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Stressed out - The role of oxidative stress in airway smooth muscle dysfunction in asthma and COPD

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ABSTRACT

The airway smooth muscle (ASM) surrounding the airways is dysfunctional in both asthma and chronic obstructive pulmonary disease (COPD), exhibiting; increased contraction, increased mass, increased inflammatory mediator release and decreased corticosteroid responsiveness. Due to this dysfunction, ASM is a key contributor to symptoms in patients that remain symptomatic despite optimal provision of currently available treatments.

There is a significant body of research investigating the effects of oxidative stress/ROS on ASM behaviour, falling into the following categories; cigarette smoke and associated compounds, air pollutants, aero-allergens, asthma and COPD relevant mediators, and the anti-oxidant Nrf2/HO-1 signalling pathway. However, despite a number of recent reviews addressing the role of oxidative stress/ROS in asthma and COPD, the potential contribution of oxidative stress/ROS-related ASM dysfunction to asthma and COPD pathophysiology has not been comprehensively reviewed.

We provide a thorough review of studies that have used primary airway, bronchial or tracheal smooth muscle cells to investigate the role of oxidative stress/ROS in ASM dysfunction and consider how they could contribute to the pathophysiology of asthma and COPD. We summarise the current state of play with regards to clinical trials/development of agents targeting oxidative stress and associated limitations, and the adverse effects of oxidative stress on the efficacy of current therapies, with reference to ASM related studies where appropriate. We also identify limitations in the current knowledge of the role of oxidative stress/ROS in ASM dysfunction and identify areas for future research.

1. Introduction

Asthma and COPD are both chronic diseases of the lungs. Asthma affects 1–18% of the population worldwide; amounting to an estimated 358 million asthma sufferers [1] with 461,000 asthma-related deaths in 2019 [2]. Asthma is defined by a history of classical asthma symptoms including wheeze, shortness of breath, chest tightness and cough accompanied by variable airflow obstruction, and typically associated with airway inflammation, and airway hyper-responsiveness (AHR) [1]. COPD affects ~10% of the population worldwide [3], amounting to an estimated 212 million COPD sufferers with 3.28 million COPD-related deaths, placing it 3rd in the causes of global deaths, in 2019 [2]. COPD is characterised by respiratory symptoms including breathlessness, cough and sputum production accompanied by airflow limitation resulting from pathophysiological changes to the airways and/or alveoli [3].

1.1. ASM dysfunction in asthma and COPD

The ASM is present throughout the bronchial tree surrounding the airways in two opposing spirals, as well as the posterior of the trachea [4]. Although its role in healthy adults remains unclear, the dysfunction of ASM is known to contribute to symptoms and decline in lung function in asthma and COPD via contractile dysfunction, release of inflammatory mediators and increased ASM mass, and is a key contributor to symptoms in patients that remain symptomatic despite optimal provision of currently available treatments [4–6].

1.1.1. ASM contractile dysfunction in asthma and COPD

The airways are hyper-responsive in asthma meaning that they are more likely to narrow in response to a bronchoconstricting stimuli than in health. In addition, the ASM is hyper-contractile in asthma and is a key contributor to this AHR [6–8]. The ASM is also infiltrated by mast

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cells in asthma, with the number increasing with disease severity and correlating with increased AHR [9,10].

Dysregulation of ASM contraction in COPD is not as widely documented, however COPD patients with airflow obstruction were shown to be very sensitive to inhaled methacholine [11], and airway rings from patients with obstructive lung disease (with all but one characterised as having COPD) demonstrated significantly increased maximal isometric force and isometric stress, which correlated to lung function decline [12].

1.1.2. Inflammatory mediator release by ASM in asthma and COPD

The ASM can also contribute to the inflammatory milieu in both asthma and COPD, being capable of releasing various cytokines and chemokines in response to asthma and COPD relevant stimuli [13].

In asthma, compared to health, the ASM has been shown to release more thymic stromal lymphopoietin (TSLP), C–C Motif Chemokine Ligand (CCL)-2, CCL11, C-X-C Motif Chemokine Ligand (CXCL)-8, and CXCL10 at baseline and/or in response to asthma relevant stimuli such as; interleukin (IL)-1β, tumour necrosis factor (TNF)-α and interferon (IFN)-γ [14–19].

Likewise in COPD the ASM has also been shown to release increased amounts of inflammatory mediators; including IL-6 and IL-8, both under basal conditions and following stimulation with TNF-α and/or transforming growth factor (TGF)-β1 [20–23]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) release from ASM from COPD patients versus non-smokers was increased in one study, however it was decreased in another [20,21].

1.1.3. Increased ASM mass in asthma and COPD

An increase in the amount of ASM surrounding the airways is a key hallmark of asthma, shown to be related to disease severity, but not duration, and to correlate with decline in lung function [24–30]. This increase in muscle mass contributes to increased shortening and narrowing of the airways during an asthma attack and can contribute to more persistent airway narrowing in more severe asthma [30]. However, the mechanism driving the increase in ASM mass in asthma has still not been fully elucidated, with both ASM hypertrophy and hyperplasia being implicated depending on airway size and severity of asthma, as well as an increase in extracellular matrix (ECM) deposition [10,25,31–33]. The ASM hyperplasia could arise from increased proliferation of ASM cells however evidence for this is contradictory in both *ex vivo* and *in vitro* studies [34,35]. Alternatively, ASM could exhibit delayed apoptosis, this has not been widely studied although a number of relevant mediators have been shown to be anti-apoptotic for ASM *in vitro* [13,36]. In addition, ASM cells or progenitors could migrate to the ASM bundle from the circulation or elsewhere in the airways, as reviewed by Berair et al., 2013 [34].

An increase in ASM mass in the central and peripheral airways in COPD has also been observed and like in asthma is related to disease severity and correlates with lung function decline [37–40]. The mechanism behind this increased ASM mass has not been thoroughly investigated, however there is some evidence for hyperplasia, associated with increased markers of proliferation [39], hypertrophy [31] and increased ECM deposition, namely fibronectin and laminin, associated with lung function decline [39,41–43].

1.2. Clinical unmet needs in asthma and COPD

Uncontrolled asthma and asthma exacerbations remain a widespread problem [44]. Although a number of novel treatments are available for T2-high asthma (predominantly eosinophilic with high T2 cytokines) [45], ~50% of asthmatics are T2-low (non-eosinophilic with/without neutrophilia) and not all patients respond to these treatments [46–48]. There is an increasing understanding of T2-low mechanisms with some novel treatments in the pipeline for such asthma [45], however none of these specifically target the ASM, with ASM dysfunction contributing to asthma symptoms in these patients via the hyper-contractility, increased mass and inflammatory mediator release described above [7,8,29,49]. Thus, targeting ASM dysfunction is particularly important in patients with T2-low asthma whose symptoms are not driven by eosinophilic inflammation.

In COPD the mainstay of therapy remains various combinations of short-acting β-agonists (SABA), long-acting β-agonists (LABA), short-acting muscarinic antagonists (SAMA), long-acting muscarinic antagonists (LAMA) and inhaled corticosteroids (ICS), with only one drug, roflumilast, developed belonging to a novel drug class, that of phosphodiesterase (PDE)-4 inhibition, in the last 20 years [50]. In addition, the majority of drugs currently under development belong to existing drug classes [50]. However, these drugs only provide relief of the symptoms of COPD and do not address the underlying pathophysiology of the disease having little impact on disease progression and mortality, with none specifically targeting the cause of ASM dysfunction [51]. Indeed, Polverino and Celli identify disease progression, prevention and treatment of exacerbations and reduction in mortality as key areas for the development of novel therapeutics in COPD [52].

Of the drugs available for asthma and COPD; current bronchodilator treatments used in asthma and COPD have limitations, including problems with effective inhaler use [53], side effects, drug tolerance and safety concerns associated with β-agonists [44,54,55], and the association of long-term oral corticosteroid (OCS) treatment with side effects such as osteoporosis, diabetes, cataracts and dyspeptic disorders [56]. In addition, insensitivity to corticosteroids is common in COPD and present in up to 10% of asthmatic patients [57] with some evidence that ASM cells themselves are resistant to the anti-inflammatory effects of corticosteroids in COPD. The anti-inflammatory activity of the glucocorticosteroid dexamethasone in ASM from COPD patients was compromised with respect to; inhibition of IL-8 and GM-CSF release in response to TNF-α compared to ASM from smokers and non-smokers [20–22] as well as inhibition of IL-6 release in response to TGF-β1 compared to ASM from healthy individuals [23]. In contrast, the ability of the glucocorticosteroid fluticasone to inhibit TNF-α induced IL-6 and IL-8 release was not compromised in ASM from mild or severe asthmatics compared to healthy individuals [58].

With regards to increased ASM mass in asthma there is a lack of therapeutic options, with only one non-medicinal intervention licensed for use in the clinic; bronchial thermoplasty [59–63], and three investigational medicinal products; fevipiprant, benralizumab and gallapomil [64–66], showing any promise in targeting increased ASM mass in clinical trials. However, this has not translated into their use in the clinic. Thus, there is a clinical unmet need for novel therapies targeting ASM dysfunction in both asthma and COPD.

1.3. Oxidative stress

In normal physiology, anti-oxidant agents generate a reducing environment preventing damage mediated by free radicals [67]. However, in several chronic diseases oxidative stress is present resulting from an imbalance between oxidant clearing mechanisms, such as; catalase, glutathione peroxidase (GPx), superoxide dismutase (SOD) and glutathione S-transferase (GST) and oxidant generating mechanisms that produce ROS; such as superoxide, hydrogen peroxide (H_20_2), peroxyl and hydroxyl radicals. This results in cells being exposed to harmful levels of ROS, which can interact with cellular molecules to cause DNA, lipid and protein damage and can affect cell growth, apoptosis, migration, inflammation, and contraction to contribute to the clinical expression and pathophysiology of diseases. Indeed, oxidative stress plays a key role in a number of diseases, including cardiovascular disease, cancer, diabetes and Alzheimer's disease [68,69].

However, ROS are also beneficial to a point and are essential for certain physiological processes, including redox regulation of protein activity, formation of intramolecular protein bonds and involvement in signalling pathways [70]. This has to be considered when developing

drugs that target oxidative stress, because indiscriminately reducing ROS can also tip the balance the other way and cause reductive stress, which can affect redox dependent signalling and protein folding, which can then amplify oxidative stress to form a positive feedback loop [70]. This could explain the contraindications and/or disappointing results with anti-oxidant therapies described in section 6.

1.4. Evidence for oxidative stress asthma and COPD

There is evidence of oxidative stress being present in both asthma and COPD. In asthma oxidative stress markers including; H_20_2, malondialdehyde (MDA) and isoprostanes are elevated in plasma and exhaled breath (EB), and linked to asthma severity or poorly controlled asthma [71]. A recent meta-analysis of oxidative stress markers in exhaled breath condensate (EBC) from adult asthmatics showed that in most cases H_20_2 and 8-isoprostanes were elevated in asthma compared to in health, with some evidence of a link to poorer lung function [72], however comparisons across studies are hampered by differences in methodology and the need for standardisation [72]. In addition, markers of oxidative stress MDA, 8-isoprostane, and the oxidative DNA damage marker 8-oxo-7,8-dihydro-29-deoxyguanosine (8-OHdG) have been shown to be increased in sputum from asthmatics compared to non-asthmatic controls in several studies [73] and epithelial cells from patients with neutrophilic asthma demonstrated elevated levels of 8-OHdG [74].

In COPD several studies show that markers of oxidative stress; such as; H_20_2, myeloperoxidase (MPO) and 8-isoprostane, are elevated in EB from COPD patients compared to healthy individuals [75] and MDA, 8-isoprostane, 8-OHdG and MPO are elevated in sputum from patients with COPD [73,76]. In addition, the markers of oxidative damage: 8-oxo-7,8-dihydroguanosine (8-OHG) in RNA and 8-OHdG in DNA are also elevated in alveolar lung fibroblasts from emphysematous COPD patients [77].

With regards to the ASM itself, ASM from mild/moderate and severe asthmatics was shown to contain higher levels of 8-OHdG staining compared to non-asthmatics, with the level of staining correlating with both AHR and lung function decline [8]. In addition, although not a direct measure of oxidative stress, in ASM from COPD patients there was increased expression of the ROS generating enzyme NADPH oxidase (NOX)-4, which increased with disease severity and correlated with lung function decline [39]. Thus, oxidative stress could play a role in ASM dysfunction in asthma and COPD.

Indeed, there is a significant body of research investigating the effects of oxidative stress/ROS on ASM behaviour, however, this has not been comprehensively reviewed. We provide a thorough review of studies that have used primary airway, bronchial or tracheal smooth muscle cells to investigate the role of oxidative stress/ROS in ASM dysfunction in relation to exposure to cigarette smoke and associated compounds, air pollutants, aero-allergens, asthma and COPD relevant mediators, and the role of the anti-oxidant nuclear factor-erythroid factor 2-related factor 2 (Nrf2)/haem oxygenase (HO)-1 signalling pathway and consider how they could contribute to the pathophysiology of asthma and COPD. We summarise the current state of play with regards to clinical trials/development of agents targeting oxidative stress and associated limitations, and the adverse effects of oxidative stress on the efficacy of current therapies, with reference to ASM related studies where appropriate. We also identify limitations in the current knowledge of the role of oxidative stress/ROS in ASM dysfunction and identify areas for future research.

2. Effect of cigarette smoke and associated compounds on measures of ASM dysfunction

Cigarette smoke (CS) is a complex mixture of noxious substances that can contribute to oxidative stress in its own right due to the high levels of oxidants and ROS within it, as well as stimulating downstream ROS production in cells [78]. Indeed, cigarette smoke extract (CSE) has been shown to induce ROS production by ASM in several studies [79–85]. Cigarette smoke is a major contributory factor in the development of COPD and can also aggravate asthma symptoms [3,86,87]. We will review how CS affects ASM function related to AHR, increased ASM mass, inflammatory mediator release and Nrf2/HO-1 signalling in ASM cells.

2.1. Effect of cigarette smoke on ASM contractility

CSE is implicated in AHR in which ASM is one of the main drivers, with women more likely to experience asthma symptoms in response to CS [6,7,86–89]. However, surprisingly the mechanisms by which CSE affect ASM contraction remain to be fully elucidated. CSE has been shown to enhance intracellular calcium elevation in ASM cells in several studies [81,89,90]. Both CS and CSE resulted in elevation of cytosolic calcium in human ASM cells through stimulation of plasmalemmal calcium influx, but not store-operated and L-type calcium channels, in a transient receptor potential ankyrin 1 (TRPA1) dependent manner, leading to myosin light-chain (MLC) phosphorylation, a key step in regulating ASM contractility [90]. CSE also increased calcium elevation in response to bradykinin and histamine, both mediators of ASM contraction, involving increased store-operated calcium entry (SOCE), that occurred in a ROS dependent manner and was associated with CSE-induced upregulation of short transient receptor potential channel 3 (TRPC3), cluster of differentiation 38 (CD38), stromal interaction molecule 1 (STIM1) and ORAI Calcium Release-Activated Calcium Modulator 1 (ORAI1) expression [81]. CSE is also implicated in increasing agonist induced ASM contraction, as assessed using epithelium denuded human bronchial rings, via ROS-dependent upregulation of brain-derived neurotrophic growth factor (BDNF) secretion and expression of the tropomyosin-related kinase (Trk)B BDNF receptor by ASM cells. In support of this observation, increased histamine induced calcium elevation following CSE exposure in ASM cells, was also shown to be mediated by and enhanced by BDNF via the TrkB receptor [91]. CSE also mediated an increase in TSLP receptor (TSLP-R) and a ROS dependent increase in TSLP expression which was shown to contribute to a CSE-mediated increase in calcium elevation in response to histamine [92]. Interestingly CSE exposure reduced the inhibitory of effect of 17β-estradiol (E_2) on intracellular calcium elevation, SOCE and STIM1 phosphorylation, and inhibited E_2 mediated generation of cyclic adenosine monophosphate (cAMP) by ASM cells, which could have implications for increased AHR in women exposed to CS [89]. In contrast, despite mediating an increase in intracellular calcium levels, normal human bronchial smooth muscle (BSM) cells have been demonstrated to become hypocontractile in a ROS dependent manner following CSE exposure, however this was accompanied by a reduction in cell viability and may not be a true representation of the role of CSE in ASM contraction [80]. Indeed, the majority of studies point to a role for CSE in inducing ASM hypercontractility and thus have implications for AHR in asthma and to reduced airway calibre in COPD (see Fig. 1).

Emerging studies also suggest a role for specific constituents of CS in ASM dysfunction (see Fig. 1). Formaldehyde, a major constituent of CS and an indoor air pollutant is known to exacerbate asthma symptoms, to enhance AHR in animal models, to increase carbachol-induced contraction in precision-cut human lung slices and to be involved in ROS generation [93,94]. Within human ASM cells, although it did not affect agonist-induced intracellular calcium elevation *per se*, it is suggested to increase sensitisation of ASM cells to calcium due to enhancing basal Rho-kinase activity and myosin phosphatase targeting protein (MYPT)-1 phosphorylation [93].

Nicotine has also been shown to contribute to AHR, and can induce oxidative stress [95,96]. Indeed, conditioned media from nicotine-stimulated fibroblasts was able to mediate increased expression of the contractile protein p-MLC in ASM cells [97]. Nicotine and CSE also resulted in upregulation of nicotinic α7 acetylcholine receptor (α7nAChR) expression by ASM *in vitro,* although the functional

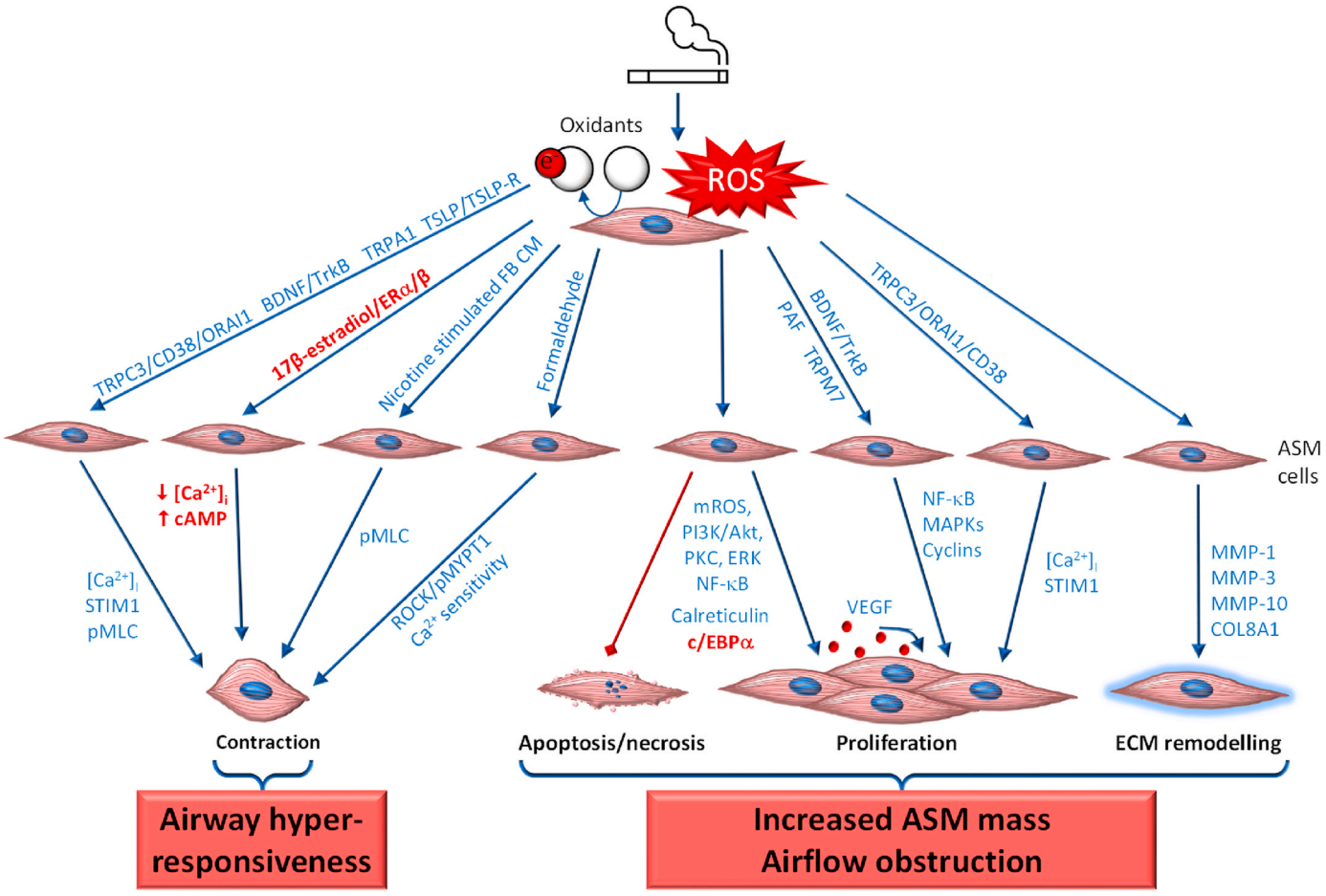

Fig. 1. Role of cigarette smoke and its constituents in increased ASM contractility and increased ASM mass. Enhancement of ASM contractility and ASM mass by CSE has implications for AHR in asthma and airflow obstruction in asthma and COPD. With regards to increased contractility, in primary ASM cells CSE and CSE induced ROS can mediate basal and agonist induced $[Ca^{2+}]_i$ elevation via several mechanisms with implications for increased contractility; upregulation of TRPC, CD38, ORAI1 and STIM1 and associated SOCE, upregulation of BDNF and its receptor TrkB, upregulation of TSLP and its receptor TSLP-R, and stimulation of TRPA1 which results in increased MLC phosphorylation. In addition, formaldehyde results in increased calcium sensitivity in ASM via ROCK and MYPT1 phosphorylation and a nicotine-induced fibroblast-derived mediator can increase MLC phosphorylation in ASM cells. CSE also counteracts the effects of 17β-estradiol, via ER receptors, in inhibiting calcium signalling and stimulating cAMP generation, shown to reduce ASM contractility, which has implications for AHR in females. With regards to increased ASM mass, in primary ASM cells CSE has been shown to inhibit apoptosis and stimulate proliferation via upregulation of calreticulin and subsequent down-regulation of C/EBPα, and via mitochondrial dysfunction induced by increased mitochondrial ROS and subsequent phosphoinositide signalling and transcriptional regulation via NF-κB. CSE can also induce proliferation via; upregulation of BDNF and its receptor TrkB, upregulation of PAF and downstream NF-κB activity, upregulation of TRPM7 expression, upregulation of TRPC, CD38, ORAI1 and STIM1 and associated SOCE and increased VEGF expression by ASM, which can also be mediated by acrolein. In addition, CSE increases COL8A1 production and levels of MMPs 1, 3 and 10 which are involved in ECM remodelling which can contribute to increased ASM mass. In contrast at high concentrations CSE can induce apoptosis and inhibit proliferation, accompanied by reduced contraction and migration, which could contribute to the ASM atrophy which can be observed in COPD. Blue arrows indicate a stimulatory effect, red diamond arrows indicate an inhibitory effect. Blue font indicates upregulation, bold red font indicates downregulation. Abbreviations used: airway hyper-responsiveness (AHR), airway smooth muscle (ASM), brain derived neurotrophic factor (BDNF), CCAAT/enhancer-binding protein alpha (C/EBPα), cigarette smoke extract (CSE), cluster of differentiation 38 (CD38), collagen type VIII alpha 1 Chain (COL8A1), cyclic AMP (cAMP), estrogen receptor (ER), extracellular matrix (ECM), extracellular signal-regulated kinase (ERK), fibroblast conditioned media (FB CM), intracellular calcium ($[Ca^{2+}]_i$), matrix metalloproteinase (MMP), mitogen-activated protein kinase (MAPK), mROS (mitochondrial ROS), myosin light chain (MLC), myosin phosphatase targeting protein (MYPT1), nuclear factor-erythroid factor 2-related factor 2 (Nrf2), nuclear factor-kappaB (NF-κB), ORAI Calcium Release-Activated Calcium Modulator 1 (ORAI1), platelet activation factor (PAF), reactive oxygen species (ROS), rho-kinase (ROCK), short transient receptor potential channel 3 (TRPC), store-operated calcium entry (SOCE), stromal interaction molecule 1 (STIM1), thymic stromal lymphopoietin (TSLP), TSLP receptor (TSLP-R), transient receptor potential ankyrin 1 (TRPA1), transient receptor potential cation channel subfamily M member 7 (TRPM7), tropomyosin receptor kinase B (TrkB), tumour necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

consequences of this upregulation were not explored [95]. Interestingly α7nAChR expression was shown to be upregulated in ASM from asthmatics versus healthy controls and in ASM from smokers versus non-smokers [95], which could have downstream consequences for ASM function following nicotine exposure.

2.2. *Effect of cigarette smoke on mechanisms relevant to ASM mass*

CS induced oxidative stress is also implicated in ASM cell apoptosis and necrosis. CSE induced a dose dependent increase in oxidative DNA damage in ASM cells accompanied by a dose dependent decrease in levels of HSP70, which protects against DNA damage and oxidative stress [98]. This was correlated with increased levels of apoptosis and necrosis in the ASM cells following CSE exposure [98]. Indeed, CSE

induced expression of pro-apoptotic Bcl-2-associated X protein (BAX), BCL2 associated agonist of cell death protein (BAD), and Fas cell surface death receptor (FAS) and inhibited expression of antiapoptotic B-cell lymphoma-2 (Bcl-2) and nuclear factor-kappaB (NF-κB p65/p50) [79] and reduced ASM cell viability at high concentrations [99]. The involvement of ROS in CSE-mediated cell death is inferred due to CSE-mediated reduction in BSM cell viability [80] and the increase in apoptotic and necrotic ASM cells [79] being inhibited in the presence of NAC.

In contrast, 10% CSE resulted in a decrease in the percentage of apoptotic cells in a study by Guan et al., accompanied by an increase in ASM cell proliferation, which would be supportive of a role of CSE in ASM remodelling in COPD [100]. However, the reported effects of CSE on ASM proliferation are not consistent. In the same study, higher concentrations of CSE have no affect on ASM cell proliferation [100], and Hu et al., 2009 show that CSE inhibits ASM growth over a 72 h period via cell cycle arrest and a reduction in expression of the proliferation marker proliferating cell nuclear antigen (PCNA) [79]. In contrast, CSE has been shown to increase bovine tracheal smooth muscle (TSM) cyclin D1 expression and DNA synthesis, and also to increase cell number in a p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) 1/2 dependent manner [101] and to increase proliferation via increased cyclin E and PCNA expression [81]. CSE has been shown to increase rat ASM DNA synthesis and cell number at a higher concentration with a lower concentration having no effect [102]. In this study CSE induced ASM proliferation was dependent on transient receptor potential cation channel subfamily M member 7 (TRPM7) which has been shown to be activated by ROS [102,103]. In addition to its effects on ASM contraction described above, BDNF is also implicated in CSE induced PCNA and cyclin E1 expression and ASM proliferation [91]. CSE and acrolein, a component of CS, also induced vascular endothelial growth factor (VEGF) release from ASM cells in a p38 MAPK dependent manner with VEGF release and p38 phosphorylation partially inhibited by NAC [104]. This could impact on ASM proliferation as VEGF has been shown to be mediate ASM proliferation [105–107]. Interestingly, in the Guan et al. study the CSE-induced increase in proliferation and decrease in apoptosis was mediated via a calreticulin-induced reduction in CCAAT/enhancer-binding protein alpha (C/EBPα) expression [100]. C/EBPα has been shown to mediate an IFN-γ induced increase in NOX1, 4 and 5 activity in aortic smooth muscle cells [108], which could implicate NOX-mediated ROS generation in protecting against CSE induced ASM proliferation and inhibition of apoptosis [100]. CS also promotes the expression of platelet activating factor (PAF) [109], which can induce proliferation of rat ASM cells, in association with increased NF-κB binding activity, in a ROS dependent manner [110].

Mitochondria are a key source of ROS in ASM cells, and their role in ASM dysfunction is extensively reviewed elsewhere [111]. With regards to the role of CS in mitochondrial dysfunction in ASM, ROS production, as a result of oxidative phosphorylation, can increase when mitochondria are exposed to damaging stimuli such as CS [112]. In ASM from non-asthmatic subjects such CSE induced mitochondrial ROS production led to mitochondrial fragmentation and disruption of mitochondrial networks via disruption of the mitochondrial fission versus fusion balance, due to upregulation of dynamin-related protein 1 (Drp1) expression (fission related) and downregulation of mitofusin (Mfn)-2 expression (fusion related), involving PI3K/Akt, PKC and ERK activation and transcriptional regulation via NF-κB [112]. Compared to ASM from non-asthmatics, ASM from asthmatic subjects at baseline had the same morphological defects and changes in Drp1 and Mfn2 expression as induced by CSE in ASM from non-asthmatics, with changes in Drp1 and Mfn2 expression exacerbated further by CSE exposure [112]. The CSE induced changes in Drp1 and Mfn2 expression were implicated in increased ASM proliferation and to a lesser extent decreased ASM apoptosis [113]. CSE has also been shown to reduce adenosine triphosphate (ATP) production [112] and increase glycolysis, which was implicated in increased ASM proliferation [113].

CSE also results in a dose dependent increase in collagen type VIII alpha 1 Chain (COL8A1) deposition by ASM cells from COPD patients, as well as enhanced matrix metalloproteinase (MMP)-1, MMP-3 and MMP-10 expression in ASM from both controls and COPD patients at higher concentrations [99]. In addition, a trend towards increased adhesion of ASM cells from COPD subjects was reduced by CSE as was the wound healing capability of ASM cells following exposure to the highest concentration of CSE [99]. In another study ASM migration was shown to be reduced in a ROS dependent manner following exposure of ASM cells to a concentration of CSE that also reduced ASM viability, so interpretation of these findings is difficult [80].

In the majority of studies CSE derived and induced ROS in ASM are implicated in mechanisms involved in increasing ASM mass, namely reduced apoptosis, increased proliferation and increased matrix deposition, which has implications for increased airflow obstruction in asthma and COPD (see Fig. 1). However, at high concentrations CSE can induce apoptosis and inhibit proliferation, accompanied by reduced contraction and migration, which could contribute to the atrophy which can be observed in COPD [114]. Further studies are required to fully understand the mechanisms involved and to determine how the effects of CSE on mechanisms of ASM dysfunction relevant to ASM mass identified *in vitro* relate to *in vivo* levels of CSE exposure.

2.3. Effect of cigarette smoke on ASM-derived inflammatory mediator expression

CSE has also been shown to be involved in pro-inflammatory mediator release by ASM cells (see Table 1) with both CSE [85,99,102,115–117], and acrolein [116] resulting in increased IL-8 release from ASM and/or BSM cells in a p38 dependent manner [116], CSE inducing TNF-α release by ASM cells [81] and CSE and nicotine resulting in increased IL-6 release by ASM cells [84,118]. Oxidative stress is implicated in CSE induced IL-8 release as in a study by Oltmanns et al., CSE induced IL-8 release from ASM was accompanied by an increase in expression of the oxidative stress induced anti-oxidant HO-1 and could be inhibited by glutathione (GSH) which quenches oxidative stress [115]. In support of this Han et al., showed that CSE resulted in increased IL-8 release from ASM cells in a ROS dependent manner with the anti-oxidant Sul-121 shown to inhibit CSE induced ROS generation, nuclear translocation of the NF-κB subunit p65 and ultimately IL-8 release, as well as inhibiting the nuclear translocation of the anti-oxidant inducer Nrf2 [85]. In addition to its role in CSE induced ASM proliferation TRPM7 is also implicated in CSE-mediated IL-8 secretion from ASM cells from rats pre-exposed to smoke, with the increase in IL-8 secretion being greater in ASM cells with higher levels of TRPM7 expression and partially inhibited by TRPM7 shRNA [102].

Inflammatory mediator release relating to CS exposure is likely to be exacerbated in a disease setting as ASM cells from smokers with COPD produced more CXCL1 in response to CSE compared to those without COPD [99]. In addition, inflammatory mediators; including IL-6 and IL-8, both under basal conditions and following stimulation with TNF-α and/or TGF-β1 [20–22] have been shown to be released in increasing amounts from ASM from smokers and to a greater extent in ASM from COPD patients compared to ASM from non-smokers. GM-CSF release was increased from ASM from COPD patients and smokers versus non-smokers in one study but was decreased in another [20,21]. As in ASM from COPD patients, the ability of dexamethasone to inhibit release of these mediators is compromised in ASM from smokers, to a level that is intermediate between the inhibition observed in ASM from non-smokers and ASM from COPD patients, as described in section 1.2 [20–22]. Resveratrol, which has anti-oxidant properties, was able to inhibit IL-8 release to an equivalent extent in ASM from COPD patients, smokers and non-smokers, and inhibited GM-CSF release to an equivalent extent in ASM from smokers and non-smokers, and to a greater extent in ASM from COPD patients [21]. Thus, targeting oxidative stress

Table 1
Effect of cigarette smoke on ASM-derived inflammatory mediator expression.

Stimulus	Mediator/signalling intermediate	Signalling pathways	References
CSE	CXCL1		[99]
	TNF-α	ROS dependent	[81]
	IL-8	p38 MAPK	[116]
		ROS/NF-κB	[85]
		TRPM7	[102]
	cPLA2	p47phox/NOX2 > p38, p42/p44, MAPK, c-Fos	[82,83]
		p47phox/NOX2 > JNK1/2, c-Jun, p300	[82,83]
	PGE2	PKC-α/c-Src/EGFR, PDGFR/PI3K/Akt > p300, NF-κB > COX2	[118]
	PGE2 and IL-6	TLR4/MyD88/TRAF6, c-Src/p47phox > p38, p42/p44 MAPK, JNK1/2, NF-κB > COX2	[84]
Nicotine	PGE2	COX2	[84]
	IL-6	COX2	[84]
Acrolein	IL-8	p38 MAPK	[116]

CSE and associated compounds stimulate release of various mediators from ASM to contribute to the inflammatory milieu. Signalling mechanisms involved in this release are listed where identified. Abbreviations used: Akt serine/threonine kinase (Akt), cellular Src (c-Src), cigarette smoke extract (CSE), c-Jun N-terminal kinase (JNK), cyclooxygenase (COX), cytosolic phospholipase A2 (cPLA2), C-X-C Motif Chemokine Ligand 1 (CXCL1), epidermal growth factor receptor (EGFR), interleukin-6 (IL-6), interleukin-8 (IL-8), mitogen-activated protein kinase (MAPK), myeloid differentiation primary response 88 (MyD88), NADPH oxidase (NOX), nuclear factor-kappaB (NF-κB), phosphoinositide 3-kinase (PI3K), platelet-derived growth factor receptor (PDGFR),prostaglandin E_2 (PGE2), protein kinase C (PKC), reactive oxygen species (ROS), toll-like receptor (TLR), TNF receptor associated factor 6 (TRAF6), transient receptor potential cation channel subfamily M member 7 (TRPM7), tumour necrosis factor alpha (TNF-α).

may represent an alternative therapeutic option to corticosteroids in COPD.

Several studies have addressed the role of CSE in activation of the pathway involved in prostaglandin E_2 (PGE2) synthesis in human TSM cells. PGE2 is synthesised following cytosolic phospholipase A2 (cPLA2) activation which leads to release of arachidonic acid from membrane phospholipids which can then be converted by cyclooxygenase (COX) 2 into PGE2 [119]. CSE exposure was shown to result in increased expression and activity of cPLA2 and ROS generation in TSM cells in a ROS dependent manner involving the NOX subunit p47phox and NOX2 [82,83]. CSE mediated ROS elevation resulted in phosphorylation of p42/p44 MAPK, p38 MAPK and c-Jun N-terminal kinase (JNK) 1/2 and a subsequent increase in activator protein (AP)-1 promoter activity [82]. In support of this CSE was shown to induce c-Fos and c-Jun (components of the dimeric AP1 transcription factor) in a ROS/p47phox/NOX2 dependent manner, with CSE induced p38 and p42 MAPK phosphorylation mediating increased c-Fos expression, and CSE induced JNK1/2 phosphorylation mediating increased c-Jun expression and ROS dependent interactions between c-Jun and p300, which ultimately resulted in cPLA2 expression by TSM cells [83].

CSE exposure was also shown to induce COX2 expression in TSM cells [84,118]. As in the above studies CSE exposure resulted in increased ROS generation [84], with the additional observations of an increase in superoxide production and p47phox translocation to the membrane in a ROS and c-Src dependent manner, with CSE also shown to induce phosphorylation of c-Src [84,118]. CSE exposure was shown to increase toll-like receptor (TLR)-4 expression, which accompanied by its binding partners myeloid differentiation primary response 88 (MyD88) and TNF receptor associated factor 6 (TRAF6), increased CSE mediated COX2 expression. TLR4 was also shown to be involved in ROS dependent CSE mediated phosphorylation of p42/p44 MAPK, p38 MAPK, and JNK1/2, which in addition to c-Src phosphorylation resulted in increased NF-κB promoter activity and induction of cytoplasmic to nuclear translocation of p65 and phosphorylation of p65, which were additionally mediated by TLR4, MyD88, TRAF6 and p47phox. This ultimately leads to increased COX2 expression which mediates increased IL-6 and PGE2 production [84,118]. Nicotine was also shown to induce COX2 expression and PGE2 production [84]. These studies do not assess the downstream effects of enhanced PGE2 release in the presence of CSE, however despite being pro-inflammatory in some contexts, PGE2 appears to have a beneficial role in the lungs, and is involved in inhibition of bronchoconstriction, mast cell activation, BSM proliferation and ASM migration [119].

2.4. Effect of cigarette smoke on Nrf2/HO-1 signalling

CS and its components are also involved in upregulation of Nrf2/HO-1 signalling in ASM cells [93,112,120] providing further evidence that exposure to noxious stimuli from CS may lead to ROS-dependent activation of compensatory protective mechanisms. Cigarette smoke particle-phase extract (CSPE) increased superoxide production in a ROS, c-Src, NOX2 and p47phox dependent manner resulting in phosphorylation of p42/p44 MAPK, p38 MAPK, and JNK1/2, translocation of Nrf2 from the cytosol to the nucleus, and ultimately induction of HO-1 expression in human TSM cells [120]. In addition, ASM cells responded to formaldehyde treatment with induction of a Nrf2-dependent anti-oxidant response involving increased expression of NAD(P)H: quinone oxidoreductase 1 (NQO1) and thioredoxin 1 (Trx1) [93]. The potential consequences of the activation of Nrf2/HO-1 signalling in relation to mechanisms of ASM dysfunction relevant to asthma and COPD are described in section 5.

Although CS and CS stimulated ROS are implicated in mechanisms that can contribute to ASM dysfunction, including; contraction, apoptosis, proliferation and pro-inflammatory mediator release, ROS generation can also be involved in the activation of pathways involved in the generation of mediators, such as; PGE2 and Nrf2/HO-1 signalling, that could confer some protection to the ASM following exposure to the noxious stimuli found in CSE. Thus, careful consideration is required with regards to targeting ROS following exposure to CS.

3. Effects of air pollutants and aero-allergens on measures of ASM dysfunction

3.1. Air pollutants

Air pollutants, including components of particulate matter; particulate matter (PM) 2.5 particles, diesel exhaust particles (DEPs) and polyaromatic hydrocarbons (PAHs), and ozone are well recognized as being oxidants in their own right or are able to induce oxidative stress and are linked to AHR, airflow obstruction and increased risk of COPD and chronic bronchitis, as well as exercise induced bronchoconstriction [121–124].

Several recent studies provide evidence for a role of PM2.5 in ASM dysfunction, relevant to AHR and airway remodelling. Higher concentrations of PM2.5 derived from ambient traffic and wood smoke resulted in an increase in human BSM cell apoptosis, however lower concentrations did not induce apoptosis or proliferation [125]. In agreement with

this, higher concentrations of PM2.5 reduced ASM viability [126], although this study and another showed that lower concentrations could increase ASM viability [126,127]. DEPs, which are a subset of PM2.5s, also have a ROS dependent cytotoxic effect on guinea pig TSM cells which is inhibitable by the anti-oxidant catalase and the free radical scavenger MK-447, but not SOD [128].

However, in several studies, at concentrations that did not affect cell viability, PM2.5 were shown to stimulate ASM migration [125,129] and ASM contraction [126,127,129], although the effect of PM2.5 was biphasic in one study resulting in inhibition of contraction at the lowest and highest concentrations and enhancement of contraction at intermediate concentrations [126]. Concentrations of PM2.5 that promoted contraction, migration and viability of ASM cells, also mediated an increase in cytoplasmic calcium concentration [126,127], and expression of contraction and remodelling related proteins; kallikrein (KLK) 14, bradykinin, the bradykinin B2 receptor (B2R), MMP-2, MMP-9 and vimentin [126,127,129] in a BRD4 dependent manner [129]. Although a direct link between PM2.5 exposure and oxidative stress is not

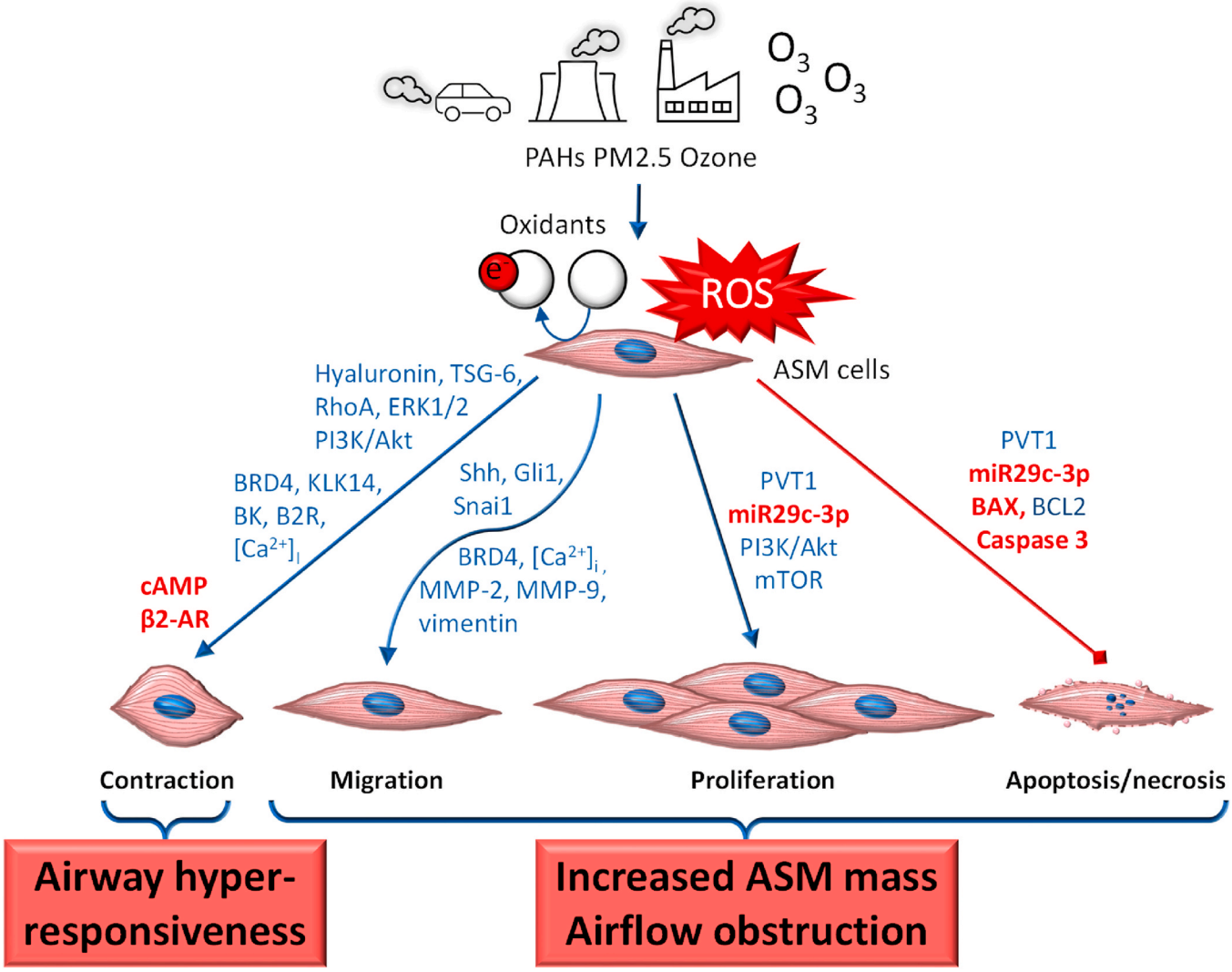

Fig. 2. Effect of pollutants on measures of ASM dysfunction. PM2.5 particles, PAH and ozone behave as both oxidants and ROS inducers, and are implicated in AHR in asthma and airflow obstruction in asthma and COPD. Whilst at high concentrations PM2.5 particles reduce ASM viability and contraction, at low concentrations that do not reduce ASM viability, PM2.5 are involved in increasing measures of ASM function implicated in AHR, with increased $[Ca^{2+}]_i$ and increased contraction associated with a BRD4 mediated increase in KLK14, BK and B2R expression. PM2.5 are also involved in increasing measures of ASM function related to increased mass which contributes to airflow obstruction, with increased viability, increased proliferation and increased migration, with the latter associated with BRD4 mediated increases in MMP-2, MMP-9 and vimentin, as well as via Shh, Gli1, Snai1 signalling. PAHs, which can bind to PM2.5 particles, are also implicated in AHR via a reduction in cAMP levels and β2-AR expression and function, which has implications for the efficacy of β-agonist based bronchodilators. In addition ozone is implicated in AHR via TSG-6 upregulation of hyaluronin in mouse models. Supporting studies in ASM show that hyaluronin treatment results in upregulation of pathways involved in contraction; rhoA, ERK1/2, PI3K/Akt in a TSG-6 dependent manner. Ozone is also implicated in increased ASM mass via upregulation of PVT1 in a mouse model. Supporting studies in ASM show that PVT1 can stimulate ASM proliferation via downregulation of miR-29c-3p and activation of PI3K-Akt-mTOR signalling and inhibit ASM apoptosis via downregulation of miR-29c-3p, upregulation of anti-apoptotic BCL2, and downregulation of pro-apoptotic BAX and caspase 3. Blue arrows indicate a stimulatory effect, red diamond arrows indicate an inhibitory effect. Blue font indicates upregulation, bold red font indicates down-regulation. Abbreviations used: airway hyper-responsiveness (AHR), airway smooth muscle (ASM), Akt serine/threonine kinase (Akt), B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (BAX), beta-2 adrenergic receptor (β2-AR), bradykinin (BK), bradykinin B2 receptor (B2R), bromodomain-containing protein 4 (BRD4), cyclic AMP (cAMP), extracellular signal-regulated kinase (ERK), glioma-associated oncogene homolog 1 (Gli1), intracellular calcium ($[Ca^{2+}]_i$), kallikrein 14 (KLK14), matrix metalloproteinase (MMP), mechanistic target of rapamycin (mTOR), particulate matter 2.5 (PM2.5), phosphoinositide 3-kinase (PI3K), plasmacytoma variant translocation 1 (PVT1) polyaromatic hydrocarbons (PAHs), Ras homolog family member A (RhoA), Snail Family Transcriptional Repressor 1 (Snai1), sonic hedgehog (Shh), TNF-stimulated gene 6 (TSG6). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

observed, bromodomain-containing protein (BRD)-4 is implicated in reduction of ROS and increased cell survival via Nrf2 signalling following exposure of a fibroblast like cell line to oxidative stress [130]. In addition, these PM2.5 mediated effects could be inhibited by IL-37 which has been implicated in protection against oxidative stress in other cells [127,131,132].

As well as promoting contraction as outlined above, components of PM2.5 can affect airway relaxation, with PAHs resulting in a reduction in cAMP, adenylyl cyclase activity and β2 adrenergic receptor (β2-AR) protein expression and function in ASM cells, which has implications for the efficacy of β-agonist based bronchodilators [133].

Ozone exposure is involved in airway narrowing and AHR in murine models and is associated with an increase in the thickness of the ASM, airway resistance and lung compliance [134,135]. In one study this is associated with expression of the long noncoding RNA plasmacytoma variant translocation 1 (PVT1) which is also increased in serum of asthma patients [134]. In support of a role for ozone-induced PVT1 in ASM dysfunction, PVT1, via reducing the activity of miR-29c-3p, was shown to be involved in promoting ASM proliferation and inhibiting ASM apoptosis, with associated inhibition of BAX and caspase 3 and upregulation of BCL-2 expression [134].

Ozone induced AHR was also shown to be mediated via a hyaluronan (HA), TNF-stimulated gene 6 (TSG-6) dependent mechanism in murine models, with AHR in tracheal rings from TSG-6 knockout mice being restored by exogenous TSG-6 [135]. Supporting studies in ASM showed that short fragment HA exposure induced signalling pathways related to contraction; activation/phosphorylation of Ras homolog family member A (RhoA), ERK1/2 and phosphoinositide 3-kinase (PI3K)/Akt serine/threonine kinase (Akt) signalling in ASM isolated from wild type but not TSG-6 knockout mice [135]. The role of pollutants in ASM dysfunction relevant to asthma and COPD is summarised in Fig. 2.

3.2. Aero-allergens

Aero-allergens; such as, house-dust mite (HDM) allergens, pollen, pet dander and fungal allergens can aggravate asthma and COPD symptoms. Aero-allergen induced ROS, co-exposure to oxidising environmental pollutants and aero-allergens, and compromised AOX defenses have all been implicated in allergic sensitisation [136,137]. In addition, oxidative stress can increase the allergenicity of proteins, and allergenic pollens can exhibit intrinsic NOX activity *per se* [136,138].

Oxidative stress is implicated in AHR and ASM thickening in numerous animal models of ovalbumin induced allergic asthma [139–143]. With regards to specific allergens; in murine models the HDM allergen *Der f* induced AHR and ASM thickening, which could be reversed by limonene, which has been reported to inhibit ROS production [144], the fungal associated allergenic protease (FAP) in combination with ovalbumin resulted in increased smooth muscle thickness and oxidative stress that could be reversed by metformin [145], and an increase in airway responsiveness and smooth muscle thickness was observed in response to artemisia pollen extract (APE) that was further increased in response to APE exposed to diesel emissions [146]. In human bronchial rings sensitised with sera from asthmatic patients the maximal contraction in response to HDM allergen was increased following exposure to acrolein and ozone [147].

In addition, in the bronchoalveolar lavage (BAL) fluid from a cohort of mild asthmatics, seven distinct oxidised phosphatidylcholines (OxPCs), that are generated by oxidation of the *sn*-2 polyunsaturated fatty acid chain of phosphatidylcholine during oxidative stress, were found to be associated with allergen challenge to cat, grass, horse, tree, HDM and ragweed allergens, with the concentration of 3 of these OxPCs shown to correlate with decline in lung function. Distinct OxPC profiles were also found to be related to AHR and decline in lung function in a separate cohort of patients. In support for a role in ASM contraction, OxPCs were able to induce airway narrowing in murine thin-cut lung slices [148].

However, investigations into the role of allergens and ROS/oxidative stress in primary ASM, BSM or TSM cells has not been studied widely. Supporting work on the role of OxPCs in ASM function showed that exposure of ASM cells to oxidised 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholines (PAPCs) but not non-oxidisable 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholines (PSPCs) resulted in enhanced secretion of various inflammatory mediators; IL-6, IL-8, and GM-CSF and increased COX-2 expression in a protein kinase C (PKC) dependent manner, as well as accumulation of various oxylipins, including prostaglandins and leukotrienes [148]. In relation to this observation, ASM cells were shown to have 5-hydroxyeicosanoid dehydrogenase (5-HEDH) activity, converting the oxylipin 5S-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) to (5-Oxo-6,8,11,14-eicosatetraenoic acid) 5-oxo-HETE, an inflammatory cell chemotaxin, in the presence of the oxidative stimulus H_2O_2, dependent on the GSH redox cycle and the generation of nicotinamide adenine dinucleotide phosphate (NADP) +, a cofactor for 5-HEDH [149].

In a guinea pig model, following ovalbumin sensitisation and subsequent challenge that induced AHR and AFO, levels of GSH measured in isolated ASM cells were lower than control and correlated inversely with AHR and AFO suggesting that the thiol anti-oxidant GSH protects against antigen-induced AHR and AFO [139], and in TSM cells isolated from ovalbumin sensitised and challenged mice the number of calcium sparks was shown to be increased compared with control mice. This was accompanied by an increase in ROS levels that were implicated in the increased calcium spikes as both were inhibited by N-acetylcysteine amide (NACA). This may have implications for ROS in antigen induced contraction as a caffeine induced increase in the calcium sparks in single TSMCs resulted in cell shortening, however the effect of NACA on this was not assessed [150].

Studies support a role for oxidative stress/ROS following exposure to PM2.5 in enhancing ASM contraction, and promoting ASM remodelling, via enhancing cell viability, proliferation and migration, and reducing apoptosis, although at high doses PM2.5 can have cytotoxic effects. Emerging evidence suggests that ozone exposure may also promote ASM contraction and remodelling. Aero-allergens are implicated in AHR, AFO, and inflammation with a limited number of studies with primary ASM cells supporting this. Further studies are required to fully elucidate the role of air pollutants and aero-allergens and associated oxidative stress/ROS in ASM dysfunction in asthma and COPD.

4. Effects of asthma and COPD relevant mediators on measures of ASM dysfunction

4.1. Alarmins

The activity of alarmins can be controlled by redox regulation [151] and they can also regulate ROS production in their own right (as described below). The ASM is more likely to be exposed to mediators such as alarmins present in the sputum in asthma and COPD due to increased mucous production, reduced mucous clearance due to epithelial dysfunction and epithelial denudation [152].

Both exogenous and endogenous S100A9 have been implicated in reducing ASM proliferation, with knock-down of S100A9 increasing platelet-derived growth factor (PDGF)-stimulated rat ASM proliferation and increasing ROS levels. Furthermore, this increase in ASM proliferation was inhibited by the anti-oxidant NAC. Thus, S100A9 appears to inhibit ASM proliferation via a reduction in ROS levels [153,154]. S100A8 is also implicated in suppressing PDGF-induced ASM proliferation, as well as suppressing PDGF induced migration in a receptor for advanced glycation endproducts (RAGE) dependent manner [155,156], and inhibiting ACh-induced ASM contraction [157], implicating S100A8/A9 in a protective role in ASM dysfunction. However, Gomes et al., 2013 detected increased levels of oxidised S100A8 and to a lesser extent oxidised S100A9 in sputum from asthmatics [158]. So whether S100A8/A9 would be present in an active form and able to play a role in

protecting from ASM dysfunction *in vivo* is uncertain.

Another alarmin who's activity undergoes redox regulation is high mobility group box 1 protein (HMGB1). HMGB1, both partially oxidised and reduced, was shown to be elevated in the sputum of patients with severe asthma versus healthy controls, with the ratio of reduced to oxidised HMGB1 increasing with asthma severity [159]. In subsequent investigations reduced HMGB1 was shown to potentiate bradykinin-mediated ASM contraction via TLR4, with a greater potentiation seen with ASM from asthmatics versus non-asthmatics. Interestingly, unlike in ASM from healthy controls, reduced HMGB1 was unable to induce ROS production in ASM from asthmatics. Thus, in health HMGB1 levels are not only lower, but it would likely be rendered inactive by ASM-derived ROS, whereas in asthma the reduced elevation of ROS in response to HMGB1 could enable HMGB1 to remain active for longer to contribute to ASM dysfunction [159]. HMGB1 has also been implicated in ASM cell proliferation following coculture with IL-2/IL-33-stimulated type 2 innate lymphoid (ILC2) cells isolated from interferon regulatory factor 7 (IRF7) −/− mice (which display impaired viral immunity) via a feed-forward amplification loop between HMGB1/TLR4 and IL-13 [160]. This could be mimicked by stimulation of ASM cells with disulphide HMGB1, but not the non-oxidisable mutant all-thiol HMGB1 [160]. Thus, HMGB1 has the potential to contribute to AHR and increased ASM mass in asthma, however its exact role appears to depend on its oxidation status, as reported for cytokine production and chemotaxis in other cell types [160–162].

Another alarmin elevated in the plasma in patients with asthma versus healthy controls is human β-defensin 3 (hBD3) [163]. hBD3 is shown to mediate general ROS production via ERK1/2, accompanied by mitochondrial ROS production and indicators of mitochondrial damage. The elevation of ROS by hBD3 is implicated in increased ASM derived IL-8 release and increased ASM apoptosis and thus has implications for airway inflammation and remodelling [163].

4.2. Interleukins

Several members of the interleukin family are implicated in stimulating ROS/oxidative stress in ASM. Increased release of 8-isoprostane, a marker of oxidative stress, from ASM cells was shown to occur following treatment with IL-1β and IL-17 in a ROS dependent manner [164,165]. In addition, IL-1β and IL-17 were shown to mediate increased expression and release of the inflammatory mediators CCL2, CCL11 and IL-8 in a ROS dependent manner [164,165]. IL-1β has also been shown to upregulate expression of vascular cell adhesion protein 1 (VCAM-1) in human TSM cells, enabling greater adherence of pro-inflammatory monocytes to the human TSM cells. This occurred in a ROS dependent manner with IL-1β shown to induce NOX activity, p47phox translocation and ROS production [166].

4.3. TNF-α

TNF-α induced ROS production is implicated in AHR, with TNF-α inducing an increased contractile response to carbachol in guinea pig tracheal rings in a ROS dependent manner [167]. In support of this TNF-α has been shown to induce ROS production by ASM cells in several studies [167–169], leading to ROS-dependent phosphorylation of the contractile protein MLC in primary guinea pig ASM [167]. The NOX family of enzymes are implicated as the source of TNF-α stimulated ROS as at concentrations similar to the TNF-α found in BAL from asthmatics, TNF-α stimulated ROS production by guinea pig ASM cells which was inhibited by the flavoprotein inhibitor diphenyleneiodonium (DPI), the pan-NOX inhibitor apocynin and p22phox knock down, and potentiated by nicotinamide adenine dinucleotide phosphate (NADPH) [167]. The cells were also shown to express p22phox, p47phox and cytochrome *b*558, with the former two also shown to be expressed by human ASM cells. Interestingly, TNF-α was shown to increase expression of the ROS generating enzyme NOX4 in ASM from COPD patients to a greater extent than in ASM from asthmatics [170].

Both TNF-α, and TNF-α inducing lipopolysaccharide (LPS), induce cPLA2 expression and subsequent PGE2 synthesis in ASM cells in a ROS dependent manner, the former via a mechanism involving NOX2 and p47phox with downstream activation of MAPKs, NF-κB and p300 [168] and the latter via a mechanism involving p67phox/p47phox with downstream MAPK-dependent activation of human antigen R (HuR) [171]. ROS dependent HuR signalling is also implicated in TNF-α mediated upregulation of suppressor of cytokine signalling 3 (SOCS-3) expression via a mechanism involving TNF receptor-1 (TNFR1)/MAPKs/p47phox and Nox2/ROS-dependent HuR signalling in human TSM cells. Overexpression of SOCS-3 markedly inhibited TNF-α and LPS-induced VCAM-1 expression in human TSM cells, and decreased monocyte adhesion to LPS challenged human TSM cells [169].

TNF-α is also involved in stimulating release of various inflammatory mediators from ASM cells, including CXCL10, shown to be dependent on HO-1 [172] and CCL5 and CCL11 shown to be dependent of NF-κB activation [173]. TNF-α treatment of ASM cells results in degradation of IκBα, enabling activation of NF-κB, NF-κB p65 nuclear accumulation, NF-κB/DNA binding and histone H3 phosphorylation. Dimethylfumarate (DMF) reduces cellular GSH levels and can inhibit all of the above effects, as well as TNF-α induced CCL5 and CCL11 secretion. Conversely the effects of DMF can be reversed by the supplementation of the ASM cells with GSH reduced ethyl ester (GSH-OEt), implicating elevated cellular GSH levels in the pro-inflammatory effects of TNF-α [173]. DMF is also implicated in Nrf2/HO-1 signalling in ASM cells as described in section 5.

Interestingly in a review in 2021, Dasgupta et al. propose a model whereby TNF-α triggers a homeostatic response in mitochondria that protects ASM from the oxidative stress it induces [174]. Briefly, TNF-α is known to increase force generation in ASM accompanied by increased ATP consumption and mitochondrial oxidative phosphorylation. This results in ROS generation and oxidative stress which leads to the accumulation of unfolded proteins in the endoplasmic reticulum (ER) and mitochondria and triggering of the unfolded protein response (mtUPR). TNF-α also selectively activates the phosphorylation of IRE1α (pIRE1α)/X-box binding protein 1 (XBP1) mediated ER stress pathway. Activation of these pathways leads to an increase in peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) which increases mitochondrial biogenesis and remodelling to increase efficiency of oxygen consumption and oxidative phosphorylation and reduce ROS formation to protect ASM cells from the negative impact of inflammation-induced ROS formation at a time of increased ATP demand [174].

4.4. Growth factors

By far the most studied growth factor with respect to oxidative stress/ROS dependent ASM dysfunction is TGF-β1. TGF-β1 induces ROS production by ASM cells, and this has been shown to contribute to various elements of ASM behaviour that could contribute to ASM dysfunction in asthma and COPD; namely increased ASM proliferation, cell size, IL-6 release, α-smooth muscle actin (α-SMA) expression and collagen I production [175–177]. A role for ROS in TGF-β1 mediated proliferation and IL-6 release is supported by the fact that these responses were attenuated by activation of the anti-oxidant transcription factor Nrf2 [175], and by the fact that TGF-β1 can induce expression of the ROS generating enzyme NOX4 [175–177] and inhibit expression of the ROS clearing enzymes manganese (Mn)-SOD and catalase, with NOX4 upregulation implicated in ROS generation and partial inhibition of MnSOD expression [175,176]. ROS are also implicated in the upregulation of connective tissue growth factor (CTGF) expression and mothers against decapentaplegic family member 3 (SMAD3) phosphorylation following TGF-β1 treatment, as both are inhibited following treatment with γ-glutamylcystein ethyl ester (γ-GCE, a precursor of GSH

biosynthesis) and subsequent increases in GSH levels in BSM cells, although the consequences of this upregulation are not investigated [178]. The upregulation of NOX4 by TGF-β1 was shown to be dependent on ROS and mediated by SMAD3/PI3K and dependent on Myc-Max interactions [175,177], and to be involved in TGF-β1 induced IL-6 release, ASM proliferation, hypertrophy and phosphorylation of the cell cycle regulators retinoblastoma protein (Rb), cell division control protein 42 homolog (Cdc2), and eukaryotic translation initiation factor 4E-binding protein (14E-BP1) which regulates protein synthesis [177]. TGF-β1-induced SMAD3-dependent upregulation of NOX4 was also shown to be involved in upregulation of α-SMA expression and collagen I production via phosphorylation of p38 MAPK and Akt [176]. NOX4 expression could also be induced by activin A, TGF-β2 and 3, although the functional consequences of this were not explored [177]. In support of the above roles of TGF-β1 in NOX4 expression and subsequent NOX4 mediated cellular responses, increased TGF-β1 expression is associated with increased NOX4 expression by ASM in COPD [39,176], which, coupled with the inhibitory effect of TGF-β1 on Nrf2/HO-1 signalling described in section 5, has implications for elevation of ROS production by ASM.

With both TGF-β1 and TNF-α being reported to increase NOX4 expression it is interesting to note that expression of NOX4, which has been shown to be the most prominent NOX isoform expressed by ASM, is elevated in the ASM in both asthma and COPD, where it correlates with decline in lung function and/or disease severity [8,39,170,176]. In addition, NOX4 expression has been implicated in the increased contraction observed by ASM cells from asthmatics versus healthy controls in culture [8] and correlates with increased ASM thickness, ASM area, and number of proliferating ASM cells in COPD, implicating it in increased ASM mass [39].

TGF-β1 treatment, in combination with foetal bovine serum (FBS) (TGF-β1/FBS), has also been shown to stimulate proliferation of ASM cells from patients with COPD to a greater extent than ASM from healthy controls with measures of the increased proliferation correlating with decline in the subject's lung function [20,179]. Mitochondrial derived ROS were shown to play a role in this increased proliferation, as well as in TNF-α-induced IL-8 release by ASM [20]. Coupled with the presence of mitochondrial dysfunction and enhanced basal and H_2O_2 stimulated mitochondrial ROS production from ASM from COPD patients versus healthy control subjects this implies a role for mitochondrial ROS in enhanced ASM proliferation and inflammatory mediator release in COPD. In support of this basal levels of GM-CSF, IL-8 and IL-6 release were greater from ASM from COPD patients versus healthy controls and stimulated further by H_2O_2 [20]. The mitochondrial dysfunction was accompanied by a decrease in oxidative phosphorylation in ASM from COPD patients versus healthy controls [20], with an impaired energy balance and accumulation of glycolysis related products suggesting a shift towards glycolysis in ASM from COPD patients versus healthy controls [179]. Indeed, the inhibition of TGF-β1/FBS induced DNA synthesis by the glycolytic inhibitor 2-deoxy-D-glucose implicates a role for this metabolic shift in enhanced ASM proliferation in COPD. Although mitochondrial ROS are implicated in TGF-β1/FBS induced proliferation [20], TGF-β1/FBS treatment was shown to result in lower mitochondrial ROS levels and an increase in the GSH/glutathione disulfide (GSSG) ratio in ASM cells from COPD patients versus healthy controls, with the GSH inhibitor buthionine sulfoximine increasing mitochondrial ROS levels in the presence of TGF-β1/FBS and inhibiting the increase in COPD ASM cell number following TGF-β1/FBS treatment in this study [179]. Thus, the role of mitochondrial ROS in ASM proliferation in COPD is unclear.

Besides TGF-β1, PDGF has long been shown to be involved in ROS dependent ASM proliferation via mechanisms including Cdc42/RAC1, PI3K or STAT signalling [180–184], and by p22phox activation of NF-κB [185]. More recently ROS dependent PDGF-induced ASM proliferation has been demonstrated to be mediated by PDGF-mediated, ROS/p22-phox/signal transducer and activator of transcription 3 (STAT3) dependent induction of IL-13 expression by ASM cells [186]. PDGF and epidermal growth factor (EGF) have also been shown to mediate ASM proliferation via ASK-1, a ubiquitously expressed MAP kinase kinase kinase (MAP3K) enzyme which can be activated by ROS amongst other stress stimuli, and is upregulated in ASM from COPD patients versus healthy controls [187]. ASK-1 was also shown to mediate TGF-β1 induced migration of ASM cells from COPD patients [187]. PDGF induced ASM proliferation can also be inhibited by an S100A9 mediated reduction in ROS, as described in section 4.1 [153,154], as well as activation of anti-oxidant HO-1 signalling (see section 5).

EGF can also induce proliferation of ASM cells *per se*, and this EGF dependent ASM proliferation could be potentiated by LTD4, via upregulation of EGF receptor (EGFR) phosphorylation, with both occurring in a ROS dependent manner [188]. Both EGF and EGF/LTD4 induced ASM proliferation could be inhibited by rosuvastatin [189] which has been shown to inhibit oxidative stress in other cell types [190].

In addition, the release of VEGF, which has been shown to mediate ASM proliferation in several studies [105–107], by ASM could be stimulated by CSE and acrolein as described in section 2.2 [104], as well as by the oxidant H_2O_2 in a p38 MAPK dependent manner and by 4-hydroxynonenal (4-HNE), a product of lipid peroxidation [104]. This implicates oxidative stress in enhanced VEGF release by ASM, which could result in increased ASM proliferation.

Another growth factor, BDNF, which can be upregulated by CSE-induced ROS, as described in section 2.1, was also shown to increase agonist induced ASM contraction *per se*, as assessed using epithelium denuded human bronchial rings, as well as enhancing histamine induced calcium elevation in ASM cells via the TrkB BDNF receptor [91]. The role of growth factors in ASM dysfunction relevant to asthma and COPD is summarised in Fig. 3.

4.5. Exogenous oxidative stress derived mediators

Oxidative stress leads to changes in DNA, lipids and proteins that can have a downstream effect on ASM cell function. One such example is DNA damage, with guanine being the primary ROS target resulting in 8-oxoguanine base lesions. During base excision repair free 8-OHdG base is generated which binds to 8-oxoguanine DNA glycosylase 1 with high affinity triggering expression of genes that can contribute to inflammatory mediator release, mast cell activation and ASM contraction, thus having implications for airway inflammation and bronchoconstriction, and exercise induced asthma [191,192]. Indeed 8-OHdG was detected at a higher level in ASM from mild-moderate and severe asthmatics compared to healthy controls, and was correlated with decline in lung function and increased AHR [8].

Oxidative stress also results in the generation of isoprostanes from arachidonic acid via non-enzymatic oxidation. Isoprostanes are involved in ASM contraction and remodelling, as reviewed by Clarke et al. [193] with 8-iso-PGF2α stimulating ASM contraction via activation of thromboxane (TP) receptors [193,194]. Further investigations into the signalling mechanisms behind this implicate SOCE and phosphatidylcholine-specific phospholipase C (PC-PLC) mediated calcium mobilisation and rho-kinase mediated calcium sensitisation in 8-iso-PGF2a mediated ASM contraction [194].

4.6. Miscellaneous mediators

The wider role of leukotrienes and lysophospholipids on ASM function is reviewed by Clarke et al. [193]. With respect to ROS, leukotriene (LT) D4 and S1P can also influence ASM function in a ROS dependent manner. In addition to potentiating EGF induced ASM proliferation LTD4 was able to induce ASM proliferation *per se*, via EGFR phosphorylation, in a ROS dependent manner [188], which as for EGF and EGF/LTD4 induced ASM proliferation could be inhibited by rosuvastatin [189]. S1P induced ROS and NOX activity in ASM cells as well as COX2 and PGE2 expression and ASM migration in a ROS dependent manner,

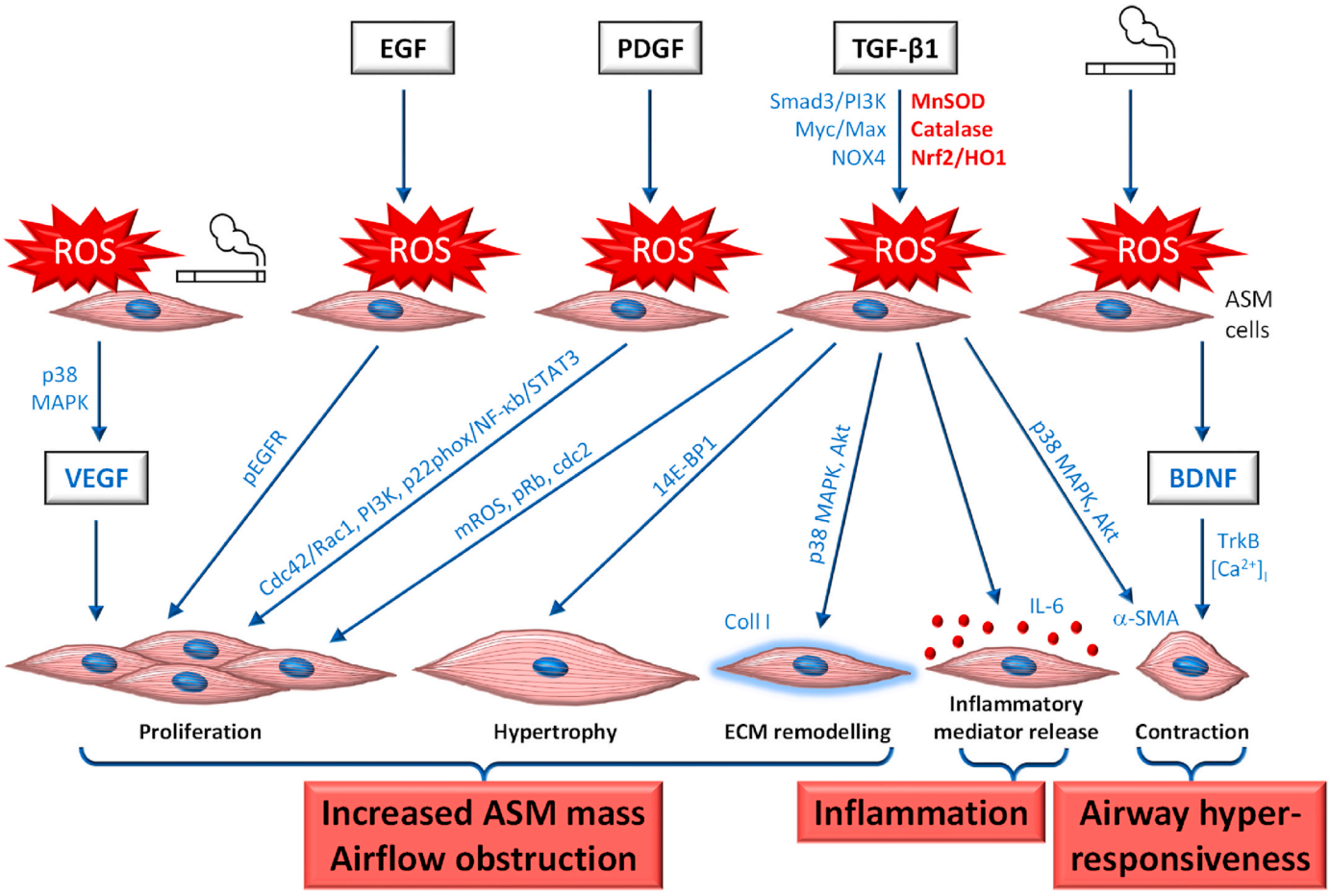

Fig. 3. The role of growth factors in oxidative stress induced ASM dysfunction. ROS are implicated in the effects of growth factors on mechanisms related to increased ASM mass, contractility and inflammatory mediator release, which could contribute to the AHR, airflow obstruction and inflammation present in asthma and/or COPD. TGF-β1 can increase ROS production by ASM via; upregulation of mitochondrial ROS, upregulation of the ROS generating enzyme NOX4, down regulation of the ROS clearing enzymes MnSOD and catalase, and down regulation of Nrf2/HO-1 signalling. This enhanced ROS leads to; increased ASM proliferation via pRb and cdc2, ASM hypertrophy via 14E-BP1, and ASM-derived Coll I production via p38 MAPK and Akt signalling that can contribute to increased ASM mass and airflow obstruction, increased α-SMA via p38 MAPK and Akt signalling that can enhance ASM contraction, and enhanced IL-6 release that can lead to inflammation. PDGF can mediate ASM proliferation in a ROS dependent manner via several signalling mechanisms, including cdc42/Rac1, PI3K and p22phox/NF-κB and/or STAT3. ASM proliferation can also be mediated via ROS dependent EGF-mediated phosphorylation of EGFR and stimulation of VEGF release from ASM by the oxidants CSE and H_2O_2 and the lipid peroxidation product 4-HNE, in a p38 MAPK dependent manner. BDNF and its receptor TrkB are also upregulated in a ROS dependent manner following CSE exposure and can contribute to ASM dysfunction via elevation of agonist induced $[Ca^{2+}]i$ and increased contraction, which can contribute to AHR. Blue arrows indicate a stimulatory effect. Blue font indicates upregulation, bold red font indicates downregulation. Abbreviations used: airway hyper-responsiveness (AHR), airway smooth muscle (ASM), Akt serine/threonine kinase (Akt), alpha-smooth muscle actin (α-SMA), brain derived neurotrophic factor (BDNF), cell division control 2 (cdc2), cell division control protein 42 homolog (Cdc42), cigarette smoke extract (CSE), epidermal growth factor (EGF), eukaryotic translation initiation factor 4E-binding protein (14E-BP1), extracellular matrix (ECM), haem-oxygenase 1 (HO-1), hydrogen peroxide (H_2O_2), interleukin 6 (IL-6), intracellular calcium ($[Ca^{2+}]_i$), manganese superoxide dismutase (MnSOD), mROS (mitochondrial ROS), mitogen-activated protein kinase (MAPK), mothers against decapentaplegic family member 3 (SMAD3), NADPH oxidase (NOX), nuclear factor-erythroid factor 2-related factor 2 (Nrf2), nuclear factor-kappaB (NF-κB), phosphoinositide 3-kinase (PI3K), phosphorylated epidermal growth factor receptor (pEGFR), platelet-derived growth factor (PDGF), phosphorylated retinoblastoma protein (pRb), reactive oxygen species (ROS), signal transducer and activator of transcription 3 (STAT3), transforming growth factor-beta (TGF-β), tropomyosin receptor kinase B (TrkB), vascular endothelial growth factor (VEGF). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with NOX2/p47phox implicated in the former [195].

5. The role of Nrf2/HO-1 signalling in protecting against ASM dysfunction

Nrf2 is believed to protect against oxidative stress with Nrf2 genetic deletion leading to T2 inflammation, oxidative stress and AHR in a mouse model [196]. Indeed, in human ASM cells Nrf2 was shown to mediate expression of the anti-oxidant genes HO-1, NOQ1, and MnSOD, with activation of HO-1 reciprocally resulting in Nrf2 translocation from cytosol to nucleus in human TSM cells [198]. The potential contribution of Nrf2/HO-1 signalling in protecting against ASM dysfunction in asthma and COPD is described below and summarised in Fig. 4.

5.1. The role of Nrf2/HO-1 signalling in ASM proliferation

Nrf2 activation was shown to mediate a reduction in ASM proliferation via an increase in expression of the cell cycle arrest mediator $p21^{Waf1}$, which correlated with Nrf2-ARE binding in the ASM [197]. In addition, HO-1, which is induced by Nrf2, has been shown to be involved in inhibition of foetal calf serum (FCS) and PDGF induction of human ASM proliferation in several studies [197–201]. HO-1 activation or inhibition were shown to reduce or enhance FCS and PDGF induced human ASM proliferation, ROS production and ERK1/2

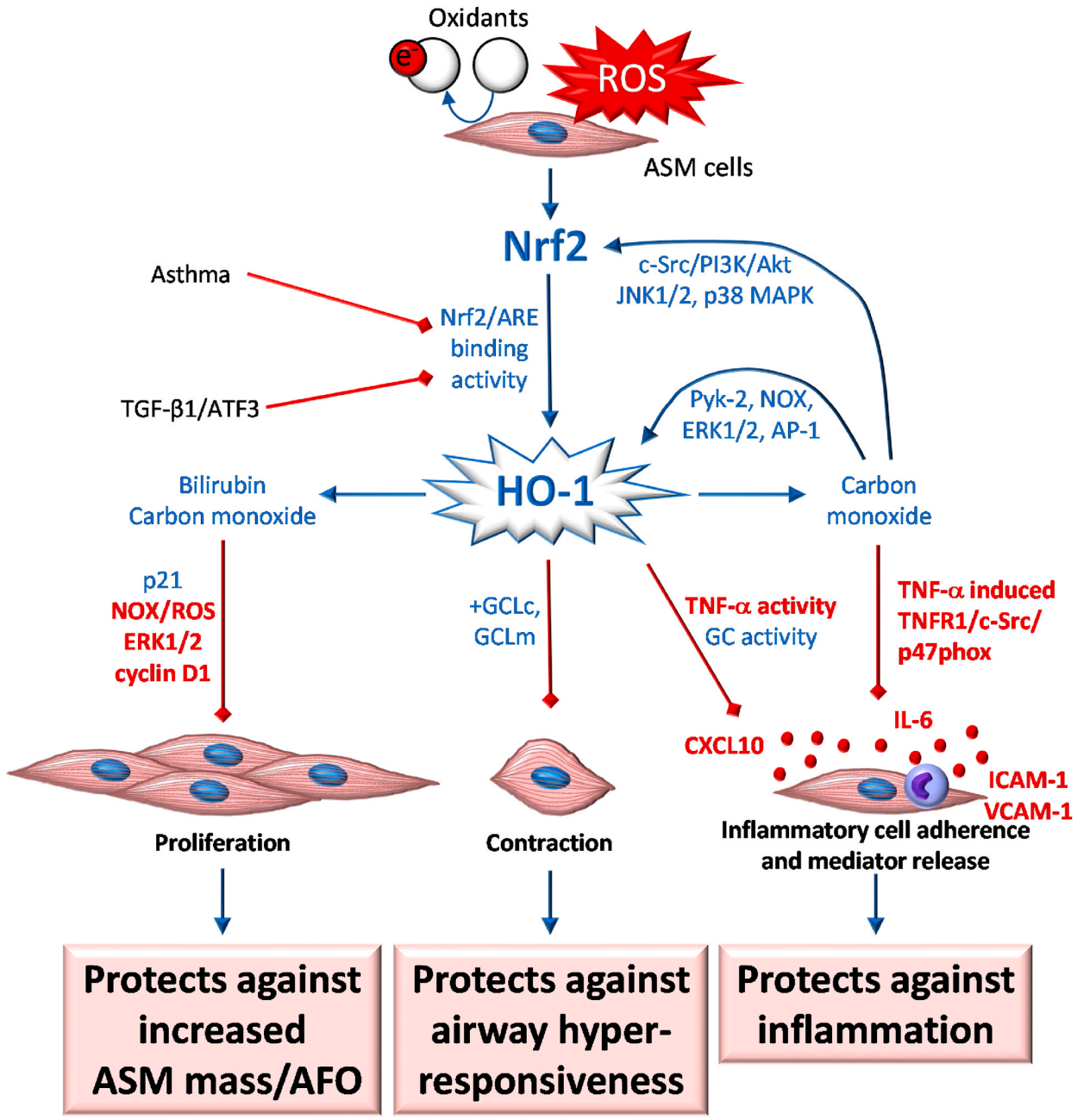

Fig. 4. The potential role of Nrf2/HO-1 signalling in protecting against ASM dysfunction. Nrf2/HO-1 signalling is activated following exposure of cells to oxidants and ROS and is implicated in negative regulation of ASM proliferation, contraction and inflammatory mediator release in ASM cells, which has implications for protecting against AHR, airflow obstruction and inflammation following exposure of ASM cells to oxidative stress. Inhibition of ASM proliferation can be mediated by both bilirubin and carbon monoxide, products of HO-1's enzymatic activity, via downregulation of NOX or ROS, ERK1/2 and cyclin D1 and upregulation of p21. With regards to inflammation; HO-1 inhibits CXCL10 release by ASM *per se* and increases the efficacy of the corticosteroid fluticasone in inhibiting TNF-α induced CXCL10 release from ASM cells, and carbon monoxide is implicated in reducing TNF-α induced IL-6 release by ASM, VCAM-1 and ICAM-1 expression by ASM and THP-1 cell adhesion to ASM. The Nrf2 induced AOX proteins GCLc and GCLm alongside HO-1 can also reduce the contractility of ASM cells. In addition, carbon monoxide also forms a positive feedback loop to upregulate expression of Nrf2 and HO-1 in ASM cells to perpetuate activation of the protective AOX pathways following exposure to oxidative stress. However, Nrf2/ARE binding is compromised in the ASM in asthma and the asthma and COPD relevant mediator TGF-β1, via ATF3, can inhibit Nrf2/ARE binding activity in ASM cells to reduce the efficacy of Nrf2/HO-1 signalling and its downstream protective effects. Thus, although the Nrf2/HO-1 pathway has the capability to negatively regulate measures of ASM dysfunction, the dysregulation of Nrf2/HO-1 signalling in the ASM in asthma and COPD could contribute to measures of ASM dysfunction. Blue arrows indicate a stimulatory effect, red diamond arrows indicate an inhibitory effect. Blue font indicates upregulation, bold red font indicates downregulation. Abbreviations used: activating transcription factor-3 expression (ATF3), activator protein 1 (AP-1), airway hyper-responsiveness (AHR), airway smooth muscle (ASM), Akt serine/threonine kinase (Akt), anti-oxidant (AOX), anti-oxidant response element (ARE), cellular Src (c-Src), c-Jun N-terminal kinase (JNK), C-X-C Motif Chemokine Ligand (CXCL10), extracellular signal-regulated kinase (ERK), glucocorticosteroid (GC), glutamate-cysteine ligase catalytic subunit (GCLc), glutamate cysteine ligase modifier subunit (GCLm), haem-oxygenase 1 (HO-1), intercellular adhesion molecule 1 (ICAM-1), interleukin 6 (IL-6), mitogen-activated protein kinase (MAPK), NADPH oxidase (NOX), nuclear factor-erythroid factor 2-related factor 2 (Nrf2), phosphoinositide 3-kinase (PI3K), PKCα/Pyruvate kinase 2 (Pyk-2), reactive oxygen species (ROS), TNF receptor-1 (TNFR1), transforming growth factor-beta (TGF-β), tumour necrosis factor alpha (TNF-α), vascular cell adhesion molecule 1 (VCAM-1). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

phosphorylation, respectively. This was shown to be mediated in a ROS dependent manner via ERK1/2 activation and bilirubin, an anti-oxidant end product of HO-1 activity [199]. In this study a role for carbon monoxide (CO), another by-product of HO-1 activity, is ruled out as myoglobin, a CO scavenger, has no effect on HO-1 mediated inhibition of PDGF/FBS induced ASM proliferation [199]. In contrast CO was implicated in inhibiting FBS induced pERK1/2 dependent human ASM cell proliferation in another study, associated with upregulation of p21 and downregulation of cyclin D1 expression [198]. In support of this study the CO-releasing molecule (CORM)-2 was shown to mediate inhibition of PDGF induced ASM cell proliferation [200]. Although CORM-2 inhibits PDGF induced cytochrome *b*558 activity and NOX activity, treatment with CORM-2 resulted in a net increase in ROS production by ASM cells, proposed to be of mitochondrial origin. In addition, the involvement of mitochondrial ROS is implicated further as the effects of CORM2 on PDGF induced ASM cell proliferation, net ROS production, ERK1/2 phosphorylation and cyclin D1 expression were mimicked following treatment with rotenone which increases mitochondrial ROS by blocking the respiratory chain at complex 1 [200]. The involvement of enhanced ROS in inhibition of PDGF induced ASM proliferation contrasts with the Taille et al., 2003 study where HO-1 activation was shown to be involved in inhibition of mitogen induced ASM proliferation via inhibition of ROS production [199].

5.2. The role of Nrf2/HO-1 signalling in inflammatory processes in ASM

HO-1 has also been linked to inflammatory processes in human TSM cells via Nrf2 and CO. HO-1 activation resulted in inhibition of TNF-α-induced THP-1 cell adherence, as well as TNF-α-induced intercellular adhesion molecule-1 (ICAM-1) and VCAM-1 expression and generation of IL-6 via suppression of TNF-α induced superoxide and H_2O_2 generation, NOX activity, p47phox translocation from the cytosol to the membrane and formation of a TNFR1/c-Src/p47phox complex [202]. CO is implicated in the effects of HO-1 on TNF-α induced ICAM-1 and VCAM-1 expression, ROS generation, IL-6 secretion and p47phox translocation as the effects could be mimicked by the CO releasing molecule CORM-2 and blocked by the CO scavenger hemoglobin [202]. Indeed, the involvement of CO and/or ROS in HO-1 upregulation has demonstrated in several studies in human TSM cells, with CORM-2 shown to lead to HO-1 upregulation via PKCα/Pyruvate kinase (Pyk)-2 dependent Nox/ROS/ERK1/2/AP-1 [203] and HO-1 upregulation and Nrf2 activation via the c-Src/PI3K/Akt/JNK1/2 and p38 MAPK pathways [204]. Further support for the role of ROS in HO-1 upregulation is provided by Lee et al., 2008 who demonstrate that lipoteichoic acid (LTA)-induced ROS generation was mediated through the TLR2/MyD88/TRAF6/c-Src/NOX pathway, which in turn initiates the activation of Nrf2, and ultimately induces HO-1 expression in human TSM cells [205].

5.3. The role of DMF in promoting HO-1 signalling in ASM

HO-1 expression in ASM cells can also be induced by DMF. This induction could be inhibited by GSH and p38 MAPK inhibition [172,201]. Downstream of HO-1 induction DMF treatment resulted in inhibition of PDGF-induced ASM proliferation in a HO-1 dependent manner. TNF-α and TNF-α/IFN-γ induced CXCL10 and G-CSF release by ASM cells could be inhibited by DMF, the former demonstrated to be in an HO-1 dependent manner. In support of this, induction of HO-1 by haemin and CoPP mimicked the reduction in TNF-α induced CXCL10 from ASM cells by DMF. DMF was also able to further reduce the inhibition of CXCL10 secretion seen in response to fluticasone, in an HO-1 dependent manner, suggesting that it acts independently of the glucocorticoid pathway and may offer steroid sparing properties [172]. Indeed, further investigation into the mechanisms behind the anti-inflammatory effects of DMF show that DMF induces IκBα glutathionylation (IκBα-SSG), resulting in subsequent inhibition of IκBα degradation, NF-κB p65 nuclear entry and NF-κB/DNA binding, as well as inhibition of histone H3 phosphorylation to reduce accessibility of NF-κB binding sites within promoters [173]. Interestingly, DMF reduces intracellular GSH levels and the effects of DMF on ASM proliferation and inflammatory mediator release were reversed by GSH, suggesting that reduction in GSH levels leads to induction of HO-1 expression and subsequent inhibition of ASM proliferation and inflammatory mediator release [172,173,201]. The effects of DMF on HO-1 signalling, coupled with its inhibitory effects on TNF-α signalling described in section 4.3 has implications for the therapeutic potential of DMF in asthma and COPD, as discussed in section 6.1.

5.4. The role of Nrf2/HO-1 signalling in ASM contraction

As the Nrf2 pathway is implicated in protecting against AHR in animal models [196] the role of Nrf2 in mouse ASM contraction was investigated. When cells were adhered to a polyacrylamide gel substrate the Nrf2 activator sulforaphane was shown to induce the anti-oxidant genes GCLc, glutamate cysteine ligase modifier subunit (GCLm) and HO-1 and decrease the net contractile moment. This is further supported by the observation that in ASM from Nrf2 knockout mice versus ASM from Nrf2 wild type mice the expression of GCLc, GCLm and HO-1 was decreased and net contractile moment was increased, with the latter reversed in the presence of the anti-oxidant NAC, thus, supporting a role for the Nrf2 pathway in protecting against enhanced ASM contractility [206]. In the same study the contractile scope of the ASM cells was shown to increase following adherence to fibronectin, laminin, and collagen I, and decrease on adherence to collagen IV and collagen V. Interestingly expression of the Nrf2 regulated genes GCLc, GCLm and HO-1 were shown to increase upon adherence to all these ECM proteins [206].

Although the above studies implicate a role for Nrf2 and HO-1 in protecting against ASM proliferation, inflammatory mediator release and contraction, the dermal sensitiser dinitrochlorobenzene (DNCB), despite mediating an increase in the Nrf2 mediated anti-oxidant response in ASM cells, had no concomitant effect on ASM agonist-induced intracellular calcium elevation, precision cut lung slice (PCLS) carbachol-induced bronchoconstriction or PCLS release of inflammatory mediators, including IL-6, IL-8 and CCL11 [207].

5.5. Dysregulation of Nrf2/HO-1 signalling in ASM

Nrf2 and HO-1 have been shown to be expressed in the ASM within human bronchial biopsies [197,199]. No difference in Nrf2 expression was observed between ASM from patients with non-severe or severe asthma versus healthy individuals. However, Nrf2 binding to anti-oxidant response elements (AREs) was reduced in ASM from patients with severe asthma compared with ASM from patients with non-severe asthma and healthy controls and HO-1 expression was reduced in ASM cells from patients with both non-severe and severe asthma compared with healthy individuals [197].

TGF-β1 is implicated in this dysregulated Nrf2 anti-oxidant response in asthma [197]. This is supported by the fact that, although TGF-β1 had no effect on Nrf2 protein expression or ARE binding, after an initial increase in HO-1 expression, expression of the Nrf2 targets NQO1 and HO-1 were inhibited by TGF-β1, with the latter shown to be mediated via the Nrf2 repressor activating transcription factor-3 (ATF3). Nrf2-ARE binding activity was also inhibited by TGF-β1, which, in addition to the reduction in HO-1, could be overridden by Nrf2 activation by sulforaphane. TGF-β1 mediated ASM proliferation, IL-6 production and release, with the repression of the anti-oxidant response by TGF-β1 implicated in these effects as they were also attenuated by Nrf2 activation by sulforaphane [197]. The dysregulation of Nrf2-ARE binding in asthma, and the suppression of Nrf2/HO-1 signalling by the asthma and COPD relevant mediator, TGF-β1, could mean that the potential protective effects of Nrf2/HO-1 signalling against oxidative stress

induced ASM dysfunction are compromised in asthma and COPD.

Further studies into the regulation of Nrf2 signalling in human ASM cells demonstrate that bromodomain and extraterminal (BET) proteins, known to be involved in regulation of gene transcription, are implicated in the negative regulation of Nrf2 dependent transcription [208]. The BET protein bromodomain inhibitor JQ1+ led to increased Nrf2 expression, Nrf2-ARE activity and expression of the classical Nrf2 targets; HO-1, NQO1, and glutamate-cysteine ligase catalytic subunit (GCLc), predominantly via inhibition of the Brd2 BET protein isoform and reduction in expression of Kelch-like ECH-associated protein 1 (Keap-1) protein, which binds to Nrf2 and promotes its degradation by the ubiquitin proteasome pathway [208,209]. Whether BET proteins are differentially expressed in the ASM between health and asthma to contribute to compromised Nrf2 responses, or whether BET protein inhibition could help restore Nrf2-ARE binding in the ASM asthma, and thus whether BET proteins represent a viable therapeutic target in ASM dysfunction requires further investigation.

6. Implications of oxidative stress for therapeutic approaches in asthma and COPD

6.1. Clinical trials of anti-oxidants in asthma and COPD

Several different approaches have been used to try to boost anti-oxidant defences in lung disease (see Table 2), however despite some clinical benefit being observed the results have not been considered beneficial enough to have led to routine clinical use of the agents tested.

6.1.1. Thiol based anti-oxidants

NAC is a thiol containing anti-oxidant which has been the subject of various clinical trials in both asthma and COPD. A recent meta-analysis has shown that high and low dose NAC for >6 months reduced the number of COPD patients with at least one exacerbation [210]. Subsequent post-hoc analysis of the PANTHEON trial included in the aforementioned meta-analysis shows that NAC is more effective in reducing the rate of COPD exacerbations in patients with a significant smoking history, and in those that are not being treated with ICS [211] in agreement with the findings of the BRONCUS study [212]. In another study imaging parameters used to assess lung resistance and volume were found to correlate with levels of GSH and GPx following NAC treatment, which the authors propose could be used in determining subsets of patients likely to benefit from long term NAC therapy [213]. In asthma NAC treatment was shown to have no significant benefit for the treatment of asthma exacerbation on top of usual asthma medication [214], but did result in a reduction in baseline AHR and protection against DE induced increase in AHR [215].

Other thiol based anti-oxidants that have been assessed in clinical trials for COPD include carbocysteine and erdosteine. A meta-analysis of 4 studies showed that carbocysteine reduced the rate of exacerbations, the number of patients with at least one exacerbation and improved the quality of life [216]. A meta-analysis of 10 studies, plus a subsequent post-hoc analysis of the RESTORE study included in this meta-analysis, showed that erdosteine could reduce the rate of exacerbations, length of time to first COPD exacerbation, duration of exacerbation [217,218] as well as reducing the number of patients with at least one exacerbation and the risk of hospitalisation [217].

However in addition to their anti-oxidant activities thiol based anti-oxidants also have mucolytic activity and whether their effects are truly anti-oxidant or via their mucolytic activity remains unclear [219,220]. In addition, by virtue of being thiol-based these anti-oxidants can be inactivated by higher levels of ROS in the lungs which could limit their efficacy [220]. Interestingly, NAC has been shown to reverse a number of ROS mediated effects in *in vitro* ASM based studies described in this review.

Another potential anti-oxidant therapeutic approach is supplementation with the thiol-based anti-oxidant GSH, however this was shown to induce bronchoconstriction when administered to asthma patients via nebulisation, which the authors postulate is due to its metabolism into leukotrienes or the formation of sulphites, both of which have bronchoconstrictor activity [221]. Another reason for caution regarding GSH

Table 2
Summary of the clinical benefits of anti-oxidant agents in asthma and COPD.

Class of agent	Agent	Disease	Type of study	Effects	References
Thiol anti-oxidant	NAC	COPD	Clinical trial	Reduced exacerbations	[210]*, [211, 212]
		Asthma	Clinical trial	Reduced AHR	[215]
			Clinical trial	No effect on exacerbations	[214]
	Carbocysteine	COPD	Clinical trial	Reduced exacerbations Improved quality of life	[216]*
	Erdocysteine	COPD	Clinical trial	Reduced exacerbations and risk of hospitalisation	[217]*
	GSH	Asthma	Clinical trial	Induced bronchoconstriction	[221]
Nrf2 activation	Sulforaphane	COPD	Clinical trial	No change in anti-oxidant gene transcription	[227]
		Asthma	Clinical trial	Reduced AHR	[228]
	DMF	Multiple sclerosis/psoriasis patients with asthma	Self-reported	Improved asthma symptoms and quality of life	[201]
	Lipoic acid	Asthma	Pre-clinical	Reduced AHR, oxidative stress and airway remodelling	[225]
Anti-oxidant vitamins	Vitamin D	COPD	Clinical trial	Improved lung function, symptoms and quality of life Reduced exacerbations	[230]*, [231],~
		Asthma	Clinical trial	Reduced exacerbations	[232]~, [233]*
	Vitamin A	COPD	Clinical trial	Improved lung function Decreased COPD risk	[234]
		Asthma	Clinical trial	No clinical benefit	[236]~
	Vitamin C	COPD	Clinical trial	Decreased COPD risk	[235]*~
		Asthma	Clinical trial	Reduced lung function decline	[236]~
	Vitamin E	COPD	Clinical trial	No clinical benefit	[235]*~
		Asthma	Clinical trial	No clinical benefit	[236]~
	β-carotene	COPD	Clinical trial	No clinical benefit	[235]*~

Summary of the clinical benefits of anti-oxidant agents in asthma and COPD that have undergone pre-clinical or clinical trials, or are self-reported associated with treatments other illnesses. Abbreviations used: airway hyper-responsiveness (AHR), chronic obstructive pulmonary disease (COPD), dimethyl fumarate (DMF), glutathione (GSH), N-acetyl cysteine (NAC), nuclear factor-erythroid factor 2-related factor 2 (Nrf2). * meta-analysis, ~ comprehensive/systematic review.

supplementation is that it can reduce the efficacy of chemotherapeutic drugs for lung cancer [222].

As described by McCarty et al., GSH levels can also be increased by phase 2 inducers, such as lipoic acid (LA), which stimulate expression of the anti-oxidant enzymes involved in GSH synthesis, via increasing Nrf2 activity to boost cellular anti-oxidant defences [223,224]. Indeed, LA has shown promise in preclinical models of asthma, resulting in decreased AHR, oxidative stress and airway remodelling [142,223,225], and has been shown to be safe and therapeutically effective in diabetic neuropathy [223,226].

6.1.2. Nrf2 activators

Sulforaphane, which has been shown to override TGF-β1 mediated responses in ASM cells (see section 5), is another Nrf2 activator that has been tested in clinical trials for COPD and asthma. In COPD, treatment with sulforaphane for 4 weeks did not result in an increase in the transcription of anti-oxidant genes regulated by Nrf2 [227]. Some clinical benefit was observed following treatment of asthmatics with sulforaphane which resulted in a reduction in AHR in 60% of patients [228]. DMF also activates Nrf2 in ASM cells. DMF is a fumaric acid ester used to treat psoriasis and multiple sclerosis, which has also been shown to reduce asthma symptoms and improve quality of life in those who also suffer with asthma and to inhibit proliferation in several cell types [201]. Although it is implicated in reducing ASM proliferation and inflammatory mediator release as described in section 5 [172,173,201], its potential as a therapy for asthma and COPD has not been assessed in preclinical studies or clinical trials [229]. DMF also reduces ASM cellular GSH levels and the inhibitory effects of DMF on ASM proliferation and inflammatory mediator release are mitigated by exogenous GSH [172,173,201], which calls into question the validity of GSH supplementation as a therapeutic strategy for asthma and COPD.

6.1.3. Antioxidant vitamins in asthma and COPD

Various anti-oxidant vitamin supplements have been tested for clinical efficacy in treating asthma and COPD. The most interesting results are for vitamin D. In COPD, both a recent meta-analysis and comprehensive review suggest clinical benefit of vitamin D supplementation, with improvement in lung function, exacerbations, symptoms and quality of life, particularly in those with vitamin D deficiency [230,231]. Likewise in asthma vitamin D was shown to have some clinical benefit; reducing exacerbation rate in adult asthmatics with vitamin D deficiency [232] as well as in asthmatic patients being treated with corticosteroids, although lung function and symptoms were unaffected, as reported in a recent meta-analysis [233].

Regarding other anti-oxidant vitamins in COPD vitamin A was shown to increase lung function and decrease COPD risk in male smokers [234]. In a more recent meta-analysis vitamin C was shown to confer some reduction in COPD risk with vitamin E and β-carotene shown to have no benefit [235]. With regards to asthma a recent systematic review including studies of vitamin A, C and E in asthma showed a reduction in lung function decline following vitamin C supplementation, with no clinical benefit reported for vitamins A and E [236]. However, a study of vitamin A supplementation with β-carotene for reducing lung cancer incidence, was implicated in increased risk of death, including from lung cancer [237].

6.1.4. Anti-oxidant vitamins and ASM

Although the results of anti-oxidant vitamins in clinical trials has not led to their use as therapeutic agents to date, evidence if emerging for a role of these vitamins in ASM dysfunction relevant to asthma and COPD. The potential impact of vitamin D on asthmatic human ASM has been reviewed by Hall et al. [238] which indicates a role for vitamin D in suppressing mitogen induced proliferation, and expression of proteins involved in airway remodelling, including MMP-9 and disintegrin and metalloproteinase domain-containing protein 33 (ADAM-33), as well as inhibiting CCL5 release, both by itself and in an additive manner to fluticasone, suggesting that vitamin D may help target ASM remodelling and inflammatory mediator release. Indeed, in recent studies vitamin D was shown to inhibit TNF-α induced collagen and fibronectin deposition [239] and to reduce the ASM mass, collagen deposition, and airway inflammation in ovalbumin sensitised rats [240]. Vitamin D was also shown to inhibit the TNF-α induced potentiation of calcium elevation in response to histamine, and to counteract the inhibitory effects of IL-13 on ASM histone deacetylase (HDAC) activity and expression [239], which could have implications for ASM hyper-contractility and response to corticosteroids.

The effect of other vitamins on ASM function has not been as widely studied. All-trans retinoic acid (ATRA), a derivative of vitamin A, and vitamin A receptor agonists were shown to inhibit PDGF-induced human BSM cell migration and Akt activation [241], and mitogen induced ASM cell proliferation [242]. In support of a protective role of vitamin A in ASM dysfunction, ASM cells from mice on a vitamin A deficient diet and/or a diet including a pan-retinoic acid receptor inhibitor were found to be hypertrophic, hypercontractile, with enhanced collagen 1A2 expression, although they were not hyperproliferative [243]. Members of the vitamin E family have been shown to have various effects on ASM cell function. Gamma-tocotrienol inhibits TGF-β1 induced increases in α-SMA, fibronectin, collagen I [244] and PDGF-BB induced ASM proliferation and migration [245] with inhibition of RhoA activation implicated in both studies. In contrast, TGF-β1 induced proliferation was unaffected by α-tocopherol [246].

6.1.5. Naturally derived anti-oxidant compounds and ASM

Various naturally derived anti-oxidant compounds have been implicated as having therapeutic potential in lung diseases *in vitro* and in animal models [247]. With regards to ASM studies various herbal derived compounds have been shown to alleviate CS or ovalbumin induced oxidative stress, inflammation and/or airway remodelling in murine or rat models [248–251] with findings supported by parallel *in vitro* ASM based experiments. Artesunate inhibited CSE induced human BSM proliferation and α-SMA expression [248], rhynchophylline restores catalase and SOD levels in TGF-β1 treated ASM cells, and inhibits TGF-β1 mediated ASM proliferation, IL-6 release and reduced markers of autophagy [249], tetrandrine increased Nrf2 and HO-1 expression, and inhibited ASM cell proliferation in TGF-β1 treated ASM cells [250] and galangin inhibited TGF-β1-induced proliferation, ROS production and NOX4 expression and TGFβ-1-induced suppression of catalase and SOD expression in ASM cells [251]. In addition, caffeic acid phenethyl ester (CAPE) reduced AHR, inflammation, fibrosis, α-SMA expression and oxidative stress in ovalbumin sensitised mice, with parallel *in vitro* work showing that CAPE reduced TGF-β1 induced ROS production and TGF-β1 and H_20_2 induced proliferation of human ASM cells [252].

6.2. Targeting excessive ROS production

6.2.1. Targeting mitochondrial ROS

An alternate approach is to target excessive ROS production in asthma and COPD. Mitochondrial anti-oxidants, particularly mitoQ, are in clinical trials for a range of diseases, however whether they have clinical benefit in asthma and COPD has yet to be tested. Coenzyme Q is an endogenously produced anti-oxidant key to the mitochondrial electron transport chain involved in the production of mitochondrial ROS. Coenzyme Q10 supplementation, alongside vitamins C and E, was reported to reduce corticosteroid usage and markers of oxidative stress in asthma patients with low serum levels of coenzyme Q10 [253] and in short-term studies coenzyme Q was shown to restore SOD levels and improve airflow obstruction in asthmatics versus controls [254], and improve several measures of COPD, including exercise tolerance, breathlessness and quality of life [255]. An alternative approach for targeting mitochondrial ROS is the use of induced pluripotent stem cell-derived mesenchymal stem cells (iPSC-MSCs) which were shown to inhibit ozone induced mitochondrial dysfunction, AHR and

inflammation in mouse lungs. Parallel *in vitro* studies showed that coculture of iPSC-MSCs with ASM cells inhibited CSE induced mitochondrial dysfunction, mitochondrial ROS levels and apoptosis via a mechanism including mitochondrial transfer [256].

6.2.2. Targeting NOX enzymes

Of the ROS generating enzymes the NOX family of enzymes represent a good therapeutic target as unlike other ROS generating enzymes their sole role is to produce ROS and therefore targeting them is likely to lead to less side effects. Limited clinical studies have been performed with the pan-NOX inhibitor apocynin. In COPD patients apocynin reduced markers of oxidative stress in exhaled breath condensate 60 and 120 min post administration via nebulisation with no adverse effects, but its therapeutic benefit in COPD has not been assessed [257,258]. In mild asthmatics aerosolised apocynin administered pre and post ozone exposure was shown to reduce AHR and maximal airway narrowing [259]. NOX1 and 4 have been identified as particularly interesting targets for lung diseases [260], with evidence presented in this review suggesting a role for NOX4 in ASM dysfunction in asthma and COPD. However clinical development of NOX inhibitors is plagued by their lack of specificity [260]. The specific NOX1/4 inhibitor GKT137831/setanaxib has advanced the furthest in clinical trials, being shown to have a good safety profile in clinical trials in healthy subjects and for diabetic nephropathy and biliary cholangitis [261]. GKT137831/setanaxib is currently being investigated as a potential therapeutic for idiopathic pulmonary fibrosis (NCT03865927) however whether GKT137831/setanaxib has clinical benefit in asthma and COPD has yet to be tested.

6.3. Potential role of current therapies in reducing ROS levels

PDE inhibitors have also been implicated in reducing oxidative stress. Roflumilast, a PDE4 inhibitor has been approved for patients with more severe COPD, and a recent meta-analysis in asthma has shown that roflumilast improves lung function, asthma control and exacerbations with variable effects on AHR [262]. In ASM cells, roflumilast N-oxide, a metabolite of roflumilast, has in combination with formoterol been shown to enhance the anti-inflammatory effect of dexamethasone [263]. Roflumilast has been demonstrated to reduce ROS in cortical neurons [264], however whether anti-oxidant effects contribute to roflumilast's therapeutic benefits in asthma and COPD requires further investigation. Another PDE4 inhibitor, CHF6001, which is currently in clinical trials for asthma and COPD [265] was shown to upregulate genes involved in negative regulation of oxidative stress in sputum from patients with COPD and bronchitis when administered on top of inhaled maintenance triple therapy [266]. Theophylline is another PDE4 inhibitor which can be used in asthma and COPD with caution [267]. Interestingly when administered alongside corticosteroids theophylline treatment resulted in an increase in anti-oxidant enzymes and a decrease in lipid peroxidation in blood and improved lung function, which could explain the reported effects of theophylline of restoring responsiveness to steroids via upregulation of HDAC activity [268,269]. However, this should be interpreted with caution as a recent meta-analysis of use of theophylline as an add on therapy to ICS indicated higher hospitalisation rates and mortality [270], and theophylline had no effect on COPD exacerbation rates or other clinically important measures when administered with low dose OCS [271].

6.4. Effect of oxidative stress on the efficacy of current therapeutic approaches

The presence of oxidative stress also has implications for the efficacy of current therapeutic approaches. Bronchial thermoplasty is a licensed treatment which aims to reduce ASM mass via heat treatment in those with severe asthma. S-glutathionylation of proteins, which is known to occur during oxidative stress [272], is implicated in protecting ASM cells from heat-induced death. This is proposed to be via glutathionylation of major vault protein (MVP), shown to protect against cell death, resulting in preferential binding to myosin 9, to counteract the stimulatory effects of myosin 9 on heat-induced ASM death [273]. As the ASM shows evidence of exposure to oxidative stress in asthma [8], this study could have implications for the efficacy of bronchial thermoplasty in reducing ASM mass in asthma.

Corticosteroid insensitivity is common in COPD and present in up to 10% of asthmatic patients [57], with the role of oxidative stress in such insensitivity reviewed by Lewis et al. [274]. Studies into the mechanisms of oxidative stress in corticosteroid insensitivity have centred around cell types other than ASM, such as; epithelial cells, macrophages and peripheral blood mononuclear cells (PBMCs) [274–276], however ASM from patients with COPD and smokers versus non-smokers was shown to be less sensitive to the corticosteroid dexamethasone, with a role for ROS in this indicated by the fact that inhibition of mediator release could be mediated by the anti-oxidant resveratrol [21]. In relation to this, CSE was shown to inhibit GC-induced leucine zipper (GILZ) transactivation induced by the corticosteroids beclomethasone monopropionate and fluticasone propionate in BSM cells [277]. However, the β-agonists formoterol and salmeterol were able to counteract this inhibitory effect of CSE on GILZ transactivation, which is important for the anti-inflammatory actions of corticosteroids [277]. In support of these observations the PRACTICAL clinical trial showed that the Symbicort inhaler which contains the corticosteroid budesonide and formoterol used as needed for symptom relief was better at preventing severe exacerbations than maintenance doses of budesonide plus the β-agonist terbutaline as needed [278].

In contrast to this the anti-inflammatory effects of formoterol can be compromised by CS. CS reduced expression of A-kinase anchoring proteins (AKAP) 5 and 12 in primary ASM cells, both of which can interact with the β2-AR, with AKAP5 expression shown to be reduced in the ASM of patients with moderate COPD versus controls [117]. AKAPs signal via protein kinase A (PKA) with disruption of AKAP-PKA interactions both augmenting CSE induced IL-8 release from ASM, and diminishing the suppression of this release by formoterol, suggesting that CSE induced changes in AKAP expression could compromise the anti-inflammatory effects of formoterol [117]. ROS are also involved in the down regulation of β-adrenergic signalling [279], which could have implications for the bronchodilatory effects of β-agonists, confounding the effects of ROS on promoting ASM contractility described in this review.

7. Summary and conclusions

In this review we have summarised studies using data from primary airway, bronchial or tracheal smooth muscle cells that assess the role of oxidative stress and ROS in ASM dysfunction relevant to asthma and COPD. The effectors, mediators and consequences of oxidative stress and ROS exposure are many and varied. Despite this, the evidence presented shows clear potential for a role of oxidative stress and ROS induced by asthma and COPD relevant stimuli, and for dysregulated ROS generation and clearance, in ASM dysfunction relating to AHR, increased ASM mass, ASM derived inflammatory mediator release and ASM corticosteroid resistance.

However, many of the studies are performed in ASM derived from individuals without asthma or COPD, so whether these findings would extrapolate to ASM derived from asthmatics or COPD patients should be investigated further, as well as the effect of disease severity on the findings. In addition, studies to identify any gender or age specific effects of oxidative stress on ASM dysfunction would be very informative, as oxidative stress is known to increase with age [280] and studies suggest CSE may have gender specific effects on AHR, as CSE has been shown to abrogate the effects of 17β-estradiol on calcium signalling and cAMP generation [89].

It also has to be considered that ROS can be beneficial. A certain level of ROS are important for normal physiological processes [70], and although CSE-induced ROS were implicated in ASM dysfunction, CSE

was also shown to be involved in stimulating signalling pathways such as; COX2/PGE2 and Nrf2/HO-1 which may act in a compensatory manner to help protect from damage following CSE exposure. Thus, the use of non-specific antioxidants could result in reduction of such beneficial ROS, leading to side effects. Due to these complexities, it is perhaps not surprising that approaches to target oxidative stress in asthma and COPD have so far not translated into the clinic. However, these have generally been delivered by oral administration and thus have systemic effects, and they do not specifically target the ASM. Development of inhaled therapies against oxidative stress could help to reduce side effects and increase efficacy by targeting the airways specifically and identification of biomarkers of oxidative stress in the ASM could help to stratify patients for treatment based on levels of oxidative stress, which may help to improve the results of trials with current anti-oxidant agents.

In addition, development of drugs targeting NOX enzymes, particularly NOX4, which is implicated in oxidative stress in the ASM in both asthma and COPD, are hindered by their lack of NOX isoform specificity and ROS scavenging properties [260]. The most highly characterised ROS clearance mechanism in ASM is the Nrf2/HO-1 pathway, which is shown to be compromised in asthma, and can be suppressed by the asthma and COPD relevant mediator TGF-β1 [197]. Interestingly, agents that boost Nrf2 show some degree of clinical benefit in asthma but not COPD [201,227,228]. A further understanding of the mechanisms involved in ROS generation and clearance in ASM, their potential dysregulation and the consequences of such dysregulation across ASM derived from asthma and COPD patients compared to healthy individuals may help to identify novel more specific therapeutic targets within the ASM.

Furthermore, ROS have been implicated in compromising the efficacy of currently available treatments in *in vitro* ASM based studies [117,273,277,279]. Further characterisation of the mechanisms behind the role of oxidative stress in; protecting ASM from heat-induced death during bronchial thermoplasty, the down regulation of β-adrenergic signalling in ASM, and in corticosteroid insensitivity in ASM may help our understanding of why some patients do not respond fully to therapy and may enable alternative therapeutic approaches to be taken. Likewise, further work is required to investigate whether current therapies, that in addition to their recognized mode of action are implicated in reducing markers of oxidative stress in other cell and sample types [264,266,268], can also reduce oxidative stress in ASM cells. This could ultimately lead to repurposing of current therapies for a new and/or wider patient population.

Declaration of competing interest

CEB serves on advisory boards for GlaxoSmithKline, AstraZeneca, Boehringer Ingelheim, Cheisi, Roche, receives honoraria from Novartis, and receives research support from GlaxoSmithKline, AstraZeneca, Chiesi, Novartis, Boehringer Ingelheim and Roche and has received grant funding from NIHR. RS has no conflicts of interest. YA has no conflicts of interest. MB has no conflicts of interest.

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