Polymerase (Fermentas). The primers were specific to the gene fragment of interest (HO-1, HO-2, EP_2 receptor, EP_4 receptor, COX-1, COX-2 and mPGES-1); primer information, annealing temperatures and references are described in Table 2. Sterile water was substituted for cDNA for an additional negative control and the housekeeping gene HPRT was used for a positive control to test the integrity of the cDNA.

2.5 Agarose Gel Electrophoresis

PCR products (amplicons) were viewed by agarose gel electrophoresis. A 1.5% agarose gel was made by adding 50 ml of 1XTBE buffer (TBE; Appendix B) to 0.75 g of agarose (Fisher Scientific) and microwaved for approximately 2 min. 2.5 µl of 0.5 µg/µl Ethidium Bromide (Sigma-Aldrich) was added. This mixture was poured into a gel box, with either 8 or 15 well combs, and allowed to set for at least 20 min. 5 µl of a 100 bp DNA Ladder (Fermentas) was added to the first lane in the gel and PCR products were mixed with 5 µl of 6X loading dye (Fermentas) and 8 or 12 µl of each sample (depending on the comb size) were added to the next lanes. The gel was placed in the gel electrophoresis apparatus and set to 90 V and run for 45 min. Images were viewed and captured with a UV Transilluminator UVP BioDoc-It system (Upland, CA, USA) to determine the size of the amplicons.

2.6 Gel Extraction

In order to extract and purify the cDNA amplicons from an agarose gel, a FisherBiotech UV Transilluminator (Fisher Scientific) was used to view the gel and the Qiagen minElute Gel Extraction Kit (Qiagen Sciences, MD, USA) was used to extract and purify the cDNA, by following the manufacturers protocol. Briefly, to bind the DNA, 3 gel volumes of solubilization and binding buffer (buffer QG) was added and the sample was incubated at 50°C for 10 min. 1 gel volume of isopropanol was added and the sample was transferred into a MinElute column with a 2 ml collection tube and centrifuged for 1 min at 13,000 rpm. With the flow-through fluid discarded, another 500 µl of buffer QG was added and the sample centrifuged for 1 min. To rinse, 750 µl of washing buffer (buffer PE) was added to the column and centrifuged for a total of 2 min. To elute the DNA, 10 µL of 10 mM Tris-Cl (buffer EB) was added to the center of the membrane within the MinElute column for 1 min and then centrifuged for 1 min. The 10 µl of DNA extract was transferred into a new mini-centrifuge tube and stored at -20°C. In order to quantify the concentrations of purified PCR amplicons, they were diluted to 1 in 50 and 1 in 100. 10 µl of the amplicon dilutions and 1 µl of 1/200 picogreen fluorescent dye (Invitrogen) were added together in sterile glass capillaries. The amount of fluorescence in each dilution was determined on a Roche Light Cycler (Roche Diagnostics, Laval, Que, Canada) and the concentration of DNA extrapolated from a standard curve of different concentrations of known lambda (λ) phage DNA. The concentration of amplicon DNA was converted to copies/µl using the following equation:

copies/µl = concentration of amplicon (g/µl) / estimated molecular mass of each amplicon (# of basepairs X 660 g/mole) x Avogadro's number ($6.022X10^{23}$ copies/mole)

Standards for each amplicon, ranging from 10^8-10^2 copies/µl were then made and used for quantification in qPCR.

2.7 Quantitative Polymerase Chain Reaction (qPCR)

To quantify potential changes in mRNA expression of HO-1, HO-2, COX-2 and mPGES-1 in HBE following the various treatments, two different commercially available kits were used. For initial experiments, the FastStart DNA Master SYBR Green I Kit

(Roche Diagnostics) was used. A mastermix was made containing 11.6 µl H_20, 2.4 µl of 25 mM $MgCl_2$, 1 µl of 10 µM specific sense primer and 1 µl of 10 µM specific antisense primer, and 2 µl of DNA FastStart DNA Master SYBR Green I. However, for the majority of the experiments, GoTaq DNA polymerase qPCR kit (Promega) was used as it newly became available and was the economical choice. Both kits were tested on the same set of samples in order to ensure consistency in our laboratory. The reaction contained 10 µl 2X GoTaq mastermix, 6 µl nuclease-free H_2O, 1 µl of 10 µM specific sense primer and 1 µl of 10 µM specific antisense primer. For each kit, 2 µl of each cDNA sample was added to a sterile capillary tube, and 18 µl of mastermix added. The PCR capillary tubes were capped, centrifuged, and then placed into the LightCycler carousel (Roche Diagnostics). The carousel was placed into the Roche LightCycler system, where it underwent an incubation period at 95°C for 10 min in order to activate the DNA polymerase and denature the cDNA. This was followed by 40-45 cycles of a denaturation period at 95°C for 15 s, a primer annealing period at the specific primer annealing temperature (Table 2) for 5 s and an elongation period at 72°C for 10 s. Fluorescence measurements were taken after each elongation period. To finish the PCR reaction, a melting curve analysis was performed as follows: samples were heated to 95°C for 1 s, cooled to 65°C, then heated to 95°C at a rate of 2°C/s, while the amount of fluorescence was continuously measured in each sample. The melting curve analysis was performed to estimate the size of the amplicon in each PCR reaction, and to make sure that only one fragment of DNA was being amplified in each reaction. A cooling period rounded out the final segment at 40°C. The mRNA expression was normalized to that of the reference gene as a ratio of the gene of interest:reference gene and relative mRNA expression was calculated using the delta threshold cycle (C_T) method (Livak and Schmittgen, 2001) and expressed as fold change ($2^{-\Delta\Delta C_T}$) relative to the expression values for untreated control samples. Multiple reference genes (18-S, HPRT, GAPDH & β-actin) were tested for potential expression changes in our system and as a result of the various drug treatments. Negative RT and cDNA controls were performed during each experiment. For all qPCR experiments, statistical significance was tested by first performing a one-way analysis of variance (ANOVA), followed by *post hoc* Student's *t*-

test analysis to compare controls to the treatment groups. A P-value of <0.05 was considered significant.

2.8 Caspase-3 Apoptosis Assay

Caspase-3 apoptosis assays (Sigma-Aldrich) were performed in order to measure the potential cell death, specifically via apoptosis, in response to oxidant stress via H_2O_2 and to various drug pre-treatments (Table 1). Caspase-3 is a critical member of the caspase family that regulates programmed cell death (PCD or apoptosis). It is known to cleave most caspase related substrates, as well as mediate chromatin condensation, DNA fragmentation and cell blebbing, which are all mechanisms that lead to cell death (Porter and Janicke, 1999). This assay is based on the hydrolysis of a peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNa) by caspase-3, resulting in the formation of p-nitroaniline (pNA), which has a high absorbance at 405 nm. Approximately 200,000 HBE were seeded in 6-well cell culture plates and grown for 5 days. After a specific treatment protocol (Tables 1 and 2), cell lysates were made as follows: the cells were incubated on ice for 30 min with 100 µl 1X lysis buffer after they were washed with PBS for 30 sec. The cells were then detached from the wells using a cell scraper (Sarstedt, Inc, Newton, NC, USA) and the cell lysate was transferred into 1.5 ml micro-centrifuge tubes, vortexed for 10 sec and centrifuged at 4°C for 30 min at 13,000 rpm. The supernatants were transferred into new 0.5 ml mini-centrifuge tubes and used immediately or stored at -20°C for no longer than a week. To determine the protein concentration, the supernatants were analyzed using the Bradford dye method (Bio-Rad, Hercules, CA, USA) and a micro-plate reader (Beckman Coulter Canada, Inc.). Using a 96-well polysterene tissue culture plate, 60 µg of cell lysate was transferred to a corresponding well in triplicate, and enough 1X assay buffer was added to each well in order to reach a final volume of 90 µl. Finally, 10 µl of 2 mM substrate (Ac-DEVD-pNa) was added to each well to start the reaction. The plate was incubated at 37°C with 5% CO_2 for 90 min and the absorbance was read at 405 nm using a micro-plate reader. To ensure the accuracy of the assay, a negative cell lysate control, caspase-3 inhibitor (Ac-DEVD-CHO) and caspase-3 positive control sample were included in each experiment.

2.9 Lactate Dehydrogenase (LDH) Cytotoxicity Assay

LDH cytotoxicity assays (Cayman Chemical, Michigan, USA) were performed in order to measure total cell death in response to oxidant stress H_2O_2 and other various drug treatments (Table 1). LDH is a soluble enzyme found in the cytosol of cells, which is released into the surrounding culture medium when the cell membrane loses its integrity due to necrosis or apoptosis, and is therefore commonly used as an indicator of cell cytotoxicity. LDH present in the culture medium is measured following a coupled two-step reaction. First, LDH catalyzes the reduction of NAD+ to NADH and H+ by oxidation of lactate to pyruvate. Diaphorase then uses the new NADH and H+ to catalyze the reduction of a tetrazolium salt (INT) to highly coloured formazan that absorbs strongly at 490-520 nm. Approximately 200,000 or 50,000 HBE cells were seeded in 6-well or 24-well cell culture plates respectively and grown for 5 days. After a specific treatment protocol (Table 1), 500 µl of culture medium from each well was transferred into 1.5 ml micro-centrifuge tubes and centrifuged at 400 x g relative centrifugal force (RCF) for 5 min to separate the cellular debris. 100 µl of each sample was transferred to a well in a 96-well polysterene tissue culture plate in triplicate. A reaction solution was prepared containing 100X of each NAD^+, lactic acid, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium (INT), LDH diaphorase in assay buffer. 100 µl of reaction solution was added to each well, gently shaken at room temperature for 30 min and the absorbance was read at 490 nm on a micro-plate reader.

2.10 Lipid Hydroperoxide (LPO) Assay

LPO assays (Cayman Chemical) were performed in order to measure potential oxidative injury via lipid peroxidation reactions in response to oxidant stress H_2O_2 and to various drug pre-treatments (Table 1). Following lipid peroxidation, highly reactive and unstable hydroperoxides are created from both saturated and unsaturated lipids. This assay allows the extraction of these hydroperoxides in chloroform, which can then be directly used in the assay. This assay measures the hydroperoxides by using redox reactions with ferrous ions to form ferric ions. The hydroperoxides undergo oxidation/reduction reactions with the ferrous ions (Fe^{2+}) contained in FTS reagent 1 to form ferric ions (Fe^{3+}), these ferric ions then react with the thiocyanate ions ($5SCN^-$)

from FTS reagent 2 to form a chromogen ($Fe(SCN)_5^{2-}$) which can be measured via colorimetric spectrometry. Approximately 200,000 HBE were seeded in 6-well cell culture plates and grown for 5 days. After a specific treatment protocol (Table 1), the cells were washed twice with PBS and harvested in 300 µl PBS via cell scraping. The cells were homogenized by pipetting and transferred into 1.5 ml micro-centrifuge tubes. An equal volume (300 µl) of methanol saturated extract R and two volumes (600 µl) of cold chloroform (stored at 4°C) was added, the solution was vortexed for 15 sec and centrifuged at 0°C for 5 min at 1500 x *g* (RCF). The 500 µl bottom chloroform layer containing the hydroperoxides was carefully transferred into a new 1.5 ml micro-centrifuge tubes. The two assay solutions; a 2:1 chloroform to methanol mixture and a 1:1 FTS reagent 1 to FTS reagent 2 mixture were prepared and 450 µl and 50 µl were added to the hydroperoxide extracts respectively. After a 5 min incubation period at room temperature, 300 µl of each sample was transferred to a corresponding well in a 96-well glass plate (Cayman Chemical) in triplicate and the absorbance was read at 490 nm using a micro-plate reader. A negative hydroperoxide control was performed during each experiment.

2.11 Western Blotting

To detect protein and measure potential changes in protein expression, western blotting was performed. HBE cells were grown to confluency in 6-well culture plates or 10 cm dishes. After a specific treatment protocol (Table 1), total or nuclear cellular protein was extracted. To investigate HO-1, HO-2, COX-1, COX-2 and mPGES-1 protein expression, total cellular protein was extracted, culture media was removed and the cells rinsed with 2-10 ml PBS and 150-300 µl RIPA buffer (Appendix B), with 10X HALT protease inhibitor cocktail (Fisher Scientific) added. The cells were incubated on ice for 30 min and via cell scraping, a cell solution was transferred into 1.5 ml micro-centrifuge tubes and centrifuged at 4°C for 30 min at 13,000 rpm in order to pellet cellular debris and collect the cellular protein lysate (supernatant) into 0.5 ml mini-centrifuge tubes. To investigate NF-κB and phospho-NF-κB protein expression, nuclear protein extractions were performed. Cells were grown on 10 cm culture dishes in order to extract sufficient protein. Cells were harvested in 500 µl PBS containing 10X HALT

protease inhibitor cocktail via cell scraping, homogenized by pipetting and transferred into 1.5 ml micro-centrifuge tubes. Samples were centrifuged at 4°C for 5 min at 3000 rpm and the supernatant discarded. Cells were re-suspended in ice-cold hypotonic buffer (Appendix B) and incubated on ice for 15 min in order to promote cell swelling and membrane fragility. 100X Nonident P-40 (Sigma-Aldrich) was added to disrupt the outer plasma membrane and after centrifugation at 13,000 rpm for 30 sec, the top layer containing the cytosolic protein fraction was collected. The resulting nuclear pellet was then re-suspended in 100 µl extraction buffer (Appendix B), vortexed for 20 sec every 10 min for 1 hr and centrifuged at 4°C for 10 min at 13,000 rpm in order to collect the nuclear protein fraction (final supernatant). Total cellular protein lysates were stored at -20°C for no longer than a month and cytosolic and nuclear fractions were stored at -80°C. To determine the protein concentration, the cell lysates were analyzed using the Bradford dye method and a micro-plate reader. To carry out Western blot analysis, 10-60 µg of HBE and CFBE protein was diluted with 2X Laemelli loading buffer (Bio-Rad) with 5% ($^{v}/_{v}$) β-mercaptoethanol and heated for 5 min at 95°C. Proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide (Sigma-Aldrich) electrophoresis gels (Appendix B). The proteins were run through the a 5% stacking gel at 50V for approximately 45 min and then at 100V for approximately 1.5 hr once the proteins reached the 8-12% resolving gel in 1X running buffer (Appendix B). After protein migration was complete, the stacking gel was removed and the protein was transferred onto a nitro-cellulose membrane at 100V for 1 hr in transfer buffer (Appendix B) kept cool on ice. The nitro-cellulose membranes were then blocked for 1 hr at room temperature with 10% ($^{w}/_{v}$) skim milk powder in Tris-base saline (TBS) containing 0.1% ($^{v}/_{v}$) Tween 20 (TBS-T, Bio-Rad, Appendix B). The name, company, host, and dilution of primary and secondary antibodies used are shown in Table 3. The membranes were incubated on a shaker with specific primary antibodies in 5% ($^{w}/_{v}$) milk or bovine serum albumen (BSA, Sigma-Aldrich) overnight at 4°C. In the morning, the membranes were washed 3 times for 5-15 min with TBS-T and once for 5-15 min in TBS. Membranes were then incubated for 2 hr with secondary antibody at room temperature in 5% ($^{w}/_{v}$) milk, followed by the washing procedure described above. In order to visualize the protein bands on the nitrocellulose membrane, the Amersham Enhanced Chemiluminescense Plus Western blotting detection

system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) was used following the manufacturers instructions, followed by exposure on Kodak X-OMAT film (Kodak, Rochester, NY) and film development using Konica Minoita SRX-101A medical film processor (Konica Minoita Medical and Graphic Inc., Wayne, NJ). In order to detect multiple proteins, including loading controls GAPDH or β-actin, the membranes were stripped of all anti-bodies using Restore PLUS Western Blot Stripping Buffer (Fisher Scientific) for 15 min, then rinsed with TBS and blocking and incubating procedures repeated. A densometric analysis was performed by scanning the film using a UMAX Powerlook III (UMAX, Dallas, TX) and the band images quantified using ImageJ software (version 1.39, National Institute of Health). For protein quantification studies, a standard curve was performed via Western Blots containing serial dilutions of control samples, in order to determine the linear dynamic range of protein expression in our system.

2.12 Drugs Used

Table 1 outlines the drugs used, their proposed action, dilution solvents, stock and treatment concentrations and company information. For many experiments, drug concentration-response curves were performed in order to test the minimal and optimal concentrations. Stock solutions of at least 1000-fold were made up in EtOH or DMSO so that the final concentration of solvent added to cells never exceeded 0.1%.

2.13 Statistics

For caspase-3, LDH and LPO assays, as well as quantitative Western Blotting experiments, the data is expressed as the mean ± standard error of the mean (SEM) normalized by percent of the control. Each experiment was performed at least 3 times on 3 different passages of HBE cells. For all quantitative experiments, statistical significance was tested by first performing a one-way analysis of variance (ANOVA), followed by *post hoc* analysis. A Student's *t*-test was used for single comparisons, a Dunnett's multiple comparisons test was used to compare treated groups with a single control, whereas a Tukey's multiple comparisons test was used to compare all possible means. A P-value of <0.05 was considered significant.

CHAPTER 3: RESULTS

3.1 HBE Cells Express mRNA Transcripts for Heme Oxygenase Isoforms, Prostanoid Synthases and Receptors

To study the expression of mRNA transcripts for a number of genes of interest in HBE cells, cDNA prepared from extracted HBE RNA was used in RT-PCR reactions. PCR amplicons of the expected size for the HO isoforms HO-1 and HO-2, prostanoid synthases COX-1, COX-2 and mPGES-1 and the EP_2 and EP_4 prostanoid receptors were detected (Figure 5). Amplicons were not detected in negative RT or water control samples (not shown). Primer sequences and annealing temperatures are listed in Table 2.

3.2 HBE Cells Express Protein for Heme Oxygenase Isoforms and Prostanoid Synthases

To confirm that the mRNA transcripts detected for the genes of interest were translated into protein, western blotting was performed. Total cell lysates were extracted and probed with antibodies as described in Table 3. Protein was detected at the predicted size for two HO isoforms HO-1 and HO-2, and three prostanoid synthases COX-1, COX-2 and mPGES-1 (Figure 6). A peptide corresponding to the binding epitope of the mPGES-1 primary antibody (binding peptide) was used as a negative control. No protein was detected in the presence of the peptide, providing additional confirmation of specificity (Figure 6E). Unfortunately, binding peptides were not commercially available for the other antibodies used.

3.3 Treatment with Colbalt Protoporphyrin IX (CoPP) Induces HO-1 and HO-2 mRNA Expression, with no Change in mPGES-1 or COX-2 mRNA Expression

It has yet to be elucidated whether PGE_2 is beneficial or harmful to the airways. It has been reported in osteoarthritic chondrocytes that HO-1 may exert its beneficial affects during inflammation by down-regulating COX-2 and mPGES-1 and reducing subsequent PGE_2 production (Megías *et al.,* 2009). Indeed, although COX enzymes have already been targeted via nonsteroidal anti-inflammatory drugs, mPGES-1 is increasingly being investigated as a potential anti-inflammatory agent (Samuelsson *et al.,* 2007). Therefore, I wished to investigate whether induction of HO-1 and potentially HO-2 (although not

previously investigated) via the known HO-1 inducer CoPP, would lead to a similar down-regulation of both COX-2 and mPGES-1. Via quantitative (q) RT-PCR, I initially confirmed that 24 hr treatment of CoPP (10 μM) significantly increased both HO-1 and HO-2 mRNA expression by 323.4 ± 40.1 ($P<0.05$) and 4.7 ± 0.9 ($P<0.05$) fold change relative to the expression values for vehicle treated control samples respectively (Figure 7). As induction of the HO system was required to test for changes in COX-2 and mPGES-1 mRNA expression, as well as serve as a positive control for future experiments, I used western blotting to confirm that this increase in HO-1 and HO-2 mRNA correlated to an increase in protein. Following 24hr treatment with CoPP (between 0-20 μM), western blotting was performed and HO-1 protein expression quantified. Expression was significantly increased by 20 μM (465.5 ± 81.7%) CoPP ($P<0.05$) over the vehicle treated controls when normalized to GAPDH (Figure 8). However, no significant changes were detectable in HO-2 protein levels ($P=0.807$). Although HO-1 mRNA and protein expression increased following CoPP treatment, this induction did not lead to a significant decrease in COX-2 ($P=0.138$) or mPGES-1 ($P=0.330$) mRNA expression (Figure 7).

3.4 Treatment with Colbalt Protoporphyrin IX (CoPP) Increases p-NF-κB Protein Expression, with no Change in NF-κB Protein Levels

The signal transduction pathways and transcription factors that regulate HO-1 gene activity are very complex, due to the multitude of inducing conditions and transcription factor binding sites (Ryter *et al.,* 2002). The transcriptional functions of NF-κB are diverse, however it has become known to be especially important to the development and maintenance of the immune system, skeletal system, and epithelium. In these locations, the NF-κB pathway contributes to the control of cell survival, differentiation, and proliferation (Hayden and Ghosh, 2012). Thus, because of its roles in the immune system and the epithelium, I wished to investigate if the NF-κB p65 (RelA) subunit, which provides the gene regulatory function, is involved in mediating the induction of HO-1 after CoPP treatment. This transcription factor can also be phosphorylated once it reaches the nucleus; therefore, I also probed for p-NF-κB p65. NF-κB signal transduction typically takes place over a short period of time; therefore,

following 30 min treatment with 10 μM CoPP (the lowest dose to show HO-1 mRNA induction) western blotting was performed using the nuclear protein fraction. p-NF-κB protein was significantly increased (174.8 ± 21.1%; $P<0.05$) over the vehicle treated controls when normalized to β-actin (Figure 9). β-actin was used as a control for the nuclear fraction experiments because the GAPDH signal was undetectable. No significant changes were seen in NF-κB protein levels ($P=0.108$; Figure 9).

3.5 Activation of the EP_2 or EP_4 Receptor does not Result in HO-1 Induction

Recently, it was demonstrated that PGE_2 can protect cells against oxidant-induced apoptosis via upregulation of HO-1, mediated by the EP_2 receptor (Park *et al.,* 2009). Also, our laboratory has established a body of evidence to suggest that the EP_4 receptor plays an important role in mediating the effects of oxidant stress in the airways (Jones *et al.,* 2012). Therefore, using qRT-PCR and western blotting, I wished to determine if pharmacological activation of the EP_2 and/or EP_4 receptor via the agonists butaprost (10 μM) and PGE_1-OH (10 μM) for 24 hrs would lead to induction of HO-1 mRNA and protein. However, following this treatment protocol, both quantitative RT-PCR and western blotting showed that neither butaprost nor PGE_1-OH treatments resulted in an increase in HO-1 or HO-2 mRNA (Figure 10) or protein (Figure 11) expression ($P>0.05$). Although these results do not support the original hypothesis, it has been shown that PGE_2 or activation of its receptors have independent protective properties (Regan, 2003) and therefore, warranted further investigation.

3.6 Hydrogen Peroxide (H_2O_2) Treatment Leads to the Induction of Apoptosis in HBE Cells

In order to investigate the potential protective properties of PGE_2 and which receptor might be responsible, it was necessary to first establish a treatment protocol to induce apoptosis via the oxidant stressor H_2O_2, without causing significant cell death in other forms, such as necrosis. This treatment protocol was determined by treating HBE cells with between 0-1000 μM H_2O_2 for 3, 6 and 24 hrs and first measuring apoptosis using the caspase-3 assay (Figure 12). After 3 hrs of incubation, no significant changes were detected between control and H_2O_2 treated samples. Following 6 hrs of H_2O_2

incubation, apoptosis was significantly increased at 400 μM (188.5 ± 30.0%; P<0.05) H_2O_2. Lastly, after 24 hrs of H_2O_2 incubation, there was a significant increase in apoptosis with 400 (138.4 ± 12.7%; P<0.05), 800 (202.4 ± 31.8%; P<0.05) and 1000 (176.1 ± 15.8%; P<0.05) μM H_2O_2. By successfully inducing apoptosis with 400 μM H_2O_2 for 6 hrs, as well as 400, 800 and 1000 μM H_2O_2 for 24 hrs, it was then necessary to determine if these doses corresponded to cytotoxicity.

3.7 Cytotoxicity from Hydrogen Peroxide (H_2O_2) Treatment in HBE Cells

In order to determine if a dose of H_2O_2 that induces apoptosis (see section 3.6) was appropriate for further experiments, HBE cells were treated with between 0-1000 μM H_2O_2 for 3, 6 and 24 hrs and total cell death was measured using LDH cytotoxicity assays (Figure 12). After 3 hrs of H_2O_2 incubation, cell death was significantly increased with 1000 μM (107.9 ± 2.5%; P<0.05) H_2O_2. Following 6 hrs of H_2O_2 incubation, a significant increase in cell death was also detected at 1000 μM (122.1 ± 1.0%; P<0.05) H_2O_2. Lastly, after 24 hrs of H_2O_2 incubation, there was a significant increase in cell death with 800 (218.0 ± 10.3%; P<0.05) and 1000 (228.6 ± 6.7; P<0.05) μM H_2O_2. Therefore, although apoptosis was induced within a range of H_2O_2 after incubation for 24 hrs, this induction was matched with significant cell death. As a result, for all subsequent experiments a treatment protocol of 6 hrs with 400 μM H_2O_2 was used to induce apoptosis.

3.8 CoPP, PGE_1-OH and PGE_2, but not Butaprost are Protective Against H_2O_2-Induced Apoptosis

In order to investigate if HO-1 induction (CoPP), PGE_2 treatment or activation of the EP_2 and/or EP_4 receptors could protect against H_2O_2-induced apoptosis, HBE cells were first pre-treated with CoPP (10 μM), PGE_2 (1 μM), butaprost (10 μM) or PGE_1-OH (10 μM) for 24 hrs, followed by exposure to 400 μM H_2O_2 for 6 hrs (see sections 3.6 and 3.7). After the cell lysates were collected, the samples were assayed for apoptosis. CoPP pre-treatment significantly reduced the oxidant-induced apoptosis (91.5 ± 5.9% vs. 219.3 ± 26.6%; P<0.05; Figure 13A). However, butaprost pre-treatment had no effect on the H_2O_2-induced apoptosis (173.6 ± 13.1% vs. 204.4 ± 11.7%; P>0.05; Figure 13B). As with CoPP treatment, both PGE_1-OH (94.5 ± 5.2% vs. 212.5 ± 16.6%; P<0.05; Figure

13C) and PGE_2 (93.4 ± 1.6% vs. 193.9 ± 6.3%; $P<0.05$; Figure 13D) pre-treatments both resulted in a reduction in apoptosis when exposed to H_2O_2, compared to H_2O_2 treated controls. The mechanism by which HO-1 induction exerts its protective properties remains heavily investigated; however, it is widely accepted that the benefits of HO-1 induction primarily comes from the products of heme degradation, CO and biliverdin. However, because PGE_2 exerts its effects through four different receptors, the mechanism of its actions is not always clear. Therefore, further investigation into receptor specificity and receptor signaling was required.

3.9 COX-1 or mPGES-1 Inhibition has no Affect on the EP_4 Receptor Mediated Protection Against Apoptosis

In order to determine if the EP_4 receptor mediated reduction in oxidant-induced apoptosis is a result of PGE_2 synthesis and its subsequent protective properties (Figure 13D), COX-1 and mPGES-1 inhibitors were used. Using another human airway epithelial cell line, Calu-3, it was previously determined that prolonged activation of the EP_4 receptor resulted in increased production of PGE_2 (Li *et al.,* 2011), and although this has yet to be confirmed in HBE cells, I wished to investigate if the use of PGE_2 synthase inhibitors would provide evidence of this occurrence. Therefore, I pretreated HBE cells with the EP_4 receptor agonist PGE_1-OH (10 μM) along with the selective COX-1 inhibitor SC-560 (1 μM) or the mPGES-1 inhibitor CAY10589 (5 μM) for 24 hrs, followed by the H_2O_2 apoptosis protocol described previously. As seen in Figure 14A, apoptosis was significantly reduced when cells were pre-treated with PGE_1-OH and the COX-1 inhibitor SC-560, followed by H_2O_2 treatment (99.9 ± 1.5% vs. 133.5 ± 7.8), as well as cells treated with only the COX-1 inhibitor SC-560 and H_2O_2 (100.2 ± 6.1% vs. 133.5 ± 7.8) when compared to H_2O_2 treated controls ($P<0.05$). Shown in Figure 14B, cells pre-treated with PGE_1-OH and the mPGES-1 inhibitor CAY10589, followed by H_2O_2 (99.5 ± 5.3% vs. 131.5 ± 7.3%) or treated with only the mPGES-1 inhibitor CAY10589 and H_2O_2 (100.1 ± 6.9% vs. 131.5 ± 7.3%), apoptosis was significantly reduced when compared to H_2O_2 treated controls ($P<0.05$).

3.10 EP_4, not EP_2 Receptor Antagonism, Diminishes the PGE_2 Mediated Protection Against H_2O_2-Induced Apoptosis

To investigate the mechanism of PGE_2 protection against oxidant-induced apoptosis (Figure 13D), EP_2 and EP_4 receptor antagonists were employed. Literature suggests that there is a positive feedback loop, in which PGE_2 activates its EP_4 receptor resulting in further PGE_2 release (Li *et al.,* 2011; Jones *et al.,* 2012; Chen *et al.,* 2012). As seen in Figure 15A, cells pre-treated with PGE_2 (1 μM) and the EP_2 receptor antagonist AH6809 (10 μM) for 24 hrs, followed by the H_2O_2 protocol described previously reduced apoptosis to control levels and apoptosis was significantly decreased compared to H_2O_2 treated controls (103.6 ± 1.3% vs. 121.6 ± 4.3%; $P<0.05$). This reduction in the apoptosis was not detected in cells pre-treated with only the EP_2 receptor antagonist AH6809, followed by the H_2O_2 protocol (118.3 ± 1.4% vs. 121.6 ± 4.3%). As Figure 15B shows, cells pre-treated with PGE_2 (1 μM) and EP_4 receptor antagonist AH23848 (10 μM) followed by H_2O_2 treatment showed no significant reduction in apoptosis (113.6 ± 2.6% vs. 120.1 ± 1.9%) when compared to H_2O_2 treated controls. Interestingly, compared to control samples, the treatment of the EP_4 receptor antagonist AH23848 itself had some affect, as it significantly induced apoptosis with (132.4 ± 4.7%) or without (132.9 ± 6.8%) the H_2O_2 treatment ($P<0.05$). Additionally, when cells were treated with PGE_2 and the EP_4 receptor antagonist AH23848, without H_2O_2 treatment, this resulted in apoptosis (129.2 ± 1.2%) that was significantly increased ($P<0.05$) when compared to controls.

3.11 Hydrogen Peroxide (H_2O_2) Treatment Leads to the Induction of Lipid Peroxidation in HBE Cells

Exposure to oxidants results in the dysfunction of many important physiological processes (Rahman *et al.*, 2006), including the induction of apoptosis (Chandra *et al.*, 2000). This dysfunction arises when oxidants damage macromolecules such as DNA, proteins and lipids (Halliwell and Cross, 1994; Rahman *et al.*, 2006). Therefore, I wished to investigate whether the protective properties of HO-1 induction (CoPP), as well as PGE_2 and EP_4 receptor activation included reducing the initial oxidative damage. Using lipid peroxidation as an assay to detect oxidizing damage, I first treated HBE cells with 0-

800 μM H_2O_2 for 90 min and performed a lipid hydroperoxide assay (LPO) in order to determine an appropriate treatment protocol for further experiments (Figure 16A). A shorter treatment time was chosen for this assay when compared to the apoptosis assay (6 hrs) because lipid peroxidation is the quick, initial process in oxidative damage, as the H_2O_2 can come into direct contact with the lipid-dominated cell membrane immediately following treatment (reviewed in Girotti, 1998). Following the 90 min treatment, 400 μM H_2O_2 resulted in a significant increase (131.3% ± 6.0; $P<0.05$) in lipid peroxidation. Therefore, for all subsequent LPO experiments this treatment protocol was used to induce lipid peroxidation.

3.12 CoPP, Butaprost, PGE_1-OH and PGE_2 do not Reduce H_2O_2-Induced Lipid Peroxidation

In order to investigate if HO-1 induction (CoPP), PGE_2 treatment or activation of the EP_2 and/or EP_4 receptors could reduce H_2O_2-induced lipid peroxidation, HBE cells were first pre-treated with CoPP, PGE_2, butaprost or PGE_1-OH for 24 hrs, followed by exposure to 400 μM H_2O_2 for 90 mins. After lipid hydroperoxide extraction, the samples were assayed. However, no drug pre-treatment reduced the lipid peroxidation, when compared to the H_2O_2 treated controls (Figure 16A-D). Indeed, even without the H_2O_2 treatment, butaprost (122.9 ± 2.4; $P<0.05$; Figure 16C) significantly increased lipid peroxidation, compared to vehicle treated controls, suggesting this drug can cause some degree of oxidative damage on its own.

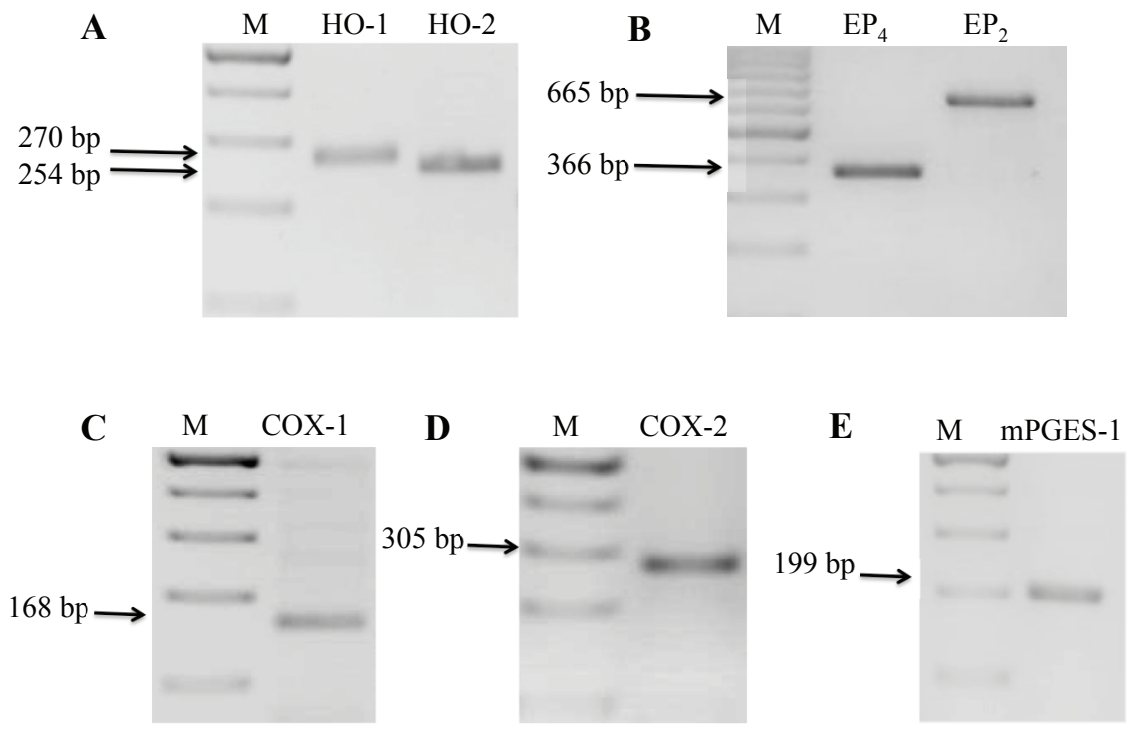

Figure 5. HBE Cells Express mRNA Transcripts for HO-1, HO-2, EP_2 and EP_4 Prostanoid Receptors and PGE_2 Synthesis Enzymes COX-1, COX-2 and mPGES-1. Using RT-PCR, mRNA transcripts for two heme oxygenase isoforms HO-1 (270 bp; Panel A) and HO-2 (254 bp; Panel A), prostanoid receptors EP_2 (655 bp; Panel B) and EP_4 (366 bp; Panel B) and PGE_2 synthesis enzymes COX-1 (168 bp; Panel C) and COX-2 (305 bp; Panel D) and mPGES-1 (199 bp; Panel D) were detected in HBE cells (n=3). A one hundred base pair DNA marker is shown (M). A negative RT and a negative water sample were run with each gel (not shown). The primer sequences and annealing temperatures are listed in Table 2.

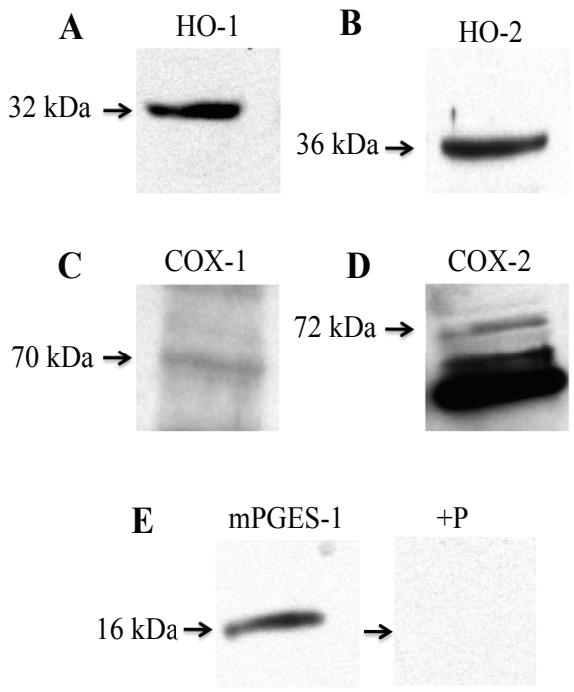

Figure 6. HBE Cells Express Protein for HO-1, HO-2, COX-1, COX-2 and mPGES-1. Western blots were performed on total cell lysates and protein was detected for HO-1 at 32 kDa (Panel A), HO-2 at 36 kDa (Panel B), COX-1 at 70 kDa (Panel C), COX-2 at 72 kDa (Panel D) and mPGES-1 at 16 kDa (Panel E) in HBE cells after incubation with antibodies against each protein (n=3). When available (mPGES-1, Panel E), the membrane was incubated with excess of a blocking peptide (+P) specific for that primary antibody. Details for the primary and secondary antibodies are described in Table 3.

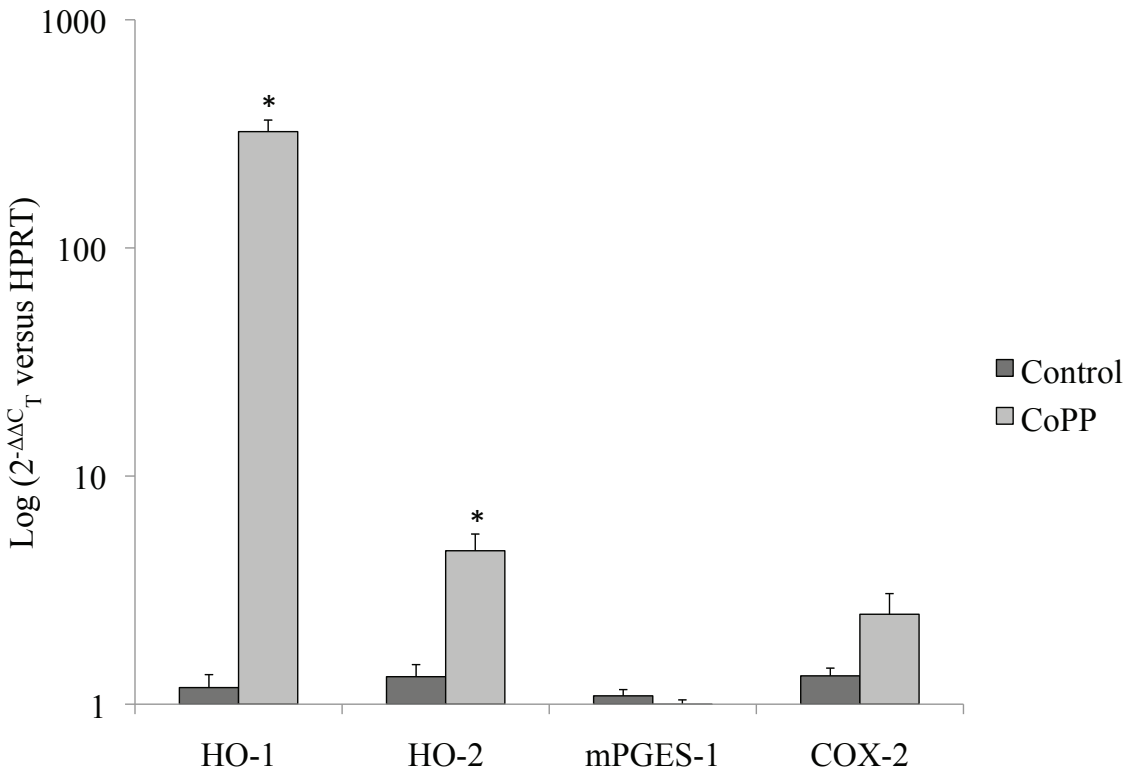

Figure 7. Quantitative mRNA Expression of HO-1, HO-2, mPGES-1 and COX-2 in Control and Colbalt Protoporphyrin IX (CoPP) Treated HBE Cells. Quantitative mRNA expression was measured for genes HO-1, HO-2, mPGES-1 and COX-2 after 24 hr treatment with CoPP (10 μM) in HBE cells. Data was normalized to that of the reference gene HPRT and relative mRNA expression was calculated using the C_T method and expressed as the log fold change ($2^{-\Delta\Delta C_T}$) relative to the expression values for vehicle treated control samples. Data are means ± SEM; n=3 trials, * represents $P < 0.05$ when compared to control, using a Student's *t*-test.

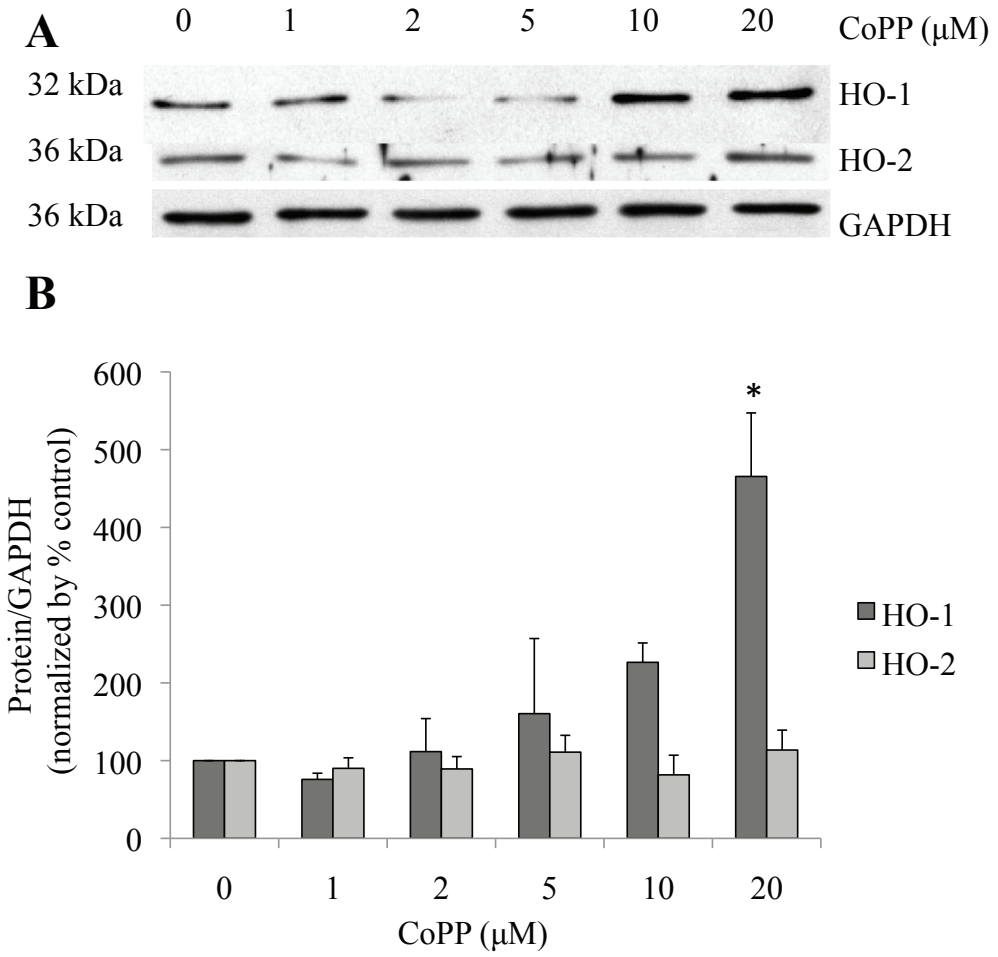

Figure 8. Increase in HO-1 Protein Expression After Treatment with Colbalt Protoporphyrin IX (CoPP) in HBE Cells. HBE cells were treated with 0-20 μM CoPP (24hr). Western blotting was performed using antibodies against HO-1, HO-2 and the control protein GAPDH (Panel A). Densometric analysis revealed increased HO-1 protein in the 20 μM CoPP treated samples, while no significant changes were detected in HO-2 protein (Panel B). Data are means ± SEM; n=3 trials, * represents P <0.05 when compared to vehicle treated control, using an ANOVA, followed by a Dunnett's multiple comparisons test.

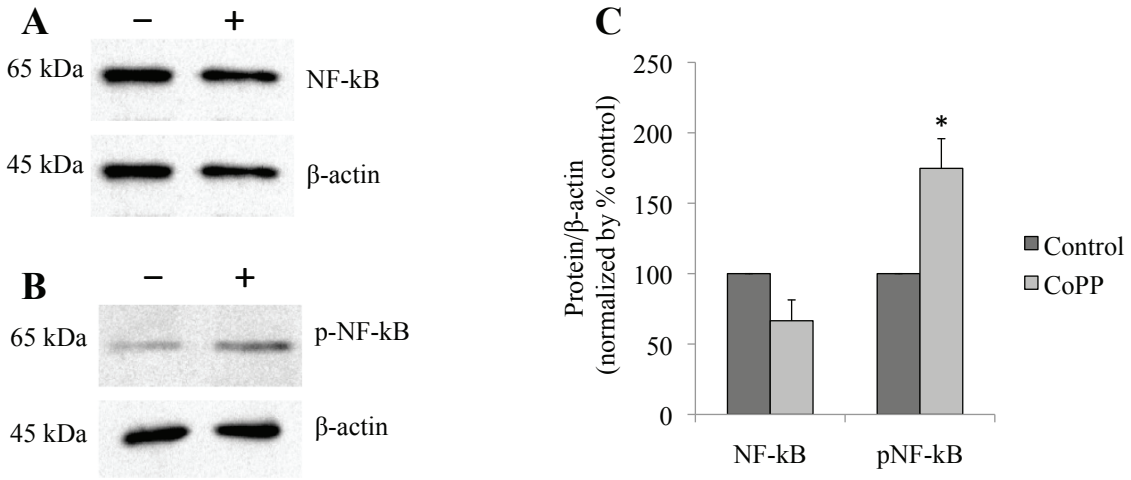

Figure 9. p-NF-κB Protein Expression is Increased After Treatment with Colbalt Protoporphyrin IX (CoPP) in HBE Cells. Signal transduction of NF-κB is fast and transient, therefore, HBE cells were treated with 10 μM CoPP for 30 min and the nuclear protein fraction extracted. Western blotting was performed using antibodies against NF-κB p65 and p-NF-κB p65 and control protein β-actin (Panels A-B). Densometric analysis revealed increased p-NF-κB protein, while no significant changes were detected in NF-κB protein (Panel C). "+" indicates the presence of CoPP. Data are means ± SEM; n=4 trials, * represents $P < 0.05$ when compared to vehicle treated control, as determined by a Student's *t*-test.

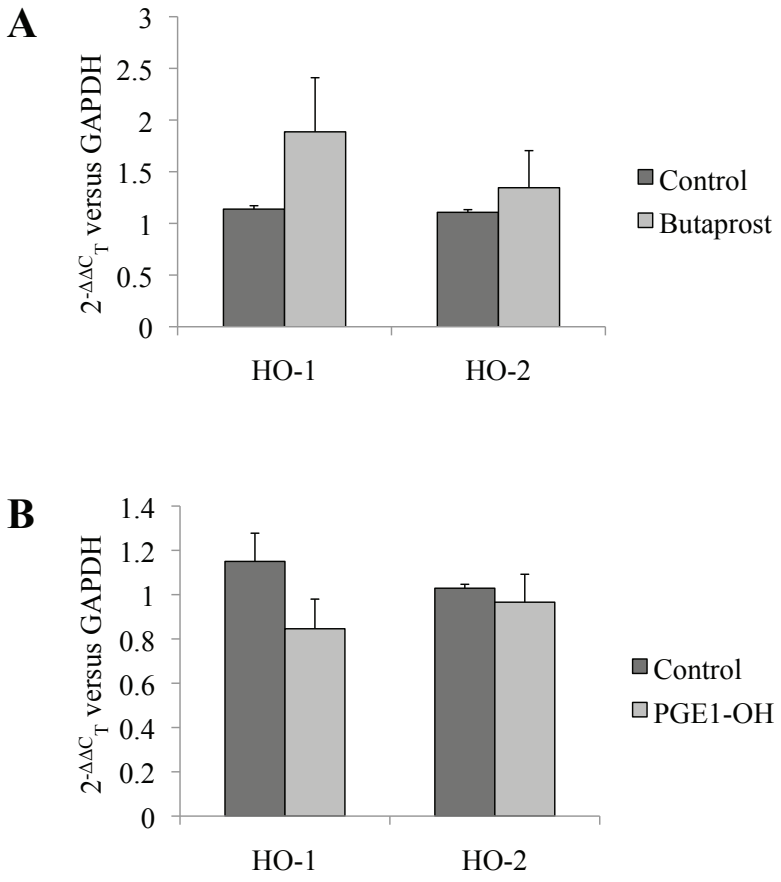

Figure 10. Neither Butaprost nor PGE_1-OH Treatment Affects HO-1 or HO-2 mRNA Expression. Quantitative mRNA expression was measured for genes HO-1 and HO-2 after 24hr treatment of either the EP_2 receptor agonist butaprost (10 μM) or the EP_4 receptor agonist PGE_1-OH (10 μM) in HBE cells. Data was normalized to that of the reference gene GAPDH and relative mRNA expression was calculated using the C_T method and expressed as fold change ($2^{-\Delta\Delta C_T}$) relative to the expression values for vehicle treated control samples. Data are means ± SEM; n=4 trials.

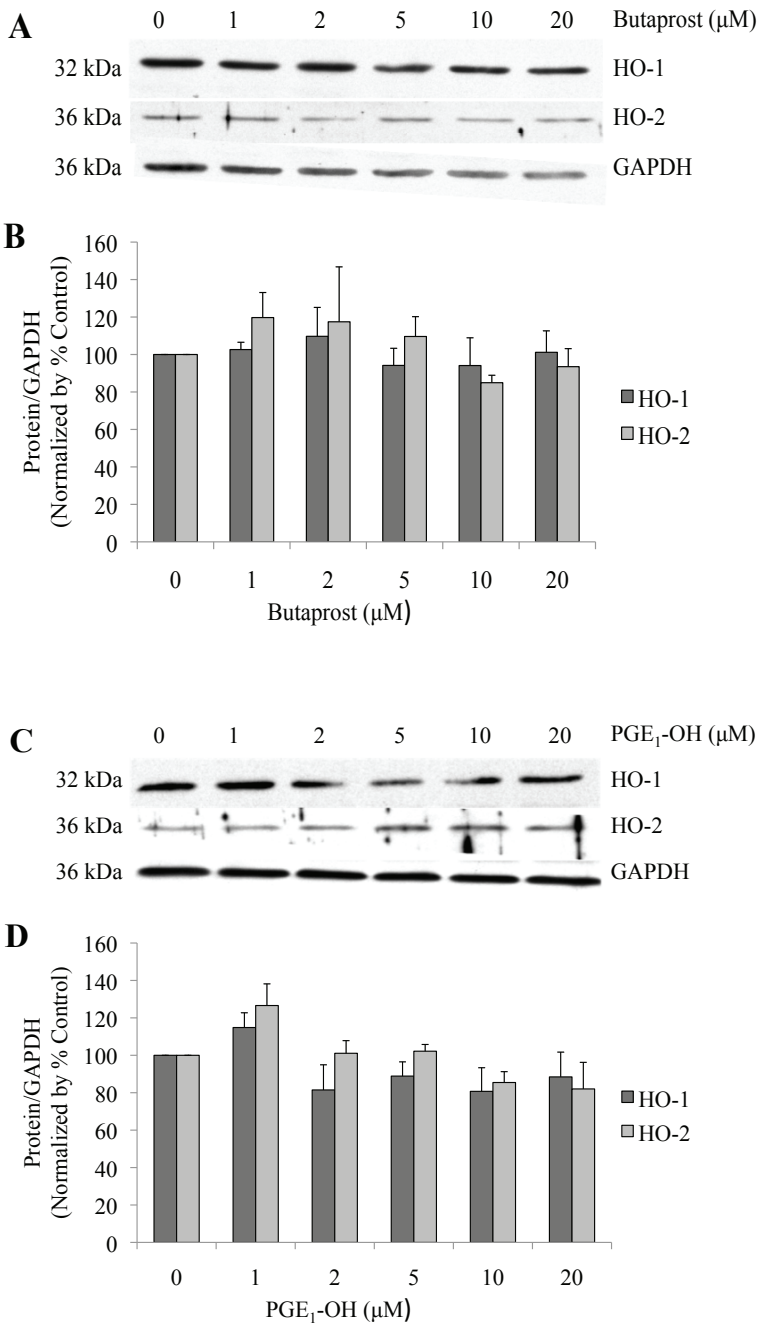

Figure 11. Butaprost and PGE_1-OH do not Affect HO-1 or HO-2 Protein Expression. HBE cells were treated with 0-20 μM butaprost (Panels A-B) or 0-20 μM PGE_1-OH (Panels C-D) for 24 hr. Western blotting was performed using antibodies against HO-1, HO-2 and the control protein GAPDH (Panels A, C). Densometric analysis revealed no significant changes in either HO-1 or HO-2 protein expression compared to vehicle treated controls (Panels B, D). Data are means ± SEM; n=3 trials.

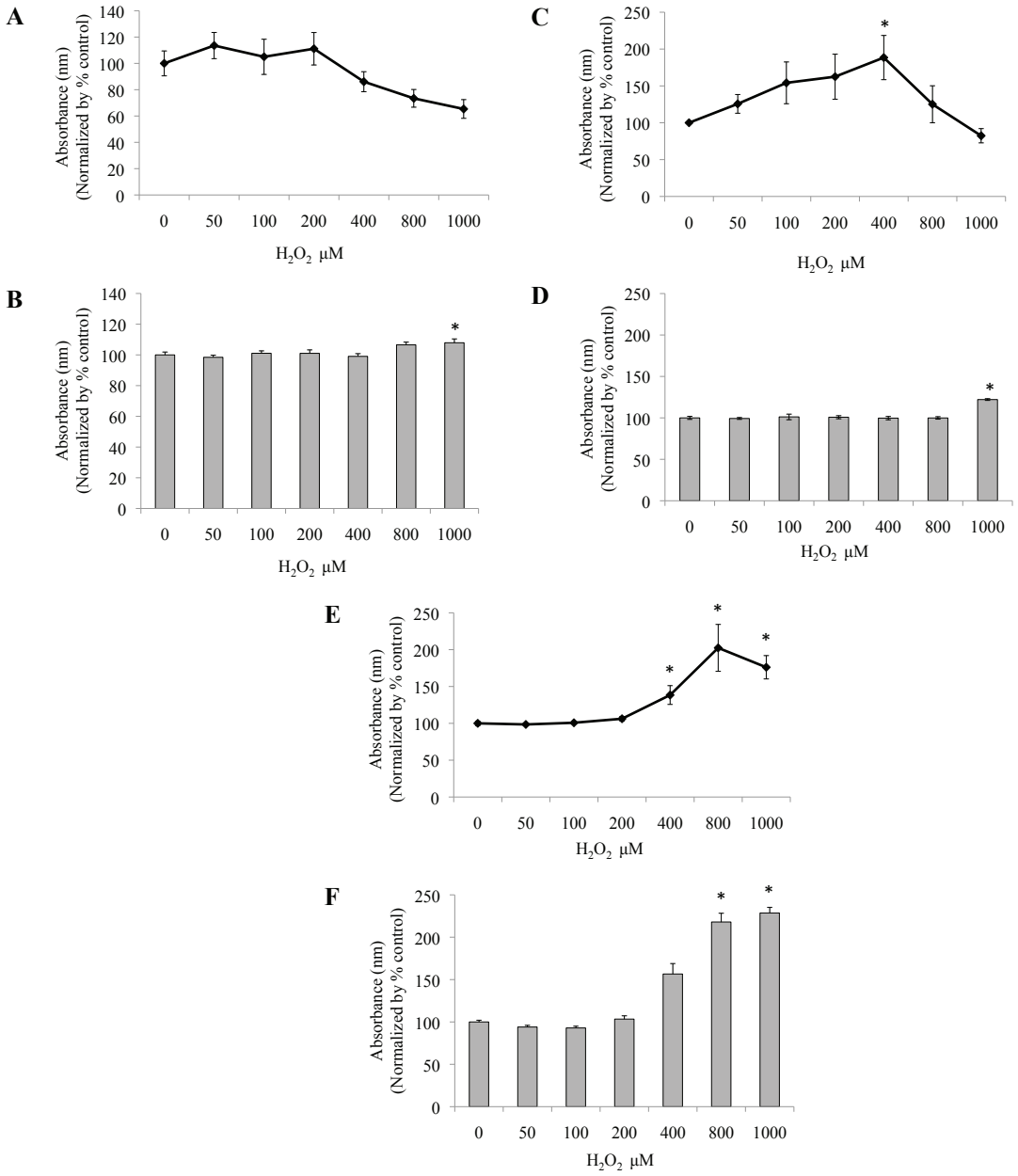

Figure 12. Determination of an Appropriate Treatment Protocol for Oxidant-Induced Apoptosis Using Hydrogen Peroxide (H_2O_2). HBE cells were treated with between 0-1000 µM H_2O_2 for 3 hrs (Panels A-B), 6 hrs (Panels C-D) and 24 hrs (Panels E-F). Caspase-3 assays (Panels A, C and E) and lactate dehydrogenase (LDH) assays (Panels B, D and F) were performed in order to determine an appropriate treatment protocol to induce apoptosis without causing significant cell death via necrosis. Data are means ± SEM; n=3 trials, * represents $P < 0.05$ when compared to control (0 µM), using an ANOVA, followed by a Dunnett's multiple comparisons test.

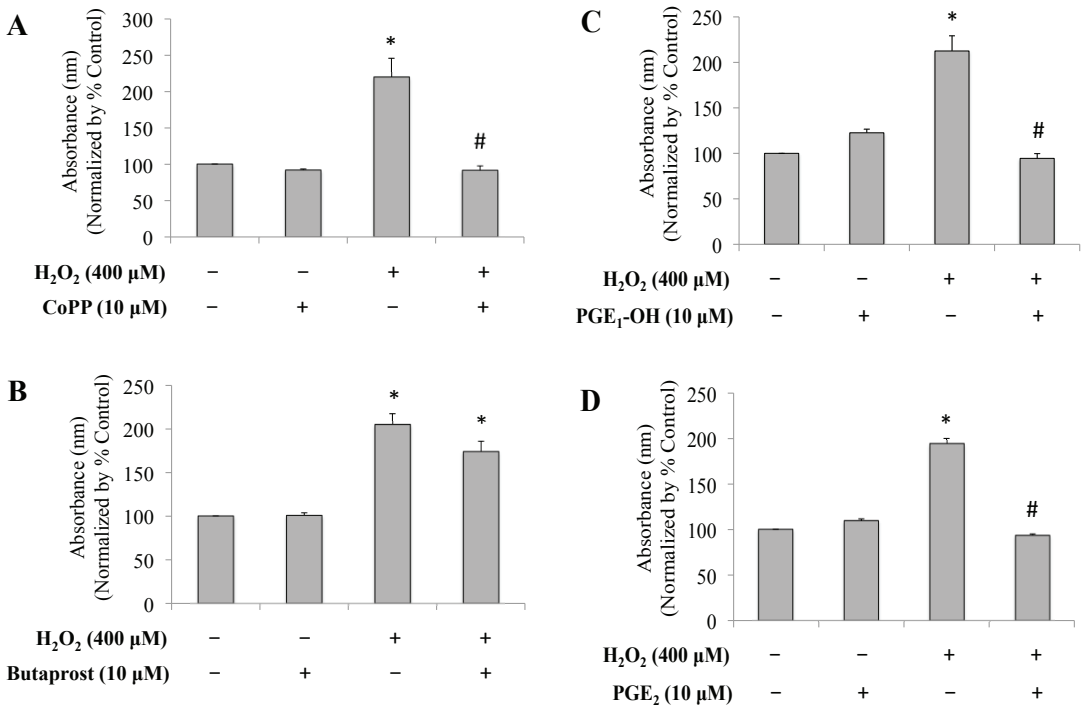

Figure 13. CoPP, PGE_1-OH and PGE_2, but not butaprost are protective against H_2O_2-induced apoptosis. HBE cells were pre-treated with either CoPP (10 µM; Panel A), Butaprost (10 µM; Panel B), PGE_1-OH (10 µM; Panel C) or PGE_2 (1 µM; Panel D) for 24 hrs, followed by a 6 hr treatment with H_2O_2 (400 µM). Caspase-3 assays were then performed. Data are means ± SEM; n=3 trials, * represents $P < 0.05$ when compared to vehicle treated, no H_2O_2 controls, # represents $P < 0.05$ when compared to vehicle treated, H_2O_2 controls, using an ANOVA, followed by a Tukey's multiple comparison test.

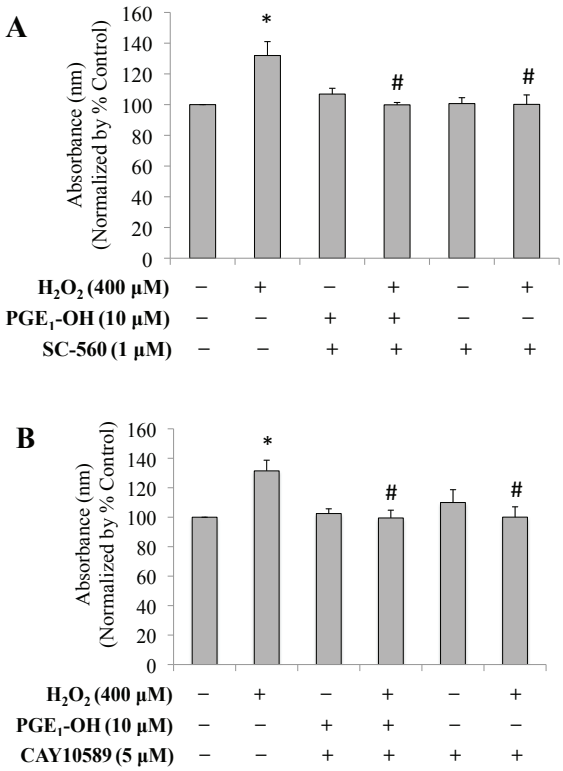

Figure 14. COX-1 or mPGES-1 Inhibition has no Affect on the EP_4 Receptor Mediated Protection Against Apoptosis. HBE cells were pre-treated with either COX-1 inhibitor SC-560 (1 µM) or mPGES-1 inhibitor CAY10589 (5 µM) alone or along with PGE_1-OH (10 µM) for 24 hrs, followed by a 6 hr treatment with H_2O_2 (400 µM). Caspase-3 assays were then performed. Data are means ± SEM; n=3 trials, * represents P <0.05 when compared to vehicle treated, no H_2O_2 controls and # represents P <0.05 when compared to vehicle treated, H_2O_2 treated control samples, using an ANOVA, followed by a Tukey's multiple comparisons test.

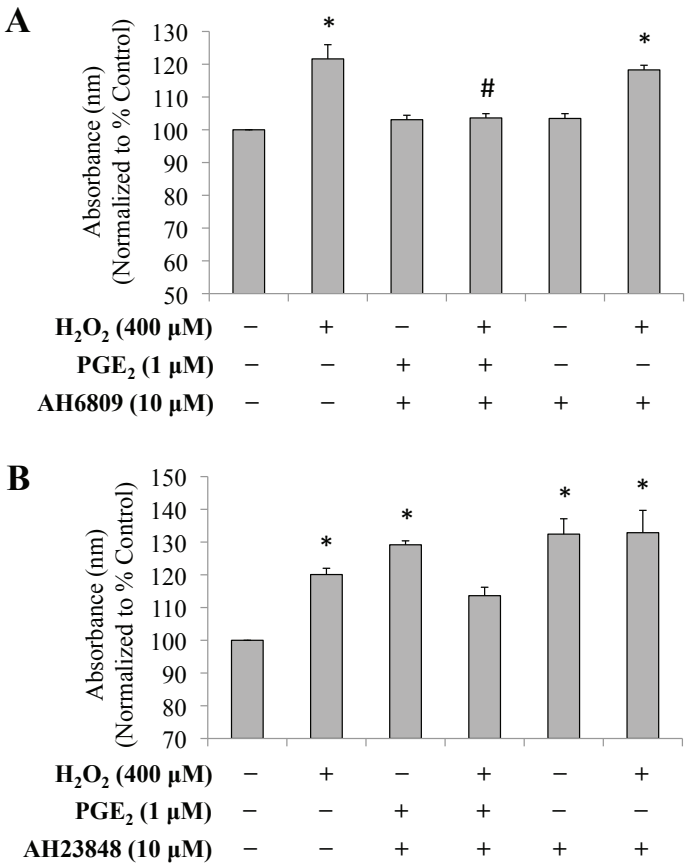

Figure 15. EP_4, not EP_2 Receptor Antagonism, Diminishes the PGE_2 Mediated Protection Against H_2O_2-Induced Apoptosis. HBE cells were pre-treated with either the EP_2 receptor antagonist AH6809 (10 µM) or the EP_4 receptor antagonist AH23848 (10 µM) alone or together with PGE_2 for 24 hrs, followed by a 6 hr treatment with H_2O_2 (400 µM). Caspase-3 assays were then performed. Data are means ± SEM; n=3 trials, * represents $P < 0.05$ when compared to vehicle treated, no H_2O_2 controls, # represents $P < 0.05$ when compared to vehicle treated, H_2O_2 treated controls as performed by an ANOVA, followed by a Tukey's multiple comparisons test.

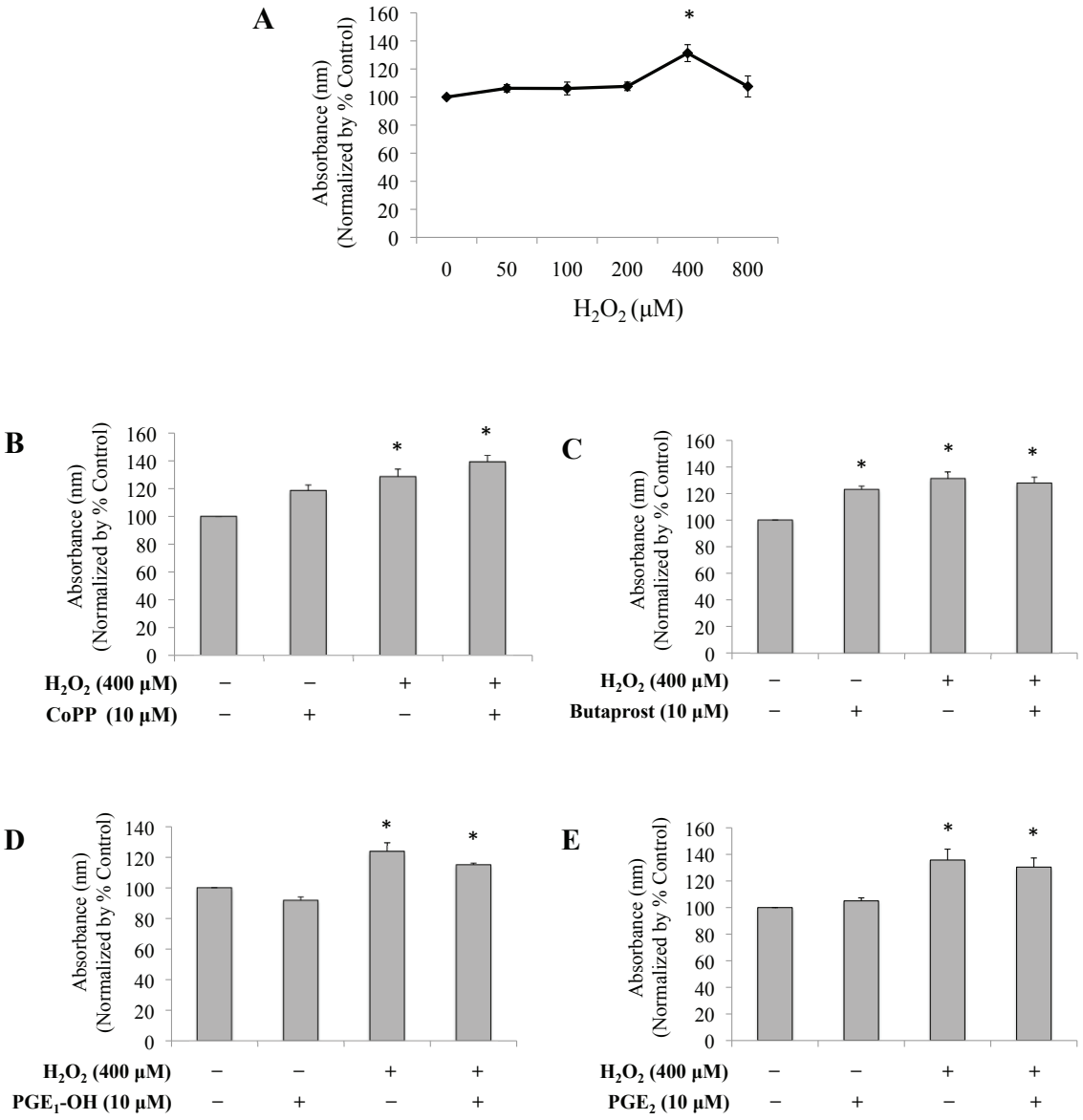

Figure 16. CoPP, Butaprost, PGE_1-OH and PGE_2 do not Reduce H_2O_2-Induced Lipid Peroxidation. HBE cells were treated with between 0-800 μM H_2O_2 for 90 mins (Panel A), lipid hydroperoxides extracted and a lipid peroxidation assay performed in order to find a dose of H_2O_2 that would cause significant lipid peroxidation. Using that dose (400 μM), HBE cells were pre-treated with CoPP (Panel B), Butaprost (Panel C), PGE_1-OH (Panel D) and PGE_2 (Panel E) for 24 hr, followed by H_2O_2 treatment. Data are means ± SEM; n=3 trials, * represents $P < 0.05$ when compared to vehicle treated, no H_2O_2 controls, using an ANOVA, followed by either a Dunnett's (Panel A) or Tukey's (Panels B-E) multiple comparisons tests.

CHAPTER 4: DISCUSSION

The airway epithelium plays a critical role in protecting lung tissue by organizing the complex interworkings between mechanical (MCC), innate and acquired host defense systems, which are tremendously underappreciated in healthy individuals. Oxidative stress is a major degenerative and functional component in the development of inflammatory pulmonary diseases, but provides us with great opportunity to intervene by researching potential therapeutic options. HO-1 is acknowledged for its anti-inflammatory, anti-oxidant and anti-apoptotic properties and is upregulated by several different stimuli, including PGE_2 (Carter *et al.*, 2004; Rahman *et al.*, 2006; Ryter *et al.*, 2007; Park *et al.,* 2009). Our laboratory is interested in the roles prostanoid receptors play in mediating the effects of oxidant stress in the airways. This study increases our understanding of how airway epithelial cells cope with oxidant stress.

I hypothesized that activation of the EP_2 and/or EP_4 receptors would lead to the induction of HO-1 expression and that this induction would protect the cells against oxidant-induced damage. Although CoPP did induce HO-1 at both the mRNA and protein level and proved to be cytoprotective, activation of either EP receptor had no affect on HO-1 expression. Using a common method for oxidant-induced apoptosis, I demonstrated that PGE_2 is capable of eliciting a survival response by reducing apoptosis in HBE cells and that the response is likely mediated by the EP_4 receptor. No treatments were able to reduce oxidant-induced lipid peroxidation. Using CoPP to induce HO-1, I determined that the upregulation of HO-1 has no effect on the gene expression of PGE_2 synthases in airway epithelial cells.

4.1 The Induction of Heme Oxygenase Isoforms via Colbalt Protoporphyrin IX

Colbalt protoporphyrin IX (CoPP) is a well-known HO-1 inducer, first described more than thirty years ago (Maines and Kappas, 1974). This drug treatment remains a commonly used method for HO-1 induction (Clérigues *et al.,* 2011; Elmarakby *et al.,* 2012; George and Arany, 2012), and its mechanism of action is becoming clearer. Therefore, CoPP was chosen to increase HO-1 (and possibly HO-2) expression in HBE cells in order to study the affects of that induction, was well as serve as a positive control for future experiments.

Initially, I found that treatment with 10 μM CoPP for 24 hrs significantly increased both HO-1 and HO-2 mRNA expression (Figure 7). This treatment protocol was chosen by reviewing the literature and using a protocol that was previously successful in several studies (Megías *et al.,* 2009; Clérigues *et al.,* 2011; Lawson *et al.,* 2011). While HO-2 is primarily considered constitutively expressed, it was of interest to include it in this study because little is known about HO-2 in the airways. Indeed, I found HO-2 mRNA expression increased after CoPP treatment. Using a mouse model of spinal cord injury, Panahian and Maines (2001), found elevated HO-2 mRNA levels in cells above the injury, using northern blot analysis. Also, Ewing and Maines (2006), showed increased HO-2 mRNA in whole rat brain tissue after treatment with Neotrophin™ (a cognition enhancing and neuroprotective drug), again using northern blot analysis. Therefore, my finding of increased HO-2 mRNA expression is not without precedent and this gene is clearly inducible.

I next wanted to confirm that the induction of HO-1 and HO-2 mRNA via CoPP resulted in increased protein translation. Via western blot analysis, I found that HO-1 protein increased following 20 μM CoPP for 24 hrs (Figure 8A-B). However, unlike the qRT-PCR results for HO-2 (Figure 7), I did not detect any changes in HO-2 protein (Figure 8A-B). Protein quantification via western blotting is intrinsically more prone to error; however, several measures were taken in order to perform high-quality quantitative western blotting, including extensive antibody optimization and sample-dilution standard curves in order to delineate the linear dynamic range. Therefore, although technique remains a possible source for error, other reasons for this discrepancy must be considered. An extensive study of over 150 yeast proteins by Gygi *et al.,* (1999) found that the correlation between mRNA and protein levels to be inefficient to predict protein expression from quantitative mRNA data and vice versa. More recent publications on the topic have come to similar conclusions studying yeast (Foss *et al.,* 2007), plants (Arabidopsis; Fu et al., 2009) and most recently, mammals (mouse; Ghazalpour *et al.,* 2011). Through this work it has become clear that assumptions made about cell biology in the past, such as the linear flow of biological information from DNA to RNA to protein to phenotype, must now be questioned, as it is now known that the process is much more complex. Protein levels depend on many other factors other than mRNA

levels, such as post-transcriptional, post-translational processing and protein stability (Ghazalpour *et al.,* 2011). Therefore, a result based only from mRNA is insufficient, and a correlation between the two measures is not a guarantee. Indeed, inconsistent results were found in a study using isolated rat neonatal cardiomyocytes, where treatment with isoproterenol strongly increased HO-2 protein expression, with no changes in HO-2 mRNA detected using qRT-PCR analysis (Ding *et al.,* 2011). This present study has only investigated HO-1 and HO-2 gene and protein expression; however, it would be important and useful to also measure HO activity. One way to do this is via HO activity assays, which measure HO derived CO or bilirubin.

The mechanism by which CoPP induces HO-1 remains to be fully elucidated. Primarily, the Bonkovsky group has done work on this subject using primary chick embryo cells, initially accumulating evidence that CoPP does not induce HO-1 by creating an oxidative stress environment nor mimicking heme (as some metalloporphyrins do), but rather involves short lived inducible transcription factors (Cable *et al.,* 1997). AP-1 binding elements have been shown to be the mechanism behind sodium arsenite, cadmium and heat shock mediated HO-1 induction (Elbirt *et al.,* 1998); however, through site-directed mutagenesis Shan *et al.,* (2000) determined these binding elements were not involved in CoPP-mediated HO-1 induction. Shan *et al.,* (2006) provided evidence that post-transcriptional up-regulation of Nrf_2 protein, as well as repression of Bach1 played key roles in the induction of HO-1 by CoPP. Nrf_2 is part of the Cap'n'Collar basic leucine zipper (bZip) family of proteins and has previously been shown to be vital for the anti-oxidant response (Moi *et al.,* 1994; Raval and Lee, 2010). Under normal conditions, Nrf_2 is retained in the cytosol by Kelch-like ECH-associated protein 1 (Keap1; Itoh *et al.,* 1999). Bach1 is also a Cap'n'Collar basic leucine zipper protein (Oyake *et al.,* 1996) that occupies MARE sites on the HO-1 promoter, and thereby represses HO-1 gene transcription and aids in the maintenance of low levels of HO-1 protein during basal conditions (Ogawa *et al.,* 2001). During oxidative stress, Bach1 is released from MARE sites and Nrf_2 is released from Keap1, allowing for nuclear translocation and HO-1 gene activation (Sun *et al.,* 2004). Further implications of the importance of Nrf_2 and Bach1 in regulating HO-1 in the lung can be seen through the use of knockout studies (Reddy *et al.,* 2007; Kassovska-Bratinova *et al.,* 2009; Reddy *et*

al., 2009), but will not be discussed here. As stated previously (section 1.4), the regulation of the human HO-1 gene is very complex and although Nrf_2 is thought to be a major player, several other transcription factors have also been linked to HO-1 induction. Therefore, when the quantification of Nrf_2 protein via western blotting was unsuccessful in this study (not shown), I wished to investigate if the NF-κB p65 (RelA) subunit, which provides the gene regulatory function, as well as its phosphorylated form, were involved in mediating the CoPP mediated induction of HO-1. Following 30 min treatment with 10 μM CoPP, western blotting was performed using the nuclear protein fraction and p-NF-κB protein was significantly increased, whereas no significant changes were seen in NF-κB protein levels. NF-κB is typically found as heterodimers composed of p50 and p65 subunits that are kept in the cytosol by IκB. IκB kinase phosphylates IκB and induces its degradation, freeing p50/p65 to translocate to the nucleus and bind to DNA (Scher and Pillinger, 2009; Hayden and Ghosh, 2012). Surprisingly, NF-κB family members lack enzymatic activity. Instead, it is through the recruitment of several transcriptional co-regulators, such as histone acetyltransferases (HATs) and histone deacetylases (HDACs), that NF-κB regulates transcription. Inducible phosphorylation of NF-κB p65 subunit at Serine 276 was found to be essential for recruitment of specific HATs, CBP and p300 and is thought to promote specificity of its target gene (Dong *et al.,* 2008). Indeed, it has been hypothesized that because NF-κB is involved in such a wide range of transcriptional targets, more regulatory factors are necessary and simply looking at subunit status is insufficient, as the NF-κB family is much more complicated than previously thought (Hayden and Ghosh, 2012). Therefore, even though no changes were detected in NF-κB p65 protein in my study, the fact that an increase in the phosphorylated form was found suggests that NF-κB may be involved in the induction of HO-1 by CoPP. However, its involvement may require specific co-regulators that are only recruited by p-NF-κB protein. If more time were available, I would more thoroughly investigate Nrf_2, as well as Bach1, by trying new antibodies and optimization techniques.

The role of HO-1 induction has been widely accepted to be cytoprotective. Induction of HO-1 by CoPP did in fact protect HBE cells from oxidant-induced apoptosis (Figure 13A). As described earlier (section 1.4), HO-1 exerts its beneficial characteristics from the products of heme oxidation. The specific mechanisms by which these

molecules, especially biliverdin, bilirubin and CO, directly produce their anti-oxidant, anti-inflammatory and anti-apoptoic effects remain unclear. However, there is plenty of evidence that show that these mechanisms do exist. For example, Stocker *et al.* (1989) demonstrated that biliverdin and bilirubin have powerful anti-oxidant properties in many *in vitro* models. More recently, Baranano *et al.,* (2002) showed that the addition of 10 nM bilirubin to cultured HeLa cells protected the cells against 100 μM (10,000 fold excess) H_2O_2-induced cytotoxicity. This dramatic action was accounted for by the fact that bilirubin acts in a catalytic fashion, similar to the potent anti-oxidant glutathione, in which bilirubin is oxidized into biliverdin and then is rapidly reduced back to bilirubin by biliverdin reductase. To confirm the role of biliverdin reductase as key player in the redox cycle, Baranano *et al*. (2002) used small inference (si)RNA targeted to reduce the levels of biliverdin reductase, which resulted in increased levels of intracellular ROS and promoted apoptosis in HeLa cells. These findings suggest that it is indeed through redox cycling, with the aid of biliverdin reductase, in which biliverdin/bilirubin exert their affects. By using *in vivo* models, Tang *et al.,* (2007) were able to show evidence that in rat liver grafts, biliverdin may be more directly involved in mediating apoptosis than simply reducing oxidant levels, as exogenous biliverdin suppressed pro-inflammatory and pro-apoptotic tumor necrosis factor-α (TNF-α) expression, down-regulated pro-apoptotic molecules (cytochrome C and caspase-3) and inhibited the pro-apoptotic JNK/AP-1 signaling pathway. Although these *in vivo* results are promising, these mechanisms may be tissue, cell or stress dependent and require further investigation in the airways. Studies concerning the role of CO in the lungs have uncovered some mechanistic evidence for its reported anti-inflammatory and anti-apoptotic properties. Mice treated with CO during hyperoxia treatment exhibited increased survival, reduced pro-inflammatory cytokines including TNF-α, IL-1β and IL-6, as well as decreased inflammatory cell recruitment. These protective effects were determined to be mediated by the MKK3/p38 MAP kinase pathway (Otterbein *et al.,* 1999; Otterbein *et al.,* 2003).

4.2 HO-1 Induction had no Affect on COX-2 or mPGES-1 mRNA Expression

It has been reported in osteoarthritic chondrocytes that HO-1 may exert its beneficial affects during inflammation by down-regulating COX-2 and mPGES-1 and

reducing subsequent PGE_2 production (Megías *et al.,* 2009). Therefore, I wished to investigate whether induction of HO-1 and potentially HO-2 via the known HO-1 inducer CoPP, would lead to a similar down-regulation of both COX-2 and mPGES-1. CoPP treatment did induce HO-1 mRNA and protein (see section 4.1); however, this induction did not lead to a down-regulation of COX-2 or mPGES-1 mRNA as originally hypothesized. Unfortunately, this mRNA expression result was not confirmed with protein analysis. Although the antibodies for COX-2 had been used successfully for publication (Megías *et al.,* 2009), in our hands, the quality of the western blots was insufficient for quantification (Figure 6D). The distinction between Megías *et al.,* (2009) and the results presented here could be due to various reasons. Firstly, the model used by Megías *et al.,* (2009) was primary chondrocytes from human cartilage specimens, whereas this study used a cell culture line of human bronchial epithelial cells. It is well documented that prostaglandins, especially PGE_2, are extremely versatile in their actions and their action is dependant on tissue and cell type (Sugimoto and Narumiya, 2007). It has also been suggested that PGE_2 may exert differential functions depending on the inflammatory status (Scher and Pillinger, 2009). This raises an issue when comparing these two studies, as Megías *et al.,* (2009) performed all experiments after the induction of inflammation by treatment with IL-1β, which strongly increased PGE_2 synthesis. Therefore, it is possible that in order to detect a down-regulation of the PGE_2 synthases COX-2 and mPGES-1, the system must first be induced by inflammatory mediators. Unlike other PGE_2 synthases COX-1, cytosolic (c)PGES-1 and mPGES-2 that are thought be expressed constitutively and promote basal PGE_2 synthesis, COX-2 and mPGES-1 expression is coupled and involved in PGE_2 synthesis during inflammation as they are both reportedly inducible by cytokines and growth factors and share common signaling pathways (Park et al., 2006; Samuelsson *et al.,* 2007). Therefore, because it is plausible that HO-1 has a regulatory role in PGE_2 synthesis during inflammation as biliverdin, bilirubin and CO have anti-inflammatory properties (Ryter *et al.*, 2006) and inflammation is a serious concern in CF lung disease, it would be useful to continue to investigate this research question, for example by stimulating inflammation using known COX-2 and mPGES-1 inducers IL-1β (Jakobsson *et al.,* 1999) or the bacterial endotoxin lipopolysaccharides (LPS; Murakami *et al.,* 2000).

4.3 Activation of the PGE_2 EP_2 or EP_4 Receptors had no Affect on HO-1 Expression

Recently, it was demonstrated that PGE_2 can protect cells against oxidant-induced apoptosis via upregulation of HO-1, mediated by the EP_2 receptor (Park *et al.,* 2009). Our laboratory has established a body of evidence to suggest that the EP_4 receptor plays an important role in mediating the effects of oxidant stress in the airways (Jones *et al.,* 2012; section 1.6). However, both qRT-PCR and western blotting analysis showed that neither EP_2 nor EP_4 activation resulted in an increase in HO-1 or HO-2 mRNA (Figure 10) or protein (Figure 11) expression. Again, differences in cell model arise as Park *et al.,* (2009) used rat C6 brain cells, whereas this study used a cell culture line of human bronchial epithelial cells. Furthermore, differences in experimental design are evident as Park *et al.,* (2009) based their experiments on induction of HO-1 via PGE_2 itself, rather than pharmacological activation of EP receptors, then used inhibitors of the EP_2 receptor as well as inhibitors of specific signaling pathways in order to delineate their findings. In order to fully understand if a relationship exists between PGE_2, its receptors and HO-1, HBE cells must be treated with PGE_2 itself followed by qRT-PCR and western blotting analysis to investigate the expression of HO-1, as well as PGE_2 receptors and synthases.

4.4 Cell Death

There are multiple ways that a cell can die and cell death is often a heterogeneous pool of necrosis, apoptosis and the more recently described necroptosis (Degterev *et al.,* 2005; Teng *et al.,* 2005). Necrosis results from a fast, accidental or non-physiological chemical or physical trauma that is characterized by a rapid depletion of cellular ATP, cell swelling that leads to disruption of the cell membrane and the release of the cellular contents into the external environment. Within the body, necrosis can promote local inflammation and further damage to surrounding tissue (Chandra *et al.,* 2000; Henriquez *et al.,* 2008; Morse *et al.,* 2009). Apoptosis is distinct from necrosis as it is a complex, tightly regulated and controlled process that is highly conserved, as along with cell proliferation, it is important in maintaining cellular homeostasis. Along with its critical function during pathological conditions, apoptosis is also essential during growth and development, immunity, aging and the removal of damaged/infected cells (Chandra *et al.,* 2000; Moffitt *et al.,* 2010). Morphological features of apoptosis include cell shrinkage,

chromatin condensation, DNA fragmentation, membrane blebbing and the formation of apoptotic bodies. In order for apoptosis to be induced, a host of molecular players including the B-cell lymphoma 2 (Bcl-2) protein family and the cysteine proteases of the caspase family must take part in various pro-apoptotic signaling cascades (Henriquez *et al.,* 2008). Several mechanisms for oxidant-induced apoptosis have been proposed. In one, H_2O_2 causes disruption of the mitochondrial membrane and the release of the pro-apoptotic molecule cytochrome c. Once cytochrome c is in the cytosol, it initiates the assembly of the apoptosome complex, which initiates the caspase cascade by activating caspase-9 (Bauer *et al.,* 1994; Stridh *et al.,* 1998). Alternatively, H_2O_2-induction of apoptosis may (Cabaner *et al.,* 1999) or may not (Hug *et al.,* 1994; Dumont *et al.,* 1999) involve the up-regulation of the Fas/Fas L system. Finally, it is thought that H_2O_2 may be more indirectly involved in apoptosis signaling by modifying the expression or activation of pro-apoptotic transcription factors such as p53, NF-κB and AP-1 (Uberti *et al.,* 1999). There is evidence that caspase-3 activation is essential for H_2O_2-induced apoptosis (Matsura *et al.,* 1999), which could be a result of any of the mechanisms described above.

Many studies implicate the induction of apoptosis at low concentrations of ROS, such as H_2O_2, and necrosis at higher concentrations (Gardner *et al.,* 1997; Hampton and Orrenius, 1997; Davies, 1999; Houot *et al.,* 2001; Miyoshi *et al.,* 2006). Therefore, I wished to measure both apoptosis via caspase-3 assays and total cell death (necrosis and end-stage apoptosis) via LDH assays following treatment with between 0-1000 μM H_2O_2 for short, moderate and long time periods (3, 6 and 24 hrs). After 3 hrs cell death was increased with 1000 μM H_2O_2. Following 6 hrs, apoptosis was increased at 400 μM H_2O_2, while cell death was increased with 1000 μM H_2O_2. Lastly, after 24 hrs, both apoptosis and cell death was increased with 800 and 1000 μM H_2O_2. As discussed in Cowley and Linsdell (2002) and Jones *et al.,* (2012), the estimates of potential H_2O_2 generation by neutrophils (Test and Weiss, 1984), along with the neutrophil counts from bronchoalveolar lavage fluid from CF patients (Konstan *et al.,* 1994), deems it likely that localized accumulations of activated neutrophils around infection sites could generate H_2O_2 concentrations equal to those I have found induces apoptosis (400 μM) and are therefore physiologically relevant.

Although the exact mechanism by which H_2O_2 induces apoptosis is not fully understood, I believe that using H_2O_2 as the oxidant stressor for this study was a good choice. Not only is it economical, H_2O_2 has great physiological relevance, as it is a tool commonly used by the immune system. The use of only one oxidant could be limiting, as there are several other oxidants that could be used (i.e. *tert*-Butyl hydroperoxide); however, the fact that H_2O_2 itself has been heavily studied and is the most commonly cited oxidant used throughout the literature with investigations of oxidative stress provides further reassurance.

The family of caspase proteases plays a central role in both the extrinsic and intrinsic pathways of apoptosis. The caspase cascade involves the activation of initiator caspases (i.e. caspase-8/9/10), followed by proteolytic activation of effector caspases (caspase-3/7). As proteolysis is irreversible, caspases are synthesized as precursors with little enzymatic activity. Activation of effector caspases leads to execution phase of apoptosis, in which the classic morphological changes begin (Movassagh and Foo, 2008). Therefore, using an assay that measures caspase-3 is a good choice for tracking changes in apoptosis, especially since all of the proposed mechanisms of H_2O_2-induced apoptosis eventually lead to caspase-3 activation. There are other techniques to measure caspase-3 activity other than an assay (i.e. western blotting, flow cytometry) and several other ways to assess apoptosis (i.e. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, DNA laddering agarose gel electrophoresis or cellular DNA fragmentation enzyme-linked immunosorbent assay (ELISA)). However, this assay technique seemed reasonably simple, required very little additional equipment (plate reader) and proved to be a good means of accumulating large amounts of data in a relatively short amount of time. The other techniques for assessing apoptosis range from assessing DNA fragmentation, detecting apoptosis with mitochondrial dyes (i.e. cytochrome c) to measuring changes in the cell membrane (change in phospholipid asymmetry). Therefore, although caspase-3 assays are a useful tool to assess apoptosis, it only looks at one molecule in the signaling pathway leading to apoptosis, also it is an end-stage protease that provides more confidence that the cells are undergoing apoptosis, techniques looking at morphological changes would be a good experiment to perform in parallel to the caspase-3 assay to ensure the results.

4.5 Prosaglandin E_2 and its Receptors

I investigated if PGE_2 treatment or activation of the EP_2 and/or EP_4 receptors could protect against H_2O_2-induced apoptosis. EP_2 receptor agonist butaprost pre-treatment had no effect on the H_2O_2-induced apoptosis (Figure 13B); whereas, both the EP_4 receptor agonist PGE_1-OH (Figure 13C) and PGE_2 (Figure 13D) pre-treatments resulted in a reduction in H_2O_2-induced. Several studies have also reported that PGE_2 can act as cell survival molecule, mediating via the EP_4 receptor, using various models including Jurkat human T-cells (George *et al.,* 2007) and glomerular epithelial cells (Aoudjit *et al.,* 2006). In order to determine if the EP_4 receptor mediated reduction in oxidant-induced apoptosis is a result of PGE_2 synthesis and its subsequent protective properties (Figure 13D), COX-1 and mPGES-1 inhibitors were used. Cells pre-treated with the EP_4 receptor agonist PGE_1-OH along with the selective COX-1 inhibitor SC-560 (Figure 14A) or the mPGES-1 inhibitor CAY10589 (Figure 14B) displayed no evidence that interruption of PGE_2 synthesis interfered with PGE_1-OH mediated protection against H_2O_2-induced apoptosis. The drug doses used for these experiments were based on previous work in our laboratory (Jones *et al.,* 2012); however, the experiments in Jones *et al.,* (2012) were looking at short-term changes and as a result may not be applicable for longer-term studies. As there is evidence that a positive feedback loop exists between PGE_2 and the EP_4 receptor (Li *et al.,* 2011; Regan, 2003), I would continue this investigation in order to be confident in my results. To do this, I would closely investigate a more appropriate dose or add the drug multiple times for the duration of the treatment period. Because PGE_2 exerts its effects through four different receptors, its actions are complex and not always clear. Therefore, investigation into receptor specificity using EP_2 and EP_4 receptor antagonists were used. Cells pre-treated with the EP_4 receptor antagonist AH23848 diminished the PGE_2 reduction in H_2O_2-induced apoptosis (Figure 15B); whereas treatment with the EP_2 receptor antagonist AH6809 had no effect (Figure 15A).

While the potential roles of PGE_2 in cell survival or apoptosis have not been well studied in the airways, other protective functions have been identified. PGE_2 has been positively implicated in the regulation of wound closure in the airway epithelium, inhibition of fibrosis, production/suppression of several pro-inflammatory cytokines and airway remodeling (Vancheri *et al.,* 2004). Airway epithelial cells are a major contributor

of PGE_2 synthesis, and it is thought that when airway epithelial cell damage occurs (i.e. CF, asthma, pulmonary fibrosis), the loss of a huge source of PGE_2 diminishes the lungs ability to repair itself (Wilborn *et al.,* 1995). Increased understanding of receptor expression and function may provide us with the potential to intervene in the treatment of lung diseases using selective PGE_2 receptor signaling pathways.

Although there are reports of both the EP_2 and EP_4 receptors having protective properties, in this study, despite both receptors being expressed in HBE cells (Figure 5B) it was only EP_4, not EP_2 receptor activation that resulted in protection against oxidant-induced apoptosis. The primary functions of the EP_2 receptor include facilitating ovulation and fertilization in female reproductive organs (Hizaki *et al.,* 1999). In the lungs it is important in the relaxation of bronchial smooth muscle and therefore, has been a major therapeutic target for the treatment of asthma (Regan *et al.,* 1994; Sugimoto and Narumiya, 2007; Roca-Ferrer *et al.,* 2011). The EP_2 receptor is coupled to the stimulatory G-protein, G_s (Figure 4; Regan, 2003; Sugimoto and Narumiya, 2007) and increases intracellular cAMP and protein kinase A (PKA) via activation of adenylate cyclase (AC; Regan, 2003). Along with involvement of EP_2 in C6 cell survival in the study by Park *et al.,* (2009). Sugiura *et al.,* (2007) showed PKA-dependent activation of the EP_2 resulted in protection against cigarette smoke extract-induced apoptosis in human lung fibroblasts. The current opinion on cAMP/PKA signaling on apoptosis is controversial (reviewed in Insel *et al.,* 2012), as some reports show anti-apoptotic (phosphylation of PI3K/Akt, Dynamin-related protein 1 or Epac (Exchange protein activated by cAMP)) mechanisms while others report it to be pro-apoptotic (dephosphylation of PI3K/Akt, intrinsic & mitochondria-dependent mechanisms, phosphorylation of protein targets). However, in my study, activation of the EP_2 receptor did not result in cytoprotection; therefore, the EP_2 receptor and cAMP/PKA signaling seems unlikely to be involved in this process in HBE cells.

Although the motivation for this study was initially related to the EP_2 receptor (Park *et al.,* 2009), in our laboratory, the EP_4 receptor has been a continued source of interest. The EP_4 receptor is strongly involved in mediating protection in inflammatory bowel diseases, including colitis. Severe susceptibility to the dextran sodium sulfate experimental model of colitis (Elson *et al.,* 1995) in the absence of EP_4 signaling (either

using $EP_4^{-/-}$ mice or use of an EP_4-selective antagonist) showed impaired mucosal barrier function, induced epithelial cell loss and accumulation of inflammatory cells (Kabashima *et al.,* 2002). These findings show the importance of EP_4 signaling in the maintenance of homeostasis in a mucosal-dependant organ and have lead the research to the exciting advancement of clinical trials (Nakase *et al.,* 2010). The EP_4 receptor similarly couples to G_s, and leads to the same increase in cAMP seen after activation of the EP_2 receptor (Figure 4; Regan, 2003; Sugimoto and Narumiya, 2007). Since both receptors share this signaling pathway, there are some functions that are shared. For example, activation of both EP_2 and EP_4 via PGE_2 induce receptor activator of NF-κB ligand (RANKL) through cAMP in osteoclastogenesis (Li *et al.,* 2000; Ono *et al.,* 2003). However, there are several instances in which EP_2 and EP_4 serve distinct roles. This can arise because activation of the EP_4 receptor can also cause an increase in phosphatidylinositol 3-kinase (PI3K) activity. In fact, there is strong evidence that the EP_4 receptor signals through the PI3K pathway to a greater extent than through activation of AC to increase cAMP (Honda *et al.,* 1993; Regan *et al.,* 1994; Fujino *et al.,* 2003). PI3K signaling can lead to activation of a variety of downstream signaling molecules involved in cell survival including protein kinase B (PKB; also known as Akt) or extracellular signal-related kinases (ERK1/2, a MAPK). It has also been reported that EP_4 can activate PI3K through an inhibitory G-protein (Fujino and Regan, 2006).

Activation of Akt kinase can lead to phosphorylation of pro-apoptotic Bcl-2-associated death promoter (BAD), among others. Following dephosphorylation by an apoptotic stimulus, BAD initiates apoptosis by inactivating anti-apoptotic Bcl-2 family proteins (Bcl-2, Mcl-2 and Bcl-XL) leading to the release of cytochrome c and activation of the caspase cascade (reviewed in Hers *et al.,* 2011). Therefore, although these signaling pathways have not yet examined in HBE cells, work has been done investigating the involvement of the EP_4 in these pathways in another human epithelial cell line, Calu-3. Joy and Cowley (2008) determined that the isoprostane 8-iso-PGE_2 stimulated CFTR by activation of the EP_4 receptor via PI3K-mediated events. Also, Li *et al.,* (2011) detected increases in the phosphylation status of downstream signaling effectors including ERK1/2, following activation of the EP_4 receptor. Activation of the ERK1/2 MAPK signaling pathway can similarly lead to the phosphorylation of BAD,

however ERK1/2 can also phosphorylate and consequently suppress pro-apoptotic proteins such as Bim and transcription factors such as FOX03a (Forkhead family), which leads to the down-regulation of pro-apoptotic genes such as FasL (reviewed in Mebratu and Tesfaigzi, 2009). Although initially observed by Fujino and Regan (2006), (using Calu-3 cells) Li *et al.,* (2011) replicated the finding that through activation of the EP_4 receptor and subsequent ERK1/2 activation, induction of the transcription factor early growth response factor-1 (Egr-1) occurs in Calu-3 cells. Egr-1 is implicated in mediating the expression of a number of genes involved in inflammation, cell growth and apoptosis (Li *et al.,* 2011), including PGE_2 synthases (Regan, 2003). Li *et al.* (2011) found increased levels of PGE_2 following EP_4 activation, providing evidence of a positive feedback loop via Egr-1 upreguation of PGE_2 synthases. As a consequence of these interesting results, I believe that these mechanisms provide us with a good working hypothesis (Figure 17) that must be investigated in HBE cells in order to elucidate the signaling pathway that links PGE_2 and EP_4 receptor activation to cell survival.

This study made use of many pharmacological tools, including inhibitors and receptor agonists and antagonists; therefore, the issue of specificity must be discussed. The EP_2 receptor agonist butaprost has become of the most frequently used tools to study EP_2 function (i.e. Sugiura *et al.,* 2006; George *et al.,* 2007; Chun and Langenbach, 2011). Butaprost has approximately $1/10^{th}$ the affinity to the EP_2 receptor compared to PGE_2, with great selectivity, as it does not bind to any other prostanoid receptors (Regan *et al.,* 1994; Kiriyama *et al*., 1997). PGE_1-OH is not as specific, as it binds to both the EP_3 and EP_4 receptors; however, it remains a common method of EP_4 receptor activation (i.e. George *et al.,* 2007; Li *et al.,* 2011) as PGE_1-OH binds more readily to EP_4 than EP_3 (Kiriyama *et al.,* 1997). The EP_2 receptor antagonist AH6809 is not selective as it binds to the EP_1, EP_2, EP_3-III and DP_1 receptors, however it has the highest affinity for the EP_2 receptor and has been shown to block the PGE_2-induced accumulation of cAMP that is elicited by the EP_2 receptor (Woodward *et al.,* 1995; Kiriyama *et al.,* 1997). The EP_4 receptor antagonist AH23848 is more selective that AH6809 as it only binds the EP_4 (Coleman et al., 1994) and TP (Brittain *et al.,* 1985) receptors and also suppresses cAMP generation (Sanchez and Moreno, 2002). The COX-1 inhibitor SC-560 is reported to be extremely selective (Mardini and FitzGerald, 2001; Jawabrah Al-Hourani, 2011) and

while the mPGES-1 inhibitor also inhibits 5-Lipoxygenase (5-LO; initiates the synthesis of leukotrienes), it has been shown to effectively inhibit PGE_2 synthesis and has minor effects on COX inhibition (Koeberle *et al.,* 2008). Therefore, although these pharmacological tools are extremely useful, because they are not all specific the results may come from a combination of inhibitory targets.

4.6 Lipid Peroxidation

Lipid peroxidation is one of the main degenerative processes resulting from oxidative stress (section 1.3). Products of lipid peroxidation have been found in several bodily fluid and tissue samples and their elevated levels commonly correlate to pathological conditions (Higdon *et al.,* 2012). As previously discussed, lipids such as AA can be oxidized by enzymes such as COX to produce powerful signaling molecules such as PGE_2 (Sugimoto and Narumiya, 2007); however, non-enzymatic oxidation of lipids yields a broad range of products whose actions are beginning to grow in appreciation (Higdon *et al.,* 2012). Polyunsaturated fatty acids (PUFAs) are a main target of lipid peroxidation (although cholesterol and lipoproteins have gained interest), due to the presence of unsaturated double bonds that contain hydrogen atoms that are more readily available for capture by ROS such as $^{\bullet}OH$ to create lipid radicals. This initiation step is followed by propagation as lipid radicals react with available oxygen and other PUFAs to create lipid hydroperoxides and new lipid radicals. In order for the lipid peroxidation reaction to terminate, addition of two radicals (lipid-lipid or lipid-protein) must occur (Sevanian and Hochstein, 1985; Halliwell and Chirico, 1993; Higdon *et al.,* 2012). The purpose of measuring lipid peroxidation in this study was to assess oxidative damage; however, these experiments were preliminary as our laboratory and several others are also interested in the fact that products of non-enzymatic lipid peroxidation (i.e. isoprostanes, isolevuglandins, isothromboxanes and isoleukotrienes; Janssen, 2001) are not simply markers of oxidative stress, but rather are mediators of cellular activity (reviewed in Crankshaw and Rangachari, 2003; Higdon *et al.,* 2012) and provide an exciting area of research.

I wished to investigate whether the protective properties of HO-1 induction (CoPP), as well as PGE_2 and EP_4 receptor activation included reducing oxidative damage. Using a

lipid hydroperoxide assay as a means to asses lipid peroxidation and oxidizing damage, I first determined that treating HBE cells with 400 μM H_2O_2 for 90 min increased lipid peroxidation (Figure 16A). No drug pre-treatment reduced the lipid peroxidation, when compared to the H_2O_2 treated controls (Figure 16A-D) and butaprost (Figure 16C) alone also increased lipid peroxidation, suggesting this drug cause some degree of oxidative damage on its own. These results were somewhat unexpected, HO-1 induction (CoPP) has been shown in several studies to exert potent anti-oxidant properties (reviewed in Haines *et al.,* 2012), especially through the actions of biliverdin and bilirubin (section 4.1). Also, Cable *et al.,* (1997) determined that CoPP does not induce HO-1 by altering the oxidative status; therefore, CoPP itself increasing lipid peroxidation was unanticipated. My initial hypothesis was that activation of the PGE_2 EP_2 or EP_4 receptors would lead to induction of HO-1, and that this would provide a mechanism for potential protection against apoptosis and oxidative damage; however, investigating this potential remained important even after this hypothesis was rejected. Modest evidence existed that PGE_2 and its EP_2 and EP_4 receptors play a role in protecting against oxidative damage, as Echeverria *et al.,* (2005) found that activation of both EP_2 and EP_4 receptors resulted in a decrease in amyloid b-peptide (a core component of neuritic plaques present in the brains of patients with Parkinson's disease), mediated production of ROS in postnatal mouse cortical neurons. Therefore, although this presented as an interesting hypothesis, the results from my study show that neither induction of HO-1 (CoPP), PGE_2, nor activation of the EP_2 or EP_4 receptors reduced H_2O_2-induced lipid peroxidation.

As described above, lipid peroxidation is a complex process, involving several steps and resulting in the production of multiple products. The assay used in this study used the measurement of lipid hydroperoxides, which are the products of the intermediate stage in lipid peroxidation, as a means to detect lipid peroxidation and oxidative damage. However, although this assay is useful, it has many limitations that must be taken into consideration. Firstly, the measurement of lipid hydroperoxides is only one way to assess lipid peroxidation. Although similar and more indirect, the TBARS (thiobarbituric acid reactive substances) assay is one of the best-established methods for the general screening of lipid peroxidation (Yagi, 1998; Halliwell and Chirico, 1993). This assay works by colorimetrically quantifying the formation of malondialdehydes (MDAs) that

come from the decomposition of lipid hydroperoxides. This assay was not an option for this study, as it requires a filter that is not available on our plate reader. Other common techniques range from more physiological in nature such as measuring biomarkers like isoprostanes, to more specific biochemical methods such as high-performance liquid chromatography (HPLC; Halliwell and Chirico, 1993). Although lipids are a large component of cells and therefore would be a large target for oxidation, in order to have a complete assessment of oxidative damage, oxidation of proteins and DNA must also be evaluated. Aldehyde site detection assays, which measure free aldehydes on DNA and proteins that can result from oxidative stress in cells (Nakamura *et al.,* 1998), may be useful to deal with this issue.

The use of the common experimental ROS H_2O_2 as an inducer of lipid peroxidation was of interest because of its physiological relevance. However, in order for the pre-treatments to show their potential anti-oxidant properties, a method to generate intracellular ROS such as copper *N*-(2-hydroxyacetophenone) glycinate (Mookerjee *et al.,* 2006) or hypoxanthine–xanthine oxidase (Aitken *et al.,*1993), instead of directly treating the cells with an ROS that could oxidize lipids in the plasma membrane (although H_2O_2 crosses cell membranes easily; Halliwell and Chirico, 1993) may have been a superior option. Also, it has been shown that, as H_2O_2 is a less potent oxidant, it reactivity is more limited to DNA damage (reviewed in Halliwell and Aruoma, 1991). Therefore, H_2O_2 must first be converted into $^{\bullet}OH$ or other highly reactive radicals in order to cause the initiation of lipid peroxidation (Halliwell and Chirico, 1993) and as a result we are likely not looking at the direct effects of H_2O_2-mediated damage.

4.7 The 16HBE14o- Cell Model

This study used the model human 16HBE14o- (HBE) airway epithelial cell line. HBE cells are widely used for studying normal airway epithelial physiology as they have many desirable characteristics for laboratory research; furthermore, HBE cells are commonly used as a wild type control to compare to similar CF cell lines (Weiser *et al.,* 2011; Wright *et al.,* 2011; Chen *et al.,* 2012), such as CFBE41o- cells (Ehrhardt *et al*, 2005). The HBE cell line was initially derived from normal bronchial epithelial cells and remain differentiated as HBE cells develop tight junctions and are able to become

polarized under appropriate culture conditions (Ehrhardt *et al*, 2002). HBE cells have been shown to possess both mRNA and functioning CFTR protein (Ehrhardt *et al*, 2002), which is essential when studying CFTR-mediated airway epithelial physiology. Cell lines are useful because they are easily maintained and allow for high reproducibility. However, although HBE cells have many characteristics of native human bronchial epithelial cells and are a reasonable model in place of primary cells or *in vivo* models for certain investigations, it is essential to remember that they are not primary cultures and therefore, do not fully represent true bronchial epithelial cells. Cell culture experiments are done in strictly controlled environments; this does not correspond to the dynamic conditions experienced by cells inside the body or through external environmental insults. Also, cell lines are derived from a single initial primary sample, and therefore, generalization of the results must be avoided. Therefore, thorough work must be done, using various models and techniques, to fully determine if the results are valid. If interesting findings arise, it would be valuable to compare them to other similar airway epithelial cell culture models such as NuLi-1 cells (Zabner *et al.,* 2003) or primary human airway epithelial (HAE) cells (Randell *et al.,* 2011), as well as cell lines derived from other epithelial cell types such as submucosal gland Calu-3 cells (reviewed in Zhu *et al.,* 2010). The aim of this study was to demonstrate the mechanisms present in wild-type airway epithelial cells, so that experiments can then be replicated in the CFBE41o- CF cell line, in order to show if these mechanisms are compromised by the CF phenotype as seen in previous work (Chen *et al.,* 2012). Ideally, it would be useful to compare HBE and eventually CFBE41o- results to primary cell cultures isolated from healthy subjects as well as patients suffering from CF, however, these resources were not available for this study. However, these preliminary studies promote a promising continuation of research to more complex *in vivo* models of research, which are ultimately required to fully understand what roles molecules such as HO-1 or PGE_2 play in the greater context of the normal airway epithelial physiology and pathogenesis and progression of CF.

4.8 Implications for Inflammatory Lung Diseases, Including Cystic Fibrosis

Although the primary cause of CF is a mutated CFTR gene, which results in absent or nonfunctioning membrane bound CFTR Cl^- channels, the fact that vital pulmonary

defense mechanisms are compromised is important in the pathogenesis and progression of CF. It is widely accepted that inefficient MCC causes the major symptoms of CF to arise, namely a build up of thick, viscous mucus and chronic bacterial infections with opportunistic pathogens such as *Pseudomonas aeruginosa*. Thus it is important to investigate the fundamental mechanisms that keep airway epithelial cells viable throughout stressful periods. Cytoprotective molecules such as HO-1 are thought to play an essential role in CF by lessening cellular damage caused by oxidant stress as a result of chronic bacterial infections (Zhou *et al*, 2004), reduced absorption of lipid soluble vitamins from the diet (Farrell *et al.*, 1977) as well as decreased glutathione transport via defective CFTR chloride channels (Linsdell and Hanrahan, 1998). Indeed, oxidative stress is a common feature in the progression and pathogenesis of all inflammatory lung diseases. The results of this study reveal that there is still much to learn about HO-1, as its induction via CoPP did protect HBE cells against oxidant-induced apoptosis, although it may not be related to prostanoid receptor function. A better understanding of how HO exerts its effects in airway epithelia could potentially lead to new therapeutic developments for CF as well as other inflammatory lung diseases.

It is clear that there is a relationship between PGE_2, its receptors and other arachidonic metabolites and CF, especially concerning oxidative stress. Several high impact papers have been published on the subject in the last decade, confirming continued interest and the hope for therapeutic intervention. Patients with CF show increased levels of PGE_2 (Strandvik *et al.,* 1996) and oxidant stress markers (Lucidi *et al.,* 2008). In Calu-3 cells, oxidant stress has been shown to acutely activate CFTR via isoprostanes and PGE_2, both mediated by the EP_4 receptor (Jones *et al.,* 2012); however oxidant stress has also been shown to suppress CFTR expression and function on a more long-term basis in the same cell line (Cantin *et al.,* 2006). The authors of these papers hypothesized that these processes are part of the innate host defense system as the acute stimulation of CFTR may provide sufficient efflux to get rid of the oxidant stressing the local cell population; however, long-term suppression of CFTR may aid in preserving intracellular glutathione. In fact, Ludwig *et al.,* (2010) have recently shown that the regulation of CFTR via PGE_2 is a complex gap junction-dependant processes involving CFTR, adenosine receptors (ADO-R), protease-activated receptors (PARs) and the PGE_2

EP_4 receptor. As these processes are CFTR-dependant and therefore, would be dysfunctional in CF disease, providing more evidence into why oxidative stress is a problem in the disease will help develop future therapeutic strategies. Rottner *et al.,* (2011) have delineated which classical anti-oxidant processes (section 1.3) are dysfunctional in CF cells, which included increased superoxide anion production, reduced expression of manganese (Mn)-SOD and Copper/Zinc (Cu/Zn)-SOD and reduced activity of extracellular (EC)-SOD. The authors also found that CF cells were more susceptible to pro-apoptotic agent-induced apoptosis, which was rescued by SOD treatment, thus indicating oxidative stress is a large contributor to apoptosis of CF cells. The hypothesis of a positive feedback loop between PGE_2, EP receptor activation and increased expression of PGE_2 synthases has been proposed to be CFTR and NF-κB dependent (Chen *et al.,* 2012). Indeed, Weber *et al.,* (2001) have also shown a strong relationship between CFTR and NF-κB activation. Therefore, although the results of this study have yet to be replicated in a CF cell line, based on the evidence presented, I believe that the anti-apoptotic affects of PGE_2 and the EP_4 receptor shown in this study may be compromised in the CF lung, providing further indication of the dysfunctional innate host defense mechanisms that plaque CF patients.

It is impossible to perform research involved in clarifying the mechanisms of mediating oxidant stress in inflammatory lung diseases such as CF without considering the simple solution of anti-oxidant therapy. As more knowledge is accumulated about techniques for measuring oxidative stress markers (i.e. 8-isoprostane) and the fact that they are becoming less invasive (i.e. exhaled breath condensate; Lucidi *et al.,* 2008), should lead us to the ability to correlate these markers to the patients' disease status and prescribe anti-oxidant therapy accordingly. However, although oxidative stress is thought of as a simple imbalance between oxidants and anti-oxidants, it appears to be much more complicated than previously thought. For example glutathione is heavily regulated by CFTR, not only by its transportation, but also by the expression of subtypes (Linsdell and Hanrahan, 1998; Cantin *et al.,* 2006). Indeed, depending on the sampling method different conclusions can be made concerning the severity of CF disease (Lucidi *et al.,* 2008), which needs to be more thoroughly investigated in order to understand these differences and combine of multiple sampling techniques. The realization of a poor

understanding of oxidative stress in CF makes the negative results from anti-oxidant therapy clinical trials (reviewed in Galli *et al.,* 2012) less surprising, albeit disappointing.

4.9 Conclusions

In conclusion, the main finding in this study is that application of the physiologically relevant oxidant stressor H_2O_2 results in induction of apoptosis and that pre-treatment with HO-1 inducer CoPP, PGE_2 and the EP_4 receptor agonist PGE_1-OH protects HBE cells against this insult. Because chronic activation of the EP_4 receptor leads to an increase in inflammatory mediators and PGE_2 synthesis (Li *et al.,* 2011), indications of a pro-survival pathway mediated by the same receptor in another airway epithelial cell line suggests that the EP_4 receptor is heavily involved in mediating inflammation and oxidant stress in the airways. Evidence shows activation of the EP_4 receptor promotes involvement of the immune system as well as attempts to keep cells viable to prevent serious damage at a tissue level (i.e. wounds). Therefore, although more work is required to fully comprehend the mechanism behind the potential protective properties of the EP_4 receptor, continuation of this work may lead to a significant therapeutic strategy to combat oxidative stress in the CF lung.

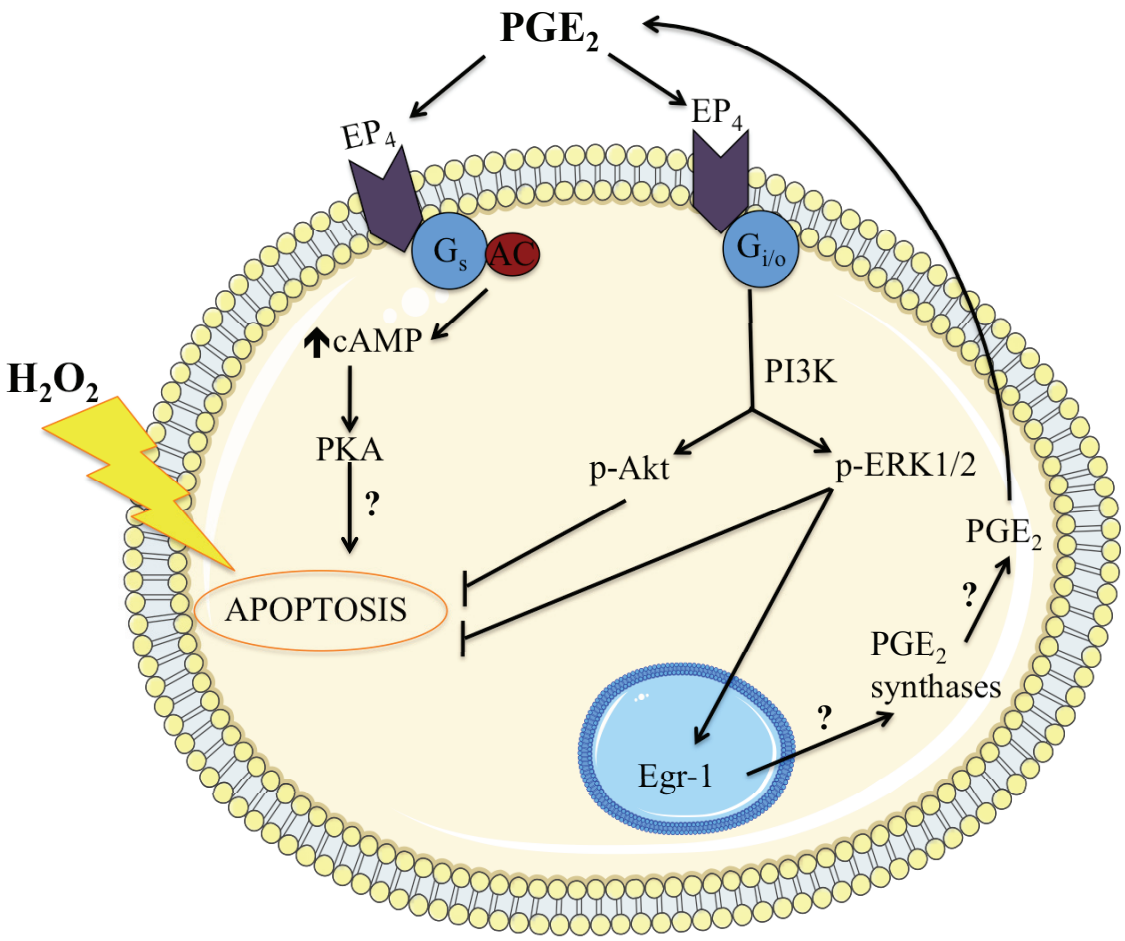

Figure 17. Proposed Mechanism of PGE_2 and EP_4 Receptor Protection Against Oxidant-Induced Apoptosis. Based on literature review, I have proposed a potential mechanism for the results found from this study and provide a working hypothesis for future experiments. PGE_2 binds to EP_4 receptors that are coupled to both G_s and $G_{i/o}$ G-proteins. Stimulation of G_s results in cAMP/PKA signaling via activation of adenylate cyclase. It remains unclear whether this signaling pathway is anti-apoptotic or pro-apoptotic. Stimulation of $G_{i/o}$ results in PI3K activation and consequential phosphorylation of the Akt and ERK1/2 cell survival-signaling pathways. ERK1/2 can also increase phosphylation of Egr-1 transcription factor, which has been shown to upregulate PGE_2 synthases, leading to the synthesis of further PGE_2, creating a positive feedback loop (section 4.5; Regan, 2003).

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APPENDIX A: TABLES

Table 1. Drug Information

Drug Name	Role	Vehicle	Stock [C]	Treatment [C]	Storage	Company
AH23848	EP_4 receptor antagonist	Ethanol	10 mM	10 μM	-20°C	Cayman Chemical
AH6809	EP_2 receptor antagonist	Ethanol	10 mM	10 μM	-20°C	Cayman Chemical
Butaprost	EP_2 receptor agonist	Methyl acetate	24.5 mM	1 – 20 μM	-20°C	Cayman Chemical
CAY10589	mPGES-1 inhibitor	Ethanol	10 mM	5 μM	-20°C	Cayman Chemical
Cobalt (III) Protoporphyrin IX chloride (CoPP)	HO-1 inducer	Ethanol	5 mM	1 – 20 μM	-20°C	Frontier Scientific
Hydrogen Peroxide (H_2O_2)	Oxidant	H_2O	30% (w/v)	50 uM – 1 mM	4°C	Sigma-Aldrich
Prostaglandin E_1 Alcohol (PGE_1-OH)	EP_2 receptor agonist	Ethanol	10 mM	1 – 20 μM	-20°C	Cayman Chemical
Prostaglandin E_2 (PGE_2)	Ligand	Ethanol	10 mM	1 μM	-20°C	Cayman Chemical
SC-560	COX-1 inhibitor	Ethanol	10 mM	1 μM	-20°C	Cayman Chemical

Table 2. PCR Primer Sequences and Information

Gene Name	Primer Sequence	Annealing Temp (°C)	Base Pair Size (bp)	Reference
HO-1	**Forward** 5'-CAGGCAGAGAATGCTGAGTTC-3' **Reverse** 5'-GCTTCACATAGCGCTGCA-3'	62	271	Megías *et al*., 2009
HO-2	**Forward** 5'- GTGGCCCAGCGAGCACTGAAACTC -3' **Reverse** 5'- AGGGAACCCATCCTCCAAGGTCTC-3'	60	254	Megías *et al*., 2009
COX-1	**Forward** 5'-TACTCACAGTGCGCTCCAAC-3' **Reverse** 5'-GCAACTGCTTCTTCCCTTTG-3'	60	168	Sobrino *et al.,* 2009
COX-2	**Forward** 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3' **Reverse** 5'-TTCTATGAGTCCGTCCGTCTCTACTAGA-3'	58	305	Picado *et al.,* 1999
mPGES-1	**Forward** 5'-GAAGAAGGCCTTTGCCAA-3' **Reverse** 5'-GGAAGACCAGGAAGTGCATC-3'	64	199	Megías *et al*., 2009
EP_2 receptor	**Forward** 5'-GCCACGATGCTCATGAAATCCGCC-3' **Reverse** 5'-CTTGTGTTCTTAATGAAATCCGAC-3'	58	655	Timoshe-nko *et al.,* 2003
EP_4 receptor	**Forward** 5'-CCTCCTGAGAAAGACAGTGCT-3' **Reverse** 5'-AAGACACTCTCTGAGTCCT-3'	58	366	Timoshe-nko *et al.,* 2003
HPRT	**Forward** 5'-GCCAGACTTTGTTGGATTG-3' **Reverse** 5'-CTCTCATCTTAGGCTTTGTATTTTG -3'	60	141	Roy *et al*., 2006
GAPDH	**Forward** 5'-GAAGGTGAAGGTCGGAGTC-3' **Reverse** 5'-GAAGATGGTGATGGGATTTC -3'	60	225	Lu and Kang, 2009

Table 3. Antibody Information

Antibody	Protein Name	Protein Size (kDa)	Host	Dilution	Incubation time	Company
Primary (1°)	HO-1	32	Mouse	1:2000	Over-night	Enzo Life Sciences, Inc.
	HO-2	36	Rabbit	1:30 000	Over-night	
	COX-1	70	Rabbit	1:500	Over-night	Cayman Chemical
	COX-2	72	Rabbit	1:1000	Over-night	
	mPGES-1	16	Rabbit	1:1000	Over-night	
	β-actin	45	Rabbit	1:2000	1 hr	Cell Signaling Technology
	GAPDH	36	Mouse	1:40 000	1 hr	Abcam, Inc.
	NF-κB	65	Rabbit	1:3000	Over-night	Cell Signaling Technology
	p-NF-κB	65	Rabbit	1:2000	Over-night	
Secondary (2°)	Anti-Mouse IgG	N/A	Goat	1:4000	2 hr	Cell Signaling Technology
	Anti-Rabbit IgG	N/A	Goat	1:4000	2 hr	

APPENDIX B: RECIPES

1.5% Agarose Gel
0.75 g agarose powder
50 ml 1XTBE
2.5 µl Ethidium Bromide
Dissolve agarose power in 1XTBE by microwaving for 2 minutes
Add Ethidium Bromide and pour into gel holder

Extraction Buffer
20 mM HEPES pH 7.9
1.5 mM $MgCl_2$
0.42 M NaCl
0.2 mM EDTA
25% (v/v) Glycerol
Add 1 uL DTT (0.1 M) and 1 uL protease inhibitor to 98 uL buffer before use

Fibronectin coating solution (100 ml)
97.8 ml LHC basal medium
0.2 ml BSA (1 mg/ml)
1 ml Vitrogen 100
1 ml Human fibronectin (1 mg/ml)

Hypotonic buffer (10X)
100 mM HEPES, pH 7.9
15 mM $MgCl_2$
100 mM KCl
Add 5 uL of 0.1 M DTT and 5 uL protease inhibitor to 1X before use

Phosphate buffered saline (10X, 1L)
8 g NaCl
0.2 g KCl
13.40 Na_2HPO_4
0.24 g KH_2PO_4
900 ml H_2O
Stir until completely dissolved; bring up to 1L with H_2O; pH to 7.4; filter to sterilize

Resolving gel (7.5%, 1 gel)
3.6 ml H_2O
1.9 ml 30% acrylamide mix
1.9 ml 1.5 M Tris (pH 8.8)
0.075 ml 10% SDS
0.075 ml 10% ammonium persulfate (APS)
0.006 ml TEMED
Add APS and TEMED immediately before pouring the gel

Resolving gel (12%, 1 gel)
3.3 ml H_2O
4 ml 30% acrylamide mix
2.5 ml 1.5 M Tris (pH 8.8)
0.1 ml 10% SDS
0.1 ml 10% ammonium persulfate (APS)
0.004 ml TEMED
Add APS and TEMED immediately before pouring the gel

RIPA buffer
4 mg Deoxycholic acid
5 ml Triton-X-100
5 ml SDS
4.38 g NaCl
12 ml 1mM EDTA
0.606 g Tris-HCl
Stir until completely dissolved; pH to 7.4

Running buffer (10X, 1L)
700 ml H_2O
144 g Glycine
30.3 g Tris base
10 g 1% SDS
Stir until completely dissolved; bring up to 1L with H_2O

Stacking gel (5%, 1 gel)
2.7 ml H_2O
0.67 ml 30% acrylamide mix
0.5 ml 1.0 M Tris (pH 6.8)
0.04 ml 10% SDS
0.04 ml 10% ammonium persulfate (APS)
0.004 ml TEMED
Add APS and TEMED immediately before pouring the gel

TBS (10X, 1L)
700 ml H_2O
292.2 g NaCl
24.22 g Tris base
Stir until completely dissolved; bring up to 1L with H_2O

TBS-T (1L)
100 ml 10X TBS
890 ml H_2O
10 ml 10% Tween

Transfer buffer (10X, 1L)
700 ml H_2O
144 g Glycine
30.3 g Tris base
1.0 g 1% SDS
Stir until completely dissolved; bring up to 1L with H_2O

Tris boric acid EDTA (TBE) (10X, 1L)
108 g Tris base
55 g Boric acid
40 ml 0.5M EDTA
800 ml H_2O
Stir until completely dissolved; bring up to 1L with H_2O