DNA strand breakage and base modification induced by hydrogen peroxide treatment of human respiratory tract epithelial cells

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Abstract Treatment of human respiratory tract epithelial cells with H_2O_2 led to concentration-dependent DNA strand breakage that was highly-correlated with multiple chemical modifications of all four DNA bases, suggesting that damage is due to hydroxyl radical, OH•. However, the major base damage occurred to adenine. Hence, conclusions made about the occurrence and the extent of oxidative DNA damage on the basis only of changes in 8-hydroxyguanine should be approached with caution.

Key words: Human respiratory tract epithelial cell; DNA damage; DNA base modification; Strand breakage; H_2O_2; GC-MS

1. Introduction

Oxygen-derived species formed in vivo by normal cellular metabolism or as a result of exogenous exposure to toxins, such as air pollutants, are thought to contribute to the development of cancer by promoting chemical changes in DNA that are potentially mutagenic [1–4]. Damage to DNA, usually measured as strand breakage, has been shown to occur in many cell types in response to oxidative stress [1–5]. However, little is known about the mechanism of this damage. Possible mechanisms include rises in intracellular free Ca^{2+} that are sufficient to activate endonucleases [6] and direct attack on DNA by highly reactive radicals, such as hydroxyl, OH• [2,7,8]. Consistent with direct free radical attack on DNA, several groups have reported increases in 8-hydroxy-2′-deoxyguanosine (8OHdG) in mammalian cells exposed to oxidative stress [1,5,9–11]. However, the extent of the increase is often small (sometimes less than the reported variability between measurements of 'normal' levels of 8OHdG in cells [12]), which has led several authors to doubt the significance of oxidative base modification in causing DNA damage. For example, Higuchi and Linn [13] found no significant increase in 8OHdG in HeLa cells treated with H_2O_2, even at lethal levels.

In the present paper, we examined the effect of H_2O_2 upon human respiratory tract epithelial cells, measuring both DNA strand breakage and oxidative base modification. H_2O_2 is a simple and widely used source of oxidative stress [2] and is physiologically relevant because the respiratory tract can be exposed to H_2O_2 generated by activated phagocytes, pathogenic micro-organisms (e.g. *Mycoplasma* [14,15]), and by oxidizing air pollutants such as cigarette smoke and O_3 [16]. H_2O_2 also contributes to carcinogenic processes in the respiratory tract [17] and other [18] cells, and oxidative DNA damage has been suggested to contribute to this action [17,18]. Rather than measure a single DNA base damage product, such as 8OHdG, we used gas chromotography-mass spectrometry to measure a wide range of products from all four DNA bases [2,12,19,20]. This is important because when a reactive radical such as OH• attacks DNA, the actual end products obtained depend very much on the local environment [2,19,21]. For example, exposure of DNA to OH• generated by ionizing radiation yields much less 8OHdG under hypoxic than under aerobic conditions conditions. In H_2O_2/metal iron (Fenton-type) OH•-generating systems, copper ion/H_2O_2 systems produce much more 8-OH-guanine (as a percentage of total base modification products) than do iron ion-dependent systems [22].

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). Alamar blue was from Alamar Biosciences (Sacramento, CA). 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., USA. Distilled water passed through a purification system (Elga, High Wycombe, Bucks, UK) was used for all purposes. Ham's F12 supplemented medium was purchased from Gibco (Grand Island, NY).

2.2. Cell culture

The papillomavirus-immortalized human bronchial epithelial cell line, HBE1 [23], at passage between 18 and 20, was used. This cell line was routinely cultured in a serum-free hormone-supplemented medium [24], which was Ham's F12 nutrient medium supplemented with insulin,

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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; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

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transferrin, epidermal growth factor (EGF), hydrocortisone, cholera toxin, and bovine hypothalamus extract as previously described [24].

2.3. Exposure of HBE cells to H_2O_2

Cells of approximately 90% confluency were used. Growth medium was removed and cells were washed twice with sterile, filtered PBS. Because the serum-free hormone supplemented culture medium was found to scavenge H_2O_2 over the time course of the experiment (probably due mainly to pyruvate present in the F12 [25]), a Hank's balanced salt solution (HBSS) was used for the exposure experiments. Incubation of cells with this new medium did not affect viability. Hydrogen peroxide stock solutions were made up immediately before experiments and volumes of this stock were added to HBSS to achieve the required final concentrations. The H_2O_2 mixtures (15 ml) were added to cell plates containing 3–5 million cells and a 60 min incubation at 37°C followed. After incubation the H_2O_2 was removed and cells were washed twice in filtered PBS.

2.4. Cell viability

Cell viability after H_2O_2 treatment was determined by the Alamar blue assay [26].

2.5. DNA isolation

The method and volumes of reagents are for batches of 3–5 million cells grown as a monolayer; a minimum of 15–20 million cells was extracted in each experiment. Exposed cells were thoroughly washed with cold filtered PBS and lysed by adding 1.5 ml of cell lysis buffer (Tris-HCl 20 mM, NaCl 200 mM, EDTA 20 mM, SDS 0.5%). The viscous lysate was scraped to one edge of the culture dish and then transferred to a disposable 15 ml centrifuge tube. Proteinase K (0.1 mg/ml final concentration) was added to the lysate and the mixture shaken until homogenous. A 2 h incubation at 50°C ensured digestion of all cellular and nuclear protein. The protein was then precipitated by shaking vigorously for 15 s with NaCl (6.0 M). Centrifugation at 1300 × *g* for 15 min pelleted the protein precipitate and the supernatant containing the DNA was removed. Two more NaCl treatments of the supernatant ensured a clear, protein free supernatant. DNA was precipitated with two volumes of 100% ethanol and transferred to a 1 ml Eppendorf tube by winding around a very fine glass rod. The precipitated DNA was washed twice with 70% ethanol and then dried under vacuum. DNA was redissolved in 1 ml of CT buffer (Tris-HCl 10 mM, EDTA 0.2 mM, pH 7.5). Recovery of DNA from 15–20 million cells was usually between 100–140 μg. After dialysis the concentration of the DNA (μg/ml) extracted from each cell sample was calculated before 1 nmol of 6-azathymine and 2 nmol of 8-azaadenine were added as internal standards. RNA contamination in the isolated DNA samples was assayed by the ethidium bromide/ribonuclease technique [27], and was found not to be significant [27].

2.6. Analysis of oxidative DNA base damage

Extracted DNA was hydrolysed in formic acid, derivatized and analysed by GC-MS as described previously [19,20,22].

2.7. Measurement of DNA strand breaks

DNA strand breaks were detected by exposing crude cell lysates to alkaline pH and monitoring the rate of strand unwinding, as followed using the fluorescent dye ethidium bromide, which binds selectively to double-stranded DNA [28,29].

Cells were washed with PBS and then pelleted to achieve a concentration of 5–10 million cells/ml. Aliquots of this cell suspension (0.2 ml) were placed in 12 glass tubes labelled T (total), P (test) or B (blank) in groups of 4. To each was added 0.2 ml of cell lysis and chromatin disruption buffer (urea 9.0 M, NaOH 10 mM, CDTA 2.5 mM, SDS 0.1%), and tubes were incubated at 0°C for 10 min to allow total disruption of chromatin. To tubes P and B, both 0.1 ml of alkaline unwinding solution A (0.45 vol. of cell lysis buffer in 0.2 M NaOH) and 0.1 ml of alkaline unwinding solution B (0.4 vol. of cell lysis buffer in 0.2 M NaOH) were added without mixing. During the subsequent incubation at 0°C for 30 min, the alkali diffuses into the viscous lysate to give a final pH of about 12.8. Before the alkali solutions were added to tube T, 0.4 ml of denaturation prevention solution (glucose 1 M, mercaptoethanol 14 mM) was added. All tubes were then incubated for 60 min at 15°C and the process was stopped by addition of 0.4 ml denaturation prevention solution and chilling to 0°C. To each tube was added 1.5 ml of ethidium bromide solution (ethidium bromide 6.7 μg/ml, NaOH 13.3 mM) and the fluorescence read at room temperature (excitation: 520 nm; emission: 590 nm).

The extent of DNA unwinding after a given time of exposure of cell extracts to alkali was calculated from the formula:

$$\% \text{ DS DNA} = (P - B)/(T - B) \times 100$$

3. Results

Exposure of human respiratory tract epithelial cells to H_2O_2 led to a concentration-dependent increase in DNA strand breakage (Fig. 1). These levels of H_2O_2 produced little effect on cell viability measured just before DNA extraction (Fig. 2). However, further incubation of the cells for 24 h led to significant losses of cell viability. Thus the lethality of H_2O_2 to these cells is not immediately apparent, but is revealed after a longer period of incubation.

Levels of oxidative DNA base modification in the control (not H_2O_2-exposed) cells were low, as expected (Table 1). Exposure of the cells to H_2O_2 produced significant concentration-dependent changes in 8-hydroxyguanine (Table 1 and Fig. 3A). However, the change in 8-hydroxyguanine was dwarfed by a much bigger change in FAPy-adenine and FAPy-guanine, products derived from the ring opening of adenine- and guanine-derived radicals after initial free radical addition to the bases (Fig. 3A). There were also significant rises (although from

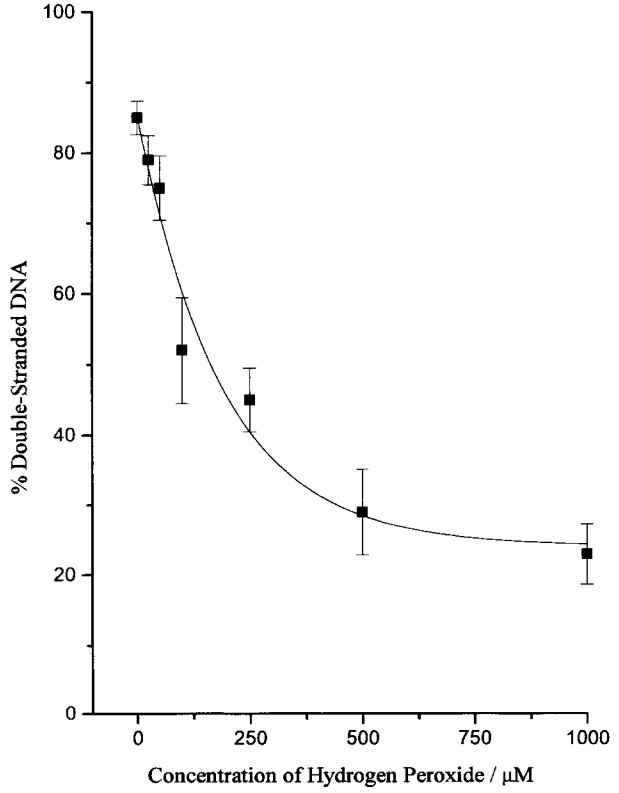

Fig. 1. Effect of increasing H_2O_2 concentrations on DNA strand breakage in cells exposed to H_2O_2 for 60 min, represented as the loss of double-stranded DNA. Experiments were performed as in section 2. Data points are means of three separate experiments plotted with standard deviation from the mean.

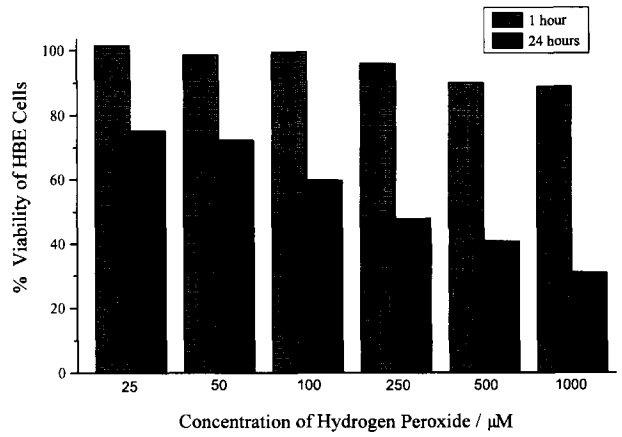

Fig. 2. Effect of increasing H_2O_2 concentrations on cell viability. Viability tests were conducted as described in section 2. Means are the result of three separate experiments. Percentage values were calcuated using untreated cells as the 100% value.

a lower baseline level) in 5-hydroxyuracil and 8-hydroxyadenine (Fig. 3B; note the different scale).

The extent of DNA strand breakage was well correlated both to the sum of DNA base modification ($r = 0.938$) and to the sum of the levels of adenine damage products ($r = 0.949$).

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 [28,29]. When human respiratory tract epithelial cells are incubated for one hour with increasing concentrations of H_2O_2, the percentage of double stranded DNA measured after alkali treatment decreases (Fig. 1) and so the number of single-strand breaks in the DNA must have risen. The extent of DNA strand breakage is concentration-dependent in the range 0–250 μM H_2O_2, but reaches a plateau at concentrations above 500 μM. H_2O_2 also damages these cells in a way that does not immediately cause loss of viability, but leads to delayed cell death. Similar observations have recently been reported in human fibroblasts; H_2O_2 at less than 300 μM did not affect viability, but prevented later division of the cells and induced senescence [30].

DNA strand breakage was accompanied by oxidative base DNA modification and closely correlated with it, suggesting that both are caused by the same mechanism. The overall pattern of base damage is suggestive of OH• attack upon the DNA, since, of the various reactive oxygen species, only OH• is known to produce such a wide range of base modifications

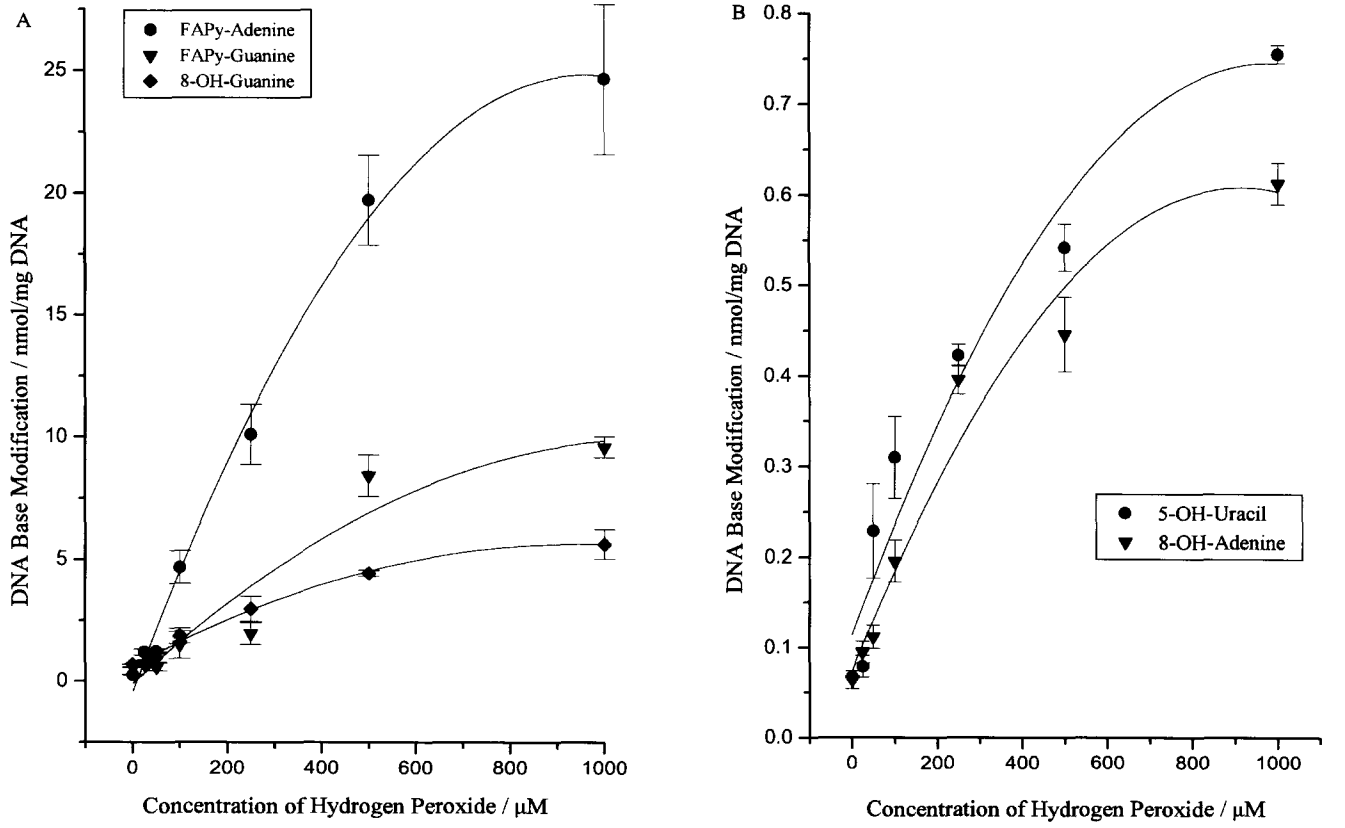

Fig. 3. (A) Effect of increasing H_2O_2 concentrations on the concentration of FAPy-adenine, FAPy-guanine and 8-OH-guanine in DNA from cells exposed to H_2O_2 for 60 min. Experiments were conducted as described in section 2. Data points are mean ± S.D. ($n = 4$). (B) Effect of increasing H_2O_2 concentrations on the concentration of 5-OH-uracil and 8-OH-adenine in DNA from cells exposed to H_2O_2 for 60 min. Experiments were conducted as described in section 2. Data points are mean ± S.D. ($n = 4$).

Table 1
Baseline levels of DNA base modification in control HBE cells and the increase in amount after exposure of the cells to 1 mM H_2O_2

Human RT epithelial cells		
Modified base product measured	Baseline level in DNA (nmol/mg DNA)	Increase in amount of modified base (nmol/mg DNA)
5-Hydroxyuracil	0.067 ± 0.020	0.687 ± 0.021
5-Hydroxymethyluracil	0.003 ± 0.000	0.017 ± 0.002
8-Hydroxyguanine	0.700 ± 0.015	4.431 ± 0.561
FAPy-guanine	0.580 ± 0.009	9.012 ± 0.611
8-Hydroxyadenine	0.064 ± 0.014	0.550 ± 0.041
FAPy-adenine	0.258 ± 0.018	24.405 ± 2.452

Results are means of three separate experiments ± S.D.

[2,12,19,27]. All of the base products identified and quantified were observed in DNA isolated from untreated cells (Table 1) and probably arose due to physiological levels of oxidative stress in these cultured cells. We minimized possible artefactual oxidative damage to DNA by avoiding potentially pro-oxidant processes, such as phenol extraction of DNA [31]. The largest percentage increases over background levels were observed in 8-OH-adenine, FApy-adenine and 5-OH-uracil. Smaller percentage increases were noted in 8-OH-guanine and FApy-guanine. The increase in hydroxymethyluracil was not significant at any H_2O_2 concentration (Table 1).

In quantative terms, the biggest increases were in FApy-adenine and FApy-guanine, not 8-OH guanine (Table 1). If we assume from the overall pattern of DNA base damage that $OH^\bullet$ is the species responsible, it seems that more $OH^\bullet$ is reacting with adenine than guanine (8-OH-adenine + FAPy-adenine > 8-OH guanine + FAPy-guanine) and that the $OH^\bullet$ that does attack guanine leads to ring-opening rather than hydroxylation. The results obtained here are very different from studies in which isolated DNA was exposed to Fenton-type systems, especially those involving copper ions [2,22,27]. In parallel to the experiments reported in this paper, we performed experiments exposing isolated DNA to Fenton systems and confirmed the previously-reported pattern of base modification [22,27,32]. However, as already emphasised, end-products resulting from $OH^\bullet$ attack on purines and pyrimidines are very much affected by the local environment. Perhaps the intracellular environment of the cells used by us is sufficiently reducing to disfavour oxidation of the OH-adduct radicals. If we assume that Fenton-type reactions within the nucleus of H_2O_2-treated cells generate $OH^\bullet$, causing strand breakage and base modification, it seems unlikely, by comparison with the data in [22], that copper ions are invoved. Iron ions, perhaps somehow preferentially bound to adenine-rich sequences, could conceivably play a role [27].

Overall, we have shown that the pattern of DNA damage produced in H_2O_2 treated human respiratory tract epithelial cells is as complex as it is extensive. Our data suggest that great care must be taken when using only one base product as a marker of oxidative DNA damage. To date, 8OHdG and 8-OH-guanine have been the principal products of DNA damage searched for in vivo and in vitro. Our data show that these products are *not* always the major product formed in mammalian cells treated with an oxidative stress (in this case H_2O_2). Hence one must be cautious in concluding that oxidative DNA damage is minimal or absent on the basis of small or zero rises in 8OHdG or 8-OH-guanine alone.

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