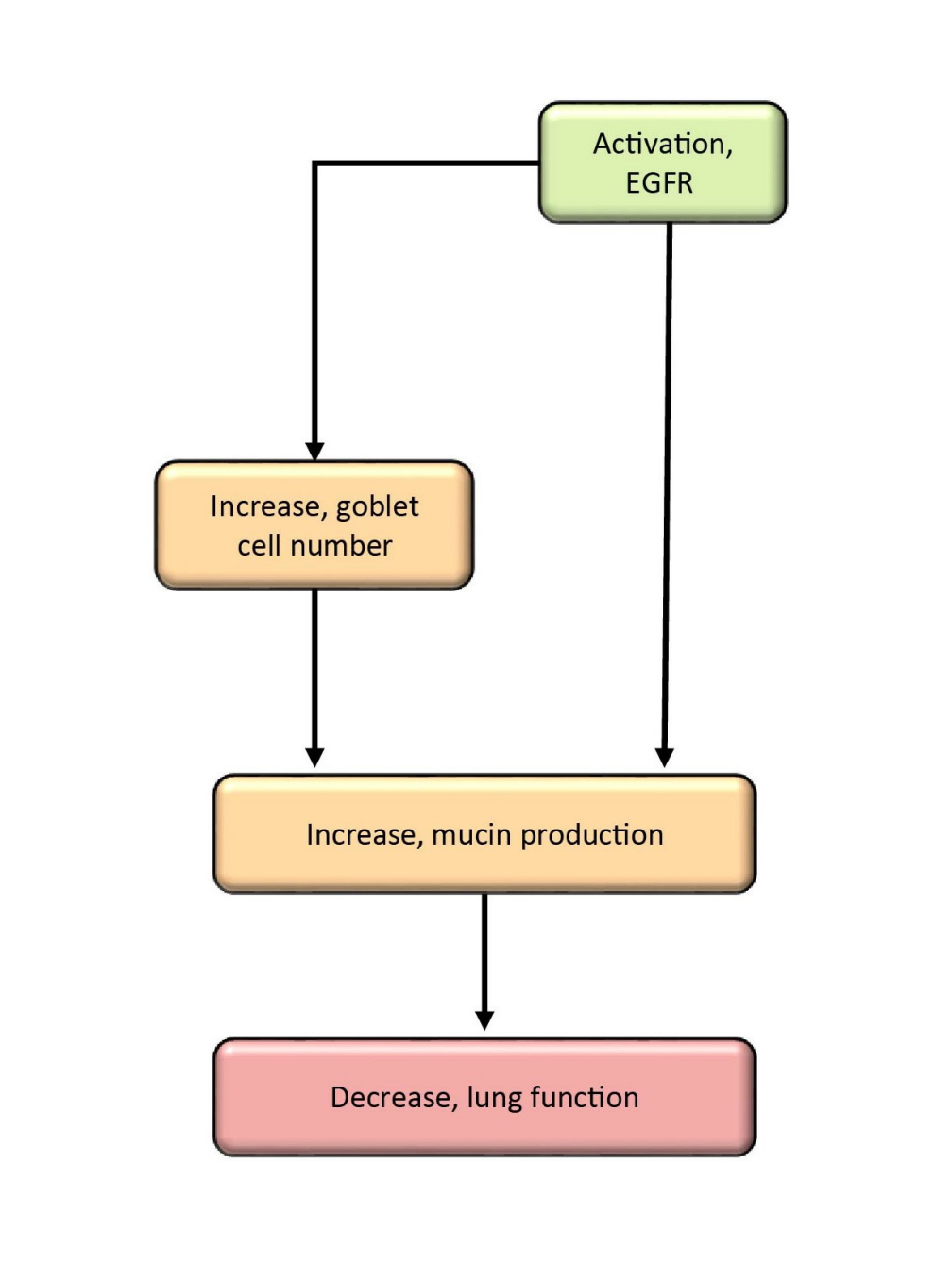
AOP ID and Title:

AOP 148: EGFR Activation Leading to Decreased Lung Function
Short Title: Decreased lung function

Graphical Representation



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Status

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Abstract

Increase in mucin production and consequent mucus hypersecretion in the airways are key attributes of many lung diseases, including asthma, cystic fibrosis and chronic bronchitis, all of which are characterized by decreased lung function (Yoshida and Tuder, 2007). Mucus hypersecretion is characterized by an increase in the number of goblet cells, mucin synthesis and mucus secretion which can result in airway obstruction and lung function decline (Kim and Criner, 2015, Yoshida and Tuder, 2007). Epidermal growth factor receptor (EGFR)-mediated signaling has been identified as the key pathway that leads to airway mucin production (Burgel and Nadel, 2008). This AOP for decreased lung function originates in EGFR activation in the airway epithelium. It describes the subsequent key events on the cellular and organ level that need to take place to culminate in the adverse outcome. The causal relationships in this AOP, including EGFR activation leading to increased number of mucin-producing goblet cells and to increased mucin production, are substantiated by multiple lines of evidence in studies performed using different model systems and approaches. Understanding how the inhaled toxicant-induced EGFR activation leads to pulmonary function impairment will be relevant to risk assessment of airborne pollutant exposure and how they contribute to the development and progression of the disease. Additionally, understanding the molecular underpinnings of these processes can aid in informing regulatory decision-making to assess the impact of inhalation toxicants on public health outcomes.

Background

This AOP delineates a sequence of key events initiating with stressor-induced activation of EGFR and resulting in decreased lung function through increased production of mucins. Excessive mucin production and consequent mucus hypersecretion are characteristic features of chronic diseases such as chronic obstructive pulmonary disease (COPD), cystic fibrosis, chronic bronchitis, and asthma, which pose a significant public health burden. Of note, exposure to cigarette smoke, occupational respiratory hazards, and air pollutants are clearly linked to the development of COPD, which is predicted to become the third leading cause of death worldwide by 2030 (Viegi et al., 2007, WHO, 2008). Mucus hypersecretion during the disease course can result in airway obstruction, decreased peak expiratory flow, respiratory muscle weakness, leading to decreased lung function (Kim and Criner, 2015, Yoshida and Tuder, 2007). Lung function decrease can have serious consequences and is associated with increased mortality (Panizza et al., 2006). This AOP is aimed to compile and organize the vast knowledge around molecular and cellular events and their relationships leading to lung function decrease with an overarching goal to facilitate the prediction and assessment of decreased pulmonary function. In vitro assays spanning from cell culture to organ system assays, with an aid of in silico methodology, all performed in human context, could be applied to measure each KE for inhaled toxicant assessments and adverse outcome predictions, and would contribute to eventual replacement of in-vivo tests in animals. This concept of hazard assessment and AO prediction aligns with integrated approach to testing and assessment (IATA) framework as a mechanistic support for regulatory decision-making (Clippinger et al., 2018).

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
1	MIE	941	Activation, EGFR	Activation, EGFR
	KE	2117	Increase, goblet cell number	Increase, goblet cell number
7	KE	962	Increase, Mucin production	Increase, Mucin production
8	AO	1250	Decrease, Lung function	Decreased lung function

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Activation, EGFR	adjacent	Increase, goblet cell number	High	High
Activation, EGFR	adjacent	Increase, Mucin production	High	High
Increase, goblet cell number	adjacent	Increase, Mucin production	High	Moderate
Increase, Mucin production	adjacent	Decrease, Lung function	Moderate	Moderate

Stressors

Name	Evidence
Reactive oxygen species	High

Reactive oxygen species

Various sources of ROS, including glucose oxidase, xanthine/xanthine oxidase, acrolein, H_2O_2, cigarette smoke extract, phorbol 12-myristate 13-acetate (PMA), 2,3,7,8-tetrachlorodibenzodioxin (TCDD), and supernatant from activated neutrophils or eosinophils cause a measurable, rapid increase in EGFR phosphorylation in human airway epithelial cells and the lungs of F344 rats (Ravid et al., 2002; Hewson et al., 2004; Casalino-Matsuda et al., 2006; Casalino-Matsuda et al., 2004; Deshmukh et al., 2008; Qi et al., 2010; Takeyama et al., 2001;Takeyama et al., 2000; Burgel et al. 2001; Kim et al. 2008; Yu et al. 2015; Yu et al., 2011; Lee et al.; 2011).

Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult	High
Juvenile	Low

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mouse	Mus musculus	Moderate	NCBI
rat	Rattus norvegicus	Moderate	NCBI

Sex Applicability

Sex	Evidence
Unspecific	

Life Stage Applicability

EGFR activation leading to increased mucin production and decreased lung function is predominantly studied in adults; however, it has been shown to alsooccur in pediatric asthma and bronchitis (Parker et al., 2015, Rogers, 2003). Nevertheless, the environmental exposures that induce EGFR activation and ultimately lead to lung function decline may apply more to adults who are more likely to be exposed to these stimulants over time (cigarette smoke, particulate matter).

Taxonomic Applicability

The evidence presented here is derived from both human patient, cell culture and animal model biological systems.In vitroandin vivostudies in these systems have been performed to clarify the mechanisms of EGFR activation leading to mucus hyperproduction by studying the increase in goblet cells and upregulation in mucin transcript and protein expression. There are several clinical studies on mucus hypersecretion and how it affects lung function in humans with chronic bronchitis, asthma and other chronic lung diseases. The use of laboratory animals in human disease phenotype modelling enhances the understanding of disease mechanisms but also has limitations, e.g. due to anatomic differences between human and animal airways, differences in disease severity, difficulty of lung function measurements (Nikula and Green, 2000, Fricker et al., 2014). In summary, assembled data suggest that the KEs of this AOP are preserved across rodents and humans and there is good evidence supporting the occurrence of KERs in these species.

Sex Applicability

At times, clinical evidence linked to occupational exposures is derived from a majority of male subjects, which could be related to a male predominance in certain professions (Eng et al., 2011; Kennedy et al., 2007). Similarly, in most Western countries, cigarette smoking is still more prevalent in men than in women, although this gap has been closing steadily over the past decades (Syamlal et al., 2014; Hitchman and Fong, 2011). Nevertheless, the availablein vivoand clinical evidence suggest that there is no remarkable gender difference.

Essentiality of the Key Events

MIE: EGFR activation

EGFR signaling is considered critical for mucin production (Vallath et al., 2014). Large amount of studies indicate that activation of EGFR through stressors and receptor ligands increase goblet cell numbers and mucin production while inhibition of EGFR

decreases mucin production or goblet cell numbers (Barbier et al., 2012, Casalino-Matsuda et al., 2006, Choi et al., 2021, Deshmukh et al., 2008, Hao et al., 2014, Huang et al., 2017, Jia et al., 2021, Kato et al., 2022, Lee et al., 2000, Lee et al., 2011, Memon et al., 2020, Parker et al., 2015, Perrais et al., 2002, Shim et al., 2001, Song et al., 2016, Takeyama et al., 1999, Takeyama et al., 2001, Takeyama et al., 2008, Takezawa et al., 2016, Tyner et al., 2006b, Val et al., 2012, Wang et al., 2019, Yu et al., 2012a, Yu et al., 2012b). As for downstream AO, several studies indicate positive correlation between EGFR pathway activation and lung function decrease (Singanayagam et al., 2022, Feng et al., 2019, Lin et al., 2021). Taken together, and considering the strong causal link of EGFR activation on adjacent KEs, we propose high essentiality for the MIE “Activation, EGFR” in the AOP148.

KE: Increase, mucin production

Increased airway mucin production is a necessary condition for mucus hypersecretion. The stressor exposure maintenance and goblet cell number increase are important for sustained mucin production increase, otherwise the mucus hypersecretion resolves following re-establishment of airway homeostasis by anti-inflammatory mechanisms (Rose and Voynow, 2006). Mucus hypersecretion is a key feature of many lung diseases, including chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis and chronic bronchitis, all of which are characterized by decreased lung function. Evidences from literature indicate that sustained increased mucin production with consequent mucus hypersecretion correlate with lung function decrease (Caramori et al., 2009, Innes et al., 2006, Vestbo and Rasmussen, 1989, Vestbo et al., 1996, Ramos et al., 2014). Overall, increased mucin production is necessary but not always sufficient for leading to downstream events. Given the requirement of the KE for AO to occur we suggest high essentiality for the KE “Increase, mucin production” in the AOP148.

KE: Increase, goblet cell number

Goblet cells are specialized cells for mucin expression. Following stressor exposure, goblet cell numbers can increase which provides capacity to increase mucin production. Increased goblet cell numbers which can result from goblet cell hyperplasia and/or metaplasia sustain airway mucin overproduction contributing to airway obstruction and consequent lung function decline (Rose and Voynow 2006). Several studies show positive correlation between increase in goblet cell number and decrease in lung fuction (Innes et al., 2006, Raju et al., 2016, Ma et al., 2005, Celly et al., 2006). Considering above-mentioned, we propose high essentiality for the KE “Increase, goblet cell number” in the AOP148.

Weight of Evidence Summary

Biological Plausibility

KER: EGFR activation leads to Increase, mucin production

Large number of studies using EGFR-activating ligands and EGFR inhibitors consistently show causal link leading from EGFR activation to increased production of mucin proteins (Barbier et al., 2012, Casalino-Matsuda et al., 2006, Choi et al., 2021, Deshmukh et al., 2008, Hao et al., 2014, Huang et al., 2017, Jia et al., 2021, Kato et al., 2022, Lee et al., 2000, Lee et al., 2011, Liu et al., 2013, Memon et al., 2020, Parker et al., 2015, Perrais et al., 2002, Shim et al., 2001, Song et al., 2016, Takeyama et al., 1999, Takeyama et al., 2001, Takeyama et al., 2008, Takezawa et al., 2016, Val et al., 2012, Wang et al., 2019, Yu et al., 2012a, Yu et al., 2012b). EGFR activation as a leading pathway for increased mucin production has broad acceptance in the scientific community and has been discussed also in review articles (Burgel and Nadel, 2004, Lai and Rogers, 2010). Therefore, we propose high biological plausibility for this KER.

KER: EGFR activation leads to Increase, goblet cell number

EGFR ligands and variety of stressors such as oxidative stress, cigarette smoke, allergens, viruses and bacterial endotoxins increase goblet cell number in an EGFR-dependent manner (Casalino-Matsuda et al., 2006, Gu et al., 2008, Hirota et al., 2012, Jia et al., 2021, Parker et al., 2015, Shatos et al., 2008, Song et al., 2016, Takeyama et al., 1999, Takeyama et al., 2001, Takezawa et al., 2016, Tyner et al., 2006a). Given the strong empirical evidence for involvement of EGFR in regulating the number of goblet cells and high reproducibility demonstrated in both in vitro and in vivo studies, we suggest high biological plausibility for this KER.

KER: Increase, goblet cell number leads to Increase, mucin production

Goblet cells are specialized cells for mucin production. The increase in the number of goblet cells is needed to accommodate the increased need for mucin production indicating that this KER is an inferred relationship, i.e. the occurrence of the downstream KE is inferred from the fact of occurrence of the upstream KE. Many studies demonstrate the correlation between increase in goblet cell numbers and mucin production (Zuhdi Alimam et al., 2000, Takezawa et al., 2016, Hao et al., 2012, Innes et al., 2006, Liang et al., 2017, Lee et al., 2000, Casalino-Matsuda et al., 2006, Tyner et al., 2006b), in fact the accepted measure of goblet cell number increase is the enhanced staining for mucus in the tissues. Thus, we judge the KER as highly plausible.

KER: Increase, mucin production leads to Decrease, lung function

Increased mucus production and hypersecretion is a physiological response to harmful exposures. This response is typically of short duration and does not pose a major problem to normal lung function. However, in the presence of sustained mucus production and secretion, maintained and promoted through increased number of mucin producing goblet cells, airways can become obstructed and result in lung function decline. In addition, impaired mucociliary clearance contributes to airway obstruction (Whitsett, 2018) and it is currently unclear whether chronic mucus hypersecretion alone is sufficient to elicit a decrease in lung function. Clinical studies and model animal research showed that MUC5AC production was inversely correlated with parameters of lung function (FEV1 (% predicted), FEV1/FVC ratio, inspiratory capacity) (Caramori et al., 2009, Innes et al., 2006, Raju et al.,

2016), and epidemiological evidence indicates a link between mucus hypersecretion and decreased lung function (Allinson et al., 2016, Pistelli et al., 2003, Vestbo et al., 1996). As a cause-effect relationship cannot be conclusively proven, we suggest moderate biological plausibility for this KER.

Quantitative Consideration

There is good quantitative understanding of how EGFR signaling influences mucus production and goblet cell number increase. In the majority of these studies, the summary evidence indicates dose-response relationships, time-response relationships, and causality for EGFR activation leading adjacent downstream KEs, lending strong support for these KERs. However, data for increased mucin production and mucus hypersecretion leading to lung function decline at the organism level are mainly derived from surrogate measures, and while those may not adequately reflect quantitative mucus production, they are accepted in the clinical community as an indicator of lung diseases, such as COPD, chronic bronchitis and asthma.

Considerations for Potential Applications of the AOP (optional)

The future application of this AOP lies in its potential for predicting decreased lung function in humans exposed to potentially harmful inhaled substances. This becomes especially pertinent as impaired lung function carries a significant risk of morbidity and mortality. Owing to the long latency period between exposure and detectable decreases in lung function for most environmental pollutants, together with the fact that lung function tests alone may not be sufficiently sensitive to account for early lung damage that remains asymptomatic, means for early identification of potentially hazardous exposures are critical for the development of appropriate public health interventions. The AOP could provide a framework for mapping out suitable in vitro models and tests for evaluation of distinct KEs in different exposure contexts thus contributing to eventual replacement of in-vivo tests in animals. The predictive power of AOP aligns well with IATA framework to integrate diverse sources of information as a mechanistic support on chemical hazard characterization.

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Appendix 1

List of MIEs in this AOP

Event: 941: Activation, EGFR

Short Name: Activation, EGFR

Key Event Component

Process	Object	Action
epidermal growth factor-activated receptor activity	epidermal growth factor receptor	occurrence
phosphorylation	epidermal growth factor receptor	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:148 - EGFR Activation Leading to Decreased Lung Function	MolecularInitiatingEvent

Stressors

Name
Cigarette smoke
Acrolein
Hydrogen peroxide
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)
Nicotine
benzo[a]pyrene
PM 2.5
Wood smoke
2,3-Butanedione

Name
Carbon nanotubes
Ozone
1,2,5,6,9,10-Hexabromocyclododecane
Tetrabromobisphenol A

Biological Context

Level of Biological Organization
Molecular

Cell term

Cell term
epithelial cell

Organ term

Organ term
lung

Evidence for Perturbation by Stressor

Overview for Molecular Initiating Event

EGFR activation in lung epithelial cells can be triggered by exposure to H_2O_2 (Goldkorn et al., 1998; Takeyama et al., 2000), naphthalene (Van Winkle et al., 1997), cigarette smoke (Takeyama et al., 2001; de Boer et al., 2006; Marinaş et al., 2011; Yu et al., 2011; Yu et al., 2015), acrolein (Deshmukh et al., 2008), and TCDD (Lee et al., 2011). Mechanistically, this process is dependent on ROS-mediated activation of metalloproteinases or ADAMs which cleave membrane-bound EGFR ligand precursors, making them locally available to bind to and transactivate EGFR in an autocrine manner (Deshmukh et al., 2009; Kim et al., 2004; Val et al., 2012; Yoshisue and Hasegawa, 2004). Furthermore, ligand binding to EGFR itself was shown to lead to H_2O_2 production, thereby facilitating receptor activation and downstream signaling (DeYulia et al., 2005; DeYulia and Cárcamo, 2005; Truong and Carroll, 2012).

Cigarette smoke

EGFR phosphorylation increased in lungs of Sprague-Dawley rats that were whole-body exposed (inExpose smoking system; SCIREQ, Montreal, Canada) at a total particulate matter (TPM) concentration of 2000 mg/m^3 for 1 h (20 cigarettes) daily for 56 days (Chen et al., 2020); in lungs of Sprague-Dawley rats exposed to 12 cigarettes daily for 40 days (Nie et al., 2012); inlungs of Sprague-Dawley rats that were whole body-exposed to six nonfiltered cigarettes per day, 5 d/wk, for 2 to 28 days (Hegab et al., 2007); in lungs of Sprague-Dawley rats exposed to 10 cigarettes per h, 6 h per day for 60 days (Wu et al., 2011); in lungs of C57Bl6/J mice exposed to cigarette smoke at a TPM concentration of 100 mg/m^3 (Teague Enterprises, Davis, CA) for 6 h a day, 5 days a week for two weeks (Mishra et al., 2016); in lungs of A/J mice that were exposed to cigarette smoke at a TPM concentration of 80 mg/m^3 (Teague Enterprises, Davis, CA) for 4 h a day, 5 days per week for 1 year (Geraghty et al., 2014); in lungs of Balb/c mice exposed to mainstream cigarette smoke for 2 h twice daily, 6 days per week for 4 weeks (Wang et al., 2018); in primary human bronchial epithelial cells and NuLi-1 bronchial epithelial cell monolayers following exposure to cigarette smoke (Mishra et al., 2016); in primary bronchial epithelial cell monolayers following treatment with cigarette smoke extract (Zhang et al., 2012); in primary human airway epithelial cells differentiated at the air-liquid interface following treatment with cigarette smoke extract (Zhang et al., 2013; Hussain et al., 2018; Cortijo et al., 2011; Chen et al., 2010) or exposure to whole mainstream cigarette smoke (Amatngalim et al., 2016); in human small airway epithelial cell monolayers following treatment with cigarette smoke extract (Geraghty et al., 2014; Agraval and Yadav, 2019); in human NCI-H292 lung cancer cells following treatment with cigarette smoke extract (Takeyama et al., 2001; Shao et al., 2004; Lee et al., 2006; Yang et al., 2012; Wang et al., 2018); in human A549 lung cancer cells following treatment with cigarette smoke extract for 15 min (Dey et al., 2011) or 3 h (Agraval and Yadav, 2019); in immortalized human bronchial epithelial 1HAEo cells following exposure to cigarette smoke (Zhang et al., 2005); human immortalized 16HBE bronchial epithelial cells following treatment with 10% cigarette smoke extract for 24 h (Yu et al., 2015) or 5% cigarette smoke extract for up to 6 h (Heihjink et al., 2012); A549 lung adenocarcinoma and HBE1 papilloma virus-immortalized human bronchial epithelial cells following exposure to cigarette smoke (Khan et al., 2008).

EGFR phosphorylation was approx. two-fold higher in lung tissues, alveolar type II and bronchial epithelial cells of healthy smokers compared to non-smokers and was also elevated in the lungs and lung epithelial cells of COPD smokers (Mishra et al., 2016).

Acrolein

EGFR activation was seen in human NCI-H292 lung cancer cells following treatment with 0.03 µM acrolein for 1 h (Deshmukh et al., 2005; Deshmukh et al., 2008).

Treatment of human normal oral keratinocytes with 5 µM acrolein for 3 h also increased EGFR phosphorylation (Tsou et al., 2021).

Hydrogen peroxide

H_2O_2 treatment increased EGFR tyrosine phosphorylation in NCI-H292 lung cancer cells (Takeyama et al., 2000) and in normal human nasal epithelial cells (Kim et al., 2008; Kim et al., 2010).

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

Phosphorylation of EGFR was significantly increased in normal human bronchial epithelial cells differentiated at the air-liquid interface following treatment with 10 nM TCDD for 0.5, 3, 4, and 6 h (Lee et al., 2011).

Nicotine

Increased EGFR phosphorylation was seen in normal human bronchial epithelial cells following treatment with 500 µM nicotine for 1 h (Martínez-García et al., 2008) or with 1 nM nicotine for 48 h (Lupacchini et al., 2021). EGFR activation is also seen in human lung cancer cells (A549, H1975) following treatment with 100 nM nicotine (Wang et al., 2020), human dysplastic oral keratinocytes following treatment with up to 10 µM nicotine (Wisniewski et al., 2018).

benzo[a]pyrene

EGFR phosphorylation increased in A549 lung cancer cells treated with 1 µM benzo[a]pyrene for 4 or 2 weeks (Kometani et al., 2009).

Treatment of immortalized human bronchial epithelial HBEC-2 and BEAS-2B cells with BPDE for 2 h increased EGFR activation in a dose-dependent manner (Xu et al., 2012).

PM 2.5

Intratracheal instillation of PM 2.5 (collected at a major city of central China; 4 mg/kg body weight) in Balb/c mice once a day for 5 consecutive days induced phosphorylation of EGFR (Tyr1068) in lung tissues (Jin et al., 2016).

Intratracheal instillation of PM 2.5 (collected at Seoul, Korea), either as an aqueous extract or a dichloromethane extract at high concentrations (164 µg/50 µL), in Balb/c mice once a week for 4 weeks induced phosphorylation of EGFR (Tyr1068) in lung tissues (Jeong et al., 2017).

Treatment of human immortalized bronchial epithelial BEAS-2B cells with with 0, 20, 50, 100 and 150 µg/mL PM 2.5 (collected on the roof of Science and Technology Building on the campus of Xinxiang Medical University, China) for 6 h increased EGFR Y1068 phosphorylation in a concentration-dependent manner (Wang et al., 2020).

Wood smoke

EGFR activation (increased phospho-EGFR (Y1068)) was seen in primary human lobar bronchial epithelial cells incubated with 20 µg/cm^2 pine wood smoke particulate matter (WSPM) for 6 h, but not at 2 h (Memon et al., 2020).

In NCI-H292 lung cancer cells stimulated with WSPM2.5 (8 µg/mL), EGFR phosphorylation increased continuously over time, with a significant increase observed at 60 min (Huang et al., 2017).

2,3-Butanedione

EGFR phosphorylation increased in H292 lung cancer cells following treatment with diacetyl (2,3-butanedione) (Kelly et al., 2019).

Carbon nanotubes

Treatment of rat RLE-6TN lung epithelial cells with 10 µg/cm^2 carbon nanoparticles (CNP Printex 90, Degussa, Essen, Germany) for 5 min significantly increased EGFR Tyr845 phosphorylation (Stöckmann et al., 2018).

Ozone

Exposure to O_3 (0.25–1.0 ppm) concentration- and time-dependently increased EGFR Y1068 and Y845 phosphorylation in human immortalized bronchial epithelial BEAS-2B cells (Wu et al., 2015).

Exposure of Balb/c mice to 0.25, 0.5, or 1.0 ppm ozone for 3 h a day, for 7 days increased EGFR Y1068 phosphorylation in the bronchial epithelium in a concentration-dependent manner (Feng et al., 2016).

1,2,5,6,9,10-Hexabromocyclododecane

EGFR phosphorylation increased significantly in human immortalized bronchial epithelial BEAS-2B cells following exposure to 10 µg/mL hexabromocyclodecane for 15 min (Koike et al., 2016).

Tetrabromobisphenol A

EGFR phosphorylation increased significantly in human immortalized bronchial epithelial BEAS-2B cells following exposure to 10 µg/mL tetrabromobisphenol A for 15 min (Koike et al., 2016).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	High

Sex Applicability

Sex	Evidence
Mixed	Moderate

EGFR activation in human, mouse and rat is well documented, and EGF ligands and EGFR are orthologous in these species. EGFR is a driver of human cancer in various tissues and numerous drugs are approved that inhibit EGFR activation (Ciardiello and Tortora, 2008). Although EGFR and its ligands are expressed in human, mouse and rat, species differences have been noted in binding and structure (Nexø and Hansen, 1985), and even can have opposite downstream effects in mouse and rat (Kiley and Chevalier, 2007).

Key Event Description

The epidermal growth factor receptor (EGFR, also referred to as ERBB1/HER1) is part of the ERBB family of receptor tyrosine kinases comprising another three distinct receptors, ERBB2/NEU/HER2, ERBB3/HER3 and ERBB4/HER4 (Yarden and Sliwkowski, 2001), all of which are transmembrane glycoproteins with an extracellular ligand binding site and an intracellular tyrosine kinase domain. Receptor-ligand binding induces dimerization and internalization, subsequently leading to activation of the receptor through autophosphorylation (Higashiyama et al., 2008).

ERBB family of receptors are expressed in tissues of epithelial, mesenchymal and neuronal origin, and EGFR pathway is involved in wide range of processes such as reproduction, growth and development (Wong, 2003, Yano et al., 2003). EGFR signaling is central to airway epithelial maintenance and mucin production (Burgel and Nadel, 2008), and EGFR expression has been demonstrated in lung epithelial cells under physiological (albeit weakly) as well as pathological conditions in vitro and in vivo (Aida et al., 1994, Burgel and Nadel, 2008, Polosa et al., 1999, O'donnell et al., 2004). Of note, lung epithelial cell EGFR phosphorylation (i.e., activation) was increased under conditions of oxidative stress including exposure to H2O2 (Goldkorn et al., 1998), naphthalene (Van Winkle et al., 1997), cigarette smoke (Marinaş et al., 2011) and in the presence of neutrophils or neutrophil elastase (Kohri et al., 2002, Shao and Nadel, 2005, Shim et al., 2001, Takeyama et al., 2000). EGFR activation by oxidative stress may have a number of root causes: ROS were shown to increase production of EGF, the prime EGFR ligand, by lung epithelial cells (Casalino-Matsuda et al., 2004). Similarly, expression and secretion of TGF-α and AREG, also EGFR ligands, were elevated in human bronchial epithelial cells in response to fine particulate matter (PM2.5) and cigarette smoke exposure (Blanchet et al., 2004, Lemjabbar et al., 2003, Rumelhard et al., 2007). Mechanistically, this process is dependent on activation of metalloproteinases or ADAMs which cleave membrane-bound EGFR ligand precursors, making them locally available to bind to and transactivate EGFR in an autocrine manner (Deshmukh et al., 2005, Val et al., 2012, Yoshisue and Hasegawa, 2004). Furthermore, ligand binding to EGFR itself was

shown to lead to H2O2 production, thereby facilitating receptor activation and downstream signaling, partly also through inhibition of EGFR phosphatase PTP1B (DeYulia et al., 2005, DeYulia Jr. and Cárcamo, 2005, Truong and Carroll, 2012). In addition, multiple lines of evidence suggest that oxidative modification, specifically EGFR sulfenylation, contributes to enhanced tyrosine phosphorylation of the receptor and downstream signaling (Paulsen et al., 2011, Truong and Carroll, 2012, Truong et al., 2016).

Classical EGFR downstream signaling involves activation of RAS which subsequently initiates signal transduction through the RAF1/MEK/ERK cascade (Hackel et al., 1999). The activation of this pathway promotes airway epithelial cell proliferation and differentiation, and facilitates epithelial wound repair (Chambard et al., 2007, Berlanga-Acosta et al., 2009). Another principal signaling cascade downstream of EGFR is phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) pathway, which promotes cell proliferation and inhibits apoptosis (Goffin and Zbuk, 2013).

Evidence for Perturbation by Stressor

EGFR activation in respiratory tract epithelial cells can be triggered by exposure to hydrogen peroxide (Goldkorn et al., 1998, Takeyama et al., 2000, Kim et al., 2008, Kim et al., 2010b), ozone (Wu et al., 2015, McCullough et al., 2014, Feng et al., 2016), naphthalene (Van Winkle et al., 1997), cigarette smoke (Takeyama et al., 2001, Yu et al., 2012a), nicotine (Wang et al., 2020b, Martínez-García et al., 2008), benzo[a]pyrene and its diol epoxide metabolite (Kometani et al., 2009, Xu et al., 2012), acrolein (Deshmukh et al., 2008), fine particulate matter (PM 2.5) (Jin et al., 2017, Jeong et al., 2017, Huang et al., 2017, Jiao et al., 2022, Tung et al., 2021, Wang et al., 2020a), carbon nanoparticles (Stöckmann et al., 2018), (Shang et al., 2020), bacterial lipopolysaccharide (LPS) (Takezawa et al., 2016), 2,3-butanedione (Kelly et al., 2019), and other chemical stressors such as hexabromocyclododecane and tetrabromobisphenol A (Koike et al., 2016), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Lee et al., 2011). Some of these stressors induce EGFR pathway activation also in other cell models. In addition to respiratory epithelium, acrolein activated EGFR in human normal oral keratinocytes (Takeuchi et al., 2001, Tsou et al., 2021) as well as in mouse J774A.1 macrophage cell line (Kim et al., 2010a), PM 2.5 induced EGFR activation in human thyroid follicular epithelial Nthy-ori 3-1 cells (Moscatello et al., 2022). Following nicotine treatment EGFR was shown to be activated in MCF10A and MDA-MB-231 breast cancer cells (Nishioka et al., 2011) and in human dysplastic oral keratinocytes (Wisniewski et al., 2018). LPS activates EGFR in several different model systems such as intestinal epithelial cells, RAW 264.7 macrophages, mammary epithelial cells, human intrahepatic biliary epithelial cells (HIBECs), etc (McElroy et al., 2012, Lu et al., 2014, De et al., 2015, Liu et al., 2013). Pro-inflammatory cytokines (e.g. SDF-1α) induce EGFR activity in IMR90 cells and human umbilical vein endothelial cells (HUVECs) (Shang et al., 2020).

How it is Measured or Detected

- Proof of EGFR activation can be derived from protein-analytical techniques such as Western blots of e.g. untreated and treated cell or tissue lysates using specific antibodies targeting the phosphorylated EGFR epitopes (Casalino-Matsuda et al., 2006, Hao et al., 2014).
- Phosphorylated, hence active EGFR can be detected and quantified also by Enzyme-Linked Immunosorbent Assay (ELISA) (Barbier et al., 2012, Knudsen et al., 2014). Detailed method description and different types of ELISA can be found in Tabatabaei and Ahmed research method article (Tabatabaei and Ahmed, 2022).
- Suppression of EGFR activity with EGFR inhibitors such as AG1478 and BIBX 1522 or neutralizing antibodies is well suited to demonstrate EGFR's involvement in signaling (Memon et al., 2020, Perrais et al., 2002, Val et al., 2012, Wang et al., 2019, Yu et al., 2012b).

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List of Key Events in the AOP

Event: 2117: Increase, goblet cell number

Short Name: Increase, goblet cell number

Key Event Component

Process	Object	Action
	goblet cell	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:148 - EGFR Activation Leading to Decreased Lung Function	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
rat	Rattus norvegicus		NCBI
mouse	Mus musculus		NCBI
rabbit	Oryctolagus cuniculus		NCBI

Term	Scientific Term	Evidence	Links
guinea pig	Cavia porcellus		NCBI
human	Homo sapiens		NCBI

Sex Applicability

Sex	Evidence
Unspecific	

The increased number of goblet cells in response to stressors can be found in rats, mice, rabbits, guinea pigs, and humans.

Key Event Description

Goblet cell is a mucus secreting cell type that can be found in epithelial mucosa of the intestine, lung, and eye. Goblet cells are necessary for mucosal epithelial homeostasis as well as for the appropriate function of both innate and adaptive immunity. Alterations in goblet cell numbers are characteristics of some pathologies. In the airway, the increased number of goblet cells is generally associated with diseases, such as asthma, cystic fibrosis, and chronic obstructive pulmonary disease (COPD). While some disorders of the intestine and conjunctiva are associated with the decrease in goblet cell numbers, Crohn's disease, cystic fibrosis, allergic conjunctivitis, and inverted mucoepidermoid papilloma have increased number of goblet cells (McCauley & Guasch, 2015). Goblet cell hyperplasia (GCH) can arise following airway injury and is defined by otherwise intact epithelium with an increase in the number of goblet cells (Hao et al, 2012; SAETTA et al, 2000). Pathologists define goblet cell metaplasia as apparent loss of ciliated or club cells with an increase of goblet cells, without an apparent increase in the total number of epithelial cells (Lumsden et al, 1984; Reader et al, 2003; Shimizu et al, 1996). The increased number of goblet cells via proliferation has been demonstrated in the rat intestine (Hino et al, 2012) and eye (Gu et al, 2008; Li et al, 2013; Shatos et al, 2008) in response dietary fiber and EGFR stimulation, respectively.

Evidence for Perturbation by Stressor

Several studies have shown that the number of goblet cells increase in response to various stressors. Cigarette smoke exposure resulted in the increase in the number of goblet cells in the airway of rats (Kato et al, 2020; Xiao et al, 2011), mice (Mebratu et al, 2011; Yang et al, 2020), dogs (Park et al, 1977), monkeys (Manevski et al, 2022), and in human airway epithelial cells cultured in air-liquid interface (Haswell et al, 2010; Haswell et al, 2021). Similarly, exposure of mice or rats to nebulized acrolein resulted in goblet cell metaplasia in the airways (Chen et al, 2010; Liu et al, 2009; Wang et al, 2009) and the treatment of primary human bronchial epithelial cells differentiated at the air-liquid interface with up to 1 µM acrolein induced a concentration dependent increase in the percentage of MUC5A-positive cells (Haswell et al., 2010). Ozone has also been shown to contribute to the increased number of goblet cells in the airways of mice (Jang et al, 2006; Larsen et al, 2010) and rats (Wagner et al, 2003). The goblet cell numbers also increased in the intestine of rats infected with Hymenolepis diminuta (tapeworm) (Webb et al, 2007) and mice infected with Nippostrongylus brasiliensis (hookworm) (Turner et al, 2013). Finally, air pollution was shown to trigger GCH in the eye (Novaes et al, 2007).

How it is Measured or Detected

There are few standard ways to measure the increased number of goblet cells in a tissue specimen or cultured cells:

- The mucin-producing secretory granules of goblet cells can be identified easily by light or electron microscopy (Rogers, 1994). The stages of metaplastic transformation can be identified as early cilia-goblet cells, late cilia-goblet, and mature goblet cells using transmission electron micrographs (Tyner et al, 2006). In laboratory animals, GCH may be identified by a pathologist as an increase in the number of goblet cells in an epithelium which normally contains only few goblet cells (Harkema & Hotchkiss, 1993).
- The increased number of goblet cells can be measured by staining the tissue or ALI culture with antibody recognizing MUC5 and counting the number of labeled cells/mm of epithelium or percentage of positive cells in the epithelium (Casalino-Matsuda et al, 2006; Jia et al, 2021; Lou et al, 1998; Tyner et al., 2006).
- Alternatively, many researchers use hematoxylin and eosin to stain the entire epithelial area (total number of nuclei) and Alcian blue (AB)-periodic acid-Schiff (PAS) to stain the intracellular mucous glycoconjugates, marking goblet cells. The change in goblet cell numbers is defined by the change in the proportion of AB-PAS-stained surface of the entire epithelial cell area over a length of 2 mm of the basal lamina (Takeyama et al, 2008).
- AB staining can be combined with goblet cell marker, Clca3, expressed as the goblet cell area / bronchial basement membrane (Leverkoehne et al, 2006; Song et al, 2016).
- Bromo-deoxyuridine (BrDU) incorporation can be used to identify the proliferating goblet cells in tissue specimens (GRANT & Specian, 1998; Hino et al., 2012).
- Proliferating Cell Nuclear Antigen 19A2 (PCNA) staining was used to identify proliferating goblet cells in the crypt of the intestinal wall in rabbits (GRANT & Specian, 1998).
- In a culture that consist solely of goblet cells (e.g., from conjunctiva), increase in goblet cells via proliferation was measured by Ki-67 immunofluorescent staining (Gu et al., 2008).

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Event: 962: Increase, Mucin production

Short Name: Increase, Mucin production

Key Event Component

Process	Object	Action
gene expression	mucin-5AC	increased
translation	mucin-5AC	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:148 - EGFR Activation Leading to Decreased Lung Function	KeyEvent

Stressors

Name
Acrolein
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)
Wood smoke
Cigarette smoke

Biological Context

Level of Biological Organization
Cellular

Cell term

Cell term
goblet cell

Organ term

Organ term
lung

Evidence for Perturbation by Stressor

Acrolein

Exposure of Sprague-Dawley rats to 3 ppm acrolein for 6 h a day, for 12 days significantly increased lung Muc5ac gene and protein expression (Chen et al., 2013).

Bronchoalveolar lavage fluid mucin content as well as Muc5ac gene and protein expression were significantly increased in the lungs of Sprague-Dawley rats that were exposed to 3 ppm of acrolein for 6 h a day, 7 days a week, for up to 2 weeks (Liu et al., 2009).

Exposure of Sprague Dawley rats to 3 ppm acrolein for 6 h a day, 5 days a week, for up to 12 days significantly increased Muc5ac gene expression in trachea and lung (Borchers et al., 1998).

Exposure of Sprague Dawley rats to 3 ppm acrolein for 3 h a day, 7 days a week, for up to 4 days significantly increased Muc5ac gene and protein expression in the lungs (Wang et al., 2009).

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

Treatment of primary normal human bronchial epithelial cells and immortalized human bronchial epithelial HBE1 cells with 10 nM TCDD for up to 48 h increased MUC5AC gene expression in a time-dependent manner. TCDD treatment (10 nM) of primary normal human bronchial epithelial cells also significantly increased MUC5AC protein levels (Lee et al., 2011).

Wood smoke

In the tracehas of rats that were nose-only exposed to smoke from burning Douglas fir wood (25 g) for up to 20 min, Muc5ac gene expression was increased at 24 h post-exposure (Bhattacharyya etal., 2004).

Cigarette smoke

Treatment of immortalized human bronchial epithelial 16HBE cells with cigarette smoke extract increased MUC5AC gene and protein expression in a concentration-dependent manner (Yu et al., 2011; Yu et al., 2015). Treatment of NCI-H292 lung cancer cells with cigarette smoke extract increased MUC5AC gene and protein expression in a concentration- and time-dependent manner (Takeyama et al., 2001; Shao et al., 2004; Baginski et al., 2006; Lee et al., 2006; Montalbano et al., 2014). Cigarette smoke extract treatment of A549 lung cancer cells (2 h) and primary human bronchial epithelial cells differentiated at the air-liquid interface (6 h

and 16 h) increased MUC5AC gene and protein expression (Di et al., 2012).

Whole-body exposure (TE-10 Teague Enterprises, Davis, CA) of rats to smoke from 1R1 research cigarettes (University of Kentucky; increasing dose between 123 to 323 mg/m3 total smoking particulate matter) for 2 h per day, 5 days per week, for 8 weeks significantly elevated Muc5ac levels in the bronchoalveolar fluid (Kato et al., 2020).

Muc5ac gene expression increased in the lungs of male Sprague-Dawley rats that were whole-body exposed to the smoke of 5 cigarettes a day, for 5 consecutive days (Takeyama et al., 2001).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	High

Sex Applicability

Sex	Evidence
Mixed	High

Evidence in support of this KE derives from in vitro studies with human cell systems (Casalino-Matsuda et al., 2009, Dohrman et al., 1998, Hao et al., 2014, Hewson et al., 2004, Lee et al., 2011, Val et al., 2012, Zhu et al., 2009), while corroborating in vivo evidence comes from studies in rodents (mouse or rat) (Hao et al., 2014, Song et al., 2016, Takeyama et al., 2001, Wagner et al., 2003).

Key Event Description

Mucins are a family of highly glycosylatedproteins produced by epithelialtissues and constitute major macromolecular components of mucus which protects epithelium from chemical and mechanical damage (Dhanisha et al., 2018). Mucin production in healthy airway provides an important role in trapping and removing bacterial and viral pathogens and particulates. Similarly, mucus layer in the intestinal epithelium provides first line of defense against physical and chemical hazards, notably ingested food and bacteria (Kim and Ho, 2010). In airways, major gel-forming secreted mucins MUC5AC and MUC5B are primarily involved in defensivefunction. MUC2 is the major intestinal mucin but is also expressed in the airway epithelium, and MUC19 is the major mucin in salivary glands (Lillehoj et al., 2013). Specialized mucin-producing goblet cells increase mucin production in respiratory tract in response to various irritants and stressors (Rogers, 2003). Many stressors specifically induce mucin mRNA and protein production through activation of the epidermal growth factor receptor (EGFR) pathway (Nadel, 2013). However, other signaling pathways, not necessarily requiring EGFR activation,via STAT6, FOXA2 and SPDEF havealso been implicated in mucin overexpression (Turner and Jones, 2009).

Evidence for Perturbation by Stressor

Various stressors such as cigarette smoke (Shao et al., 2004, Takeyama et al., 2001), reactive oxygen species (Yu et al., 2011, Casalino-Matsuda et al., 2009), phorbol 12-myristate 13-acetate (PMA) (Hewson et al., 2004), 2,3,7,8-tetrachlorodibenzodioxin (TCDD) (Lee et al., 2011), ozone (Wagner et al., 2003), fine particulate matter (Val et al., 2012), allergens such as ovalbumin (Song et al., 2016), as well as bacteria and viruses (Dohrman et al., 1998, Hao et al., 2014, Zhu et al., 2009) increase mucin production in respiratory airways. Wide range of inflammatory cytokines such as interleukin (IL) 1B, IL4, IL6, IL9, IL13, TNF, induce mucin production in different tissues, including respiratory and intestinal epithelium (Linden et al., 2008). Injection of the urban particulate matter into the middle ear cavity of rats increased MUC5AC and MUC5B expression in the middle ear mucosa (Park et al., 2014). Bacterial lipopolysaccharide (LPS) induced mucin expression in human intrahepatic biliary epithelial cells (HIBECs), colon adenocarcinoma cell line HT29, etc (Liu et al., 2013, Smirnova et al., 2003).

How it is Measured or Detected

In the literature, increased mucin production is frequently equated with increased MUC5AC mRNA and protein expression and less frequently with changes in MUC5B, MUC2 mRNA and protein levels. Due to high molecular weight and extensive glycosylated nature of mucins, conventional polyacrylamide gel-based protein analytic approaches can be challenging for mucin measurements (Kesimer and Sheehan, 2012). Strategies and methods for measuring airway mucins are thoroughly described in a review by Atanasova and Reznikov (Atanasova and Reznikov, 2019). Below we list the methods commonly used for mucin production detection and measurement.

- Alterations in mucin genes (MUC5AC, MUC5B) expression in cell and tissue lysates are commonly assessed by RT-PCR or RT-qPCR (Yu et al., 2011, Shao et al., 2004, Lee et al., 2011, Wagner et al., 2003, Hao et al., 2014, Zhu et al., 2009, Val et al., 2012). For absolute quantification of MUC5AC and MUC5B transcript copy numbers droplet digital PCR can be performed (Okuda et al., 2019).
- In situ hybridization is used in some studies for mucin (MUC5AC, MUC5B, MUC2) mRNA quantification (Takeyama et al., 2001, Dohrman et al., 1998, Okuda et al., 2019).
- For mucin mRNA detection and quantification RNase protection assay (RPA) is also used (Dohrman et al., 1998).
- Northern blot of mucin mRNAs can also be applied for mucin gene expression measurement (Chen et al., 2006, Zuhdi Alimam et al., 2000).
- In addition, assessment of mucin gene promoter activity by reporter gene expression (e.g. luciferase assay) allows assumptions on mucin expression levels (Chen et al., 2006).
- Changes in mucin protein levels can be detected by Western blot in cell and tissue lysates using suitable antibodies (Lee et al., 2011, Okuda et al., 2019, Ramsey et al., 2016).
- As a quick alternative to Western blot, dot-blot /slot-blot assay can be performed (Thornton et al., 1989).
- Secreted mucin protein levels can be detected and quantified by Enzyme-Linked Immunosorbent Assay (ELISA) (Yu et al., 2011, Shao et al., 2004, Wagner et al., 2003, Dohrman et al., 1998, Song et al., 2016). ELISA method description for detection and quantification of mucin molecules can be found in the article from Steiger and colleagues (Steiger et al., 1994).
- Analytical techniques such as immunocyto/histochemistry/immunofluorescence in cytological preparations or histological tissue sections with an appropriate antibody are also common methods of mucin protein level quantification (Zhu et al., 2009, Okuda et al., 2019). For immunofluorescent assays fluorescent dyes such as fluorescein isothiocyanate, Alexa488, Alexa555 are applied with subsequent visualization (e.g. confocal laser scanning or fluorescence microscopy) (Yu et al., 2011, Casalino-Matsuda et al., 2009, Val et al., 2012).
- Immunoassay of MUC5AC protein is also used as mucin protein detection method, as described in the study of Takeyama and colleagues (Takeyama et al., 2001).
- MUC5AC positive cell number determination through flow cytometry is another method for comparing and quantifying stressor-treated samples to control samples (Val et al., 2012).
- For in vivo studies and clinical samples, an experienced pathologist may judge the presence and severity of mucin production on histological tissue sections stained with hematoxylin/eosin and Alcian blue and/or periodic acid Schiff stains (Song et al., 2016, Atanasova and Reznikov, 2019, Okuda et al., 2019).
- Mass spectrometric approaches could be utilized for targeted identification of mucins and their quantification in cell and tissue samples (Kesimer and Sheehan, 2012).

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List of Adverse Outcomes in this AOP

Event: 1250: Decrease, Lung function

Short Name: Decreased lung function

Key Event Component

Process	Object	Action

Process	Object	Action
respiratory function trait		decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:148 - EGFR Activation Leading to Decreased Lung Function	AdverseOutcome
Aop:302 - Lung surfactant function inhibition leading to decreased lung function	AdverseOutcome
Aop:411 - Oxidative stress Leading to Decreased Lung Function	AdverseOutcome
Aop:418 - Aryl hydrocarbon receptor activation leading to impaired lung function through AHR-ARNT toxicity pathway	KeyEvent
Aop:419 - Aryl hydrocarbon receptor activation leading to impaired lung function through P53 toxicity pathway	AdverseOutcome
Aop:424 - Oxidative stress Leading to Decreased Lung Function via CFTR dysfunction	AdverseOutcome
Aop:425 - Oxidative Stress Leading to Decreased Lung Function via Decreased FOXJ1	AdverseOutcome

Stressors

Name
Ozone
Nitric oxide
Cigarette smoke
Diesel engine exhaust
PM10

Biological Context

Level of Biological Organization
Individual

Evidence for Perturbation by Stressor

Ozone

Acute exposure of healthy young adult subjects (aged 19 to 35 years, non-smokers) to 0.06 ppm ozone for 6.6 h resulted in a 1.71 ± 0.50% (mean ± SEM) decrease in FEV1 and a 2.32 ± 0.41% decrease in FVC compared with air exposure (Kim et al., 2011).

A US-based study found inverse associations between increasing lifetime exposure to ozone (estimated median: 36; interquartile range 29–45; range 19–64) and FEF75 and FEF25–75 in adolescents (aged 18–20 years) (Tager et al., 2005).

Nitric oxide

In a Dutch cross-sectional study in school children (aged 7–13 years), NOx exposure from industrial emissions per interquartile range of 7.43 µg/m^3 had a significantly lower percent predicted peak expiratory flow (PEF) (-3.67%, 95%CI -6.93% to -0.42%). Children exposed to NOx (per interquartile range of 7.43 µg/m^3) also had a significantly lower percent forced vital capacity (FVC) and percent predicted 1-s forced expiratory volume (FEV1) (– 2.73 95%CI -5.21 to -0.25) (Bergstra et al., 2018).

The European Study of Cohorts for Air Pollution Effects (ESCAPE), a meta-analysis of 5 cohort studies on the association of air pollution with lung function, found that a 10 µg/m^3 increase in NO_2 exposure was associated with lower levels of FEV1 (–14.0 mL, 95% CI –25.8 to –2.1) and FVC (–14.9 mL, 95% CI –28.7 to –1.1), and an increase of 20 µg/m^3 in NOx exposure was associated with a lower level of FEV1, by –12.9 mL (95% CI –23.87 to –2.0) and of FVC, by –13.3 mL (95% CI –25.9 to –0.7) (Adam et al., 2015).

Cigarette smoke

A smoking history of > 20 pack-years decreased pulmonary function including forced vital capacity (FVC), forced expiratory volume

in one second (FEV1), FEV1/FVC, and forced expiratory flow at 25–75% (FEF25–75%) (Kuperman and Riker, 1973).

In the Framingham Heart Study, cigarette smoking showed an inverse association with FVC and FEV1% (Ashley et al., 1975).

In the international Seven Countries Study, there was a dose-effect relationship between pack-years and forced expiratory volume in 0.75 s (FEV0.75) in continuous smokers without chronic bronchitis (Pelkonen et al., 2006).

In 34 male subjects aged between 15–18 years who smoked FVC was lower than in an age-matched male group that did not smoke. The most common duration of cigarette smoking was 1–3 years (47%) and the maximal number of cigarettes smoked per day was less than or equal to 10 cigarette(s) per day (88%) (Tantisuwat and Thaveeratitham, 2014).

A dose–response relation was found between smoking and lower levels of FEV1/FVC and FEF25–75 in children between 10-18 years of age (Gold et al., 1996).

In a study of 147 asthmatics, FEV1%predicted was significantly lower in ex-smokers and current smokers compared with never-smokers (Broekema et al., 2009).

In a 6-year longitudinal study in Japanese-American men, FEV1 was lowest in current smokers (2702 mL) and in former smokers (2817 mL) at baseline. These 2 groups experienced a steeper annual decline in FEV1 (-34.4 and -22.8 mL/year, respectively, adjusted by height and age at baseline) compared with never-smokers (-20.3 mL/year) (Burchfiel et al., 1995).

Diesel engine exhaust

In a study of 733 adult females who had lived in the Tokyo metropolitan area for more than 3 years, the higher the level of air pollution, the more significantly the FEV1 was reduced (Sekine et al., 2004).

In a study in 29 healthy subjects, exposure to DE inside diesel-powered trains for 3 days was associated with reduced lung function (Andersen et al., 2019).

In workers who tested diesel engines in an assembly unit of a manufacturing plant, FEV1, FEV1/FVC, FEV25-75 and MEF were significantly reduced compared to non-exposed workers (Zhang et al., 2017).

PM10

A Taiwanese study in 1016 children between 6 and 15 years of age reported that lifetime exposure to to 25–85 µg/m^3 PM10 were associated with lower FEV1, FVC, and FEF25-75 (Tsui et al., 2018).

The Swiss Study on Air Pollution and Lung Diseases in Adults (SAPALDIA) found that an increase of 10 µg/m^3 in annual mean concentration of PM10 was associated with 3.4% lower FVC and 1.6% lower FEV1 (Ackermann-Liebrich et al., 1997).

In the Health Survey for England, a 10 mg/m^3 difference in PM10 across postcode sectors was associated with a lower FEV1 by 111 mL, independent of active and passive smoking, social class, region and month of testing (Forbes et al., 2009).

A 7 µg/m^3 increase in five year means of PM10 (interquartile range) was associated with a 5.1% (95% CI: 2.5%–7.7%) decrease in FEV1, a 3.7% (95% CI: 1.8%–5.5%) decrease in FVC in the German SALIA study (Schikowski et al., 2005).

The ESCAPE project, a meta-analysis of 5 European cohorts/studies from 8 countries, reported that an increase of 10 µg/m^3 in PM10 was associated with a lower level of FEV1 (–44.6 mL, 95% CI:–85.4– –3.8) and FVC (–59.0 mL, 95% CI: –112.3– –5.7) (Adam et al., 2015).

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	High

Sex Applicability

Sex	Evidence
Mixed	High

Pulmonary function tests reflect the physiological working of the lungs. Therefore, the AO is applicable to a variety of species, including (but not limited to) rodents, rabbits, pigs, cats, dogs, horses and humans, independent of life stage and gender.

Key Event Description

Lung function is a clinical term referring to the physiological functioning of the lungs, most often in association with the tests used to assess it. Lung function loss can be caused by acute or chronic exposure to airborne toxicants or by an intrinsic disease of the respiratory system.

Although signs of cellular injury are typically exhibited first in the nose and larynx, alveolar-capillary barrier breakdown may ultimately arise and result in local edema (Miller and Chang, 2003). Clinically, bronchoconstriction and hypoxia are seen in the acute phase, with affected subjects exhibiting shortness of breath (dyspnea) and low blood oxygen saturation, and with reduced lung function indices of airflow, lung volume and gas exchange (Hert and Albert, 1994; and How it is Measured or Detected;). When alveolar damage is extensive, the reduced lung function can develop into acute respiratory distress syndrome (ARDS). This severe compromise of lung function is reflected by decreased gas exchange indices (PaO_2/FIO_2 ≤200 mmHg, due to hypoxemia and impaired excretion of carbon dioxide), increased pulmonary dead space and decreased respiratory compliance (Matthay et al., 2019). Acute inhalation exposures to chemical irritants such as ammonia, hydrogen chloride, nitrogen oxides and ozone typically cause local edema that manifests as dyspnea and hypoxia. In cases where a breakdown of the alveolar capillary function ensues, ARDS develops. ARDS has a particularly high risk of mortality, estimated to be 30-40% (Gorguner and Akgun, 2010; Matthay et al., 2018; Reilly et al., 2019).

Lung function decrease due to reduction in lung volume is seen in pulmonary fibrosis, which can be linked to chronic exposures to e.g. silica, asbestos, metals, agricultural and animal dusts (Meltzer and Noble, 2008; Cheresh et al., 2013; Cosgrove, 2015; Trethewey and Walters, 2018). Additionally. decreased lung function occurs in pleural disease, chest wall and neuromuscular disorders, because of obesity and following pneumectomy (Moore, 2012). Decreased lung function can also be a result of narrowing of the airways by inflammation and mucus plugging resulting in airflow limitation. Decreased lung function is a feature of obstructive pulmonary diseases (e.g. asthma, COPD) and linked to a multitude of causes, including chronic exposure to cigarette smoke, dust, metals, organic solvents, asbestos, pathogens or genetic factors.

How it is Measured or Detected

Pulmonary function tests are a group of tests that evaluate several parameters indicative of lung size, air flow and gas exchange. Decreased lung function can manifest in different ways, and individual circumstances, including potential exposure scenarios, determine which test is used. The section outlines the tests used to evaluate lung function in humans (https://www.nhlbi.nih.gov/health-topics/pulmonary-function-tests, accessed 22 March 2021) and in experimental animals.

Lung function tests used to evaluate human lung function

The most common (“gold standard”) lung function test in human subjects is spirometry. Spirometry results are primarily used for diagnostic purposes, e.g. to discriminate between obstructive and restrictive lung diseases, and for determining the degree of lung function impairment. Specific criteria for spirometry tests have been outlined in the American Thoracic Society (ATS) and the European Respiratory Society (ERS) Task Force guidelines (Graham et al., 2019). These guidelines consist of detailed recommendations for the preparation and conduct of the test, instruction of the person tested, as well as indications and contraindications, and are complemented by additional guidance documents on how to interpret and report the test results (Pellegrino et al., 2005; Culver et al., 2017).

Spirometry measures several different parameters during forceful exhalation, including:

- Forced expiratory volume in 1 s (FEV1), the maximum volume of air that can forcibly be exhaled during the first second following maximal inhalation
- Forced vital capacity (FVC), the maximum volume of air that can forcibly be exhaled following maximal inhalation
- Vital capacity (VC), the maximum volume of air that can be exhaled when exhaling as fast as possible
- FEV1/FVC ratio
- Peak expiratory flow (PEF), the maximal flow that can be exhaled when exhaling at a steady rate
- Forced expiratory flow, also known as mid-expiratory flow; the rates at 25%, 50% and 75% FVC are given
- Inspiratory vital capacity (IVC), the maximum volume of air that can be inhaled after a full expiration

A reduced FEV1, with normal or reduced VC, normal or reduced FVC, and a reduced FEV1/FVC ratio are indices of airflow limitation, i.e., airway obstruction as seen in COPD (Moore, 2012). In contrast, airway restriction is demonstrated by a reduction in FVC, normal or increased FEV1/FVC ratio, a normal spirometry trace and potentially a high PEF (Moore, 2012).

Lung capacity or lung volumes can be measured using one of three basic techniques: 1) plethysmography, 2) nitrogen washout, or 3) helium dilution. Plethysmography consists of a series of sequential measurements in a body plethysmograph, starting with the measurement of functional residual capacity (FRC), the volume of gas present in the lung at end-expiration during tidal breathing. Once the FRC is known, expiratory reserve volume (ERV; the volume of gas that can be maximally exhaled from the end-expiratory level during tidal breathing, i.e., the FRC), vital capacity (VC; the volume change at the mouth between the positions of full inspiration and complete expiration), and inspiratory capacity (IC; the maximum volume of air that can be inhaled from FRC) are determined, and total lung capacity (TLC; the volume of gas in the lungs after maximal inspiration, or the sum of all volume compartments) and residual volume (RV; the volume of gas remaining in the lung after maximal exhalation) are calculated (Weinstock and McCannon, 2017).

The other two techniques used to measure lung volumes—helium dilution and nitrogen washout—are based on the principle of conservation of mass: [initial gas concentration] x [initial volume of the system] = [final gas concentration] x [final volume of the system]. The nitrogen washout method is based on the fact that nitrogen is present in the air, at a relatively constant amount. The subject is given 100% oxygen to breathe, and the expired gas, which contains nitrogen in the lung at the beginning of the test, is collected. When no more nitrogen is noted in the expirate, the volume of air expired and the entire amount of nitrogen in that volume are measured, and the initial volume of the system (FRC) can be calculated. In the helium dilution method, a known volume and concentration of helium is inhaled by the subject. Helium, an inert gas that is not absorbed significantly from the lungs, is diluted in proportion to the lung volume to which it is added. The final concentration of helium is then measured and FRC calculated (Weinstock and McCannon, 2017).

Measurements of lung volumes in humans are technically more challenging than spirometry. However, they complement spirometry (which cannot determine lung volumes) and may be a preferred means of lung function assessment when subject compliance cannot be reasonably expected (e.g. in pediatric subjects) or where forced expiratory maneuvers are not possible (e.g. in patients with advanced pulmonary fibrosis). There are recommended standards for lung volume measurements and their interpretation in clinical practice, issued by the ATS/ERS Task Force (Wanger et al., 2005; Criée et al., 2011).

Finally, indices of gas exchange across the alveolar-capillary barrier are tested by diffusion capacity of carbon monoxide (DLCO) studies (also referred to as transfer capacity of carbon monoxide, TLCO). The principle of the test is the increased affinity of hemoglobin to preferentially bind carbon monoxide over oxygen (Weinstock and McCannon, 2017). Complementary to spirometry and lung volume measurements, DLCO provides information about the lung surface area available for gas diffusion. Therefore, it is sensitive to any structural changes affecting the alveoli, such as those accompanying emphysema, pulmonary fibrosis, pulmonary edema, and ARDS. Recommendations for the standardization of the test and its evaluation have been outlined by the ATS/ERS Task Force (Graham et al., 2017). An isolated reduction in DLCO with normal spirometry and in absence of anemia suggests an injury to the alveolar-capillary barrier, as for example seen in the presence of pulmonary emboli or in patients with pulmonary hypertension (Weinstock and McCannon, 2017; Lettieri et al., 2006; Seeger et al., 2013). Reduced DLCO together with airflow obstruction (i.e., reduced FEV1) indicates lung parenchymal damage and is commonly observed in smokers and in COPD patients (Matheson et al., 2007; Harvey et al., 2016), whereas reduced DLCO with airflow restriction is seen in patients with interstitial lung diseases (Dias et al., 2014; Kandhare et al., 2016).

Lung function tests used to evaluate experimental animal lung function

Because spirometry requires active participation and compliance of the subject, it is not commonly used in animal studies. However, specialized equipment such as the flexiVent system (SCIREQ®) are available for measuring FEV, FVC and PEF in anesthetized and tracheotomized small laboratory animals. Other techniques such as plethysmography or forced oscillation are increasingly preferred for lung function assessment in small laboratory animals (McGovern et al., 2013; Bates, 2017).

In small laboratory animals, plethysmography can be used to determine respiratory physiology parameters (minute volume, respiratory rate, time of pause and time of break), lung volume and airway resistance of conscious animals. Both whole body and head-out plethysmography can be applied, although there is a preference for the latter in the context of inhalation toxicity studies, because of its higher accuracy and reliability (OECD, 2018a; Hoymann, 2012).

Gas diffusion tests are not frequently performed in animals, because reproducible samplings of alveolar gas are difficult and technically challenging (Reinhard et al., 2002; Fallica et al., 2011). Modifications to the procedure employed in humans have, however, open possibilities to obtain a human-equivalent DLCO measure or the diffusion factor for carbon monoxide (DFCO)—a variable closely related to DLCO, which can inform on potential structural changes in the lungs that have an effect on gas exchange indices (Takezawa et al., 1980; Dalbey et al., 1987; Fallica et al., 2011; Limjunyawong et al., 2015).

Regulatory Significance of the AO

Established regulatory guideline studies for inhalation toxicity focus on evident clinical signs of systemic toxicity, including death, or organ-specific toxicity following acute and (sub)chronic exposure respectively. In toxicological and safety pharmacological studies with airborne test items targeting the airways or the lungs as a whole, lung function is a relevant endpoint for the characterization of potential adverse events (OECD, 2018a; Hoymann, 2012). Hence, the AO “decreased lung function” is relevant for regulatory decision-making in the context of (sub)chronic exposure (OECD, 2018b; OECD, 2018c).

Regulatory relevance of the AO “decreased lung function” is evident when looking at the increased risk of diseases in humans following inhalation exposure, and because of its links to other comorbidities and mortality.

To aid diagnosis and monitoring of fibrosis, current recommendations include both the recording of potential environmental and occupational exposures as well as an assessment of lung function (Baumgartner et al., 2000). The latter typically confirms decreased lung function as demonstrated by a loss of lung volume. As the disease progresses, dyspnea and lung function worsen, and the prognosis is directly linked to the decline in FVC (Meltzer and Noble, 2008).

Chronic exposure to cigarette smoke and other combustion-derived particles results in the development of COPD. COPD is diagnosed on the basis of spirometry results as laid out in the ATS/ERS Task Force documents on the standardization of lung function tests and their interpretation (Pellegrino et al., 2005; Culver et al., 2017, Graham et al., 2019). Rapid rates of decline in the lung function parameter FEV1 are linked to higher risk of exacerbations, increased hospitalization and early death (Wise et al., 2006; Celli, 2010). Reduced FEV1 also poses a risk for serious cardiovascular events and mortality associated with cardiovascular

disease (Sin et al., 2005; Lee et al., 2015).

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 2857: Activation, EGFR leads to Increase, goblet cell number

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
EGFR Activation Leading to Decreased Lung Function	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI
Mus musculus	Mus musculus	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI

Sex Applicability

Sex	Evidence
Unspecific	

Evidences for this KER are derived from studies in human cell cultures as well as mouse and rat *in vitro* and *in vivo* systems. No

sex-specific observations were noted.

Key Event Relationship Description

Mucus hypersecretion in the airway epithelium is a key characteristic of many lung diseases, including chronic obstructive pulmonary disease (COPD), asthma and cystic fibrosis (Yoshida & Tuder, 2007). Epidermal growth factor receptor (EGFR)-mediated signaling has been identified as the key pathway that leads to increased mucus production in the airways (Burgel & Nadel, 2004). The KER is restricted to EGFR activation that results in the increase in the number of goblet cells based on evidence of goblet cell metaplasia, hyperplasia, or increased proliferation/differentiation of goblet cells. In metaplasia, the increase in goblet cells occurs at the expense of other cell types, such as ciliated cells (Curran & Cohn, 2010). EGFR-mediated metaplasia or hyperplasia has been demonstrated in response to allergens (Song et al, 2016; Takeyama et al, 1999), viruses (Tyner et al, 2006), reactive oxygen species (ROS) (Casalino-Matsuda et al, 2006), and cigarette smoke (Takeyama et al, 2001a) in the respiratory epithelium. The EGFR mediated proliferation of the goblet cells has been demonstrated in the mucus and tear producing conjunctiva of the rat and human eye (Gu et al, 2016; Li et al, 2013; Shatos et al, 2008). Finally, EGFR has been shown to also augment goblet cell maturation in colon organ cultures (Duh et al, 2000) and favor goblet cell differentiation from human airway basal cells (Parker et al, 2015).

Evidence Supporting this KER

Biological Plausibility

The involvement of EGFR in regulating the number of goblet cells has been demonstrated in both in vitro and in vivo studies. The treatment of human bronchial epithelial cells differentiated and cultured in air-liquid interphase with EGFR ligands (e.g., amphiregulin or HB-EGF) significantly increased the number of goblet cells (Hirota et al., 2012; Jia et al., 2021). Moreover, ALI cultures of bronchial epithelia from asthmatic children differentiated without EGF had less goblet cells than those differentiated in the presence of EGF (Parker et al., 2015). EGFR ligands also stimulated proliferation of cultured rat and human conjunctival goblet cells (Gu et al., 2008; Li et al., 2013; Shatos et al., 2008). EGFR augments goblet cell maturation and increases colonic goblet cell index during the colonic organ culture (Duh et al., 2000). EGFR also mediates the increase in goblet cells number by a variety of stressors. Xanthine oxidase increased the number of Muc5Ac positive cells in bronchial epithelial ALI cultures, and this increase was blocked by anti-EGFR antibodies (Casalino-Matsuda et al., 2006). Moreover, EGFR mediated the increase in goblet cells in the mice airways challenged with allergens (Jia et al., 2021; Le Cras et al., 2011; Song et al., 2016), and viruses (Tyner et al., 2006). Finally, challenging the rat airway with allergens (Takeyama et al., 1999), bacterial endotoxin (Takezawa et al., 2016), agarose plug (Lee et al., 2000), or cigarette smoke (Takeyama et al., 2001b) increased goblet cell numbers in a EGFR dependent manner.

Empirical Evidence

The studies that provide strong empirical evidence for the KER are listed below:

In vitro systems

- Treatment of bronchial epithelial cells grown in ALI with xanthine plus xanthine oxidase increased EGFR activation (measured by pEGFR/EGFR ratios) and the number of Muc5Ac positive cells. The increase in goblet cell numbers was prevented by anti-EGFR monoclonal antibodies (Casalino-Matsuda et al., 2006).
- Treatment of human primary bronchial epithelial cells with amphiregulin or HB-EGF significantly induced goblet cell differentiation demonstrated by CLCA1 (marker of goblet cells) expression in NHBE cells cultured in ALI (Hirota et al., 2012).
- Differentiated human bronchial epithelial ALI cultures treated basolaterally with human bronchial epithelial growth factor (HBEGF) resulted in a remarkable increase in the number of cells expressing MUC5AC (Jia et al., 2021).
- EGFR inhibitor AG1478 treatment of bronchial epithelial cell ALI cultures from asthmatic children showed significant reductions in goblet cell numbers. In addition, the cultures differentiated in EGF-negative conditions had reduced goblet cell numbers compared with the cultures differentiated in the presence of EGF (Parker et al., 2015).
- The treatment of rat and human conjunctival goblet cells with EGF significantly increased proliferation via PI3K/AKT pathway (Li et al., 2013).
- Treatment of rat conjunctival goblet cells with EGF significantly increased phosphorylation of EGFR and increased proliferation. Pre-treatment of goblet cells stimulated with EGF with AG1478 significantly inhibited the EGF-induced proliferation (Shatos et al., 2008).
- Treatment of rat conjunctival goblet cells with EGF, TGF-α, or HB-EGF stimulated the phosphorylation of EGFR and resulted in increased proliferation, demonstrated with the WST-1 assay and the increase of Ki-67 positive cells (Gu et al., 2008).
- In colon culture, EGF treatment increased the goblet cell index (number of goblet cells/total colonic epithelial cells) compared with untreated cultures (Duh et al., 2000).

In vivo systems

- The irreversible EGFR inhibitor, EKB-569, blocked goblet cell metaplasia in the Sendai virus-infected mouse model (Tyner et

al., 2006).

- Induction of airway inflammation with house dust mite resulted in goblet cell metaplasia as evidenced by extensive AB staining and increase in Clca3-positive cells in mouse airways. Concomitant treatment with EGFR inhibitor, erlotinib, reduced the number of Clca3- (Le Cras et al., 2011) Muc5AC- positive cells (Jia et al., 2021) in the mouse lung.
- Chronic treatment with EGFR inhibitor, gefitinib, decreased goblet cell hyperplasia in the airways of ovalbumin-challenged mice (Song et al., 2016).
- Agarose plug instillation in the rat bronchial epithelium decreased the basal, ciliated and secretory cells, and increased the number of pregoblet and goblet cells. Intraperitoneal injection of the EGFR inhibitor, BIBX1522, reduced epithelium stained with Alcian blue-PAS in the agarose plug-induced rat airway (Lee et al., 2000).
- Intranasal instillation of LPS induced increase in the number of goblet cells (AB/PAS staining) in the rat nasal epithelium, and the AB/PAS staining was reduced by injection of EGFR inhibitor, AG1478 (Takezawa et al., 2016).
- Ovalbumin sensitization increased the numbers of goblet and pre-goblet cells in the rat airway epithelium, and EGFR inhibitor, BIBX1522, prevented the observed goblet cell increase (Takeyama et al., 1999).
- Cigarette smoke exposure increased the number of goblet cells (AB-PAS staining) in the rat airway epithelum and this increase was blocked by concomitant treatment with EGFR tyrosine kinase inhibitor, BIBX1522 (Takeyama et al., 2001b).

Quantitative Understanding of the Linkage

Below we list some quantitative aspects of the response relationship.

Response-response relationship

- Rats who underwent small bowel resection had significantly more goblet cells (number of cells/mm villus/crypt in the ileum compared with sham operated animals. The rats that were treated with an EGFR inhibitor, ZD1839 50 mg/kg per day) starting at the day of surgery did not show the increase in goblet cells following small bowel resection (Jarboe et al, 2005).
- The removal of EGF (human recombinant, 0.5 ng/ml) from the culture medium of human bronchial epithelial cells during differentiation in ALI reduced the number of goblet cells to 6.7 ± 0.8% from 15.5 ± 1.5 in cultures differentiated in the presence of EGF (Atherton et al, 2003).
- A 30-min treatment of primary human bronchial epithelial cells at the air-liquid interface with 0.6 mM xanthine and 0.5 units xanthine oxidase resulted in a 2-fold increase in EGFR phosphorylation. Daily 30-min treatments of primary human bronchial epithelial cells at the air-liquid interface with 0.6 mM xanthine and 0.5 units xanthine oxidase for 3 days resulted in goblet cell metaplasia as evidenced by an increase in the numbers of MUC5AC-positive cells from 3.3 ± 1.2% to 21.6 ± 3.4%, a decrease in ciliated cell numbers, and increased MUC5AC protein expression (32.5 + 9.3% above PBS control). This effect could be inhibited by EGFR blockade with neutralizing antibodies (Casalino-Matsuda et al., 2006).
- ALI cultures from bronchial epithelial cells of asthmatic children supplemented with 10ng/ml EGF showed a higher percentage of goblet cells (mean 23.4%) compared with cultures without EGF (mean 13.9%). Treatment of the EGF-supplemented cultures simultaneously with 0.2µg/ml or 2µg/ml AG147 resulted in decrease in the percentage of goblet cells (mean 13.1% and 7.7%, respectively) compared with cultures supplemented with EGF alone (Parker et al., 2015).
- Treatment of mouse gut in organ culture, which do not have goblet cells at day 6, with 10 ng/mL EGF increased the colonic goblet cell index (number of goblet cells/total colonic epithelial cells) by 1.8-fold compared with untreated cultures (Duh et al., 2000).
- The OVA-induced goblet cell hyperplasia (~33 mm2/mm) was significantly attenuated by 50 mg/kg gefitinib treatment for 12 h each day during days 14–20 (~12 mm2/mm, as measured by goblet cell area / perimeter of the bronchial basement membrane (Song et al., 2016).
- Cigarette smoke exposure at 8 cigarettes (nonfiltered cigarettes; 1.2 mg nicotine, 12 mg condensate) per day for 5 days markedly increased AB/PAS staining in airway epithelia of male Sprague-Dawley rats and goblet cell numbers from 40 ± 19 to 167 ± 19 cells/mm of epithelium, while decreasing the number of ciliated cells (not quantified). Treatment with the EGFR inhibitor BIBX1522 during exposure dose-dependently decreased goblet cell numbers, with a maximal decrease seen for 3 mg/kg inhibitor (51 ± 19 cells/mm epithelium) (Takeyama et al., 2001b).
- Intranasal instillation of 0.1 mg LPS (E.coli 0111:B4) once a day for 3 consecutive days induced increased number of goblet cells in the nasal epithelium (as judged by histopathology), with an approx. 50% increase in AB/PAS-stained epithelium compared to untreated controls. Intranasal instillation of EGFR inhibitor AG1478 1 hr after LPS instillation dose-dependently decreased the % AB/PAS-stained epithelium, with a maximal decrease seen at 10 mg/kg (Takezawa et al., 2016).
- Sensitization of rats with 10 mg ovalbumin (OVA) intraperitoneally (i.p.) on days 0 and 10 followed by intratracheal OVA instillations (0.1%, 100 ml) on days 20, 22, and 24 significantly increased the numbers of goblet cells in the airway epithelium on day 26. OVA sensitized rats were also treated with i.p. administration (10 mg/kg 1 h before the intratracheal instillation of ovalbumin), intratracheal instillation (1025M, 100 ml on days 20, 22 and 24), and i.p. injection (10 mg/kg of every 24 h until

the day before the rats were euthanized) of BIBX1522. This treatment with the EGFR inhibitor reduced the Alcian blue/PAS stained area from ~18% to ~4 % (Takeyama et al., 1999).

- Treatment of human primary bronchial epithelial cells with 1 ng/mL amphiregulin or HB-EGF for 24 h significantly induced goblet cell differentiation in NHBE cells cultured in ALI demonstrated by CLCA1 (marker of goblet cells) expression (Hirota et al., 2012).
- Stimulation of human bronchial epithelial cells with human bronchial epithelial growth factor (20 ng/mL) for 24 h, resulted in a remarkable increase in the number of cells expressing MUC5AC. Treatment of the house dust mite-induced asthma mouse model with Erlotinib (during day 14–day 23)-resulted in decreased MUC5AC density in the lung (Jia et al., 2021).
- 105 PFUs of Sendai virus was intranasally administered to mice to reach maximal viral tissue levels at 3–5 days after inoculation and viral clearance by day 12. The blocking of EGFR by oral administration of 20 mg/kg EKB-569 daily during postinfection days 10–21 decreased the number of Muc5AC positive cells to ~2 compared with ~7 (cells/mm basement membrane) in lungs of virus only infected mice (Tyner et al., 2006).
- Instillation of agarose plugs (0.7-0.8 mm diameter, 4% agarose II) in Fischer rats caused a time-dependent increase in goblet cell area (by AB/PAS staining), which was detectable as early as 24 h and was greatest 72 h post-instillation. The AB/PAS-stained area increased from 0.1 ± 0.1% in control animals to 4.7 ± 1.4, 13.3 ± 0.7, and to 19.1 ± 0.7% at 24, 48, and 72 h post-instillation, respectively. Goblet cell numbers increased from 0 to 13.1 ± 5.6, 25.7 ± 15.0, and 51.5 ± 9.0 cells/mm basal lamina at 24, 48, and 72 h post-instillation, respectively. Concurrently, the numbers of basal, ciliated, and secretory cells decreased. Treatment of the animals prior and after instillation with 80 mg/kg/day BIBX1522 resulted in a marked decrease in the AB/PAS-stained area (<5% at 72 h). Of note, the AB/PAS staining in the airway epithelia coincided with EGFR staining (Lee et al., 2000).

Time-scale

The following studies show that EGFR activation (upstream KE) occurs earlier than the increase of goblet cell (downstream KE), complying with temporal concordance terms of upstream KE occurring before downstream KE.

- EGFR phosphorylation was detected in histamine stimulated NHBE cells in 2 hours and goblet cell differentiation marker CLCA1 mRNA was increased 5 days after histamine treatment (Hirota et al., 2012).
- Treatment of rat conjunctival goblet cells with 0.1 µM EGF significantly increased phosphorylation of the EGFR by 28.6 ± 7.6- and 29.2 ± 3.2-fold at 1 and 5 minutes, respectively. At the same concentration, 24-hour EGF treatment increased proliferation 4.9 ± 1.8-fold. Similar observations were made with human conjunctival goblet cells: Treatment with 0.1 µM EGF significantly increased proliferation 1.5 ± 0.3-fold above basal (Li et al., 2013). In another study in rat conjunctival goblet cells, treatment with 0.1 µM EGF, TGF-α, or HB-EGF for 5 min significantly stimulated the phosphorylation of EGFR by 21.1 ± 2.5, 22.2 ± 6.7, and 19.9 ± 6.0 fold above basal level, and 24-h treatment stimulated cell proliferation 1.3 ± 0.1 fold, 1.2 ± 0.02 fold, and 1.1 ± 0.04 fold compared to untreated cells (WST-1 assay). These latter results were also confirmed by Ki-67 immunofluorescent staining, showing increases in positive cells by 61.4%, 38.1%, 27.8% following EGF, TGF-α, and HB-EGF treatment compared to untreated cells (Gu et al., 2008).
- EGFR phosphorylation was observed in cultured rat conjunctival goblet cells after incubation with EGF for 1 or 5 minutes, and increase in number of goblet cells was measured at 24 hours after incubation with EGF for 20 minutes (Shatos et al., 2008).

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Relationship: 986: Activation, EGFR leads to Increase, Mucin production

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
EGFR Activation Leading to Decreased Lung Function	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	Moderate

Sex Applicability

Sex	Evidence
Unspecific	

EGFR activation leading to mucus hypersecretion was shown in human primary and cultured airway epithelial cell models (Casalino-Matsuda, Monzón, and Forteza 2006; Y.C. Lee et al. 2011; Takeyama et al. 1999; Val et al. 2012), in mouse (Deshmukh et al. 2008; Song et al. 2016), rat (H.M. Lee et al. 2000; Shim et al. 2001), canine and feline respiratory epithelium (Choi et al. 2021).

Key Event Relationship Description

Increased mucin production which leads to mucus hypersecretion in the airway epithelium is a key characteristic of many lung diseases, including chronic obstructive pulmonary disease (COPD), asthma and cystic fibrosis (Yoshida and Tuder 2007). In airways, major gel-forming secreted mucins MUC5AC and MUC5B are produced in specialized mucin-producing goblet cells. Epidermal growth factor receptor (EGFR)-mediated signalling has been identified as the key pathway that leads to increased mucin production (Burgel and Nadel 2004). Following ligand binding in response to wide variety of stimuli (such as bacterial or viral infections, oxidative stress, particulate matter, etc) EGFR undergoes receptor dimerization and autophosphorylation resulting in activation of downstream pathways that lead to increase in mucin gene expression as well as result in increased number of goblet cells (Vallath et al. 2014). The increase in goblet cell numbers is out of the scope of this KER. As a result of EGFR signalling activation, mucin (MUC5AC, MUC5B) gene and protein expression is increased in airway epithelial cells leading to mucus overproduction and hypersecretion. The relationship between EGFR activation and downstream increase in mucin production is well established in studies of numerous laboratories, and interference with the EGFR signalling represents great pharmacological potential for treatment of airway mucus hyperproduction (Lai and Rogers 2010; Burgel and Nadel 2004).

Evidence Supporting this KER

EGFR can be activated by bacterial infection, EGFR ligands, exposure to cigarette smoke and other sources of ROS, leading to increased mucin production via Ras/Raf-1/MEK/ERK-mediated activation of the Sp1 transcription factor, which can be suppressed at least partially in the presence of EGFR inhibitors (Sydlik et al., 2006; Casalino-Matsuda et al., 2006; Takeyama et al., 2008; Perrais et al., 2002; Hewson et al., 2004; Wu et al., 2007; Barbier et al., 2012; Lee et al., 2011).

Biological Plausibility

Numerous studies on different mammalian model systems conclusively demonstrate the positive role of EGFR activation in increased mucin expression. EGFR signalling upregulates the expression of mucin genes, such as MUC2 and MUC5AC, through activation of SP1 transcription factor (Y.C. Lee et al. 2011; Perrais et al. 2002). In addition, hypoxia inducible factor 1 subunit alpha (HIF1A) was also shown to be implicated in MUC5AC expression downstream of EGFR pathway (Yu, Li, et al. 2012). In one study, EGFR was shown to promote mucus production through antagonism with Claudin1 (Jia et al. 2021). Abundant evidence in studies from many laboratories suggests highly biologically plausible causal relationship between EGFR activation and increased mucin production.

Empirical Evidence

Below we list (not exhaustive) studies providing evidence of increase in mucin production as a result of EGFR activity.

- Stimulation of EGFR by its ligands, EGF and transforming growth factor alpha (TGFA), increases MUC5AC gene and protein expression. Pretreatment of cells with selective kinase inhibitors for EGFR (BIBX1522, AG1478, Compound 56) prevented the ligand-induced staining for MUC5AC protein (Takeyama et al. 1999). Ovalbumin sensitization resulted in EGFR protein expression and mucous glycoconjugate production (AB-PAS-positive staining) in rat airways which was inhibited by pretreatment with EGFR inhibitor BIBX1522 (Takeyama et al. 1999). The same research group has demonstrated upregulation in EGFR-mediated MUC5AC mRNA and protein production after cigarette smoke exposure in NCI-H292 cells and in vivo in Sprague-Dawley rats (Takeyama et al. 2001).
- IL13 treatment causes EGFR-dependent (diminished through EGFR-selective inhibitor BIBX1522 pretreatment) MUC5AC staining and AB/PAS-staining of mucous glycoconjugates in rat airway tissue (Shim et al. 2001).
- Instillation of agarose plugs on rat airways resulted in EGFR-dependent (diminished through EGFR-selective inhibitor BIBX1522 pretreatment) MUC5AC gene expression and mucus glycoconjugate production (H.M. Lee et al. 2000).
- Daily treatment of primary human bronchial epithelial cells with xanthine and xanthine oxidase for 3 days increased the levels of phosphorylated EGFR and induced expression of MUC5AC mRNA and protein. These responses could be at least partially prevented by pre-incubation with anti-EGFR antibodies and by tissue kallikrein (TK) inhibitor (TK processes pro-EGF) (Casalino-Matsuda, Monzón, and Forteza 2006).
- MUC5AC and MUC5B gene and protein expressions were induced in Mycoplasma pneumoniae M129 infection in human NCI-H292 cells, primary human bronchial epithelial cells and mouse airways. EGFR phosphorylation and thus signalling was induced by M. pneumoniae, and EGFR inhibition by AG1478 attenuated M. pneumonia-induced mucin overproduction (Hao et al. 2014).
- Chronic treatment with EGFR inhibitor gefitinib downregulated the expression of EGFR and markedly decreased the levels of MUC5AC in BALF of ovalbumin-challenged mice. The ovalbumin-induced mucus secretion was significantly ablated by chronic gefitinib treatment in mouse lung tissue (Song et al. 2016).

- TGFA increased MUC5AC protein production and mucous glycoconjugates in NCI-H292 cells, and pre-treatment with EGFR inhibitor AG1478 prevented the increase in the staining and MUC5AC production (Takeyama et al. 2008).
- Increased MUC5AC expression was seen in the tracheal epithelium of C57Bl/6 mice after instillation of PM2.5 (particulate matter with a diameter below 2.5 um). PM2.5 increased MUC5AC expression in human H292 lung cancer cells and primary human bronchial epithelial cells grown as monolayer. Pretreatment with EGFR inhibitor or neutralizing anti-EGFR antibody reduced this response (Val et al. 2012).
- Acrolein exposure of FVB/NJ mice increased lung MUC5AC RNA and protein levels. EGFR inhibitor erlotinib gavage after every exposure abolished this effect (Deshmukh et al. 2008).
- Infection of H292 cells with influenza A virus (IAV) resulted in increased EGFR phosphorylation. This was correlated with increases in MUC5AC gene and protein levels. Cell treatment with anti-EGFR antibody or EGFR inhibitor PD168393 abolished MUC5AC increase. EGFR ligand TGFA neutralization with a specific antibody ablated IAV-induced MUC5AC up-regulation (Barbier et al. 2012).
- Treatment of primary normal bronchial epithelial (NHBE) cells and HBE1 cell cultures with TCDD resulted in increased MUC5AC gene and protein levels. EGFR phosphorylation was also increased. TCDD-induced MUC5AC expression was significantly decreased when cells were pretreated with EGFR inhibitor AG1478 (Y.C. Lee et al. 2011).
- Treatment of NCI-H292 cells with EGFR ligands with EGF, TGFA or TNF increased MUC2 and MUC5AC promoter activity, mRNA levels, and apomucin expression. Pretreatment with EGFR inhibitor AG1478 resulted in complete inhibition of MUC2 and MUC5AC gene up-regulation by EGF and TGFA (Perrais et al. 2002).
- EGFR activation promoted mucus production in mice (Jia et al. 2021). EGFR inhibitor erlotinib decreased MUC5AC levels and mucus secretion in the HDM-induced asthma model (Jia et al. 2021).
- Virulence factor flagellin purified from human respiratory pathogen Pseudomonas aeruginosa increases MUC5AC mRNA and protein levels in 16HBE cells. Reactive oxygen species (involved in EGFR activation) scavenging, TGFA (EGFR ligand) neutralization with an antibody, TACE (metalloprotease involved in TGFA release) inhibition with TAPI-1, and treatment with EGFR neutralizing antibody which blocks EGFR ligand binding and inhibits EGFR phosphorylation, all decreased flagellin-induced MUC5AC production (Yu, Zhou, et al. 2012).
- Blastomyces dermatitidis increased MUC5AC and MUC5B levels in immortalized canine airway carcinoma (BACA) cells. B. dermatitidis also increased phosphorylation levels of EGFR in BACA cells. Pretreatment of BACA cells with EGFR inhibitor AG1478 before challenge by B. dermatitidis reduced expression of MUC5AC and MUC5B (Choi et al. 2021).
- Treatment of primary human lobar bronchial epithelial cells (HLBECs) with pine wood smoke particulate matter (WSPM; 2.5 µm and smaller fractions) increased EGFR phosphorylation levels. MUC5AC protein levels in the cells and MUC5AC secretion into media was also increased after pine WSPM treatment. In addition, pine WSPM induced MUC5AC expression in mouse airways and in primary human small airway epithelial cells (SAECs), immortalized HBEC-3KT cells, as well as human airway epithelial cell models from unique donors. Inhibition of EGFR AG1478 prevented pine WSPM-induced MUC5AC expression (Memon et al. 2020).
- Particulate matter (PM) treatment of human bronchial epithelial cells (HBECs) significantly upregulated MUC5AC expression. PM also increased the levels of EGFR ligand Amphiregulin (AREG) as well as EGFR phosphorylation. AREG silencing through siRNA alleviated PM-induced EGFR phosphorylation and mucus hypersecretion in HBECs, while exogenous AREG enhanced PM-induced EGFR pathway activation and mucus hypersecretion. EGFR inhibitor AG1478 pretreatment significantly inhibited MUC5AC expression in PM-stimulated HBECs (Wang et al. 2019).
- Exposure to wood smoke PM with a diameter less than 2.5um (WSPM2.5) increased MUC5AC production in the rat airways, primary human airway epithelial cells and the NCI-H292 cell line. EGFR-selective inhibitor AG1478 pretreatment prevented MUC5AC expression in NCI-H292 cells (Huang et al. 2017).
- In SARS-CoV-2-infected human bronchial epithelial (HBE) cultures MUC5B/MUC5AC gene expression peaked 7-14 days post inoculation, MUC5B protein levels were significantly up-regulated at day 14 and MUC5AC protein showed tendency of up-regulation. SARS-CoV-2 infection of HBE cultures induced expression of EGFR ligands (AREG, HBEGF). Inhibiting EGFR pathway with EGFR-tyrosine kinase inhibitor (Gefitinib) or with EGFR monoclonal antibody (Cetuximab) reduced SARS-CoV-2-induced MUC5B and MUC5AC gene expressions in HBE cultures (Kato et al. 2022).
- Lipopolysaccharide (LPS)-induced increases in MUC5AC mRNA and protein levels and increased Alcian blue/PAS staining in rat nasal epithelium were significantly inhibited by EGFR inhibitor AG1478 intranasal instillation (Takezawa et al. 2016).
- EGFR inhibitor AG1478 treatment of bronchial epithelial cell cultures from asthmatic children showed significant reductions in mucus (MUC5AC) secretion. In addition, bronchial epithelial cell cultures from asthmatic children differentiated in EGF-negative conditions had reduced mucus secretion compared to the cultures differentiated in the presence of EGF (Parker et al. 2015).
- LPS induces increased MUC5AC expression in human intrahepatic biliary epithelial cells (HIBECs). EGFR inhibitor AG1478 treatment inhibits LPS-induced MUC5AC overexpression (Liu et al. 2013).
- Cigarette smoke increased MUC5AC expression in immortalized human bronchial epithelial 16HBE cells. This increase was prevented by pretreatment with EGFR inhibitor gefitinib (Yu, Li, et al. 2012).

Uncertainties and Inconsistencies

A study carried out in three airway epithelial cell systems (normal human bronchial epithelial primary culture, immortalized normal bronchial epithelial cell line HBE1, and human lung adenocarcinoma cell line A549) showed increased MUC5AC and MUC5B expression via SP1-mediated mechanism whereby MUC5AC expression was diminished by EGFR inhibition, but MUC5B expression was not sensitive to EGFR inhibition (Yuan-Chen Wu et al. 2007).

Quantitative Understanding of the Linkage

Below we list some quantitative aspects of the response relationship.

Response-response relationship

- Stimulation of EGFR by its ligands, 25 ng/ml EGF and 25 ng/ml TGFA, causes mucous glycoconjugate production in human pulmonary mucoepidermoid carcinoma cell line NCI-H292 (assessed by volume density of Alcian blue/periodic acid–Schiff (AB–PAS)). EGF and

TGFA induce MUC5AC gene (Northern blot) and protein (ELISA) expression. Pretreatment of cells (30 min) with selective kinase inhibitors for EGFR (10 µg/ml BIBX1522, 10 µM AG1478, 10 µM Compound 56) prevented the ligand-induced staining for MUC5AC protein (Takeyama et al. 1999). Ovalbumin sensitization resulted in EGFR protein expression and mucous glycoconjugate production (AB-PAS-positive staining) in rat airways which was inhibited by pretreatment with EGFR inhibitor BIBX1522 (10 mg/kg, i.p.) (Takeyama et al. 1999). The same research group has demonstrated upregulation in EGFR-mediated MUC5AC mRNA and protein production after cigarette smoke exposure in NCI-H292 cells and in vivo in Sprague-Dawley rats (Takeyama et al. 2001).

- IL13 treatment causes EGFR-dependent (diminished through EGFR-selective inhibitor BIBX1522 pretreatment) MUC5AC staining and AB/PAS-staining of mucous glycoconjugates in rat airway tissue. 50 ng IL-13 were necessary to significantly raise the % mucin-expressing epithelial area above background (<10% in controls vs 15% in treated rats), and % mucin-expressing epithelial area was maximal at the highest tested concentration, 500 ng. Mechanistically, IL13 induced TNF expression in airway epithelium and infiltrating neutrophils through IL8-like chemoattractant. TNF subsequently induced EGFR expression (Shim et al. 2001).
- Instillation of agarose plugs (0.7- to 0.8-mm diameter 4% agarose type II medium electroendosmosis in PBS) on rat airways resulted in EGFR-dependent (diminished through EGFR-selective inhibitor BIBX1522 pretreatment) mucus glycoconjugate production and MUC5AC gene expression. Mechanistically, agarose plugs caused TNF release in the airway epithelium and inflammatory cells, thus inducing EGFR (H.M. Lee et al. 2000).
- Daily treatment of primary human bronchial epithelial cells with 0.6 mM xanthine and 0.5 units xanthine oxidase for 3 days nearly doubled the levels of phosphorylated EGFR and increased expression of MUC5AC mRNA by approx. 2-fold and that of MUC5AC protein by ca. 30%. These responses could be at least partially prevented by pre-incubation with anti-EGFR antibodies and by tissue kallikrein (TK) inhibitor (TK processes pro-EGF) (Casalino-Matsuda, Monzón, and Forteza 2006).
- MUC5AC and MUC5B gene and protein expressions were induced in Mycoplasma pneumoniae M129 infection in human NCI-H292 cells, primary human bronchial epithelial cells and mouse airways. EGFR phosphorylation and thus signalling was induced by M. pneumoniae, and EGFR inhibition (10uM AG1478) attenuated M. pneumonia-induced mucin overproduction (Hao et al. 2014).
- Chronic treatment with EGFR inhibitor gefitinib (50 mg/kg, for 12 h each day, days 14-20) downregulated the expression of EGFR and markedly decreased the levels of MUC5AC (ELISA) in BALF of ovalbumin-challenged mice. The ovalbumin-induced mucus secretion (PAS staining) was significantly ablated by chronic gefitinib treatment in mouse lung tissue (Song et al. 2016).
- TGFA increased MUC5AC protein production and the Alcian blue/PAS staining (mucous glycoconjugates) in NCI-H292 cells within 24 h, and pre-treatment with EGFR inhibitor AG1478 (10uM) prevented the increase in the staining and MUC5AC production (Takeyama et al. 2008).
- Increased *MUC5AC* expression (ca. 5-fold) was seen in the tracheal, but not the lung epithelium of C57Bl/6 mice 48 h after instillation of PM2.5 (particulate matter with a diameter below 2.5 um). This effect was significant with 50 µg, but not with 10 µg PM2.5. PM2.5 dose-dependently increased MUC5AC expression in human H292 lung cancer cells and primary human bronchial epithelial cells grown as monolayer, with significant increases of ca. 10- and 8-fold above that of control at PM2.5 concentrations > 5 µg/cm2. At a concentration of 10 µg/cm2, *MUC5AC* mRNA level in H292 cells peaked at >30 times that of control after 24 h of treatment. When primary bronchial epithelial cells differentiated the air-liquid interface were treated with PM2.5, MUC5AC expression also increased in a dose-dependent manner. However, 10 µg/cm2 PM2.5 were necessary to induce a significant, maximal response (ca. 3-fold increase), and pretreatment with 10 µM AG1478 or 0.5 µg/µL neutralizing anti-EGFR antibody reduced this response by ca. 50% (Val et al. 2012).
- Acrolein exposure of FVB/NJ mice at 2 ppm for 6 h per day, 5 days a week, for 4 weeks increased lung MUC5AC RNA and protein levels approx. 4-fold. Gavage of 100 mg/kg EGFR inhibitor erlotinib after every exposure abolished this effect (Deshmukh et al. 2008).
- Infection of H292 cells with influenza A virus (IAV) at MOI=1 resulted in increased EGFR phosphorylation, peaking at 24 h. This was correlated with activation of ERK and Sp1 and increases in MUC5AC gene and protein levels. Cell treatment with anti-EGFR antibody or EGFR inhibitor PD168393 abolished MUC5AC increase. EGFR ligand TGFA neutralization with a specific antibody ablated IAV-induced MUC5AC up-regulation (Barbier et al. 2012).
- Treatment of primary normal bronchial epithelial (NHBE) cells and HBE1 cell cultures with 10 nM TCDD resulted in increased MUC5AC gene and protein levels. EGFR, ERK, p38 MAPK phosphorylation was also increased. TCDD-induced MUC5AC expression was significantly decreased when cells were pretreated with EGFR inhibitor AG1478, MEK/ERK inhibitor PD98059, and p38 inhibitor SB203580. SP1 involvement was demonstrated by treatment with the Sp1 inhibitor mithramycin A (Y.C. Lee et al. 2011).
- Treatment of NCI-H292 cells with EGFR ligands with EGF (25 ng/ml), TGFA (20 ng/ml), or TNF (40 ng/ml) resulted in increase of MUC2 and MUC5AC promoter activity, mRNA levels, and apomucin expression. Pretreatment with EGFR inhibitor AG1478 resulted in complete inhibition of MUC2 and MUC5AC gene up-regulation by EGF and TGFA. Mechanistically, EGF and TGFA-induced increases in MUC2 and MUC5AC were mediated by Sp1 on transcriptional level (Perrais et al. 2002).
- Jia and colleagues demonstrated that EGFR activation promotes mucus production and exacerbates asthma in mice through Claudin1 (CLDN1) decrease (Jia et al. 2021). Claudin1 knockdown promoted MUC5AC gene and protein expression in human bronchial epithelial 16HBE cells, ALI cultures, and exacerbated house dust mite (HDM)-induced asthma in mice (notably, increased MUC5AC protein levels and mucus secretion). EGFR ligand HBEGF (heparin binding EGF like growth factor) significantly decreased mRNA levels of CLDN1 in 16HBE cells and promoted MUC5AC expression which was reversed by CLDN1 overexpression. EGFR inhibitor erlotinib restored the expression of CLDN1 and decreased MUC5AC levels and mucus secretion in the HDM-induced asthma model (Jia et al. 2021).
- Virulence factor flagellin purified from human respiratory pathogen Pseudomonas aeruginosa increases MUC5AC mRNA and protein levels in a concentration-dependent manner reaching a peak at 10 ug/ml in 16HBE cells. Reactive oxygen species (involved in EGFR activation) scavenging, TGFA (EGFR ligand) neutralization with an antibody, TACE (metalloprotease involved in TGFA release) inhibition with TAPI-1, and treatment with EGFR neutralizing antibody which blocks EGFR ligand binding and inhibits EGFR phosphorylation, all decreased flagellin-induced MUC5AC production (Yu, Zhou, et al. 2012).
- Blastomyces dermatitidis–infected canine and Histoplasma capsulatum–infected feline airway epithelia exhibited increased expression of mucins MUC5AC and MUC5B which was inversely correlated with expression of FOXA2. Similarly, B. dermatitidis increased mucins and reduced FOXA2 in immortalized canine airway carcinoma (BACA) cells. B. dermatitidis also increased phosphorylation levels of EGFR, AKT and ERK1/2 in BACA cells. Pretreatment of BACA cells with inhibitors of EGFR (AG1478), AKT (LY294002), and ERK1/2 (PD98059) before challenge by B. dermatitidis reduced expression of MUC5AC and MUC5B and restored the expression of FOXA2, indicating that pulmonary blastomycosis activates both EGFR-ERK1/2 and EGFR–AKT signalling pathways leading to FOXA2

suppression and excessive mucus production (Choi et al. 2021).

- Treatment of primary human lobar bronchial epithelial cells (HLBECs) with pine wood smoke particulate matter (WSPM; 2.5 µm and smaller fractions) for 24 h induced MUC5AC expression 20–30-fold. MUC5AC protein levels in the cells and MUC5AC secretion into media was also increased after pine WSPM treatment. In addition, pine WSPM induced MUC5AC expression in mouse airways and in primary human small airway epithelial cells (SAECs), immortalized HBEC-3KT cells, as well as human airway epithelial cell models from unique donors. At 6 h after pine WSPM treatment, increased EGFR phosphorylation was observed in HLBECs. Transcript abundance of EGFR ligands EPGN and HB-EGF was increased up to approximately 20- and 2.5-fold, respectively, in 24 h following pine WSPM treatment. Inhibition of EGFR with 10 uM AG1478 prevented pine WSPM-induced MUC5AC expression. Inhibitor treatments of GSK3β (TWS119) and p38 MAPK (PD16936) also effectively inhibited WSPM-induced MUC5AC expression suggesting p38 MAPK and GSK3β implication downstream of EGFR (Memon et al. 2020).
- Particulate matter (PM (50, 100, and 300 µg/cm3)) treatment of human bronchial epithelial cells (HBECs) over 24 h significantly and dose-dependently upregulated MUC5AC expression. PM also increased the levels of EGFR ligand Amphiregulin (AREG) as well as EGFR and AKT/ERK phosphorylation. AREG silencing through siRNA alleviated PM-induced EGFR (and AKT, ERK) phosphorylation and mucus hypersecretion in HBECs, while exogenous AREG enhanced PM-induced EGFR/AKT/ERK pathway activation and mucus hypersecretion. EGFR inhibitor AG1478 pretreatment significantly inhibited EGFR-AKT/ERK pathway activation and MUC5AC expression in PM-stimulated HBECs (Wang et al. 2019).
- Yet another study on effect of wood smoke PM with a diameter less than 2.5um (WSPM2.5), show increase in MUC5AC production in the rat airways, primary human airway epithelial cells and the NCI-H292 cell line. EGFR-selective inhibitor AG1478 pretreatment prevented MUC5AC expression in NCI-H292 cells (Huang et al. 2017).
- MUC5B and variably MUC5AC RNA levels were increased in both proximal and distal airways in COVID-19 autopsy lungs. MUC5B-dominated mucus accumulation was observed in bronchioles, microcysts, and in damaged lung alveolar spaces in 90% of COVID-19 subjects. In SARS-CoV-2-infected human bronchial epithelial (HBE) cultures MUC5B/MUC5AC gene expression peaked 7-14 days post inoculation, MUC5B protein levels were significantly up-regulated at day 14 and MUC5AC protein showed tendency of up-regulation. SARS-CoV-2 infection of HBE cultures induced expression of EGFR ligands (AREG, HBEGF). Inhibiting EGFR pathway with EGFR-tyrosine kinase inhibitor (Gefitinib) or with EGFR monoclonal antibody (Cetuximab) reduced SARS-CoV-2-induced MUC5B and MUC5AC gene expressions in HBE cultures (Kato et al. 2022).
- LPS-induced increases in MUC5AC mRNA and protein levels and increased Alcian blue/PAS staining in rat nasal epithelium were significantly inhibited by EGFR inhibitor AG1478 intranasal instillation (Takezawa et al. 2016).
- EGFR inhibitor AG1478 treatment of bronchial epithelial cell cultures from asthmatic children showed significant reductions in mucus (MUC5AC) secretion. In addition, bronchial epithelial cell cultures from asthmatic children differentiated in EGF-negative conditions had reduced mucus secretion compared to the cultures differentiated in the presence of EGF (Parker et al. 2015).
- Treatment with 100 µg/mL of LPS for 24 h increased MUC5AC protein and phosphorylated EGFR levels in HIBECs. LPS-induced MUC5AC overexpression was significantly inhibited by treatment with 10 µg/mL of EGFR inhibitor AG1478 (Liu et al. 2013).
- Cigarette smoke treatment (9 puffs) for 24 hours increased MUC5AC expression in 16HBE cells in a HIF1A-dependent manner. HIF1A knockdown with specific siRNA significantly inhibited cigarette smoke-induced MUC5AC mRNA and protein increase. EGFR inhibitor gefitinib inhibited cigarette smoke-induced HIF1A production and HIF1A activity, and decreased MUC5AC mRNA and protein levels in a concentration-dependent manner (Yu, Li, et al. 2012).

Time-scale

In many studies the measurements of EGFR activity and mucin levels were routinely done simultaneously at same time points after stressor introduction. Several examples below demonstrate that various stressors or EGFR ligands increase mucin levels as early as 12 hour time-point, usually reaching higher levels of expression at later time-points such as 24 hours.

- EGF (25 ng/ml) alone or TGFα (25 ng/ml) alone caused an ≈2-fold increase in MUC5AC protein production (ELISA) in NCI-H292 cells (routinely done 12 or 24 hours). Incubation with EGF or TGFα increased MUC5AC gene expression beginning at 12 h and reaching a maximum at 24 h (Takeyama et al. 1999).
- IL13 treatment caused MUC5AC staining and Alcian blue-PAS -positive staining of mucous glycoconjugates in rat airway tissue after IL13 instillation. EGFR protein staining started to occur at 16h and increased at 24h. Similarly, AB/PAS staining was clearly detectable at 16h and reached higher levels at 24h (Shim et al. 2001).
- Instillation of agarose plugs on rat airways resulted in EGFR-dependent mucus glycoconjugate production and MUC5AC gene expression detectable as early as 24 h and greatest 72 h after instillation. Authors also show a clearly detected immunostaining with an antibody to EGFR in cells that stained positively with AB/PAS (H.M. Lee et al. 2000).
- M. pneumoniae M129 infection led to respectively 6.8- and 5-fold increase in mucins MUC5AC and MUC5B in NHBE cells after 18h of infection. In NCI-H292 cells exposure to M. pneumoniae M129 for 18h stimulated MUC5AC and MUC5B expressions to 2.9- and 3-fold, respectively. Phosphorylated EGFR was also detected at the 18h postinfection in NCI-H292 cells. After 3 days of infection with M. pneumoniae, the conducting airways of mice displayed mucus hypersecretion with 8.8-fold higher expression of MUC5AC and 9.4-fold higher levels of MUC5B protein (Hao et al. 2014).
- Infection of NCI-H292 cells with influenza A virus at MOI=1 resulted in increased EGFR phosphorylation (and related MUC5AC increase), peaking at 24 h (Barbier et al. 2012).
- Treatment of NCI-H292 cells with EGF (25 ng/ml), TGFA (20 ng/ml), or TNF (40 ng/ml) resulted in EGFR-dependent increases of MUC2 and MUC5AC promoter activity, mRNA levels, and apomucin expression assessed at 24 hours (Perrais et al. 2002).
- Daily intranasal insitillation of 0.1 mg LPS (E.coli 0111:B4) for 3 consecutive days increased rat nasal epithelium mucus production which was inhibited by intranasal instillation of 10 mg/kg AG1478 (Takezawa et al. 2016).

The following studies show that EGFR activation (upstream KE) occurs earlier than mucin expression (downstream KE), complying with temporal concordance terms of upstream KE occurring before downstream KE.

- Treatment of NHBE cells with 10 nM TCDD resulted in significantly increased EGFR phosphorylation after 30 min. TCDD treatment led to a time-dependent increase in MUC5AC promoter activity, peaking between 6 and 12 h. The results demonstrate that TCDD activates

EGFR within 30 minutes, activates downstream ERK and p38 within 3–4 hours, followed by Sp1 phosphorylation increase at 4–6 hours, leading to increase in MUC5AC transcription (Y.C. Lee et al. 2011).

- At a concentration of 10 µg/cm2 PM2.5, MUC5AC mRNA level in NCI-H292 cells peaked at >30 times that of control after 24h of treatment and decreased to approx. 20-fold that of control at 36h; the increase after 8h time point was not significant. EGFR ligand AREG release was significantly induced by 10 µg/cm2 PM2.5 from 8h of exposure to 36h. AREG ligand was tested alone and shown to induce MUC5AC expression at concentrations found in the supernatants of PM2.5-treated cells after 24h exposure (Val et al. 2012).
- Treatment of primary HLBECs with pine wood smoke particulate matter (WSPM) for 24 h induced MUC5AC expression 20–30-fold, earlier time-points were not tested. EGFR phosphorylation was already increased following pine WSPM treatment at 6 h (but not at 2 h). The mRNA expression of EGFR ligands EPGN and HB-EGF rapidly increased within 2–4 h of pine WSPM exposure, and HLBEC treatment with EPGN and HB-EGF induced MUC5AC expression, albeit to a lesser degree than pine WSPM (Memon et al. 2020).
- HBEC treatment with particulate matter (PM) for 24 h significantly induced MUC5AC expression through EGFR/AKT/ERK pathway whereby EGFR phosphorylation increase was observed at 15 minutes after PM treatment and reached highest levels at 1 hour post-treatment. However, MUC5AC levels were not measured at earlier time-points (Wang et al. 2019).

Known Feedforward/Feedback loops influencing this KER

Wood smoke particulate matter with a diameter less than 2.5um (WSPM2.5) induced MUC5AC production in the rat airways, primary human airway epithelial cells and the NCI-H292 cell line. Mechanistic investigation in NCI-H292 cells showed that MUC5AC production occurs through amphiregulin (AREG)-EGFR-ERK signalling (a. AREG neutralization suppressed most of the WSPM2.5-induced EGFR and ERK phosphorylation, b. pretreatment with EGFR-selective inhibitor AG1478 abolished ERK activation and MUC5AC expression, c. ERK–selective inhibitor PD98059 pretreatment inhibited WSPM2.5-induced MUC5AC expression). In turn, AREG-EGFR-ERK pathway activation was shown to contribute to the *de novo* synthesis of AREG (a. pretreatment with AG1478 or PD98059 significantly reduced WSPM2.5-induced AREG mRNA expression and AREG release, b. exogenous AREG treatment increased AREG mRNA expression, an effect that was inhibited in the presence of AG1478 or PD98059). Thus this positive feedback loop sustains mucus production (Huang et al. 2017).

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Relationship: 2858: Increase, goblet cell number leads to Increase, Mucin production

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
EGFR Activation Leading to Decreased Lung Function	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens		NCBI
Mus musculus	Mus musculus		NCBI
Rattus norvegicus	Rattus norvegicus		NCBI
Mustela furo	Mustela putorius furo		NCBI

Life Stage Applicability

Life Stage	Evidence
Not Otherwise Specified	

Sex Applicability

Sex	Evidence
Unspecific	

Increase in goblet cell numbers and concomitant increased mucin production developing after exposure to noxious agents, such as allergens, cigarette smoke, pollution, or pathogens has been described in human independently of sex and age. Experimental models exist in mice, rats, guinea pigs, rabbits, dogs, and ferrets.

Key Event Relationship Description

An increase in goblet cell numbers arises from proliferation of this particular cell population (goblet cell hyperplasia) and/or from transdifferentiation of other specialized cell types, such as ciliated cells and club cells, into goblet cells (goblet cell metaplasia; Reader et al, 2003; Evans et al., 2004; Tesfaigzi, 2006). Goblet cell hyperplasia (GCH) is a common feature of airway epithelia in asthma and other respiratory diseases and can arise from airway injury following exposure to, for example, allergens, pathogens, or cigarette smoke (Miyabara et al., 1998; Nagao et al., 2003; Saetta et al., 2000; Walter et al., 2002; Hao et al., 2012, 2013, 2014; Lukacs et al., 2010; Yageta et al., 2014; Hegab et al., 2007; Silva and Bercik, 2012; Kim et al., 2016). Goblet cell metaplasia (GCM) is a key feature of remodeled airways observed in both asthma and chronic obstructive pulmonary disease (COPD; Kato et al., 2020; Kuchibhotla and Heijink, 2020; Nie et al., 2012). Since goblet cells are mucin-producing cells, an increase in goblet cell numbers will consequently lead to an increase (from basal levels) in mucin production, in fact methods for goblet cell detection and quantification include measurement of mucin levels with specific antibodies or staining of mucous glycoconjugates (Alcian Blue/periodic acid Schiff (AB/PAS) stain). Correlation and co-incidence between increase in goblet cell numbers and increased mucin production is shown in multiple studies.

Evidence Supporting this KER

This KER is inferred in that goblet cells are specialized mucin production cells and widely accepted measurement methods for counting goblet cells are based on staining of mucous glycoconjugates. There is indirect evidence demonstrating an increase in mucin production along the presence of GCH or GCM in airway epithelia following stressor exposures, judged by increased MUC5AC mRNA and protein expression, histopathological examination, increase in AB/PAS staining, and/or MUC5AC-positive antibody staining (Alimam et al, 2000; Hegab et al., 2007; An et al., 2013; Zhou et al., 2016). Increase in goblet cell number and mucin overproduction are also linked experimentally through genetic modification. For example, conditional deletion of transcription factor Foxa2 in respiratory epithelial cells of the developing mouse lung results in goblet cell hyperplasia in bronchi and bronchioles at post-natal day 16 and later (evidenced by histology), which was accompanied by extensive AB/PAS and MUC5AC staining (Wan et al., 2004). Similarly, Muc1-knockout rats exposed to cigarette smoke were protected from goblet cell metaplasia and MUC5AC overproduction (Kato et al., 2020).

Biological Plausibility

This KER is inferred from the functional characteristic of the goblet cells whose primary role is mucin production, hence the assumption that increase in goblet cell numbers also increases mucin production is highly plausible. Studies in human cells, mice and rats demonstrate that mucin content or MUC5AC mRNA and protein expression increase in the presence of histologically confirmed GCH or GCM. While both events are measured in parallel and causal evidence is missing, our confidence remains high for the plausibility of this relationship.

Empirical Evidence

Increase in goblet cell numbers and associated increase in mucin production can be induced by a variety of insults including cigarette smoke (Lee et al., 2006; Kato et al., 2020, An et al., 2013; Ning et al., 2013; Xu et al., 2015; Liang et al., 2017), acrolein (Chen et al., 2010), bacterial products (Kim et al., 2004; Takezawa et al., 2016), neutrophil elastase (Voynow et al., 2004; Park et al., 2013), ozone (Harkema and Hotchkiss, 1993), allergens (Le Cras et al., 2011, Habibovic et al., 2016), interleukin 13 (Atherton et al., 2003; Tanabe et al., 2011; Mishina et al., 2015). Parallel increase in goblet cell numbers and MUC5AC gene and protein

expression levels were also reported in lung tissues of asthmatic and COPD patients, in nasal polyp tissues (Burgel et al., 2000; Ordonez et al., 2001; Xia et al., 2014) as well as in mouse models of asthma (Evans et al., 2004; Takeyama et al., 2008, van Hove et al., 2009). Increases in goblet cell numbers were confirmed by morphometric analysis (enumeration of goblet cells, % PAS-stained area) and correlated with increases in mucin gene and protein expression levels.

Uncertainties and Inconsistencies

MUC5B, mainly present in submucosal glands, is the other main mucin found in human airways (Rose and Voynow, 2002). MUC5AC and MUC5B often both increase with goblet cell numbers increase in patients with respiratory diseases (Burgel et al., 200). However, depending on the causative agent, dominant MUC5B immunophenotypes are observed with no induction in MUC5AC (Silva and Bercik, 2012). Sprague-Dawley rats receiving one intratracheal dose of LPS developed GCH in their terminal bronchioles that was not MUC5AC and PAS-positive. An analysis in human bronchus epithelial cells confirmed that when challenged with supernatant from LPS-stimulated macrophages, goblet cells induced MUC5B levels but MUC5AC was inhibited.

Quantitative Understanding of the Linkage

Our quantitative understanding of this KER is limited by the fact that few studies investigate causality between increase in goblet cell number and increased mucin production. To our knowledge, there is no comprehensive, systematic study of the dose-response relationship. This may be primarily due to the fact that GCM and GCH are qualitative findings on histopathological examination and cannot be as easily quantified as, for example, the number of cells that stain positively with an anti-MUC5AC antibody as a marker for mucin production.

Response-response relationship

The following examples all describe the parallel increase in goblets cells and mucin production after exposure to a noxious agent. In some of these examples, the response-response relationship is reinforced by the use of an antagonist or inhibitor treatment that attenuates or blocks the stressor-induced goblet cell proliferation and concomitantly reduces mucin mRNA and protein expression.

- Intranasal instillation of 0.1 mg LPS (E.coli 0111:B4) once a day for 3 consecutive days induced GCM in rat nasal epithelium (as judged by histopathology), with an approx. 50% increase in AB/PAS-stained epithelium compared to untreated controls. A treatment (intraperitoneal (i. p.) injection of 1 or 10 mg/kg) with of the epithelial growth factor receptor (EGFR) inhibitor AG1478 one hour before each LPS administration significantly inhibited LPS-induced GCM (histology assessment) and mucus production. Intranasal instillation of AG1478 one hour after LPS instillation resulted in a similar inhibition of both GCM and mucus production (Takezawa et al., 2016).

- Intratracheal instillation of agarose plugs (0.7- to 0.8-mm diameter; 4% agarose type II) in male Fischer 344 rats caused GHC, evidenced by histology and AB/PAS staining. In the airways containing the plugs, goblet cell numbers increased from 0 cells/mm basal lamina to 13.1±5.6, 25.7±15.0, and 51.5±9.0 cells/mm basal lamina after 24, 48, and 72 h, respectively. The percentage of the total length of epithelium staining positively with AB/PAS increased from 0.1 ± 0.1% in control animals to 4.7 ± 1.4, 13.3 ± 0.7, and to 19.1 ± 0.7% at 24, 48, and 72 h, respectively. Muc5ac gene expression was found preferentially in cells that were AB/PAS-positive and increased in a time-dependent manner (Lee et al., 2000).

- Analysis of lungs from BALB/c mice sensitized with five i. p. injections of 100 µg ovalbumin (OVA) followed by intranasal instillation of 100 µg OVA, as well as from BALB/c mice treated with 5 µg IL-13 (intranasal instillation on three consecutive days) revealed clear airway GCM 24 h post treatment. Marked MUC5AC mRNA and protein expression (apomucin and glycosylated mucin) were observed in lungs from OVA- and IL-13-treated mice but not in lungs from control saline-treated mice (Alimam et al. 2000).

- BALB/c mice were sensitized to OVA with 4 i. p. injections (20 µg each) administered at weekly intervals. They were subsequently exposed for 30 min to an aerosol containing 2.5% OVA. At 3 days post challenge, ca. 60% of the cells in proximal airways were AB-PAS-positive. The number of these cells peaked at day 7 (> 30-fold increase compared with unsensitized controls). The volume density of mucin concomitantly increased on day 3 post challenge with peak measures on day 7 (15-fold increase over baseline) (Evans et al., 2004).

- Brown–Norway rats were sensitized by an i. p. injection with 1 mg OVA and 200 mg Al(OH)3 in 1mL of sterile saline on days 0 and 7. The rats were then exposed to aerosolized 1% (w/v) OVA or sterile saline for 30 min on days 14–16. Distinct GCH (evidenced by AB/PAS staining) was observed in the epithelium throughout the airways of the OVA-sensitized and -challenged rats 24 h after the final OVA challenge (on day 17), and the staining progressed to 7-fold increase further on day 24. Marked MUC5AC immunoreactivity was observed in goblet cells similar to the AB/PAS staining pattern. (Takeyama et al., 2008).

- Oropharyngeal inspiration of neutrophil elastase (50 µg (43.75 units/40 µl PBS)) by BALB/c mice on day 1, 4, and 7, resulted in GCM as observed on days 8, 11 and 14. The histological mucus index (HMI, defined a grading system of PAS-positive staining, from 0 (no PAS staining) to 4 (>75% of airways epithelium stained) increased from 1 (25% stained area) on day 8 to ca. 2 (26-50%) on day 11, then decreased to 1.3 on day 14, while the HMI remained close to 0 in controls. Muc5ac mRNA expression qualitatively corresponded to AB/PAS histology evolving form 1.64 ± 0,49 on day 8, to 13.53 ± 3.28 on day 11, and 8.62 ± 1.48 on day 14. Muc5ac protein expression followed the same trend (Voynow et al., 2004).

- Lungs of C57BL6/J mice treated with house dust mite (HDM) extract (intranasal instillation of 50 µg HDM, 5 days per week for 3 weeks) showed increase in goblet cell number as judged by increased PAS staining in the airway epithelium with concurrent ca. 10-fold increase in Muc5ac expression (Habibovic et al., 2016).

- Pyocyanin, a redox-active exotoxin of Pseudomonas aeruginosa, caused increase in goblet cell numbers in mouse airways after 3-week daily intranasal inoculation (25 µg/day). Development of GCM in small terminal bronchioles (88-fold more PAS-stained cells) was paralleled with a 6.4-fold and a 11.4-fold increase in MUC5B-positive cells in large bronchi and terminal bronchioles respectively (Hao et al., 2012).
- Male Sprague–Dawley rats that were exposed to 3 ppm acrolein for 6 h a day, for 2 x 5 days separated by a 2-day rest, developed GCM (as judged by histopathology), increasing the % AB/PAS-positive stained epithelium from ca. 5% (in air controls) to 35%. This was accompanied by a nearly 15% increase in Muc5ac-positive stained cells, a ca. 3-fold increase in Muc5ac mRNA expression and a ca. 4-fold increase in protein expression. A treatment with simvastatin, a statin inhibitor of EGFR and extracellular signal-regulated kinase (ERK) activation one day prior exposure to acrolein, significantly inhibited the increase of AB/PAS staining in airway epithelium in a dose-dependent matter. The number of Muc5ac-positive cells was also significantly attenuated, as well as the Muc5ac protein levels in lung homogenates (Chen et al., 2010).
- Exposure of female Sprague-Dawley rats to wood smoke (total of 40 g of China fir sawdust smoldered) for 1 h four times per day, five days per week, for three months caused GCM in the airways (as judged by histopathology), a 2-fold increase in Muc5ac gene expression, an increase in the % AB/PAS-positive stained epithelium from approx. 6% (air controls) to ca. 17%, an increase in Muc5ac-positive stained cells from approx. 5% (air controls) to ca. 25% (Huang et al., 2017).
- In Sprague-Dawley rats that were whole-body exposed to 4% (v/v air) cigarette smoke (CS) for 1 h daily, for 56 days, the number of goblet cells in the bronchial epithelium significantly increased (ca. 10 cells/mm epithelium in air controls vs 60 cells/mm in CS-treated animals), and the number of Muc5ac-positive cells increased from ca. 20 cells/mm to ca. 80 cells/mm. A treatment with (-)-Epigallocatechin-3-gallate (EGCG, major catechin in green tea, 50 mg/kg oral gavage every other day) significantly reduced the number of goblet cells (PAS-stained) as well as the number of MUC5AC positive cells (Liang et al., 2017).
- Bronchial biopsies and epithelial brushings of smokers revealed a significantly larger number of goblet cells compared with healthy control subjects, leading to a 2.2-fold increase in the volume of stored mucin in the epithelium per surface area of basal lamina (4.32 ± 0.55 µm3/µm2 vs 1.94 ± 0.31 µm3/µm2 in controls) (Innes et al., 2006).
- The large airways of mice that were whole body exposed to CS of 10 cigarettes (160–180 mg/m3 TPM; TE-10, Teague Enterprises) for 2 h a day, 5 days a week, for up to 12 weeks, exhibited GCH and increased mucus production, evidenced by histology and increases in the numbers of PAS-positive goblet cells (approx. 15% compared to control), Mu5ac mRNA expression (approx. 5-fold increase compared to controls), and Muc5ac-positive cells (approx. 50% compared to control; Zhou et al., 2016).
- Ferrets that were exposed to CS (3R4F reference cigarette) for 1 h, twice daily for 6 months developed GCH and GCM in medium and small airways, evidenced by histopathological examination of AB/PAS-stained lung tissues. Mucus expression measured by PAS-positive goblet cell area, normalized by the size of the airway lumen to account for cell variation due to airway diameter, was 60% (0.042% ± 0.025% smoke vs. 0.025% ± 0.013% air control; P = 0.06) higher in smoke-exposed airways than in control airways. Muc5b and Muc5ac staining was greater in smoke-exposed ferrets, but patchy staining made quantification impossible (Raju et al., 2016).
- In primary human bronchial epithelial cells differentiated at the air-liquid interface, basolateral treatment with 10 ng/mL IL-13 increased the number of goblet cells from 0.2 ± 0.1 to 15.9 ± 1.1, the number of PAS-positive cells from 2.5 ± 1.5 to 28.2 ± 0.7, and the number of MUC5AC-positive cells from 0.1 ± 0.1 to 25.7 ± 1.0. Reversely, addition of clarithromycin to the IL-13 treatment reduced in a dose dependent manner (maximum with 32 µg/mL) the number of goblet cells from 15.9 ± 1.1 to 4.1 ± 2.5, the number of PAS-positive cells from 28.2 ± 0.7 to 10.7 ± 3.6, and the number of MUC5AC-positive cells from 25.7 ± 1.0 to 5.2 ± 2.9 (Tanabe et al., 2011).
- Similarly, a treatment of 3D bronchial organotypic cultures with 5 ng/mL IL-13 for 14 days induced GCH (histopathology assessment). MUC5AC mRNA expression significantly increased (ca. 10-fold) compared with cells treated with DMSO, and MUC5AC protein concentration measured in the supernatant also increased (1.8-fold) compared with DMSO-treated control (Mishina et al., 2015).
- In nasal polyp tissues from 8 patients with nasal polyposis, hyperplastic epithelium occupied a mean of 75% (range, 44%-100%). Nasal polyp tissue contained a significantly greater percentage of AB/PAS-and MUC5AC-stained area (approx. 51%) than in control epithelium (approx. 20 %). In nasal polyps, hyperplastic epithelium contained significantly larger numbers of MUC5AC-stained areas (ca. 40%) than in normal pseudostratified epithelium (ca. 15%; Burgel et al., 2000).
- Similarly, increase in goblet cell numbers was seen in nasal polyp tissues from 25 patients but not in healthy controls, as evidenced by more PAS-positive epithelial cells (PAS staining index 1.9 [1.3, 2.2] vs 0.7 [0.4, 1.2] in controls). This was accompanied by increased MUC5AC staining, with a mean staining score of 2.2 [1.7, 3.0] in polyp tissues vs 0.6 [0.4, 1.1] in normal controls, and increased MUC5AC gene expression, with levels of 4.4 [2.3, 6.3] in polyp tissues vs 1.2 [0.4, 2.2] in normal controls (Xia et al., 2014).
- In patients with COPD, goblet cell increase in lung tissues was confirmed with DAB/PAS staining, a staining specifically targeting mucosubstances such as mucin in cells, (goblet cell rate 0.20 ± 0.10% vs 0.13 ± 0.06% in healthy controls). The rate of MUC5AC expression was also significantly higher in COPD patients (0.27 ± 0.09%) than in healthy control (0.20 ± 0.10%) (Ma et al., 2005).
- Healthy smokers had greater goblet cell density (9.80±3.49 cells/mm) than nonsmokers (2.31±1.81 cells/mm) revealed by

PAS staining in endobronchial mucosal biopsies. Healthy smokers also had a greater mucin volume density (26.35±10.96 µL/mm2) compared with nonsmokers (5.77±4.34 µL/mm2) (Kim et al., 2015).

- Intragastric administration in Sprague-Dawley rats of the thromboxane A2 receptor antagonist seratrodast prior exposure to CS (1h/day, 6 days/week for 4 weeks) significantly attenuated the CS-induced increase in AB/PAS-stained goblets cells and Muc5ac expression in airways (An et al., 2015).
- An i. p. treatment of AG1478 (EGFR inhibitor) or/and niflumic acid (calcium activated chloride channels (CLCAs) inhibitor) inhibited CS (6 non-filtered cigarettes/day, 5 days/week, for 2 to 28 days) -induced increase in percentage area of goblet cells (measured by mucin staining) and MUC5AC mRNA expression in rat respiratory epithelium (Hegab et al, 2007)
- Airway GCM induced by intratracheal instillation of LPS (200-300 µg) in Sprague-Dawley rats was significantly reduced by daily gavage of a matrix metalloproteinase inhibitor (MMPI, 20 mg/kg) starting 3 days prior of LPS administration and until euthanasia. Area of AB/PAS-stained goblet cells was 3.37 ± 2.36% in control, 71.6 ± 2.56% in LPS and 14.7 ± 4.33% in LPS + MMPI groups. MUC5AC expression was also significantly reduced (6.5-fold) in LPS+MMPI group compared with the LPS group (Kim et al., 2004).
- In Sprague-Dawley rats, CS (5 cig twice daily for 4 weeks)- induced increase in goblet cells significantly decreased with i. p. hydrogen-rich saline treatments applied 30 min prior to CS exposure. The AB/PAS-stained area as well as MUC5AC levels were decreased by approx. 50% by hydrogen-rich saline pretreatment (Ning et a., 2013).
- CS exposure (5 cig twice daily for 4 weeks) increased goblet cell numbers in mouse airways as shown by an increased area of AB/PAS-staining and Muc5ac-positive staining. Berberine, a strong anti-inflammatory plant alkaloid, administered i. p. every other day (5 and 10 mg/kg) significantly attenuated CS-induced effects on goblets cells and mucin production (Xu et al., 2015).
- C57BL/6 wild-type mice exposed to CS (10 cig daily for 4 days) and intranasally inoculated with 50PFU of influenza A/PR8/34 virus showed significantly increased AB/PAS–positive area in the bronchial epithelium. Administration of carbocisteine (mucoregulatory drug) significantly reduced both the AB/PAS-positive areas in bronchial epithelium, and MUC5AC levels in bronchoalveolar lavage (BAL) fluids compared with CS/virus exposed mice (Yageta et al., 2013).

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Relationship: 2859: Increase, Mucin production leads to Decreased lung function

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
EGFR Activation Leading to Decreased Lung Function	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI
Rattus norvegicus	Rattus norvegicus	Moderate	NCBI
Mustela furo	Mustela putorius furo	Moderate	NCBI

Sex Applicability

Sex	Evidence
Unspecific	

Increased mucin production correlating with decreased lung function was shown in human patients and animal models (ferret and rodents).

Key Event Relationship Description

Increased mucin production and mucus hypersecretion following acute exposure are thought to contribute to innate airway defenses and are most likely limited by anti-inflammatory mechanisms aimed at resolving the exposure-related stress (Ramos, Krahnke, and Kim 2014; Rose and Voynow 2006). However, under chronic exposure conditions, with a support of increased number of specialized mucin-expressing goblet cells, mucus production sustains. When intracellular mucin is secreted into the lumen, it gets hydrated and expands massively (Verdugo 1991) leading to airway narrowing which ultimately decreases the airflow to lungs. This process may lead to airway obstruction and progressive decline in lung function (Aoshiba and Nagai 2004; Victor Kim and Criner 2015; Vestbo, Prescott, and Lange 1996). The association between increased mucin production and lung function decrease is correlative and is described in human patients as well as in animal models. The link between mucus hypersecretion and decreased lung function as well as increased hospitalization / mortality rates is shown in various clinical studies (Ekberg-Aronsson et al. 2005; Vestbo and Rasmussen 1989; Lahousse et al. 2017; Corhay et al. 2013). Lung function is commonly tested through spirometry by measuring forced expiratory volume in 1 s (FEV1) – the maximum volume of air that can forcibly be exhaled during the first second following maximal inhalation and forced vital capacity (FVC) – the maximum volume of air that can forcibly be exhaled following maximal inhalation.

Evidence Supporting this KER

Biological Plausibility

Clinical studies showed that MUC5AC expression in bronchial epithelium was inversely correlated with FEV in 1 s (% predicted) and with FEV1/FVC ratio (Caramori et al., 2009; Innes et al., 2006), model animal studies support the link between increased production of mucins and decrease in lung function (Feng et al. 2019; He et al. 2017; Raju et al. 2016), and epidemiological evidence indicates an association between mucus hypersecretion and decreased lung function (Allinson et al., 2015; Pistelli et al., 2003; Vestbo et al., 1996). The cause-effect relationship between increased mucin production and decreased lung function cannot be conclusively proven, but the link between mucus hypersecretion and airway obstruction / lung function decline is clinically accepted. However, increased mucin production needs to be persistent in order to result in sustained mucus hypersecretion. In addition, impaired mucociliary clearance contributes to airway obstruction (Whitsett 2018) and it is currently unclear whether chronic mucus hypersecretion alone is sufficient to elicit a decrease in lung function. Considering all above-mentioned, we suggest moderate biological plausibility for this KER.

Empirical Evidence

The evidences compiled below show association between increased mucin production (upstream KE) and decreased lung function (downstream KE). We present several studies on this association that represent the general consensus in the scientific and medical field, more information on the correlation between mucin/mucus concentration and lung function decrease parameters can be derived also from number of review articles (Button, Anderson, and Boucher 2016; Pistelli, Lange, and Miller 2003; Ramos, Krahnke, and Kim 2014; Shen et al. 2018).

- The volume of epithelial mucin stores, assessed as volume of mucin per surface area of basal lamina, was larger in bronchial biopsies from smokers with airflow obstruction than in those without and correlated with the FEV1 (Forced expiratory volume in 1 s) /FVC (Forced vital capacity) ratio. The increase in stored mucin occurs because of an increase in MUC5AC levels and despite a decrease in MUC5B (Innes et al. 2006).
- MUC5AC protein and mucus glycoconjugate levels were increased in the airways of COPD patients compared with non-COPD patients. Lung function tests of COPD patients suggested function deterioration compared with non-COPD group (Ma et al. 2005).
- MUC5AC expression in bronchial epithelium was inversely correlated with FEV1 (% predicted) in COPD patients (Caramori et al. 2009).
- Increased MUC5AC concentrations in sputum were reliably associated with parameters of lung function decline, including decreased FEV1, FEF25-75% (forced expiratory flow, midexpiratory phase), RV/TLC (residual volume/total lung capacity ratio) (Radicioni et al. 2021).
- The MUC5AC levels in the bronchoalveolar lavage fluid of patients with interstitial lung disease were negatively correlated with FEV1/FVC (r=-0.761, p=0.000), FEV1 predicted value (r=-0.668, p=0.002), and diffusing capacity (r=-0.606, p=0.006) (Wei et al. 2019).
- In bronchial tissues of COPD patients MUC5AC mRNA levels were negatively correlated with FEV1/FVC (P = 0.01), FEV1% predicted (P = 0.01), V(50)% predicted and V(25)% predicted data (markers of small airway obstruction) (r = -0.53, r = -0.53, r = -0.48, r = -0.43, P < 0.01) (Wang et al. 2007).
- Cigarette smoke (CS)-exposed ferrets displayed greater MUC5AC and MUC5B staining in the airway epithelium. PAS-positive goblet cell area was also higher in CS-exposed ferret airways. Inspiratory capacity, a sensitive marker of airway obstruction, was significantly reduced in CS-exposed ferrets (Raju et al. 2016).
- Rats exposed to biomass fuel (BMF) and motor vehicle exhaust (MVE) had increased levels of MUC5AC immunostaining and AB/PAS staining in the lung sections compared to the controls at 3 months. After 7 months of exposure to BMF or MVE significant reductions in lung function were observed, manifested by increased resistance and functional residual capacity

(FRC), and decreased dynamic pulmonary compliance (Cdyn), peak expiratory flow (PEF) and FEV at 20 ms/FVC (He et al. 2017).

- In allergen challenged rats statistically significant changes is lung function parameters were observed by 1 to 3 days of challenge (reductions in inspiratory capacity, functional residual capacity, FVC, PEF, maximum mid-expiratory flow and increases in respiratory system resistance and lung elastance). Mucin content (measured by electrophoretic mobility shift assay) in the bronchoalveolar lavage fluid samples was increased from day 1, and up to 6 days after antigen challenge and correlated with the increases in PAS-positive cells in the bronchial epithelium (Celly et al. 2006).

The following evidences support the notion of causality between upstream KE and downstream KE.

- The authors used a peptide derived from the myristoylated alanine-rich C kinase substrate protein NH2-terminal sequence (MANS) which was shown to selectively block methacholine (MCh)-induced mucin hypersecretion in mouse model of asthma (Singer et al. 2004). MCh-induced mucin secretion was significantly inhibited in MANS-pretreated mouse airways. MCh-induced fall in specific airway conductance (sGaw) was partially inhibited by MANS peptide (Agrawal et al. 2007).

- In acute exacerbation of chronic obstructive pulmonary disease (AECOPD) rat model MUC5AC mRNA and protein levels as well as mucous glycoconjugates (AB/PAS staining) were elevated, and increased phosphorylations of EGFR, PI3K and AKT were observed. Concomitantly, lung function parameters were decreased. Administration of Louqin Zhisou decoction (LQZS; Chinese herbal formula) to AECOPD rats attenuated the levels of phosphorylated EGFR, PI3K and AKT, decreased elevated MUC5AC levels and AB/PAS staining, and resulted in improved maximal voluntary ventilation (Feng et al. 2019). Similarly, in the study by Lin and colleagues, EGFR, p38MAPK and MUC5AC levels were increased along with decreases in lung function parameters (FVC, FEV0.1, FEV0.3, FEV0.1/FVC and FEV0.3/FVC) in COPD model rats whereas electroacupuncture at "Zusanli" (ST36) reversed both the increased levels of EGFR, p38MAPK and MUC5AC and the decreased levels of the lung function parameters (Lin et al. 2021). These data combined with the accepted knowledge in the field suggest causal relationship across the axis EGFR/increased mucin production/decreased lung function.

The following evidences show association between chronic mucus hypersecretion and decreased lung function. We include these evidences as supporting information based on assumption that increased mucin production is a prerequisite for chronic mucus hypersecretion.

- Chronic mucus hypersecretion was significantly and consistently associated with decline in lung function, i.e. FEV1 and an increased risk of subsequent hospitalization from COPD (Vestbo, Prescott, and Lange 1996).

- Chronic mucus hypersecretion was associated with both a lower FEV1 and faster FEV1 decline (Allinson et al. 2016).

- Several other patient studies of COPD and chronic bronchitis show correlations between chronic mucus hypersecretion symptoms such as long-term (at least 3 months and usually up to 2 years and more) cough, sputum production, presence of phlegm on most days, with lower FEV1% and FVC% (de Oca et al. 2012; Lahousse et al. 2017; Corhay et al. 2013; V. Kim et al. 2016; Liang et al. 2017).

Uncertainties and Inconsistencies

Physiological response to stressors that increase mucin production often is resolved after stressor exposure is eliminated, and the normal function of the airway is restored. For this KER to occur, sustained mucin production should ensue. Moreover, the KER is based on assumption that increased mucin production logically leads to mucin hypersecretion. However, when mucin secretion is inhibited (e.g. through MANS peptide (Singer et al. 2004)), increase in mucin production might not translate into mucin hypersecretion. A study of endobronchial biopsies from patients with mild and moderate asthma showed an increase in stored mucin compared with healthy controls. Stored mucin levels were similar in mild and moderate asthma patients, however secreted mucin was significantly lower in mild asthma patients than in moderate asthma patients (28.4 ± 6.3 versus 73.5 ± 47.5 µg/ml). These data add uncertainty to the KER by signifying the role of mucin secretion which is needed for downstream KE to occur (Ordoñez et al. 2001).

Quantitative Understanding of the Linkage

Below we list some quantitative aspects of the response relationship.

Response-response relationship

The evidences below are correlative and are not sufficient to conclude on causality of the KER.

- COPD patients with decreased lung function had increased mucin expression. MUC5AC protein levels detected by immunohistochemistry were significantly higher (0.27%±0.09%) in COPD group compared with non-COPD group (0.20%±0.10%) Similarly, histopathological analysis of goblet cells with AB/PAS staining (detection of mucus glycoconjugates) revealed significantly higher amount of goblet cells (0.20%±0.10%) in COPD group than in the non-COPD group (0.13%±0.06%). Lung function tests operated on patients indicated significantly lower FEV1/FVC ratio and FEV1% in COPD group (FEV1/FVC: 63.78% ±6.60%, FEV1%: 77.56%±12.74%) compared to non-COPD group (FEV1/FVC: 79.80%±4.47%, FEV1%: 92.05%±15.17%). (Ma et al. 2005).

- The area occupied by AB/PAS stained cells in the bronchial submucosal glands was significantly increased in COPD patients [20% (5.5–31.7%) gland area] in comparison to smokers with normal lung function [9.5% (2.5–17.5%)] and non-smokers [2%

(0.4–6.2%)]. The area occupied by MUC5AC stained cells in the bronchial epithelium was also increased in smokers (with/without COPD) [73.5% (25–92%) epithelial area] compared with non-smokers [15% (2.7–32%)]. MUC5AC expression inversely correlated with FEV1 (% of predicted), indicating a potential role of MUC5AC in the pathogenesis of airflow obstruction in COPD (Caramori et al. 2009).

- Following exposure to smoke from 3R4F research cigarettes for 1 h twice daily for 6 months, ferrets showed increased mucin production, increased number of goblet cells and chronic mucus hypersecretion (histology, PAS staining, MUC5AC and MUC5B staining). Mucus expression measured by PAS-positive goblet cell area, normalized by the size of the airway lumen to account for cell variation due to airway diameter, was 60% higher in smoke-exposed than in air-exposed animals (0.042% ± 0.025% smoke vs. 0.025% ± 0.013% air control). Inspiratory capacity, a sensitive marker of airway obstruction, was significantly reduced in smoke-exposed ferrets (79.5 ± 9.4 mL vs. 85.9 ± 5.9 mL air control) (Raju et al. 2016).
- Increased MUC5AC concentration in sputum was associated with lung function decrease parameters such as decreased FEV1, FEF25-75%, RV/TLC. Statistical modelling of 3-year longitudinal data indicated that baseline MUC5AC (but not MUC5B) concentration is a significant predictor for lung function decrease (FEV1 (p=0.010), FEV1/FVC (p=0.013), FEF25–75% (p=0.0005), FVC (p=0.14), CAT (COPD assessment test) score decline (p<0.0001)). Current smokers at-risk for COPD with raised baseline visit MUC5AC concentrations showed decline in lung function over 4 years whereas former smokers at-risk for COPD with normal baseline MUC5AC concentrations did not show lung function decline during the same observational period (Radicioni et al. 2021).
- AECOPD rat model showed declines in lung function parameters such as forced expiratory volume in 0.3 second (FEV0.3), FEV0.3/FVC% and maximal voluntary ventilation MVV ($P < 0.01$) measured with pulmonary functionality test machine AniRes2005. AB/PAS-staining in rat airway epithelium was 18.73 ± 2.38% compared to 0.02 ± 0.02% in control animals. Similarly, MUC5AC mRNA and protein levels were increased in AECOPD rats. Administration of LQZS to AECOPD rats decreased AB/PAS staining to 1.49 ± 1.18%, abolished AECOPD-related MUC5AC mRNA and protein upregulation, and resulted in improved MVV parameter (Feng et al. 2019).

Time-scale

An observational longitudinal study showed that raised MUC5AC concentrations at initial monitoring visit of smokers at-risk for COPD resulted in significant lung function decline (FEV1) over 4 years (Radicioni et al. 2021).

In longitudinal follow-up studies of patients with chronic mucus hypersecretion an excess decline in FEV1 was recognized throughout years, suggesting that mucus hypersecretion may lead to progressive lung function decline in time (Sherman et al. 1992; Vestbo, Prescott, and Lange 1996). An analysis of the National Survey of Health and Development (NSHD) data indicated that chronic mucus hypersecretion was associated with smoking status, and that the longer it was present, the faster was the decline in FEV1, corresponding to an additional decrement of 3.6 ± 2.5 ml/yr per occasion (Allinson et al. 2016).

Generally, it is observed that the prevalence of chronic mucus hypersecretion increases with age (Viegi et al. 2007) indicating that long-term exposures are needed for the stressor-induced increase in mucin production to develop into chronic mucus hypersecretion with an eventual risk of lung function decline.

Known modulating factors

Modulating Factor (MF)	MF Specification	Effect(s) on the KER	Reference(s)
Mucociliary clearance (MCC)	Mucus removal through ciliary movement	Impaired MCC contributes to decreased lung function (see also AOPs 411, 424, 425)	Ramos, Krahnke, and Kim 2014

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