

Review

Oxidative Stress, Chromatin Remodeling and Gene Transcription in Inflammation and Chronic Lung Diseases

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Inflammatory lung diseases are characterized by chronic inflammation and oxidant/antioxidant imbalance. The sources of the increased oxidative stress in patients with chronic inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) derive from the increased burden of inhaled oxidants, and from the increased amounts of reactive oxygen species (ROS) generated by several inflammatory, immune and various structural cells of the airways. Increased levels of ROS produced in the airways is reflected by increased markers of oxidative stress in the airspaces, sputum, breath, lungs and blood in patients with lung diseases. ROS, either directly or via the formation of lipid peroxidation products such as 4-hydroxy-2-nonenal may play a role in enhancing the inflammation through the activation of stress kinases (JNK, MAPK, p38) and redox sensitive transcription factors such as NF- κ B and AP-1. Recent evidences have indicated that oxidative stress and pro-inflammatory mediators can alter nuclear histone acetylation/deacetylation allowing access for transcription factor DNA binding leading to enhanced pro-inflammatory gene expression in various lung cells. Understanding of the mechanisms of redox signaling, NF- κ B/AP-1 regulation, the balance between histone acetylation and deacetylation and the release and expression of pro- and anti-inflammatory mediators may lead to the development of novel therapies based on the pharmacological manipulation of antioxidants in lung inflammation and injury. Antioxidants that have effective wide spectrum activity and good bioavailability, thiols or molecules which have dual antioxidant and anti-inflammatory activity, may be potential therapeutic agents which not only protect against the direct injurious effects of oxidants, but may fundamentally alter the underlying inflammatory processes which play an important role in the pathogenesis of chronic inflammatory lung diseases.

Keywords: AP-1, Chronic obstructive pulmonary disease, Cigarette smoke, Deacetylases, Histone acetylation, NF- κ B, Reactive oxygen species, Lungs

Introduction

Oxidative stress has been implicated in the pathogenesis of several inflammatory lung disorders. Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($\cdot OH$) are unstable molecules with unpaired electrons, capable of initiating oxidation. Biological systems are continuously exposed to oxidants either generated endogenously by metabolic reactions (e.g., from mitochondrial electron transport during respiration, during activation of phagocytes) or exogenously (such as air pollutants or cigarette smoke). The lung exists in a high-oxygen environment and, together with its large surface area and blood supply, is susceptible to injury mediated by ROS. Increased ROS production has been directly linked to oxidation of proteins, DNA, and lipids which may cause direct lung injury or induce a variety of cellular responses through the generation of secondary metabolic reactive species. ROS may alter remodeling of extracellular matrix, apoptosis and mitochondrial respiration, cell proliferation, maintenance of surfactant and the antiprotease screen, effective alveolar repair response and immune modulation in the lung (Rahman and MacNee 1999, 2000b). Furthermore, increased levels of ROS have been implicated in initiating the lung inflammatory response through the activation of transcription factors such as nuclear factor-*kappa*B (NF- κ B) and activator protein-1 (AP-1), signal transduction, chromatin remodeling (histone acetylation/deacetylation) and gene expression of pro-inflammatory mediators (Rahman and MacNee 1998). It is proposed that ROS produced by phagocytes that have been recruited to sites of inflammation, are a major cause of the cell and tissue damage associated with many chronic inflammatory lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF) and

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adult respiratory distress syndrome (ARDS) (Rahman and MacNee 1996, 1999, 2000a). All these diseases involve the recruitment of immune and inflammatory cells to the lungs. These cells are activated and produce mediators of inflammation including reactive oxygen species (ROS) and cytokines, such as the pro-inflammatory cytokine tumour necrosis factor- α (TNF- α).

This review describes the evidence for the role of ROS in the pathogenesis of chronic inflammatory lung diseases, discuss the ROS-mediated molecular mechanisms (cell signaling, chromatin remodeling and gene expression). Understanding of the molecular mechanisms of ROS-mediated cell signaling pathways would provide information for development of novel antioxidant therapeutic targets in inflammation and injury.

Cell-derived ROS

A common feature of all inflammatory lung diseases is involvement of an inflammatory-immune response, characterised by activation of epithelial cells, and resident macrophages, and the recruitment and activation of neutrophils, eosinophils, monocytes, and lymphocytes. Inflammatory cells once recruited in the airspace become activated and generate ROS in response to a sufficient level of a secretagogue stimulus (threshold concentration). The activation of macrophages, neutrophils and eosinophils generates $O_2^{\cdot-}$, which is rapidly converted to H_2O_2 by superoxide dismutase (SOD), and $\cdot OH$, formed non-enzymatically in the presence of Fe^{2+} as a secondary reaction. ROS can also be generated intracellularly from several sources such as mitochondrial respiration, the NADPH oxidase system and xanthine/xanthine oxidase. However, the primary ROS-generating enzyme is NADPH oxidase, a complex enzyme system that is present in phagocytes and epithelial cells.

Several transition metal salts react with H_2O_2 to form $\cdot OH$. Most studies have focused on iron for the generation of $\cdot OH$ *in vivo*. Iron is a critical element in many oxidative reactions. Free iron in the ferrous form catalyses the Fenton reaction and the superoxide driven Haber-Weiss reaction, which generate the $\cdot OH$, a ROS which damages tissues, particularly to cell membranes by lipid peroxidation. ROS, may also be released by lung epithelial cells (Rochelle *et al.*, 1998) and also stimulate inflammatory cells directly thereby, amplifying lung inflammatory and oxidant events. ROS interact with a variety of molecules and donate electrons in biological systems. Reactive oxygen species also act on certain amino acids in proteins (e.g. enzymes, kinases) such as methionine, tyrosine and cysteine, profoundly altering the function of these proteins in inflammatory lung diseases.

Inhaled Oxidants and Cigarette Smoke

Cigarette smoking, inhalation of airborne pollutants, either oxidant gases (such as ozone, nitrogen dioxide NO_2 , sulphur dioxide SO_2) or particulate matters results in direct lung damage as well as in the activation of lung inflammatory responses. Cigarette smoke is a complex mixture of over 4,700 chemical compounds, including high concentrations of oxidants 10^{14} per puff (Church and Pryor 1985). Short-lived oxidants such as $O_2^{\cdot-}$ and nitric oxide (NO) are predominantly found in the gas-phase. NO and $O_2^{\cdot-}$ immediately react to form highly reactive peroxynitrite (ONOO \cdot). The radicals in the tar phase of cigarette smoke are organic in nature, such as long-lived semiquinone radicals which can react with $O_2^{\cdot-}$ to form $\cdot OH$ and H_2O_2 (Pryor and Stone, 1993). This aqueous phase of the cigarette smoke condensate may undergo redox recycling for a considerable period of time in epithelial lining fluid (ELF) of smokers (Zang *et al.*, 1995). The tar phase is also an effective metal chelator and can bind iron to produce tar-semiquinone + tar- Fe^{2+} , which can generate H_2O_2 continuously. Furthermore, since both cigarette tar and lung epithelial lining fluid contain metal ions, such as iron, Fenton reaction will result in the production of the $\cdot OH$ which is a highly reactive and potent ROS.

ROS and Membrane Lipid Peroxidation

Reactive oxygen species such as $O_2^{\cdot-}$ and $\cdot OH$, generated and released by activated immune and inflammatory cells are highly reactive, and when generated close to cell membranes oxidise membrane phospholipids (lipid peroxidation) which may initiate a chain reaction. Thus, a single $\cdot OH$ can result in the formation of many molecules of lipid hydroperoxides in the cell membrane. The peroxidative breakdown of polyunsaturated fatty acids impair membrane function and inactivate membrane-bound receptors and enzymes, increase tissue permeability which have been implicated in the pathogenesis of many forms of lung injury. There is increasing evidence that aldehydes, generated endogenously during the process of lipid peroxidation, are involved in many of the pathophysiological effects associated with oxidative stress in cells and tissues. Compared with free radicals, lipid peroxidation aldehydes are generally stable, can diffuse within, or even escape from the cell and attack targets far from the site of the original free radical event. In addition to their cytotoxic properties, lipid peroxides are increasingly recognised as being important in signal transduction for a number of important events in the inflammatory response (Fig. 1).

Isoprostanes (a member of F_2 -isoprostane family) are ROS catalysed isomers and stable end-products of non-enzymatic

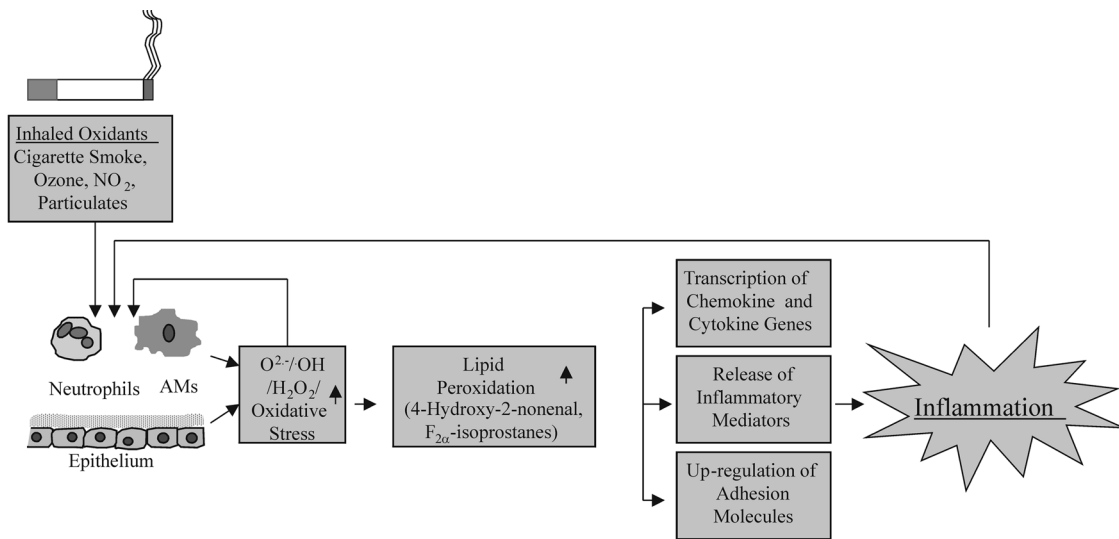


Fig. 1. Mechanisms of ROS-mediated lung inflammation. Inflammatory response is mediated by oxidants either inhaled and/or released by the activated neutrophils, alveolar macrophages, eosinophils and epithelial cells leading to production of ROS and membrane lipid peroxidation. Activation of transcription of the pro-inflammatory cytokine and chemokine genes, up-regulation of adhesion molecules and increased release of pro-inflammatory mediators which is involved in the inflammatory responses in patients with chronic inflammatory diseases.

lipid peroxidation of arachidonic acid. F₂-isoprostane is a potent smooth muscle cell constrictor and a mitogen and modulates platelet as well as other cell functions *in vitro* via membrane receptors (thromboxane A₂) for prostaglandins (Morrow and Roberts, 1997). 4-hydroxy-2-nonenal (4-HNE), a highly reactive diffusible end product of lipid peroxidation, is known to induce/regulate various cellular events in particular proliferation, apoptosis and activation of signaling pathways (Parola *et al.*, 1999; Uchida *et al.*, 1999). 4-HNE has a high affinity towards cysteine, histidine and lysine residues, alters protein function and forms direct protein-adducts. Many of the effects of ROS in airways may be mediated by the secondary release of inflammatory lipid mediators such as 4-HNE. Inhibition of lipid peroxidation, specifically the pathways leading to the production of 4-HNE and F₂-isoprostane, may be used as targets for antioxidant therapy in inflammation and injury in patients with chronic lung diseases such as asthma, COPD, IPF and ARDS.

ROS-mediated DNA Damage

Cigarette smoke has been shown to induce sister chromatid exchanges, micronuclei, cell transformations *in vitro* and tumor induction *in vivo* (Perera *et al.*, 1987). Cigarette smoke-derived free radicals/oxidants have been shown to damage DNA, e.g. incubation of bacteriophage DNA with buffered aqueous extracts of cigarette tar results in a dose-dependent production of single-strand breaks in DNA (Boris 1987). Cigarette smoke condensate (CSC)-mediated DNA strand break is protected by [•]OH radical scavengers suggesting that

the [•]OH is responsible for DNA nicks caused by cigarette smoke condensate (Pryor, 1992). It has been shown that cigarette smoke tar radical (quinone) complexes with or becomes bonded to the DNA suggesting that CSC acts as a site- or base-sequence-specific DNA cleavage agent. Bermudez *et al* have shown that tar component in sidestream cigarette smoke produces DNA nicks which was prevented by glutathione in rat alveolar macrophages (Bermudez *et al.*, 1994). Similarly Izzotti *et al* have shown that N-acetylcysteine inhibited the carcinogen-DNA adducts in the tracheal epithelium of rats exposed to cigarette smoke (Izzotti *et al.*, 1995).

Chronic cigarette smoking increases the number of neutrophils in lung fluid and organic compounds such as catechol and hydroquinone which may activate these cells to produce increased amount of ROS. Leanderson and Tagesson have reported that neutrophils can cause oxidative DNA damage (formation of 7-hydro-8-oxo-2 deoxyguanosine (8-oxodG) in alveolar epithelial cells through the generation of ROS. Similarly, Asami *et al* have demonstrated increased levels of 8-hydroxyguanine in leukocytes obtained from smokers as compared to nonsmokers (Asam *et al.*, 1996). Furthermore, increased levels of cigarette smoke-mediated carcinogen-DNA have been demonstrated in lung tissue of smokers, which may be associated with increased iron burden in lower respiratory tract (Thompson *et al.*, 1991; Philips *et al.*, 1998). This suggests that the recruitment of inflammatory cells may cause oxidative DNA damage in lung cells which may be a contributing factor in the pathogenesis of lung cancer.

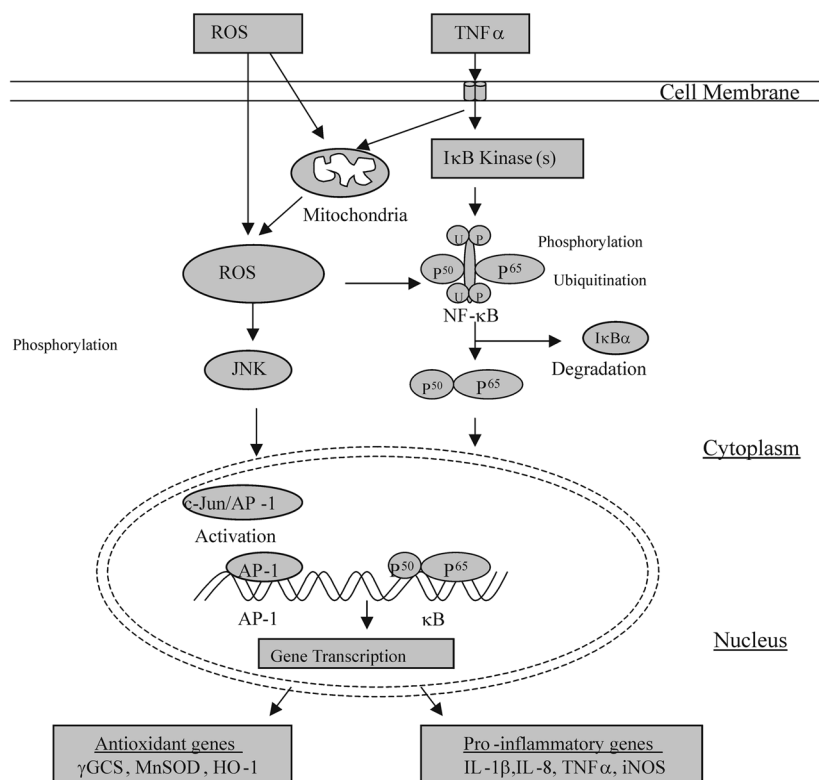


Fig. 2. Model for the ROS-mediated mechanism of NF- κ B and AP-1 activation leading to the gene transcription. ROS directly or indirectly (its generation via mitochondria) are involved in the activation of NF- κ B and AP-1. Activation of NF- κ B involves the phosphorylation, ubiquitination, and subsequent proteolytic degradation of the inhibitory protein I κ B. Free NF- κ B then translocates into the nucleus and binds with its consensus sites. Similarly, AP-1 either c-Jun/c-Jun (homodimer) or c-Fos/c-Jun (heterodimer) is activated by the phosphorylation of JNK pathway leading to the activation AP-1 and binds with its TRE consensus regions. Activation of NF- κ B/AP-1 leads to the co-ordinate expression of antioxidant protective and pro-inflammatory genes.

Role of ROS in Signal Transduction

The expression of inflammatory mediators can be regulated by the activation of redox-sensitive transcription factors AP-1 and NF- κ B stimulated in response to ROS and TNF- α (Rahman and MacNee, 1998; Rahman *et al.*, 2001c) (Fig. 2). In addition to ROS, cellular redox status, particularly intracellular thiol status can be directly involved in the activation of AP-1 and NF- κ B, signal transduction and gene expression involved in cellular pathophysiological activities. Both environmental or inflammatory cell-derived ROS can lead to the activation and phosphorylation of the mitogen activated protein kinase (MAPK) family, including extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 kinase, and the phosphoinositide 3 signaling protein (PI-3K) via sensitive cysteine rich domains, sphingomyelinase-ceramide pathway, leading to increased gene transcription (Thannickal and Fanburg, 2000). Activation of members of the MAPK family leads to the transactivation of transcription factors such as c-Jun, activating factor-2 (ATF2), cyclic AMP response element binding proteins (CREB)-binding protein (CBP) and Elk-1 (Adler *et al.*, 1999). This eventually results in chromatin remodeling and

expression of genes regulating a battery of distinct pro-inflammatory and antioxidant genes involved in several cellular events including apoptosis, proliferation, transformation and differentiation.

The exact intracellular molecular mechanism of ROS action has not been completely characterised. Redox sensitive molecular targets usually contain highly conserved cysteine residues, and their oxidation, nitration, and formation of disulfide links are crucial events in oxidant/redox signaling. It is hypothesized that oxidation of those sulfide groups in signaling proteins causes structural modifications, resulting in the exposure of active sites and protein activation. Such molecular targets include transcription factors (NF- κ B and AP-1), signaling molecules such as ras/rac or JNK, protein tyrosine phosphatases and p21^{ras}. Thiol molecules such as intracellular glutathione (GSH) and thioredoxin are of central therapeutic importance in the regulated control of such redox signaling pathways, by reducing disulfide bridges or oxidised cysteine residues (Rahman and MacNee, 2000a).

Recent studies have shown that in response to tumour necrosis factor (TNF α) and lipopolysaccharide (LPS), which are relevant stimuli for the inflammatory response in chronic inflammatory lung diseases, airway epithelial cells can

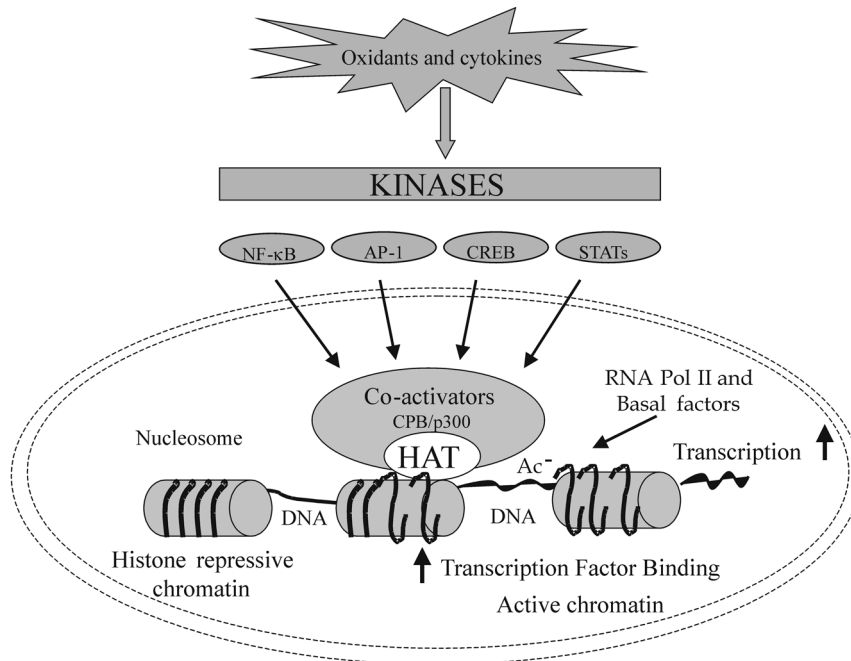


Fig. 3. Co-activator transcriptional complex. The intrinsic HAT activity of master co-activator CBP/p300 and other transcription factors/co-activators acetylate lysine moiety of histone proteins which leads to the loosening of chromatin (gene transcription).

concurrently produce increased amounts of intracellular ROS. This intracellular production of oxidants and the subsequent changes in intracellular GSH redox status is important in the molecular events controlling the expression of genes for inflammatory mediators. The signaling pathways and activation of transcription factors in response to ROS are subject of rigorous investigation.

Role of ROS in Chromatin Remodeling and Gene Transcription

Chromatin remodeling Gene transactivation is controlled by multimeric complexes of transcriptional coactivators and co-repressors which bind to consensus sites within gene promoters and regulate transcription. Many factors, including specific DNA sequences, histones, non-histone chromosomal proteins, transcriptional activators/repressors and the transcription machinery are all necessary for the establishment of an active transcription complex. Condensation of eukaryotic DNA in chromatin suppresses gene activity through the coiling of DNA on the surface of the nucleosome core and the folding of nucleosome assemblies, thus decreasing the accessibility to the transcriptional apparatus (Wu, 1997). The basic unit of chromatin, the nucleosome, consists of 146 base pairs of DNA wrapped around two subunits each of highly conserved core histones H2A, H2B, H3 and H4. Tightly bound DNA around a nucleosome core (histone proteins H2A, H2B, H3 and H4), suppresses gene transcription by decreasing the accessibility to transcription

factors, such as NF- κ B and AP-1 to the transcriptional complex. Acetylation of lysine residues in the N-terminal tails of the core histone proteins results in uncoiling of the DNA, allowing increased accessibility for transcription factor binding. Acetylation of lysine (K) residues on histone 4 (K5, K8, K12, K16) is thought to be directly related in the regulation of gene transcription (Imhof and Wolffe, 1998). However, core histones may also be modified by phosphorylation, methylation, ADP-ribosylation, or ubiquitination of a specific amino acid residue (Davie and Spencer, 2001). Histone acetylation is reversible and is regulated by a group of acetyltransferases (HATs) which promote acetylation, and deacetylases (HDACs) which promote deacetylation.

The nuclear receptor coactivators, steroid receptor co-activator 1 (SRC-1), cyclic AMP response element binding (CREB)-binding protein (CBP)/adenoviral protein E1A (p300) protein, CBP/p300 associated factor (P/CAF), and Activator Transcription Factor-2 (ATF-2), all possess intrinsic HAT activity (Ogryzko *et al.*, 1996; Kawasaki *et al.*, 2000; Pham *et al.*, 2000). Of these, CBP/p300 and ATF-2, which are regulated by MAP kinase pathways, are vital for the co-activation of several transcription factors including NF- κ B and AP-1 in the transcription machinery (Thomson *et al.*, 1999). These activation complexes act with RNA polymerase II to initiate transcription (Fig. 3). Thus, it is likely that acetylation of H3/H4 via CBP/p300 and/or ATF-2 has a significant role in the activation of NF- κ B/AP-1-mediated gene expression for pro-inflammatory mediators, although the precise molecular mechanisms are still not fully understood.

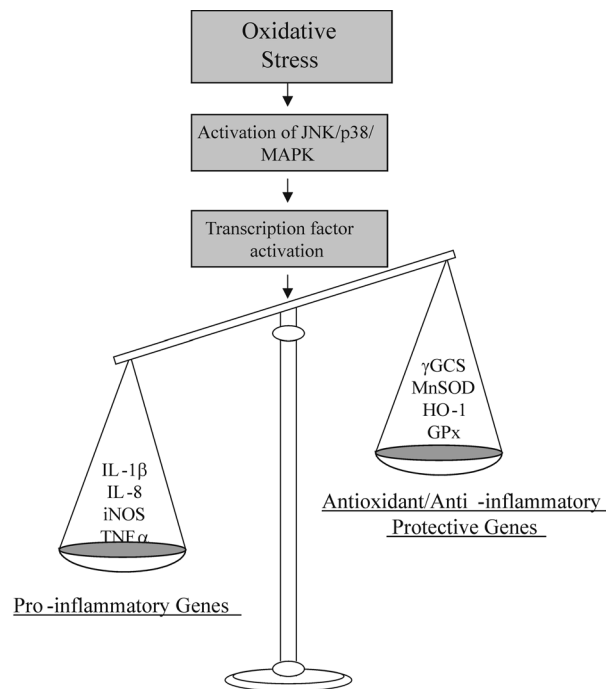


Fig. 4. Activation of transcription factors NF- κ B and AP-1 by oxidants and/or pro inflammatory cytokines activate MAP kinase pathways that lead to the induction of both pro-inflammatory and anti-inflammatory/antioxidant genes in lung cells. In inflammation, the balance appears to be tipped in favour of increased pro-inflammatory mediators, either because of release of inflammatory mediators, or amplification of the pro-inflammatory effects. Induction of antioxidant protective genes may be a delayed response and declined sharply or overtaken by pro-inflammatory effects.

Disruption of the nucleosome or DNA unwinding caused by deacetylation inhibitors facilitates AP-1 binding (Ng *et al.*, 1997). It has been suggested that oxidant generating systems and proinflammatory mediators influence histone acetylation/phosphorylation via a mechanism dependent on the activation of the MAPK pathway (Bohm *et al.*, 1997; Tikoo *et al.*, 2001; Miyata *et al.*, 2002). Recent evidences have shown that oxidative stress induced by H_2O_2 and TNF- α increases the activation of AP-1 and NF- κ B, and may regulate chromatin remodeling leading to IL-8 expression (Rahman *et al.*, 2001b). This may have role in cell proliferation, apoptosis and imbalance in gene transcription for pro-inflammatory mediators and antioxidant protective genes.

The family of HDAC enzymes consists of ten distinct deacetylases. HDACs remove the acetyl moieties from the ϵ -acetamido groups of lysine residues of histones (restoration of positive charges) causing rewinding/condensation of DNA associated with displacement of transcription factors from their cognitive DNA binding sites leading to silencing of gene transcription. HDACs associate in multimeric complexes which serve to direct these proteins to specific promoter sites. HDACs represent a super family of molecules sharing a 390-amino acid region of homology known as the deacetylase core (Finnin *et al.*, 1999). HDACs are found complexed to corepressor molecules like Sin3, N-CoR, and SMRT. Recently, the role of HDACs (1 and 2) has been shown in the

regulation of cell proliferation and corticosteroid-mediated inhibition of pro-inflammatory mediators (Sambucetti *et al.*, 1999; Ito *et al.*, 2000). Several distinct HDACs are now recognized, and these are differentially expressed and regulated in different cell types. This may contribute to the responsiveness to corticosteroids between different stimuli, genes and cell types.

Gene transcription Inflammatory mediators play a crucial role in chronic inflammatory processes and appear to determine the nature of the inflammatory response by directing the selective recruitment and activation of inflammatory cells and their perpetuation within the lungs. In *in vitro* studies, using macrophage, alveolar, and bronchial epithelial cells, ROS have been shown to cause increased gene expression of inflammatory mediators such as IL-1 and TNF- α . Direct or indirect oxidant stress to the airway epithelium and alveolar macrophages may also generate cytokines such as TNF- α which in turn can activate airway epithelial cells to induce proinflammatory genes such as TNF- α , IL-8, IL-1, inducible NO synthase, COX-2, ICAM-1, IL-6, MIP-1, GM-CSF, stress response genes (HSP-27, 70, 90, HO-1) and antioxidant enzymes (γ -glutamylcysteine synthetase γ -GCS, MnSOD, thioredoxin). The genes for these inflammatory mediators are regulated by redox-sensitive transcription factors such as NF- κ B and AP-1 (Fig. 4).

An important effect of oxidative stress and inflammation is the upregulation of protective antioxidant genes. Among the antioxidant enzymes, GSH and its redox enzymes appear to have an important protective role in the airspaces and intracellularly in epithelial cells. Oxidative stress causes upregulation of γ -GCS, an important enzyme involved in the synthesis of GSH, as an adaptive mechanism against subsequent oxidative stress. Important protective antioxidant and stress response genes such as mRNA for MnSOD, γ -GCS, heme oxygenase-1 (HO-1), glutathione peroxidase, thioredoxin reductase and metallothionein are induced by various oxidative stresses including hyperoxia and inflammatory mediators such as TNF- α and lipopolysaccharide in lung cells.

Thus oxidative stress, including redox modulation, causes increased gene expression of both pro-inflammatory genes by oxidant-mediated activation of transcription factors such as AP-1 and NF- κ B and also activation of stress response protective genes such as γ -GCS-HS, HO-1 and MnSOD in lungs. Both ROS, and redox modulation cause increased gene expression of both pro-inflammatory genes by ROS-mediated activation of redox-sensitive transcription factors and also activation of stress response and antioxidant protective genes in the lungs. A balance may therefore exist between pro- and anti-inflammatory gene expression and the levels of GSH in response to ROS and during inflammation, which may be critical to whether this leads to cell injury or protection against the injurious effects of inflammation.

Smokers and Patients with Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is a slowly progressive condition characterised by airflow limitation, which is largely irreversible. Airway inflammation in COPD, is characterized by predominantly by neutrophils and macrophages. COPD is characterized by chronic inflammation of the respiratory tract, even in ex-smokers. The increased oxidant burden derives from the fact that cigarette smoke, contains an estimated 10^{14} oxidants per puff and many of these are relative long-lived such as tar-semiquinone which can generate \cdot OH and hydrogen peroxide (H_2O_2) by the Fenton reaction (Pryor and Stone, 1993; Zang *et al.*, 1995). It is reported that more than 90% of patients with COPD are smokers, but not all smokers develop COPD (American Thoracic Society, 1995; British Thoracic Society, 1997). 15-20% of cigarette smokers appear to be susceptible to its effects and show a rapid decline in forced expiratory volume in one second (FEV₁) and develop the disease. The major risk factor for COPD is cigarette smoke, which is one of the most potent oxidants. Other factors that may exacerbate COPD, such as air pollutants, infections, and occupational dusts, are also potential oxidants. Thus, oxidants present in cigarette smoke and environmental pollutants may play an important role in the pathogenesis of COPD.

ROS in Alveolar Space

The ROS burden in the lungs is enhanced in smokers by the release of ROS from macrophages and neutrophils. Oxidants present in cigarette smoke can stimulate alveolar macrophages to produce ROS and to release a host of mediators, some of which attract neutrophils and other inflammatory cells into the lungs. Both neutrophils and macrophages, which are known to migrate in increased numbers into the lungs of cigarette smokers, compared with non-smokers can generate ROS via the NADPH oxidase complex system. Moreover, the lungs of smokers with airway obstruction have more neutrophils than smokers without airway obstruction (Bosken *et al.*, 1992). Circulating neutrophils from cigarette smokers and patients with exacerbations of COPD release more $O_2^{\cdot-}$ (Rahman *et al.*, 1996b). Cigarette smoking is associated with increased content of MPO in neutrophils (Aaron *et al.*, 2001), which is correlated with the degree of pulmonary dysfunction (Fiorini *et al.*, 2000). MPO activity was also found to be correlated negatively with FEV₁ in patients with COPD, suggesting that neutrophil MPO-mediated oxidative stress and inflammatory response play an important role in the pathogenesis of COPD (Gompertz *et al.*, 2001).

ROS in Blood

The circulating neutrophil appears to be a critical cell in the pathogenesis of COPD. Previous epidemiological studies have shown a relationship between circulating neutrophil numbers and the FEV₁ (Chan-Yeung *et al.*, 1988). Moreover, a relationship has also been shown between the change in peripheral blood neutrophil count and the change in airflow limitation over time. Similarly, a correlation between $O_2^{\cdot-}$ release by peripheral blood neutrophils and bronchial hyperreactivity in patients with COPD has been shown, suggesting a role for systemic ROS in the pathogenesis of the airway abnormalities in COPD (Postma *et al.*, 1988). Another study has shown a relationship between peripheral blood neutrophil luminol enhanced chemiluminescence, as a measure of the release of ROS and measurements of airflow limitation in young cigarette smokers (Richards *et al.*, 1989).

Various studies have demonstrated increased production of $O_2^{\cdot-}$ from peripheral blood neutrophils obtained from patients during acute exacerbations of COPD, which returned to normal when the patients were restudied when clinically stable (Rahman *et al.*, 1997). Other studies have shown that circulating neutrophils from patients with COPD show upregulation of their surface adhesion molecules, which may also be an oxidant-mediated effect (Rahman *et al.*, 1996b; Noguera *et al.*, 1998). Activation may be even more pronounced in neutrophils which are sequestered in the pulmonary microcirculation in smokers and in patients with COPD, since neutrophils which are sequestered in the pulmonary microcirculation in animal models of lung

inflammation release more ROS than circulating neutrophils (Brown *et al.*, 1995). Thus neutrophils, which are sequestered in the pulmonary microcirculation may be a source of ROS, and may have a role in inducing endothelial adhesion molecule expression in COPD.

Generation of ROS-mediated Lipid Peroxidation Products

Isoprostanes are products of non-enzymatic lipid peroxidation and have therefore been used as markers of oxidative stress. The isoprostanes (members of F₂-isoprostane), are ROS catalysed isomers of arachidonic acid and are stable lipid peroxidation products, which circulate in plasma and are excreted in the urine. The levels of lipid peroxides, such as 8-isoprostane and the hydrocarbons ethane and pentane, are increased in exhaled air condensate in smokers and in patients with COPD (Montuschi *et al.*, 2000; Paredi *et al.*, 2000). Furthermore, the increased levels of these markers of lipid peroxidation products have been correlated with airway obstruction (decline in FEV₁). We have recently shown that the plasma and BALF levels of F₂-isoprostane were increased in smokers and patients with COPD. Furthermore, the level of F₂-isoprostanes was more pronounced in plasma during exacerbations of COPD. Thus it indicates that there is increased lipid peroxidation in patients with COPD, implicating direct oxidative damage of epithelial cells and an enhanced airway inflammatory response. Lipid peroxidation may also have a role in the signaling events in the molecular mechanisms involved in the lung inflammation in COPD. However, it is not known whether the increased level of lipid peroxidation products found in these diseases are the result of primary lung-associated processes, such as alveolar macrophage activation, neutrophil activity, or caused by the ongoing lipid peroxidative chain reaction in the alveoli, parenchyma, or airways, which are induced by inhaled oxidants/cigarette smoke.

Indirect and non-specific measurements of lipid peroxidation products, such as thiobarbituric acid reactive substances (TBARS), have also been shown to be elevated in breath condensate and in lungs of patients with stable COPD (Morrison *et al.*, 1999; Nowak *et al.*, 1999). The levels of plasma lipid peroxides (TBARS) have been shown to be elevated in COPD and negatively correlated with the FEV₁, suggesting that increased oxidative stress occur in patients with COPD (Rahman *et al.*, 1996b). Thus the crude measurement of the levels of TBARS might not be a marker to discriminate between asthma and COPD. Oxidative stress, measured as lipid peroxidation products in plasma, has also been shown to correlate inversely with the % predicted FEV₁ in a population study (Britton *et al.*, 1995). This indicates that in patients with COPD lipid peroxidation may play a role in the progression of the disease.

Specific end products of lipid peroxidation may be a good

marker of COPD. 4-HNE is a highly reactive and specific diffusible end-product of lipid peroxidation. Our preliminary data indicate increased 4-HNE-modified protein levels in airway and alveolar epithelial cells, endothelial cells and neutrophils in subjects with airway obstruction compared to subjects without airway obstruction (Rahman *et al.*, 2001a). This demonstrates not only the presence of 4-HNE but that 4-HNE modifies proteins in lung cells to a greater extent in patients with COPD. The increased level of 4-HNE-adducts in alveolar epithelium, airway endothelium and neutrophils was inversely correlated with FEV₁, suggesting a role for 4-HNE in the pathogenesis of COPD.

Cigarette Smoke-mediated Lipid Peroxidation, Cell Signaling and Gene Transcription

Lipid peroxidation products, in particular aldehydes, derived from cigarette smoke have been shown to act as a signal for activation of transcription factors and gene expression leading to inflammatory response. Cigarette smoke extract stimulates protein kinase C, possibly by the formation of aldehydes/lipid peroxidation products in human bronchial epithelial cells (Wyatt *et al.*, 1999). Cigarette smoke can activate epidermal growth factor (EGF) receptors by tyrosine phosphorylation, resulting in the induction of mucin synthesis in epithelial cells and *in vivo* in lungs (Takeyama *et al.*, 2001). Levels of ras^{p21} oncoprotein were higher in plasma of patients with COPD, suggesting cytogenetic damage and abnormal signal transduction by smoking (Cebulska *et al.*, 1999). *In vitro* experiments have demonstrated that cigarette smoke condensate induces a distinct pattern of stress response in cultured epithelial cells, which may be related to the reported pro-inflammatory activities of CSC (via the formation of ROS/lipid peroxidation products) *in vitro* and *in vivo*.

Oxidative stress has been implicated in the expression of both pro-inflammatory and protective antioxidant genes. The *c-fos* gene belongs to a family of growth and differentiation-related immediate early genes, the expression of which generally represents the first measurable response to a variety of chemical and physical stimuli. Studies in various cell lines have shown enhanced gene expression of the *c-fos* in response to cigarette smoke (Muller, 1995). These effects of cigarette smoke can be mimicked by peroxy nitrite and smoke-related aldehydes (4-HNE/F₂-isoprostanes, acrolein, acetaldehyde) in concentrations that are present in cigarette (Muller *et al.*, 1997). AP-1 (c-Fos/c-Jun) DNA binding is increased in epithelial and endothelial cells in response to cigarette smoke condensate (CSC), whereas CSC causes an initial decrease in the DNA binding of NF-κB following by a subsequent 2-fold increase in DNA binding (Muller and Gebel, 1998; Freed *et al.*, 2001; Gebel and Muller, 2001). This is associated with the lack of phosphorylation and degradation of IκB-α in CSC treated cells. NF-κB and AP-1, and its components are increased in lungs of smokers and patients with COPD.

Recently, Mochida-Nishimura *et al.* have demonstrated that increased activation of NF- κ B and MAP kinase pathways by LPS occurs in BAL cells from smokers than non-smokers (Mochida-Nishimura *et al.*, 2001). They found that activation of p38 was more rapid in BAL cells from smokers compared to non-smokers. However, Chang *et al.* have shown that MAP kinases (MEK1 and ERK2) were increased without any change in the levels of p38 in terminal bronchioles in the lungs of rats exposed to cigarette smoke (Chang *et al.*, 2001). Furthermore, recent animal studies have shown that cigarette smoking induces neutrophil influx to the airspace, increased IL-8 release and NF- κ B activation in the lungs. These effects were abolished by recombinant SOD treatment suggesting cigarette smoke-mediated oxidative stress regulate the molecular events in lung inflammation (Nishikawa *et al.*, 1999). The level of IL-8 was also increased in bronchial epithelial cells of smokers which was associated with airway inflammation (Mio *et al.*, 1997). In fact, cigarette smoke extract treatment of cultured human bronchial epithelium increased the levels of IL-8 and this effect was mimicked by acrolein and acetaldehyde (major components of cigarette smoke). This study suggested that lipid peroxidation products may increase the levels of IL-8 in lungs of smokers which may contribute to lung inflammation (Mio *et al.*, 1997). Similarly Zhang *et al.* showed that interleukin-6 was induced in mouse spleen and liver in response to sidestream cigarette smoke. The elevated level of IL-6 was blocked by dietary antioxidants (Zhang *et al.*, 2001). In an interesting study conducted by Rusznak and co-workers, bronchial epithelial cells from smokers with COPD showed greater susceptibility to the effects of cigarette smoke, releasing greater levels of IL-1 β and sICAM-1 compared with smokers without COPD (Rusznak *et al.*, 2000). This study provides some evidence as to why only a proportion of smokers develop COPD. The increased burden of proinflammatory mediators and upregulation of adhesion molecules may be involved in the recruitment and trafficking of inflammatory cells into the airway epithelium seen in smokers and patients with COPD.

Cigarette smoke-mediated lipid peroxidation has been shown to be involved in epithelial remodeling during lung injury. The levels of 4-HNE-adducts are increased in epithelial cells in patients with COPD, which may be of relevance for the understanding of epithelial changes in this disease. In addition to its ability to increase the expression of proinflammatory mediators, 4-HNE has also been shown to induce apoptosis in T cells (Liu *et al.*, 2000) and cause activation of the epidermal growth factor receptor (EGFR) in human epidermoid carcinoma cells, which results in growth inhibition (Liu *et al.*, 1999). Previous studies have shown that cigarette smoke increases mucin production in epithelial cells, a mechanism in which the EGFR was implicated (Takeyama *et al.*, 1993). Therefore, it is tempting to speculate that enhanced 4-HNE levels may contribute to mucus cell hyperplasia (induction of muc5ac mRNA), as observed in COPD. In turn, this points/implicates to a potential role for 4-

HNE in the signaling events involved in lung inflammation leading to the development of COPD.

Oxidative stress and 4-HNE have been shown to induce cellular stress responses, such as cell signaling via the MAP kinase pathways, leading to the induction of AP-1-mediated genes (Uchida *et al.*, 1999; Leonarduzzi *et al.*, 2000), for example, the antioxidant γ -GCS mRNA in alveolar epithelial cells (Liu *et al.*, 2001). γ -GCS expression is increased in lungs of patients with COPD (Rahman *et al.*, 2000) and in response to cigarette smoke in alveolar epithelial cells (Rahman *et al.*, 1996a). The induction of γ -GCS may be an important adaptive response of the alveolar epithelium to oxidative stress. Cigarette smoke-mediated oxidative stress has also been implicated in the expression of other protective antioxidant genes. The expression of a number of antioxidant genes was increased in the bronchial epithelial cells in rats exposed to whole cigarette smoke for up to 14 days (Gilks *et al.*, 1998). These include the mRNA of manganese superoxide dismutase (MnSOD), metallothionein (MT) and glutathione peroxidase, suggesting the oxidant/peroxide-mediated upregulation of a battery of antioxidant genes which may be important protective mechanism against the effects of cigarette smoke. Cigarette smoke-induced oxidative stress also causes upregulation of HO-1 and heat shock protein-70 in both human monocytes and endothelial cells as well as in the human premonocytic line U937 cells (Favatier and Polla, 2001; Vayssier-Taussat *et al.*, 2001). An imbalance of an array of redox-regulated antioxidant versus pro-inflammatory genes might be associated with the susceptibility or tolerance to disease (Fig. 4).

Role of ROS and Cigarette Smoke-induced Oxidative Stress in Chromatin Modeling: Role for Histone Acetylation and Deacetylation

The role of the nucleosome remodeling in the control of gene transcription co-activator and transcription factor access to the target promoter sites of genes is increasingly viewed as an important regulatory mechanism for the transcriptional activation of genes. Levels of histone acetylation have been directly related to the levels of gene transcription. Oxidative stress and proinflammatory mediators have been suggested to influence histone acetylation and phosphorylation by ADP-ribosylation, via a mechanism dependent on the activation of MAPK pathway (Miyata *et al.*, 2001; Tikoo *et al.*, 2001). Recently, we and other investigators have shown that both H₂O₂ and TNF- α (relevant stimuli for cigarette smoke-mediated inflammatory response) caused an increase in histone acetylation (HAT activity) in alveolar epithelial cells (Ito *et al.*, 2001; Rahman *et al.*, 2001b). The exact mechanism of increased histone acetylation in response to these agents is not clear. It has been reported that oxidants and TNF- α activate MAPK pathways, specifically ERK and JNK, and by activation of these redox-dependent pathways these agents

may regulate ATF-2 and CBP co-activators that possess intrinsic HAT activity (Thomson *et al.*, 1999; Kawasaki *et al.*, 2000).

ROS and TNF- α increase the activation of AP-1 and NF- κ B, and regulate chromatin remodelling leading to IL-8 and IL-6 expression in lung cells (Lakshminarayanan *et al.*, 1998; Berghe *et al.*, 1999). Recently Ito and co-workers have shown a role for histone acetylation and deacetylation in IL-1 β -induced TNF- α release in alveolar macrophages derived from cigarette smokers (Ito *et al.*, 2001). They have also suggested that oxidants may play an important role in the modulation of HDAC and inflammatory cytokine gene transcription. Our preliminary data indicated that cigarette smoke condensate increased the acetylation of histone 4 associated with decreased levels of HDAC 1 and 2 levels in alveolar epithelial cells (Rahman *et al.*, unpublished data). This observation is confirmed in our laboratory in sputum neutrophils where HDAC 1 and 2 expression was associated with NF- κ B activation in COPD patient (Drost *et al.*, unpublished data). We also showed that inhibiting HDACs alone resulted in enhanced activation of AP-1 and NF- κ B and increased histone acetylation culminating in increased IL-8 release (Rahman *et al.*, 2001b). This observation corroborated with previous studies showing acetylation of histone proteins is associated with increased binding of the transcription factor AP-1 and NF- κ B (Ng *et al.*, 1997; Chen *et al.*, 2001). Thus cigarette smoke-mediated oxidative stress may produce chronic inflammatory response in the airways. IL-8 release was also augmented when trichostatin A (histone deacetylase inhibitor) was combined with TNF- α or H₂O₂, associated with NF- κ B binding (Fig. 5). This suggests that inhibition of HDAC allows NF- κ B to retain in the nucleus and triggers augmented TNF- α or H₂O₂-mediated gene transcription. In return, NF- κ B itself is acetylated, which participates in active transcription machinery for prolong transcription (Chen *et al.*, 2001).

It is now known that p65, a component of the NF- κ B transcription factor, has intrinsic HAT activity and transactivation of p65 is independent of nuclear translocation (Ashburner *et al.*, 2001; Ito *et al.*, 2001). It has been recently shown that HDAC1 can interact directly with the p65 subunit of NF- κ B to exert its corepressor function in the nucleus. Therefore, NF- κ B interaction with HDAC (1 and 2) proteins may be a further mechanism whereby NF- κ B can regulate transcription. HDACs may be dissociated/phosphorylated from binding to nuclear p65 by oxidants leading to enhanced p65 acetylation/phosphorylation resulting in IL-8 gene expression. This pathway may not require classic NF- κ B/I κ B kinase pathway.

It has been reported that IL-8 and IL-6 release are enhanced by HDAC inhibitors in intestinal epithelial cells and in murine fibrosarcoma L929sA cells (Berghe *et al.*, 1999; Fusunyan *et al.*, 1999), which also enhance the effect of IL-1 or TNF- α treatments. Similarly, Pender and co-workers have recently demonstrated that HDAC inhibitors (increasing the overall level of acetylation of the histone proteins) enhanced the

A model proposing modulation of histone acetylation and deacetylation in gene transcription

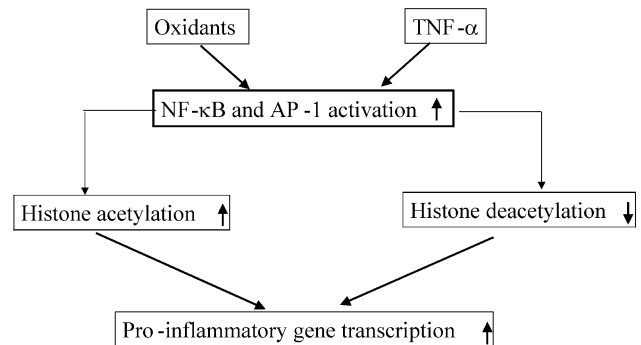


Fig. 5. Schematic diagram showing ROS and TNF- α -mediated activation of NF- κ B and AP-1 leading to alteration in histone acetylation/deacetylation and increased pro-inflammatory gene transcription.

levels of stromelysin-1 (matrix metalloproteinase-3) by augmenting histone acetylation by TNF- α or IL-1-stimulated mesenchymal cells (Pender *et al.*, 2000). Shankaranarayanan and co-workers have shown that acetylation of histone 3 and STAT6 by CBP/p300 is associated with 15-lipoxygenase-1 gene expression induced by IL-4 in A549 lung epithelial cells (Shankaranarayanan *et al.*, 2001). Moreover, it is reported that HDAC inhibitors enhance pulmonary cells responsiveness to a subsequent stressor, such as H₂O₂ and TNF- α , leading to increased transcription factor DNA-binding and enhanced gene expression (Rahman *et al.*, 2001b). This has an implication in inflammatory lung disease states where the HDAC enzyme is inactivated. In these cases, ROS and TNF- α would lead to a further increased inflammatory response from the tissue.

ROS and CSC-mediated inhibition of HDAC-2 levels is also supported by the observations that various proinflammatory mediators such as intercellular adhesion molecule-1 (ICAM-1), IL-8, IL-6, TNF- α , IL-1 β , monocyte chemoattractant protein-1, matrix metalloproteinases and heat shock proteins, which are increased in BAL fluid of smokers, are also induced by inhibition of histone deacetylases. Inhibition of HDAC in tumour cell lines leads to specific chromatin acetylation and alteration of cell cycle proteins, including upregulation of p21^{waf1/cip1} and cyclin E (Sambucetti *et al.*, 1999). Recent data from our laboratory showed that cigarette smoke-mediated inhibition of HDAC may upregulate p21^{waf1/cip1} in alveolar epithelial cells suggesting another role of cigarette smoke in cell cycle regulation (Marwick *et al.*, unpublished data). It has been shown that glucocorticoid suppression of inflammatory genes requires recruitment of HDAC-2 to the transcription activation complex by the glucocorticoid receptor (Ito *et al.*, 2000). This results in deacetylation of histones and a decrease in inflammatory gene transcription. Our preliminary data also suggested that H₂O₂

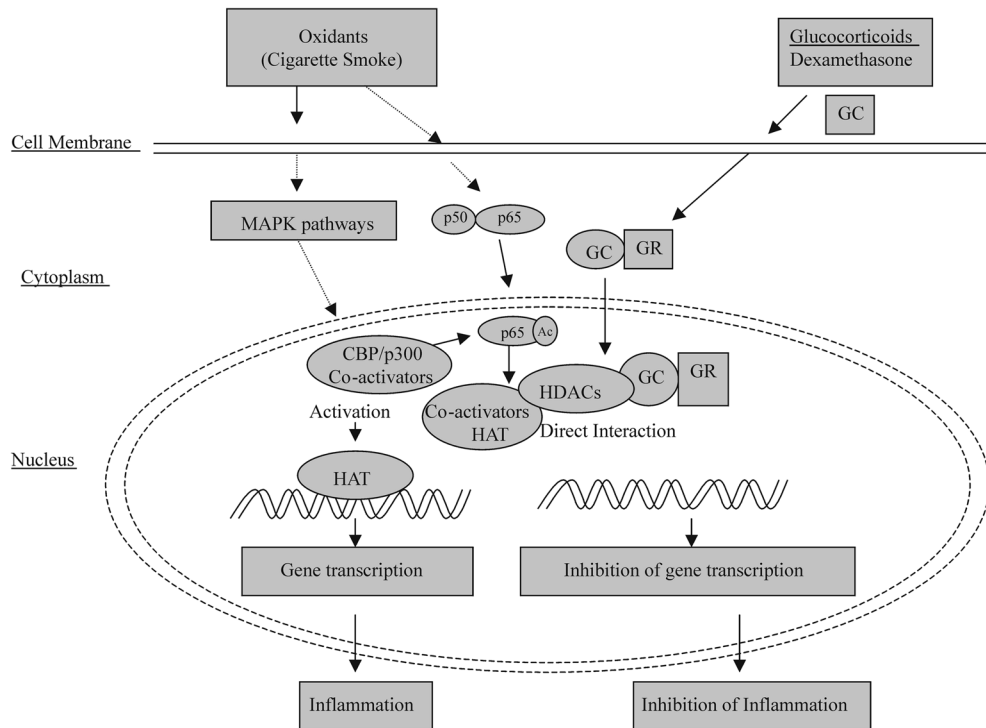


Fig. 6. Model showing the possible mechanism of histone acetylation by oxidative stress and its repression by corticosteroids (GCs) leading to inhibition of gene transcription. MAP kinase signaling pathways may be activated by oxidative stress leading to histone acetylation. Direct interaction between co-activators (HAT), histone deacetylase and the glucocorticoid receptor (GR) may result in repression of the expression of pro-inflammatory genes. HDAC forms a bridge with HAT to inhibit gene transcription. However, when the HDAC is inhibited by oxidants or the NF- κ B subunit p65 is acetylated, steroids may not be able to recruit HDACs into the transcriptional complex to inhibit pro-inflammatory gene expression.

and TNF- α -mediated induction of IL-8 was partially blocked by dexamethasone when the HDAC activity was inhibited by TSA in alveolar epithelial cells (Rahman *et al.*, unpublished data).

The reduced level of HDAC-2 was associated with increased proinflammatory response and reduced responsiveness to glucocorticoids in alveolar macrophages obtained from smokers (Ito *et al.*, 2001). The cigarette smoke/oxidant-mediated reduction in HDAC-2 levels in alveolar epithelial cells will not only increase inflammatory gene expression but will also cause a decrease in glucocorticoid function (Fig. 6). This may be one of the potential reasons for the failure of glucocorticoids to function effectively in reducing inflammation in COPD. The signaling mechanisms involved in the cigarette smoke-mediated chromatin remodeling and glucocorticoid insensitivity are currently unknown. It may be possible that cigarette smoke-mediated formation of potential aldehydes/acetaldehydes is responsible for oxidation/nitrosylation/phosphorylation of HDACs during inflammation. Nevertheless, oxidative stress results in an imbalance between histone acetylation and deacetylation, which may account for the enhanced expression of inflammatory mediators leading to amplification of lung inflammation. This may serve as a potential mechanism for

therapeutic intervention to ameliorate the chronic inflammatory response which occurs in the development of smoking-induced chronic inflammatory lung disease such as COPD.

Conclusions

ROS may be critical to the inflammatory response to cigarette smoke/environmental oxidants, through the upregulation of redox-sensitive transcription factors, alteration in histone acetylation/deacetylation and hence pro-inflammatory gene expression; but is also involved in the protective mechanisms against the effects of cigarette smoke by the induction of antioxidant genes. Further understanding of the effects and roles of ROS in basic cellular functions as amplification of pro-inflammatory and immunological responses, signalling pathways, activation of transcription factors, chromatin remodeling (histone acetylation and deacetylation) and gene expression will provide important information regarding basic pathological processes contributing to chronic lung diseases such as COPD. Identification of genes that predispose to the development of chronic lung diseases may identify novel therapeutic targets.

The effective wide spectrum antioxidant therapy that has good bioavailability and potency/long lasting action is urgently needed to control the underlying oxidative and inflammatory processes that occur in the pathogenesis of chronic inflammatory lung diseases. Therefore, study of the protective role of antioxidant compounds on inhibition of the inflammatory response and correcting the fundamental oxidant/antioxidant imbalance in patients with lung diseases are an important area of further research. Understanding of the molecular mechanisms of antioxidants on ROS-mediated cell signaling pathways would provide information for development of novel antioxidant therapeutic agents to prevent the progression of chronic lung diseases such as COPD. The proof of concept for the role of oxidative stress in the pathogenesis of chronic lung diseases will come from clinical studies on the effectiveness of antioxidant therapy.

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