Biomarkers, 2009; 14(S1): 90–96

informa
healthcare

REVIEW ARTICLE

The role of oxidative stress in the biological responses of lung epithelial cells to cigarette smoke

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Abstract

The mechanism(s) by which cigarette smoke contributes to lung diseases, such as cancer, remains unclear. Recent developments in our knowledge of cell signalling events suggest that cigarette smoke causes oxidative stress and proinflammatory responses in cells of the lung. Cigarette smoke is a complex mixture of over 4000 compounds and high levels of oxidants and reactive oxygen species (ROS) have been detected in both mainstream and sidestream smoke. Oxidative stress that ensues, when the antioxidant defences are depleted, is accompanied by increases in ROS production in lung epithelial cells. Cigarette smoke-mediated oxidative stress produces DNA damage and activates survival signalling cascades resulting in uncontrolled cell proliferation and transformation. Intervention studies using antioxidants have provided compelling evidence that oxidative stress plays a critical role in the aetiology of smoking-related disorders.

Keywords: *Cigarette smoke; oxidative stress; survival signalling; cancer*

Introduction

Cigarette smoking results in direct lung damage, as well as in the activation of lung inflammatory responses important in the aetiology in the development of lung cancer. Cigarette smoke is a complex mixture of over 7000 chemical compounds (Rodgman & Perfetti 2009), including a high concentration of oxidants (e.g. 10^{14}–10^{16} per puff; Church & Pryor 1985). Short-lived oxidants, such as superoxide anion (O_2^-) and nitric oxide, are predominantly in the gas-phase, but react immediately to form highly reactive peroxynitrite. The radicals in the tar-phase of cigarette smoke are organic, and include long-lived semiquinone radicals that can form the hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) on reaction with the O_2^- anion (Pryor & Stone 1993). The aqueous-phase of the cigarette smoke may undergo redox-cycling for a considerable period of time with the lung-lining fluid (LLF) (Zang et al. 1995). The tar-phase is an effective metal chelator and can bind iron to produce tar-semiquinone and tar-Fe^{2+}, which can continuously generate H_2O_2. In addition, as cigarette smoke and LLF contain metal ions, especially iron, the Fenton reaction will occur with H_2O_2, producing reactive oxygen species (ROS), and the highly reactive $^{\bullet}OH$ radical (Pryor & Stone 1993).

ROS are produced during a variety of biochemical reactions and cellular functions. The steady-state formation of pro-oxidants is normally balanced by a similar rate of consumption by antioxidants. Oxidative stress results from an imbalance between formation and neutralization of pro-oxidants. Various pathological processes disrupt this balance by increasing the formation of ROS in proportion to the available antioxidants (thus, oxidative stress). In humans, a pro-oxidant state has been shown to occur during inflammation and cancer (Cerutti 1985). ROS are highly reactive, unstable molecules that have an unpaired electron in their outer shell. They react with (oxidize) various cellular components including DNA, proteins, lipids/fatty acids and advanced glycation end products (e.g. carbonyls). These reactions between cellular components and ROS lead to DNA damage,

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ISSN 1354-750X print/ISSN 1366-5804 online © 2009 Informa UK Ltd
DOI: 10.1080/13547500902965047

mitochondrial malfunction, cell membrane damage, cell proliferation and apoptosis (Sies 1997).

This review describes the evidence for the role of cigarette smoke-mediated ROS production in the pathogenesis of lung diseases, such as cancer, and discusses the molecular mechanisms of ROS-mediated cell signalling pathways in inflammation and lung injury.

Exposure of cells to cigarette smoke *in vitro*

There are three different methods for exposing cells *in vitro* to cigarette smoke components. Cells can be exposed to cigarette smoke total particulate matter (TPM), aqueous cigarette smoke extract (CSE) containing components of the particulate and vapour phase or whole smoke. TPM is prepared by trapping the particles from the mainstream smoke on a Cambridge filter pad, a glass-fibre filter that retains 99% of particles larger than 0.1 μm, and extracting the particles with dimethyl sulphoxide (DMSO) (Pryor 1997). CSE is prepared by bubbling smoke from one cigarette into culture medium (Carp & Janoff 1978). Cells cultured on Transwells at the air–liquid interface are exposed to freshly generated whole smoke diluted with air in a Perspex chamber (Massey et al. 1998). The cells are maintained at 37°C and fed with a continuous supply of culture medium for exposure periods of 30 min to 3 h which delivers a biologically relevant dose of cigarette smoke (0.1–4 μg cm^{-2} per cigarette) (Massey et al. 1998).

Formation and measurement of ROS by cigarette smoke in cells

ROS can be measured in cells *in vitro* by the use of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). H_2DCFDA is taken up within cells and is cleaved by cellular esterases, to form the oxidant-sensitive form, 2′,7′-dichlorodihydrofluorescein (H_2DCF) which is non-fluorescent. In the presence of ROS, H_2DCF is oxidized and converted into 2′,7′-dichlorofluorescein (DCF) which is fluorescent and can easily be detected and quantified using a fluorescence plate reader (Hempel et al. 1999).

Recent studies in a human lung carcinoma cell line, H292 cells, have shown that cigarette smoke TPM produces ROS in cells. These studies used a derivative of H_2DCFDA called 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) that contains a thiol-reactive chloromethyl group of H_2DCFDA that allows for covalent binding to intracellular components, permitting an even longer retention within the cell. Treatment of H292 cells with TPM produced a time- and dose-dependent increase in intracellular ROS generation (Tai et al. 2008) (Figure 1). Cell-free wells containing CM-H_2DCFDA alone were used as negative (fluorescence) controls and demonstrated no fluorescence (data not shown). Treatment with medium and DMSO alone had very little effect on ROS generation. H292 cells treated with TPM showed increases in fluorescence intensity compared with untreated control cells with 100 μg ml^{-1} TPM and an exposure period of 60 min showing the largest generation of ROS (Figure 1).

Reduction of glutathione levels *in vitro* by cigarette smoke

Glutathione (GSH) is an important antioxidant in the lung *in vivo*, but it also performs other functions in signal transduction, metabolism and in the detoxification of electrophilic compounds (Brown 1994, Meister & Anderson 1983). A dose-dependent decrease in measurable GSH was observed after incubation with CSE (Wickenden et al. 2003). These authors employed an assay that measures both reduced (GSH) and oxidized (GSSG) glutathione. If oxidation had occurred then no reduction in total GSH would have taken place and suggests that cigarette smoke electrophilic components may conjugate with GSH. This was correlated by the findings that both GSH and DTT, but not the antioxidant mannitol, were able to inhibit the effects of CSE pointing to the effects being due to electrophilic compounds and not oxidants (Wickenden et al. 2003).

Proinflammatory effects of cigarette smoke *in vitro*

The proinflammatory effects of cigarette smoke have been assessed in a number of cellular systems *in vitro*. Kode et al. (2006) assessed the effects of CSE on the levels of proinflammatory cytokines, interleukin (IL)-8 and IL-6, and on nuclear translocation of nuclear factor-κB (NF-κB) in human small airway epithelial cells (SAEC).

IL-8 and IL-6 were both significantly released in human SAEC exposed to CSE for 24 h in a dose-dependent fashion (Table 1). Expression of proinflammatory cytokines are under the transcriptional control of the redox-sensitive transcription factor NF-κB. Activation of NF-κB in human SAEC by CSE was assessed by immunofluorescence analysis of NF-κB RelA/p65 localization in cells and by Western blot analysis of nuclear protein levels of RelA/p65 (Kode et al. 2006). Using immunocytochemistry, human SAEC showed a significant increase in the level of nuclear NF-κB localization in response to a 20 min exposure to CSE compared

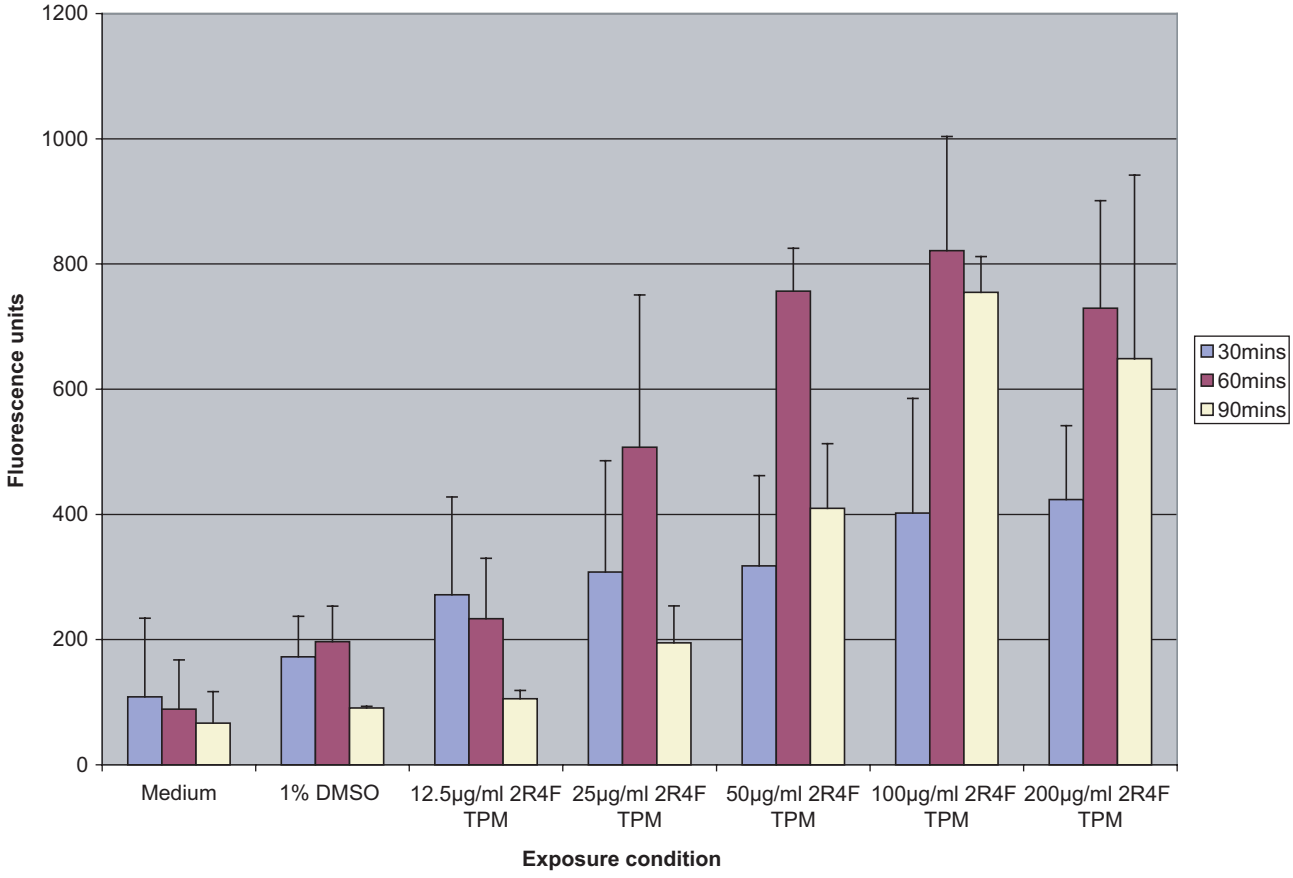

Figure 1. Concentration and time-dependent oxidation of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) in H292 cells following exposure to total particulate matter (TPM). The data are the mean ± SD of three independent experiments (Tai et al. 2008). DMSO, dimethyl sulphoxide.

Table 1. Cigarette smoke extract (CSE) causes a dose-dependent increase in proinflammatory cytokine (interleukin (IL)-8 and IL-6) release from human small airway epithelial cells.

	Treatment				
Proinflammatory cytokine (pg ml^{-1})	Control	CSE (0.2%)	CSE (0.5%)	CSE (1.0%)	TNF-α (10 ng ml^{-1})
IL-8	56.2 ± 7.1	126 ± 40.6***	171 ± 21.8***	418 ± 52.3 ***	591 ± 76.2***
IL-6	87.3 ± 7.2	187 ± 43.5***	275 ± 31.6***	476 ± 54.8***	623 ± 51.7***

TNF, tumour necrosis factor.
Data represent the mean ± SEM of three individual experiments. ***p <0.001 compared with control values (Kode et al. 2006).

with untreated controls (Figure 2). To confirm these observations, nuclear extracts were prepared and dose-dependent increases in nuclear NF-κB RelA/p65 protein levels were observed in human SAEC exposed to CSE for 1 h compared with untreated control cells (Figure 3).

Oxidative stress-mediated DNA damage and cell transformation by cigarette smoke *in vitro*

There are a number of diseases that are caused by cigarette smoke, namely chronic obstructive pulmonary disease (COPD), cardiovascular disease (CVD) and cancer. It is beyond the scope here to describe the contribution of oxidative stress in all these diseases and this review will concentrate on some of the evidence for the involvement of oxidative stress in cigarette smoke-mediated lung carcinogenesis.

To achieve this, we must understand the multistage model of chemical carcinogenesis in which normal cells are transformed into cancer cells. Cell division is a physiological process that occurs in almost all tissues and under many circumstances. Normally, the balance between proliferation and programmed cell death, usually in the form of apoptosis, is maintained

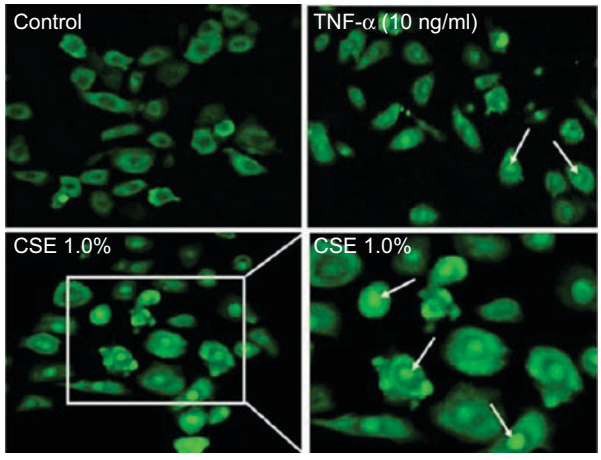

Figure 2. Cigarette smoke extract (CSE) causes nuclear factor (NF)-κB RelA/p65 nuclear translocation in primary human small airway epithelial cells (Kode et al. 2006). TNF, tumour necrosis factor.

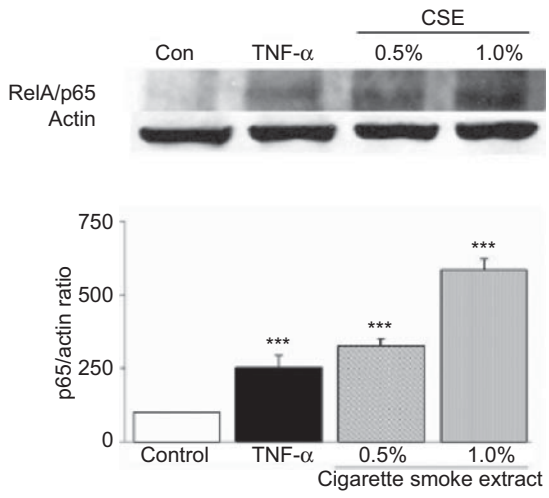

Figure 3. Cigarette smoke extract (CSE) mediates nuclear translocation of nuclear factor (NF)-κB RelA/p65 protein as seen by increased nuclear levels of NF-κB RelA/p65 protein in nuclear extracts isolated from human primary human small airway epithelial cells. Data represent the mean ± SEM of three individual experiments. ***$p < 0.001$ compared with control values (Kode et al. 2006). TNF, tumour necrosis factor.

by tightly regulating both processes to ensure the integrity of organs and tissues. Mutations in DNA that lead to cancer (only certain mutations can lead to cancer and the majority of potential mutations will have no bearing) disrupt these orderly processes by disrupting the programming regulating the processes. Carcinogenesis is caused by these mutations of the genetic material of normal cells, which upsets the normal balance between proliferation and cell death. Tumour promotion results in uncontrolled cell division. This uncontrolled and often rapid proliferation of cells leads to cell (conversion) transformation and tumorigenesis.

Initiating events in carcinogenesis can be investigated *in vitro* by analysis of DNA damage in the form of DNA strand breaks, chromosomal aberrations and DNA adduct formation. The Comet assay or 'single cell gel electrophoresis assay' is a relatively inexpensive and simple assay to detect DNA damage in individual cells (Kumaravel et al. 2009). Treated cells or isolated nuclei are embedded in agarose and lysed. The DNA is allowed to unwind in alkaline conditions and the DNA is electrophoresed and stained with ethidium bromide. The assay is based on the ability of negatively charged loops or fragments of DNA to be drawn through an agarose gel in response to an electric field. The extent of DNA migration in the direction of the anode out of the cell or nucleus is dependent directly on the DNA damage present and appears like the tail of a 'comet'. The size and shape of the 'comet' correlates with the extent of DNA damage (Kumaravel et al. 2009).

A slight modification of the technique incorporating a lesion-specific endonuclease, formamidopyrimidine DNA-glycosylase (FPG), provides the opportunity to measure oxidative lesions in DNA (Collins et al. 1993). FPG specifically recognizes oxidized purines and creates additional breaks in the DNA where these lesions are present. The modified Comet assay has been used with H292 cells exposed to mainstream cigarette smoke (whole smoke) (Thorne et al. 2009). In these studies, considerable DNA damage in terms of strand breaks, alkali labile sites (ALS) and oxidative DNA lesions were observed. The initial measurements of oxidative lesions were obscured by high levels of strand breaks and ALS. Following a 24 and 48 h recovery period, the strand breaks and ALS, but not oxidative lesions, were repaired which allowed for the semiquantitative measurement of oxidative DNA lesions in this assay (Figure 4).

The Syrian hamster embryo (SHE) cell transformation assay can be used to assess the carcinogenic potential of a chemical (Breheny et al. 2005). These authors have developed a two-stage transformation assay using SHE cells separating initiation (I) and promotion (P) stages of carcinogenesis and have shown that this two-stage assay responded to cigarette smoke and induced the morphological transformation (MT) in SHE cells at both I and P stages. The role of oxidative stress in MT mediated by cigarette smoke TPM has been examined by using the antioxidant curcumin. SHE cells were exposed to cigarette smoke TPM for 24 h (I stage) and TPM for 7 days (P stage) in the presence and absence of curcumin. Curcumin was found to have an inhibitory effect on TPM induced MT at both I and P stages and was found to have the greatest inhibition when present at both stages (I+P) (Figure 5) (Tai et al. 2007).

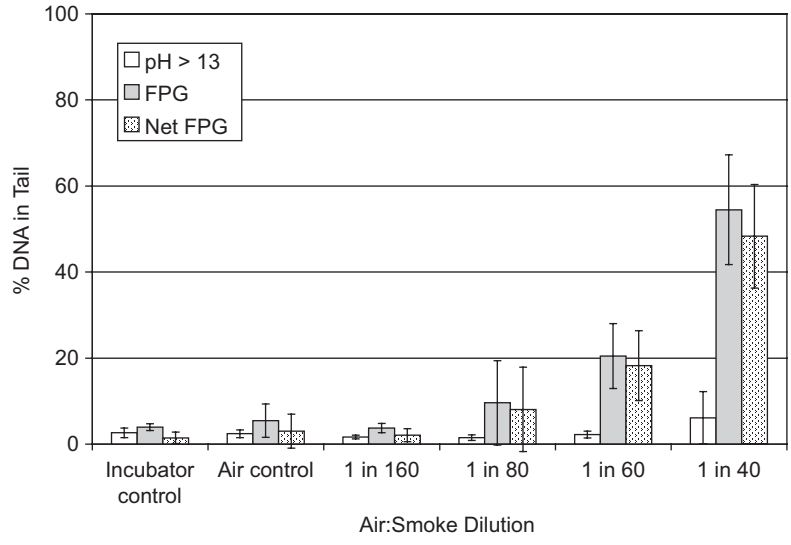

Figure 4. Oxidative DNA damage in H292 cells exposed to 30 min mainstream cigarette smoke, following a 20 h recovery period. Strand breaks have repaired at all dilutions quicker than oxidative lesions, allowing their semiquantitative measurement. Standard deviation based on 100 scored nuclei per dose (Thorne et al. 2009). FPG, formamidopyrimidine DNA-glycosylase.

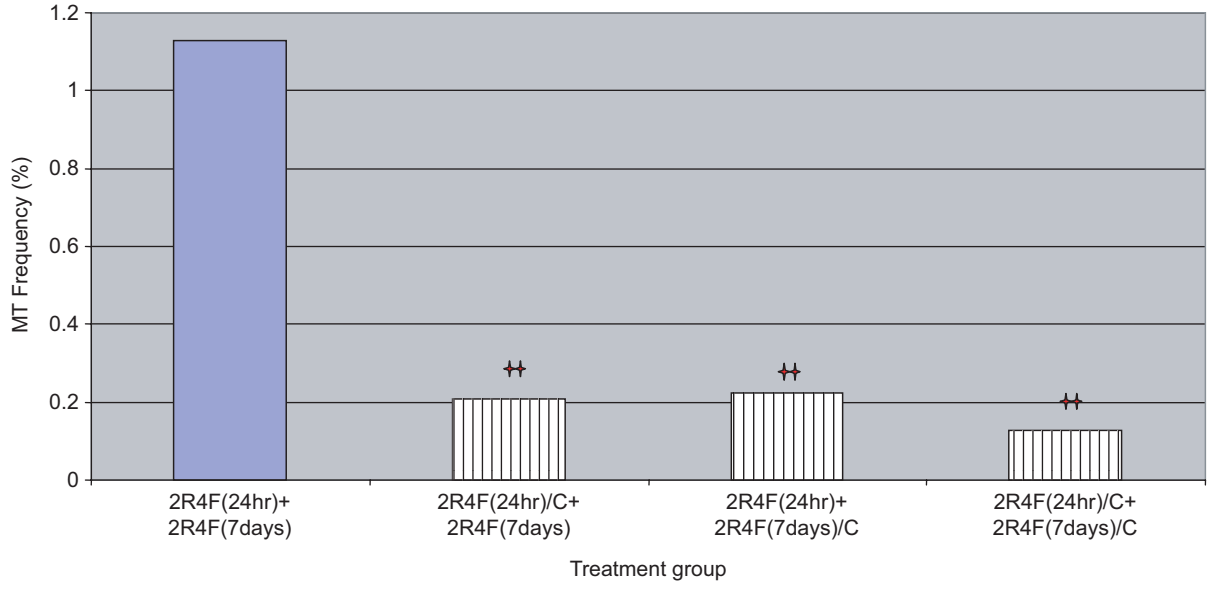

Figure 5. Effect of curcumin on morphological transformation (MT) of SHE cells treated with total particulate matter (TPM) (I+P). MT frequency was calculated in SHE cells incubated with 0.33 ng ml^{-1} TPM (2R4F) for either 24 h or 7 days with and without 1 μM curcumin. $^{**}p<0.01$ significance relative to TPM (I) and TPM (P) (Tai et al. 2007).

Modulation of cell survival signalling by cigarette smoke-mediated oxidative stress

The epidermal growth factor receptor (EGFR) is implicated as being important in the aetiology of a number of cancers, including lung cancer (Franklin et al. 2002). Previous studies have shown that oxidative stress induced by H_2O_2 causes aberrant phosphorylation of the EGFR (Ravid et al. 2002). Under H_2O_2-induced oxidative stress the EGFR is not only activated but is also stabilized due to its inability to undergo clathrin-mediated endocytosis and enter lysosomal degradation pathways (Ravid et al. 2002).

Among the plethora of deleterious chemicals in cigarette smoke, H_2O_2 has been reported to be abundant in the gas phase of mainstream smoke (Nakayama et al. 1984). Recent studies have shown that cigarette smoke causes aberrant phosphorylation of the EGFR and

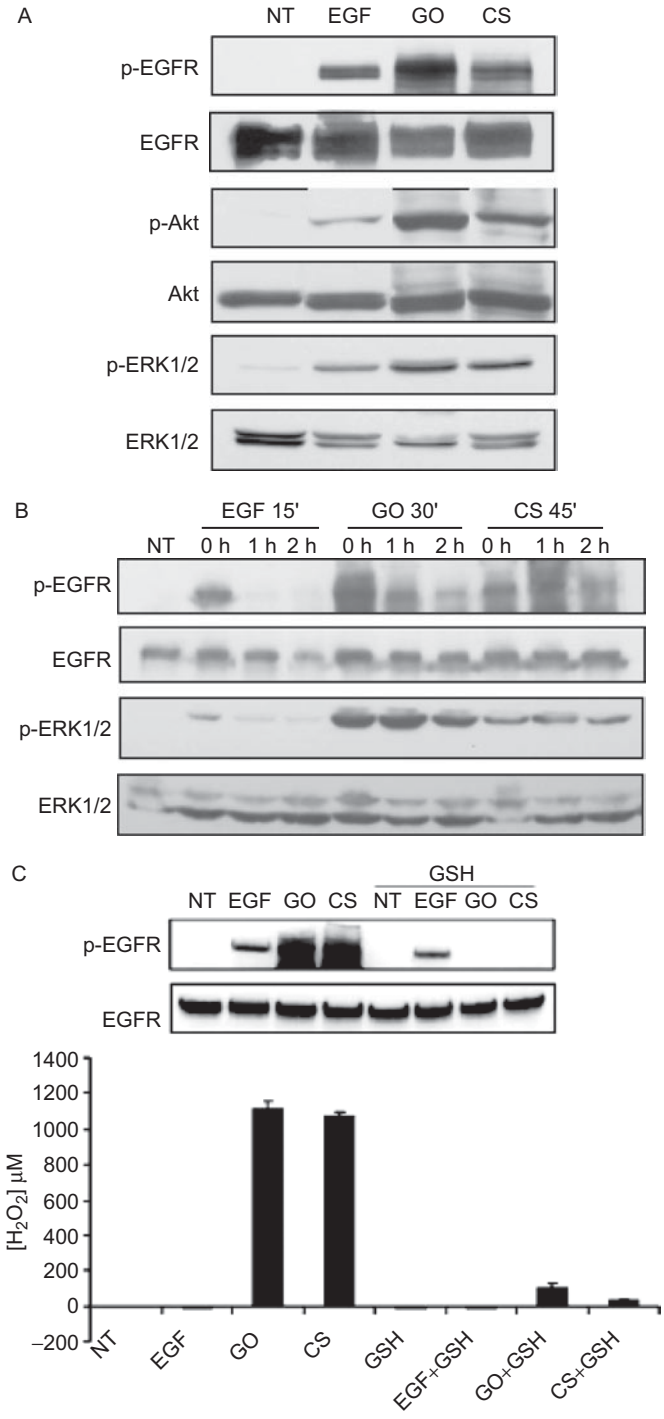

Figure 6. Exposure of human airway epithelial (HAE) cells to cigarette smoke results in prolonged epidermal growth factor receptor (EGFR) activation and downstream proliferation signalling. Serum starved HAE cells were untreated (NT) or incubated with 100 ng ml^{-1} EGF (15 min), 1 U ml^{-1} glucose oxidase (GO; 30 min) or smoke from one cigarette (CS; 45 min). (A) Cell lysates were separated by SDS-PAGE and immunoblotted with antiphosphotyrosine (PY20), anti-EGFR, anti-Akt, antiphospho-Akt, anti-ERK1/2 and antiphospho-ERK1/2 antibodies. (B) Cells were either lysed immediately or given fresh media and further incubated for 1 and 2 h at 37°C before cell lysis. Lysates were separated by SDS-PAGE and immunoblotted with PY20, anti-EGFR, anti-ERK1/2 and antiphospho-ERK1/2 antibodies. (C) Serum-starved HAE cells were not treated (NT) or pretreated with 10 mM GSH for 30 min followed by treatment with EGF (±GSH), GO (±GSH) or CS from one cigarette (±GSH). Aliquots of media (1 ml) from each treatment were taken and analysed in triplicate for H_2O_2 determination. Cell lysates were prepared and separated by SDS-PAGE and immunoblotted with PY20 and anti-EGFR antibodies (Khan et al. 2008).

subsequent activation of survival and proliferative signalling molecules such protein kinase B (Akt) and extracellular signal-regulated kinase 1/2 (ERK1/2), through prolonged EGFR activation in human airway epithelial (HAE) cells (Figure 6A, B) (Khan et al. 2008). In these experiments, there were significant increases in H_2O_2 produced by HAE exposed to cigarette smoke (Figure 6C). To determine whether H_2O_2 produced in cigarette smoke was involved in the activation of EGFR, cells were pretreated with GSH. H_2O_2 produced by cigarette smoke is almost completely abrogated in the presence of GSH. Furthermore, there is a concomitant inhibition of cigarette smoke-induced EGFR phosphorylation, whereas activation by EGF is not affected (Figure 6C), indicating different mechanisms of action (Khan et al. 2008).

These results parallel those with H_2O_2 from previous studies where the EGFR is stabilized through loss of c-*Cbl* binding and receptor ubiquitination. In addition, the levels of apoptosis were investigated and following a 48 h recovery period from the cigarette smoke exposure, only 30% of the cells were viable compared with 90% following glucose oxidase (GO) exposure (Khan et al. 2008). The apparent greater toxicity after smoke exposure can be attributed to the complexity of cigarette smoke. These results would suggest that the surviving population of cells with prolonged EGFR activation, 48 h post-exposure, may have the potential to become hyperplastic and this will contribute to the cellular events that may ultimately lead to tumorigenesis.

Conclusions

In summary, oxidative stress mediated by ROS may be critical to the inflammatory responses to cigarette smoke through the upregulation of redox-sensitive transcription factors leading to proinflammatory gene expression. Cigarette smoke-induced oxidative stress results in DNA damage in the form of strand breaks and oxidative modifications. Through the activation of survival signalling cascades, e.g. EGFR/NF-κB/Akt, oxidative stress causes uncontrolled cell proliferation and cell transformation. There is compelling evidence through intervention studies with antioxidants that oxidative stress plays a critical role in the effects of cigarette smoke *in vitro*. More studies are needed in cells exposed to whole smoke to understand more exactly the role of oxidative stress in the aetiology of smoking-related disorders.

Acknowledgements

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Breheny D, Zhang H, Massey ED. (2005). Application of a two-stage Syrian hamster embryo cell transformation assay to cigarette smoke particulate matter. *Mutat Res* 572:45–57.

Brown LA. (1994). Glutathione protects signal transduction in type II cells under oxidant stress. *Am J Physiol* 266:172–7.

Carp H, Janoff A. (1978). Possible mechanisms of emphysema in smokers. *In vitro* expression of serum elastase-inhibitory capacity by fresh cigarette smoke and its prevention by antioxidants. *Am Rev Respir Dis* 118:617–21.

Cerutti PA. (1985). Pro-oxidant states and tumour promotion. *Science* 227:375–81.

Church DF, Pryor WA. (1985). Free radical chemistry of cigarette smoke and its toxicological implications. *Environ Health Perspect* 64:111–26.

Collins AR, Duthie SJ, Dobson VL. (1993). Direct enzyme detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis* 14:1733–5.

Franklin WA, Veve R, Hirsch FR, Helfrich BA, Bunn PA Jr. (2002). Epidermal growth factor receptor family in lung cancer and premalignancy. *Sem Oncol* 29:3–14.

Hempel SL, Buetter GR, O'Malley YQ, Wessels DA, Flaherty DM. (1999). Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison with 2′,7′-dichlorodihydrofluorescein diacetate, 5(and 6)-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate and dihydrorhodamine 123. *Free Rad Biol Med* 27:146–59.

Khan EM, Lanir R, Danielson AR, Goldkorn T. (2008). Epidermal growth factor receptor exposed to cigarette smoke is aberrantly activated and undergoes perinuclear trafficking. *FASEB J* 22:910–17.

Kode A, Yang S-R, Rahman I. (2006). Differential effects of cigarette smoke on oxidative stress and pro-inflammatory cytokine release in primary human airway epithelial cells and in a variety of transformed alveolar epithelial cells. *Respir Res* 7:132–52.

Kumaravel TS, Vilhar B, Faux SP, Jha AN. (2009). Comet assay measurements: a perspective. *Cell Biol Toxicol* 25:53–64.

Massey ED, Aufderheide M, Koch W, Lodding H, Pohlmann G, Windt H, Jarck P, Knebel JW. (1998). Micronucleus induction in V79 cells direct exposure to whole cigarette smoke. *Mutagenesis* 13:145–9.

Meister A, Anderson ME. (1983). Glutathione. *Ann Rev Biochem* 52:711–60.

Nakayama T, Kodama M, Nagata C. (1984). Generation of hydrogen peroxide and superoxide anion radical from cigarette smoke. *Gann* 75:95–8.

Pryor WA, Stone K. (1993). Oxidants in cigarette smoke: radicals, hydrogen peroxide, peroxynitrite and peroxynitrite. *Ann N Y Acad Sci* 686:12–27.

Pryor WA. (1997). Cigarette smoke radicals and the role of free radicals in chemical carcinogenicity. *Environ Health Perspect* 105:875–82.

Ravid T, Sweeney C, Gee P, Carraway KL, Goldkorn T. (2002). Epidermal growth factor receptor activation under oxidative stress fails to promote c-*Cbl* mediated down-regulation. *J Biol Chem* 277:31214–19.

Rodgman A, Perfetti TA. (2009). The chemical components of tobacco and tobacco smoke. Boca Raton, FL: CRC Press Inc.

Sies H. (1997). Oxidative stress: oxidants and antioxidants. *Exp Physiol* 82:291–5.

Tai T, Xu Y, Breheny D, Gaca M. (2007). The effect of cigarette smoke and the antioxidants resveratrol and curcumin on a two-stage cell transformation assay. *Free Rad Biol Med* 43 (Suppl. 1):S60.

Tai T, Carr T, Faux SP, Gaca MD. (2008). Detection of cigarette smoke induced reactive oxygen species (ROS) using a CM-H_2DCFDA fluorescence probe. *Free Rad Biol Med* 45 (Suppl. 1):S68–9.

Thorne D, Wilson J, Kumaravel TS, Massey ED, McEwan M. (2009). Measurement of oxidative DNA damage induced by mainstream cigarette smoke in cultured NCI-H292 pulmonary carcinoma cells. *Mutat Res* 673:3–8.

Wickenden JA, Clarke MCH, Rossi AG, Rahman I, Faux SP, Donaldson K, MacNee W. (2003). Cigarette smoke prevents apoptosis through inhibition of caspase activation and induces necrosis. *Am J Respir Cell Mol Biol* 29:562–70.

Zang LY, Stone K, Pryor WA. (1995). Detection of free radicals in aqueous extracts of cigarette tar by electron spin resonance. *Free Radic Biol Med* 19:161–7.