DNA damage in human respiratory tract epithelial cells: damage by gas phase cigarette smoke apparently involves attack by reactive nitrogen species in addition to oxygen radicals

Jeremy P.E. Spencer[a], Andrew Jenner[a], Ken Chimel[b], Okezie I. Aruoma[a], Carroll E. Cross[c], Reen Wu[b,c], Barry Halliwell[a,c,*]

[a]*Neurodegenerative Disease Research Centre, University of London King's College, Manresa Road, London, SW3 6LX, UK*
[b]*Pulmonary Research Group, Californian Regional Primate Research Centre, UC Davis, Davis, California, 95616, USA*
Division of Pulmonary/Critical Care Medicine, Department of Internal Medicine, UC Davis Medical Centre, Sacramento, California, 95817, USA

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Abstract Treatment of human respiratory tract tracheobronchial epithelial cells with gas-phase cigarette smoke led to dose-dependent DNA strand breakage that was highly correlated with multiple chemical modifications of all four DNA bases. The pattern of base damage suggests attack by hydroxyl radicals (OH•). However, by far the most important base damage in quantitative terms was formation of xanthine and hypoxanthine, presumably resulting from deamination of guanine and adenine respectively. Hence, DNA damage by cigarette smoke may involve reactive nitrogen species as well as reactive oxygen species.

Key words: Human respiratory tract cell; DNA damage; DNA base modification; Strand breakage; Cigarette smoke; GC-MS

1. Introduction

Several epidemiological studies have strongly indicated that smoking is a major cause of human cancers, especially lung cancer, and other respiratory diseases (e.g. [1,2]). Damage to DNA, usually measured as strand breakage, has been shown to occur in several human and other mammalian cell types exposed to cigarette smoke [3–6]. However, the mechanism of damage to mammalian cell DNA by cigarette smoke is not clear. Cigarette smoke is a complex mixture consisting of more than 3500 chemicals, many of them known to be mutagens and/or carcinogens [7,8]. In addition, reactive oxygen species such as superoxide radical ($O_2^{\bullet-}$), H_2O_2 and hydroxyl radical (OH•) have been shown to be generated from both the gas and tar phases of cigarette smoke [7,9]. It is frequently suggested that damage by these species accounts for the DNA strand breakage observed in cells exposed to cigarette smoke [6,7,9,10], which could contribute to the increased risk of cancer in smokers. Consistent with oxidative DNA damage, several authors have reported increases in 8-hydroxy-2′-deoxyguanosine (8OHdG), a well known product of oxidative damage to DNA (reviewed in [11,12]) in cells exposed to cigarette smoke [6,10].

In the present paper, we examined the effect of cigarette smoke upon human respiratory tract epithelial cells, measuring both strand breakage and modification of the purine and pyrimidine bases. Rather than measure a single DNA base damage product, such as 8OHdG, we used gas chromatography-mass spectrometry to measure a wide range of products from all four DNA bases [11,13]. This is important because when a reactive species such as OH• attacks DNA, the actual end products obtained from the initial reaction products depend very much on the local environment [13,14].

In addition, different species modify the DNA bases in different ways, ie. OH• modifies all four bases whereas singlet O_2 is selective for guanine [11,13]. Hence, measurement of a wide range of base products gives much more molecular information about the species responsible for DNA damage [13–15].

2. Materials and methods

2.1. Reagents

8-Azaadenine, 6-azathymine, 8-bromoadenine, 5-hydroxyuracil (isobarbituric acid), 4,6-diamino-5-formamidopyrimidine (FAPy-adenine), 2,5,6-triamino-4-hydroxypyrimidine and 5-(hydroxymethyl)uracil were purchased from Sigma Chemical Co (Poole, Dorset, UK). 2-Amino-6,8-dihydroxypurine (8-hydroxyguanine) was from Aldrich (Gillingham, Dorset, UK). Silylation grade acetonitrile and bis(trimethylsilyl) trifluoracetamide (BSTFA) (containing 1% trimethylchlorosilane) were obtained from Pierce Chemical Co (Rockford, IL, USA). 6-Amino-8-hydroxypurine (8-hydroxyadenine) was synthesised by treatment of 8-bromoadenine with concentrated formic acid (95%) at 150°C for 45 min and purified by crystallisation from water. 2,6-Diamino-4-hydroxy-5-formamidopyrimidine (FAPy-guanine) was synthesised by treatment of 2,5,6-triamino-4-hydroxypyrimidine with concentrated formic acid and recrystallised from water. Dialysis membranes with a relative molecular mass cut off of 3500 were purchased from Spectrum supplied by Pierce Chemical Co. Distilled water passed through a purification system (Elga, High Wycombe, Bucks, UK) was used for all purposes. Ham's F12 nutrient medium was purchased from Gibco (Grand Island, New York). Alamar blue was from Alamar Biosciences (Sacramento, CA).

2.2. Sample preparation and assays

The culturing of the human bronchial epithelial cell line HBE 1 [16,17], the Alamar blue assay (a measure of cell viability based on mitochondrial dehydrogenase activity[18]), DNA isolation and assessment of RNA contamination [19], analysis of oxidative DNA base damage [11,13,15] and measurement of DNA strand breaks [20,21] were carried out as previously described [22].

*Corresponding author. Neurodegenerative Disease Research Centre, University of London King's College, Manresa Road, London, SW3 6LX, UK. Fax: (44) (171) 333 4949.

Abbreviations: 5-OH-uracil, 5-hydroxyuracil; 5-OHMe-uracil, 5-(hydroxymethyl)uracil; FAPy-adenine, 4,6-diamino-5-formamidopyrimidine; 8-OH-adenine, 8-hydroxyadenine; FAPy-guanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH-guanine, 8-hydroxyguanine; 8OHdG, 8-hydroxy-2′-deoxyguanosine.

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2.3. Exposure of cells to cigarette smoke

Cells of approximately 90% confluency were used. Cells were grown in T75 flasks to obtain an enclosed system (Fig. 1) into which a quantitative amount of gas phase cigarette smoke could be pumped. It was expected that the average puff of a cigarette was about 35–50 ml and that reactive components of the gas would react completely in under 2 min [7–9]. Hence cells were exposed to multiples of this puff volume for 2 min. After exposure, flasks were well flushed with nitrogen and the cells were washed with filtered PBS; DNA extraction took place immediately afterwards. The Cambridge filter removes the majority of the particulate phase of the cigarette smoke, allowing cells to be exposed to only the gas phase.

3. Results

Exposure of human respiratory tract tracheobronchial epithelial cells to gas phase cigarette smoke led to a dose-dependent increase in DNA strand breakage (Fig. 2). These volumes of cigarette smoke produced little effect on cell viability (as measured by the Alamar blue assay) one hour after the experiment (Fig. 3). However, further incubation of the cells for 24 h led to significant losses of cell viability, with the exception of the 35 ml exposure group, which showed an apparent small increase in viability, presumably the consequence of continuing cellular replication. Thus the loss in viability caused by higher doses of cigarette smoke in these cells is not immediately obvious, but becomes apparent after a longer period of incubation.

Levels of oxidative DNA base modification in the control (not cigarette smoke exposed) cells were low, as expected (Table 1). Exposure of the cells to cigarette smoke produced significant changes in 8-hydroxyguanine (Fig. 4A, Table 1). However, the increase was only about 100%, comparable with previous studies [6,10]. There were somewhat greater rises in FAPy-guanine and smaller rises in the amounts of hydroxyuracil, hydroxyadenine (Fig. 4A,B) and FAPy-adenine (Table 1). However, in quantitative terms these changes were dwarfed by much larger changes in xanthine and hypoxanthine (Fig. 4A).

The extent of DNA strand breakage was well correlated to the increase in levels of 8-OH-guanine ($r = 0.91$), hypoxanthine ($r = 0.91$), xanthine ($r = 0.95$) and to the sum of the levels of total base damage products ($r = 0.94$).

4. Discussion

When double-stranded DNA is exposed to moderately alkaline solutions, hydrogen bonds are broken and the two strands unwind. Strand breaks present in the DNA molecule increase the rate of this unwinding, so that an increased rate of DNA unwinding can be used as a sensitive measure of strand breaks [20,21]. When human respiratory tract epithelial cells are incubated for one hour with increasing volumes of filtered cigarette smoke, the percentage of double strand DNA decreases (Fig. 1) and so the number of single-strand breaks in the DNA must rise. Using our exposure system, the extent of DNA strand breakage is dose related in the range 0–210 ml of smoke. Cigarette smoke also damages these cells in a way that does not immediately cause loss of viability, but leads to delayed loss of cell metabolic activity. Comparable observations have recently

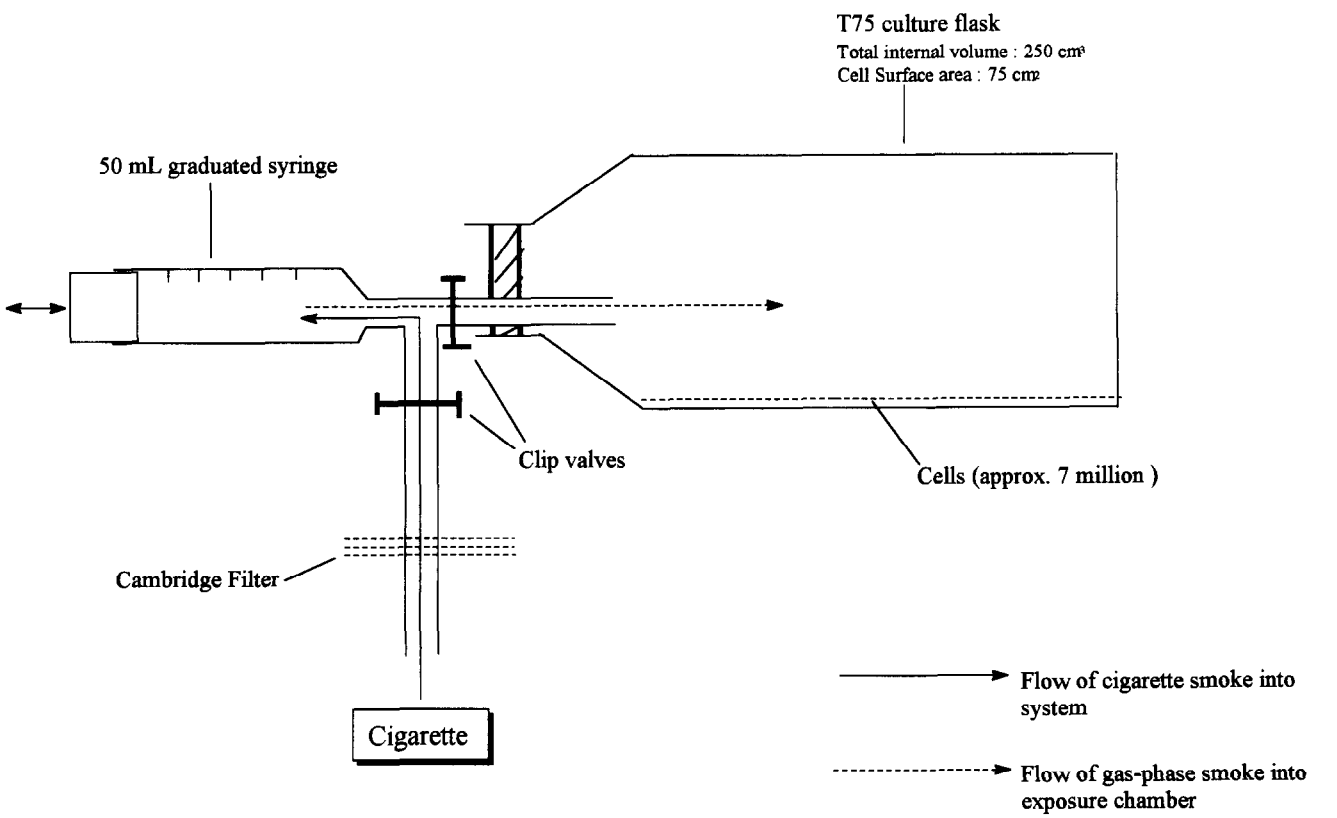

Fig. 1. Diagram of cigarette smoke exposure chamber. Cigarette smoke was drawn into the system through a Cambridge filter using a manual syringe. The required volume of gas-phase cigarette smoke was then pumped into the exposure chamber, (the dead volume in the system was calculated prior to experiments so that accurate volumes of gas-phase smoke could be delivered to cells). After the 2 minute exposure the chamber was flushed with nitrogen after removing the syringe.

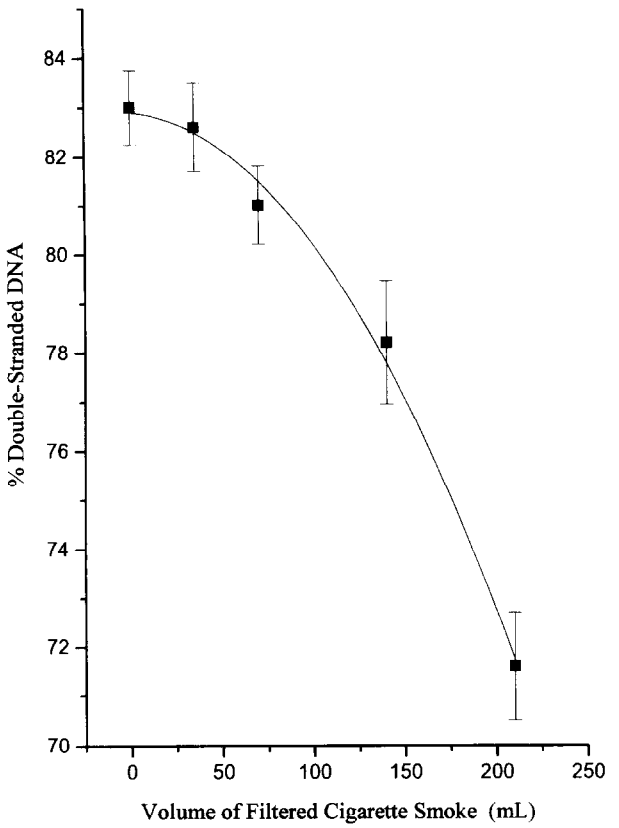

Fig. 2. Effect of gas phase cigarette smoke on DNA strand breakage epresented as the loss of double-stranded DNA. Experiments were erformed as in section 2. Data points are means of three separate xperiments plotted with standard deviation from the mean.

been reported in human fibroblasts exposed to H_2O_2; H_2O_2 at ess than 300 μM did not affect viability, but prevented later livision of the cells and induced senescence [23].

Our data confirmed previous reports of a modest rise of 8-OHdG in cells exposed to cigarette smoke [6,10]. The levels of several other products of oxygen free radical attack upon DNA also increased. This pattern of multiple base damage uggests that OH• attack upon the DNA is involved since, of the various reactive oxygen species, only OH• is known to produce such a wide range of base modifications [11,13,14]. All of these base products were observed in DNA isolated from untreated cells and probably arose due to normal levels of oxidative stress in this cell line. We attempted to minimize artefactual oxidative damage to DNA by avoiding potentially pro-oxidant processes, such as phenol extraction of DNA [24].

The most striking increases in base damage products were in xanthine and hypoxanthine, deamination products of guanine and adenine respectively [25,26]. Although we have no direct evidence that these products were produced by deamination, our data suggest that the major mechanism of damage by cigarette smoke may indeed involve deamination reactions [26], especially as treatment of the cells with H_2O_2 produced higher levels of strand breakage and oxidative DNA base damage products, but much lower rises in xanthine and hypoxanthine, than did cigarette smoke exposure (Table 1). Deaminating species may include HNO_2, NO_2, N_2O_3 and peroxynitrite ($ONOO^-$) [25–28]. Formation of deamination products such as xanthine or hypoxanthine could lead to depurination and subsequent strand breakage due to the relative instability of these products. Indeed, changes in DNA resulting from deamination are consistent with the types of mutations observed in many cancers, e.g. in the p53 gene [25].

Overall, our data confirm that exposure of cells to gas-phase cigarette smoke can lead to oxidative DNA damage and demonstrate for the first time that this is probably mediated by the attack of OH•. Hence constituents of the smoke must penetrate to the nucleus and there generate OH•, since this reactive species combines with molecules at its site of formation. However, our data suggest that hypoxanthine and xanthine (presumably resulting from deamination reactions) are quantitatively much more important and perhaps therefore more likely to contribute to the various mutations that can eventually lead to cancer. Indeed, the concentration of various reactive nitrogen species in smoke is much higher than that of oxygen-derived species [8,29]. Hence, the role of oxidative damage in smoke-induced carcinogenicity may have been over-emphasised, and more attention might be paid to means of protecting cells against the reactive nitrogen species present.

Table 1
Baseline levels of DNA base modification in control human respiratory ract epithelial cells and the increase in amount after exposure of the ells to 210 ml cigarette smoke for 2 min or to H_2O_2 for 60 min. Results re means of three separate experiments ± S.D.

Human RT epithelial cells			
Modified Base	Baseline level in extracted DNA (nmol/mg DNA)	Increase in amount in smoke exposed cells (nmol/mg DNA)	Increase in amount in cells treated with 1 mM H_2O_2 (nmol/mg DNA)
5-Hydroxyuracil	0.034 ± 0.01	0.051 ± 0.004	0.687 ± 0.02
8-Hydroxy-guanine	0.898 ± 0.01	1.818 ± 0.10	4.431 ± 0.56
FAPy-guanine	0.814 ± 0.16	3.240 ± 0.30	9.012 ± 0.61
8-Hydroxyaden-ine	0.154 ± 0.02	0.500 ± 0.04	0.550 ± 0.04
FAPy-adenine	0.532 ± 0.09	1.024 ± 0.06	24.405 ± 2.45
Xanthine	1.210 ± 0.13	5.913 ± 0.41	1.025 ± 0.54
Hypoxanthine	0.717 ± 0.05	4.517 ± 0.41	1.644 ± 0.23

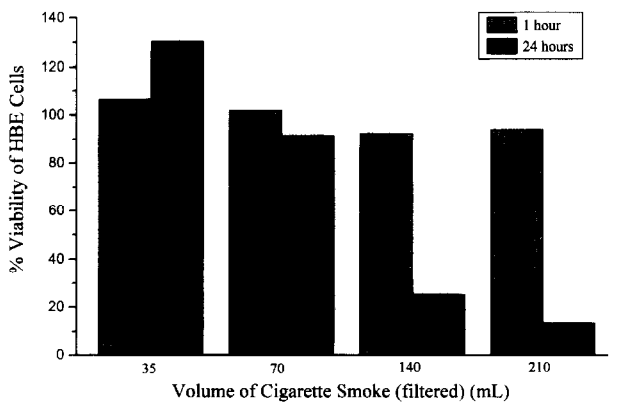

Fig. 3. Effect of gas phase cigarette smoke on HBE cell viability. Viability tests were conducted using the Alamar blue assay, which measures mitochondrial metabolic activity, as described in [18,22]. Means are the result of three separate experiments. % values were calculated using untreated cells at 1 and 24 h as 100% values.

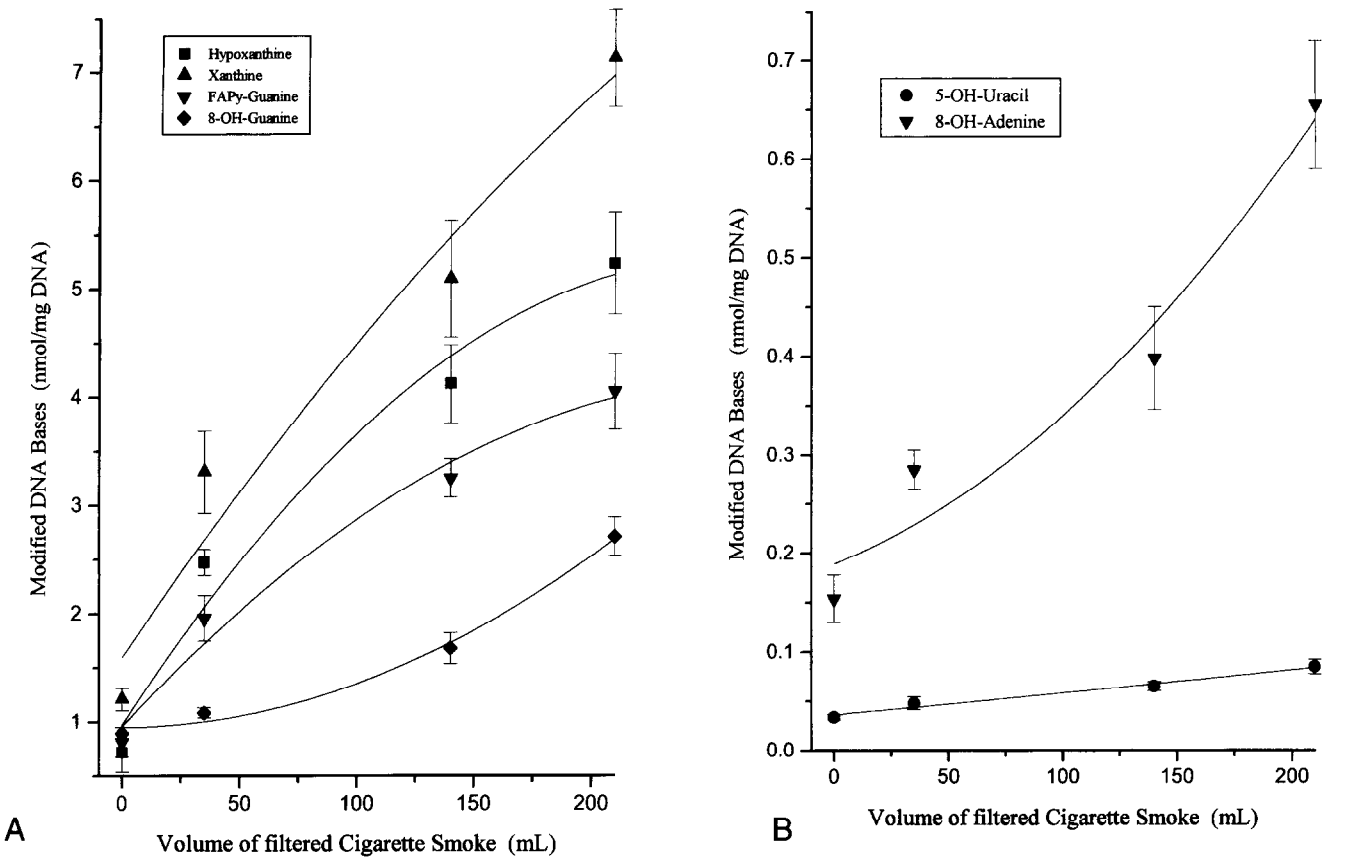

Fig. 4. (A) Effect of exposing cells to gas phase cigarette smoke on the amount of xanthine, hypoxanthine, FAPy-guanine and 8-OH-guanine in their DNA. Experiments were conducted as described in section 2. Data points in the Figure are mean ± S.D. (n = 3 separate experiments). (B) Effect of exposing cells to gas phase cigarette smoke on the concentration of 5-OH-uracil and 8-OH-adenine in their DNA. Experiments were conducted as described in section 2. Data points in the Figure are mean ± S.D. (n = 3 separate experiments).

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