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Epithelial mechanisms in airway responses induced by hyperosmolarity

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Epithelial Mechanisms in Airway Responses Induced by Hyperosmolarity

Yi Jing

Dissertation submitted to the School of Medicine
at West Virginia University
in partial fulfillment of the requirements
for the Degree of

Doctor of Philosophy
in Pharmacology and Toxicology

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2007

Keywords: airway epithelium, airway smooth muscle, cell volume, epithelium-derived relaxing factor, exercise-induced asthma, guinea-pig trachea, hyperosmolarity, ion transport, kinase inhibitor, phosphorylation

Abstract

Epithelial Mechanisms in Airway Responses Induced by Hyperosmolarity

Yi Jing

There is airway dilation in normal people but obstruction in asthmatic patients during exercise; this has been thought to be initiated by hyperventilation causing evaporative water loss and hyperosmolarity of the airway surface liquid (ASL). In animal experiments the epithelium can detect osmolarity increases in the ASL and mediate relaxation of airway smooth muscle by releasing epithelium-derived relaxing factor (EpDRF), while in asthmatic patients this local regulatory function could be altered because of damage to epithelium. Challenged by hyperosmolarity, epithelial cells undergo shrinkage, volume-related ion transport, and release regulatory mediators, such as EpDRF. The changes in epithelial ion transport and EpDRF-mediated airway relaxation in response to hyperosmolarity have been found to be functionally linked in the guinea-pig isolated, perfused trachea. The two responses could be regulated through common stress-sensitive signaling pathways, and the epithelial shrinkage could be involved in the detecting hyperosmolar stress, leading to EpDRF release. We hypothesize that hyperosmolar challenge activates stress-sensitive protein kinases (PK)s, e.g. MAP kinases, in epithelium, and that the activity of PK(s) regulates epithelial bioelectric events and EpDRF release. The guinea-pig isolated, perfused trachea apparatus was used to investigate the effects of kinase and ion transport inhibitors on airway epithelial bioelectric and smooth muscle relaxation responses induced by hyperosmolarity. Western blots were used to examine the phosphorylation of p38 and JNK signaling proteins. Epithelial cell volume responses to hyperosmolarity were investigated. The results indicate that a physiologically-relevant increase in osmolarity elicits concentration-dependent epithelial hyperpolarization and smooth muscle relaxation, as well as epithelial shrinkage. The rapid and prolonged shrinkage is similar to the quick and long-lasting hyperpolarization and relaxation responses, suggesting a possible link between the epithelial shrinkage and EpDRF release. However, a common signaling pathway regulating both of the bioelectric and relaxation responses was not identified. Therefore, in terms of kinase signaling, there may not be a cause-effect relationship between the two responses. Inhibition of p38 potentiated the airway relaxation elicited by hyperosmolarity, suggesting that p38 inhibitors have the potential for the treatment of bronchoconstriction induced by exercise through the potentiation of EpDRF release.

Dedication

To my family,

My wife Lian Chang and son Ray Jing

(By giving me love and a warm family,

You made it more meaningful for me to pursue my dreams.)

My parents, Yuexian Jing, Mushao Yin and sister, Song Jing

(Your unconditional support means more to me than you will ever know.)

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List of Abbreviations

ACh	Acetylcholine
ASL	Airway surface liquid
CaM-K-II	Calmodulin kinase-II
D-M	D-mannitol
DMSO	Dimethyl sulphoxide
EC ₅₀	Concentration producing 50% of the maximal effect
ECL	Enhanced chemiluminescence
EIA	Exercise-induced asthma
EL	Extraluminal
EMEM	Minimum essential medium eagle
EpDRF	Epithelium-derived relaxing factor
ERK	Extracellular-regulated kinase
FBS	Fetal bovine serum
IL	Intraluminal
IL-8	Interleukin-8
I_{sc}	Transepithelial short-circuit current
JNK	c-Jun N-terminal kinase
MAP kinase	Mitogen-activated protein kinase
MCh	Acetyl- β -methylcholine chloride (methacholine)
MKH	Modified Krebs-Henseleit
MLCK	Myosin light chain kinase

mosM	Milliosmolar
PI-3-K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
RANTES	Regulated on Activation, Normal T-cell Expressed and Secreted
R_t	Transepithelial resistance
SE	Standard error
V_t	Transepithelial potential
ΔP	Inlet minus outlet perfusion pressure difference

General Introduction

Oxygen (O_2) is needed for metabolism in the body to produce energy, so a continuous supply of O_2 must be available for their cell functioning. Carbon dioxide (CO_2), as a by-product of this aerobic metabolism, must be removed from the vicinity of the metabolizing cells (Levitzky, 2003). The human respiratory system is developed to perform this gas exchange, and is composed of the thoracic wall, airways, lung and its blood circulation system. The primary function of the airways is to deliver O_2 to the lung gas-exchanging surfaces and exhaust CO_2 . Under normal circumstances the distribution of air and blood to the gas-exchanging surfaces is nicely matched, although they are regulated separately (Weibel, 1980). Any pathological condition, such as asthma, which causes narrowing or obstruction of the major airways will increase their resistance. Asthma is a respiratory disorder that is associated with reversible airway obstruction, inflammation, airway hyperresponsiveness and remodeling (McFadden, 1999). Over the past 20 years asthma has been diagnosed with increasing frequency and is now recognized as a major medical problem in the United States (Slavin and Reisman, 2002). It has been estimated that asthma costs at least 6 billion dollars per year for physician visits, medication and laboratory evaluation, and over 10.7 billion dollars of costs in days missed from work (Weiss et al., 2000).

Airway Structure and Physiology

The human airway is a tracheobronchial tree branching system (Fig. GI-1). Air

is inspired from nostrils, and sometimes from mouth when needed, passing through the pharynx, larynx, trachea, bronchi, bronchioles, and eventually reaches the gas-exchanging zone alveoli. From the trachea to the alveolar sacs human airway branches into 23 generations (Fig. GI-2). The first 16 generations are purely conductive; transitional airways lead into the respiratory zone made of alveoli (Weibel, 1963). Along with the diameter decrease from bronchus to alveolus the structure of airway wall is also shifted from thicker to thinner in three major patterns (Fig. GI-3); and the epithelial cells gradually reduce their thickness from pseudostratified to cuboidal and eventually to squamous at the gas-exchanging area. The airway wall contains cartilage only in bronchi and gradually becomes thinner as the alveolus is approached. The airways in the conducting zone have many elements, but basically there are three important structures: the epithelium, basement membrane and smooth muscle. The smooth-muscle layer disappears in the alveoli (Weibel and Burri, 1973).

In the conducting airways epithelia are composed primarily of two cell types, ciliated cells and goblet cells (Fig. GI-4), as well as some other types of cells interspersed in the epithelium, including undifferentiated or basal cells, brush cells, Clara cells, Kulchitsky cells and serous cells (Kuhn, 1976; Nadel, 1980; Weibel, 1980). Each type of cell has some specific functions, e.g., undifferentiated or basal cells serve as stem cells for the replacement of other types of cells; brush cells may have sensory function; and Clara cells are rich in cytochrome P-450 and many other metabolic enzymes (Kuhn, 1976; Weibel, 1980; Goldie and Hay,

1997). The ciliated cells line most of the surface of the airways; their chief characteristic is the presence of a rich tuft of cilia (Fig. GI-5). The synchronized beating of cilia propels the overlying mucous blanket towards the larynx and pharynx. The goblet cells are the secretory cells that together with the submucous glands synthesize polysaccharide-rich mucus in their Golgi apparatus and secrete it onto the epithelial surface (Kuhn, 1976).

Airway Surface Liquid (ASL) and Epithelial Ion Transport

ASL is the protective fluid on the apical side of airway epithelium. The ASL is elaborated in two layers; a high viscosity gel layer (mucus) lies on the top of a low viscosity solution (sol) hypophase in which an aqueous electrolyte solution is maintained (Weibel, 1980). The depth and molecular composition of the ASL is tightly regulated by epithelial ion transport (Widdicombe et al., 1997; Tarran, 2004). The osmolarity of the ASL is carefully maintained at an isotonic level or slightly higher than that of the interstitium, 285 mosM (Boucher, 1999). The average thickness of the sol hypophase is about 5-7 μm in depth, which is approximately the same length of the cilia extending. The gel layer is a discontinuous mucus blanket (Clarke and Boucher, 1993; Widdicombe and Widdicombe, 1995; Boucher, 1999). Epithelial cilia beat within the flexible hypophase with low viscous resistance while the sticky surface layer traps foreign microorganisms and particles. The movement of the hypophase can flow up the airways as a coherent sheet, which allows for the effective clearance of the foreign particles out of the airway system (Weibel, 1980).

The model of airway epithelial ion transport has been described (Boucher, 1994a, b). For most of the mammalian species, epithelial cells in the conducting airways actively extrude Na^+ through the Na^+-K^+ -pump located at the basolateral membrane, which creates the drive for the entry of Na^+ into the epithelium through Na^+ channels on the apical membrane (Boucher, 1999). The Na^+ extrusion makes intracellular environment more negative, which favors Cl^- to be secreted down its electric gradient to the apical surface through Cl^- channels. Cl^- secretion could also be caused secondarily by the activity of $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter located at the basolateral membrane (Willumsen et al., 1989). Because the tight junction structure in the paracellular pathway limits the free movement of ions by providing a transepithelial resistance (R_t), a transepithelial potential (V_t) could be maintained (Fig. GI-6).

Airway Epithelium: A Multifunctional Protective Barrier

The airway epithelium plays multifunctional roles in maintaining the stability of internal and external milieu of the airways (Munakata et al., 1989; Hamilton et al., 2001). Together with the intercellular tight junctions it forms a barrier to the entry of inhaled substance, microorganisms and particles (Gumbiner, 1987; Schneeberger and Lynch, 1992; Godfrey, 1997b). In addition to this primary function, airway epithelium is much more than a passive diffusion barrier (Folkerts and Nijkamp, 1998; Hamilton et al., 2001). It is a metabolic site processing both endogenous and exogenous substances (Goldie and Hay, 1997); it recognizes foreign particles (antigens) and secretes inflammatory mediators including

RANTES, IL-8, -16, prostaglandins and nitric oxide (Slavin and Reisman, 2002); it actively transports ions and fluid to maintain the ASL (Boucher, 1999; Widdicombe, 2002); and it serves as a sensor and transducer that detects osmolarity increases in the ASL and regulates underlying smooth muscle tone by releasing epithelium-derived relaxing factor (EpDRF) (Dortch-Carnes et al., 1999; Fedan et al., 1999).

The idea that respiratory epithelium could modulate the airway smooth muscle tone was rendered for the first time in 1985. Flavahan et al. (1985) reported that mechanical removal of the epithelium from canine bronchial rings increased the sensitivity of its underlying smooth muscle to airway contractile agonists and transmural nerve stimulation. In epithelium-intact preparations, the peak airway contraction elicited by a particular electrical frequency was followed by a decrease in tone as the stimulation continued; but with epithelium-denuded preparations the decrease in muscle tone was abolished. In epithelium-denuded preparations precontracted with acetylcholine (ACh) isoproterenol-induced relaxations were attenuated (Flavahan et al., 1985). Later, the increased smooth muscle sensitivity to airway contractile agonists, ACh, histamine, and 5-hydroxytryptamine on epithelium-removed bovine tracheal strips was observed (Barnes et al., 1985). In the following months and years, the fact that airway epithelium modulates the tone of its underlying smooth muscle was confirmed on preparations made from guinea-pig (Farmer et al., 1986; Hay et al., 1986; Murlas, 1986), human and rabbit (Raeburn et al., 1986a, b) airways. All these results

suggested the possibility that the increased sensitivity of airway smooth muscle to contractile agonists after removal of the epithelium was due to the loss of an epithelium-derived inhibitory factor, or EpDRF, which normally inhibits smooth muscle reactivity to contractile stimulation (Hay et al., 1987a, b).

EpDRF and the Perfused Trachea Apparatus

The first convincing evidence supporting the EpDRF hypothesis came with the development of the coaxial bioassay technique. In this system, a rat endothelium-denuded aorta strip (recipient tissue) was positioned coaxially within the lumen of an epithelium-intact guinea-pig trachea acting as a donor of EpDRF or a denuded trachea. The aorta strip was attached to a force transducer so that the tension of this tissue could be measured. The aorta strip was precontracted with an α_1 -adrenoceptor agonist, phenylephrine, then treated with ACh, which subsequently relaxed the aorta strip. The relaxation of the aorta strip was abolished when the assay was performed with preparations in which the epithelium was removed (Ilhan and Sahin, 1986). In the following studies similar results were reported when the guinea-pig tracheal smooth muscle and rat anococcygeal muscle were used as the recipient tissues (Güc et al., 1988a, b), and when the human bronchus was used as a donor tissue (Fernandes et al., 1990a). Further pharmacological characterization of EpDRF demonstrated that EpDRF was neither a product of nitric oxide synthase, cyclooxygenase, lipoxygenase and cytochrome P450; nor related to free radical production, soluble guanylate cyclase, β -adrenoceptors, platelet-activating factor and metabolites of

arachidonic acid (Barnes et al., 1985; Munakata et al., 1988, 1990; Goldie et al., 1990; Fernandes and Goldie, 1990b). Although great efforts have been made to understand the mechanism of EpDRF, the identity of EpDRF remains elusive. Because hemoglobin attenuates airway smooth muscle relaxation induced by hyperosmolarity through tightly binding with carbon monoxide (CO), CO may have a potential role in this epithelium-mediated airway relaxation (Fedan et al., 2004b).

In the late 1980s, the guinea-pig isolated, perfused trachea apparatus was developed. The advantage of this preparation is that the intraluminal (IL) and extraluminal (EL) sides of the trachea are completely separated. The contractile agonists can be applied to the EL side to get a direct access to the airway smooth muscle, while pharmacological agents added to the IL must diffuse across the epithelium before interacting with the underlying smooth muscle. Because the epithelial tight junction barrier is rather impermeable to many molecules (Fedan and Frazer, 1992), any responses observed of the smooth muscle will be affected by the epithelium. This development provided a useful apparatus for the studies of epithelium-mediated airway responses (Munakata et al., 1988). Studies performed on this preparation demonstrated that hyperosmolarity of the IL perfusing solution was a major stimulus for the release of EpDRF. Application of hyperosmolar solutions made with NaCl, KCl, urea, D-mannitol (D-M) and other solutes to the IL side of carbachol-contracted trachea elicited the smooth muscle relaxation; the relaxation response was attenuated if the epithelium was removed.

These results indicate that the relaxation induced by hyperosmolar solutions was epithelium-dependent (Munakata et al., 1988).

Exercise-induced Asthma (EIA): Two Theories

Exercise-induced asthma attacks have intrigued scientists for a long period of time. Over the last 20 years there have been considerable debates about the mechanism of exercise-induced airway narrowing in asthmatic patients (Anderson and Daviskas, 2000). The two major theories are the thermal hypothesis and the osmotic hypothesis. EIA was originally thought to be related to the consequences of heating and humidifying large volume of inspired air during exercise. The thermal hypothesis developed from the earlier airway cooling theory put forward in 1979 (Deal et al., 1979), and revised to the thermal theory in 1986 (McFadden et al., 1986). It was concluded that “the magnitude of EIA is directly proportional to the thermal load placed on the airways-respiratory heat exchange.” There is some evidence to support this hypothesis (McFadden, 1990, 1999). The thermal hypothesis proposes that the narrowing of the airways is a direct consequence of microvascular events; and does not embrace a role of airway epithelium and smooth muscle.

The osmotic hypothesis was developed and has been sustained since 1984 (Anderson, 1984) with airway epithelium involvement being added in 1989 (Anderson et al., 1989) and the submucosal involvement in signaling bronchial blood flow in 1992 (Anderson and Daviskas, 1992). It has been calculated that the volume of ASL is less than 1 ml in the first 10 generations of airways (Anderson,

1984). Mathematic models developed to estimate the water loss from airways suggest that only the first 12 generations of airways are needed to humidify the inspired air (Daviskas et al., 1990, 1991); about 7.39 mg/L water is calculated to be the net loss from airways during exercise under the author's experimental conditions. Although this value seems small, it is relatively large compared to the volume of fluid available in the first 12 generations. Therefore, the rate of water loss is great enough to cause a rapid osmolarity increase in ASL (Anderson et al., 1989, 1997b). Measuring the osmolarity of ASL in a canine model, Freed and Davis (1999) found a 130 mosM increase during hyperventilation of dry air, and a 40 mosM increase 4-6 min after the hyperventilation.

Asthma is a chronic inflammatory disorder of the airways. The pathogenesis of airway inflammation is characterized by inflammatory cell infiltration, airway smooth muscle proliferation and epithelial damage (Elias et al., 2003; Pelaia et al., 2005). As mentioned above, exercise-induced airway obstruction in the asthmatic patients is thought to be triggered by hyperventilation and increased osmolarity of the ASL (Hallstrand et al., 2005a; Anderson, 2006). Inhalation of hypertonic saline or D-M powder to elevate the osmolarity of the ASL in asthmatic patients activates mast cells and triggers the release of histamine and leukotriene (Taube et al., 2001; Brannan et al., 2003), leading to obstruction of the airways. Based on the osmotic theory, a method has been developed to diagnose the potential asthmatic individuals through the bronchoconstriction that results after the inhalation of D-M dry powder (Anderson et al., 1997a; Brannan et al., 1998).

Epithelium-mediated Airway Responses to Hyperosmolarity

Although the osmotic theory has been developed for years, the epithelial mechanism in the response of airway smooth muscle to hyperosmolarity is still poorly understood. Studies performed on the guinea-pig perfused trachea have demonstrated that airway epithelium can perceive an osmolarity increase in the ASL and elicit underlying smooth muscle relaxation by releasing EpDRF (Munakata et al., 1988; Fedan et al., 1990). The fact that the epithelium behaves as an osmometer and signaling transducer was also observed in human airways (Willumsen et al., 1994; Matsui et al., 2000). A more recent study presented evidence that the epithelium appears to detect the change in osmolarity rather than the osmolar concentration *per se* (Fedan et al., 2004a). The details about how the epithelium senses changes in osmolarity and initiates regulatory signaling are not clear. Previous studies in other cells suggested that cell volume change in response to hyperosmolarity could activate stress sensitive mitogen-activated protein (MAP) kinases (Roger et al., 1999; Duzgun et al., 2000; Zhang and Bourque, 2003).

Challenged by hyperosmolarity, airway epithelium reacts by releasing regulatory mediators. Studies *in vivo* demonstrated that hyperosmolar solution applied directly to the airway surface induced mucosal vasodilation mediated by epithelium-derived nitric oxide (Smith et al., 1993; Prazma et al., 1994). Experiments *in vitro* with guinea-pig isolated, perfused trachea demonstrated that hyperosmolar challenge elicited transepithelial depolarization; the V_t change was

followed by EpDRF-mediated airway relaxation (Dortch-Carnes et al., 1999). The epithelial bioelectric and smooth muscle mechanical responses to hyperosmolar challenge are thought to be functionally linked, because blockade of epithelial ion transport with Na^+ and Cl^- channel blockers attenuated the epithelium-mediated airway relaxation (Fedan et al., 1999). Their findings suggest that the bioelectric and mechanical responses are both epithelium-dependent, and the bioelectric response may be involved in the process of EpDRF release.

Hyperosmolar challenge causes cell shrinkage and volume-related ion transport (Lang et al., 1998). Studies with many types of cells demonstrated that hyperosmolarity or cell shrinkage activates MAP kinases (Shrode et al., 1998; Roger et al., 1999; Duzgun et al., 2000; Sheikh-Hamad and Gustin, 2004). The volume-related ion transport is regulated directly or indirectly by protein phosphorylation (Klein et al., 1993, 1999; Davis et al., 2001; Heinzinger et al., 2001; Liedtke and Cole, 2002a). The release of regulatory mediators in response to hyperosmolarity could be modulated by protein kinases (Hashimoto et al., 1999; Furuichi et al., 2002). Therefore, the hypothesis of the present study was hyperosmolar challenge activates protein kinases in epithelium, and the activity of protein kinases regulates epithelial bioelectric events and EpDRF release.

To test this hypothesis, specific kinase inhibitors were used to investigate possibly involved kinase pathways in epithelial signaling. Because the stress-sensitive MAP kinases play an important role in airway inflammation, and the MAP kinase inhibitors are being tested in the clinic for their therapeutic effects

to asthma (Pelaia et al., 2005), the involvement of the three MAP kinases, p38, c-Jun N-terminal kinase (JNK) and extracellular-regulated kinase (ERK), was examined using two structurally different inhibitors in each case (Fig. GI-7, Table GI-1). Moreover, the involvement of four other kinases, protein kinase C (PKC), phosphatidylinositol 3-kinase (PI-3-K), calmodulin kinase II (CaM-K-II) and myosin light chain kinase (MLCK) (Table GI-1), were examined, because they have been reported to be involved in the regulation of epithelial ion transport (Shrode et al., 1998). To get a better understanding for the role of these kinases, the phosphatase inhibitor, Na_3VO_4 , and the stress inducer, NaAsO_2 , were also examined (Table GI-1).

Fig. GI-1. Anatomy of human tracheobronchial tree and pulmonary subsegments. Trachea branches into bronchi and smaller airways of left and right lung lobes. Reproduced with permission from McGraw-Hill, Inc.; Pulmonary Diseases and Disorders by Alfred P. Fishman, Copyright 1980.

Conducting zone	Generation			Diameter, cm	Length, cm	Number	Total cross-sectional area, cm ²
	trachea		0	1.80	12.0	1	2.54
	bronchi		1	1.22	4.8	2	2.33
			2	0.83	1.9	4	2.13
			3	0.56	0.8	8	2.00
	bronchioles		4	0.45	1.3	16	2.48
		5	0.35	1.07	32	3.11	
	terminal bronchioles		16	0.06	0.17	6×10^4	180.0
Transitional and Respiratory zones	respiratory bronchioles		17	↓	↓	↓	↓
			18	↓	↓	↓	↓
			19	0.05	0.10	5×10^5	10^3
	alveolar ducts	T ₃	20	↓	↓	↓	↓
		T ₂	21	↓	↓	↓	↓
		T ₁	22	↓	↓	↓	↓
	alveolar sacs	T	23	0.04	0.05	8×10^6	10^4

Fig. GI-2. Schematic representation of airway branching in the human lung with approximate dimensions. The first 16 generations are purely conducting; transitional airways lead into the respiratory zone made of alveoli. (Figure after Weibel, 1963. Data from Bouhuys, 1977). Reproduced with permission from McGraw-Hill, Inc.; Pulmonary Physiology, Sixth Edition, by Michael G. Levitzky, Copyright 2003.

Fig. GI-3. Change of airway wall structure at the three principal levels. The epithelial layer gradually becomes reduced from pseudostratified to cuboidal and then to squamous, but retains its lining structure. The smooth-muscle layer disappears in the alveoli. The airway wall contains cartilage only in bronchi and gradually becomes thinner as the alveolus is approached. (Figure originally from Weibel and Burri, 1973). Reproduced with permission from McGraw-Hill, Inc.; Pulmonary Diseases and Disorders by Alfred P. Fishman, Copyright 1980.

A

B

Fig. GI-4. Ultrastructure of tracheal, bronchial and bronchiolar epithelium. (A) Trachea and large bronchi. (B) Bronchioles. There are distinct differences in epithelial morphology along proximal and distal airways. Reproduced with permission from Novartis Medical Education; The CIBA Collection of Medical Illustrations, Respiratory System, Volume 7, Illustrated by Frank H. Netter, Copyright 1997.

Fig. GI-5. Cilia of airway epithelial cells. Surface view of bronchiolar epithelium shows tufts of cilia forming on individual ciliated cells and microvilli on other cells. Note secretion droplet (arrow). Reproduced with permission from McGraw-Hill, Inc.; Pulmonary Diseases and Disorders by Alfred P. Fishman, Copyright 1980.

Fig. GI-6. Illustration of airway epithelial ion transport. The ion channels and transporters are asymmetrically distributed at the apical and basolateral surfaces. The specific inhibitors of ion channels and transporters are shown in parenthesis. The tight junction structures between epithelial cells form a resistant barrier to the movement of ions and help to maintain a transepithelial potential. The big arrows indicate general transcellular movement of Na^+ and the passive paracellular movement of Cl^- . Figure is illustrated by and provided as a courtesy of Jeffrey S. Fedan, Ph.D.

Fig. GI-7. MAP kinase signaling pathways and the examined inhibitors. EpDRF release was assumed to be regulated by stress-sensitive MAP kinases. The examined specific inhibitors SB 203580, SKF 86002, SP 600125, dicumarol, PD 98059 and U 0126 block either up- or down-stream of p38, JNK and ERK pathways, as indicated in the scheme.

Table GI-1. Properties of the kinase and phosphatase inhibitors and the stress inducer, NaAsO₂

Enzymes	Agents	EC ₅₀ (μM)	¹ Conc. (μM)	Mechanisms
p38	SB 203580	0.6 ^a	30	Competitive binding in the ATP pocket ^k
	SKF 86002	1 ^b	30	Competitive binding in the ATP pocket ^k
JNK	SP 600125	0.19 ^a	30	Competes with ATP to inhibit the enzyme ^a
	Dicumarol	5 ^c	15	Blocks stress signaling by the inhibition of quinone reductase ^c
ERK	PD 98059	2 ^a	50	Inhibits activation of ERK by blocking upstream enzyme MEK ^l
	U 0126	0.5 ^d	30	Inhibits activation of ERK by blocking upstream enzyme MEK ^l
PKC	Chelerythrine	0.66 ^e	20	Competitive binding to the phosphate acceptor of the enzyme ^e
PI-3-K	LY 294002	1.4 ^f	50	Blocks the ATP-binding site of the enzyme ^f
CaM-K-II	KN-62	1 ^g	20	Binds directly to the calmodulin binding site of the enzyme ^m
MLCK	ML-7	0.3 ^h	30	Inhibits the binding of ATP to MLCK ⁿ
Phosphatase	Na ₃ VO ₄	30-50 ⁱ	300	Analog to phosphate intermediates ^o
² SAPKs	NaAsO ₂	60 ^j	300	Stress inducer (activates stress-sensitive protein kinases) ^p

¹Concentrations commonly reported in literature were used in this study. ²Stress-activated protein kinases.

^aWoo et al. (2005). ^bPrabhakar et al. (1993). ^cCross et al. (1999). ^dHirst et al. (2002). ^eHerbert et al. (1990). ^fVlahos et al. (1994). ^gMasse and Kelly (1997). ^hGoggel and Uhlig (2005). ⁱPugazhenthii et al. (1996). ^jFlatman and Creanor (1999). ^kPelaia et al. (2005). ^lEnglish and Cobb (2002). ^mTokumitsu et al. (1990). ⁿShen et al. (2002). ^oGordon (1991). ^pLudwig et al. (1998).

Chapter I

Epithelial Kinase Signaling in Airway Bioelectric and Mechanical Responses Induced by Hyperosmolarity

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Abbreviations: ASL, airway surface liquid; EpDRF, epithelium-derived relaxing factor; V_t , transepithelial potential; ΔP , inlet minus outlet perfusion pressure difference; MKH, modified Krebs-Henseleit; MCh, methacholine; D-M, D-mannitol; IL, intraluminal; EL, extraluminal.

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Abstract

Hyperventilation during exercise causes evaporative water loss and hyperosmolarity of the airway surface liquid (ASL). Mucosal hyperosmolar challenge elicits airway transepithelial potential (V_t) changes followed by smooth muscle relaxation mediated by epithelium-derived relaxing factor (EpDRF). In guinea-pig isolated, perfused trachea we examined the relationship of V_t and relaxation responses induced by hyperosmolarity, osmolarity was increased by adding D-mannitol (D-M). Using intraluminally-applied kinase inhibitors, the kinase-interfering agent, NaAsO₂, and the phosphatase inhibitor, Na₃VO₄, we investigated epithelial signaling roles of mitogen-activated protein (MAP) kinases (p38, JNK and ERK) and four other kinases (PKC, PI-3-K, CaM-K-II and MLCK) in response to hyperosmolarity. Western blots were used to confirm the activation of two MAP kinases, p38 and JNK, which were implicated in functional studies. When the increased osmolarity was less than 80 mosM, D-M-induced hyperpolarization and relaxation responses shared the same concentration-dependence. Challenging the tracheal epithelia with 30 mosM D-M (~EC₅₀), the p38 inhibitors, SB 203580 and SKF 86002, potentiated the relaxation, while the JNK inhibitors, SP 600125 and dicumarol, the PKC inhibitor, chelerythrine, as well as NaAsO₂ and Na₃VO₄, inhibited the hyperpolarization. There was an osmolarity concentration-dependent activation of p38 and JNK in western blots. The PI-3-K inhibitor, LY 294002, and Na₃VO₄ inhibited methylcholine (MCh)-induced contraction, while SP 600125 and dicumarol

potentiated and chelerythrine inhibited MCh-induced hyperpolarization. The results indicate that JNK, PKC and phosphatase affect the hyperosmolarity-induced hyperpolarization; p38 plays a role in the regulation of EpDRF-mediated relaxation to hyperosmolarity. A common kinase signaling pathway coordinating both of the responses was not identified.

Introduction

The airway epithelium plays many important roles for the normal functioning of the pulmonary system (Munakata et al., 1989; Hamilton et al., 2001). In addition to its primary function as a protective barrier, it regulates the depth (normally 5-10 μm) and molecular composition of the airway surface liquid (ASL) (Widdicombe, 2002). Under normal circumstances, the osmolarity of the ASL is finely maintained at an isotonic level, or slightly higher than that of the interstitium, 285 mosM (Boucher, 1999; Tarran, 2004). Hyperventilation during exercise causes evaporative water loss and hyperosmolarity of the ASL (Anderson and Daviskas, 2000). Using a canine model, about 130 and 40 mosM increase in osmolarity were calculated during and shortly after hyperventilation of dry air, respectively (Freed and Davis, 1999). In non-asthmatic subjects, respiratory resistance declines due to bronchial dilation following exercise (Silverman et al., 2005); but in asthmatics, exercise causes airway obstruction (Anderson and Daviskas, 2000). Studies on guinea-pig perfused trachea demonstrated that airway epithelium could detect an increase in lumen osmolarity and elicit airway relaxation by releasing epithelium-derived relaxing factor (EpDRF) (Munakata et al., 1988; Fedan et al., 1990). This epithelium-dependent regulatory mechanism could participate in bronchodilation that occurs during exercise, a mechanism which may be defective in asthmatic patients.

Asthma is currently considered as a chronic inflammatory disorder of the airways characterized by airway hyperreactivity to a variety of stimuli (Hamilton et

al., 2001; Elias et al., 2003). Exercise-induced airway obstruction in asthmatic patients is thought to be triggered by the hyperventilation and increased osmolarity of the ASL (Hallstrand et al., 2005a; Anderson, 2006). The pathogenesis of airway inflammation involves inflammatory cell infiltration, epithelial damage, subepithelial fibrosis and airway smooth muscle hyperplasia (Elias et al., 2003; Pelaia et al., 2005). Because the alteration of airway epithelium is prominent in severe asthmatic patients, asthma has been also considered as an epithelial disease (Chanez, 2005). The epithelial damage would result in two important consequences: 1) Epithelial shedding would expose the underlying airway wall to a variety of contractile mediators and environmental agents (Brannan et al., 2003; Anderson and Kippelen, 2005; Anderson, 2006); 2) epithelial damage would alter epithelium-mediated inflammatory suppression (Holgate et al., 2003; Hallstrand et al., 2005b) and hyperosmolarity-induced airway relaxation through EpDRF (Fedan et al., 2004a). The precise mechanism of EpDRF-mediated airway relaxation is poorly understood. The objective of this study was to investigate the epithelial signaling mechanism(s) involved in hyperosmolarity-induced EpDRF release.

The airway epithelium behaves as an osmometer that sensitively perceives osmolarity changes in the ASL (Matsui et al., 2000; Fedan et al., 2004a), and regulates the tone of underlying smooth muscle and mucosal vasculature. Experiments *in vivo* showed epithelium-mediated vasodilation in response to mucosal hyperosmolarity (Smith et al., 1993; Prazma et al., 1994). Experiments *in*

vitro with guinea-pig perfused trachea and tracheal segment in the Ussing apparatus demonstrated that hyperosmolar challenge elicited depolarization and decreased transepithelial short-circuit current (I_{sc}) (Dortch-Carnes et al., 1999; Wu et al., 2004); the depolarization response preceded epithelium-dependent airway relaxation (Dortch-Carnes et al., 1999). The hyperosmolarity-induced bioelectric and mechanical responses also were thought to be functionally linked because blockade of epithelial ion transport with Na^+ and Cl^- channel blockers inhibited epithelium-mediated airway relaxation (Fedan et al., 1999).

Challenged by hyperosmolarity, airway epithelium undergoes shrinkage, volume-regulatory ion transport and releases mediators. Commonly involved signaling pathways underlying these adjustments in other types of cells are the three mitogen-activated protein (MAP) kinases, p38, c-Jun N-terminal kinase (JNK) and extracellular-regulated kinase (ERK) (Puddicombe and Davies, 2000; Sheikh-Hamad and Gustin, 2004). The induction of some epithelial-derived mediators in response to hyperosmolarity are regulated by stress-sensitive p38 and JNK (Hashimoto et al., 1999; Furuichi et al., 2002). Moreover, the activities of cell volume-related ion transport are regulated directly or indirectly by MAP kinases and other kinases, i.e., protein kinase C (PKC), calmodulin kinase-II (CaM-K-II), myosin light chain kinase (MLCK) and phosphatidylinositol 3-kinase (PI-3-K) (Shrode et al., 1998; Zhao et al., 2004).

The hypothesis of this study was hyperosmolar challenge activates signaling kinases in epithelia, and that the activities of kinases or phosphatases

regulate epithelial bioelectric events and EpDRF release. Our aims were to elucidate the relationship of airway bioelectric and mechanical responses and epithelial signaling mechanism in response to hyperosmolar challenge. The guinea-pig isolated, perfused trachea apparatus, which allows simultaneously measuring epithelial bioelectric and smooth muscle mechanical responses, was utilized to investigate the role of appropriate kinases using specific inhibitors. The involvement of MAP kinases implicated in asthma (Pelaia et al., 2005) was examined using two structurally different inhibitors in each case and further confirmed by immunoblotting. To get a better understanding for the involvement of these kinases, the stress inducer, NaAsO_2 , and the phosphatase inhibitor, Na_3VO_4 , were also examined.

Materials and Methods

Animals. These studies were conducted in facilities accredited fully by the Association for the Assessment and Accreditation of Laboratory Animal Care International and were approved by the institutional Animal Care and Use Committee. Male guinea pigs (550 - 700 g), HsdPoc:DH, from Harlan (Indianapolis, IN), monitored free of endogenous viral pathogens, parasites, and bacteria were used in all experiments. The animals were acclimated before use and were housed in filtered ventilated cages on Alpha-Dri virgin cellulose chips and hardwood Beta-chips as bedding, provided HEPA-filtered air, Teklad 7006 diet and tap water ad libitum, under controlled light cycle (12 h light) and temperature (22 - 25 °C) conditions. The animals were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and sacrificed by thoracotomy and bleeding before removing the trachea.

Materials. Modified Krebs-Henseleit (MKH) solution (pH 7.4 at 37 °C) contained 113 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, and 5.7 mM glucose, and was saturated with 95% O₂ and 5% CO₂. The osmolarity of MKH was 281 ± 5 mosM. Acetyl-β-methylcholine chloride (methacholine, MCh) and D-mannitol (D-M) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of MCh and D-M were prepared in saline.

Kinase inhibitors, SB 203580, SKF 86002, SP 600125, dicumarol, PD 98059, U 0126, chelerythrine, LY 294002, KN-62 and ML-7 were purchased from

Calbiochem-Novabiochem (La Jolla, CA) and dissolved in dimethyl sulphoxide (DMSO, final concentration in MKH solution was $\leq 0.04\%$). NaAsO_2 and Na_3VO_4 were obtained from Mallinckrodt Laboratory Chemicals (Phillipsburg, NJ) and Sigma-Aldrich (St. Louis, MO), respectively, and prepared in MKH solution. RIPA cell lysis buffer was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Laemmli protein sample buffer and 10% SDS-polyacrylamide ready gels were purchased from Bio-Rad Laboratories (Hercules, CA). Phosphorylated and nonphosphorylated p38, JNK and ERK control proteins and their primary antibodies, horseradish peroxidase (HRP)-linked anti-rabbit secondary antibody and β -actin antibody were purchased from Cell Signaling Technology (Beverly, MA). BCA protein assay reagents and western-blot stripping buffer were purchased from Pierce Biotechnology (Rockford, IL). Enhanced chemiluminescence (ECL) solution was obtained from GE Healthcare Life Sciences (Piscataway, NJ).

Trachea Perfusion Preparation. The guinea-pig isolated, perfused trachea preparation (Dortch-Carnes et al., 1999) was utilized to measure the airway epithelial bioelectric and muscle mechanical responses induced by the various agents used in this study. A 4.2-cm segment of guinea-pig trachea was removed and mounted at its natural length onto a custom-designed plastic perfusion holder which allows indwelling cannulas with side-opening holes to be inserted into the lumen from either end of the trachea. The trachea holder was placed into an extraluminal (EL) bath filled with MKH solution. The trachea lumen

was perfused at constant flow (24 ml/min) with MKH solution from another bath, referred to as the intraluminal (IL) bath; the transmural pressure was adjusted to zero. MKH solution in the IL and EL baths was kept at 37 °C and saturated with 95% O₂ and 5% CO₂ gas. Agents may be added separately to the IL or EL baths so that they can act directly on epithelia or airway smooth muscle, respectively. The inlet minus outlet perfusion pressure difference (ΔP) caused by tracheal diameter change was sampled through the side-holes in the indwelling cannulas, which were connected to a differential pressure transducer. Airway smooth muscle contraction decreases tracheal diameter and increases ΔP , whereas ΔP decreases during relaxation. Voltage electrodes (filled with 4% agar/saline) were connected across the IL and EL baths to record transepithelial potential (V_t). Thus, the trachea perfusion preparation allows simultaneously monitoring both V_t and ΔP in the same trachea.

Concentration-Response Curves for Hyperosmolar D-M-induced V_t and ΔP responses. The tracheal preparation was equilibrated by perfusing MKH solution for 2.5 – 3 h to allow stabilization of basal V_t and ΔP . To prepare the trachea for relaxation responses, the smooth muscle was precontracted by adding MCh (3×10^{-7} M, EC₅₀) to the EL bath. MCh elicited epithelial hyperpolarization. When the MCh-induced V_t and ΔP responses reached their plateaus, the osmolarity of the perfused solution was elevated cumulatively with stepwise addition of D-M to the IL bath. D-M was used to increase osmolarity of the MKH solution because it is an impermeant osmolyte. Each addition was made

after the V_t and ΔP responses to the previous concentrations reached their plateaus. After all the responses were concluded the preparation was washed with fresh MKH solution to allow V_t and ΔP recovering to their basal levels.

Effects of Kinase Inhibitors, NaAsO_2 , and Na_3VO_4 , in the Trachea Perfusion Preparation. To avoid variability between individual animals and different shipments, a paired design was used, i.e., control responses and the effects of inhibitors were obtained from the same preparation. After stabilization of basal V_t and ΔP , the control responses were obtained after incubating with vehicle (DMSO) or MKH for 30 min in the IL bath. MCh was then added to the EL bath. When the MCh-responses reached their plateaus, the osmolarity of IL perfused MKH solution was elevated by adding 30 mosM ($\sim\text{EC}_{50}$) D-M. After the D-M-induced V_t and ΔP responses became stable, the preparation was washed with fresh MKH solution to allow V_t and ΔP recover to their basal levels. Ninety min after the V_t and ΔP baselines were re-established, the trachea was incubated with a test agent in the IL bath for 30 min, after which the trachea was challenged again with MCh and D-M in the presence of the agent. The possible effects of DMSO on the two sets of responses to MCh and D-M were evaluated in a separate group of control preparations.

Western-Blot Analysis. The tracheas were challenged the same way as in the D-M concentration-response experiments except there was no exposure to MCh. The epithelia of control and test groups were obtained from the same trachea. Tracheas were removed and cut longitudinally into 3 strips. They were

randomly assigned into 3 groups: 1) controls incubated in MKH solution, 2) D-M-exposed (20, 40, 80, 160, 240 and 320 μ M), and 3) NaAsO₂-exposed (500 μ M) as a positive control. Each condition was investigated at least four times. After a 2-h period of equilibration the strips were exposed to MKH, D-M, or NaAsO₂ for 20 min. The exposure was stopped by transferring the strips into ice-cold MKH solution. At 0 °C the epithelia on the strips were scraped off with a scalpel blade, collected and centrifuged 600 x g for 3 min at 4 °C; the pellets were sonicated in 50 μ l RIPA lysis buffer to lyse the cells and shear DNA. The protein concentration of the samples was measured with the BCA protein assay.

The samples were loaded, 20 μ g/well, on a 10% SDS-polyacrylamide gel together with phosphorylated and nonphosphorylated p38 or JNK standards and subjected to electrophoresis. The proteins were then electrophoretically transferred to a nitrocellulose membrane. After blocking the nonspecific protein binding by incubating the membrane in phosphate-buffered saline (PBS) with 0.1% triton X-100 and 5% nonfat milk at room temperature for 1 h, the membrane was incubated with phosphorylated p38 or JNK primary antibodies at 4 °C overnight, followed by incubation with the HRP-linked anti-rabbit secondary antibody at room temperature for 1 h. After washing the membrane in PBS, the blots were incubated with ECL for 1 min and exposed to Kodak BioMax XAR film. In order to determine the total amount of p38 and JNK proteins on the blots, the membrane was stripped and re-probed with nonphosphorylated p38 or JNK antibodies. At the same time the accuracy of sample loading was also determined

using β -actin immunoblotting.

Data Analysis. The epithelial bioelectric responses were quantified as V_t in mV. MCh contractions were measured as ΔP elevation in cm H₂O. D-M-induced relaxations were normalized as the percent relaxation of the MCh response (% MCh). The EC₅₀ values for V_t and ΔP responses to D-M were derived from D-M concentration-response curves using least-squares analysis of four-parameter logit curve fit and are given along with 95% confidence intervals. The effects of the test agents on basal V_t and ΔP were also noted and quantified. The results are presented as means $\pm$ SE, and differences were analyzed statistically using Student's paired *t*-test. $p < 0.05$ was considered significant. The western-blot bands on the films were scanned with a General Electric Molecular Dynamics scanner and quantitated with image analysis program ImageQuant 5.2 from the intensity of their pixels. The effects of agents on the phosphorylated and total amounts of p38 and JNK were plotted as fold increases compared to their controls.

Results

Concentration-Response Relationships for Epithelial Bioelectric and Smooth Muscle Mechanical Responses Induced by Hyperosmolarity. To understand the relationship between epithelial bioelectric and muscle mechanical responses induced by hyperosmolarity, and to ascertain an appropriate D-M concentration to be used for testing the effects of kinase or phosphatase inhibitors, concentration-response curves for V_t and ΔP responses induced by D-M were obtained (Fig. CI-1). When the osmolarity increase was below 80 mosM, the bioelectric hyperpolarizing and relaxant responses shared the same concentration-dependence. When the osmolarity increase was higher than 80 mosM, the bioelectric response changed to depolarization, and the relaxation reached nearly the maximum response. The EC_{50} values obtained, 16.6 (13 - 21.2) and 24.4 (19.2 - 31.3) mosM for the hyperpolarization and relaxation responses, respectively, were relevant to the osmolarity increase in the ASL that accompanies exercise hyperventilation and were not significantly different. It was decided to use 30 mosM D-M in subsequent experiments. This concentration of D-M always produced reliable relaxation and hyperpolarization responses.

Effects of DMSO, Kinase Inhibitors, $NaAsO_2$, and Na_3VO_4 , on Basal V_t and ΔP . The average basal V_t in these experiments was -12.1 ± 0.6 mV ($n = 88$). In the concentration range, 0.02 - 0.2%, DMSO had no effect on basal ΔP but hyperpolarized V_t by 0.57 ± 0.09 mV when its concentration reached 0.1% ($n = 3$). At a concentration of 0.04%, DMSO had no effect on basal V_t or ΔP ; therefore,

this concentration was used in subsequent controls. Of the six MAP kinase inhibitors SB 203580, SKF 86002, SP 600125, dicumoral, PD 98059 and U 0126, only SB 203580 decreased basal V_t (by 0.8 ± 0.3 mV); the other five inhibitors had no effect. SB 203580, dicumarol and PD 98059 slightly decreased basal ΔP (cm H₂O) by 0.06 ± 0.02 , 0.46 ± 0.13 and 0.16 ± 0.04 , respectively; the other three inhibitors had no effect on ΔP (Table 1). The other kinase inhibitors, chelerythrine, LY 294002, KN-62 and ML-7 decreased basal V_t (mV) by 1.53 ± 0.29 , 3.92 ± 0.61 , 0.47 ± 0.14 and 0.60 ± 0.46 , respectively, while, Na₃VO₄ and NaAsO₂ increased basal V_t (mV) by 0.70 ± 0.22 and 1.69 ± 0.67 , respectively. Chelerythrine increased basal ΔP by 0.22 ± 0.06 cm H₂O; the other kinase-interfering agents had no effect on basal ΔP (Table 2).

Effects of DMSO, Kinase Inhibitors, NaAsO₂, and Na₃VO₄, on MCh-induced Contraction and Hyperpolarization Responses. EL applied MCh (3×10^{-7} M) caused contraction accompanied by transepithelial hyperpolarization. DMSO and all the examined MAP kinase inhibitors (SB 203580, SKF 86002, SP 600125, dicumarol, PD 98059 and U 0126) had no effect on MCh-induced contraction ($n = 6, 7, 6, 10, 6, 6$, and 6 , respectively; data not shown). LY 294002 and Na₃VO₄ inhibited contractile responses to MCh (Figs. CI-2 and CI-3); chelerythrine ($p = 0.052$) and NaAsO₂ ($p = 0.093$) had a considerable but not significant inhibitory effect; and KN-62 and ML-7 had no effect (Figs. CI-2 and CI-3).

SP 600125 and dicumarol potentiated MCh-induced hyperpolarization (Fig.

CI-4), while chelerythrine inhibited this response (Fig. CI-5). DMSO and the other kinase inhibitors (Figs. CI-4 and CI-5), as well as NaAsO₂ and Na₃VO₄ (*n* = 9 and 6, respectively; data not shown) had no effect.

Effects of DMSO, Kinase Inhibitors, NaAsO₂, and Na₃VO₄, on D-M-induced Hyperpolarization and Relaxation Responses. IL-applied D-M elicited transepithelial hyperpolarization and relaxation. SP 600125 and dicumarol (Fig. CI-6), chelerythrine (Fig. CI-7), NaAsO₂ and Na₃VO₄ (Fig. CI-8) inhibited D-M-induced hyperpolarization. DMSO and the other kinase inhibitors had no effect (Figs. CI-6 and CI-7). The inhibition by NaAsO₂ was complete (Fig. CI-8); this is the first time we have observed an agent nullifying the bioelectric response to hyperosmolar challenge.

SB 203580 and SKF 86002 potentiated D-M-induced relaxation (Fig. CI-9). DMSO and the other kinase inhibitors SP 600125, dicumarol, PD 98059 and U 0126 (Fig. CI-9); chelerythrine, LY 294002, KN-62 and ML-7 (Fig. CI-10); as well as NaAsO₂ and Na₃VO₄ (*n* = 9 and 6, respectively, data not shown), had no significant effect on the D-M-induced relaxation responses.

Epithelial p38 and JNK Phosphorylation Induced by Hyperosmolarity. Immunoblotting experiments with ERK were not performed because the above results indicated that ERK is not involved in the regulation of basal V_t or responses to MCh and D-M. Western-blot analysis with specific antibodies to phosphorylated and nonphosphorylated p38 and JNK showed that after exposing epithelia to D-M (20, 40, 80, 160, 240 and 320 mosM) for 20 min, the

phosphorylation of p38 (Figs. CI-11A and CI-11B) and JNK (Figs. CI-12A and CI-12B) was increased in an osmolarity concentration-dependent manner at the two and three highest concentrations, respectively, while the total amount of p38 and JNK proteins did not change significantly (Figs. CI-11B and CI-12B). At 320 mosM D-M, the phosphorylation of p38 and JNK was increased by about 4- and 6-fold, respectively (Figs. CI-11A and CI-12A). Interestingly, the positive control, NaAsO₂ (500 μ M), not only induced an increase in p38 phosphorylation but also reproducibly increased the total amount of p38 protein (Fig. CI-11B). NaAsO₂ caused an increase in JNK phosphorylation, but the total amount of JNK protein was not changed (Fig. CI-12B).

Discussion

Hyperventilation during exercise causes evaporative water loss and hyperosmolarity of the ASL (Anderson and Daviskas, 2000). The airway epithelium regulates the tone of the underlying smooth muscle by releasing EpDRF in response to hyperosmolar challenge (Fedan et al., 2004a). This epithelium-mediated airway relaxation may protect people from experiencing airway narrowing during exercise. The mechanism(s) involved in EpDRF release is not clear. Previous studies have suggested a link between epithelial bioelectric and muscle relaxation responses elicited by hyperosmolarity (Dortch-Carnes et al., 1999; Fedan et al., 1999). This study was conducted to understand in more detail the relationship between epithelium and smooth muscle and to probe epithelial signaling mechanisms involved in EpDRF release.

We obtained concentration-response curves for simultaneously-measured V_t and ΔP responses induced by hyperosmolar D-M. When the increase in osmolarity was lower than 80 mosM, the curves for the hyperpolarization and relaxation shared the same concentration-dependence, suggesting a possible link between the two types of responses over an osmolar concentration range that is physiologically relevant to exercise. However, when the osmolarity was elevated beyond 80 mosM, the V_t response changed to depolarization, and the relaxation response reached maximum. Our earlier study conducted with 240 mosM sucrose also demonstrated a transient hyperpolarization that preceded depolarization (Dortch-Carnes et al., 1999). Thus, the V_t response was qualitatively related to the

concentration of osmolytes. The transition from hyperpolarization to depolarization occurred at around 80 mosM. The concentration-response curves revealed that the epithelium is very sensitive to small changes in luminal osmolarity. A considerable airway relaxation could be elicited in response to small osmolarity increases.

In the face of hyperosmolar challenge (lower than 80 mosM) the hyperpolarization is likely to be a consequence of Cl^- and K^+ secretion as the cells shrink (Russell, 2000). Since cells adjust their volume principally through activation of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter after shrinkage (O'Neill, 1999), the secondary Cl^- and K^+ secretion and Na^+ extrusion caused by this event may also partially contribute to the hyperpolarization. We currently do not know the precise mechanism for the large depolarization evoked by D-M higher than 80 mosM. In the Ussing apparatus, apical D-M (120 mosM) decreased I_{sc} (Wu et al., 2004), suggesting the primary driving force, Na^+/K^+ -pump, for I_{sc} or V_t was inhibited. However, the depolarization caused solely by the Na^+/K^+ -pump inhibitor, ouabain, needs much longer time to reach plateau (Chapter II, Figs. CII-2A and CII-4A), indicating that an additional mechanism must be involved. In rat hepatocytes, increased Na^+ channel conductance became prominent in response to 80 mosM sucrose and upwards (Wehner et al., 2000); the activation of Na^+ conductance and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter is mediated by PKC (Heinzinger et al., 2001). Combined Na^+ influx (perhaps with the reduction of Cl^- efflux) and Na^+/K^+ -pump inhibition could depolarize the epithelium. In addition, a decrease in V_t could also

result from a decrease in transepithelial resistance (R_t). Earlier studies using the Ussing apparatus demonstrated that the depolarization caused by hyperosmolar D-M was not due to a decrease in R_t (Wu et al., 2004).

Using kinase-interfering agents, we investigated the mechanisms of epithelial signaling involved in the regulation of basal V_t and ΔP , and their responses to MCh and D-M. Our primary focus was the stress-sensitive MAP kinases; p38, JNK and ERK were each examined using two structurally-different inhibitors. Of the six MAP kinase inhibitors only SB 203580 decreased basal V_t ; because SKF 86002 did not produce the same effect, the effect of SB 203580 might not involve p38. Other kinase inhibitors, i.e., chelerythrine, LY 294002, KN-62 and ML-7, all decreased basal V_t . In contrast, NaAsO_2 and Na_3VO_4 increased basal V_t . Although NaAsO_2 and Na_3VO_4 are not kinase-specific in their actions, their general effects are to elevate protein phosphorylation and produce responses opposite to those of the kinase inhibitors if epithelial ion transport is regulated by phosphorylation. These results support the hypothesis that PKC, PI-3-K, CaM-K-II and MLCK play some roles in regulating basal epithelial ion transport.

The MAP kinase inhibitors, SB 203580, dicumarol and PD 98059, slightly decreased basal ΔP , suggesting that the regulation of basal smooth muscle tone may involve p38, JNK and ERK. However, a solid conclusion can not be reached because SKF 86002, SP 600125 and U 0126, which are thought to inhibit p38, JNK and ERK, respectively, had no effect. Chelerythrine elevated basal ΔP in

this study; another PKC inhibitor, Ro31-8220, completely abolished guinea-pig tracheal relaxation induced by the PKC activator, PMA (Liu et al., 1998a, b), indicating that PKC plays a role in the regulation of airway smooth muscle tone. Because the epithelial diffusion barrier would reduce the concentration of IL applied agents at the smooth muscle by 400 - 800 fold (Fedan and Frazer, 1992), the basal ΔP elevation in response to chelerythrine was likely an epithelium-dependant effect.

The MAP kinase inhibitors were generally without effect on the contractile responses to MCh. The inhibition caused by LY 294002 was likely due to its inhibitory effect on L-type Ca^{2+} channels activated by M_2 receptors (Callaghan et al., 2004). Na_3VO_4 inhibited contractile responses to MCh in this study; it has been reported to inhibit contractile responses in guinea-pig vascular smooth muscle, and the mechanism was not due to its inhibition of the Na^+-K^+ -pump (Ozaki et al., 1982).

Studies performed on the Ussing apparatus investigating the mechanisms of MCh-induced hyperpolarization found that the hyperpolarization resulted primarily from Cl^- secretion and the activation of $Na^+-K^+-2Cl^-$ cotransporter (Phillips et al., 2002; Wu et al., 2004). MCh activated JNK in Chinese hamster ovary cells expressing M_3 muscarinic receptors, and the activation of JNK was negatively regulated by PKC (Wylie et al., 1999), suggesting that PKC and JNK could have differential regulatory effects on MCh-induced responses. These findings help to explain the opposite effects between SP 600125, dicumarol and

chelerythrine on the MCh-induced hyperpolarization. PKC down-regulation of the activity of JNK was also observed in Rat-1 fibroblasts (Cadwallader et al., 1997). Because the activity of $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter is modulated by phosphorylation, the mechanisms of PKC and JNK could be through their regulation of the cotransporter (Flatman, 2002).

SP 600125, dicumarol and chelerythrine inhibited D-M-induced hyperpolarization. The mechanism may have involved inhibition of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter and secondary secretion of Cl^- and K^+ , because hyperosmolar sucrose-induced activation of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter occurred through the regulation of JNK and PKC in endothelial and epithelial cells, respectively (Klein et al., 1999; Liedtke and Cole, 2002b). Our further studies conducted with simultaneous R_t analysis (Chapter II) demonstrated that the inhibited hyperpolarization caused by SP 600125 and dicumarol was not only through the inhibition of epithelial ion transport but also involved significant decreases in R_t . The effects of NaAsO_2 and Na_3VO_4 may have resulted from their interactions with multiple kinase pathways which are involved in the regulation of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter. Similar to the JNK inhibitors, the inhibition of hyperpolarization caused by NaAsO_2 also involved significant decreases in R_t (Chapter II). Although Na_3VO_4 was reported to have an inhibitory effect on the $\text{Na}^+\text{-K}^+$ -pump (Dafnis and Sabatini, 1994), it increased basal V_t during the 30 min exposure, which is evidence that the activity of the $\text{Na}^+\text{-K}^+$ -pump was not inhibited in our experiments. Because Na_3VO_4 had no effect on R_t (Chapter II), its inhibition on the

D-M-induced hyperpolarization resulted from decreased epithelial ion transport.

SB 203580 and SKF 86002 potentiated D-M-induced relaxation. This result suggests that p38 inhibition may potentiate hyperosmolarity-induced EpDRF release; conceivably, p38 inhibitors could provide therapeutic benefits for the prevention of bronchoconstriction induced by exercise. It is known that p38 signaling contributes to the pathogenesis of airway inflammation and hyperreactivity (Adams et al., 2001). Thus, the findings of this study lead support to the notion that p38 inhibitors can be developed as anti-asthma medicines (Adcock et al., 2006).

Activation of epithelial p38 and JNK induced by hyperosmolar D-M was examined in western blots. Their phosphorylation levels were elevated in an osmolarity concentration-dependant manner, reaching 4- and 6-fold at 320 mosM, respectively. However, the concentrations that elicited maximal hyperpolarization and relaxation responses in the functional perfused trachea studies were lower than those required to induce significant phosphorylation in western blots. The differences suggest that kinase signaling leading to the bioelectric and mechanical responses either do not involve p38 or JNK, or that only small increases in phosphorylation are sufficient, over the physiological osmolarity range. Meanwhile, other signaling mechanisms may also contribute to the functional responses to hyperosmolarity. We are planning to use antibodies of pan-phosphotyrosine and pan-phosphothreonine to investigate whether other kinases could also be involved. In addition, 2-dimensional PAGE analysis and

proteomics may also help us to identify the potentially involved signaling proteins.

Taken together, the results indicate that airway hyperosmolar challenge relevant to exercise elicits epithelial hyperpolarization and smooth muscle relaxation; the two responses share the same osmolarity concentration-dependence. The hyperosmolarity-induced hyperpolarization is possibly regulated by JNK, PKC and phosphatase through the activity of $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter; whereas, p38 plays a role in the EpDRF-mediated relaxation. ERK is not involved in either response. A common signaling pathway coordinating both of the responses was not identified.

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Fig. CI-1. Concentration-response relationship for D-M-induced bioelectric and mechanical responses. ΔV_t , changes in V_t from baseline (-11.33 ± 2.81 mV). Relaxation (% MCh): percent relaxation of 3×10^{-7} M MCh-induced contraction. Osmolarity elevation always induced relaxation. When the osmolarity increase was below 80 mosM D-M increased V_t , and, at higher concentrations, D-M decreased V_t . The transition from hyperpolarization to depolarization occurred at around 80 mosM. Coincidentally, this concentration also induced nearly maximum relaxation. The EC_{50} values for the D-M-induced hyperpolarization and relaxation responses were 16.6 (13 - 21.2) and 24.4 (19.2 - 31.3) mosM, respectively; and were not significantly different. $n = 6$.

Fig. CI-2. Effects of the kinase inhibitors on MCh-induced contraction. LY 294002 ($n = 6$) inhibited the contractile responses. DMSO and the other inhibitors, chelerythrine, KN-62 and ML-7 ($n = 6, 8, 6$ and 6 , respectively) had no effect ($\dagger p = 0.052$). $*p < 0.05$.

Fig. CI-3. Effects of NaAsO₂ and Na₃VO₄ on MCh-induced contraction. Na₃VO₄ (*n* = 6) inhibited the contractile responses, while the effect of NaAsO₂ (*n* = 9) was not significant (†*p* = 0.093). **p* < 0.05.

Fig. CI-4. Effects of the MAP kinase inhibitors on MCh-induced hyperpolarization. SP 600125 and dicumarol ($n = 10$ and 6 , respectively) potentiated the hyperpolarization responses. The effects of DMSO and the other inhibitors, SB 203580, SKF 86002, PD 98059 and U 0126 ($n = 6, 7, 6, 6$ and 6 , respectively) were not significant. $*p < 0.05$. $†p = 0.11$.

Fig. CI-5. Effects of the kinase inhibitors on MCh-induced hyperpolarization. Chelerythrine ($n = 8$) inhibited the hyperpolarization responses. DMSO and the other inhibitors, LY 294002, KN-62 and ML-7 ($n = 6$) had no effect. $*p < 0.05$.

Fig. CI-6. Effects of the MAP kinase inhibitors on D-M-induced hyperpolarization. SP 600125 and dicumarol ($n = 10$ and 6 , respectively) inhibited the hyperpolarization responses. The effects of DMSO and the other inhibitors, SB 203580, SKF 86002, PD 98059 and U 0126 ($n = 6, 7, 6, 6$ and 6 , respectively) were not significant. $*p < 0.05$.

Fig. CI-7. Effects of the kinase inhibitors on D-M-induced hyperpolarization. Chelerythrine ($n = 8$) inhibited the hyperpolarization responses. DMSO and the other inhibitors, LY 294002, KN-62 and ML-7 ($n = 6$) had no effect. $*p < 0.05$.

Fig. CI-8. Effects of NaAsO₂ and Na₃VO₄ on D-M-induced hyperpolarization. Both NaAsO₂ and Na₃VO₄ ($n = 9$ and 6 , respectively) inhibited the hyperpolarization responses. The inhibition by NaAsO₂ was complete. * $p < 0.05$.

Fig. CI-9. Effects of the MAP kinase inhibitors on D-M-induced airway relaxation. SB 203580 and SKF 86002 ($n = 7$ and 6 , respectively) potentiated the relaxation responses. DMSO and the other inhibitors, SP 600125, dicumarol, PD 98059 and U 0126 ($n = 6, 10, 6, 6$ and 6 , respectively) had no effect. $*p < 0.05$.

Fig. CI-10. Effects of the kinase inhibitors on D-M-induced airway relaxation. DMSO and the kinase inhibitors, chelerythrine, LY 294002, KN-62 and ML-7 ($n = 6, 8, 6, 6$ and 6 , respectively) had no significant effect on the relaxation responses ($\dagger p = 0.065$).

A

B

Fig. CI-11. Phosphorylation of p38 induced by hyperosmolar D-M. (A) After exposing epithelia to D-M for 20 min the phosphorylation of p38 was increased in an osmolarity concentration-dependant manner, while the total amount of p38 was not affected. (B) Example of p38 phosphorylation induced by 80 mosM D-M. NaAsO₂ not only induced p38 phosphorylation but also increased the total available p38 protein. *n* = 4.

A

B

Fig. CI-12. Phosphorylation of JNK induced by hyperosmolar D-M. (A) After exposing epithelia to D-M for 20 min the phosphorylation of JNK was increased in an osmolarity concentration-dependant manner, while the total amount of JNK was not affected. (B) Example of JNK phosphorylation induced by 160 mosM D-M. NaAsO₂ induced JNK phosphorylation but the total available JNK protein was not increased. *n* = 4.

Table CI-1. Effects of MAP kinase inhibitors on basal V_t (mV) and ΔP (cm H₂O)

Target	p38		JNK		ERK	
Inhibitors	SB 203580	SKF 86002	SP 600125	Dicumarol	PD 98059	U 0126
Conc ^a (μM)	30	30	30	15	50	30
<i>n</i>	7	6	10	6	6	6
V_t (mV)	Depol ^b 0.8 ± 0.3	- ^c	-	-	-	-
ΔP (cm H ₂ O)	Relaxation 0.06 ± 0.02	-	-	Relaxation 0.46 ± 0.13	Relaxation 0.16 ± 0.04	-

^aConcentration; ^bDepolarization; ^cNo effect.

Table CI-2. Effects of other kinase inhibitors, NaAsO₂ and Na₃VO₄
on basal V_t (mV) and ΔP (cm H₂O)

Effects	PKC inhibitor	PI-3-K Inhibitor	CaM-K-II inhibitor	MLCK inhibitor	Phosphatase inhibitor	Stress inducer
Agents	Chelerythrine	LY 294002	KN-62	ML-7	Na ₃ VO ₄	NaAsO ₂
Conc ^a (μM)	20	50	20	30	300	300
<i>n</i>	8	6	6	6	6	9
V_t (mV)	Depol ^b 1.53 ± 0.29	Depol 3.92 ± 0.61	Depol 0.47 ± 0.14	Depol 0.60 ± 0.46	Hyperpol 0.70 ± 0.22	Hyperpol ^c 1.69 ± 0.67
ΔP (cm H ₂ O)	Contraction 0.22 ± 0.06	- ^d	-	-	-	-

^aConcentration; ^bDepolarization; ^cHyperpolarization; ^dNo effect.

Chapter II

Epithelial Mechanisms in Airway Bioelectric and Mechanical Responses Induced by Hyperosmolarity: Studies on a Novel Tracheal Perfusion Apparatus

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Abbreviations: ASL, airway surface liquid; EpDRF, epithelium-derived relaxing factor; V_t , transepithelial potential; R_t , transepithelial resistance; ΔP , inlet minus outlet perfusion pressure difference; MKH, modified Krebs-Henseleit; MCh, methacholine; D-M, D-mannitol; IL, intraluminal; EL, extraluminal.

Section assignment: Pulmonary

Abstract

In the preceding study (Chapter I) we investigated the effects of kinase-interfering agents on hyperosmolar D-mannitol (D-M)-induced airway epithelial bioelectric response and smooth muscle relaxation mediated by epithelium-derived relaxing factor (EpDRF), using the tracheal perfusion apparatus. Some of the kinase and phosphatase inhibitors altered transepithelial potential (V_t) responses. The release of EpDRF has been thought to be linked to epithelial ion transport. Because V_t responses could result from changes in epithelial ion transport and/or transepithelial resistance (R_t), to understand better the mechanism of EpDRF release we modified the tracheal perfusion apparatus so that V_t , R_t and airway diameter changes could be monitored simultaneously. To validate the apparatus, selected ion transport inhibitors, bronchoactive agonists and osmolytes were examined for their specific pharmacological effects. The kinase-interfering agents that were observed previously to affect V_t responses were subsequently examined. The protein kinase C (PKC) inhibitor, chelerythrine, inhibited V_t responses to methacholine and D-M. The c-Jun N-terminal kinase (JNK) inhibitors, SP 600125 and dicumarol, the phosphatase inhibitor, Na_3VO_4 , and the stress inducer, NaAsO_2 , altered V_t responses as well as R_t . None of the kinase-interfering agents affected airway relaxation to D-M. The results indicate that this novel apparatus is useful for the study of airway pharmacology. PKC, JNK and phosphatase are involved in the regulation of epithelial ion transport. The permeability of tight junctions could be regulated by protein phosphorylation.

Evidence was not obtained to suggest that the bioelectric and mechanical responses to D-M are regulated by a common kinase signaling pathway.

Introduction

Hyperventilation during exercise is thought to cause evaporative dehydration and hyperosmolarity of the airway surface liquid (ASL) (Anderson and Daviskas, 2000). In a canine model the elevation of osmolarity in the ASL reached 130 mosM during and 40 mosM shortly after hyperventilation of dry air (Freed and Davis, 1999). The airway epithelium can detect an increase in osmolarity of the ASL and modulate the underlying smooth muscle tone by releasing epithelium-derived relaxing factor (EpDRF) (Munakata et al., 1988; Fedan et al., 1999). The EpDRF-mediated airway relaxation could contribute to bronchodilation during exercise. However, in asthmatic patients inhalation of mannitol dry powder to elevate the osmolarity of the ASL elicits airway obstruction (Anderson et al., 1997a; Brannan et al., 1998), indicating that the mechanism of hyperosmolarity-induced EpDRF release could be altered. In the bronchial epithelium of asthmatic patients altered protein tyrosine phosphorylation has been reported (Hamilton et al., 2005).

Exercise-induced airway obstruction is a highly prevalent disorder among asthmatics (Cabral et al., 1999; Hallstrand et al., 2002). Asthma is considered as an airway chronic inflammatory disease characterized by hyperractivity to a variety of environmental stimuli and periodic episodes of airflow obstruction caused by airway spasm (Hamilton et al., 2001; Elias et al., 2003). The inflammation results in inflammatory cell infiltration, smooth muscle proliferation, and epithelial damage and shedding (Homer and Elias, 2005; Hallstrand et al.,

2005a). Epithelial damage would expose the underlying smooth muscle to contractile mediators during hyperventilation (Brannan et al., 2003; Anderson and Kippelen, 2005; Anderson, 2006), and disrupt the EpDRF-mediated airway relaxation in response to hyperosmolar challenge (Fedan et al., 2004a).

Using the guinea-pig isolated, perfused trachea apparatus we previously observed that relaxation responses to hyperosmolarity were inhibited by blockers of epithelial Na^+ and Cl^- channels (Fedan et al., 1999). While simultaneously monitoring both transepithelial potential (V_t) and airway diameter, we observed that the epithelial bioelectric responses to hyperosmolar solution occurred before the relaxation responses (Dortch-Carnes et al., 1999). These findings suggested that the bioelectric events may initiate the release of EpDRF. Recent concentration-dependence studies (Chapter I) using D-mannitol (D-M) to elevate osmolarity in the tracheal lumen revealed that the relaxation response was nearly maximal at 80 mosM and was accompanied by a hyperpolarization response at and below this concentration. At higher D-M concentrations maximal relaxation was achieved, but V_t decreased.

Because hyperosmolar challenge of many cells stimulates activation of mitogen-activated protein (MAP) kinase pathways, p38, c-Jun N-terminal kinase (JNK) and extracellular-regulated kinase (ERK) (Hashimoto et al., 1999; Duzgun et al., 2000), and production of epithelium-derived mediators as well as cell volume-related ion transport in response to hyperosmolarity are regulated by stress-sensitive protein kinases (Shrode et al., 1998; Furuichi et al., 2002), the

roles of these MAP kinases as well as some other signaling pathways in the response to D-M were examined (Chapter I). Using kinase-interfering agents we found that protein kinase C (PKC), phosphatidylinositol 3-kinase (PI-3-K), calmodulin kinase-II (CaM-K-II), myosin light chain kinase (MLCK) and protein-phosphotyrosine phosphatases may play a role in the regulation of basal V_t . In addition, some of these agents, particularly those targeting PKC, JNK and phosphatases, modified the V_t responses to D-M. Such changes could have originated from alterations in epithelial ion transport and/or from alterations of the transepithelial resistance (R_t), i.e., the permeability of paracellular pathway across tight junctions (Tsukita et al., 2001).

To understand better whether kinase-modifying agents affected bioelectric responses to hyperosmolarity by altering epithelial ion transport or R_t , we further modified the tracheal perfusion apparatus so as to be able to measure V_t , R_t and mechanical responses of the trachea simultaneously. To validate the apparatus, we examined the effects of well-characterized ion channel blockers, ion pump and cotransporter inhibitors, bronchoactive agonists and osmolytes. The objectives of this study were: 1) to evaluate the performance of this novel perfusion apparatus with four categories of the selected pharmacological agents and to study the relationship between their bioelectric and mechanical effects on airways; and 2) to examine the effects of the JNK inhibitors, SP 600125 and dicumarol, the PKC inhibitor, chelerythrine, the stress inducer, NaAsO_2 and the phosphatase inhibitor, Na_3VO_4 . These agents were observed in our previous study to alter basal V_t and

V_t responses induced by hyperosmolar challenge (Chapter I).

Materials and Methods

Animals. These studies were conducted in facilities accredited fully by the Association for the Assessment and Accreditation of Laboratory Animal Care International and were approved by the institutional Animal Care and Use Committee. Male guinea pigs (550 - 700 g), HsdPoc:DH, from Harlan (Indianapolis, IN), monitored free of endogenous viral pathogens, parasites, and bacteria were used in all experiments. The animals were acclimated before use and were housed in filtered ventilated cages on Alpha-Dri virgin cellulose chips and hardwood Beta-chips as bedding, provided HEPA-filtered air, Teklad 7006 diet and tap water ad libitum, under controlled light cycle (12 h light) and temperature (22 - 25 °C) conditions. The animals were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and sacrificed by thoracotomy and bleeding before removing the trachea.

Materials. Modified Krebs-Henseleit (MKH) solution (pH 7.4 at 37 °C) contained 113 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃ and 5.7 mM glucose was saturated with 95% O₂ and 5% CO₂. The osmolarity of MKH solution was 281 ± 5 mosM.

Acetyl-β-methylcholine chloride (methacholine, MCh), dimethyl sulphoxide (DMSO), amiloride, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), ouabain, bumetanide, histamine, terbutaline, NaCl and D-M were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of MCh and D-M were prepared in saline. Amiloride, NPPB and bumetanide were dissolved in DMSO (final

concentration, 0.01%). Other agents were prepared in MKH. Hyperosmolar solutions were prepared by adding osmolytes to the MKH solution.

SP 600125, dicumarol and chelerythrine were purchased from Calbiochem-Novabiochem (La Jolla, CA) and dissolved in DMSO (final concentration, 0.01%). NaAsO₄ and Na₃VO₄ were obtained from Mallinckrodt Laboratory Chemicals (Phillipsburg, NJ) and Sigma-Aldrich (St. Louis, MO), respectively, and prepared in MKH solution.

Tracheal Perfusion Preparation. The preparation was further modified from the perfused trachea apparatus employed in the previous study (Chapter I) by adding current electrodes mounted across the tracheal wall to deliver current pulses so that R_t could be calculated. A 4.2-cm segment of guinea-pig trachea was removed and mounted at its natural length onto a custom-designed plastic perfusion holder which allows cannulas with side-opening holes to be inserted into the lumen from either end of the trachea. The tracheal holder was placed into an extraluminal (EL) bath filled with MKH solution. The tracheal lumen was perfused at constant flow (24 ml/min) with MKH solution from another bath, referred to as the intraluminal (IL) bath. The transmural pressure was adjusted to zero. MKH solution in the IL and EL baths was kept at 37 °C and saturated with 95% O₂ and 5% CO₂. Agents may be added separately to the IL or EL baths so that they can act directly on epithelia or airway smooth muscle, respectively. The inlet minus outlet perfusion pressure difference (ΔP) caused by tracheal diameter change was sampled through side-holes in the indwelling cannulas which were connected

to a differential pressure transducer. Airway smooth muscle contraction decreases tracheal diameter and increases ΔP , whereas ΔP decreases during relaxation. Current and voltage electrodes (filled with 4% agar/saline) were connected to the lumen and EL sides of the trachea. Current pulses (20 μA , 5 s duration and 50 s interval) were delivered through current electrodes, while V_t and the voltage deflections caused by the current pulses, were monitored under open-circuit conditions. Because the current pulse was delivered from a point source, the field of current is not evenly distributed to the whole surface area of epithelium; therefore, the value of R_t measured with this method is not connected for unit area, as measured with the Ussing apparatus. In the present study R_t was assessed as changes in R_t (ΔR_t) before and after a treatment. R_t was calculated from the voltage responses caused by 20 μA pulses using Ohm's law.

Effects of Ion Transport Inhibitors, Bronchoactive Agonists and Osmolytes on V_t , ΔP and R_t . The tracheal preparations were equilibrated by perfusing MKH solution for a 2.5 – 3 h period to allow stabilization of basal V_t and ΔP ; then current pulses were delivered. Agents dissolved either in DMSO or MKH were added to the IL or EL bath, as appropriate. Relaxation responses were elicited after the tracheas had first been precontracted with EL 3×10^{-7} M MCh and reached the plateau. Because 240 mosM NaCl and D-M added to the perfusing MKH solution elicited depolarization and relaxation of the airways (Dortch-Carnes et al., 1999), and added 294 mosM NaCl caused an increase of tight junction permeability in airway epithelium (Hogman et al., 2002), 300 mosM NaCl and D-M

were used as osmolytes in this study to evaluate the performance of the apparatus.

Effects of Kinase Inhibitors, NaAsO₂ and Na₃VO₄ on V_t , R_t and ΔP Responses Induced by Hyperosmolar D-M. To reduce variability, the control responses and responses obtained in the presence of agents were obtained successively from the same trachea, i.e., a paired design was employed. After perfusing the trachea with MKH solution for a 2.5 – 3 h period to stabilize basal V_t and ΔP , current pulses were delivered. To obtain control responses the trachea was incubated with vehicle (DMSO or MKH) for 30 min in the IL bath, and then exposed to MCh in the EL bath. When the MCh-induced contraction and hyperpolarization responses reached their plateaus, the osmolarity of IL perfused MKH solution was elevated by adding 30 mosM D-M ($\sim EC_{50}$) to induce relaxation. D-M also caused hyperpolarization at this concentration. After the D-M-induced V_t and ΔP responses reached their plateaus, the trachea was washed with fresh MKH solution to allow the V_t and ΔP recover to their basal levels.

Ninety min after the V_t and ΔP baselines were re-established, the trachea was incubated with an agent in the IL bath for 30 min. Then the trachea was exposed to EL MCh and challenged with IL D-M again in the presence of the agent. To evaluate whether changes occurred in responses to MCh and D-M in these two cycles, control experiments using tracheas exposed only to DMSO were run in parallel.

Data Analysis. V_t was quantified in mV. R_t was quantified in Ohms. The

magnitude of V_t responses induced by MCh and D-M are presented as changes in V_t (ΔV_t) before and after exposure to the agents; likewise, ΔR_t was also calculated. Airway contraction and relaxation responses were measured as changes in ΔP , in cm H₂O. Values are presented as means $\pm$ SE. Differences were analyzed statistically using Student's paired t -test. $p < 0.05$ was considered significant.

Results

At the conclusion of the 2.5 – 3 h equilibration in MKH solution, basal V_t was 14.8 ± 0.8 mV ($n = 32$).

Effects of Amiloride and NPPB on V_t , R_t and ΔP . IL-applied Na^+ and Cl^- channel blockers, amiloride (3×10^{-5} M) and NPPB (10^{-4} M), respectively, decreased V_t . The effect of amiloride was immediate, and the effect of NPPB was slower to develop and gave rise to a larger depolarization (Fig. CII-1A). Amiloride caused small but significant increase in R_t , while NPPB had no effect (Fig. CII-1B). Both amiloride and NPPB had no effect on ΔP ($n = 4$, data not shown).

Effects of Ouabain and Bumetanide on V_t , R_t and ΔP . Applied to the EL bath, the Na^+ - K^+ -pump inhibitor, ouabain (10^{-5} M), and the Na^+ - K^+ - 2Cl^- cotransporter inhibitor, bumetanide (10^{-5} M), decreased V_t . Ouabain caused an immediate depolarization, decrease in R_t and a delayed contraction; it completely depolarized the epithelium. Bumetanide caused a slight relaxation (Fig. CII-2A). The depolarization caused by bumetanide was relatively small, and it did not affect R_t (Fig. CII-2B).

Effects of Histamine and Terbutaline on V_t , R_t and ΔP . EL-applied histamine (7.4×10^{-5} M) and terbutaline (10^{-7} M), an H_1 -receptor agonist and a β_2 -receptor agonist, respectively, caused contraction and relaxation (Fig. CII-3A). Both histamine and terbutaline caused variable and small changes in V_t ; on average, they did not affect V_t appreciably. Both agonists had no effect on R_t (Figs. CII-3A and CII-3B).

Effects of Hyperosmolarity on V_t , R_t and ΔP . Hyperosmolarity elicited with IL NaCl and D-M (300 mosM each) caused relaxation and depolarization (Figs. CII-4A and CII-4B). NaCl decreased R_t , while D-M increased R_t (Fig. CII-4B).

Effects of DMSO, SP 600125, Dicumarol, Chelerythrine, NaAsO₂ and Na₃VO₄ on Basal V_t , R_t and ΔP , and Their Responses Induced by MCh and Hyperosmolar D-M.

Baseline effects on V_t , R_t and ΔP . DMSO (0.04%) and the JNK inhibitors, SP 600125 (30 μ M) and dicumarol (15 μ M), had no effect on basal V_t . The PKC inhibitor, chelerythrine (20 μ M), decreased basal V_t by 1.45 ± 0.40 mV. The phosphatase inhibitor, Na₃VO₄ (300 μ M), increased basal V_t by 2.58 ± 0.21 mV. The stress inducer, NaAsO₂ (300 μ M), caused a transient increase in V_t followed by a decrease; it also caused a $36.1 \pm 8.9 \Omega$ increase in R_t . Dicumarol decreased basal ΔP by 0.46 ± 0.13 cm H₂O. DMSO and the other agents had no effect on R_t and ΔP ($n = 6$, data not shown).

Effects of DMSO on MCh- and D-M-induced V_t and R_t responses. To evaluate if DMSO had any effect, control experiments were performed to examine whether it alters MCh- and D-M-induced V_t and R_t responses. At a concentration of 0.04%, DMSO had no effect on MCh- and D-M-induced hyperpolarization (Figs. CII-5A and CII-5C); it also had no effect on R_t (Figs. CII-5B and CII-5D).

Effects of SP 600125 on MCh- and D-M-induced V_t and R_t responses. SP 600125 potentiated MCh-induced hyperpolarization (Fig. CII-6A), and there was

no significant change in R_t (Fig. CII-6B), indicating the observed V_t responses were caused primarily by increased epithelial ion transport. SP 600125 inhibited D-M-induced hyperpolarization (Fig. CII-6C). It also caused a decrease in R_t (Fig. CII-6D). Because both decreases in R_t and epithelial ion transport could lead to the inhibition of D-M-induced hyperpolarization, the changes in V_t and R_t responses were normalized as percentage changes. The $\% \Delta V_t$ and $\% \Delta R_t$ were 11.8 ± 1.9 and 8.0 ± 3.5 , respectively, and were not significantly different. Therefore, the inhibition of D-M-induced hyperpolarization in the presence of SP 600125 may be attributable to an increase in tight junction permeability.

Effects of dicumarol on MCh- and D-M-induced V_t and R_t responses. Dicumarol potentiated MCh-induced hyperpolarization responses (Fig. CII-7A), and there was no change in R_t (Fig. CII-7B), indicating that a stimulation of ion transport was the cause of the observed V_t responses. Dicumarol inhibited D-M-induced hyperpolarization (Fig. CII-7C), and caused a decrease in R_t (Fig. CII-7D). Because the $\% \Delta V_t$ and $\% \Delta R_t$ induced by D-M were 14.09 ± 4.21 and 6.83 ± 1.55 , respectively, and were not significantly different, the inhibition of D-M-induced hyperpolarization in the presence of dicumarol was probably not attributable to a decrease in ion transport.

Effects of chelerythrine on MCh- and D-M-induced V_t and R_t responses. Chelerythrine inhibited MCh- and D-M-induced hyperpolarization responses (Figs. CII-8A and CII-8C), and it had no effect on R_t (Figs. CII-8B and CII-8D). The results suggest that the inhibited V_t responses in the presence of chelerythrine

were caused primarily by a decrease in ion transport.

Effects of NaAsO₂ on MCh- and D-M-induced V_t and R_t responses.

NaAsO₂ had a considerable but not significant inhibitory effect ($p = 0.056$) on MCh-induced hyperpolarization responses (Fig. CII-9A). At the same time, its effect on R_t was not significant (Fig. CII-9B). NaAsO₂ completely inhibited D-M-induced hyperpolarization (Fig. CII-9C); it also decreased R_t (Fig. CII-9D). The $\% \Delta V_t$ and $\% \Delta R_t$ of the D-M-induced responses were 15.6 ± 2.5 and 9.6 ± 2.2 , respectively, and were not significantly different. Therefore, the significant inhibition of D-M-induced hyperpolarization in the presence of NaAsO₂ was primarily caused by the decreased R_t, and cannot be attributed to a decrease in epithelial ion transport.

Effects of Na₃VO₄ on MCh- and D-M-induced V_t and R_t responses.

Na₃VO₄ inhibited MCh-induced hyperpolarization responses (Fig. CII-10A), and caused a decrease in R_t (Fig. CII-10B). Because the $\% \Delta V_t$ and $\% \Delta R_t$ were 9.82 ± 2.19 and 9.50 ± 3.56 , respectively, and were not significantly different, the equivalent changes in $\% \Delta V_t$ and $\% \Delta R_t$ suggest that the inhibition of MCh-induced hyperpolarization in the presence of Na₃VO₄ was caused by the decreased R_t. Na₃VO₄ inhibited D-M-induced hyperpolarization responses (Fig. CII-10C), and there was no significant change in R_t (Fig. CII-10D), indicating that the inhibited V_t response in the presence of Na₃VO₄ was caused by a decrease in ion transport.

Effects of the agents on MCh- and D-M-induced ΔP responses.

DMSO and all the kinase-interfering agents had no effect on MCh- and D-M-induced ΔP

responses ($n = 6$, data not shown).

Discussion

In order to understand the role of the airway epithelium in the hyperosmolarity-induced bioelectric response and the subsequent relaxation, we have been investigating the relationship between the two responses and the possible involvement of protein phosphorylation in epithelial signaling using the perfused trachea apparatus. Evidence was obtained earlier that epithelial bioelectric events and the EpDRF-mediated relaxation are functionally linked (Dortch-Carnes et al., 1999; Fedan et al., 1999). Kinases and phosphatases may be involved in the regulation of epithelial V_t (Chapter I). Because agents that affect R_t could secondarily affect basal V_t and evoked V_t responses, and our previously employed tracheal perfusion apparatus was unable to discriminate between changes in R_t and epithelial ion transport, we conducted the present study to examine further the mechanisms of bioelectric and mechanical responses to hyperosmolar challenge.

The Ussing chamber (Reuss, 2001) had been employed to provide R_t information in response to ion transport inhibitors and hyperosmolarity in our previous studies (Wu et al., 2004); since tracheal segments are rigidly clamped in the Ussing apparatus, responses of the tracheal smooth muscle and the possible correlations between epithelial bioelectric and muscle mechanical responses could not be made. Moreover, the basal V_t measured with the Ussing preparation was reduced by two third compare to that obtained from the perfusion apparatus (Dortch-Carnes et al., 1999; Fedan et al., 2006a). The lower potential measured

with the Ussing apparatus may be a consequence of the distortion brought about by fixing a cylindrical organ into a flat mount. Apparently, this results in a decrease in epithelial ion transport or a permeability increase across tight junctions. To better understand the airway physiology and epithelial regulatory mechanism, we further modified the tracheal perfusion apparatus so that V_t , R_t and ΔP could be obtained simultaneously under conditions in which the *in vivo* configuration of the organ is maintained. The performance of the apparatus was validated using ion transport inhibitors, bronchoactive agonists and hyperosmolar challenge. After characterization of the apparatus, we then utilized it to examine the bioelectric effects of the kinase-interfering agents.

Both amiloride and NPPB decreased basal V_t . The increase in R_t caused by amiloride, which was also observed in rabbit airways (Poulsen et al., 2006), could partially offset the decrease in V_t . The larger depolarization caused by NPPB suggests that guinea-pig tracheal epithelium is primarily a Cl^- -secreting rather than a Na^+ -absorbing epithelium (Wu et al., 2004). Both amiloride and NPPB had no effect on basal ΔP , indicating that, at rest, ion transport through Na^+ and Cl^- channels is not involved in the regulation of smooth muscle tone.

Ouabain and bumetanide depolarized basal V_t . Although ouabain also decreased R_t , it is not difficult to conclude that the abolition of V_t was primarily due to the inhibition of the Na^+/K^+ -pump. The activity of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter in guinea-pig tracheal epithelium supports ~10% of transepithelial short-circuit current (I_{sc}) in the Ussing apparatus (Wu et al., 2004). Bumetanide, which had no

effect on R_t , caused a ~20% decrease in V_t . This effect is comparable with that obtained in cultured guinea-pig tracheal epithelium (Robison and Kim, 1994). The ouabain-induced muscle contraction occurred because decreased Na^+ extrusion reduced $\text{Na}^+, \text{Ca}^{2+}$ -exchange, causing intracellular Ca^{2+} accumulation in the smooth muscle (Chideckel et al., 1987). The bumetanide-induced relaxation can be attributed, in part, to its inhibition of the $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter (Lavallee et al., 1997).

Histamine and terbutaline caused, as expected, contraction and relaxation of the tracheas, respectively. Because both receptors are located on muscle cells, their mechanisms are not mediated by epithelium. Although histamine increased V_t and I_{sc} in canine tracheas (Yamada et al., 1994) and terbutaline increased I_{sc} in cultured guinea-pig epithelia (Robison and Kim, 1994), the two agonists used in EC_{50} concentrations caused, surprisingly, small and variable changes in V_t in the guinea-pig perfused trachea. Overall, they had no effect on basal V_t and R_t .

Hyperosmolar NaCl and D-M elicited depolarization and relaxation, as reported earlier (Dortch-Carnes et al., 1999). Similar to the observations made with the Ussing apparatus (Wu et al., 2004; Poulsen et al., 2006), and microelectrode and microscopy studies (Willumsen et al., 1994; Hogman et al., 2002), NaCl decreased R_t but D-M increased R_t . Because the NaCl-induced $\% \Delta R_t$ (20.0%) was less than the $\% \Delta V_t$ (84.7%), the depolarization induced by NaCl was probably the result of decreased ion transport. In contrast, D-M induced an increase in R_t in the face of depolarization; therefore, a decrease in ion

transport is responsible for the D-M-induced depolarization. Decreased ion transport to hypertonic NaCl and D-M was also observed in other studies (Willumsen et al., 1994; Wu et al., 2004). Moreover, increased Na^+ influx, as reported in hepatocytes, may potentially contribute to the depolarization obtained at this high NaCl concentration (Wehner et al., 2000). These results indicate that hyperosmolar NaCl and D-M oppositely alter tight junction permeability of the tracheal epithelium.

To investigate the role of kinase signaling in the responses of the epithelium to MCh and physiologically-relevant increases in osmolarity using D-M (30 mosM), the effects of SP 600125, dicumarol, chelerythrine, NaAsO_2 and Na_3VO_4 were examined. MCh and D-M both induced hyperpolarization. Activation of the $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter and Cl^- secretion were reported to play a role in maintaining basal V_t and MCh-induced hyperpolarization (Phillips et al., 2002; Wu et al., 2004). Since activation of $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter is common in cells exposed to hyperosmolarity and undergoing regulatory volume increase (O'Neill, 1999), its activation and secondary Cl^- secretion could also be involved in the D-M-induced hyperpolarization.

MCh activated JNK in Chinese hamster ovary cells expressing M_3 muscarinic receptors, and the activation of JNK was negatively regulated by PKC (Wylie et al., 1999). This finding implies that PKC and JNK could have differential regulatory effects on MCh-induced responses. In accord with this observation, chelerythrine inhibited, while SP 600125 and dicumarol potentiated, MCh-induced

hyperpolarization. PKC negatively regulates activation of JNK in Rat-1 fibroblasts as well (Cadwallader et al., 1997). The inhibitory effects of chelerythrine on basal V_t and MCh-induced hyperpolarization suggest that PKC regulates epithelial ion transport, possibly through $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter (Liedtke et al., 2002a).

In our earlier investigation, NaAsO_2 significantly inhibited MCh-induced hyperpolarization (Chapter I); in the present study this effect was less pronounced ($p = 0.056$). NaAsO_2 caused a considerable increase in R_t , which offset the V_t response. Na_3VO_4 also inhibited MCh-induced hyperpolarization, but decreased R_t . Because the $\% \Delta V_t$ and $\% \Delta R_t$ were not different, the inhibited hyperpolarization was caused primarily by the decreased R_t . Na_3VO_4 was also found to decrease transepithelial resistance in kidney monolayer cells and brain endothelial cells through inhibition of phosphatase, and alteration of tyrosine phosphorylation of tight junction proteins (Collares-Buzato et al., 1998; Lohmann et al., 2004).

Although chelerythrine, SP 600125 and dicumarol inhibited D-M-induced hyperpolarization, chelerythrine did not affect R_t , while SP 600125 and dicumarol decreased R_t . Because the $\% \Delta V_t$ and $\% \Delta R_t$ caused by SP 600125 and dicumarol were comparable, the inhibited hyperpolarization was probably attributable to the decrease in R_t . JNK signaling has not been reported to be intimately involved in the regulation of transepithelial resistance. However, a recent study (Wong et al., 2005) demonstrated that the JNK inhibitor, dimethylaminopurine, worsened the CdCl_2 -induced damage to the blood-testis

barrier, indicating that in response to hyperosmolarity JNK inhibition might cause an increase in the permeability of tight junctions. The results suggest that PKC, but not JNK, regulates epithelial ion transport during D-M-induced hyperpolarization, possibly through $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter. This finding is consistent with the observation that hyperosmolar sucrose activates the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter in human tracheal epithelia, and that this activation is regulated by PKC δ and not affected by JNK (Liedtke and Cole, 2002b).

NaAsO_2 significantly inhibited D-M-induced hyperpolarization and decreased R_t . Because the $\%\Delta V_t$ and $\%\Delta R_t$ were comparable, the inhibited hyperpolarization is likely to have been caused by the decrease in R_t . We were unable to find an earlier report of an effect of NaAsO_2 on tight junction permeability. Because NaAsO_2 activates stress-sensitive kinases, the decrease in R_t could be a reflection of regulation of tight junctions by kinases. Na_3VO_4 inhibited D-M-induced hyperpolarization without affecting R_t , indicating that protein-phosphotyrosine phosphatase regulates the ion transport stimulated by D-M. Although Na_3VO_4 has an inhibitory effect on the $\text{Na}^+\text{-K}^+\text{-pump}$ (Dafnis and Sabatini, 1994), we cannot conclude that the inhibited hyperpolarization occurred as a result of decreased activity of the $\text{Na}^+\text{-K}^+\text{-pump}$, for the reason that Na_3VO_4 did not decrease basal V_t , and it hyperpolarized the epithelium during the 30 min exposure.

In summary, we have developed a useful new method for the study of airway physiology and pharmacology using tracheas that retain their *in situ* shape.

Information obtained with this apparatus has had to be collected separately from different methods previously. Using this technology we were able not only to evaluate the relationship between epithelial bioelectric and mechanical responses directly, but also to gain information on the contribution of changes in R_t to bioelectric responses. The results obtained with kinase and phosphatase inhibitors indicate that PKC is intimately involved in the regulation of epithelial ion transport. JNK and protein-phosphotyrosine phosphatase regulate the epithelial ion transport induced by MCh and D-M, respectively; they also play a role in the regulation of epithelial tight junctions. Studies with the kinase-modifying agents did not identify a common signaling pathway that regulates both of the bioelectric and mechanical responses to hyperosmolar challenge.

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A

B

Fig. CII-1. Effects of amiloride (3×10^{-5} M, IL) and NPPB (10^{-4} M, IL) on V_t , R_t and ΔP . (A) Representative tracings. Both amiloride and NPPB caused depolarization. The gap in the NPPB tracing represents 20 min. (B) Summary of the effects of amiloride and NPPB on V_t and R_t . The depolarization caused by amiloride was accompanied by an increase in R_t , while NPPB had no significant effect on R_t . Neither agent affected ΔP (data not shown). $n = 4$, $*p < 0.05$.

A

B

Fig. CII-2. Effects of ouabain (10^{-5} M, EL) and bumetanide (10^{-5} M, EL) on V_t , R_t and ΔP . (A) Representative tracings. Ouabain caused an immediate depolarization and a delayed contraction; bumetanide caused depolarization and a slight relaxation. (B) Summary of the effects on V_t and R_t . Ouabain abolished V_t and decreased R_t . Bumetanide caused a smaller depolarization and had no effect on R_t . $n = 4$, $*p < 0.05$.

A

B

Fig. CII-3. Effects of histamine (7.4×10^{-5} M, EL) and terbutaline (10^{-7} M, EL) on V_t , R_t and ΔP . (A) Representative tracings. Histamine caused contraction and variable V_t effects. Terbutaline caused relaxation and a transient depolarization in three out of four preparations followed by slow hyperpolarization. (B) Summary of the effects on V_t and R_t . Overall, histamine and terbutaline did not affect V_t appreciably. Both agents had no significant effect on R_t . $n = 4$, $*p < 0.05$.

A

B

Fig. CII-4. Effects of hyperosmolar NaCl and D-M (300 mosM each, IL) on V_t , R_t and ΔP . (A) Representative tracings. NaCl and D-M caused depolarization and relaxation in the presence of MCh. (B) Summary of the effects of V_t and R_t . Though both osmolytes caused depolarization, NaCl decreased R_t while D-M increased R_t . $n = 4$, $*p < 0.05$.

Fig. CII-5. Effects of DMSO (0.04%, IL) on MCh- and D-M-induced V_t and R_t responses. (A and B) DMSO had no significant effect on MCh-induced hyperpolarization. There was also no effect on R_t . (C and D) DMSO had no effect on D-M-induced hyperpolarization. There was no effect on R_t . $n = 6$.

Fig. CII-6. Effects of SP 600125 (30 μ M, IL) on MCh- and D-M-induced V_t and R_t responses. (A and B) SP 600125 potentiated MCh-induced hyperpolarization. There was no change in R_t . (C and D) SP 600125 inhibited D-M-induced hyperpolarization. It also caused a small but significant decrease in R_t . $n = 6$, $*p < 0.05$.

Fig. CII-7. Effects of dicumarol (15 μ M, IL) on MCh- and D-M-induced V_t and R_t responses. (A and B) Dicumarol potentiated MCh-induced hyperpolarization. There was no significant change in R_t . (C and D) Dicumarol inhibited D-M-induced hyperpolarization. There was also a decrease in R_t . $n = 6$, $*p < 0.05$.

Fig. CII-8. Effects of chelerythrine (20 μ M, IL) on MCh- and D-M-induced V_t and R_t responses. (A and B) Chelerythrine inhibited MCh-induced hyperpolarization. There was no significant change in R_t . (C and D) Chelerythrine inhibited D-M-induced hyperpolarization. R_t was not affected. $n = 6$, * $p < 0.05$.

Fig. CII-9. Effects of NaAsO₂ (300 μ M, IL) on MCh- and D-M-induced V_t and R_t responses. (A and B) NaAsO₂ had no significant effect on MCh-induced hyperpolarization ($p = 0.056$); its effect in R_t was also not significant ($p = 0.082$). (C and D) NaAsO₂ completely inhibited D-M-induced hyperpolarization, which was accompanied by a decrease in R_t . $n = 6$, * $p < 0.05$.

Fig. CII-10. Effects of Na_3VO_4 (300 μM , IL) on MCh- and D-M-induced V_t and R_t responses. (A and B) Na_3VO_4 inhibited MCh-induced hyperpolarization and decreased R_t . (C and D) Na_3VO_4 inhibited D-M-induced hyperpolarization without affecting R_t . $n = 6$, * $p < 0.05$.

Chapter III

Volume Change in Guinea-pig Airway Epithelial Cells Induced by Methacholine and Hyperosmolar Challenge

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Running title: Airway Epithelial Cell Shrinkage Induced by Hyperosmolarity

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Abbreviations: ASL, airway surface liquid; EpDRF, epithelium-derived relaxing factor; MKH, modified Krebs-Henseleit; MCh, methacholine; D-M, D-mannitol.

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Abstract

Airway epithelium modulates underlying smooth muscle tone by releasing epithelium-derived relaxing factor (EpDRF) in response to hyperosmolarity of the airway surface liquid (ASL). The mechanism by which the epithelium senses the increase in osmolarity to release EpDRF is largely unknown. Studies in other cell types have demonstrated that shrinkage occurs in response to hyperosmolar challenge. Our earlier, functional studies have suggested that cell shrinkage does not necessarily evoke EpDRF release. However, these studies were not able to assess changes in cell volume directly. Therefore, we isolated airway epithelial cells from guinea-pig trachea with protease, and examined directly the cell volume response to hyperosmolarity using a cell multisizer. Hyperosmolar challenge with D-mannitol, over a concentration range that elicits EpDRF release and airway relaxation in functional studies using the isolated, perfused trachea, induced rapid, prolonged and concentration-dependent shrinkage, suggesting a potential link between epithelial shrinkage and EpDRF release.

Introduction

We have previously performed functional studies using guinea-pig isolated, perfused trachea apparatus to investigate the epithelial mechanisms involved in the regulation of airway diameter in response to hyperosmolar challenge. Hyperosmolarity of the airway surface liquid (ASL), occurring during exercise (Freed and Davis, 1999; Anderson and Daviskas, 2000), elicits changes in epithelial ion transport and initiates airway smooth muscle relaxation mediated *via* epithelium-derived relaxing factor (EpDRF); the bioelectric and mechanical responses are thought to be functionally linked (Dortch-Carnes et al., 1999; Fedan et al., 1999). Our previous studies performed with kinase inhibitors demonstrated that transepithelial potential (V_t) changes and EpDRF release in response to hyperosmolar challenge of the epithelium involved common signaling kinases, e.g., JNK, PKC and p38 (Chapters I and II). It is well known that hyperosmolar challenge causes cell shrinkage, and osmosensing mechanisms initiate signaling events which contribute to the regulation of adaptive responses (Schliess and Haussinger, 2006).

The airway epithelium behaves as an osmometer and transducer that sensitively perceives osmolarity changes in the ASL (Willumsen et al., 1994; Matsui et al., 2000; Fedan et al., 2004a). Although the fact that epithelium regulates the underlying smooth muscle tone by releasing EpDRF has been known for years (Munakata et al., 1988; Fedan et al., 1990), the mechanisms by which the epithelium senses increase in osmolarity to release EpDRF are still

poorly understood. Several hypotheses have been postulated for other cell types, including changes in pathways involving membrane tension response elements, cytoskeletal actin filament architecture, macromolecular crowding and mechanosensitive channels (Burg, 2000; Kinnunen, 2000; Papakonstanti et al., 2000; Zhang and Bourque, 2003; Okada, 2004). Recent studies have presented new evidence that airway epithelium senses the incremental increase in osmolarity rather than the volume changes or absolute osmolarity *per se* (Fedan et al., 2004a). However, cell volume changes in response to hyperosmolar challenge were not measured directly in that study. Currently none of these mechanisms has been firmly established. The multiple sensing mechanisms could exist selectively in different types of cells or be activated by the magnitude or nature of challenges (Strange, 2004). Thus, it is important to characterize the volume effects of hyperosmolar challenge of airway epithelium using direct measurement.

To understand the relationship between epithelial volume change and EpDRF release, we investigated the effects of hyperosmolar challenge on freshly isolated epithelial cells from guinea-pig trachea. The cell volume changes were measured under osmolar conditions used previously in functional experiments involving the perfused tracheal apparatus, and the results were compared with those obtained in the functional studies.

Materials and Methods

Animals. These studies were conducted in facilities accredited fully by the Association for the Assessment and Accreditation of Laboratory Animal Care International and were approved by the institutional Animal Care and Use Committee. Male guinea pigs (550 - 700 g), HsdPoc:DH, from Harlan (Indianapolis, IN), monitored free of endogenous viral pathogens, parasites, and bacteria were used in all experiments. The animals were acclimated before use and were housed in filtered ventilated cages on Alpha-Dri virgin cellulose chips and hardwood Beta-chips as bedding, provided HEPA-filtered air, Teklad 7006 diet and tap water ad libitum, under controlled light cycle (12 h light) and temperature (22 - 25 °C) conditions. The animals were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and sacrificed by thoracotomy and bleeding before removing the trachea.

Materials. Modified Krebs-Henseleit (MKH) solution (pH 7.4 at 37 °C) contained 113 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, and 5.7 mM glucose, and was saturated with 95% O₂ and 5% CO₂. The osmolarity of MKH was 281 ± 5 mosM. EMEM (minimum essential medium eagle) and FBS (fetal bovine serum) were obtained from Cambrex Bioscience (Walkersville, MD) and Invitrogen (Carlsbad, CA), respectively. Protease, DNase I, Acetyl-β-methylcholine chloride (methacholine, MCh) and D-mannitol (D-M) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of MCh and D-M were prepared in saline.

Preparation of Cell Suspension. Tracheas were removed and opened longitudinally by cutting through the smooth muscle band. The tracheas were incubated in 2 ml 0.2% protease in EMEM at 37°C for 1 h. Digestion was stopped with 10% FBS/EMEM solution at 4°C. The epithelia were gently scraped off with a scalpel blade, rinsed, and triturated in 10 ml of EMEM solution containing 0.1% DNase I to disaggregate clumps. The suspended cells were screened through a strainer (Falcon 40 mm nylon filter) and collected by centrifugation at 800 rpm for 5 min. The pellet of cells was suspended in 1 ml MKH solution saturated with 95% O₂ and 5% CO₂ at 37°C for 1 h.

Trypan Blue Assay. 4 µl of cell suspension was diluted with 16 µl MKH and then mixed with 20 µl 0.4% trypan blue solution (Sigma-Aldrich, St. Louis, MO). After incubating for 2 min, cells were evaluated under a microscope for the active beating of cilia and ability to exclude trypan blue.

Epithelial Volume Changes Induced by MCh and Hyperosmolar D-M Solutions. The effects of MCh and D-M on cell volume were examined under similar conditions, e.g., exposure times and concentrations, that were used previously in functional studies on isolated, perfused trachea (Chapters I and II). Because the relaxation response to hyperosmolar D-M was rapid and long-lasting in functional studies, the time-course response to D-M was measured initially beginning at 0 min and continuing for 5 h. Subsequently, the concentration-dependent responses to D-M (10, 30, 80 and 120 mosM) were examined for a period of 30 min. As in the protocol used in functional studies,

epithelial suspensions were exposed to MCh (3×10^{-7} M) for 15 min before exposing to D-M (120 mosM) for another 15 min.

Cell Volume Measurement. Epithelial cell diameter was measured using a Coulter Multisizer (Model II; Coulter Scientific Instruments; Hialeah, Florida). Epithelial cells were suspended in MKH solution lacking or containing MCh and/or D-M for this measurement. Volumes expressed in picoliter (pI) were calculated from cell diameters (μm).

Data Analysis. Cell volumes measured in the presence of MCh and D-M were compared to their sizes at the starting point ($t = 0$) in the time-course and concentration-dependence experiments. The effects of MCh and D-M on cell volume were analyzed by one way repeated-measures analysis of variance. $p < 0.05$ was regarded as significant. Each experiment was repeated at least 4 times.

Results

Epithelial Cell Morphology and Cilia Movement. Epithelial cells were examined by microscopy. Epithelia were scraped off in small sheets; their cilia were actively beating after the protease digestion (Figs. CIII-1A and CIII-1B). After filtration, most of the cells were separated individually; there were some occasional doublets (Figs. CIII-2A and CIII-2B). The average diameter of the cells (at $t = 0$) was 9.5 - 10 μm . Active beating of cilia lasted for more than 5 h (Figs. CIII-2A and CIII-2B). Cell suspensions with more than 85% of cells excluding trypan blue were used for these experiments.

Time-Dependent Effects of D-M. Epithelial cells suspended in MKH solution gradually reduced their volume during a 5 h period. However, the volume change over the first 60 min was not significant (Fig. CIII-3). Therefore, the effects of MCh and D-M were examined during the initial 30-min period. D-M (120 mosM) caused a rapid decrease in volume, which reached about 22% shrinkage at 3 min, compared to their original ($t = 0$) volume. Only a small regulatory volume increase was observed during the following 5 h (Fig. CIII-3).

Concentration-Dependent Effects of D-M. Hyperosmolar D-M induced a rapid and concentration-dependent decrease in epithelial cell volume. The shrinkage induced by 10, 30, 80 and 120 mosM D-M solutions at 3 min were about 7%, 9%, 20% and 22%, respectively, compared to their original ($t = 0$) volume. No regulatory volume increase was observed (Fig. CIII-4).

Volume Changes Induced by MCh and Hyperosmolar D-M. The experimental conditions with respect to MCh and D-M were similar to those previously used in the trachea perfusion experiments. MCh (3×10^{-7} M) had no effect on the volume of epithelial cells. D-M (120 mosM) caused similar epithelial cell shrinkage in the absence and presence of MCh (Fig. CIII-5).

Discussion

Hyperventilation during exercise is thought to result in hyperosmolarity of the ASL due to evaporative water loss (Freed and Davis, 1999; Anderson and Daviskas, 2000). Airway epithelium has been recognized as an osmometer that sensitively perceives osmolarity changes in the ASL and regulates the underlying smooth muscle tone by releasing EpDRF (Matsui et al., 2000; Fedan et al., 2004a). Our functional studies demonstrated that in response to hyperosmolar challenge the epithelial bioelectric responses and EpDRF release are functionally linked (Dortch-Carnes et al., 1999; Fedan et al., 1999); and kinase signaling plays a role in regulating the epithelial ion transport and EpDRF-mediated airway relaxation (Chapters I and II). In order to understand the relationship between epithelial volume change and EpDRF release we developed a novel method with cell suspensions freshly prepared from guinea-pig trachea to investigate the epithelial volume changes under the pharmacological conditions that were previously used in the functional studies.

Under the microscope most freshly prepared epithelial cells exhibited diameters between 9.5 and 10 μm , which matched the values obtained from the Coulter Multisizer. The lengths of the cilia were a little smaller than the diameters of their cells. In normal airways the length of the epithelial cilia has been reported to be approximately 7 μm (Tarran, 2004). Suspended in MKH solution, epithelial cells could live for more than 5 h, during which active cilia beating was observed and trypan blue was excluded by most of the cells. Although the cells gradually

lost volume after being suspended in MKH solution, the volume changes were not significant over the first 60 min. This window allowed us to examine the volume effects induced by MCh and hyperosmolar D-M. In functional studies, the airway contraction and relaxation responses to MCh and D-M, respectively, require about 15 – 20 min to reach their plateaus (Chapters I and II).

Hyperosmolar challenge with D-M solutions induced a rapid, prolonged and concentration-dependent decrease in cell volume. The shrinkage reached plateau at about 3 min. Although 120 mosM hypertonic medium caused a 40% reduction in the volume of rat skeletal muscle cells (Zhao et al., 2004), the maximal cell shrinkage observed in our time-dependent and concentration-dependent studies with the highest exposure, 120 mosM D-M, was about 22%. 30 mosM D-M induced about a 10% reduction in epithelial volume, suggesting that it is close to the EC₅₀ concentration for its effect on cell shrinkage. This value is comparable to the EC₅₀ of D-M as a relaxant of the guinea-pig perfused trachea.

Like many other types of mammalian cells (O'Neill, 1999), no regulatory volume increase was observed in most of the groups of this study after hypertonic exposure. In guinea-pig colonic epithelial cells, muscarinic stimulation with carbachol induced no cell volume change in the upper region of crypts but shrinkage in the basal region of crypts within 10 min and the volume was recovered by 20 min (Manabe et al., 2004). In this study, MCh had no effect on the volume of airway epithelia at 15 min, but the cells were shrunk by hyperosmolar D-M 15 min later.

The results indicate that this novel method is useful for the study of epithelial volume changes in response to anisotonic conditions. Hyperosmolar challenge, over a concentration range that elicits EpDRF release in the functional studies, induces rapid, long lasting and concentration-dependent shrinkage in airway epithelia, suggesting a possible link between epithelial shrinkage and EpDRF release.

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A

B

Fig. CIII-1. Clump of the airway epithelial cells after protease digestion. (A) 40x objective, (B) 60x objective. Epithelia were scraped off in small sheets. Cilia were actively beating after enzyme treatment.

A

B

Fig. CIII-2. Isolated airway epithelial cells in MKH solution. (A) 60x objective, (B) 100x objective. Epithelial clumps were gently separated by trituration into single cell suspensions. More than 85% of the cells excluded trypan blue for up to 5 h, during which active cilia beating were observed.

Fig. CIII-3. Time-dependent effects of D-M (120 mosM) on the volume of isolated airway epithelial cells. The volume of un-challenged epithelial cells suspended in MKH solution was measured every 60 min for 6 h, beginning with $t = 0$. To monitor the shrinkage of epithelial cells exposed to D-M, cell volume was measured at 0, 1, 3, 5, 10, 20, 30, 45, 60, 120, 180, 240 and 300 min. Suspended in MKH solution, epithelia gradually reduced their volumes and reached plateau at 5 h. Their volume change was not significant over the first 60 min. D-M induced a rapid decrease in volume, which reached about 22% shrinkage, between 3 to 5 minutes. Only a small regulatory volume increase was observed during the following 5 h. $n = 4$, $*p < 0.05$ compared to $t = 0$.

Fig. CIII-4. Concentration-dependent effects of D-M on the volume of isolated airway epithelial cells. The time points of volume measurement were 0.5, 1, 3, 5, 10, 20 and 30 min. D-M induced a rapid decrease in cell volume at all concentrations. The percentage of the cell shrinkage induced by 10, 30, 80 and 120 mosM D-M solutions at 3 min was around 7%, 9%, 20% and 22%, respectively, compared to their original ($t = 0$) volumes. $n \geq 4$, $*p < 0.05$. No regulatory volume increase was observed.

Fig. CIII-5. Airway epithelial volume changes induced by MCh (3×10^{-7} M) and D-M (120 mosM). Exposure of suspended epithelia to MCh for 15 min (the time required for MCh-induced contraction to reach plateau in functional studies) had no effect on cell volume. Exposure of suspensions to D-M for another 15 min (the time needed for D-M-induced relaxation to reach plateau in functional studies) caused epithelial shrinkage in the absence and presence of MCh. $n = 4$, $*p < 0.05$.

General Conclusions

A physiologically-relevant osmolarity increase in the ASL caused by exercise induces airway epithelial hyperpolarization and smooth muscle relaxation; the two responses share the same osmolarity concentration-dependence. The concentrations which induce the maximal relaxation and the transition of bioelectric responses from hyperpolarization to depolarization are coincidentally around 80 mosM. The depolarization elicited by more than 80 mosM hyperosmolarity is primarily caused by an inhibition of epithelial ion transport but not by a decrease of transepithelial resistance (Fig. GC-1).

The guinea-pig trachea perfusion apparatus which allows ΔP , V_t and R_t to be monitored simultaneously is useful for the study of airway physiology and pharmacology. PKC is intimately involved in the regulation of epithelial ion transport. JNK and protein phosphatase play a role in the epithelial bioelectric responses induced by MCh and D-M, respectively. The permeability of epithelial tight junctions could be regulated by common protein kinases, such as JNK.

Although some signaling kinases, e.g., PKC, are involved in the regulation of basal V_t and ΔP , as well as V_t responses induced by MCh and D-M, only the inhibition of p38 potentiated the airway relaxation elicited by hyperosmolar challenge, suggesting that p38 inhibitors could potentially have the therapeutic effect of reducing the bronchoconstriction induced by exercise. A common signaling pathway regulating both the bioelectric and mechanical responses to hyperosmolarity was not identified; therefore, in terms of kinase signaling, there

may not be a cause-effect relationship between the two responses.

Hyperosmolar challenge elevates the phosphorylation of p38 and JNK in airway epithelia and many other types of cells. The inhibitors of p38 are being developed as anti-asthma medicines because p38 signaling contributes to the pathogenesis of airway inflammation and hyperresponsiveness. The results of this study provided additional support for the notion that p38 inhibitors also have the potential for the treatment of bronchoconstriction induced by exercise possibly through the potentiation of EpDRF release (Fig. GC-2).

Hyperosmolar challenge over a concentration range that elicits epithelial bioelectric events and EpDRF release in the functional studies also induces concentration-dependent decreases in epithelial cell volume. The rapid and prolonged shrinkage is similar to the quick and long-lasting bioelectric and mechanical responses, suggesting a possible link between epithelial shrinkage and EpDRF release.

Fig. GC-1. Proposed model of epithelial ion transport and EpDRF release induced by hypersmolarity. Hypersmolar challenge lower than 80 mosM threshold induces secretion of Cl^- and K^+ and activation of the $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter, and possibly, secondary activation of the Na^+-K^+ -pump, causing hyperpolarization. Although the activity of electroneutral $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter may remain unchanged, more than 80 mosM hypersmolar challenge could inhibit the activity of Na^+-K^+ -pump and secretion of Cl^- and K^+ , which was evidenced by the significant decrease of transepithelial short-circuit current (Wu et al., 2004), causing depolarization. This conclusion was also supported by the finding that the decreased V_t did not result from the decrease of R_t . Hypersmolarity always induces airway smooth muscle relaxation *via* EpDRF release. The maximal response was elicited by an 80 mosM increase in osmolarity.

Fig. GC-2. Effects of p38 inhibitors on hyperosmolarity-induced airway smooth muscle response. Hyperosmolar challenge activates p38 in both inflammatory and airway epithelial cells, causing contractile mediator release and inhibition of EpDRF release, respectively. Inhibitors of p38 block the activities of p38 signaling and decrease the airway smooth muscle tone, which could be useful for the treatment of exercise-induced airway obstruction.

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Curriculum Vitae

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EDUCATION

2000 – 2007	Ph.D., Pharmacology. Department of Biochemistry and Molecular Pharmacology, West Virginia University, Morgantown, West Virginia.
1989 – 1992	M.S., Pharmacology and Toxicology. Institute of Pharmacology and Toxicology, Beijing, China.
1981 – 1986	M.D., Medicine. Tianjin Medical College, Tianjin, China.

EXPERIENCE

2000 – 2007	Graduate Student. Department of Biochemistry and Molecular Pharmacology, West Virginia University. Under the guidance of Dr. Jeffrey Fedan, working on a project investigating the airway epithelial mechanisms in exercise-induced asthma. Elucidate the relationship between airway transepithelial bioelectric and smooth muscle mechanical responses evoked by luminal hyperosmolarity. Investigate epithelial signaling mechanisms in regulating airway diameter using protein kinase inhibitors and ion transport blockers. Confirm the involvement of MAP kinases in epithelial signaling using western blots. Characterize volume change of isolated epithelial cells during hyperosmolar challenge.
1999 – 2000	Research Fellow in Dr. Yao-tseng Chen's laboratory. Ludwig Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center. Involved in the immunoscreening of expressing tumor cDNA libraries and characterizing the isolated clones. Prepared cDNA libraries from tumor specimens or cell lines and screened them with autologous or allogeneic serums from cancer patients. Analyzed the selected cDNA clones by DNA sequencing and mRNA expressing in normal and tumor tissues. Expressed cDNA clones showing differential mRNA tissue expression to make specific antibodies. Identified tumor antigens with restricted tissue expression and used as targets for immunotherapy and/or immunodiagnosis.
1997 – 1999	Research Associate in Dr. Neena Singh's laboratory. Department of Pathology, Case Western Reserve University. Worked on the cell and molecular biology of prion disease, especially on the analysis of an inherited form of CJD that co-segregated with a mutation in the prion

protein gene. Investigated synthesis and processing of mutant prion protein in order to elucidate the pathogenic mechanisms of prion disease with *in vitro* models.

- 1994 – 1997 Research Assistant. Institute of Medical and Pharmaceutical Sciences. Tianjin, China. In charge of evaluating the toxicity of synthetic medical materials, and establishing the cell cultural methods on microcarrier and microencapsulation.
- 1993 – 1994 Research Assistant. Institute of Biotechnology, Beijing, China. Participated in the research of pro-urokinase (pro-UK) gene. Removed the signal peptide sequence from the human pro-UK gene; inserted non-signal peptide sequence of human pro-UK cDNA into pBV220 just under the control of temperature-induced promoter PR PL. Successfully expressed mature pro-UK in *E. coli* at 42°C.
- 1989 – 1992 Graduate Student. Institute of Pharmacology and Toxicology, Beijing, China. Investigated the carcinogenicity of tris(2-methyl-1-aziridiny)phosphine oxide (MAPO) and unsymmetrical dimethyl hydrazine (UDMH). Completed the thesis “SHE cell malignant transformation induced by MAPO and UDMH *in vitro*,” and the project “Unscheduled DNA synthesis induced by MAPO and UDMH.”
- 1986 – 1989 Research Intern. Institute of Hygiene and Environmental Medicine. Tianjin, China. For the approval of a second class new medicine, sustained-effect riboflavin injection, completed all the required evaluations for its toxicity.

PUBLICATIONS

Papers

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1. Jing Y., Dowdy J., Van Scott M. R. and Fedan J. S. Epithelial kinase signaling in airway bioelectric and mechanical responses induced by hyperosmolarity (submitting to J Pharmacol Exp Ther).
2. Jing Y., Dowdy J., Van Scott M. R. and Fedan J. S. Epithelial mechanisms in airway bioelectric and mechanical responses induced by hyperosmolarity: Studies on a novel tracheal perfusion apparatus (submitting to J Pharmacol Exp Ther).
3. Jing Y., Dowdy J., Van Scott M. R. and Fedan J. S. Volume changes in guinea-pig airway epithelial cells induced by methacholine and hyperosmolar challenge (in preparation).

PATENT

Co-inventor of the pending patent "Device to measure muscle contractile/relaxant and epithelial bioelectric responses of perfused, intact airways *in vitro*."

AWARDS

2005	Travel award to Experimental Biology meeting, American Society for Pharmacology and Experimental Therapeutics.
2004	Oral presentation award, Van Liere research convocation, Office of Research and Graduate Studies, West Virginia University.
2003 - 2005	Predoctoral Fellowship "Epithelial mechanism involved in exercise induced asthma." American Heart Association.