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Anti-Inflammatory Effect of Sulfur Dioxide and Sulfite on Cigarette Smoke Exposed Human Airway Smooth Muscle Cells

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Anti-Inflammatory Effect of Sulfur Dioxide and Sulfite on Cigarette Smoke Exposed Human Airway Smooth Muscle Cells

by

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Anti-Inflammatory Effect of Sulfur Dioxide and Sulfite on Cigarette Smoke Exposed Human Airway Smooth Muscle Cells

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Sulfur dioxide is an endogenously produced gas, once thought to be solely toxic. More recent studies have demonstrated that at physiological concentrations however it can also have beneficial effects, such as inhibition of inflammation, vasodilation, and relaxation of smooth muscle. Airway smooth muscle cells (HASMC) are involved in multiple aspects of chronic inflammatory airway diseases such as asthma and COPD, including airflow restriction and release of pro-inflammatory mediators. While the response of HASMC to cigarette smoke exposure has been studied, the effects of sulfur dioxide on the cigarette smoke-induced inflammatory mediators have not. It has been proposed that sulfur dioxide creates a hypoxic state, similar to cigarette smoke, which suggested that the hypoxia inducible factor-1α (HIF-1α) may be involved in mediating the resulting inflammation. This also suggested that the effects of cigarette smoke and sulfur dioxide, two air pollutants commonly present in the industrial areas south of

Houston, might be cumulative. This led us to hypothesize that co-exposure to cigarette smoke and sulfur dioxide would antagonize the cigarette smoke-induced inflammation and that HIF-1α would mediate the inflammatory response by its effects on the MAPK and NF-κB pathways. Although sulfur dioxide has been shown to induce inflammation and airway smooth muscle constriction, these effects primarily occur at higher concentration. The exception to this is a group of individuals with severe asthma, COPD or a sensitivity to sulfur dioxide that react negatively to low concentrations of sulfur dioxide (< 1 ppm). These individuals typically have elevated levels of pro-inflammatory cytokines and we have found that exposure of inflammatory cytokine-stimulated HASMC to sulfur dioxide does result in augmented release of GM-CSF, a pro-inflammatory cytokine that has been increasingly recognized as a major regulator of airway inflammation. However, we also found that sulfur dioxide reduced cigarette smoke-induced GM-CSF release from HASMC via down-regulation of the p38, p44/42 and NF-κB pathways. While HIF-1α activity clearly limits the NF-κB-driven induction of GM-CSF, it does not appear that sulfur dioxide is reducing GM-CSF through a HIF-1α mediated mechanism.

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List of Abbreviations

AhR	aryl hydrocarbon receptor
Akt	Protein kinase B (PKB)
ANOVA	analysis of variance
ARNT	aryl hydrocarbon receptor nuclear translocator
ASM	airway smooth muscle
ATP	adenosine triphosphate
BSA	bovine serum albumin
CO	carbon monoxide
CO_2	carbon dioxide
COPD	chronic obstructive pulmonary disease
CSE	cigarette smoke extract
DMSO	dimethyl sulfoxide
DPBS	Dubelcco's phosphate buffered saline
DTT	dithiothreitol
ELISA	enzyme linked immunosorbent assay
ERK1/2	extracellular signal regulated kinases 1 and 2
FBS	fetal bovine serum
FIH	factor inhibiting HIF
GM-CSF	granulocyte macrophage-colony stimulating factor
GSK3β	glycogen synthase kinase-3 beta
HASMC	human airway smooth muscle cells
H_2DCFDA	2'7'-dichlorofluorescein diacetate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HIF	hypoxia inducible factor
HIF-PH	HIF-1α prolyl hydroxylase
HO	heme oxygenase
H_2O_2	hydrogen peroxidase
H_2S	hydrogen sulfide

HRE	hypoxia response element
HRP	horseradish peroxidase
Ig	immunoglobulin
IL-1β	interleukin-1 beta
IP	immunoprecipitation
JNK	c-Jun terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MEKK	mitogen activated protein kinase kinase kinase (aka MAPKKK)
MKK	mitogen activated protein kinase kinase (aka MAPKK)
mRNA	messenger ribonucleic acid
Na_2SO_3	sodium sulfite
NAC-ME	N-acetyl-L-cysteine methyl ester
NF-κB	nuclear factor kappa B
NO	nitric oxide
NOS	nitric oxide synthase
PHD	prolyl hydroxylase
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SNK	Student-Newman-Keuls
SO_2	sulfur dioxide
TBS	tris buffered saline
TBST	tris buffered saline tween20
TNF	tumor necrosis factor
Tris	triaminomethane
Ub	ubiquitination
pVHL	von Hippel Landau factor

Chapter 1 Introduction to the Effects of Sulfur Dioxide and Sulfite Co-exposure with Cigarette Smoke Extract on Human Airway Smooth Muscle Cells

INTRODUCTION

Environmental toxicants are frequently studied individually to reduce experimental complexity and clearly establish the effects of a particular factor. However, humans are not normally exposed to a single toxicant at a time; instead they encounter mixtures of various pollutants and other substances in their daily lives which may have short- and long-term effects on their health. The interaction of these substances, either directly with each other or through cellular pathways, can produce results that do not correspond to the sum of the individual effects. Due to the recognition of this issue, there is a growing interest in studying the effects of pollutants and other substances in combinations that may reveal these interactions. This is of critical importance because the current experimental evidence used to establish public policy may not reflect the true hazards we encounter, resulting in less effective regulations.

Symptoms in individuals experiencing chronic inflammation can be exacerbated by exposure to environmental toxicants (1,2). Asthma and chronic obstructive pulmonary disease (COPD) are airway diseases characterized by chronic inflammation and airflow obstruction. People with these conditions are more susceptible to the harmful effects of cigarette smoke and cigarette smoke can accelerate the progression of these diseases (3-6).

Cigarette smoke is generally considered an airborne pollutant, although contact with residue on surfaces can also result in exposure. It is estimated that over 20 million people have died prematurely in the U.S. over the last 50 years due to cigarette smoking-induced effects (7). Cigarette smoke induces inflammation while dysregulating immune system function, making smokers more susceptible to infections (8-11). This is true even in individuals who do not have smoking-related diseases such as COPD (12,13).

Sulfur dioxide (SO_2), a component of urban air pollution, is a toxic, non-flammable, colorless gas with a pungent odor that is very water-soluble (14). Exposure to sulfur dioxide exacerbates asthmatic symptoms and may be associated with disease development (15). Sulfur dioxide is also associated with increased mortality in COPD patients (16-18) and has been used to create a COPD model in rats (19,20). At high doses it induces inflammation and fibrosis (21).

While historically sulfur dioxide has been viewed strictly as a toxic gas, it has more recently been found to be produced endogenously from the metabolism of L-cysteine (22). This places sulfur dioxide in the growing list of gasotransmitters- small molecule, endogenously produced gases used as cellular signaling molecules, including nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S). Most of these gases were originally thought of as toxic pollutants, but new evidence has revealed their role in intracellular and extracellular biological regulation (23-25) and they are now being investigated as potential therapeutic targets.

Sulfur dioxide has been found to promote inflammation in the airways of OVA stimulated rats (26) but suppress it in lungs damaged with oleic acid (27) and the cardiovascular system (28,29). Similarly, sulfur dioxide has displayed both anti-oxidant

(30,31) and pro-oxidant effects (32). While it has been suggested that the different effects are due to differences in sulfur dioxide dose (high versus low doses) or source (exogenous versus endogenous), our research demonstrates that the different effects are responses to different cellular stimuli – sulfur dioxide augments the effects of pro-inflammatory cytokines but inhibits cigarette smoke extract (CSE)-induced inflammation. This has important implications for the development of sulfur dioxide as a therapeutic molecule because the presence of inflammation may alter an individual's response to treatment, producing a toxic effect instead of a therapeutic one.

Industrial workers have some of the highest smoking rates in the United States (33) and many of them spend a large portion of their working life in close proximity to sulfur dioxide sources. This puts them, and their coworkers, at a high risk for co-exposure to cigarette smoke and sulfur dioxide. Although there have been a few studies that looked at the correlation between cigarette smoking and sulfur dioxide on health indicators in specific populations (34-36), to our knowledge there has been no research on the effects of acute co-exposure to both. This research contributes to the elucidation of the mechanisms of action of sulfur dioxide and cigarette smoke in the human airway.

CHRONIC OBSTRUCTIVE PULMONARY DISEASE AND ASTHMA

COPD and asthma are both airway diseases characterized by chronic inflammation, but the predominant immune cells involved are different: neutrophils, macrophages and CD8+ cytotoxic T cells for COPD versus eosinophils and CD4+ T helper cells in asthma (37). These inflammatory cells release chemokines, cytokines,

proteases and other molecules that initially assist in resolving the infection or injury but, if they persist too long, contribute to the pathogenesis of the diseases by attacking healthy tissue in addition to damaged or infected cells. While the symptoms of COPD and asthma can overlap (coughing, shortness of breath, wheezing, chest tightness), the pathophysiology is distinct in most cases. The development of COPD typically occurs in mid-life while asthma can occur at any time but usually starts at a young age. There is a subset of individuals, up to 20% of patients, who either have both diseases or have one of the diseases plus conditions that are normally associated with the other disease (38). This is known as asthma-chronic obstructive pulmonary disease overlap syndrome (ACOS) (38). Both COPD and asthma are treated with bronchodilators and corticosteroids, but COPD and steroid-resistant asthma respond poorly to corticosteroids. Currently there are no cures for COPD or asthma and our understanding of the etiology of these diseases is incomplete.

COPD

COPD is a world-wide health crisis. It is the third leading cause of mortality and the fourth main cause of disability in the U.S. (39). According to the World Health Organization (WHO), in 2016 there were 251 million cases of COPD world-wide and over 5 million deaths in 2015 (40). It is estimated that yearly health care expenses for COPD will rise to $49 billion by 2020 in the U.S. alone (39). The severity of COPD is classified using the Global Initiative for Chronic Obstructive Lung Disease (GOLD) scale: GOLD 1 (mild) to GOLD 4 (very severe) FEV_1 (forced expiratory volume in one

second) and GOLD A (low risk, low symptom burden) to GOLD D (high risk, higher symptom burden) based on the individual's exacerbation rate and symptom severity (41) .

COPD is a progressive disease involving decreased elasticity of the alveoli, which makes it difficult to expel air from the lungs, and thickening of the airway plus excess mucous production that both reduce available airspace within the airways - all of which contribute to primarily irreversible airflow restriction (42). COPD patients often have emphysema, which is destruction of the walls between the alveoli leading to enlarged air sacs. The primary cause of COPD is cigarette smoke exposure (43), but exposure to outdoor air pollution (traffic, industrial) or indoor air pollution (cooking with wood or other solid fuels) is also a risk factor for the development of COPD and asthma (44). Approximately 3% of COPD cases are due to genetic mutation resulting in α_1-anti-trypsin (AAT) deficiency which allows increased activity of neutrophil proteases, such as elastase, that destroy lung tissue (45). AAT deficiency can cause an early onset of COPD compared to cigarette smoke-induced disease and up to 85% of individuals with severe AAT deficiency will develop COPD (46). Elastase, cathepsin-G and proteinase-3 are proteases stored in neutrophil granules that are released during inflammation and degrade a variety of molecules, including collagens, proteoglycans, immunoglobulins, and protease inhibitors (47). Their activity is regulated by antiproteases such as AAT, which are oxidized and inhibited by cigarette smoke, thus contributing to the pathogenesis of COPD (47). This upset in the protease/antiprotease balance, along with a similar one in the oxidant/antioxidant balance and inflammation are believed to be responsible for the COPD-induced airway damage (48,49). Although COPD is primarily linked with alterations in the pulmonary system, it is also associated with systemic defects such as

malnutrition, peripheral muscle dysfunction, and anemia which may be due to increased levels of circulating pro-inflammatory mediators (50).

Individuals with COPD have an elevated response to the inhalation of irritants, such as pollution and cigarette smoke, compared to people without COPD who smoke, and the level of inflammation increases with the severity of COPD (51). This can cause increasing levels of lung tissue destruction by disrupting the mechanisms that should eventually resolve the inflammatory response (51). The inflammatory mediators induced by the over-response include IL-1β (52), TNF (53), IL-6 (54), IL-8 (55), TGFβ (56), reactive oxygen species (ROS) and proteases (57). The inflammation due to COPD continues even after the individual has stopped smoking (58), suggesting an autoimmunity mechanism may be involved in its pathogenesis (59).

Prolonged neutrophilia, elevated neutrophil levels, is characteristic of many inflammatory diseases, including COPD and may be the result of increased production or reduced elimination of cells (60). Neutrophils have a lifespan of approximately 5 days before apoptosis and phagocytosis by macrophages (61), however stimulation with some inflammation-promoting cytokines can extend their lifespan to allow them to perform their essential functions, which include phagocytosis of invading pathogens and release of cytokines and chemokines to attract additional immune cells to the site of injury (62). When the pro-survival factors continue unabated, neutrophilia can develop. Stimulation with granulocyte macrophage-colony stimulating factor (GM-CSF) prolongs human neutrophil survival by increasing the stability of Bcl-2 family protein Mcl-1 via a PI3K/AKT and p44/42-dependent pathway (63). GM-CSF levels are elevated in individuals with COPD (64). Ongoing release of pro-inflammatory cytokines, such as

GM-CSF, by airway cells may be partly responsible for promoting neutrophilia in COPD, making it a potential therapeutic target for treatment of this disease.

Asthma

Asthma is a serious public health concern, 1 in 13 people in the United States (65) and 300 million worldwide (66) have asthma. It is responsible for 250,000 deaths per year and its prevalence is increasing (66). In addition to the human toll, there is a large financial burden, with 1.6 million asthma-related emergency room visits and $56 billion in associated health care costs per year in the United States alone (65). Asthma severity is classified as: Intermittent, Mild Persistent, Moderate Persistent or Severe Persistent based on the frequency and intensity of symptoms both during and between exacerbations, FEV_1 lung function test results and peak flow variability (67).

Asthmatic individuals experience airflow restriction due to constriction of the airway by smooth muscle, airway thickening resulting from tissue remodeling, and blockage due to increased mucous secretion (68,69). Like COPD, there are both genetic and environmental factors that contribute to the pathogenesis of asthma; however asthma can also have an allergic component. Exposure to primary or secondhand cigarette smoke is associated with an increased risk of asthma (70). Asthmatics have increased airway hyper-responsiveness, which causes bronchoconstriction at lower irritant levels than normal individuals, when exposed to environmental triggers such as allergens, pollution, sulfur dioxide and cigarette smoke. If untreated, the disease progresses and asthmatics may eventually develop irreversible airflow obstruction from airway

remodeling. Asthmatics can have inflammation in the large and small airways but do not develop the destruction of the alveolar walls that is characteristic of COPD (49).

As with COPD, oxidative stress is present in asthmatics and may contribute to the pathogenesis of the disease, however anti-oxidant treatment has not been very successful which raises the question of whether oxidative stress is a cause or result of asthma (71).

Eosinophils are short lived, white blood cells that primarily combat allergens and parasites before undergoing apoptosis and phagocytic clearance from the site of infection or inflammation (72). IL-3, IL-5 and GM-CSF are cytokines that promote eosinophil survival (73), while TGFβ attenuates their survival-promoting effect and reduces autocrine-stimulated GM-CSF release from eosinophils (72).

Eosinophilia is a hallmark of asthma (74) and, as with neutrophils, release of cytotoxic granules from the persistent eosinophils can cause tissue damage (75,76). The level of eosinophilia is correlated with pulmonary function and asthma severity (74). GM-CSF is elevated in individuals with asthma (64) and may contribute to the prolongation of inflammation characteristic of this disease by helping to perpetrate eosinophilia. GM-CSF antibody molecules are currently in trials as treatments for several diseases including asthma (77) and rheumatoid arthritis (78,79).

GM-CSF

GM-CSF was originally identified as a hematopoietic growth factor that promoted the production and differentiation of white blood cell granulocytes (neutrophils, eosinophils) and macrophages (80). More recently it has been recognized, not only as a pro-inflammatory cytokine, but as a key regulator of inflammation that is associated with autoimmune diseases (81). GM-CSF is released from T-cells, macrophages, smooth

muscle cells, epithelial cells, fibroblasts and other cells in response to pathogens and inflammatory mediators to augment the immune response (82). It stimulates neutrophil phagocytosis, ROS generation, and degranulation (83) and is also associated with the perception of pain (84). GM-CSF molecules, for example Leukine® by Immunex and Leucomax® by Novartis, are currently sold as therapeutics for use after bone marrow transplantation and chemotherapy to induce production of myeloid cells.

Overexpression of GM-CSF leads to pathology and has been associated with rheumatoid arthritis, asthma, COPD, inflammatory bowel disease and psoriasis (81) and GM-CSF inhibitors are currently being tested for treatment of some of these diseases. However, excessive inhibition of GM-CSF causes pulmonary alveolar proteinosis (reduced surfactant clearance by macrophages) which makes the individual more susceptible to infection and poses potential problems for GM-CSF antibody therapies (85,86).

IL-2 induces GM-CSF release from T helper (T_h) cells, which allows them to induce neuroinflammation (87). An increase in GM-CSF-releasing T_h cells is associated with increased disease severity of multiple sclerosis (MS), another chronic inflammatory disease with an autoimmune etiology (88). TGFβ induced GM-CSF release from T helper (T_h) cells, which is opposite to the eosinophil response (89). In low sodium media however, TGFβ inhibited GM-CSF release (89). This indicates that TGFβ can have different effects depending on the conditions the cells are experiencing. In leiomyoma and myometrial smooth muscle, GM-CSF induced TGFβ1 (90). Elevated TGFβ1 levels are associated with the development of fibrosis in the body (90). Therefore, while TGFβ

can help resolve inflammation by inhibiting pro-inflammatory cytokines such as GM-CSF, overexpression can be harmful.

GM-CSF signaling involves a receptor with two subunits: an α subunit that is specific to GM-CSF (GMRα) and a βc subunit that is shared with IL-3 and IL-5 and transduces the extracellular signal into the cell. GM-CSF binds to the high specificity, low affinity GMRα and then βc is recruited to form a high affinity heterodimer (82). A hexamer complex consisting of two of each component (ligand, GMRα & βc) is formed but activation of the receptor requires the formation of a dodecamer from two hexamers (91). This allows the Janus kinase 2 (Jak2) molecules that dimerized with the βc subunit intracellular domains to transphosphorylate each other and the βc subunits on tyrosine residues. This subsequently recruits phosphotyrosine binding proteins, such as signal transducer and activator of transcription (STATs), PI3K and growth factor receptor-bound protein 2 (Grb2), and triggers various signaling cascades (91).

The GM-CSF receptor can act like a multiposition switch. Low levels of GM-CSF result in phosphorylation of Ser585, instead of tyrosine phosphorylation, on βc and activation of PI3K/Akt which promotes survival of granulocytes and macrophages (91). At higher concentrations (≥ 10 pM) Tyr577 is phosphorylated, possibly due to a change in conformation that brings the two Jak2 molecules closer, activating STAT5, Grb2/Ras/MAPKs and PI3K/Akt which induce survival and proliferation (91). Individuals with acute myeloid leukemia have constitutive Ser585 phosphorylation on the GM-CSF receptor intracellular βc subunit (91). Inhibitor of κB kinase β (IKKβ), along with the p85 subunit of PI3K interact with GMRα (92), providing another path for signal specificity.

A soluble form of the GMRα-subunit (sGMRα) is produced through alternative splicing which cleaves the transmembrane domain, but it can also be produced by cleavage of the cell surface-attached subunit by metalloproteases (93). Monocytes constitutively secrete sGMRα and release can be up-regulated by stimulation with GM-CSF, LPS, and other factors (93). The sGMRα, which does not bind the βc subunit, can antagonize GM-CSF receptor activation by competing for GM-CSF ligand in the extracellular space (93).

GM-CSF belongs to a family of cytokines, including IL-3 and IL-5, with similar functions – induction of inflammation and hemopoietic cell production and differentiation during insult (92). Although not necessary for most homeostatic functions, this group is needed specifically for eosinophil production (92). The genes for all 3 cytokines plus IL-4 are located on chromosome 5q23-31 in close proximity to each other, suggesting a common evolutionary heritage (94). GM-CSF and IL-3 are monomers while IL-5 forms a homodimer (92). GM-CSF is produced by a far wider range of cell types than IL-3 or IL-5, which are produced mainly by T cells and mast cells (92). GM-CSF is believed to be more effective at differentiation and IL-3 more at inducing proliferation of myeloid cells (92). IL-3 can stimulate a wide variety of cells, including neutrophils, eosinophils, basophils, macrophages, mast cells and dendritic cells (92). IL-5 has more restricted effects than the others, stimulating eosinophils and basophils, but IL-3 has a much stronger effect on basophil survival than GM-CSF or IL-5 (92). These differences are at least partially mediated by the different α–subunits for each cytokine, which are each capable of binding factors intracellularly in addition to the βc subunit.

GM-CSF transcription is induced by NF-AT, NF-κB and activator protein 1 (AP-1) transcription factors. Glucocorticoids, one of the main asthma treatments, inhibit GM-CSF production by competitive binding of the glucocorticoid receptor to nuclear factor of activated T-cells (NF-AT) and AP-1 sites on the GM-CSF enhancer (95). The mRNA half-life of GM-CSF decreases the longer the gene is being actively transcribed, however inhibition of translation with cycloheximide increased mRNA half-life (96). The GM-CSF transcript contains an AU-rich element in its 3'-UTR that reduces mRNA half-life and inhibits translation, possibly by disrupting activity of the poly(A)-binding protein (97). Effects of this element can be modulated by binding of various proteins.

GM-CSF production and release can be stimulated by a wide variety of factors and it in turn can induce numerous inflammatory mediators. Increased GM-CSF release produces an increase in IL-1β release and inhibition of GM-CSF results in decreased IL-1β release (98-100). Similarly, increased IL-1β release produces an increase in GM-CSF release (101-103). TNF induces GM-CSF release from HASMC (104) and GM-CSF can induce TNF from monocytes and osteoblasts (105,106). GM-CSF induces TGFβ receptors, but not TGFβ1, in human airway smooth muscle cells, resulting in increased connective tissue production when they are activated (107). Other airway cells such as epithelial cells are known to release TGFβ1, and in a normal response this promotes repair, but in a pathological response it can produce fibrosis in the lungs (107).

AIRWAY SMOOTH MUSCLE

While airway smooth muscle, located within the airway wall, was once viewed simply as force-generating tissue, more recent work has revealed that the cells are capable of producing a growing number of factors once thought to be the purview of immune cells. This has shown that these cells can have important effects on the development of airway diseases such as asthma and COPD. Airway smooth muscle cells are involved in the pathogenesis of asthma in multiple ways: their contraction causes airflow restriction by squeezing the airway; airway hyper-reactivity causes a faster, stronger response of the smooth muscle to irritants; increased smooth muscle mass contributes to airway thickening; and they release pro-inflammatory mediators that can promote inflammation within the airway wall (108).

Similarly, in COPD there is an increase in airway smooth muscle mass in the small airways (109,110), there is increased airway hyper-responsiveness and the smooth muscle cells release pro-inflammatory mediators that support inflammation in the airway wall (111). The inflammatory mediators released by airway smooth muscle cells are believed to recruit inflammatory cells into the airway wall leading to irreversible airway remodeling (112). Airway smooth muscle cells can secrete IL-1, TNF, GM-CSF, IL-6, IL-8, IL-10 and many other mediators that will affect the inflammatory status of the airway. Therefore, it is crucial to understand how these cells will respond to stimuli to develop a complete picture of the pathogenesis of airway diseases.

CIGARETTE SMOKE

Cigarette smoke is a pollutant which is composed of over 4000 chemicals, including carcinogens (benzo-a-pyrene, acrolein), gases (CO, SO_2, nitrogen oxides), free radicals (superoxide O_2^-), reactive oxygen species (ROS) and toxicants (nicotine, acetone, ammonia, formaldehyde, hydrogen cyanide) (113). Humans and other animals can be exposed to cigarette smoke products through inhalation, ingestion or dermal contact. The constituents of this mixture can vary depending on whether the exposure is to mainstream smoke, side-stream smoke, exhaled smoke, surface residue or vapors off-gassed from exposed surfaces. The brand and type of cigarette, method of smoking and exposure of reaction products to sunlight are additional factors that can alter the chemical makeup of the mixture.

Exposure to cigarette smoke induces inflammation and oxidative stress and disrupts the immune system response (8-11). Cigarette smoke activates toll-like receptor (TLR) 4, which induces NADPH oxidase (NOX) and subsequent ROS production (Figure 1) (114-116). The increased intracellular ROS may activate the MAPK and NF-κB pathways, inducing production of pro-inflammatory mediators, such as GM-CSF, that promote eosinophilia and neutrophilia (117,118). In addition to primary smoke, secondhand smoke can also induce inflammation and pro-inflammatory cytokines such as IL-1β and TNF while inhibiting immune functions (119). Prenatal and childhood exposure to cigarette smoke is associated with decreased lung function in the offspring and increased risk of future COPD development (120-122).

Cigarette smoke activates macrophages which subsequently release ROS, proteases, cytokines and chemokines to promote inflammation and an immunological

response (57). Cigarette smoke activates TLR4, but alveolar macrophages from smokers and individuals with COPD displayed reduced TLR4 activity, indicated by reduced p38 MAPK phosphorylation, p44/42 activity, NF-κB activity and inflammatory cytokine release (123,124). Additionally, exposure to cigarette smoke reduces the response of alveolar macrophages to other TLR ligands such as lipopolysaccharide (LPS), and contributes to suppression of the immune system in smokers which affects their ability to mount a normal response to infection or injury (125).

Individuals with asthma and COPD have elevated ROS levels in their airways (126), however it is unclear if ROS drives the disease or is a consequence of it (71). Antioxidants have not been very successful in the treatment of asthma (127). Production of ROS has been linked to activation of NF-κB (128,129) and the IL-1β/IL-18 inflammasome (NLRP3). The inflammasome can also be activated by a pathway independent of TLR4/ROS production, possibly due to an increase in caspase-1 activity (130). Exposure to cigarette smoke has been shown to reduce levels of protein components that make up the NLRP3 inflammasome by increased proteasomal degradation, which impairs the cells' ability to develop an immune response to cigarette smoke or other infectious organisms or toxicants (131).

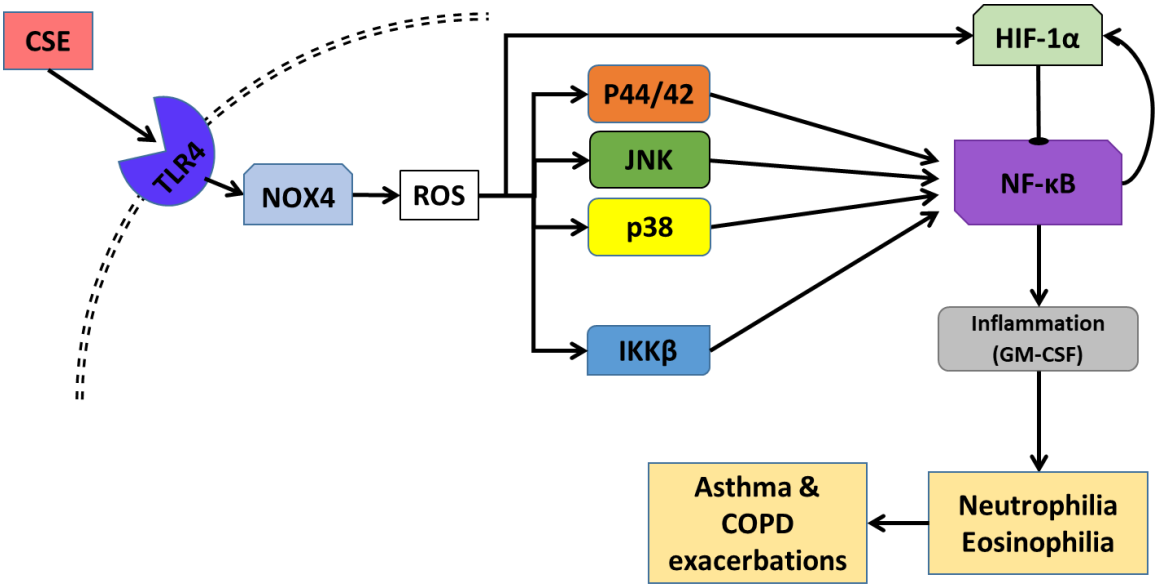

Figure 1: Cigarette smoke activates TLR4

MAP KINASES AND NF-κB IN AIRWAY DISEASE

There are currently five separate mitogen activated protein kinase (MAPK) pathways known: p44/42 (extracellular regulating kinase, ERK1/2), p38, c-Jun N-terminal kinase (JNK), ERK3 and ERK5. Of the three most characterized pathways, p44/42 is primarily activated by growth factors while p38 and JNK are activated by various stresses and inflammatory cytokines (132) (Figure 2). All three of these pathways are activated by cigarette smoke (48).

The kinases in the MAPK signaling cascades are activated by phosphorylation of key residues which in turn causes them to activate the downstream kinase by phosphorylation. For example: phosphorylated MEKK4 will phosphorylate MKK3 & MKK6, which will subsequently phosphorylate p38, and then p38 will phosphorylate its

targets. Numerous signals affect these pathways which integrate the inputs to determine the cellular response. There is considerable crosstalk between the MAPK pathways and with other pathways, which allows cells to produce varied responses despite using the same signal transduction pathways for different stimuli.

The NF-κB pathway is a major regulator of inflammation that can be activated by a variety of signals, including MAPKs, oxidative stress, and hypoxia. Activation induces the expression of numerous genes involved in survival, immunity, migration, and inflammation, including cytokines, chemokines and growth factors. In most cells, the NF-κB subunits are sequestered in the cytoplasm by being bound with the inhibitor of κB (IκB) which masks the nuclear translocation signal. The NF-κB pathway is activated by a wide variety of stimuli, which phosphorylate the inhibitor of κB kinase (IKK). IKK consists of three subunits IKKα, IKKβ, and IKKγ (or NF-κB essential modulator (NEMO)), and is activated by phosphorylation. Once activated, it phosphorylates IκB on Ser32 and Ser36 which results in the ubiquitination and proteasomal degradation of IκB (although there are alternative forms of activation which only cause dissociation from NF-κB without degradation). This reveals the nuclear localization signal, allowing NF-κB to dimerize and translocate to the nucleus to initiate gene transcription. NF-κB can form various heterodimers consisting of combinations of the p50, p65 (or RelA), p52, c-Rel and RelB subunits that target different genes, but the most common dimer is p50/p65 (133).

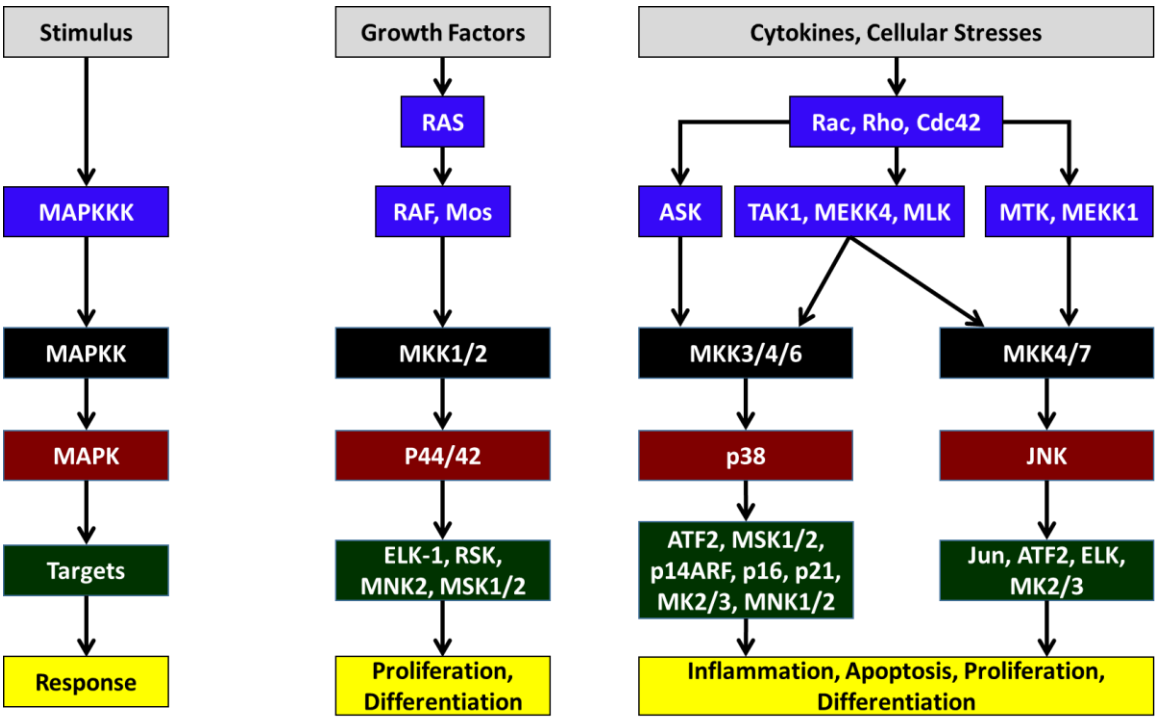

Figure 2: Mitogen Activated Protein Kinase (MAPK) Pathways The three most studied MAPK signaling cascades: p44/42, p38 and JNK with a subset of activators and targets.

Smokers with COPD have greater levels of p38 MAPK activation than non-smokers or smokers without COPD in alveolar macrophages and cells in the alveolar walls (134). There is also a correlation between increased p38 activation and increased recruitment of CD8+ T cells into the alveolar walls (134). In bronchial epithelial cells, stable COPD patients did not have elevated p38 activation, but experienced increased activation with exacerbations (135). This indicates that the cellular responses to cigarette smoke can be cell type specific. Epithelial cells from mild asthmatics also had elevated p38 phosphorylation (135).

Steroid Resistance

Corticosteroids are the primary treatment for asthma but they are not nearly as effective in COPD or for a subset of severe asthmatics (136,137). This steroid resistance may be partly due to activation of the p38 pathway. IL-8 release and p38 activation were greater in peripheral blood mononuclear cells (PBMC) of COPD patients compared to smokers without the disease (138). Corticosteroid treatment (dexamethasone) was less effective at reducing IL-8 release from COPD PBMC compared to non-COPD, but p38 inhibitors increased its effectiveness – in part by inhibiting p38-induced glucocorticoid receptor phosphorylation which blocks its activity (138). Use of a p38 inhibitor improved the effectiveness of dexamethasone in COPD alveolar macrophages (139).

Activation of p38 in asthmatics is associated with steroid resistance, and inhibition of p38 activity increased the effectiveness of corticosteroid treatment (140,141). This has also been connected to phosphorylation of the glucocorticoid receptor by p38 in steroid-resistant asthmatics (142). Activation of p38 inhibited the suppression of LPS-induced GM-CSF release by dexamethasone– a response that was reversed by p38 inhibition (142). Therefore, phosphorylation of the glucocorticoid receptor by p38 is associated with steroid resistance in both asthma and COPD.

Transcriptional Regulation: Acetylation

LPS-stimulation of alveolar macrophages increased p38 and p44/42 activation, however, while inhibition of MKK1 by PD098059 suppressed GM-CSF expression, p38 inhibition by SB203580 did not (143). GM-CSF production was NF-κB dependent which was dependent on histone acetyltransferase (HAT) activity (143). HATs acetylate lysine residues on histones and other proteins, including NF-κB, HIF-1α, p300/CBP, and TNF, which can affect binding to proteins, DNA and other molecules (144). Acetylation of histones loosens their binding to DNA, which makes DNA more accessible and up-regulates transcription, conversely histone deacetylases (HDAC) down-regulate gene expression by reducing acetylation. MKK1 inhibitor PD098059 suppressed GM-CSF production by inhibiting HAT activity without affecting binding of NF-κB to the DNA (143). This suggests that p44/42 induces acetylation. Acetylation of NF-κB can increase transcriptional activity while inhibiting ubiquitin-induced degradation of p65 by competing for the same modification sites on p65 (145). Inhibition of HDAC by Trichostatin A (TSA) resulted in an increase in LPS-induced GM-CSF release (143). Despite lacking a significant effect on LPS-induced GM-CSF production in alveolar macrophages, p38 inhibitor SB203580 augmented the TSA-induced increase in LPS-stimulated GM-CSF production (143). This suggests that increased GM-CSF expression due to hyperacetylation may be suppressed by p38 activity. However, p38 MAPK induces NF-κB gene expression by promoting HDAC degradation (146) and inhibition of HDAC resulted in decreased p38 phosphorylation (147,148). This suggests that HDAC activity induces p38 phosphorylation.

Cigarette smoke activates the EGF receptor, which dimerizes and autophosphorylates the intracellular domain (48). This triggers a Ras-Raf1-MKK1/2-p44/42 signaling cascade resulting in phospho-p44/42 translocating to the nucleus and activating transcription factors like AP-1, which induces genes such as GM-CSF (48). P44/42 has been shown to increase HAT activity by phosphorylating the p300 HAT which increased inflammatory cytokine production (149). JNK also induces p300/CBP (CREB binding protein) HAT activity (150). Therefore, all 3 MAPKs induce NF-κB transcriptional activity which produces GM-CSF: p38 by inhibiting HDACs and P44/42 & JNK by inducing HAT activity.

Cigarette smoke, and specifically acrolein, a component of cigarette smoke, inhibited HDAC activity, which induced the expression of inflammatory genes such as GM-CSF, and increased glucocorticoid resistance (151,152). Cigarette smoke-induced inflammation activated HATs which, combined with the cigarette smoke-induced oxidative stress that inhibited HDACs, increased NF-κB-induced cytokine transcription (152). Glucocorticoid activity requires the deacetylation of the glucocorticoid receptor (GR) by HDAC2 to remove the post-ligand binding acetylation that inhibits it, thereby allowing the GR to bind to NF-κB and suppress its activity (153). The GR recruits HDAC2 to suppress HAT activity and additionally it directly inhibits cAMP-response element (CREB) binding protein (CBP) which binds NF-κB and mediates transcription (154).

mRNA Stabilization

P44/42 is capable of stabilizing GM-CSF mRNA by a mechanism that involves binding of the Y Box binding protein (YB-1) to the 3'-UTR AU-rich elements (ARE) on the GM-CSF transcript (73). Tristetraprolin (TTP) binds to the 3'-UTR of cytokine mRNAs, such as GM-CSF, TNF & IL-10, and accelerates their degradation, thereby down-regulating inflammation (155). Activated p38 phosphorylates MAPKAP kinase 2 (MK2), which phosphorylates TTP, allowing 14-3-3 proteins to bind the GM-CSF mRNA (155,156). This up-regulates induction of pro-inflammatory mediators by increasing mRNA stability and efficiency of translation (155,156). TTP is subsequently dephosphorylated by protein phosphatase 2A (PP2A), thereby reactivating the enhanced mRNA degradation (157).

Pathway Interactions

Cigarette smoke induces activation of MAP kinases p38, p44/42, JNK and ERK5 and transcription factor AP-1 (158,159). Inhibition of the p38, p44/42 and JNK pathways all inhibit AP-1 transcription factor activity which is involved in survival, proliferation and differentiation (158,159). Cigarette smoke activates the p44/42 pathway in an EGF receptor-dependent manner (48). However, cigarette smoke induces activation of JNK through an EGF receptor-independent, Src-dependent pathway (160). Src activity is elevated in individuals with COPD and promotes the ongoing inflammation along with protease-driven tissue damage in this disease (161). Src is activated by cigarette smoke-induced protein kinase C (PKC) activity and subsequently phosphorylates the EGF receptor which initiates the MAPK, PI3K and STAT signaling cascades (161).

IL-1β and TNF stimulation of HASMC induces GM-CSF release via activation of JNK (162). The phosphorylation of JNK peaks at 15 mins and is reduced by JNK inhibitor, SP600125 (162). However, JNK has not been found to be very active in HASMC (163).

Glycogen synthase kinase-3 (GSK3β) is an unusual kinase in that it is constitutively active and phosphorylation on Ser9 inhibits its activity (164). GSK3β kinases include Akt/PKB (164), which is rapidly activated by cigarette smoke (165). When it is active, GSK3β binds and may phosphorylates MEKK4 which reduces its activity (Figure 3, top) (166). MEKK4 can induce MKK3/4/6 phosphorylation, which activates p38 and JNK (166). Inhibition of GSK3β will therefore result in an increase in MEKK4 activity and a subsequent increase in p38 and JNK activity (Figure 3, bottom). GSK3β also phosphorylates and inactivates eIF2B, which participates in eukaryotic translation initiation, thereby reducing translation (167). Inhibition of GSK3β, for example by Akt phosphorylation on Ser9, should therefore increase translation.

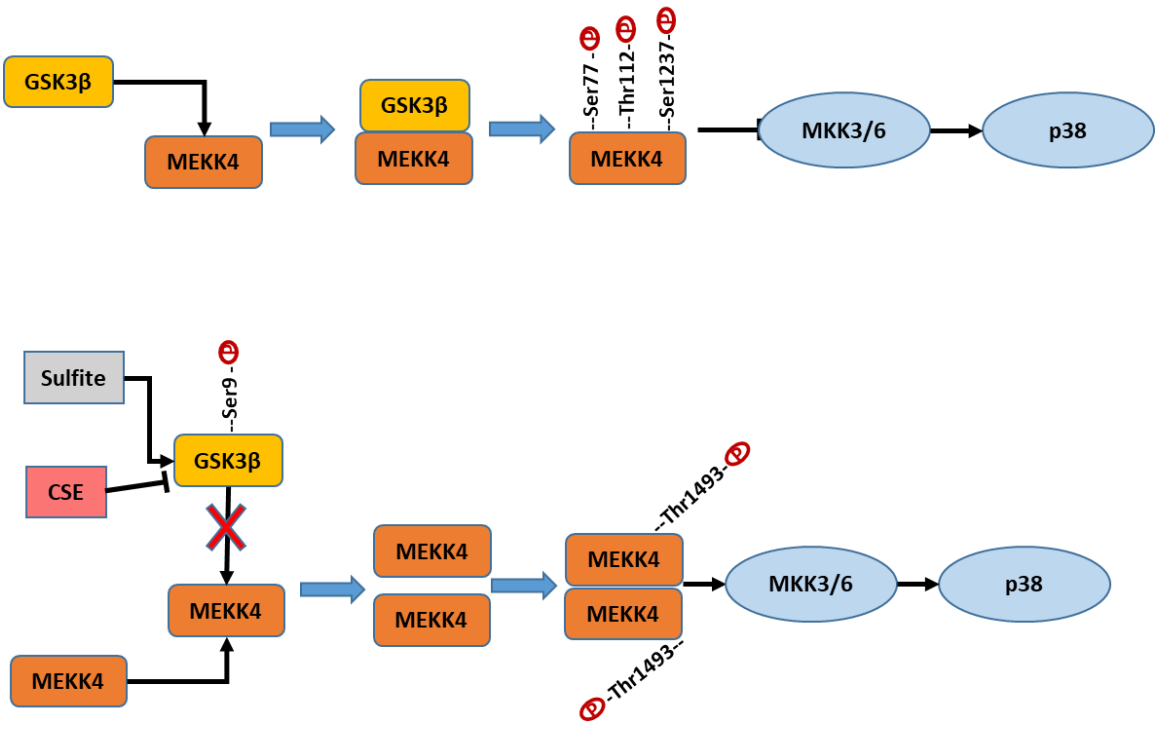

Figure 3: GSK3β regulates MEKK4 activation of p38 MAPK.

TGF-β activated kinase 1 (TAK1) is another MAPKKK that has been demonstrated to activate MKK3/4/6 and IKKβ (168,169). In unstimulated cells, TAK1 binds MEKK3 and both kinases are inactive (Figure 4, top). TAK-1 is activated by cigarette smoke, causing TAK-1 and MEKK3 to dissociate, re-dimerize with different enzymes and activate the MAPK and NF-κB pathways, which has been shown to induce pro-inflammatory mediators in HASMC (Figure 4, bottom) (170).

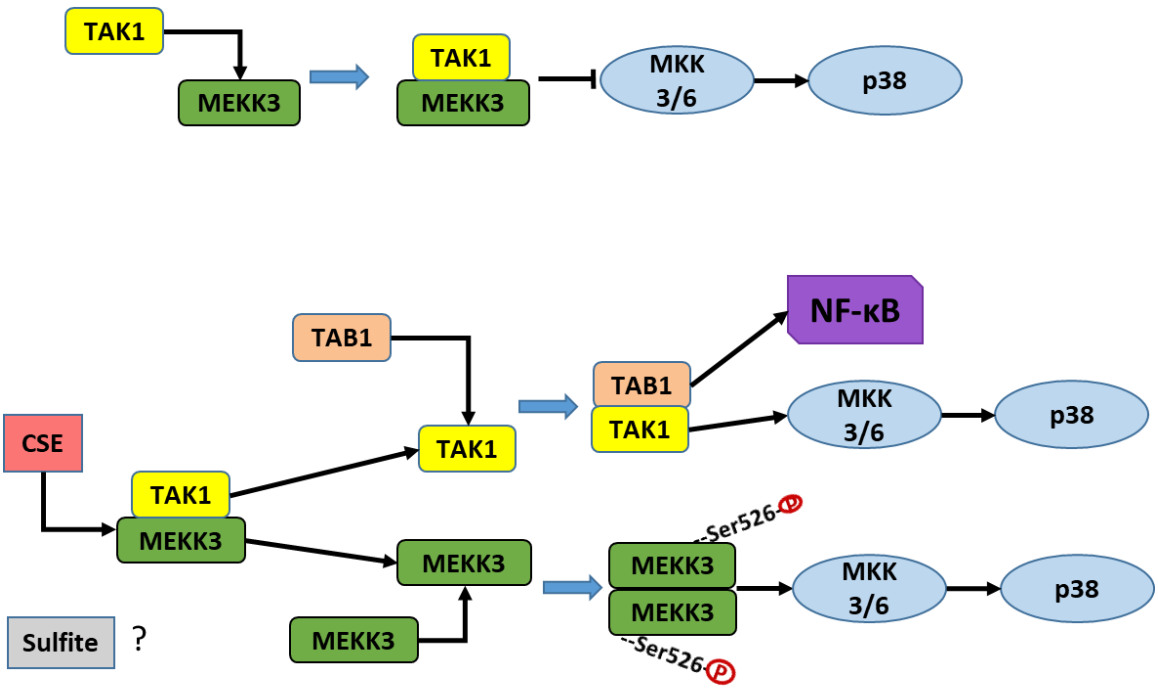

Figure 4: TAK-1 activates MAPK and NF-κB pathways

Cigarette smoke activates the aryl hydrocarbon receptor (AhR) which dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT, also known as HIF-1β), translocates to the nucleus and induces expression of genes including cytochrome p450 which detoxify the cigarette smoke (160). Cigarette smoke also induces phosphorylation of inhibitor of nuclear factor- kappa B (IκB), which causes it to release NF- κB allowing it to translocate to the nucleus and induce gene expression (171).

HIF-1 Pathway in Airway Disease

Hypoxia inducible factor (HIF) regulates the cellular response to low oxygen levels (hypoxia) and directs metabolic and vascular adaptations. In the classic pathway, when sufficient levels of oxygen are present, prolyl hydroxylases (PHDs) hydroxylate specific prolines on HIF-1α (P402 & P564), marking it for ubiquitination by the von Hippel Landau (pVHL) factor (Figure 5, top) (172,173). pVHL ubiquitination targets HIF-1α for subsequent degradation by the proteasome, thereby inhibiting HIF activity (174,175). During hypoxia, the oxygen co-factor required for hydroxylation is not available, which inhibits PHD activity and stabilizes HIF-1α levels (Figure 5, bottom). HIF-1α then dimerizes with HIF-1β, translocates to the nucleus and binds hypoxia response elements (HREs) in the DNA to induce transcription of genes that promote cell adaptation and survival (176). These genes include vascular endothelial growth factor (VEGF), which induces angiogenesis, and metabolic factors such as glucose transporter-1 (GLUT1) and glycolytic enzymes (177). Once it dimerizes, HIF-1α is less susceptible to degradation (178). On the other hand, HIF-1β, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT), has generally been considered to be constitutively active rather than inducible. However, some evidence indicates that there may be cell specific hypoxia-induced upregulation of HIF-1β (179). Factor inhibiting HIF (FIH) reduces HIF-1α transcription factor activity in hypoxia by hydroxylating asparagine 803, which interferes with HIF-1α binding to its co-activator CBP/p300 (180,181).

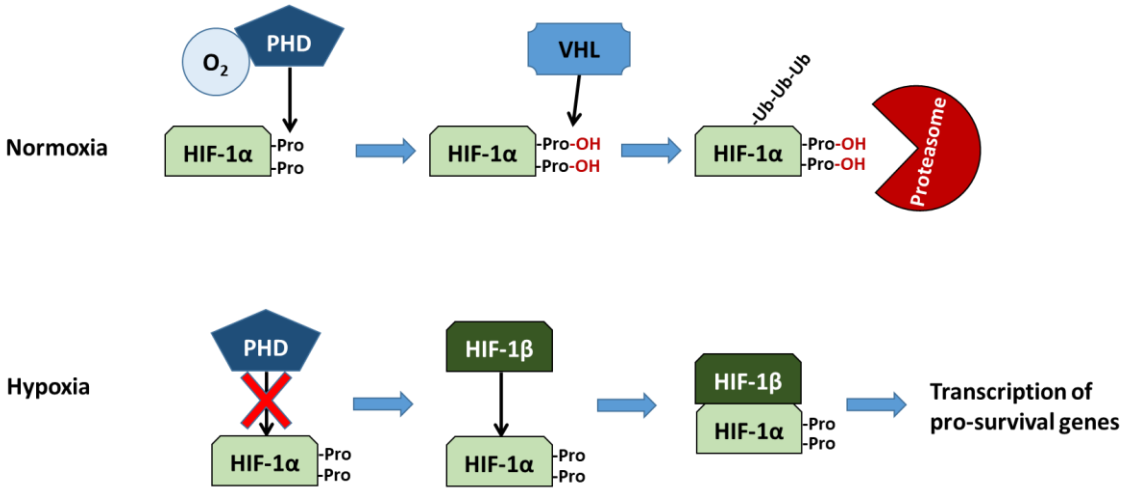

Figure 5: Canonical HIF-1α Pathway

Hypoxia rapidly induces HIF-1α activity and prolonged hypoxia induces NF-κB and CREB transcription factors (182). Constitutive NF-κB activity is needed for hypoxia-induced HIF-1α accumulation (183). During hypoxia, inhibition of PHDs include those that suppress IKKβ activity, resulting in increased NF-κB activity (183). Hypoxia-induced HIF-1α activation induces NF-κB activity to promote cell survival and inhibition of NF-κB down-regulates survival (184).

Chronic smoking can create systemic hypoxia both in the smoker and fetus (185,186), however HIF can also be induced by inflammation and infection (187). The oxygen-independent HIF-1α induction occurs in myeloid cells via TLR activation which causes IKK phosphorylation, followed by NF-κB-driven HIF-1α transcription and translation (Figure 6) (187). In T cells, antigen stimulation results in PI3K/Akt/mTOR activation which induces HIF-1α transcription and translation (187). Therefore smoking may result in HIF-1α induction via multiple pathways. Deletion of HIF-1α impairs the

inflammatory response, possibly due to metabolic changes, indicating a role for HIF-1α in regulation of inflammation (187). Once HIF-1α levels accumulate, HIF-1α inhibits NF-B activity (Figure 6) (188).

Individuals with severe COPD have reduced HIF-1α levels in their airways, despite the hypoxia and inflammation produced by their condition (189). Cigarette smoke induced HIF-1α mRNA expression, which activated expression of vascular endothelial growth factor (VEGF) in human airway epithelial cells (190). Two of the major components of cigarette smoke extract (CSE), nicotine and the aldehyde acrolein, stabilize HIF-1α, indicating that more than one constituent is involved (190). VEGF is involved in vascular remodeling and induces the formation of new blood vessels in low oxygen tissues. CSE-generated ROS, PI3K, p44/42 MAPK, and NF-κB activation are required for HIF-1α stabilization (190). GSK3β phosphorylates HIF-1α inducing its ubiquitination by pVHL and consequent degradation (191). Therefore, CSE-induced GSK3β inhibition should stabilize HIF-1α. P38 activity is also needed for HIF-1α stabilization and activity (192,193).

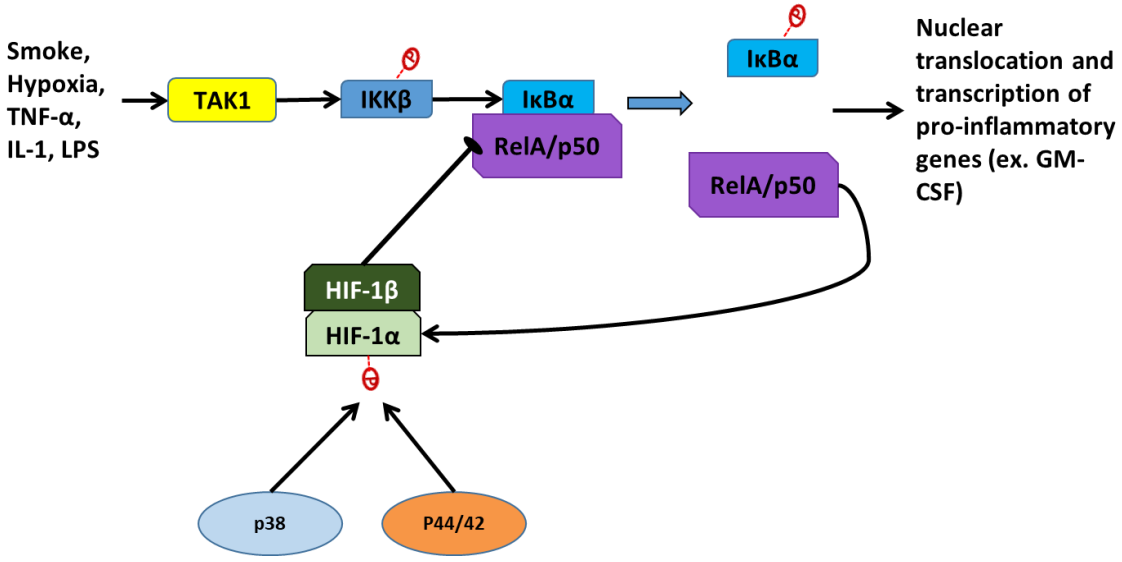

Figure 6: Crosstalk between HIF-1α and NF-κB pathways.

GASOTRANSMITTERS

Cellular metabolism produces a number of gases, including nitric oxide, carbon monoxide, hydrogen sulfide and sulfur dioxide. Originally these gases were considered toxic waste products without any positive effects on biological activity. With the discovery of the relaxation-inducing effects of endothelium-derived nitric oxide on vascular smooth muscle, there was a growing interest in the biological effects of these "waste product" gases (194). In general, they tend to be toxic at high concentrations but can have cytoprotective effects at physiological levels. Gasotransmitters are defined as: 1) small molecule gases; 2) able to freely pass through cell membranes; 3) their biological effects are not mediated by binding to a specific cell membrane receptor; 4) they are synthesized enzymatically by cells when needed and this production is regulated; 5) they have specific functions at physiological concentration which are well-characterized; and 6) they have specific molecular targets which may or may not include

second messengers (195). The three most studied gasotransmitters are nitric oxide, carbon monoxide and hydrogen sulfide. All three interact with metals and hemes in other proteins and with each other (196).

Nitric oxide was the first gasotransmitter discovered. It is produced endogenously from arginine by nitric oxide synthase (NOS), is a radical and therefore highly reactive, and has a half-life of seconds (197). It causes vasorelaxation by stimulating the production of cyclic guanosine monophosphate (cGMP) through a reaction with iron in guanylyl cyclase which relaxes the vascular smooth muscle (198). This results in blood vessel dilation that lowers blood pressure and increases blood flow, which can speed up the delivery of immune cells to sites of injury. Nitric oxide is used by the immune response in additional ways. After stimulation with endotoxins, inducible NOS (iNOS) is up-regulated in activated macrophages. The resulting NO is released by the cells to eliminate pathogens such as bacteria, fungus and helminths (199). Nitric oxide relaxes airway smooth muscle and has been shown to be therapeutic for some asthmatics (200). NO levels are elevated in the breath of individuals with chronic inflammatory conditions and there are currently efforts to develop clinical tests using NO levels for patient evaluation (201). However, NO can simultaneously produce both harmful and beneficial effects which has made its development as a therapeutic somewhat difficult (202).

Carbon monoxide, the second gasotransmitter discovered, is produced endogenously as a result of the metabolism of heme by the heme oxygenases (HO) during the degradation of old red blood cells (203). It is considered virtually chemically inactive and is eliminated from the body by exhalation (197). Individuals with chronic inflammatory diseases have elevated levels of CO in their exhaled breath, indicating an

up-regulation of HO activity (204). Like NO, CO induces vascular smooth muscle relaxation and vasodilation by stimulating cGMP production through an interaction with the iron in guanylyl cyclase (205). However, nitric oxide binds to sGC with greater affinity and can competitively inhibit CO binding (206). Carbon monoxide also causes vasodilation by activating the plasma membrane calcium-activated K^+ channels (207). CO reduces pro-inflammatory cytokine release (IL-1β & TNF) while increasing anti-inflammatory IL-10 (208). Additionally, CO causes airway smooth muscle relaxation and inhibits the cytokine-induced GM-CSF release in HASMC (209).

Hydrogen sulfide was the third molecule to be designated a gasotransmitter (210). It is produced endogenously from cysteine by cystathionine-γ-lyase (in the cardiovascular system, serum, macrophages) and cystathionine-β-synthase (in the brain) and has a short half-life (197). Hydrogen sulfide can relax smooth muscle and induce vasodilation by inhibiting cGMP phosphodiesterase, rather than interacting with the iron in guanylyl cyclase like NO and CO (211). Another mechanism it uses to induce smooth muscle relaxation and vasodilation is activation of ATP-sensitive potassium (K_{ATP}) channels (212). Hydrogen sulfide appears to work in concert with nitric oxide to produce smooth muscle relaxation and vasodilation (213). Inhalation of hydrogen sulfide induced a reversible, hibernation-like state in mice that included reduced metabolism and body core temperature (214). This may be partly accomplished by the inhibition of mitochondrial cytochrome c oxidase by higher concentrations of hydrogen sulfide (215). However, at physiological concentrations, it has also been found to inhibit inflammation and promote proliferation and angiogenesis of endothelial cells (216). It modifies numerous proteins with S-sulfhydration (R-S-SH) (211). Hydrogen sulfide is detoxified by being oxidized

to sulfite and then metabolized to sulfate which is eliminated from the body in urine (211).

Since these small gases can directly modify proteins by chemical interactions and readily diffuse through membranes, rather than binding to cell surface receptors, regulation most likely occurs by producing them on demand in the vicinity of their target molecule (197). For example, NO is regulated by the binding of NOS to a target molecule or a scaffold protein like carboxy-terminal PDZ ligand of neuronal NO synthase protein (CAPON) that directs it to the target (197).

SULFUR DIOXIDE

Sulfur dioxide is produced by industrial processes, fossil fuel combustion, volcanic activity and wildfires (15). The main contributor to atmospheric sulfur dioxide is fossil fuel combustion by electrical utilities and industry, however local elevations may occur due to additional sources such as shipping transport and port activities (15). Sulfur dioxide and its derivatives, bisulfite (HSO_3^-) and sulfite (SO_3^{2-}), are also used in wine making, food preservation, paper manufacturing and for a number of other purposes which increase potential exposure to the public through both inhalation and ingestion. While inhalation is a more efficient method of absorption, at current atmospheric sulfur dioxide levels most individuals ingest much more sulfur dioxide/sulfites than they inhale (217). Ingested sulfur dioxide/sulfite has a lower absorption rate than inhaled, which may be due to oxidation of the sulfites in the gut (217). This makes both absorption routes potential contributors to sulfur dioxide toxicology.

Sulfur dioxide is also produced endogenously from L-cysteine by aspartate aminotransferase (AAT) in the same sulfur metabolism pathway as hydrogen sulfide (Figure 7) (216). Inhibition of endogenous hydrogen sulfide producing enzymes results in an induction of sulfur dioxide production, while overexpression of hydrogen sulfide produced inhibitory sulfhydration of AAT and subsequent sulfur dioxide suppression (216). Conversely, in pulmonary hypertension, which has depressed levels of sulfur dioxide and hydrogen sulfide, a sulfur dioxide donor increases not only cellular sulfur dioxide levels but also hydrogen sulfide levels (218). This suggests that a cellular homeostasis exists between hydrogen sulfide and sulfur dioxide levels. However, while they exhibit many of the same physiological effects, the actual mechanisms are not identical (216). Sulfur dioxide has been shown to inhibit vascular smooth muscle cell proliferation by suppressing p44/42 activation (219), however we are unaware of any studies investigating the effect of sulfur dioxide/sulfite on the p38 and JNK pathways.

Humans can first detect the sulfur dioxide odor at 1-5 ppm and most individuals start experiencing discomfort above this range. Due to its high water-solubility, most sulfur dioxide is typically absorbed in the nasal cavity when an individual is at rest, and transported directly to the bloodstream, although a portion of it can be lost due to desorption (220). The increased airflow rate during exercise transports an increased amount of sulfur dioxide further into the airway, allowing it to have a greater effect on the respiratory system, therefore active individuals such as children and athletes have an elevated risk from exposure (221,222). Transport of sulfur dioxide further into the respiratory tract can also occur when it is combined with a carrier aerosol or particulates (223).

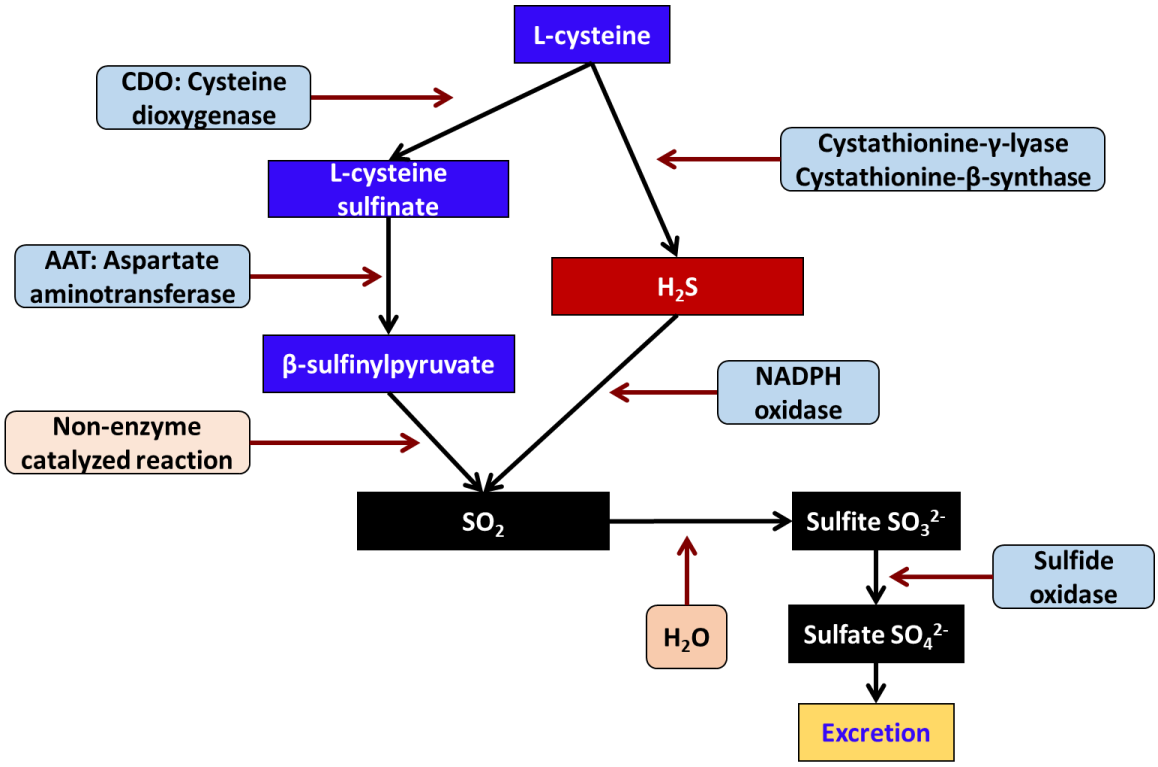

Figure 7: Sulfur dioxide (SO_2) and hydrogen sulfide (H_2S) metabolism pathway. (Simplified version of the pathway.) L-cysteine is converted to L-cysteine sulfonate by CDO and then AAT converts it to β-sulfinylpyruvate after which it undergoes non-enzymatic reactions to form sulfur dioxide and sulfite. Sulfite is metabolized into sulfate by sulfide oxidase and then the sulfate is excreted in the urine. Hydrogen sulfide is converted to sulfur dioxide by NADPH and then to sulfite demonstrating how the two gases can produce similar cellular effects.

Sulfur dioxide exposure triggers bronchoconstriction, inflammation and excess mucous secretion in asthmatic individuals at low levels that do not affect normal individuals (15,224). Additional symptoms include irritation of the eyes, nose, and throat; cough; and shallower, faster respiration. Normal resting individuals only display respiratory effects after sulfur dioxide exposure at higher concentrations (≥ 5 ppm) (225-227). However exercise or mouth breathing, which avoids absorption in the nasal cavity, produce effects as low as 1 ppm in normal individuals (228,229). Both sulfur dioxide and sodium sulfite induce bronchoconstriction in asthmatics unrelated to a change in pH (230).

Sulfur dioxide exposure reduces lung function (34) and produces a greater effect on cardiac events (35) in cigarette smokers compared to non-smokers. Also, elevated pollution levels, especially sulfur dioxide, correlate with a reduction in lung functions that is greater in smokers than non-smokers (36). Together this suggests that active smokers, who most likely have prolonged inflammation, suffer negative effects in excess of those experienced by non-smokers when exposed to sulfur dioxide. Other groups that may have an exacerbated response to sulfur dioxide are older adults and individuals with sensitivities to sulfur dioxide (231).

Sulfur dioxide in aqueous solution at a physiological pH rapidly hydrates to sulfurous acid and then dissociates to approximately equal amounts of bisulfite (HSO_3^-) and sulfite (SO_3^{2-}) with a small amount of sulfur dioxide remaining (217). Bisulfite is oxidized into sulfate by sulfite oxidase in the mitochondria and then excreted in the urine (232). Oxidation by sulfite oxidase is the body's main defense against bisulfite toxicity but these reactions produce sulfur radicals at low doses, which can have potentially

harmful effects (217). Rats, mice and monkeys exposed to sodium sulfite and sodium bisulfite eliminate 70-95% of the sulfite within 24 hours with much of the remainder eventually eliminated in the feces (233).

In vivo sulfur dioxide inhalation or *in vitro* sodium bisulfite ($NaHSO_3$) exposure in rats causes abnormal cardiac and mitochondrial function, including inhibition of cytochrome c oxidase activity and reduced ATP levels (234). Combined exposure to small particulates ($PM_{2.5}$) and sulfur dioxide induce IL-6 and TNF and down-regulate TGFβ mRNA and protein levels in rat brain (235). In the rat lung, sulfur dioxide combined with small particulates ($PM_{2.5}$) induce activation of TLR4, p38 and NF-κB; increase nitric oxide (NO); induce expression of IKKβ; and reduce inhibitor of κB (IκBα) (236) .

Sulfur dioxide can have detrimental effects on the human cardiac system, especially when combined with small particulates that carry the sulfur dioxide further into the respiratory system (237). However, it can also have beneficial effects, such as vasorelaxation and suppression of inflammation, when released endogenously or administered at low levels (28).

Inhaled sulfur dioxide causes systemic oxidative damage in mice: it suppresses the antioxidant activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT); lowers glutathione levels (GSH); and increases thiobarbituric acid-reactive substances (TBARS), an indicator of lipid peroxidation (32). However, in the lungs, low sulfur dioxide levels increase SOD and GPx activity while high levels suppress them (238). Interestingly, while exposures equivalent to 5 and 10 ppm result in significant increases in production of TNF-α and IL-6 in the lungs and serum, 20 ppm do

not (239). This suggests that the body reacts to low sulfur dioxide levels by activating inflammatory and antioxidant systems which are subsequently overwhelmed at high sulfur dioxide levels.

Sulfur dioxide has been shown to inhibit serum and PDGF-stimulated proliferation of vascular smooth muscle by activating PKA which phosphorylates Raf-1, suppressing its activation of p44/42 (219,240).

Bisulfite reacts with a large number of molecules. Prolonged sulfur dioxide inhalation (up to 120 hours at 6 ppm maximum concentration) increased protein S-sulfonate ($R\text{-}S\text{-}SO_3^-$) modifications in human plasma (241). Some of these reactions include breaking disulfide bonds in proteins such as cystine and albumin, producing one S-sulfonated protein and one thiolated protein (242). It is unclear if the S-sulfonate modifications produce a biological effect or if, as previously proposed, they are simply a method of storing sulfur dioxide in the body (217), however the reduction of oxidized enzymes could affect enzymatic activity.

Sodium sulfite has been investigated as a potential hypoxia inducer for *Caenorhabditis elegans* (*C. elegans*) research (243), indicating that HIF-1α may be involved in its mechanism of action. Since cigarette smoke also induces HIF activity, we hypothesized that the combined exposure of HASMC to cigarette smoke and sulfur dioxide/sulfite would induce higher levels of the pro-inflammatory cytokine GM-CSF than either component alone, indicating an increase in toxicity, and the induction of inflammatory mediators would be regulated by HIF-1α. Surprisingly, we found that lower levels of sulfur dioxide (5 ppm) and sulfite (200 μM) reduced cigarette smoke-induced GM-CSF release from HASMC.

Chapter 2 Materials and Methods

AIRWAY SMOOTH MUSCLE CELL ISOLATION AND CULTURE

Materials

Cellgro DMEM/F12 media (#10-090-CV or #16-405-CV for ROS measurements), Cellgro DPBS (#21-031-CV) and Cellgro 1M HEPES (#25-060-CI) were from Corning. Hyclone fetal bovine serum (#SH30396.03) was from GE Life Sciences. Penicillin (10,000 U/mL) streptomycin (10,000 µg/mL) mixture (#15140-122) and 0.25% Trypsin-EDTA (1X) (#25200-056) were from Gibco Life Technologies. Sodium sulfite (#S4672) and lipopolysaccharide (LPS, #L4391) were purchased from Sigma. IL-1β (#201-LB) was from R&D Systems. TNF was the kind gift of Dr. Bing Tian.

Table 1: Pathway inhibitors used in cell cultures.

Reagent	Description	Manufacturer	Catalog #	Solvent
NAC-ME	N-Acetyl-L-Cysteine Methyl Ester ROS scavenger	Sigma Aldrich	01042	DMEM/F12 Media without FBS
BMS-345541	NF-κB inhibitor	Sigma Aldrich	B9935	DMSO
SB203580	p38 inhibitor	AdipoGen	SYN1074M010	DMSO
U0126	ERK1/2 inhibitor	Cell Signaling	9903S	DMSO
LL-Z1640-2	TAK1 inhibitor	Enzo Life Sciences	ALX-380-267-M001	DMSO
TAT-cyclo-CLLFVY	HIF-1α dimerization inhibitor	Tocris	5582	DMEM/F12 Media, no FBS
TC-S 7009	HIF-2α dimerization inhibitor	Tocris	5243	DMSO
1,4-DCPA	Prolyl 4-hydroxylase inhibitor (HIF stabilizer)	Cayman	71220	DMSO
DMOG	Dimethyloxallyl Glycine (competitive inhibitor of HIF-PH)	Cayman	71210	DMSO

Methods

De-identified primary HASMC were obtained from multiple sources: The Mayo Clinic (Rochester, MN, USA) collected HASMC using methods (244) approved by the Mayo Foundation institutional review board. Briefly, lung tissues were obtained from surgical samples and the 3rd to 6th generation bronchi were isolated. The epithelium was removed and the tissue underwent enzymatic digestion. HASMC were collected and the cell type was confirmed by immunohistochemistry, RT-PCR for α-smooth actin, and immunostaining for sm22.

Primary HASMC were obtained at the University of Manitoba (Winnipeg, Manitoba, CAN) using methods approved by the Human Research Ethics Committee (245). Briefly, tissue samples were obtained from healthy 2nd to 4th generation bronchus in adenocarcinoma patients undergoing surgical resection and smooth muscle cells were isolated. The cell type was verified by the characteristic hill-and-valley growth pattern and staining for α–actin-2 and calponin.

The University of Pennsylvania Institutional Review Board (Philadelphia, PA, USA) approved acquisition of de-identified lung tissue from the National Disease Resource Interchange (NDRI), where it was isolated from deceased donors, and primary HASMC were isolated for use. Briefly, the tissue was dissected, enzymatically digested and the cells were collected by centrifugation. HASMC were resuspended in HAM's F12 media, grown until apparent confluence, and cell type was confirmed by staining for markers specific to smooth muscle (246).

HASMC were cultured in DMEM/F12 media supplemented with fetal bovine serum (10%), HEPES (10 mM) and penicillin/streptomycin (100 U/µg per mL) and maintained in a humidified atmosphere with 5% CO_2 at 37˚C. Cells were passaged at 70-90% confluency and utilized at passages 5-7. Experiments were conducted utilizing DMEM/F12 media with 10% FBS, except for ROS measurements which were performed using serum-free media due to serum-induced ROS generation.

Table 2: Plate seeding densities for experiments.

Plate Type	100 mm	6 well	12 well	24 well	96 well
Seeded with (# of cells/well)	600,000	100,000	40,000	20,000	3370

CIGARETTE SMOKE

HASMC cultures were initially exposed directly to cigarette smoke (results shown in Appendix). The smoke was generated by a single University of Kentucky reference cigarette (#3R4F) run in a Jaeger-Baumgartner cigarette smoking machine. It was pumped to a glass exposure chamber containing the lidless cell cultures by a Masterflex Precision peristaltic pump (model # 7520-40) at 2.6 L/minute before being exhausted into a fume hood (Figure 8). The Jaeger-Baumgartner cigarette smoking machine has 30 cigarette ports, automatic cigarette lighting and various programs to specify the smoking protocol: number of cigarettes, number of puffs per cigarette, puff length, puff intervals, etc. Half of a cigarette was smoked using the Smoke and Pause program (4 puffs, 1 puff/minute) and a brief incubation period followed during which the peristaltic pump

was turned off to keep the smoke in the exposure chamber. The exposure, from the first cigarette puff at Time 0, lasted a total of 7 minutes and then the peristaltic pump was turned on (without smoke) to purge the smoke from the exposure chamber. Following the smoke exposure, the cultures were exposed to either 30 minutes of SO_2 or air flow and then incubated at 37˚C, 5% CO_2 in a humid atmosphere until collection (Figure 9).

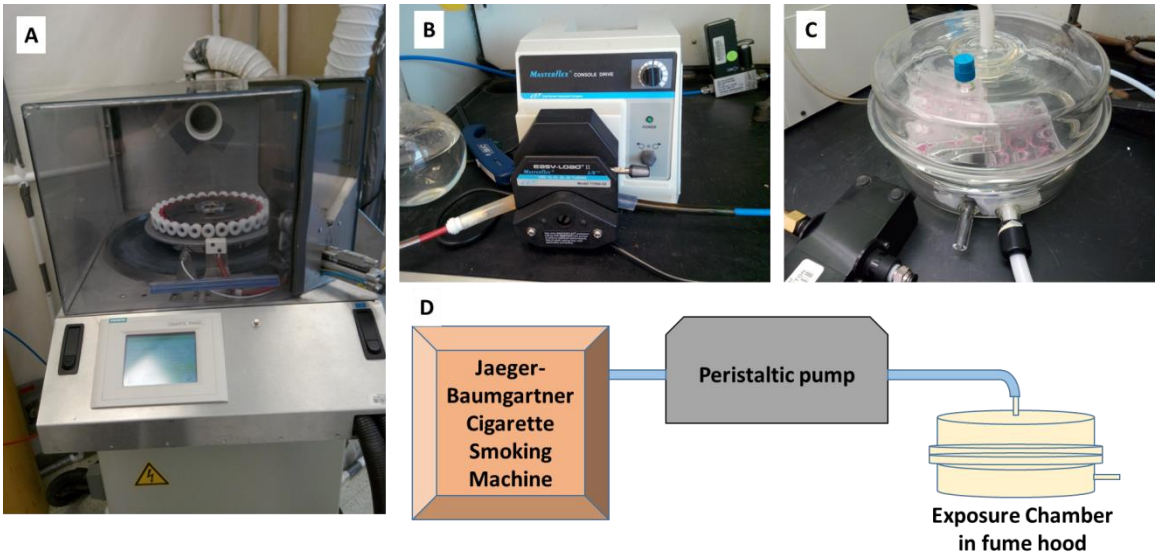

Figure 8: Direct cigarette smoke exposure schematic. **A**, Jaeger-Baumgartner cigarette smoking machine with 30 ports and automatic cigarette lighting; **B**, Masterflex Precision peristaltic pump; **C**, enclosed glass smoke exposure chamber containing cell cultures in fume hood; **D**, connection diagram.

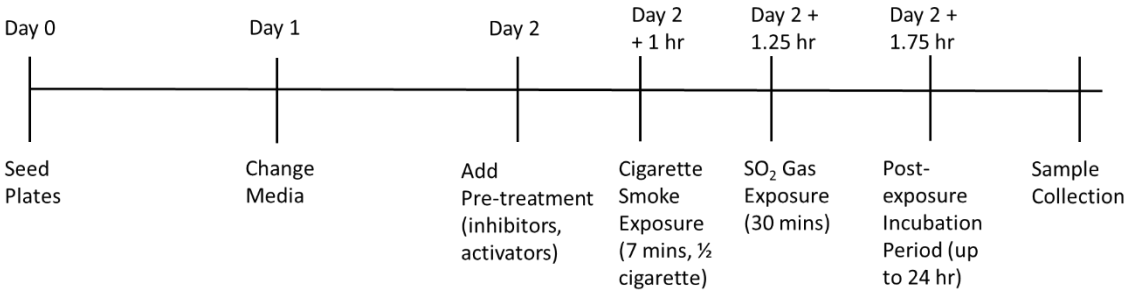

Figure 9: Direct cigarette smoke exposure protocol.

The results from the direct cigarette smoke exposures were inconsistent. This led us to investigate the repeatability of the smoking machine output. Particulate levels were measured using an EMMS Total Particulate Matter Transducer (TPM 100) in-line with the smoke flow. Ten cigarettes were individually tested. Each cigarette was smoked for 8 puffs (the entire cigarette at 2.6 L/min) using the Smoke and Pause program (1 puff/minute) and the peak particulate matter reading was recorded. This experiment revealed a wide range of output, which varied from cigarette to cigarette, and puff to puff. The issue was caused by the reliability of the automatic lighting. The cigarette did not always start burning and sometimes it started burning poorly which resulted in reduced smoke. This was at least partly traced to variability in the distance between the end of the cigarette and the lighting mechanism. Although the reliability of the cigarette lighting was greatly improved by replacing some parts, we decided to investigate the use of cigarette smoke extract to further reduce variability and create a more representative exposure.

CIGARETTE SMOKE EXTRACT

HASMC in a human respiratory system are not exposed directly to inhaled gases. They are buried under an epithelial layer and basement membrane. Therefore, HASMC were exposed to cigarette smoke extract (CSE). CSE was prepared by bubbling smoke from 50 University of Kentucky reference cigarettes (#3R4F) through 500 mL of DMEM/F12 media via a fritted glass tube with 170-220 µm pores (Figure 10). The media contained 10mM HEPES and Penicillin/Streptomycin, but no serum. The smoke was generated by a Jaeger-Baumgartner cigarette smoking machine which has 30 cigarette ports and automatic cigarette loading and lighting. The cigarettes were smoked in two runs, 25 cigarettes per run, using the Smoke and Pause program (8 puffs/cigarette, 1 puff/cigarette/minute). The smoke was pumped from the smoking machine to the fritted glass tube at 2.6 L/min by a Masterflex Precision peristaltic pump (model # 7520-40) and exhausted into a fume hood. The resulting mixture was 100% CSE. The media was thoroughly mixed, divided into 1.5 mL microcentrifuge tubes (1 mL per tube) and frozen at -80°C. CSE was thawed prior to use, the contents of all the vials were combined and mixed thoroughly and any unused portion was discarded.

The initial attempt to produce CSE using the cigarette-smoking machine used a flow rate of 1 L/min instead of 2.6 L/min. However, this flow rate proved to be too low to effectively deliver the smoke to the exposure chamber or fritted glass tube and the pump rate was returned to the 2.6 L/min used in the direct smoke exposures.

Concentrations of 2, 5, 10, 50 and 100% CSE by volume were initially tested before 10% CSE was chosen for further experiments. Serum-free DMEM/F12 media was administered to controls (0% CSE). The 50 and 100% CSE exposures resulted in

massive cell death, essentially eliminating the entire culture; therefore no additional experiments were performed at these levels. There were no visual differences in the cultures exposed to 0, 2, 5 or 10% CSE. Cell viability was assayed using trypan blue exclusion at 8 and 24 hr post-exposure. There were no statistically significant differences between 0 and 10% CSE exposures and the viability never dropped below 90% (Figure 19).

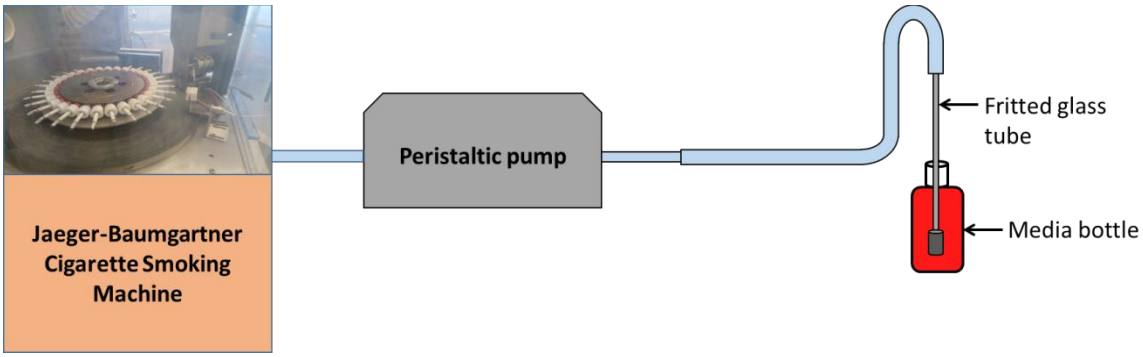

Figure 10: Production of cigarette smoke extract. Smoke was generated by a Jaeger-Baumgartner Cigarette Smoking Machine and pumped to a fritted glass tube submerged in DMEM/F12 media by a peristaltic pump.

SULFUR DIOXIDE

Cell cultures with the culture plate lids removed were placed in a glass exposure chamber. A maximum of two 6 to 96 well culture plates could be placed in the exposure chamber simultaneously. The sealed exposure chamber was placed in an incubator at 37°C for the exposure. Two gas cylinders, one containing 500 ppm SO_2 and the other containing air + 5% CO_2, were connected to mass flow valves to control the SO_2

concentration. The gases were then mixed prior to entering the exposure chamber within the incubator. The gas flowed through the exposure chamber at 855 mL/min and was exhausted into a fume hood (Figure 11). Control cultures were exposed to 855 mL/min air + 5% CO_2 gas flow in a glass exposure chamber. Since direct cigarette smoke exposure required approximately 15 minutes to complete and start the SO_2 exposure, for consistency, a 15 minute incubation with CSE was used prior to SO_2 exposure (Figure 12).

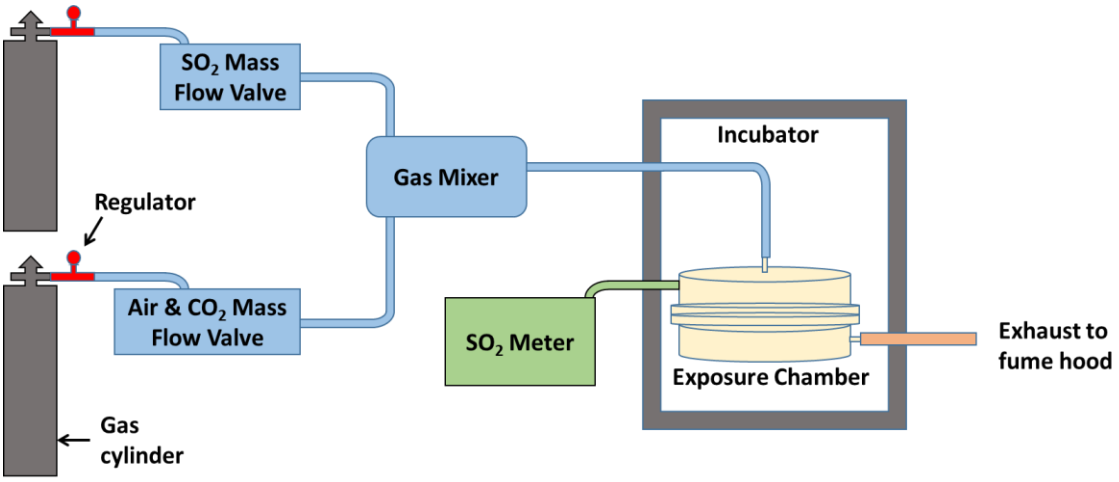

Figure 11: SO_2 exposure schematic. Two compressed gas cylinders, a 500 ppm SO_2 tank and air + 5% CO_2 tank, were connected to mass flow valves to control the flow rate and SO_2 concentration. The gases were combined in a mixer and the mixture flowed through a glass exposure chamber in an incubator and exhausted to a fume hood. The SO_2 concentration was measured using a real-time SO_2 meter.

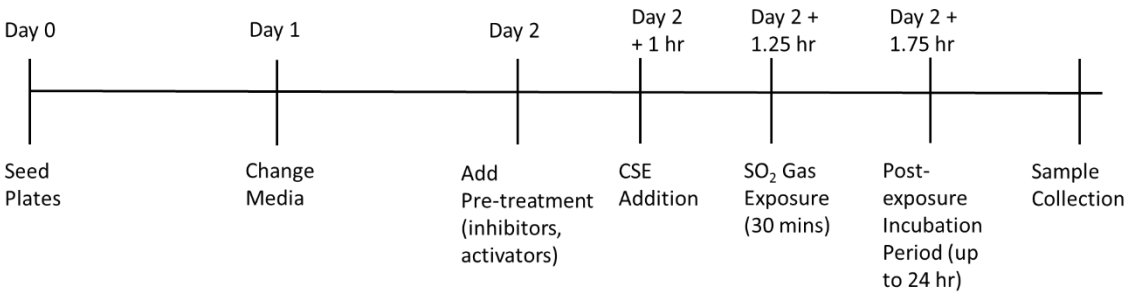

Figure 12: CSE & SO_2 exposure protocol. Pre-treatments occurred 1 hr prior to CSE addition. CSE was added 15 minutes prior to the start of the 30 minute SO_2 exposure. The end of the SO_2 exposure was the start of the post-exposure incubation period.

The cell cultures were exposed to 1, 2.5 and 5 ppm SO_2 gas flow for 30 minutes. Since the air in the glass exposure chamber would initially have 0 ppm SO_2, there would be a time lag before the target concentration was reached. The SO_2 mass flow valve settings were determined using empty culture plates in the exposure chamber and the SO_2 concentrations were monitored using a real time SO_2 meter (Environmental Sensor Co, Model # Z1300XP). To minimize the time lag, the system was primed before use by flowing the gas at steady state settings until the target ppm was reached in the empty exposure chamber. The gas was then shut off and the exposure chamber was removed from the incubator and opened to add the culture plates, thereby returning the chamber to 0 ppm. Once the exposure chamber was reconnected to the gas lines, a higher initial SO_2 mass flow value was used for the first four minutes so that the target ppm was reached in the exposure chamber within 5-6 minutes of the start of gas flow. The SO_2 mass flow valve setting was reduced to the value that produced the target ppm at steady state conditions at the 4 minute mark. A 10 mL/min SO_2 mass flow valve was used to obtain the empirical values shown (Table 3).

Table 3: Gas flow parameters for 30 min SO_2 exposures

Target Exposure Level	Air Flow (mL/min or SCCM)	SO_2 Mass Flow Valve setting – steady state to reach target exposure level (mL/min)	Higher Initial SO_2 setting – first 4 mins (mL/min)
1 ppm	850	1.15	2.15
2.5 ppm	850	2.65	4.65
5 ppm	850	5.40	8.40

The effects of SO_2 on cell viability were assayed using trypan blue exclusion. Cell viability never dropped below 90% (Figure 19). The only statistically significant differences were between 1 & 5 ppm at 8 hr and 0 & 5 ppm SO_2 at 24 hr. However, the differences were only 2-3% viability therefore they are unlikely to be biologically significant.

SODIUM SULFITE

SO_2 is very soluble in water. At physiological pH levels it quickly hydrolyzes into nearly equal concentrations of sulfite (SO_3^{2-}) and bisulfite (HSO_3^-) (217). We hypothesized that the effects of SO_2 were due to the actions of its products, sulfite and bisulfite and tested this hypothesis by treating HASMC with sodium sulfite (Na_2SO_3).

The first step was to determine the sulfite concentration that would be equivalent to the 30 minute 1, 2.5 and 5 ppm SO_2 exposures. Since SO_2 is absorbed by water, the readings on the SO_2 meter should drop by the amount of SO_2 absorbed. The SO_2 levels in the glass exposure chamber were recorded for the entire 30 minute exposure. Experiments using empty culture plates (blue line), PBS in two culture plates (black line),

DMEM/F12 media in a single culture plate (orange line) and DMEM/F12 media in two culture plates (red line) were performed. There was no difference in the SO_2 absorption in the PBS and DMEM/F12 experiments using two culture plates, indicating that the absorption was based on the amount of aqueous solution, not its specific composition. The SO_2 reading decreased 30-40% when media was added to a single culture plate and 60% when media was added to two culture plates compared to the empty plates. Approximately 3 ppm SO_2 was absorbed from the 5 ppm exposures with two filled culture plates and 1.4 & 0.6 ppm from the 2.5 and 1 ppm exposures respectively (Figure 13).

A 30 minute 5ppm SO_2 exposure has a gas flow of 855 mL/min, so the total gas volume the cells are exposed to is 25.65L. At 1 atmosphere & 70°F, 3 ppm SO_2 in air is 8.46 mg/m^3, therefore 0.217 mg SO_2 was absorbed. SO_2 has a molecular weight of 64.066 giving 3.39 x 10^{-6} mol. The combined media volume of two culture plates was 15.98 mL, producing 212 µM SO_2. The chemical reactions of SO_2 in aqueous solution are:

$$H_2O + SO_2 \leftrightarrow H_2SO_3 \quad \text{(A)}$$

$$H_2SO_3 \leftrightarrow H^+ + HSO_3^- \quad \text{(B)}$$

$$HSO_3^- \leftrightarrow H^+ + SO_3^{2-} \quad \text{(C)}$$

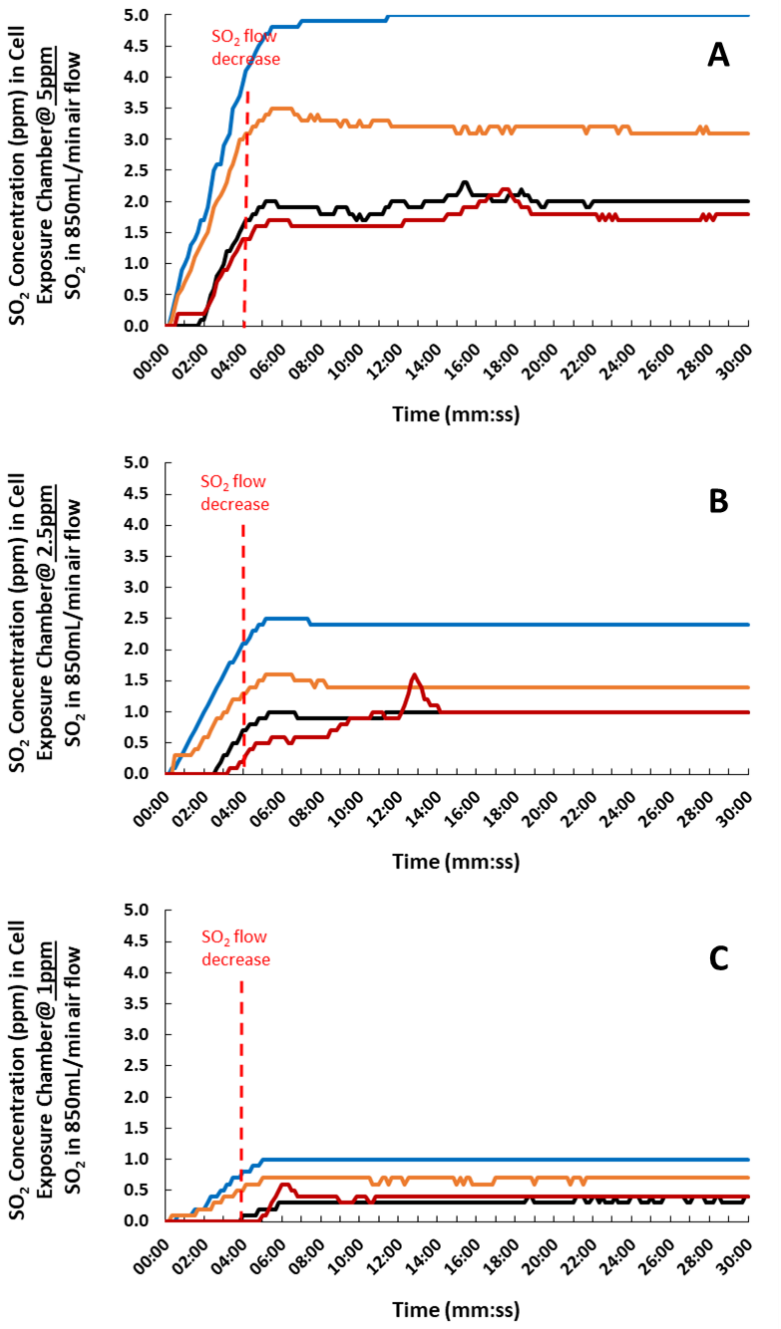

Figure 13: SO_2 absorption by aqueous solutions. Real time ppm SO_2 levels in glass exposure chamber during 30 minute exposure. Blue line, empty culture plates; Black line, two culture plates with PBS; Orange line, one culture plate with DMEM/F12 media; Red line, two culture plates with DMEM/F12 media. **A**: 5 ppm SO_2; **B**: 2.5 ppm SO_2; **C**: 1 ppm SO_2 gas flow.

Therefore, a mole of SO_2 would be equivalent to adding a mole of Na_2SO_3. SO_2 has a solubility of 200 mg/mL at room temperature in water. The 30 minute exposure to 5 ppm SO_2 results in only 0.0136 mg/mL, so the media is not saturated. Similar calculations were performed for the 1 & 2.5 ppm SO_2 exposures. The Na_2SO_3 concentrations used in subsequent experiments were: 200 µM (5ppm), 100 µM (2.5ppm) and 40 µM (1ppm).

The change from SO_2 to sodium sulfite eliminated the 30 minute period necessary for the exposure. Reversing the order of the exposures (SO_2 first followed by CSE) produced the same pattern of response (**Figure 15**D), therefore the HASMC were given CSE and sodium sulfite simultaneously to simplify the protocol (Figure 14).

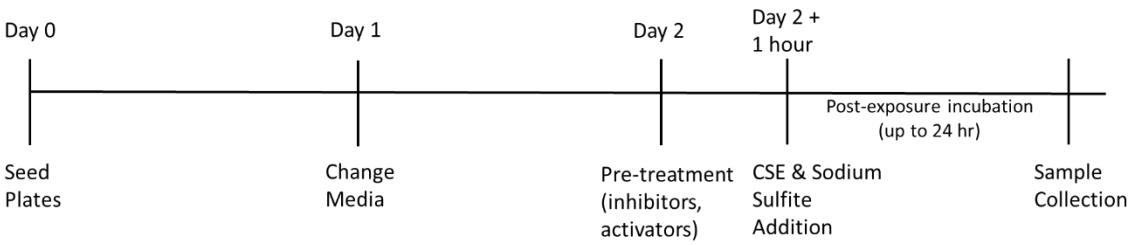

Figure 14: CSE and sodium sulfite exposure protocol.

CELL VIABILITY

Cell viability was measured at 8 and 24 hours post-exposure using trypan blue exclusion. HASMC were seeded into 6 well plates and collected by trypsinization. Cells were resuspended in 250 µL PBS, mixed with 250 µL trypan blue solution and counted on a hemocytometer (four corner squares + center square). Each sample was measured in duplicate and averaged, with three samples per condition. Viability was calculated as the percentage of viable cells/total cells.

GM-CSF ELISA

HASMC GM-CSF release into the culture media was measured using quantitative sandwich enzyme-linked immunosorbent assays (ELISA) (R&D Systems Human GM-CSF Quantikine ELISA, #SGM00 or DGM00). HASMC were exposed to CSE and SO_2/Na_2SO_3, incubated for 8 or 24 hours, and the cell culture media was collected, centrifuged and supernatant was frozen at -80ºC. The media was subsequently assayed for GM-CSF levels according to the manufacturer's protocol. Briefly, the media was added to wells containing an immobilized GM-CSF antibody. The cytokine was bound to the immobilized antibody and an enzyme-linked GM-CSF antibody was added. The enzyme produced a color change in a substrate that was proportional to the bound GM-CSF, measured at an absorbance of 450 nm on a Tecan Infinite F200 Pro plate reader. The detection limit was 3 pg/mL and the samples were diluted if necessary for the readings to fall within the 500 pg/mL upper limit. Samples from three individual wells were tested and averaged for each experiment.

ROS MEASUREMENTS

Intracellular ROS levels were measured using Molecular Probes H_2DCFDA (Invitrogen #D399). HASMC were grown on 96 well plates in DMEM/F12 media with 10% FBS. After 24 hours, the media was changed to DMEM/F12 without FBS or phenol red, and the exposures were performed in this media 24 hours later. HASMC were incubated with 5 μM H_2DCFDA for 10 mins prior to spectrophotometer measurement.

QRT-PCR

Cell cultures were lysed using Qiagen Buffer RLT and total RNA was extracted using the Qiagen RNeasy Mini kit (#74104). The RNA was quantified using an Epoch, measured in triplicate and averaged. The Invitrogen Superscript III First Strand Synthesis kit (#11752) was used to generate cDNA. The cDNA was combined with SYBR Green and primers and run in a LightCycler 96 qRT-PCR machine. Data was analyzed using LightCycler 96P software.

IMMUNOBLOTTING

HASMC lysates were collected in RIPA buffer (Cell Signaling #9806) containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific #PI78441). Total protein levels in the samples were assayed by Pierce BCA. Proteins were denatured in Laemmli buffer (BioRad #161-0747) containing β-mercaptoethanol (Sigma #M3148) and

separated on 4-15% tris-glycine SDS-PAGE gels (BioRad Criterion #567-1085) at 160V until the leading edge of the dye reached the bottom of the gel (approximately 1 hour) or the 25 kDa molecular weight marker was near the bottom. The proteins were transferred to PVDF membrane (BioRad #1620177) using 75V for 1.5 hours while packed in ice. Membranes were washed in TBST, blocked in 5% milk for 1-2 hours and incubated with primary antibodies overnight at 4˚C on a rocker. The membranes were washed three times in TBST for 5 minutes and incubated with the secondary antibody for 1 hour at room temperature. Membranes were again washed three times in TBST and then incubated with ECL for 5 minutes. The chemiluminescent signal was visualized using the BioRad ChemiDoc Touch Imaging System and quantified using BioRad Image Lab 6.0.

STATISTICAL ANALYSIS

Data are shown as either the average of multiple experiments or the average of 3 wells within a single experiment. The error bars display the standard error of the mean (SEM). Statistical analysis was performed by SigmaPlot 12.5 software using one- or two-way analysis of variance (ANOVA) or repeated measures ANOVA. Student-Newman-Keuls (SNK) posthoc test was used to determine significant differences within groups. In cases where the data failed normality or equal variance tests, the data was transformed using a logarithm function prior to analysis. Differences were considered significant if $P < 0.05$.

Table 4: Primary antibodies used in immunoblotting

Antibody	Manufacturer	Catalog #	Species	Dilution	Diluent
p38 MAPK	Cell Signaling	8690	Rabbit mAb	1:1000	3% BSA
Phospho-p38 MAPK (Thr180/Tyr182)	Cell Signaling	4511	Rabbit mAb	1:1000	3% BSA
MKK3	Cell Signaling	8535	Rabbit mAb	1:1000	3% BSA
Phospho-MKK3/6 (Ser189/Ser207)	Cell Signaling	12280	Rabbit mAb	1:1000	3% BSA
p44/42	Cell Signaling	4695	Rabbit mAb	1:1000	3% BSA
Phospho-p44/42 (Thr202/Tyr204)	Cell Signaling	4370	Rabbit mAb	1:2000	3% BSA
JNK	Cell Signaling	9252	Rabbit mAb	1:1000	3% BSA
Phospho-JNK (Thr183/Tyr185)	Cell Signaling	4668	Rabbit mAb	1:1000	3% BSA
NF-κB P65	Cell Signaling	8242	Rabbit mAb	1:1000	3% BSA
Phospho-p65 (Ser276)	Cell Signaling	3037	Rabbit mAb	1:1000	3% BSA
HIF-1α	BD Biosciences	610958	Mouse mAb	1:500	3% BSA
ARNT/HIF-1β	Cell Signaling	5537	Rabbit mAb	1:1000	3% BSA
AhR	Cell Signaling	83200	Rabbit mAb	1:1000	3% BSA
GSK3β	Cell Signaling	12456	Rabbit mAb	1:1000	3% BSA
Phospho-GSK3β (Ser9)	Cell Signaling	5558	Rabbit mAb	1:1000	3% BSA
GAPDH (loading control)	Cell Signaling	5174	Rabbit mAb	1:1000	5% milk or 3% BSA

Table 5: Secondary antibodies used in immunoblotting

Antibody	Manufacturer	Catalog #	Dilution	Diluent
XP Anti-Rabbit IgG, HRP-linked	Cell Signaling	7074	1:3000	3% BSA
m-IgGk BP-HRP Anti-Mouse IgG kappa light chain	Santa Cruz	516102	1:1000	3% BSA

Chapter 3 Inhibitory Effect of SO_2/Sulfites on Cigarette Smoke Extract-Induced GM-CSF Release

Individuals with asthma have elevated levels of IL-1β, TNF and GM-CSF in their airways (247). Individuals with COPD have elevated levels of IL-1β, TNF and GM-CSF (248,249) and the levels further increase with COPD exacerbations (52). IL-1β and TNF are used to stimulate an inflammatory state in airway cell cultures to represent the conditions present in the extracellular milieu of individuals with chronic inflammatory diseases such as asthma and COPD.

HASMC were exposed to both direct smoke and CSE. The direct smoke exposure results are provided in the appendix to verify that the cellular response was consistent whether the toxicant source was cigarette smoke or cigarette smoke extract. Due to differences in the exposure method, including the cigarette smoke dose and serum stimulation from media changes, the levels of GM-CSF release cannot be directly compared.

Likewise, smooth muscle would not be exposed directly to gaseous sulfur dioxide. Even endogenous SO_2 exists in a molecular state, not as little bubbles of gas floating around the body. Sulfur dioxide, sodium sulfite, sodium bisulfite, or sodium metabisulfite all quickly dissociate in aqueous solution into the same combination of sulfite, bisulfite and sulfur dioxide, the exact equilibrium is dependent on factors including temperature, pH and the constituents of the solution but not the source of the sulfites (217). For these cell culture experiments sulfur dioxide or an equivalent concentration of sodium sulfite were used and the exposures should be considered

comparable. They are not identical due to the 30 minute window needed to dissolve the gaseous sulfur dioxide into the culture media versus an instantaneous addition of the sodium sulfite; however any differences in response are not readily apparent.

Cigarette smoke extract (CSE) has been show to induce GM-CSF release from the airway bronchial epithelial cell line HBE-14o (250), alveolar epithelial cells A549 (251), cytotrophoblast cell line B6Tert-1(252) and lung fibroblasts (253). HASMC have previously been shown to release GM-CSF when stimulated with IL-1β, TNF, and LPS (104,209). Endogenous levels of hydrogen sulfide inhibit fetal calf serum-, TGF-β- and IL-1β-induced cytokine release (IL-6, IL-8), proliferation, and p38 and p44/42 (ERK1/2) phosphorylation in HASMC (254-256). However, the role of sulfur dioxide/sulfites in cigarette smoke extract-induced GM-CSF release in HASMC has not been investigated. Therefore, we decided to assay the effect of sulfur dioxide/sulfite on CSE-induced GM-CSF release from HASMC.

SO_2 / Sulfite Inhibits CSE-Induced GM-CSF Release in HASMC

Exposure to CSE or direct cigarette smoke stimulated the release of GM-CSF, a pro-inflammatory cytokine, from Donor 1 HASMC (**Figure 15** and Figure 36A respectively). The CSE-induced GM-CSF release was inhibited by treatment with gaseous SO_2 (Figure 15A) or sulfite (Figure 15B&C), one of the major products of the absorption of SO_2 in aqueous solutions. The response to treatment with sulfite was remarkably consistent with the sulfur dioxide exposures (Figure 15A&B). A 45 minute reduction in the exposure time produced an unexpectedly large change in GM-CSF

release, approximately a 50% reduction, indicating a very concentrated release occurred during a short time window (Figure 15B&C). Although sulfites alone also increased GM-CSF release, this effect was donor specific and so will not be explored further.

The effect of SO_2/sulfites on CSE-induced GM-CSF release was independent of the order of exposure (Figure 15D). However, at 8 hours the sulfite-induced inhibition of GM-CSF release was smaller when the cells were exposed to sulfites before CSE. The sulfite-induced inhibition at 24 hours was consistent with the CSE-first experiments, indicating that the cells recovered the phenotype with additional time, however these results were from a single experiment.

These results suggest that SO_2 and sulfite have anti-inflammatory properties, however, when used to treat Donor 1 HASMC stimulated with LPS or the pro-inflammatory cytokines IL-1β and TNF, SO_2 and sulfite actually augmented the GM-CSF release (**Figure 16**). This may partially explain why asthmatic individuals, who have elevated levels of pro-inflammatory cytokines such as IL-1β in their airways, are sensitive to low SO_2 levels that do not affect non-asthmatics. Interestingly, the effect of sulfite on cytokine-stimulated GM-CSF release in HASMC is opposite the effect of hydrogen sulfide.

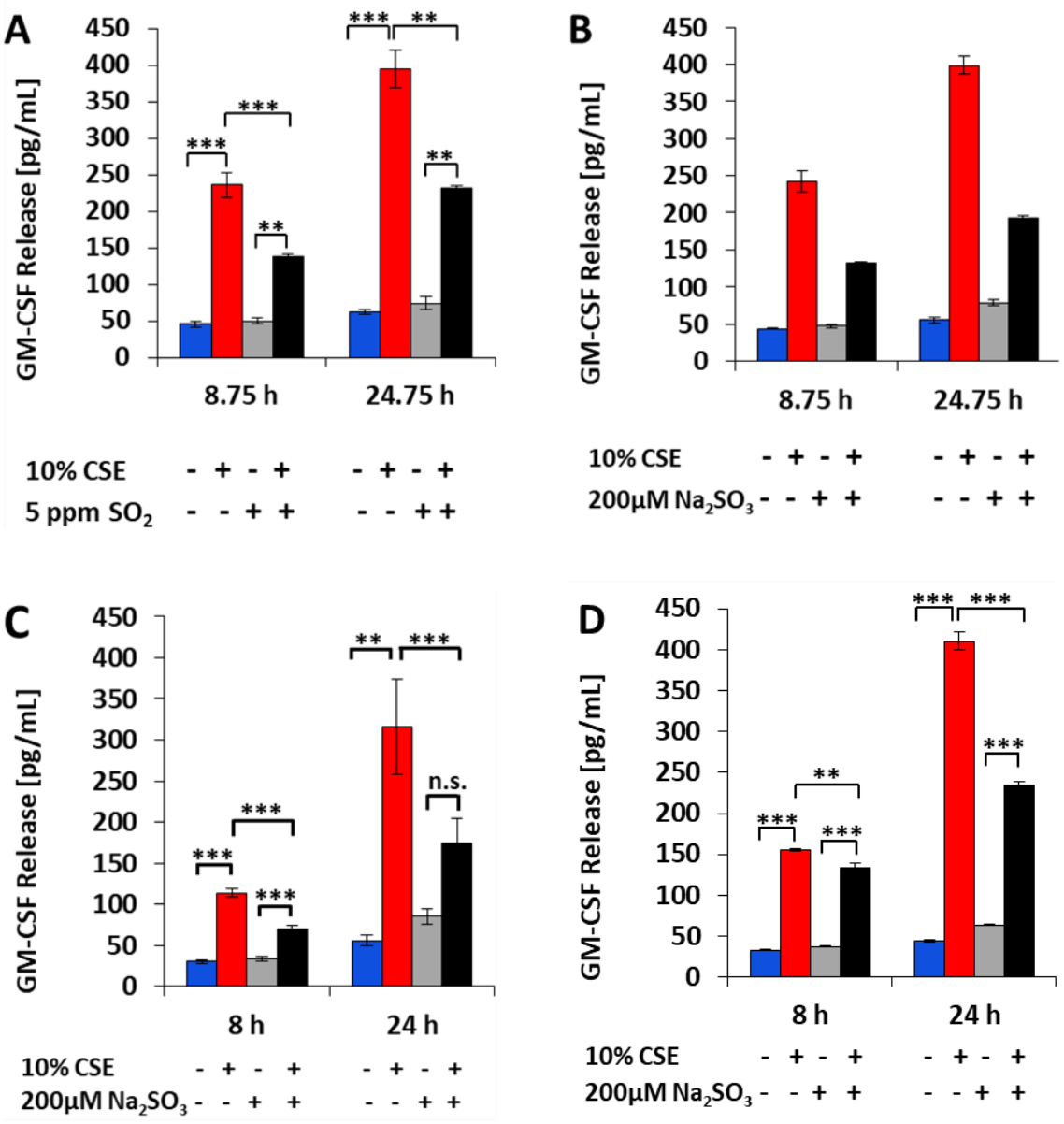

Figure 15: CSE-stimulated GM-CSF release from HASMC. **A**: CSE followed by 30 mins SO_2 for 8.75 & 24.75h (n = 3); **B**: CSE followed by Na_2SO_3 for 8.75 & 24.75h (n = 2); **C**: Simultaneous addition of CSE & Na_2SO_3 for 8h (n = 9) & 24h (n = 4); **D**: Reverse exposure order - 30 mins SO_2 followed by CSE for 8 and 24h (average of 3 wells from single experiment). Error bars: standard error of mean (SEM); * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$; n.s. indicates no significant difference.

Toll-like receptor 4 (TLR4) is activated by cigarette smoke (257) and knockout of this receptor suppresses the cigarette smoke-induced inflammatory response (258). LPS, a TLR4 ligand, did not elicit the same response to sulfur dioxide treatment that CSE produced. It appears that the cigarette smoke-induced activation of TLR4 is not mediated by the presence of lipopolysaccharide (LPS) in the cigarette smoke, or at least not by LPS alone (258). Since cigarette smoke-induced signaling is also dependent on the interleukin-1 receptor 1 (IL-1R1) (258), a combination of signals may be required. The activation of TLR4 may induce release of cytokines, such as IL-1, that activate the IL-1R1 receptor, into the extracellular milieu which would induce autocrine activation of IL-1R1. Alternatively, cigarette smoke constituents may directly activate IL-1R1 in conjunction with TLR4.

The effect of LPS on GM-CSF release reached a maximum at 100 ng/mL and then dropped off. IL-1β would produce a similar response at higher concentrations (1-10 ng/mL), however airway smooth muscle cells are very responsive to IL-1β and the GM-CSF release would increase several fold. TNF would also display a similar pattern of response.

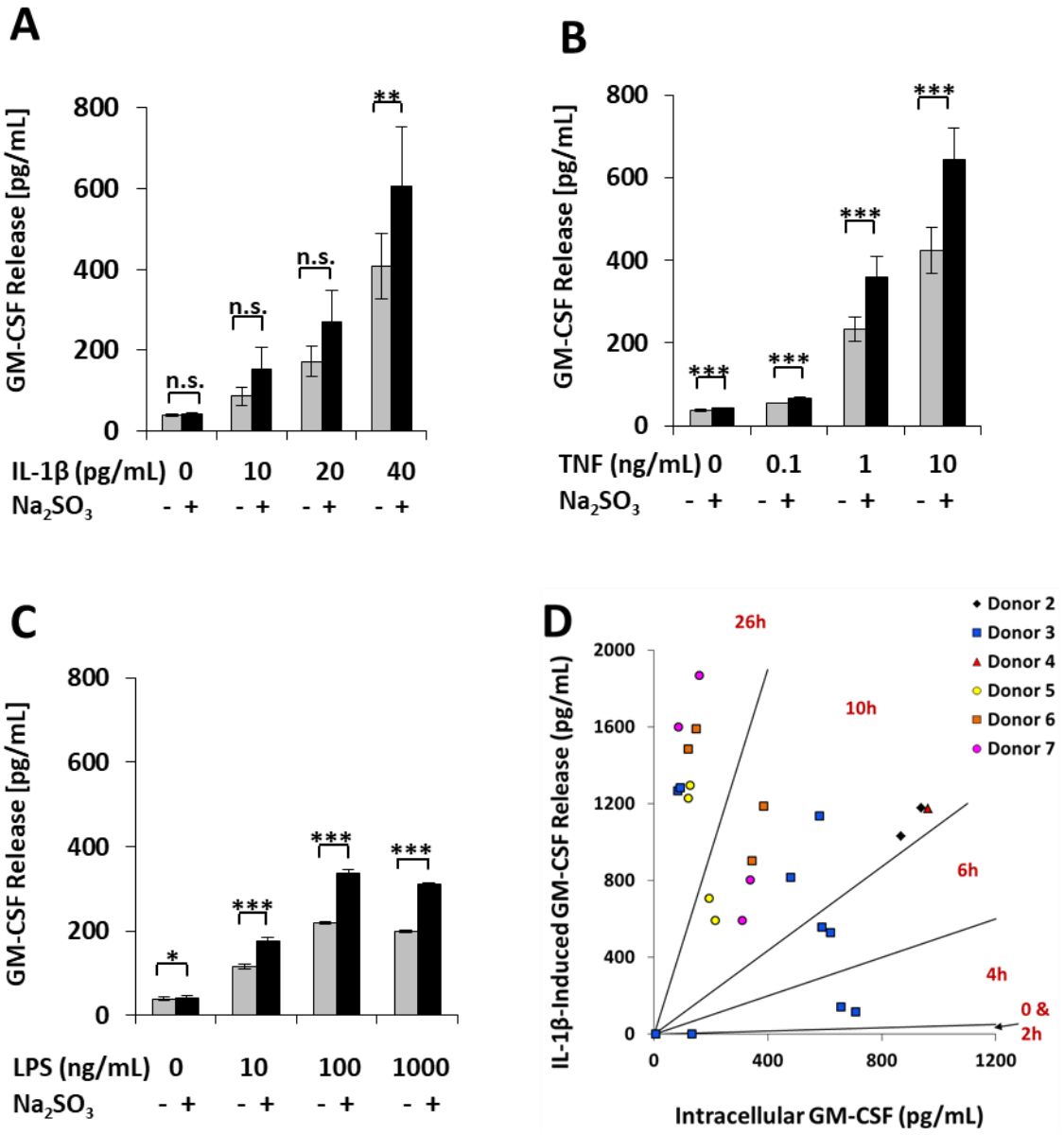

Figure 16: Cytokine and LPS- induced GM-CSF release. Effect of sulfites on (**A**) IL-1β-, (**B**) TNF- or (**C**) LPS-induced GM-CSF release at 8h; **D**: Intracellular vs extracellular (1 ng/mL) IL-1β-induced GM-CSF levels over time. Error bars: SEM; * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$; n.s. indicates no significant difference.

The induction of GM-CSF with CSE and the inhibition by sulfites was consistent across HASMC from 8 different non-asthmatic individuals (Donors 1, 2, 3, 4, 8, 9, 10, 11), although there was variability in the strength of the response to both (Figure 17A&B). Sulfites alone (without CSE) did not produce a consistent effect on GM-CSF release for all donors. Sulfites increased baseline GM-CSF for some donors and decreased it for others, but in all cases the effect was a small change. The effect of CSE and sulfites on GM-CSF was also independent of the HASMC collection site (Figure 17C&D) and donor characteristics (**Table 6**).

Interestingly, some individuals produce almost no GM-CSF without stimulation while others produced relatively high levels. Donors 2 & 3 displayed very low GM-CSF levels at all conditions and Donors 4 & 11 had very little difference due to CSE or sulfite at 8 hours. By 24 hours, though, Donors 2, 3 & 4 had significant CSE-induced GM-CSF release and the greatest levels of sulfite-induced suppression of all the donors. Surprisingly, Donor 11, which had high constitutive levels of GM-CSF release like Donor 1, produced the least GM-CSF release at 24 hours with very little difference among the 4 conditions. Despite the differences, CSE induced GM-CSF and sulfite inhibited the GM-CSF induction in all donors. Since Donor 1 HASMC produced higher levels of GM-CSF, making it easier to measure changes during the investigation, these cells were used for subsequent experiments unless otherwise specified.

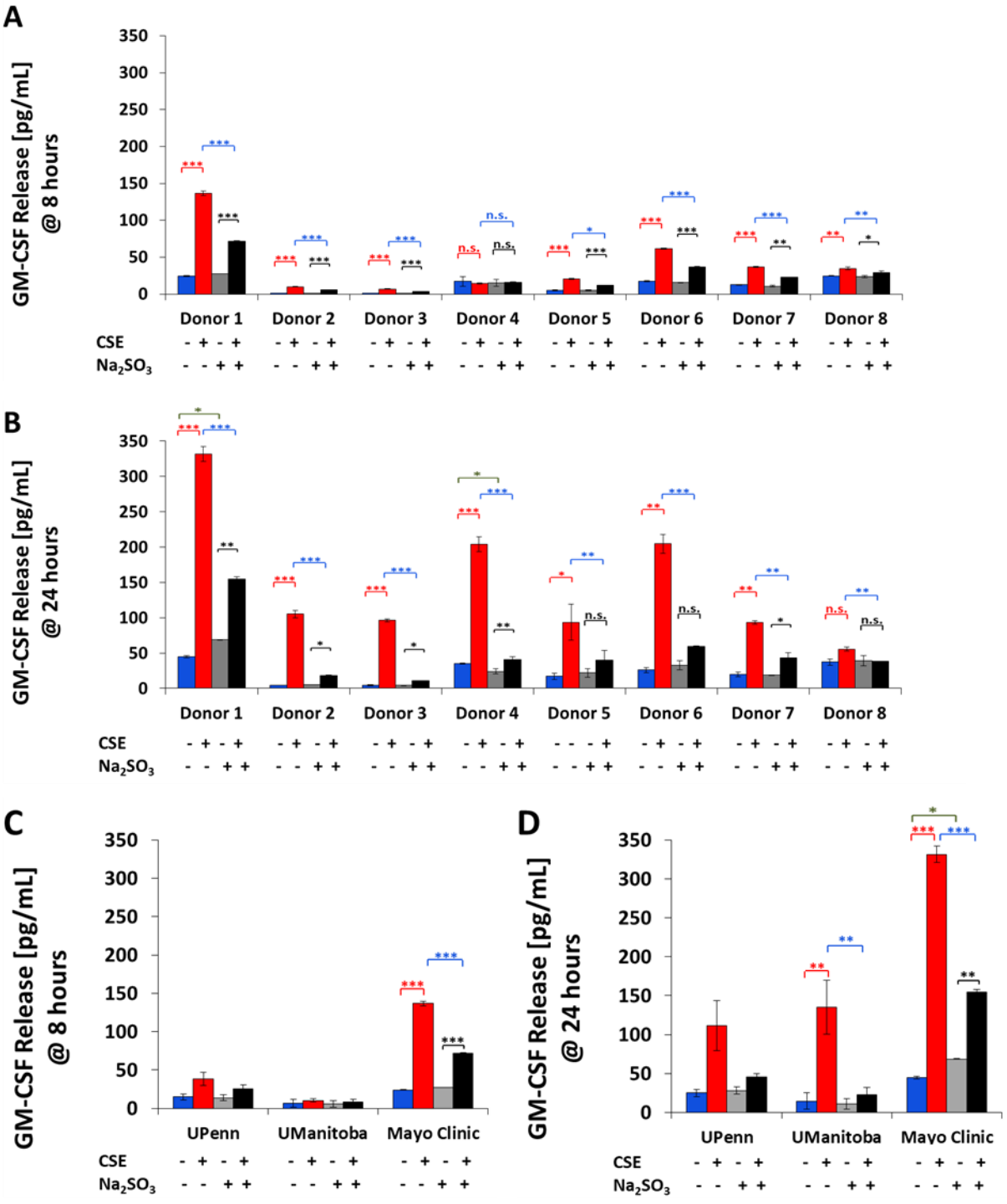

Figure 17: Response of HASMC from multiple donors to CSE and Na_2SO_3 stimulation. **A & B**: Individual donor response at 8 and 24 hours (average of 3 wells from single experiment); **C & D**: Average response of HASMC harvested at multiple locations.

Error bars: SEM; * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; n.s. indicates no significant difference.

Table 6: HASMC Donor Demographic Characteristics. None of the donors were diagnosed with COPD and one had asthma as a child.

Donor	Age	Gender	Collection Location	Race	Smoking status
1	> 40	Unknown	Mayo Clinic, Rochester, MN, USA	Caucasian	Never
2	64	Male	University of Manitoba, Winnipeg, Canada	Unknown	12 pack yrs Quit 30 yrs ago
3	63	Male	University of Manitoba, Winnipeg, Canada	Unknown	90 pack yrs Quit 15 yrs ago
4	65	Female	University of Manitoba, Winnipeg, Canada	Unknown	30 pack yrs Quit 8 yrs ago
5	63	Female	University of Manitoba, Winnipeg, Canada	Unknown	3 pack yrs Quit 25 yrs ago
6	78	Female	University of Manitoba, Winnipeg, Canada	Unknown	Never
7	65	Male	University of Manitoba, Winnipeg, Canada	Unknown	45 pack yrs Quit 21 yrs ago
8	26	Male	University of Pennsylvania, Philadelphia, PA, USA	Caucasian	Never
9	21	Male	University of Pennsylvania, Philadelphia, PA, USA	Caucasian	Never
10	46	Male	University of Pennsylvania, Philadelphia, PA, USA	Hispanic	Never
11	52	Female	University of Pennsylvania, Philadelphia, PA, USA	Caucasian	Never

The GM-CSF release in response to CSE is not a rapid process- at 4 hours post-exposure, the GM-CSF levels in the culture media are usually below ELISA detection levels (< 3pg/mL, data not shown). By 8 hours post-exposure the Donor 1 HASMC have released approximately125 pg/mL GM-CSF (Figure 15C) and this release doubles within the next 45 minutes (Figure 15A&B) but over the following 16 hours there is only an

additional 50% increase in GM-CSF levels. The majority of the release between 8 and 24 hours post-exposure occurs prior to 16 hours post-exposure (data not shown). This suggests that the time period surrounding 8 hours post-CSE exposure is a crucial release window for HASMC GM-CSF cytokine signaling.

Similar to CSE-stimulation, IL-1β-induced GM-CSF release from HASMC is 10% of the 26 hour level at 4 hours and approximately 50% or more of the 26 hour total by 10 hours (**Figure 16**D). Unstimulated HASMC have very little intracellular GM-CSF (5 pg/mL cell lysate) and, upon IL-1β stimulation, build up the intracellular levels for the first 4 hours before beginning release. While HASMC continue to produce GM-CSF after 4 hours of IL-1β stimulation, by 26 hours there is a low level of intracellular GM-CSF, indicating nearly complete release. Combined with the reduction in the GM-CSF release rate, this suggests that, despite the continued presence of the stimulus, GM-CSF production is greatly reduced by 26 hours, although it is not completely abolished.

CSE Induces & Sulfite Inhibits GM-CSF in A Dose-Dependent Manner

CSE stimulation produces a dose-dependent GM-CSF increase in HASMC (Figure 18A&B). The 10% CSE stimulation produced the strongest increase in GM-CSF release. As the concentration of SO_2 increased from 1 ppm to 5 ppm there was a dose dependent decrease in GM-CSF release which was most evident with 10% CSE stimulation (Figure 18A&B).

When HASMC were treated with concentrations of sodium sulfite equivalent to the 30 minute 1, 2.5 and 5 ppm SO_2 exposure (40, 100 & 200 µM Na_2SO_3 respectively), a

similar dose dependent decrease in CSE-induced GM-CSF release was seen (Figure 18C&D). This indicates that the effect of SO_2 on CSE-induced GM-CSF release is due to the sulfites in the culture media.

HASMC viability was assayed by trypan blue exclusion at 8 and 24 hours after stimulation. Viability did not fall below 90% with 10% CSE or 5 ppm SO_2 (Figure 19). This indicates that the cells were not dying due to toxicity of the exposures. Based on these results, subsequent experiments were conducted using 10% CSE and 5 ppm SO_2 or 200 µM Na_2SO_3.

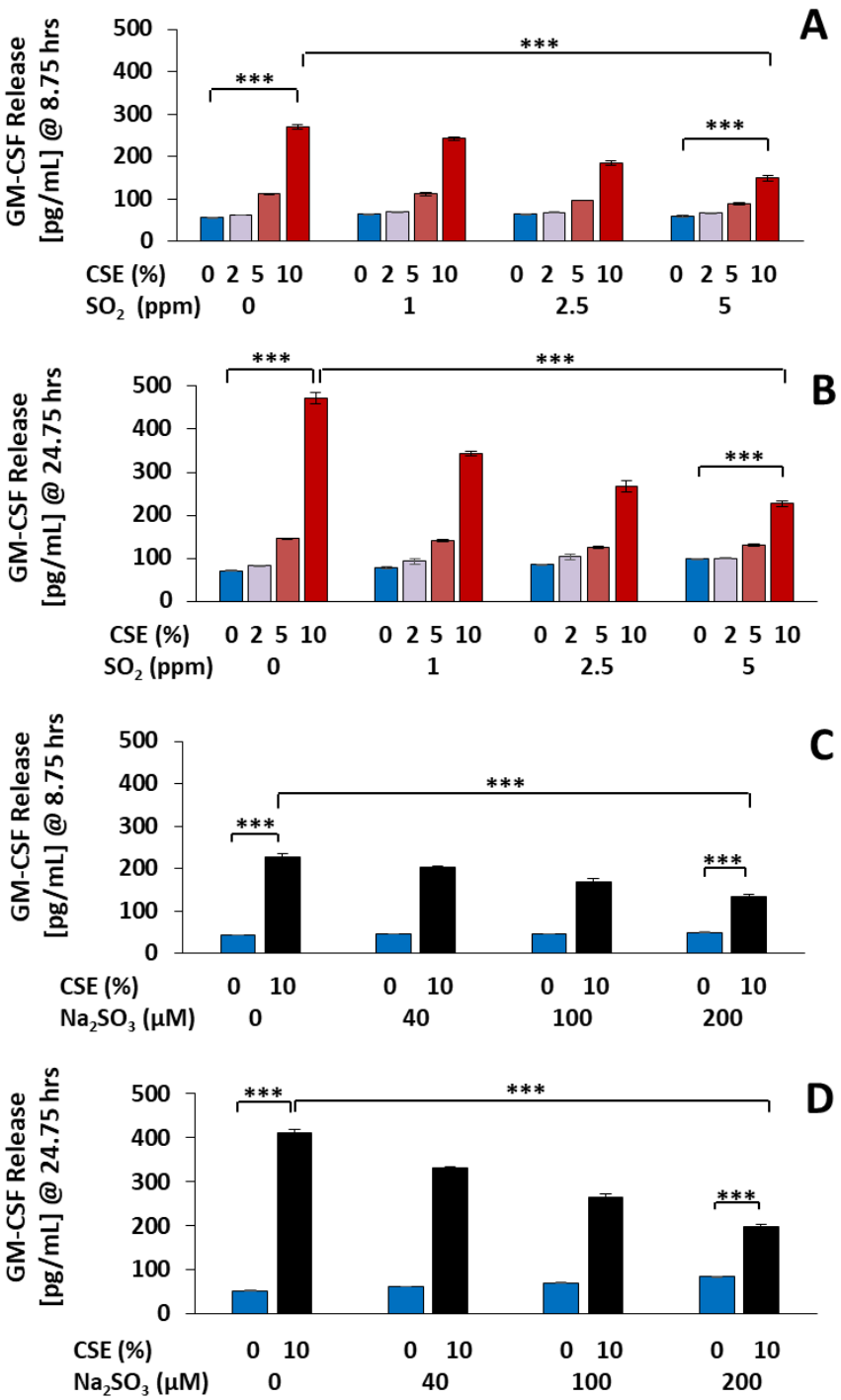

Figure 18: CSE, SO_2 & Na_2SO_3 dose response at 8.75 & 24.75 hours. **A** & **B**: CSE and SO_2-stimulated GM-CSF release; **C** & **D**: CSE and Na_2SO_3-stimulated GM-CSF release; Error bars: SEM; Average of 3 wells from single experiment; *** indicates $P < 0.001$. Note: only 0/10% CSE & 0/5ppm/200µM SO_2/Na_2SO_3 statistics shown.

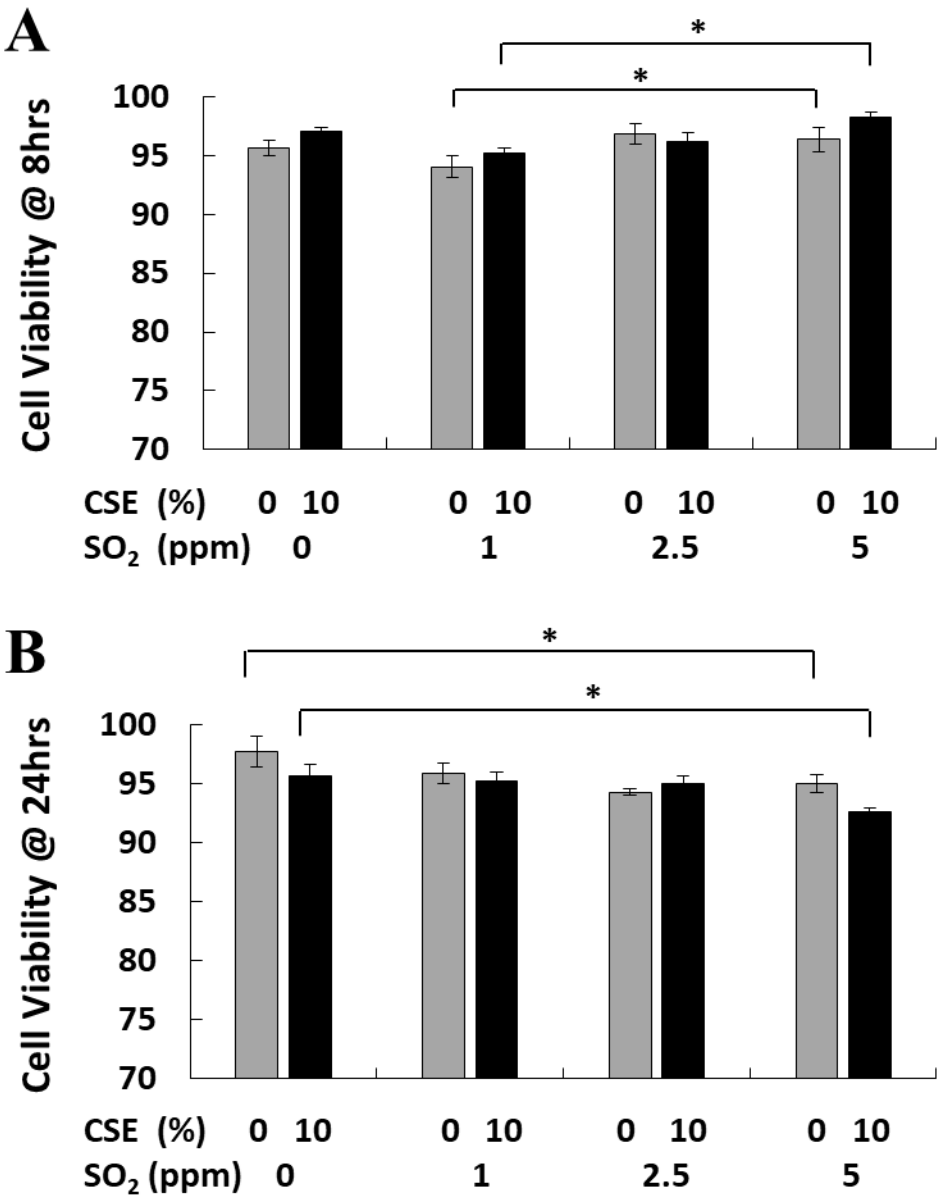

Figure 19: Viability in CSE- and SO_2-stimulated HASMC at 8 and 24 hours. Trypan blue exclusion; average of 3 wells, 2 samples per well, from single experiment. Error bars: SEM; * indicates $P < 0.05$.

CSE & SULFITE INDUCE ROS PRODUCTION

ROS such as H_2O_2 have previously been shown to induce NF-κB activity, which up-regulates GM-CSF production (117,118). CSE has previously been found to induce formation of reactive oxygen species (ROS) in HASMC and other cell types by activating Toll-Like Receptor 4 (TLR4) which subsequently activated the NADPH oxidases (NOX) that produce ROS (114-116). It has also been reported that SO_2 generates ROS. We found that stimulation with 10% CSE produced a 20-30% increase in HASMC intracellular ROS levels, which is a level that indicates a physiological response to stimulation and not an excessive response indicating cellular damage or destruction, while 200 µM Na_2SO_3 produced a maximum increase of 5-6% (Figure 20). Interestingly, the Na_2SO_3 neither increased nor decreased the CSE-induced ROS. This suggests that the effect of sulfite on GM-CSF release is not mediated by changes in ROS levels.

IL-1β stimulation does not appear to induce ROS in HASMC (Appendix Figure 38), which suggests that it produces GM-CSF from a non-ROS-induced pathway. Sulfites also had little effect, although the combined IL-1β + sulfite might produce ROS at 8 hours. Additional experiments are needed to verify this result. This further suggests that the effect of sulfites on GM-CSF production may not be linked to ROS production.

Since neither sulfite nor IL-1β is producing GM-CSF in an ROS-dependent manner, this opens the possibility that they may be stimulating the same pathway or interconnected pathways.

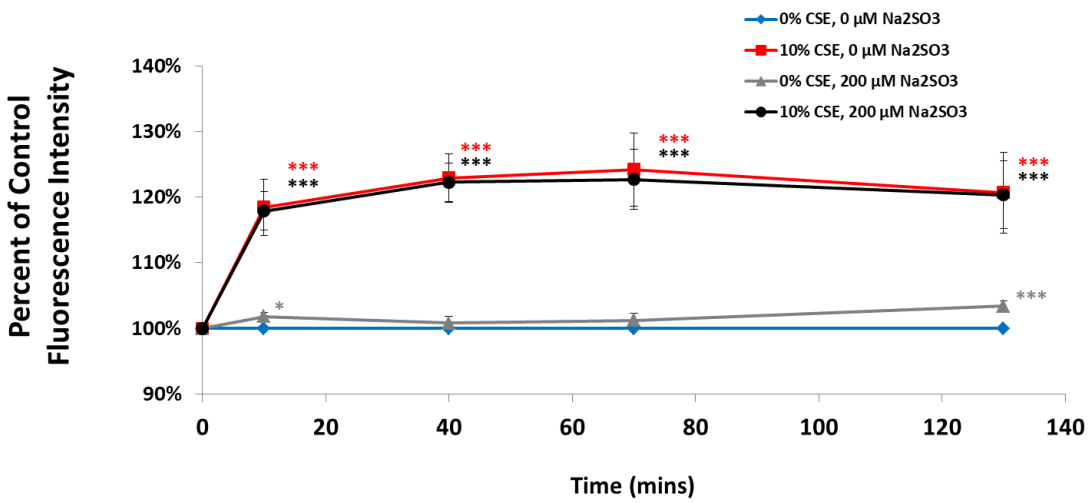

Figure 20: CSE- and Na_2SO_3-induced intracellular ROS levels as a function of H_2DCFDA fluorescence intensity compared to 0% CSE, 0 µM Na_2SO_3 control at same time point. Error bars: SEM; * indicates $P < 0.05$ compared to time 0 within same line; ** indicates $P < 0.01$ compared to time 0 within same line; *** indicates $P < 0.001$ compared to time 0 within same line; n = 6 independent experiments for all points.

The anti-oxidant NAC-ME inhibits CSE-induced GM-CSF release, indicating that CSE is inducing GM-CSF in an ROS-dependent manner (Figure 21A). However, stimulation with exogenous H_2O_2 alone does not induce GM-CSF in HASMC (Figure 21B). This indicates that the ROS present in CSE are not sufficient to induce GM-CSF release from HASMC.

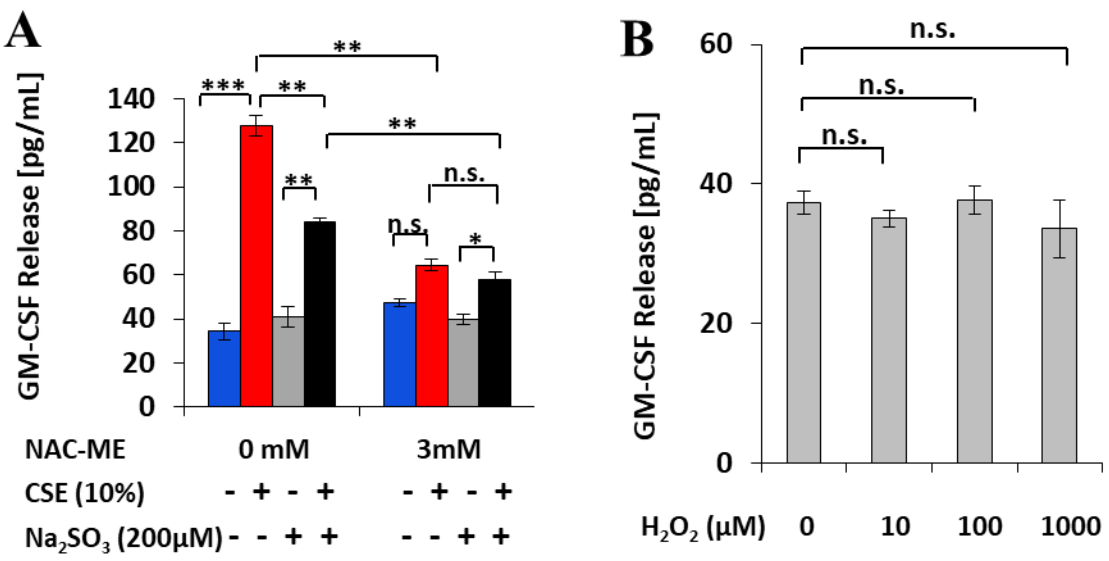

Figure 21: Effect of NAC-ME & H_2O_2 on GM-CSF release. **A**: Inhibition of CSE-induced GM-CSF release with anti-oxidant NAC-ME at 8h; **B**: GM-CSF release after 8h stimulation with exogenous H_2O_2; Error bars: SEM; * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$; n.s. indicates no significant difference; n = 3for all bars.

Chapter 4 MAPK & NF-κB Pathways

INHIBITION OF CSE-INDUCED MAPKS BY SULFITE

Cigarette smoke has been shown to activate the p38 and p44/42 pathways in HASMC (259). Others have demonstrated that sulfur dioxide inhibits p44/42 (ERK1/2) activity in vascular smooth muscle cells (240). However, the effect of sulfite on CSE-stimulated HASMC has not been investigated.

CSE induced activation of p38 quickly, at 5 minutes; the activation peaked at 30 mins and then decreased over time (Figure 22, top). Treatment with sulfite reduced p38 phosphorylation. CSE also induced phosphorylation of the kinases that phosphorylate p38: MKK3 and MKK6. This activation was weak at 5 minutes and peaked at 1-4 hours before decreasing at 8 hours. Sulfite suppressed the CSE-induced activation of MKK3/6.

The JNK pathway is not very active in HASMC, however CSE induced strong phosphorylation of JNK at 5 minutes, indicating that it may have an early rapid response in these cells (Figure 22, middle). Sulfite does not affect the phosphorylation of JNK.

Similar to JNK, the p44/42 pathway demonstrates a strong CSE-induced activation at 5 minutes (Figure 22, bottom). The inhibitory effect of sulfite on CSE-induced p44/42 activation is not evident until 2 hours, much later than the effect on p38.

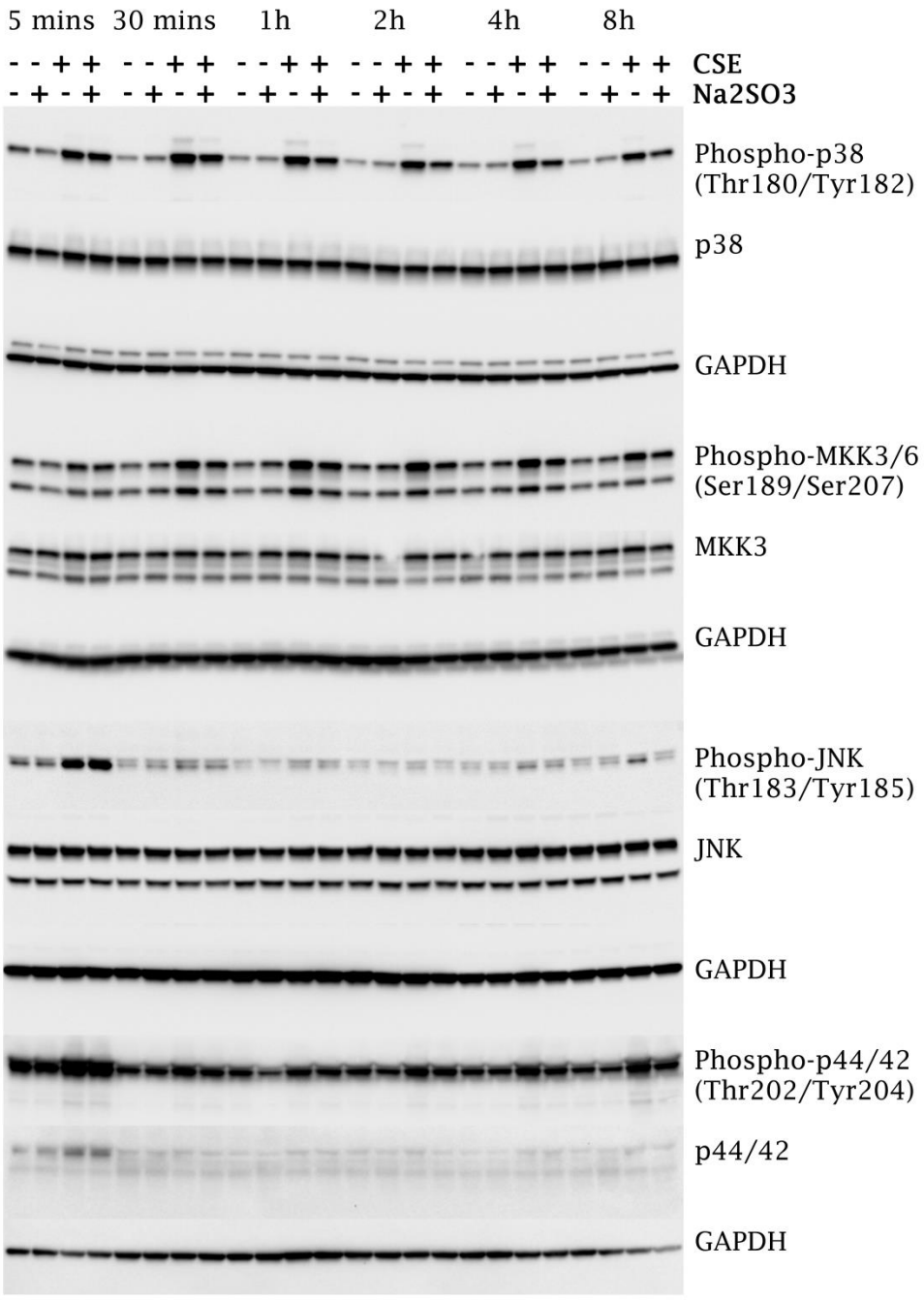

Figure 22: Time course of MAPK phosphorylation. Western blots depicting phospho-protein, total protein and GAPDH loading control for p38, MKK3/6, JNK and p44/42 (ERK1/2) MAPKs. Representative blots from 2 separate experiments.

INHIBITION OF MAPKS SUPPRESSES CSE-INDUCED GM-CSF RELEASE

Inhibition of p38 MAPK with SB203580 produced a dose-dependent decrease in CSE-induced GM-CSF release, both in the presence and absence of sulfite (Figure 23). The inhibitor did not affect constitutive GM-CSF release. Although the cells exposed to 10% CSE, 200μM Na_2SO_3 (black bars) started out with a lower GM-CSF release than the cells stimulated with 10% CSE alone (red bars), at the highest inhibitor dose the combined CSE/sulfite exposure had a higher GM-CSF release than CSE alone. This suggests that the p38 inhibitor was able to more effectively suppress the GM-CSF release from the cells stimulated with CSE alone. While sulfite suppresses p38-mediated GM-CSF release induced by CSE, it also appears to either activate an alternative form of GM-CSF induction or disrupt GM-CSF suppression at high p38 inhibitor concentrations. Alternatively, sulfite may somehow inhibit SB203580 activity, resulting in decreased efficacy of the inhibitor.

SB203580 selectively inhibits p38α and p38β activity, but not p38δ or p38γ, by binding to the ATP-binding pocket, however it also inhibits the TGFβ Type I receptor (260,261). It inhibits p38 activity but not the activation (phosphorylation) of p38 itself. This suggests that p38α/β or TGFβ receptor activation is required for CSE-induced GM-CSF release. It is possible that sulfites activate one of the other isoforms (p38δ or p38γ) in airway smooth muscle, which contributes to GM-CSF production independently of p38α or p38β.

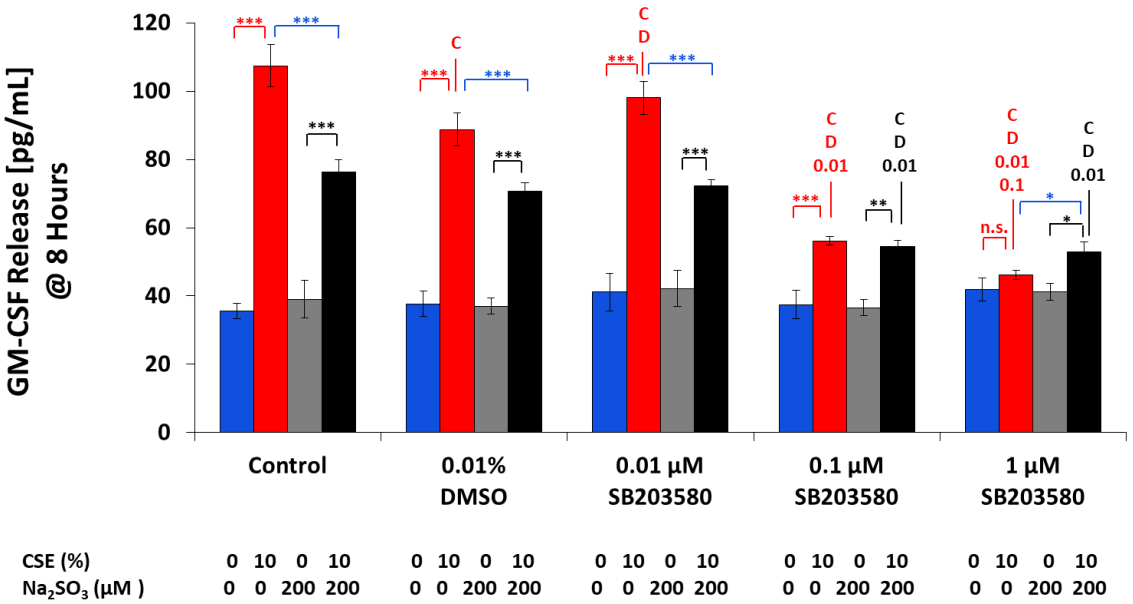

Figure 23: P38 inhibitor SB203580 dose response. Error bars: SEM; * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; n.s. indicates no significant difference; C, D, 0.01 & 0.1 indicate at least P < 0.05 compared to control, DMSO, 0.01µM, or 0.1µM, respectively, within the same CSE/Na_2SO_3 conditions; n = 3 for all bars.

Inhibition of p44/42 (also known as ERK1/2) with U0126 dose-dependently decreased HASMC CSE-induced GM-CSF release (Figure 24). This effect was independent of sulfite treatment. Unlike the p38 inhibitor SB203580, the addition of sulfite always resulted in a further decrease in CSE-induced GM-CSF compared to CSE alone. U0126 inhibits p44/42 by interfering with the activation of MKK1which subsequently phosphorylates and activates p44/42 (262). Therefore, U0126 should reduce p44/42 phosphorylation in addition to p44/42 kinase activity.

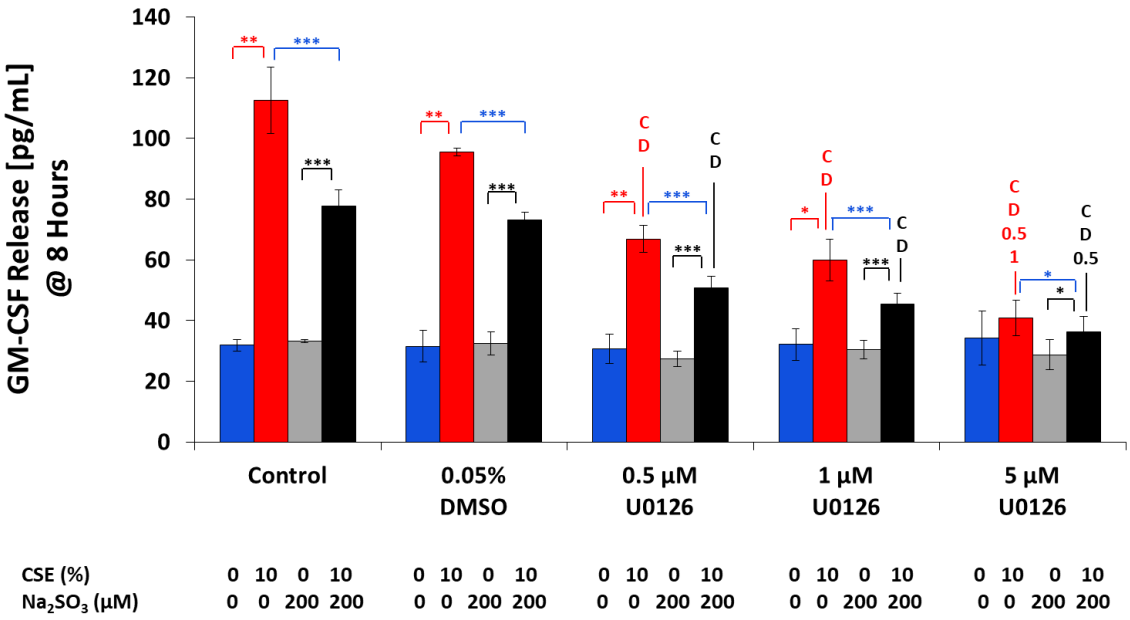

Figure 24: P44/42 inhibitor U0126 dose response. Error bars: SEM; * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; n.s. indicates no significant difference; C, D, 0.5 & 1 indicate at least P < 0.05 compared to control, DMSO, 0.5µM, or 1µM, respectively, within the same CSE/Na_2SO_3 conditions; n = 3 for all bars.

Transforming growth factor-β activated kinase 1 (TAK1) is activated by a variety of stimuli, including IL-1β, TNF, LPS and cigarette smoke, and then activates the MAPK and NF-κB pathways (168,169). TAK1 activates NF-κB by phosphorylating IKKβ, which subsequently phosphorylates IκBα, causing its degradation and freeing NF-κB to translocate to the nucleus (169). TAK1 also phosphorylates MKK3, MKK4 and MKK6, kinases that activate p38 and JNK (263,264). LL-Z1640-2 mediated TAK1 inhibition was previously shown to inhibit CSE-stimulated IL-8 release from HASMC (170).

Inhibition of TAK1 with inhibitor LL-Z1640-2 reduced CSE-induced GM-CSF release in a dose-dependent manner (Figure 25). While it reduced GM-CSF stimulated with CSE (red bars) or CSE/sulfite (black bars), like SB203580 at the highest inhibitor dose it was less effective at suppressing GM-CSF from CSE/sulfite treated cells. This suggests that both p38 and TAK1 may be involved in the mechanism that limits the reduction in CSE-induced GM-CSF release in the presence of sulfite. LL-Z1640-2 inhibits TAK1 by competitive binding in the ATP-binding pocket (265). Therefore, TAK1 inhibition should reduce the phosphorylation of its downstream targets.

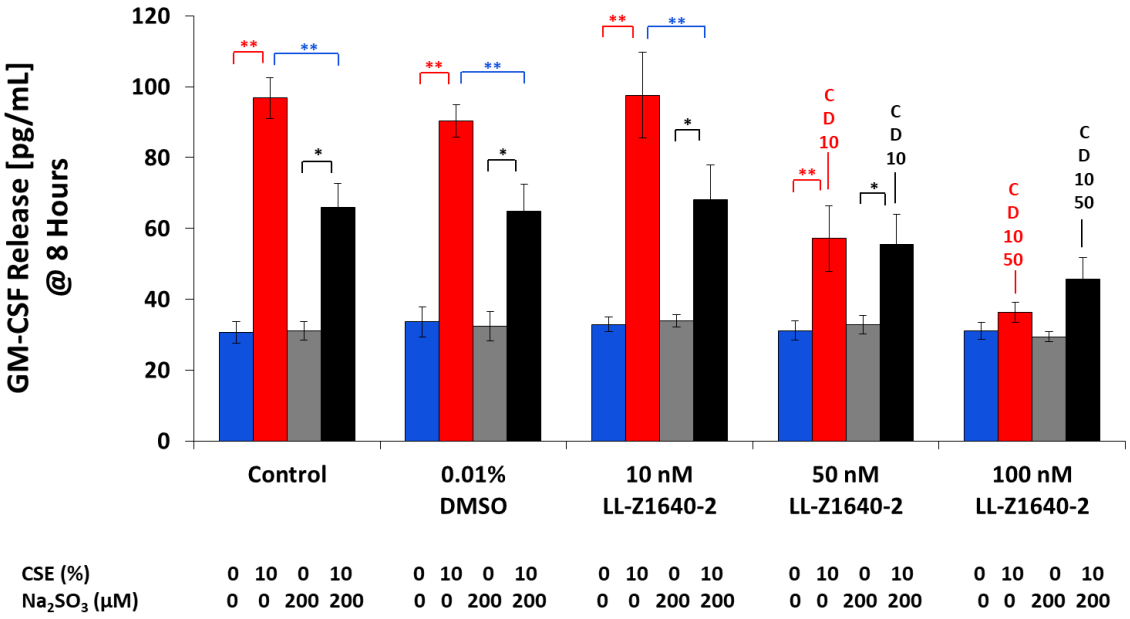

Figure 25: TAK-1 inhibitor LL-Z1640-2 dose response. Error bars: SEM; * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$; n.s. indicates no significant difference; C, D, 10 & 50 indicate at least $P < 0.05$ compared to control, DMSO, 10 nM, or 50 nM, respectively, within the same CSE/Na_2SO_3 conditions; n = 3 for all bars.

SULFITE INHIBITS CSE-INDUCED NF-κB ACTIVATION

CSE stimulation induced phosphorylation of the NF-κB p65 subunit on Ser276 starting at 30 minutes (Figure 26, top). This activation was inhibited by sulfite. These data show that NF-κB activation comes after the activation of p38 MAPK, thus suggesting that NF-κB is a target of the p38 MAPK activation. ROS has been shown to stimulate phosphorylation of NF-κB subunit p65 on Ser276 in a cAMP dependent protein kinase A (PKAc)-dependent manner (266). Ser276 phosphorylation was needed for NF-κB binding with its co-activator CBP/p300 and subsequent transcriptional activation (266). Phosphorylation of Ser536 on p65, commonly used to indicate NF-B activation, is not dependent on ROS stimulation (266).

SULFITE INHIBITS CSE-INDUCED GSK3β ACTIVATION

CSE quickly, by 5 minutes, induced phosphorylation of Ser9 on GSK3β and sulfite inhibited this phosphorylation (Figure 26, middle). GSK3β is phosphorylated by multiple kinases, including PKA, Akt/PKB, PKC, and p38 (267). Phosphorylation on Ser9 prevents GSK3β from binding its substrates, and therefore inactivates it (268). GSK3β is a kinase that is constitutively active unless it is deactivated, for example by Ser9 phosphorylation. It can bind to the MAPKKK MEKK4 and inhibits its activation of the p38 MAPK and JNK pathways. Therefore, CSE-induced Ser9 phosphorylation could inhibit GSK3β, thereby activating MEKK4 and subsequently p38. Sulfite reduced the

Ser9 phosphorylation, which can increase GSK3β activity, by down-regulating MEKK4 and p38 activity.

SULFITE INHIBITS CSE-INDUCED HIF-1A BUT NOT AHR

CSE induced HIF-1α protein accumulation which was suppressed by sulfite (Figure 26, middle). The reduction in HIF-1α resulting from the sulfite treatment suggests that sulfite does not induce hypoxia, however it is also possible that the lower protein accumulation is a result of less NF-κB stimulation from the MAPK pathways which has a stronger effect on HIF-1α transcription.

The constitutively expressed HIF-1β remained unaffected by either CSE or sulfite. The aryl hydrocarbon receptor (AhR), which shares HIF-1β /ARNT as a dimerization partner with HIF-1α, was activated by cigarette smoke but there was no apparent effect due to the sulfite (Figure 26, bottom). When the AhR is activated, it translocates to the nucleus and after completing its transcriptional functions, is quickly degraded. This suggests that a decrease in AhR protein levels is a sign of receptor activation.

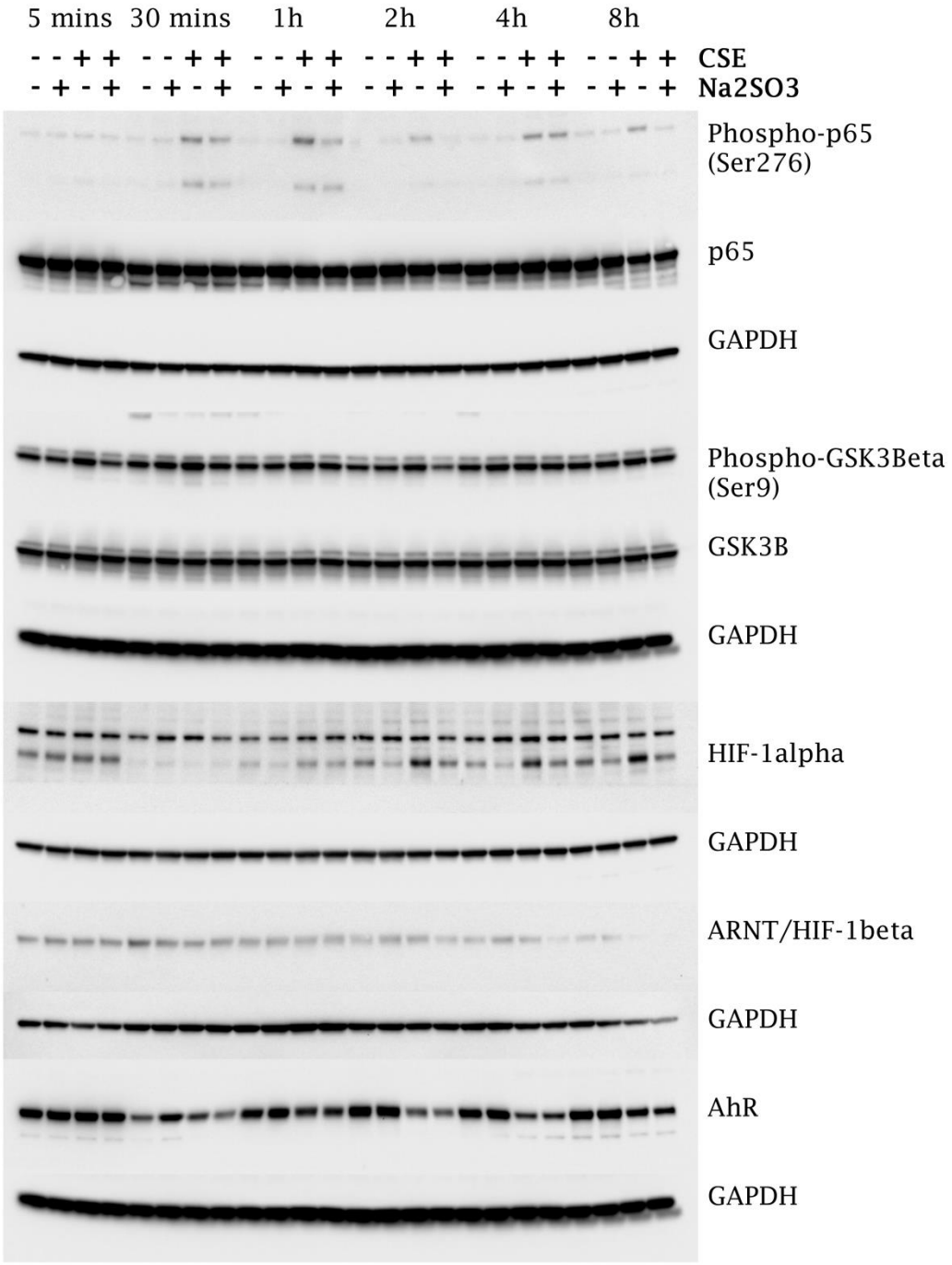

Figure 26: Time course of NF-κB, GSK3β, HIF-1α, and AhR activation. Western blots depicting phospho-protein, total protein and GAPDH loading control for NF-κB p65, GSK3β Ser9 inhibition, HIF-1α accumulation and AhR activation. Representative blots from 2 separate experiments.

INHIBITION OF NF-κB SUPPRESSES CSE-INDUCED GM-CSF RELEASE

BMS-345541 is an inhibitor of NF-κB activity that functions by binding an allosteric site in both IKKα and IKKβ, which prevents IKK from phosphorylating IκBα, triggering its degradation and releasing NF-κB (269). Inhibition should therefore reduce phosphorylation of the NF-κB p65 subunit.

Inhibition of NF-κB in HASMC with BMS-345541 reduced GM-CSF release in a dose-dependent fashion which was independent of sulfite treatment (Figure 27). Like the p44/42 inhibitor U0126, sulfite did not limit the effectiveness of BMS-345541 at the highest concentration. Combined with the effect of CSE and sulfite on p65 phosphorylation, this indicates that NF-κB activation is required for the GM-CSF release from HASMC.

The release of the pro-inflammatory cytokine GM-CSF from CSE-stimulated HASMC involves p38, p44/42, TAK1 and NF-κB activity. At the higher p38 and TAK1 inhibitor concentrations that reduced CSE-induced GM-CSF release to near constitutive levels, the addition of sulfite, which at lower inhibitor concentrations suppressed GM-CSF release, resulted in higher GM-CSF release. This suggests the presence of a p38- and TAK1-mediated mechanism, affected by sulfite, which modulates GM-CSF production. HIF-1α has been identified as a factor that inhibits NF-κB induction. This mechanism has previously been identified as also requiring TAK1, which makes it a potential candidate for the unexpected changes in GM-CSF release at high inhibitor concentrations.

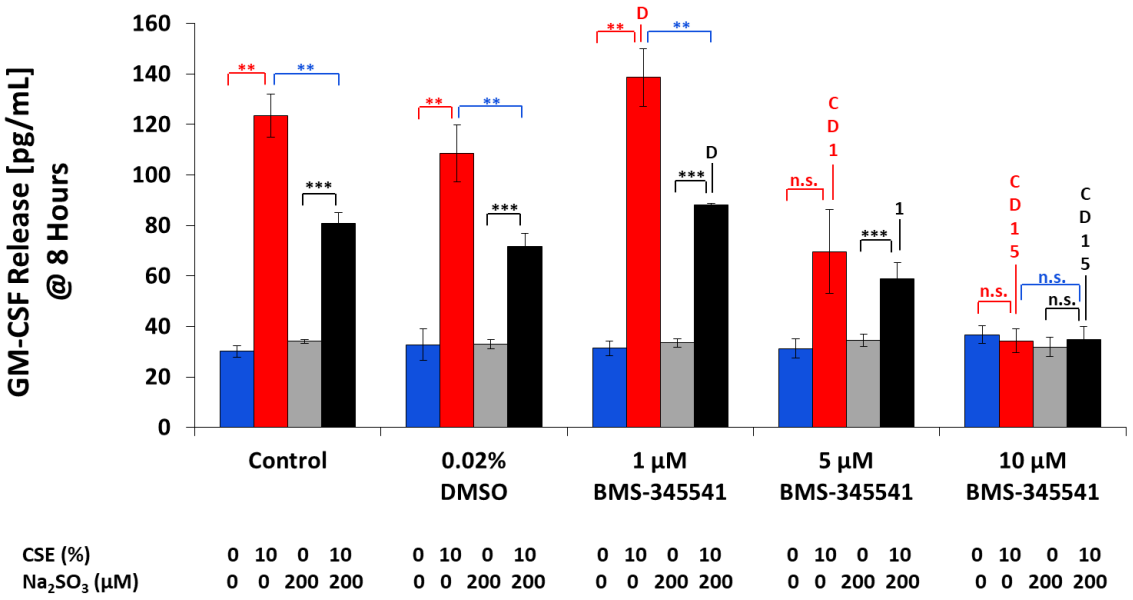

Figure 27: NF-κB inhibitor BMS-345541 dose response. Error bars: SEM; * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; n.s. indicates no significant difference; C, D, 1 & 5 indicate at least P < 0.05 compared to control, DMSO, 1 µM, or 5 µM, respectively, within the same CSE/Na_2SO_3 conditions; n = 3 for all bars.

INHIBITION OF HIF-1 AUGMENTS CSE-INDUCED GM-CSF RELEASE

TAT-cyclo-CLLFVY inhibits the dimerization of the ubiquitous HIF-1α with HIF-1β by binding the HIF-1α PAS-B domain and physically blocking the protein-protein interaction (270). This inhibits HIF-1α/β heterodimer activity, including transcriptional induction of a number of hypoxia-associated genes, but it does not affect activity of the HIF-2α isoform (270). Similarly, inhibition of the cell-type specific HIF-

2α isoform with TC-S 7009 involves physically blocking dimerization with HIF-1β by binding the PAS-B domain on HIF-2α (271).

Inhibition of HIF-1α dimerization using TAT-cyclo-CLLFVY resulted in an increase in CSE-induced GM-CSF release from HASMC (Figure 28). This effect occurred both in the presence and absence of sulfite. Additionally, the inhibitor produced a small but significant increase in GM-CSF with sulfite alone (the increase in GM-CSF release with the inhibitor in the baseline cells, which were not given CSE or sulfite, did not reach the level of statistical significance). This indicates that HIF-1α activity is suppressing GM-CSF release.

Inhibiting dimerization of the closely related HIF-2α did not cause a significant change in CSE-induced GM-CSF release, either with or without sulfite (Figure 29). This indicates that the mechanism of suppression of GM-CSF is specific to HIF-1α.

1,4 dihydrophenonthrolin-4-one-3-carboxylic acid (1,4-DPCA) stabilizes HIF-1α protein by inhibiting prolyl-4-hydroxylase (PHD). Similarly, dimethyloxalylglycine (DMOG) also stabilizes HIF-1α by inhibiting prolyl-4-hydroxylase. Inhibition of PHD with 1,4-DPCA (Figure 30) or DMOG (Figure 31) did not affect CSE-induced GM-CSF release. This suggests that inhibiting HIF-1α degradation, and accumulating HIF-1α protein, is not the only factor required to suppress CSE-induced GM-CSF production. It is possible that the inhibition mechanism is only activated above a certain level of NF-κB activation, but this does not coordinate with the effects seen at high inhibitor concentration. In these cases the inhibitor should have greatly reduced NF-κB activation and the HIF-1α-associated pathway inhibiting NF-κB would not be activated. However,

the mechanism of HIF-1α-associated NF-κB inhibition is not yet clearly defined, so the involvement of HIF-1α cannot be eliminated.

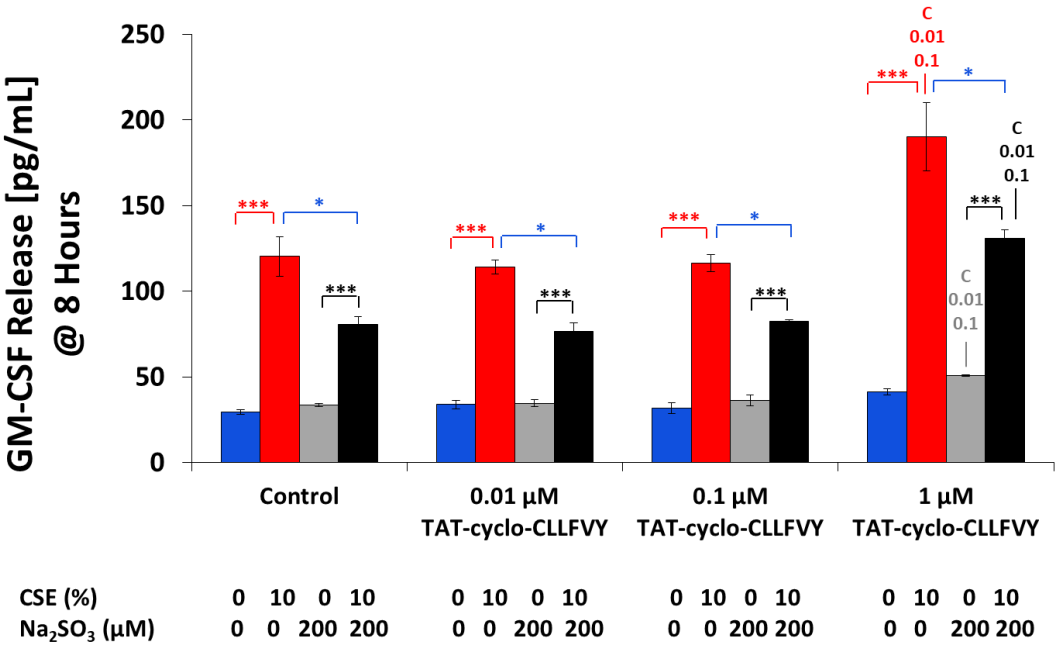

Figure 28: HIF-1α dimerization inhibitor TAT-cyclo-CLLFVY dose response. Error bars: SEM; * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$; n.s. indicates no significant difference; C, D, 0.1 & 0.01 indicate at least $P < 0.05$ compared to control, DMSO, 0.1 µM, or 0.01 µM, respectively, within the same CSE/Na_2SO_3 conditions; n = 3 for all bars.

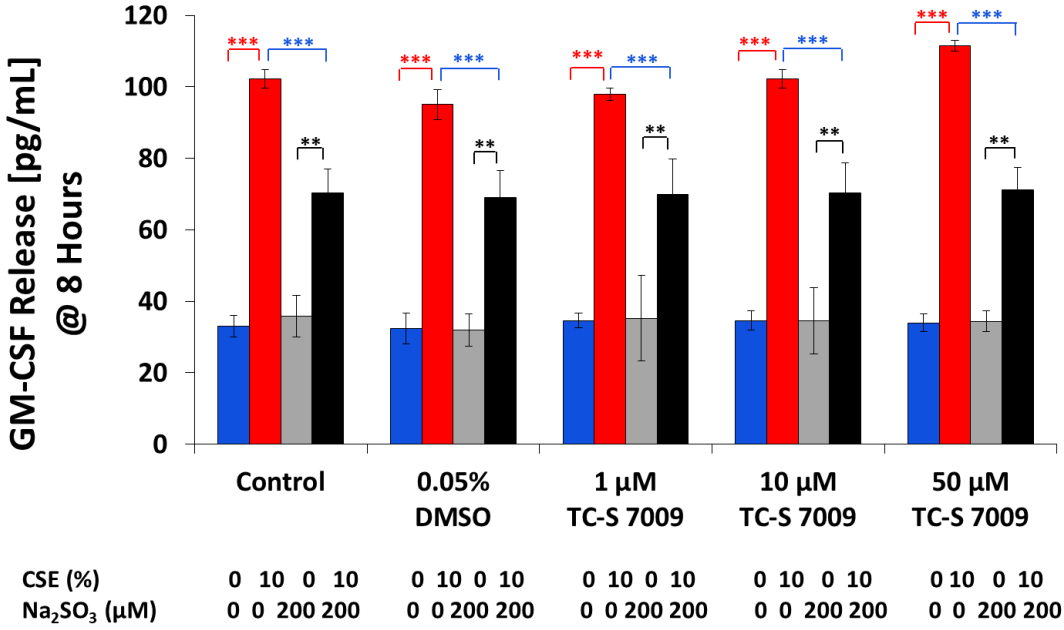

Figure 29: HIF-2α dimerization inhibitor TC-S 7009. Error bars: SEM; * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; n.s. indicates no significant difference; n = 4 for all bars.

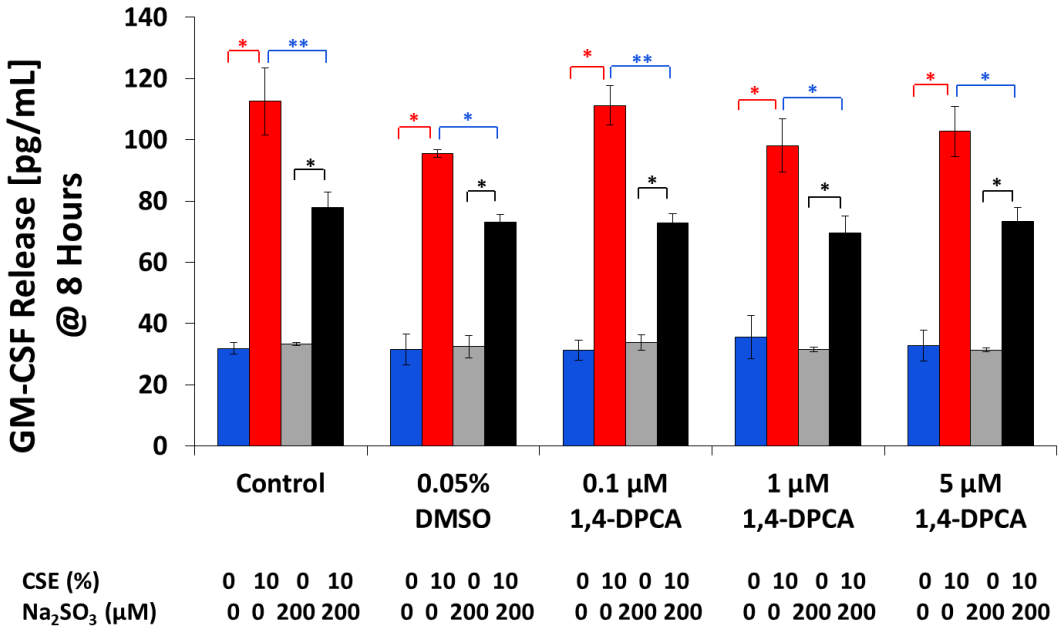

Figure 30: HIF-1α stabilizer 1,4-DPCA. Error bars: SEM; * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; n.s. indicates no significant difference; n = 3 for all bars.

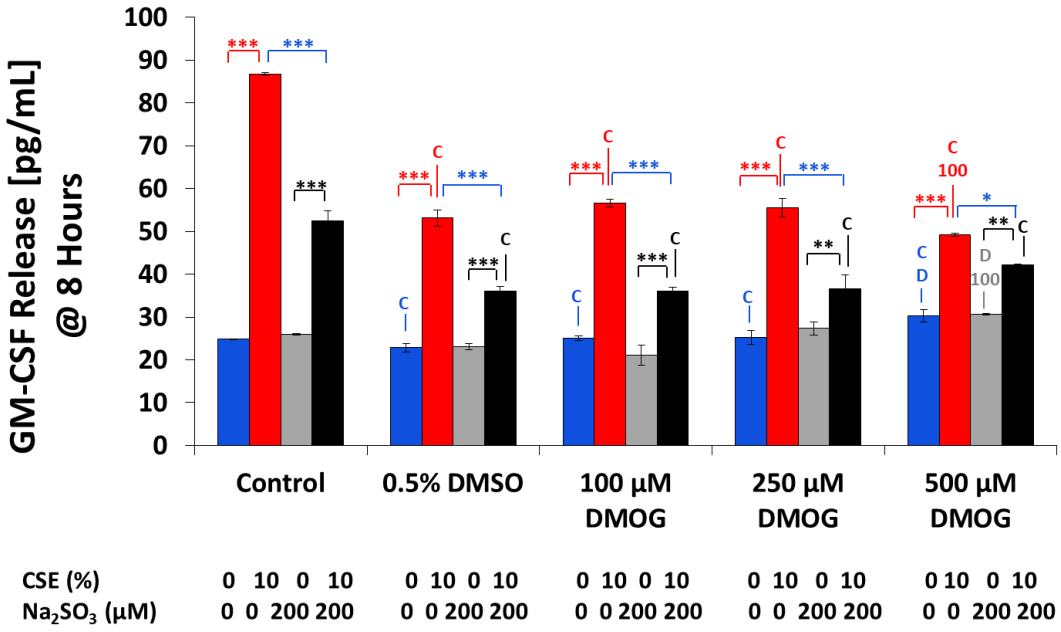

Figure 31: HIF-1α stabilizer DMOG. Error bars: SEM; * indicates P < 0.05; ** indicates P < 0.01; *** indicates P < 0.001; n.s. indicates no significant difference; C, D & 100 indicate at least P < 0.05 compared to control, DMSO, or 100 µM, respectively, within the same CSE/Na_2SO_3 conditions; average of 3 wells from single experiment.

Effect of Inhibitors on MAPK Activation

Treatment of HASMC with the NF-B inhibitor BMS-345541, the p38 inhibitor SB203580, and the TAK1 inhibitor LL-Z1640-2 appears to have had no effect on p38 phosphorylation (Figure 32, top). Unexpectedly, disruption of HIF-1α dimerization with TAT-cyclo-CLLFVY strongly inhibited p38 phosphorylation. This suggests that either HIF-1α activity is needed for p38 phosphorylation or it is needed to prevent p38

dephosphorylation. Since the phosphorylation of MKK3/6 is not affected by the disruption of HIF-1α dimerization, this mechanism appears to be working directly on p38 rather than an upstream kinase.

Although JNK is not very active in HASMC, the inhibition of NF-κB with BMS-345541 produced an increase in CSE-induced JNK phosphorylation (Figure 32, middle). This suggests that NF-κB activity is somehow suppressing JNK activation in these cells. It is possible that JNK may be active when the cellular stimulation does not activate NF-κB in HASMC. However there is no clear indication of a sulfite-based effect on the phosphorylation levels, further suggesting that JNK is not involved in the mechanism of action of sulfite in HASMC.

Inhibition of HIF-1α dimerization with TAT-cyclo-CLLFVY resulted in an increase in p44/42 (ERK1/2) activation both in unstimulated and stimulated HASMC (Figure 32, bottom). This suggests that a decrease in p38 phosphorylation may induce p44/42 phosphorylation. Since the disruption of HIF-1α dimerization results in an increase in GM-CSF release but a decrease in p38 phosphorylation, increased p44/42 activity may be the driving factor in the GM-CSF production. This suggests that p38 has an inhibitory effect on NF-κB activation, rather than an inducing effect. However, inhibition of p38 with SB203580 clearly reduced GM-CSF release which indicates that p38 activation is also needed for GM-CSF release. It is possible that p38 initially activates the mechanisms that result in GM-CSF production and later takes on an inhibitory role. This would account for the seemingly conflicting results.

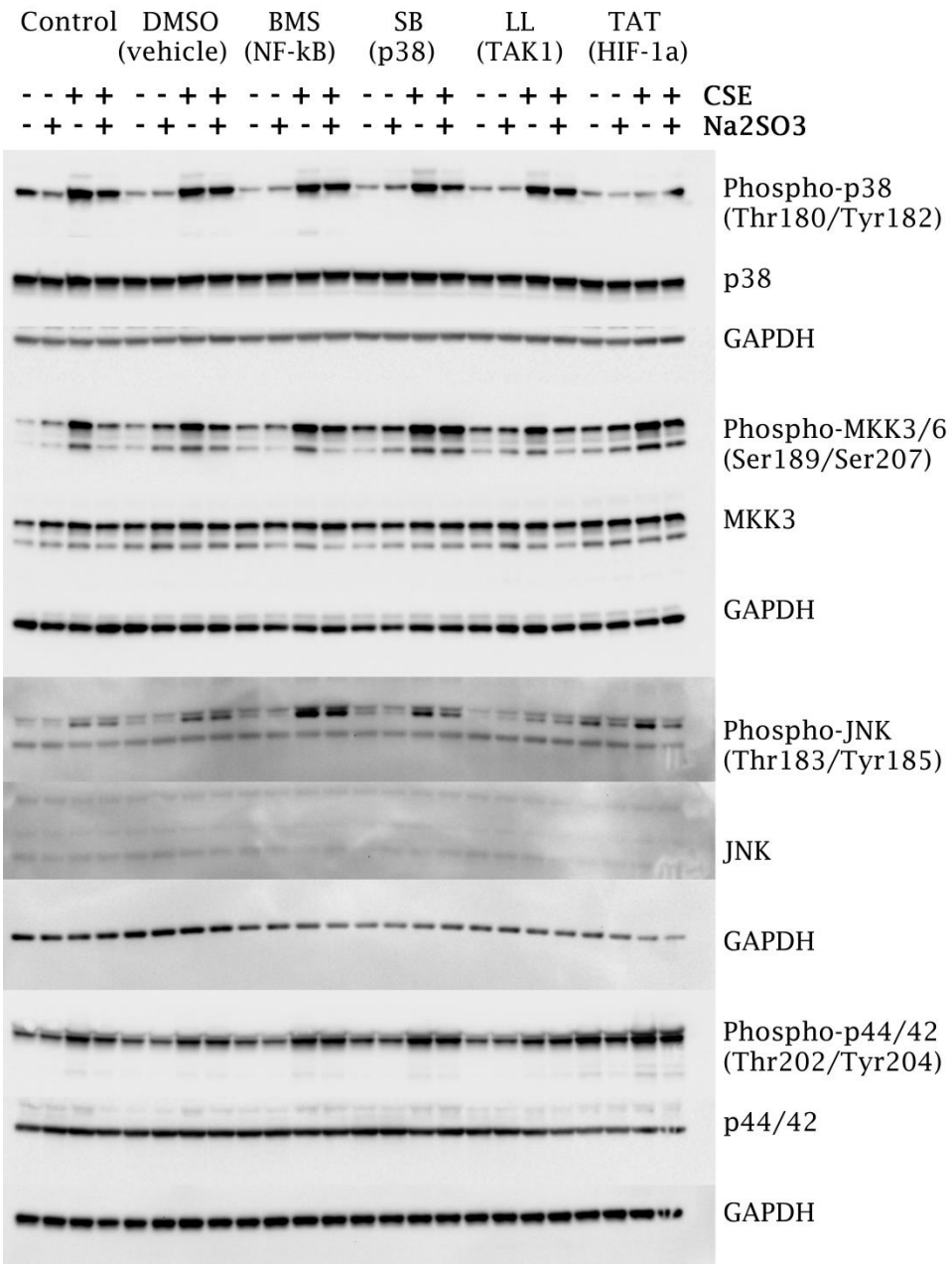

Figure 32: Effect of inhibitors on MAPK phosphorylation. Western blots depicting phospho-protein, total protein and GAPDH loading control for p38, MKK3/6, JNK and p44/42 (ERK1/2) MAPKs at 1 hour. Cells were pre-treated with inhibitor for 1 hour prior to exposures. Representative blots from 2 separate experiments.

GM-CSF mRNA abundance is induced by CSE (Figure 33). While sulfite reduced the transcript level, the difference does not appear to be large enough to account for the entire reduction in GM-CSF release. Sulfite may also affect the translation efficiency. Hydrogen sulfide induces eIF2α phosphorylation which inhibits translation (272). Since there is a connection between hydrogen sulfide, sulfur dioxide and sulfite in the cellular sulfur metabolism pathways (Figure 7), sulfite may also affect eIF2α phosphorylation causing a decrease in translation.

GSK3β has also been identified as a kinase that phosphorylates eIF2b (268). Phosphorylation inhibits eIF2b and decreases protein translation. CSE deactivates GSK3β by phosphorylating it on Ser9 and sulfite reduces this inactivating phosphorylation. Therefore, sulfite would increase eIF2b phosphorylation and reduce translation.

EFFECT OF CSE & SO_2 ON GM-CSF MRNA ABUNDANCE

Corresponding to its effect on GM-CSF release, CSE induced GM-CSF mRNA accumulation in HASMC (Figure 33). This increase in abundance was reduced by sulfite treatment, but at a lesser magnitude compared to the extracellular protein levels. This suggests that sulfite regulation of CSE-induced GM-CSF may also exist at the protein level.

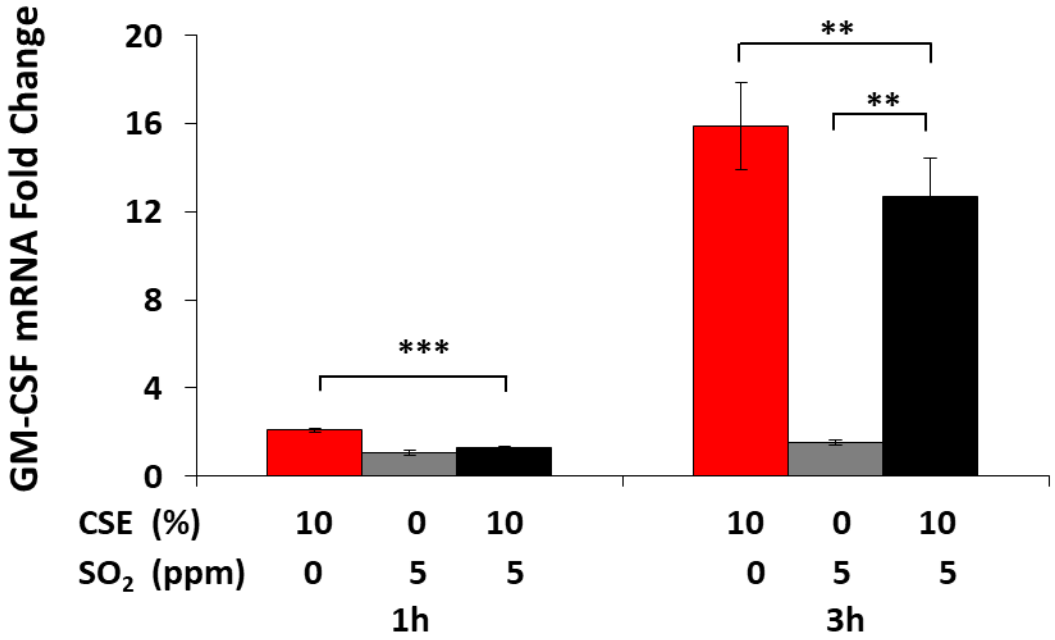

Figure 33: Effect of CSE and SO_2 on GM-CSF mRNA abundance at 1h (n = 5) & 3h (n = 4). mRNA abundance was determined by qRT-PCR using the $2^{-\Delta\Delta Ct}$ method, normalized to HPRT1, and the fold change was calculated compared to 0% CSE, 0 ppm SO_2. Error bars: SEM; ** indicates $P < 0.01$; *** indicates $P < 0.001$; n.s. indicates no significant difference.

Chapter 5 Discussion & Conclusions

Sulfur dioxide has a plethora of research demonstrating its toxic effects in humans, animals and plants. There is however a small but growing body of research that indicates that low concentrations of sulfur dioxide in certain environments is beneficial. Sulfur dioxide has been suggested to be the next gasotransmitter: small endogenously produced gases that act as signaling molecules to modulate biological responses to stimuli. Like the other gasotransmitter, nitric oxide, carbon monoxide, and hydrogen sulfide, it induces smooth muscle relaxation and vasodilation (196). These are effects particularly beneficial to the cardiovascular system. Sulfur dioxide has also been found to induce relaxation and limit proliferation of airway smooth muscle cells (219,240). However, the effect of sulfur dioxide on cigarette smoke-induced induction of pro-inflammatory mediators has not been studied.

Sulfur dioxide is converted to sulfites in aqueous solution. Sodium sulfite has been investigated as a hypoxia inducer (243). Since cigarette smoke also creates a hypoxic state and both have been reported to generate intracellular ROS that drives activation of the inflammation-inducing NF-κB pathway, we hypothesized that exposure to sulfite would augment cigarette smoke-induced inflammatory mediator production through activation of the MAPK and NF-κB pathways and the response would be mediated by HIF-1α activity. We found that p38 MAPK, MKK3/6, p44/42 (ERK1/2) and NF-κB are induced by CSE and co-exposure to sulfite inhibited the activation (**Figure 34**). Chemical inhibition of p38, p44/42, TAK-1 and NF-κB attenuated GM-CSF release from HASMC (**Figure 34**), indicating that they are required for GM-CSF induction. Inhibition of NF-κB increased CSE-induced JNK phosphorylation, but sulfite

had no effect on this activation. Inhibition of HIF-1α dimerization increased p44/42 activation and GM-CSF release but suppressed p38 MAPK phosphorylation (**Figure 34**).

Sulfite decreases the CSE-induced release of GM-CSF, a pro-inflammatory cytokine that promotes eosinophilia and neutrophilia. Elevated levels of GM-CSF are associated with chronic inflammatory diseases such as asthma, COPD, multiple sclerosis, and rheumatoid arthritis and GM-CSF is currently being investigated as a therapeutic target in these diseases (77-79). The inhibitory effect of sulfur dioxide on cigarette smoke-induced GM-CSF release in HASMC indicates it may have therapeutic effects in the airway. However, its augmentation of pro-inflammatory cytokine-induced GM-CSF release is a complicating factor and may limit its use in individuals with pre-existing chronic inflammation.

SULFITE INHIBITS CSE-INDUCED MAPK ACTIVATION

CSE induces activation of p38, JNK and p44/42 in HASMC (**Figure 34**). The activation of p38 and p44/42 extends to the final 8 hour time point investigated. Activation of JNK was rapid and transitory with phosphorylation levels returning to baseline within 30 mins. This suggests that if JNK has a role in CSE-induced pro-inflammatory mediator production, it is as a trigger that helps kick it off. Sulfite attenuates the CSE-induced activation of p38 and p44/42, but does not affect JNK activation. This may be due to the transitory nature of the JNK activation, since initial activation of p38 and p44/42 at 5 minutes was similarly unaffected by sulfite. The delay could indicate that the sulfite signal modulates other pathways first, before being

transmitted to the MAPKs, because p38 is affected within 30 minutes, while p44/42 is down-regulated by sulfite at 2 hours.

Hallsworth, et al. (2001) found that IL-1β stimulation of HASMC induced phosphorylation of p44/42, JNK and p38 MAPK and release of GM-CSF in a dose-dependent manner (273). The p44/42 inhibitor, U1026, and p38 inhibitor, SB203580, inhibited p44/42 and p38 activity respectively (273). However, while U0126 reduced GM-CSF release, SB203580 augmented it, which was unexpected and suggested the presence of a GM-CSF induction mechanism that is suppressed by p38 (273). This effect may be cell type specific because SB203580 inhibits TNF- and IL-1α-induced GM-CSF release in human airway epithelial cells (274).

Our studies demonstrated that CSE-induced GM-CSF release from HASMC was suppressed by the p38 inhibitor SB203580 and the p44/42 inhibitor U0126 (**Figure 34**). However inhibition of p38 MAPK activity did not affect p44/42 phosphorylation levels, indicating that p44/42 activation is not dependent on p38. Others have shown that cigarette smoke activates the EGF receptor, which triggers a Ras-Raf1-MKK1/2-p44/42 signaling cascade (48). This results in phospho-p44/42 translocating to the nucleus and activating the transcription factors AP-1, which induces GM-CSF (48). It has also been shown that sulfur dioxide inhibits serum and PDGF-stimulated proliferation of vascular smooth muscle by activating PKA which phosphorylates Raf-1, suppressing its activation of p44/42 (219,240). Therefore, p44/42 activation may be induced by cigarette smoke-stimulation of the EGF receptor that activates Raf-1, while sulfite stimulates PKA phosphorylation which then phosphorylates Raf-1 and inhibits the p44/42 pathway activation (Figure 35).

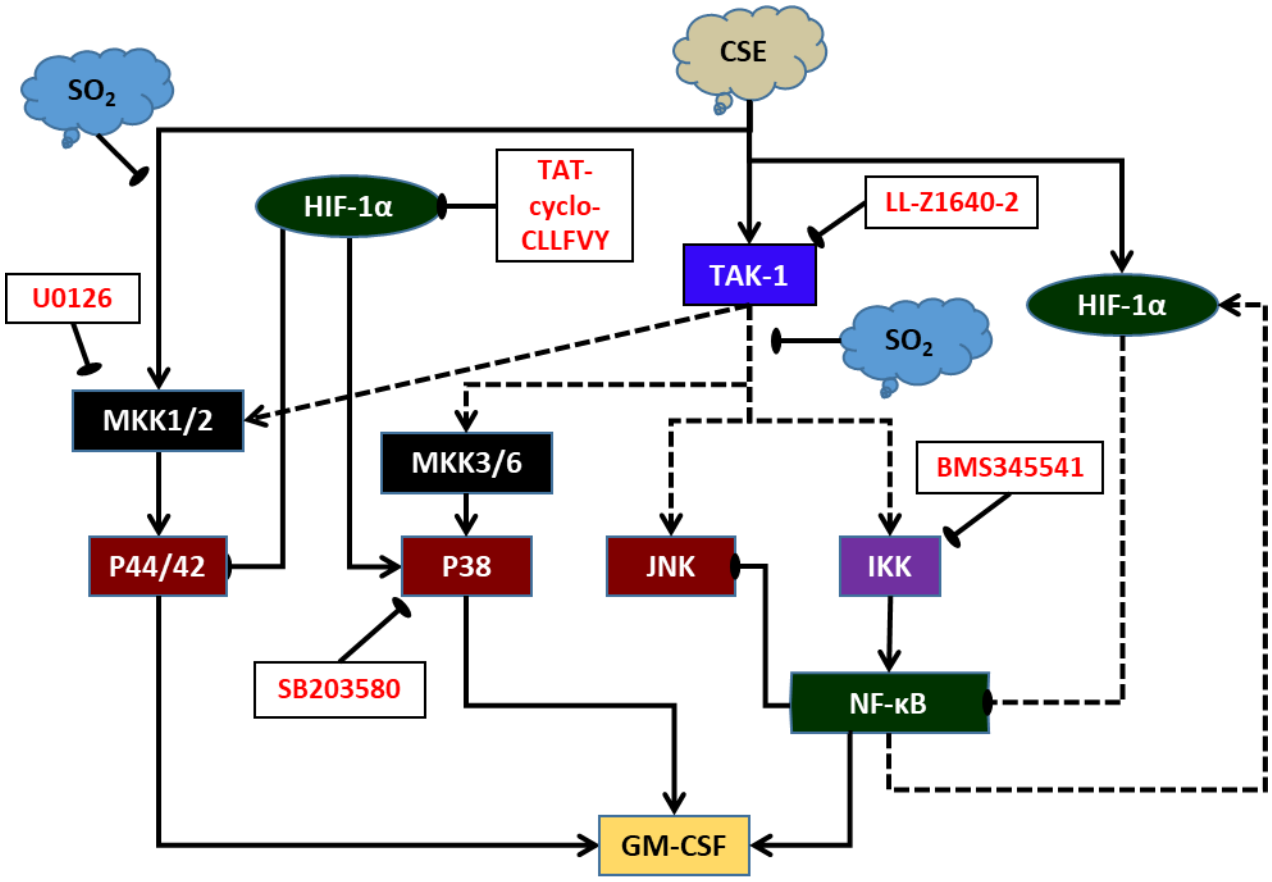

Figure 34: Proposed pathway for CSE and SO_2 effects in HASMC. Arrows indicate activation; blunt ends indicate inhibition; solid lines show demonstrated pathway steps; dashed lines indicate possible interactions requiring additional evidence. IKK: inhibitor of NF-κB kinase; JNK: c-Jun N-terminal kinase; p44/42: extracellular signal-regulated kinase (ERK); GM-CSF: granulocyte macrophage-colony stimulating factor; TAK-1: transforming growth factor β activating kinase 1. Inhibitors are shown in red text.

Others found that inhibition of TAK1 by LL-Z1640-2 inhibits p44/42 phosphorylation in CSE-stimulated airway smooth muscle cells, a result that was not seen here (170). This may be due to the use of a higher inhibitor dose (100 nM instead of 50 nM) in the other studey. LL-Z1640-2 did not inhibit TNF-stimulated MEKK4 or EGF-stimulated p44/42 but it did inhibit LPS-induced TNF, IL-1β, and IL-6 (275,276). This indicates that the specific MAPKKK which activates the p44/42 kinase cascade depends on the stimulus.

Cigarette smoke also activates the PI3K/Akt/NF-κB pathway (277), which provides a mechanism for activation of NF-κB that is not dependent on p38 MAPK. The induction of GM-CSF gene transcription is dependent on both the AP-1 and NF-κB transcription factors. The activation of AP-1 is typically associated with the MAPK pathways, so p44/42 and Akt activation should induce GM-CSF even if p38 and JNK are not activated. However, inhibition of p38 MAPK in our work demonstrated that it is needed for GM-CSF production.

This may be due to the effect of p38 on histone deacetylase (HDAC). NF-κB induced gene expression is dependent on acetylation of histones by histone acetyltransferase (HAT) and the deacetylation by HDAC. Acetylation of histones increases transcription by loosening its grip on the DNA. The MAPKs induce NF-κB activity by altering HAT and HDAC activities. P44/42 (149) and JNK (150) increase HAT activity while p38 inhibits HDAC activity by promoting its degradation (Figure 35) (146). Therefore, these 3 MAPKs promote increased acetylation and transcription and inhibition of any one could result in decreased NF-κB activity and GM-CSF transcription.

Inhibition of HDAC was also shown to decrease p38 phosphorylation (147,148). This suggests that HDAC activity induces p38 phosphorylation and may provide a pathway for the effect that disruption of HIF-1α dimerization had on p38 phosphorylation in our work (Figure 35). HDAC binds the oxygen-sensing domain in HIF-1α and promotes its stability and activity (278). HDAC inhibitors induce HIF-1α degradation (279). HIF-1α activity is inhibited by both impaired dimerization and protein degradation, and in both cases resulted in reduced p38 phosphorylation. Strangely, the reduction in p38 phosphorylation correlates with increased GM-CSF release from HASMC, but the p38 inhibitor SB203580 causes a decrease in GM-CSF release. One possible explanation is that initially p38 induces GM-CSF, but once the NF-κB pathway is strongly activated, it acquires an inhibitory role. The promotion of NF-κB activity by p38-mediated HDAC degradation would dominate early and then an inhibitory function associated with HIF-1α and TAK1 would ensue at a later time.

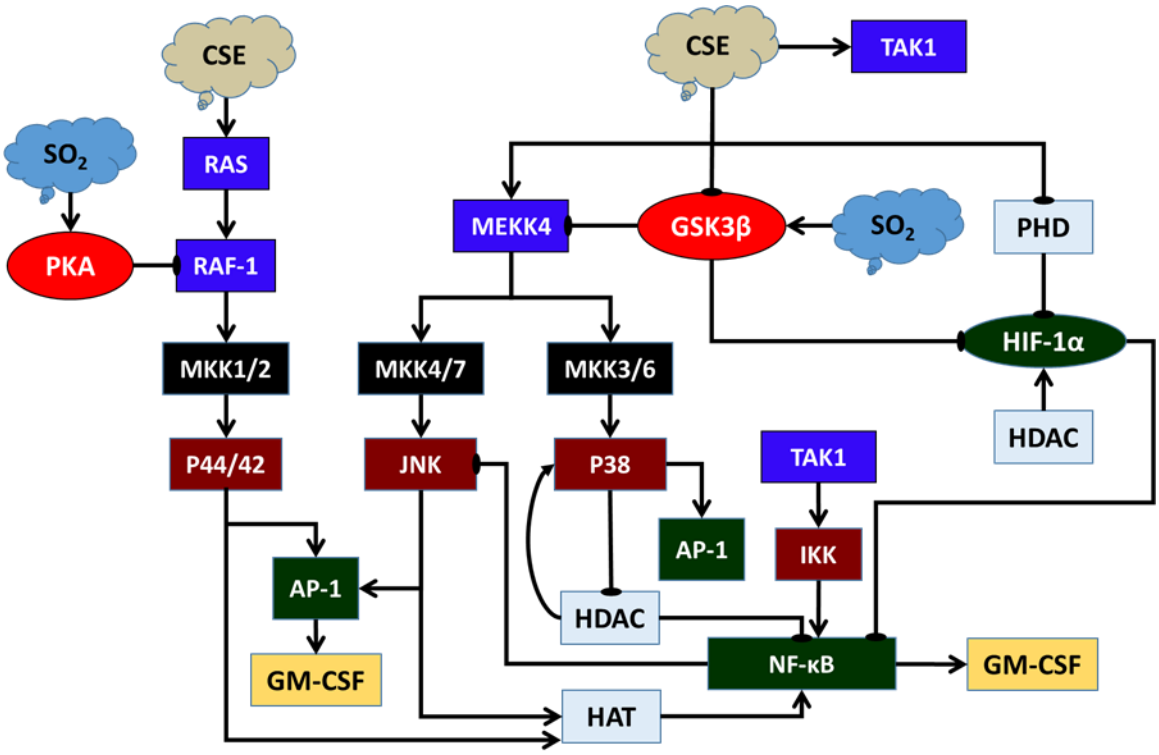

Figure 35: Expanded proposed mechanism for CSE and sulfite-mediated GM-CSF production in HASM, including potential histone acetylation, PKA and GSK3β interactions.

While the effects of sulfur dioxide/sulfite on histone acetylation have not been investigated, other gasotransmitters have been found to affect HAT and HDAC activity. Nitric oxide induces HAT activity and inhibits HDAC (280), but hydrogen sulfide decreases histone acetylation in LPS-stimulated cells (281). Nitric oxide activity would increase gene transcription, while hydrogen sulfide would reduce it. Hydrogen sulfide and sulfur dioxide do not have identical effects, but it is reasonable to predict similar effects in the absence of actual experimental data. Modulation of histone acetylation is therefore a possible mechanism for the effect of sulfite on CSE-induced GM-CSF release.

P38 also modifies mRNA stability and translational efficiency by phosphorylating mRNA binding proteins. The p38 inhibitor SB203580 reduces IL-1β and TNF protein levels by inhibiting p38 phosphorylation of 3'-UTR binding proteins which enhance translation, but it reduces GM-CSF transcription by inhibiting NF-κB transactivation without affecting DNA binding (133,282). SB203580 had no effect on the mRNA stability of thrombin-stimulated GM-CSF (133). This indicates that the regulation of expression of GM-CSF is different from some of the other pro-inflammatory cytokines.

Activation of p44/42, but not p38, stabilizes GM-CSF mRNA in TNF + fibronectin stimulated eosinophils (73). This stabilization was accomplished by binding of proteins such as Y Box binding protein (YB-1) to AU-rich elements in the 3'-UTR of GM-CSF mRNA (73).

Micro RNAs (miRNAs) have been found to regulate GM-CSF expression by binding to the 3'-UTR (283). During oxidative stress in murine alveolar epithelial cells, miR133a and miR133b binding suppressed expression of GM-CSF (283). In normoxic conditions, inhibition of these miRNAs increased basal levels of GM-CSF (283).

Hydrogen sulfide inhibits monosodium urate (MSU) crystal-induced ROS generation, NLRP3 inflammasome activation and caspase-1 activity in macrophages (284). Recently, studies on N-Acetyl-L-Cysteine (NAC) suggested that it may function as an anti-oxidant by inducing the production of endogenous hydrogen sulfide (285). As NAC is upstream of both hydrogen sulfide and sulfur dioxide in the endogenous sulfur metabolic pathway, it is logical to assume that it would also increase cellular sulfur dioxide and sulfite levels. Our work supports this proposal since the effects of NAC administration mirrored sulfite treatment.

While our results suggest that the effect of sulfite on kinase phosphorylation is propagated down through the kinase cascade, another possible explanation for the reduced phosphorylation of the various kinases should be mentioned. Cigarette smoke oxidizes protein tyrosine phosphatases that dephosphorylate the MAP kinases, leaving them unable to deactivate the kinases (286). Sulfur dioxide and sulfites may down-regulate MAP kinase activation by reducing the oxidized phosphatases so that they are capable of dephosphorylating the kinases.

The GM-CSF mRNA levels are up-regulated by cigarette smoke and there is a significant down-regulation by sulfite, but the magnitude of the mRNA change does not directly correspond to the protein inhibition. This indicates that sulfite must also affect mechanisms that regulate translation, protein stability or the rate of protein release from the cell.

There are two known potential targets for sulfite to reduce GM-CSF translation. eIF2B is involved in eukaryotic translation initiation. GSK3β phosphorylates eIF2B, which inactivates it, thereby reducing translation (167). CSE inhibits GSK3β by inducing Ser9 phosphorylation in HASMC, which should increase translation. Sulfite reduces the CSE-induced GSK3β phosphorylation which reduces translation and potentially GM-CSF protein levels. Another potential target for sulfite to reduce GM-CSF translation is eIF2α. The effect of sulfite is unknown, but hydrogen sulfide induces eIF2α phosphorylation which inhibits translation (272).

HIF-1A MEDIATION OF CSE-INDUCED GM-CSF RELEASE FROM HASMC

Like GM-CSF, HIF-1α is an essential inflammatory mediator. HIF-1α is regulated at the protein level by ubiquitin-mediated degradation induced by normoxia. Hypoxia inhibits this proteasomal degradation, but this is far from the only mechanism of HIF regulation. HIF-1α is also stabilized and activated by non-hypoxic pathways. Factor inhibiting HIF (FIH) reduces HIF-1α transcriptional activity by hydroxylating asparagine 803, which impairs binding to its co-activator CBP/p300 (180,181). P44/42 phosphorylates HIF-1α on Ser641 and Ser643inducing HIF-1-mediated transcription (287). Activation of NF-κB subunit p65 upregulates transcription of HIF-1α (288).

Hypoxia has been shown to activate Ras, p38, Akt, NF-κB, PI3K, and AP-1 and inhibition of p38 or PI3K reduced NF-κB activity (289). We found that CSE induced p38, p44/42, JNK, NF-κB, and HIF-1α in HASMC. TGFβ1 stabilizes HIF-1α protein by inhibiting PHD2 transcription (290). Hypoxia induced TGFβ1and Smad4 (291). Disruption of HIF-1α reduces TGFβ and exacerbates induction of pro-inflammatory mediators (292). TGFβ down-regulates GM-CSF production (293). Therefore, the up-regulation of TGFβ by hypoxia generates a feedback mechanism where it stabilizes HIF-1α which induces more TGFβ, and TGFβ suppresses GM-CSF. HIF-1α may also inhibit its own activity by inducing expression of miR-145 which inhibits TGFβ, thereby inhibiting the TGFβ-induced stabilization of HIF-1α protein.

GM-CSF induced phosphorylation of PI3K subunit p85 which subsequently binds to and activates the GM-CSF receptor (294). TGFβ inhibits GM-CSF-induced p44/42 activation by inhibiting PI3K activity in human leukemia cells (294). Therefore,

inhibition of HIF-1α, which would reduce TGFβ, would result in elevated GM-CSF-induced p44/42 phosphorylation. Our results displayed an increase in p44/42 phosphorylation when HIF-1α dimerization was inhibited, suggesting that the HIF-1α mediated GM-CSF suppression is actually due to induction of TGFβ rather than NF-κB inhibition. This inhibition of HIF-1α also resulted in an increase in GM-CSF, therefore the greater levels of GM-CSF and reduced levels of TGFβ should both contribute to an increase in p44/42 activation.

GSK3β phosphorylates HIF-1α resulting in increased proteasomal degradation, which should increase GM-CSF production (191). CSE inhibits GSK3β by phosphorylating it on Ser9, which would increase HIF-1α activity to limit NF-κB activation. Sulfite treatment increased GSK3β activity, which would decrease HIF-1α levels resulting in a loss of the NF-κB suppression. However, sulfite would have already reduced NF-κB activation, and the end result would be a smaller difference in CSE-induced NF-κB activation with and without sulfite. This may account for the unexpectedly small difference in GM-CSF mRNA levels between CSE and CSE/sulfite.

IL-1β stimulation stabilized HIF-1α and induced nuclear translocation via an ROS-associated mechanism that peaked at 2 hours, however high levels of IL-1β (10 ng/mL) were needed (295). IL-1β concentrations that produced a similar GM-CSF release to our CSE-stimulation did not result in ROS generation in HASMC (Figure 38). Similarly, 10 ng/mL TNF induced ROS production and HIF-1α nuclear translocation that was blocked by antioxidants (296).

TNF+IL-4 induces HIF-1α mRNA expression in BEAS-2B cells and the addition of hypoxia augments this transcription, despite the fact that hypoxia alone does not induce HIF-1α mRNA expression in these cells (297). This cytokine activation of HIF-1α is at least partially driven by PI3K and NF-κB activation. Since GM-CSF transcription, like HIF-1α, is strongly linked to activation of NF-κB, this suggests that hypoxia (sodium sulfite treatment) combined with cytokine stimulation will up-regulate GM-CSF transcription, as was seen in our work. TNF+IL-4 also increased HIF-1α translation efficiency. This results in increased HIF-1α activity as evidenced by the elevated VEGF levels from TNF+IL-4+hypoxia-stimulated cells compared to TNF+IL-4 stimulation alone (297). The non-hypoxic (cytokine) pathway that induces HIF-1α activity operates by increasing transcription and translation, while the hypoxic pathway stabilizes the existing protein.

The limitations of this study include the use of HASMC from a single donor to study the mechanisms of action of sulfite on CSE-induced pro-inflammatory mediator induction. While the inhibitory effect of sulfite on CSE-induced GM-CSF release was demonstrated for 8 different donors, mechanistic studies were conducted solely on HASMC from Donor 1. Therefore, there is a possibility some of the effects are donor specific. Additionally, the only inflammatory mediator studied was GM-CSF. While GM-CSF is recognized as a key regulator, it is possible the effect of sulfite on GM-CSF may differ from the effect of sulfite on other inflammatory regulators.

CONCLUSIONS

We have shown that CSE induced GM-CSF release from HASMC in a p38-, p44/42-, TAK1- and NF-κB-dependent manner. Sulfite reduces GM-CSF release by inhibiting activation of the MAPK and NF-κB pathways. The effects of CSE and sulfite may be partially mediated by GSK3β, which can modulate MEKK4, eIF2B and HIF-1α. However, while HIF-1α limits NF-κB activation, and thus GM-CSF transcription, it does not appear that sulfite modulates GM-CSF production through this mechanism. Instead, it appears that the effect of sulfite on CSE-induced GM-CSF release may be the result of multiple mechanisms, such as the MAPK modification of histone acetylation, GSK3β inhibition of translation via eIF2B and possibly a sulfite-induced inhibition of eIF2α.

Appendix

HASMC Response to Direct Cigarette Smoke Exposure & Media Change

Stimulation of HASMC with direct cigarette smoke exposure induces GM-CSF release and this release increases with time (Figure 36A). Changing the cell culture media greatly increases the GM-CSF release from cigarette smoke-stimulated cells (Figure 36B). The effect of media change was also evident in CSE-stimulated HASMC (Figure 36C&D). This is most likely due to the addition of fetal bovine serum (FBS) with the fresh media, although it could also be caused by the removal of inhibitory factors in the conditioned media. The initial experiments performed using direct cigarette smoke involved a media change at the end of the exposure. This resulted in substantially higher GM-CSF production than the CSE-stimulated cultures with no media change. It is highly likely that the increased GM-CSF production from the media change was the result of pathway activation not directly associated with the mechanism of action of cigarette smoke and the media change was eliminated to reduce possible confounding effects.

Interestingly, while IL-1β-stimulated GM-CSF release increased with increasing FBS levels, changing the media with cytokine addition actually reduced the GM-CSF release (Figure 37).

Stimulation with IL-1β did not induce ROS production in HASMC during the first 4 hours (Figure 38). It is possible that ROS may be produced at 8 hours or later but further experiments would be needed to confirm this.

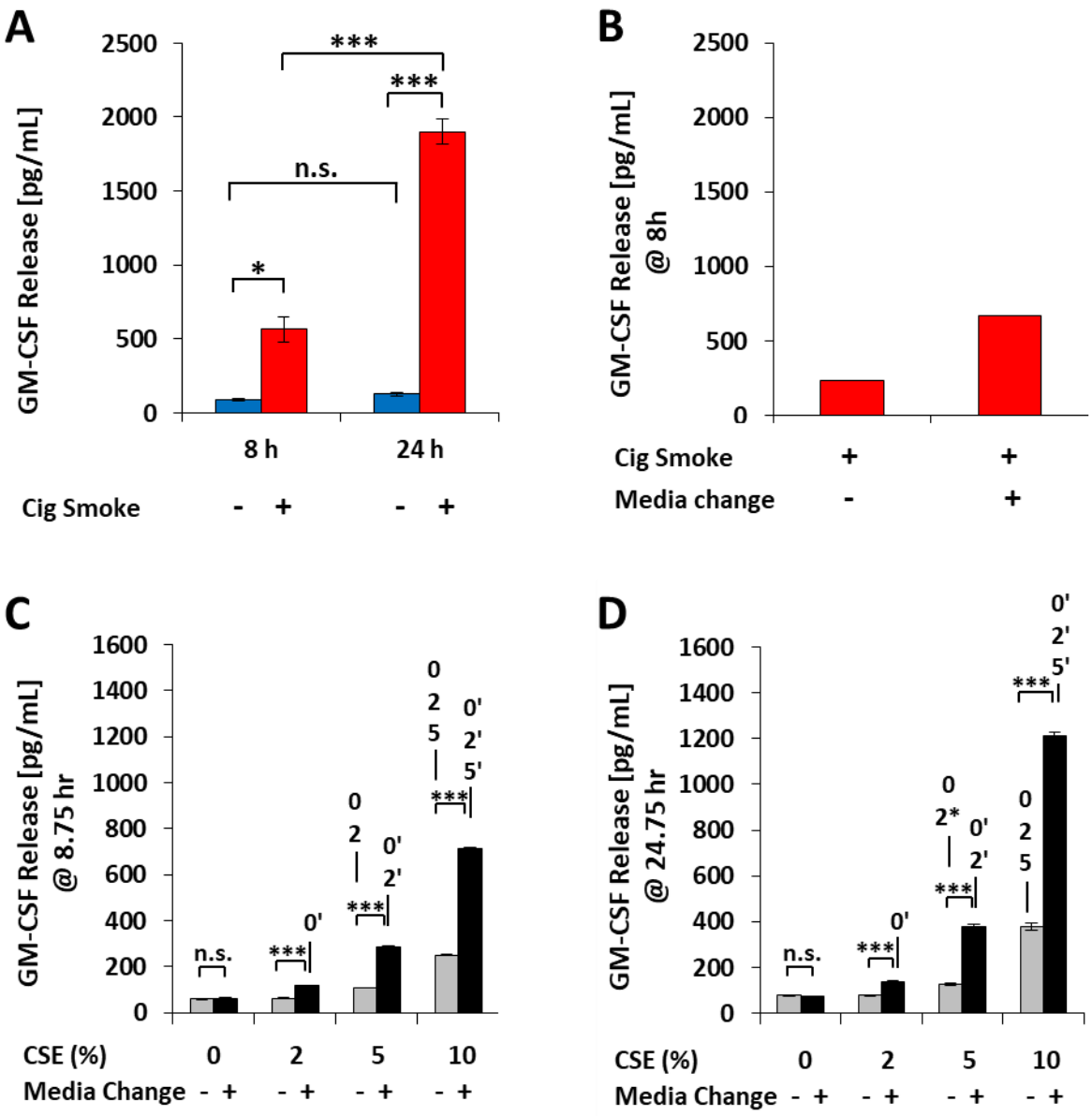

Figure 36: Effect of direct cigarette smoke on GM-CSF release. **A**: direct cigarette smoke exposure with media change at 8 & 24h (n = 6); **B**: Effect of media change 1h prior to exposure (1 well); **C** & **D**: media change with CSE addition (3 well average); ; Error bars: SEM; * indicates $P < 0.05$; *** indicates $P < 0.001$; n.s. indicates no significant difference; 0, 2 or 5 indicates difference from 0%, 2% or 5% CSE, no media change bars, respectively ($P < 0.001$); 0', 2' or 5' indicates difference from 0%, 2% or 5% CSE, media change bars, respectively ($P < 0.001$); 2* indicates difference from 2% CSE, no media change bar ($P < 0.05$).

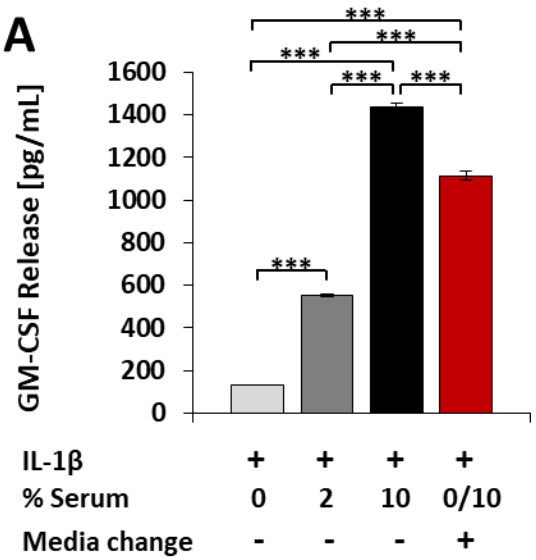

Figure 37: Effect of media change and FBS concentration on IL-1β-stimulated GM-CSF release. IL-1β-stimulation with media change & serum dose response (0/10: 24h serum starvation followed by IL-1β addition in 10% FBS media); Error bars: SEM; *** indicates $P < 0.001$; Average of 3 wells from single experiment.

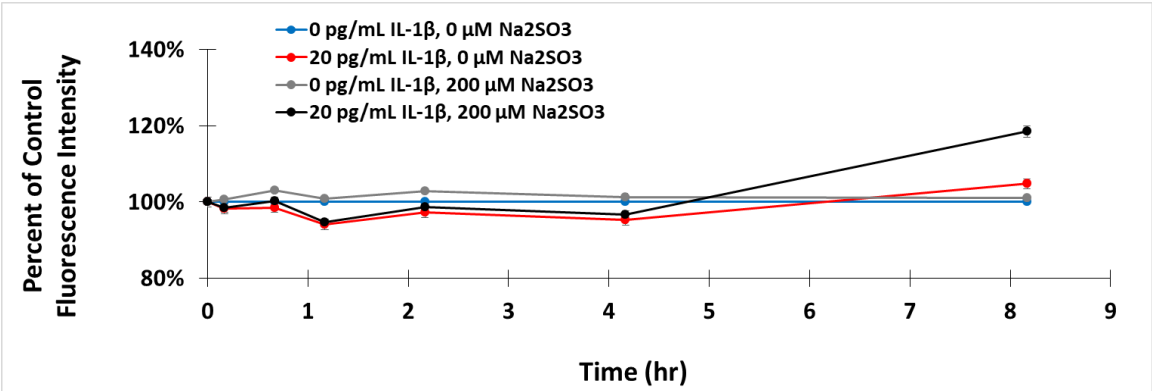

Figure 38: Intracellular ROS levels as a function of H_2DCFDA fluorescence intensity compared to 0 pg/mL, 0 µM Na_2SO_3 control at same time point. Error bars: SEM; average of 10 wells from a single experiment for all points.

Direct cigarette smoke exposure-induced GM-CSF release was reduced by NF-κB inhibition (Figure 39), replicating the results achieved with CSE. This indicates that the HASMC response is attributable to the effects of the cigarette smoke and not altered by the use of CSE.

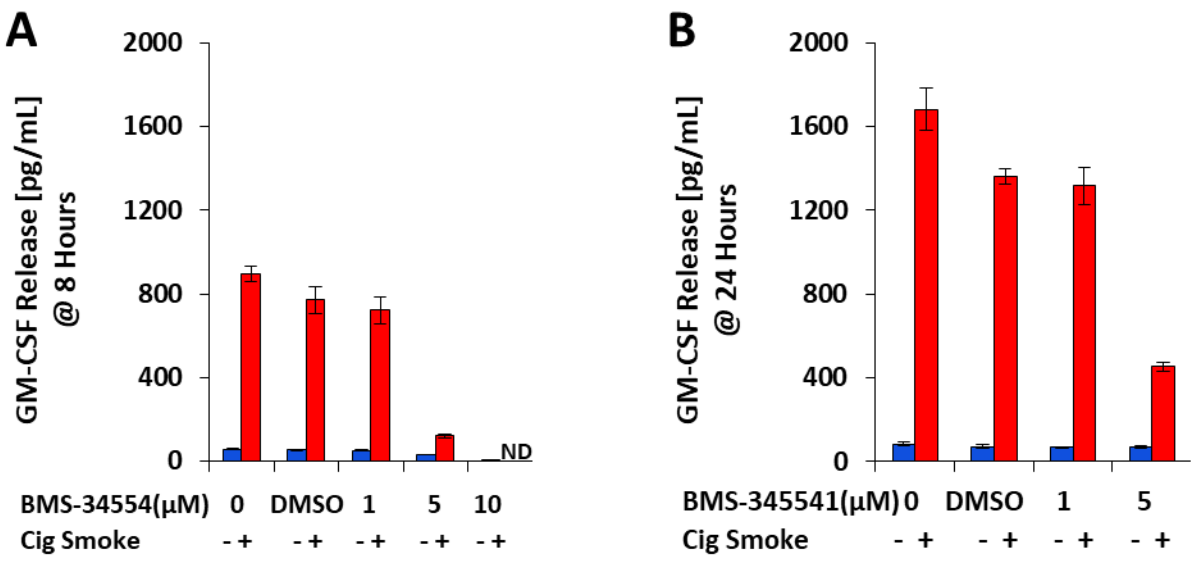

Figure 39: Effect of NF-κB inhibitor on direct cigarette smoke-induced GM-CSF release. NF-κB inhibitor BMS-345541 reduces CS-induced GM-CSF release at 8 hours (A) and 24 hours (B); average of 3 wells from single experiment; Error bars: SEM; ND indicates levels not detectable.

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Vita

Barbara Ann Rolls was born in snowy Buffalo, NY and attended the State University of New York at Buffalo where she received a Bachelor of Science in Aerospace Engineering and a Master of Science in Mechanical Engineering. She worked as an engineer in the automotive industry at several companies before returning to SUNY Buffalo to earn a Bachelor of Science in Biological Sciences. At SUNY Buffalo, she studied the phylogeny of bat parasites in the ecology laboratory of Dr. Katharina Dittmar de la Cruz. Not a big fan of insects, she moved to sunny Galveston, Texas to pursue a Ph.D. in Biomedical Science at the University of Texas Medical Branch. There she found that all things, even bugs, truly are bigger in Texas. Barbara was an active student in the Biochemistry and Molecular Biology Department. She served as chair of the Biological Chemistry Student Organization, organized seminars for several guest speakers, facilitated the Biochemistry Journal Club, and organized several skill development workshops for students. She was a Co-Director of the National Student Research Forum and a member of the GSBS Career Forum Steering Committee. She has received numerous awards and was an NIEHS Pre-doctoral Fellow. Following the completion of her doctoral research, she is returning to the North, where they do not have insects the size of small dogs, to pursue a career in industry.

Education

B.S., Aerospace Engineering, State University of New York at Buffalo

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Honors

Elias Hochman Scholarship, 2017

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Bohdan R. Nechay Scholarship, 2014

GSBS Associates Christina Fleischmann Travel Award, 2014

Kay and Cary W. Cooper, PhD Scholarship, 2014

Robert C. Brasier Award for Outstanding Performance as a Bench Tutorials: Scientific Research and Design Mentor, Galveston Independent School District Bench Tutorials Program, 2014

Dr. Barbara H. Bowman Student Travel Award, 2014

Dr. Mary Faggard Kanz Travel Award for Environmental Toxicology, 2013

Barbara Bowman Scholarship, 2013

The Biological Chemistry Student Organization Award, 2013

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National Institute of Environmental Health Sciences (NIEHS) Pre-doctoral Fellow Toxicology Scholar, 2010

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Abstracts

Rolls B, Dittmar K. 2009. Evolution of Odorant Receptor 83b in Hippoboscoidea. Rochester Academy of Science 30th Meeting. Rochester, NY

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Summary of Dissertation

Sulfur dioxide is an endogenously produced gas, once thought to be solely toxic. More recent studies have demonstrated that at physiological concentrations however it can also have beneficial effects, such as inhibition of inflammation, vasodilation, and relaxation of smooth muscle. Airway smooth muscle cells (HASMC) are involved in multiple aspects of chronic inflammatory airway diseases such as asthma and COPD, including airflow restriction and release of pro-inflammatory mediators. While the response of HASMC to cigarette smoke exposure has been studied, the effects of sulfur dioxide on the cigarette smoke-induced inflammatory mediators have not. It has been proposed that sulfur dioxide creates a hypoxic state, similar to cigarette smoke, which suggested that the hypoxia inducible factor-1α (HIF-1α) may be involved in mediating

the resulting inflammation. This also suggested that the effects of cigarette smoke and sulfur dioxide, two air pollutants commonly present in the industrial areas south of Houston, might be cumulative. This led us to hypothesize that co-exposure to cigarette smoke and sulfur dioxide would antagonize the cigarette smoke-induced inflammation and that HIF-1α would mediate the inflammatory response by its effects on the MAPK and NF-κB pathways. Although sulfur dioxide has been shown to induce inflammation and airway smooth muscle constriction, these effects primarily occur at higher concentration. The exception to this is a group of individuals with severe asthma, COPD or a sensitivity to sulfur dioxide that react negatively to low concentrations of sulfur dioxide (< 1 ppm). These individuals typically have elevated levels of pro-inflammatory cytokines and we have found that exposure of inflammatory cytokine-stimulated HASMC to sulfur dioxide does result in augmented release of GM-CSF, a pro-inflammatory cytokine that has been increasingly recognized as a major regulator of airway inflammation. However, we also found that sulfur dioxide reduced cigarette smoke-induced GM-CSF release from HASMC via down-regulation of the p38, p44/42 and NF-κB pathways. While HIF-1α activity clearly limits the NF-κB-driven induction of GM-CSF, it does not appear that sulfur dioxide is reducing GM-CSF through a HIF-1α mediated mechanism.

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