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Oxidative DNA Damage in Human Respiratory Tract Epithelial Cells. Time Course in Relation to DNA Strand Breakage

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When human respiratory tract epithelial cells were exposed to 100 μM H_2O_2, there was rapid induction of DNA strand breakage and chemical modifications to all 4 DNA bases suggestive of attack by $OH^{\bullet}$. The major products were FAPy-adenine, FAPy-guanine, and 8-OH-guanine. Some of the base modifications were removed very quickly from the DNA (e.g., 8-OH-guanine), whereas others persisted for longer (e.g., thymine glycol), probably due to differential activity of different repair enzymes. By contrast, strand breaks continued to increase over the time course of the experiment, perhaps because strand breakage is also implicated in the repair process. One should therefore be cautious in using strand breakage as a sole measure of oxidative DNA damage, and when drawing conclusions about the pattern and biological significance of oxidative DNA damage in cells the relative persistence of different lesions must be considered.

Oxygen derived species such as the superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are produced in mammalian cells during normal aerobic metabolism [1,2]. Excess generation of these species *in vivo* results in damage to many biological molecules including lipids, protein and carbohydrates [2-4]. Reactive oxygen species (ROS) are also thought to contribute to the development of cancer by promoting chemical changes in DNA which are potentially mutagenic [5-9]. Both DNA strand breakage [2,10-13] and modification of DNA bases [14-16] are frequently observed in cells subjected to oxidative stress. Such damage may result by a variety of mechanisms including rises in intracellular free Ca^{2+} that are sufficient to activate endonucleases [17] or from direct attack on DNA by highly reactive radicals, such as hydroxyl ($OH^{\bullet}$) [6,7,18,19]. It is well established that H_2O_2 may react with transition metal ions bound to DNA to form $OH^{\bullet}$ [10,20-22] and produce a pattern of base modification very similar to that produced by ionizing radiation, an established source of $OH^{\bullet}$ [7,21,14,23,24]. Consistent with direct $OH^{\bullet}$ radical attack on DNA, several groups have reported increases in products of base oxidation, particularly in 8-hydroxyguanine (or 8-hydroxydeoxyguanosine) in mammalian cells exposed to oxidative stress [8,11,12,25-27].

Chemical changes in the DNA bases are of considerable importance if repair of these changes does not occur, or if repair is such that the fidelity of the original code is lost [28]. For example, formation of thymine glycol or formamidopyrimidines in the DNA template is known to cause a block in DNA replication [29,30] and 8-hydroxyguanine causes miscoding ($G \cdot C \rightarrow T \cdot A$ transversion mutation) [31,32]. H_2O_2-derived radicals have been shown to cause $G \cdot C \rightarrow T \cdot A$ transversions in the SupF gene of *E. coli* [33] and UV light can cause tandem double $CC \rightarrow TT$ mutations in the p53 gene, implicated in the development of squamous cell

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skin carcinoma [34,35]. Therefore, counteracting the multitude of different types of ROS induced DNA damage is clearly a major challenge to organisms, and a cell without any DNA repair capacity would probably not remain viable. The excretion of as many as 10^4 oxidatively modified DNA bases per cell per day in the urine [8] highlights the importance of oxidative damage to DNA and its repair in human cells. It has been known for some time that DNA repair is activated by oxidative damage [36-38]. In mammalian systems at least two distinct glycosylases and two endonucleases (III and IV) exist [38-40] and are induced by oxidative stress. DNA glycosylases initiate repair by hydrolysing the base-sugar N-C glycosylic bond of modified bases to generate abasic (AP) sites [38,40-42]. The resulting pathway of ''base excision repair'' acts on oxidative and non-oxidative damages, determined by the specificity of individual DNA glycosylase enzymes. The N-glycosylase which acts on oxidized pyrimidines, such as *cis*- and *trans*- thymine glycol, and endonuclease III are identical [39]. Another glycosylase, formamidopyrimidine-DNA glycosylase (FPG-protein), removes oxidized purine bases which have opened imidazole rings [17,39,40,42], but has also been shown to remove 8-hydroxyguanine from oxidatively modified DNA [43,44]. All these enzymes prime the DNA for repair by DNA polymerase I [40,42].

DNA damage within mammalian cells is frequently assessed by the measurement of strand breakage [40]. In the present paper, we have determined the time course of oxidative DNA damage in relation to that of strand breakage.

MATERIALS AND METHODS

Reagents. Diaminopurine, 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) trifluoroacetamide (BSTFA) (containing 1% trimethylchlorosilane) were obtained from Pierce Chemical Co (Rockford, Illinois, 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. Thymine glycol was synthesised by reaction of 5-methyl-uracil with osmium tetroxide (OsO_4) for 1 hour at 60°C and excess OsO_4 was removed by freeze drying. 2-Hydroxyadenine, 5-hydroxycytosine, and 5-hydroxy-methyl-hydantoin were gifts from Dr. Miral Dizdaroglu (National Institute of Standards and Technology, Gaithersburg, Maryland, USA). Cellu.Sep™ dialysis membranes with a relative molecular mass cut off of 3500 were obtained from Pierce Chemical Co.(Rockford, IL, USA). Distilled water passed through a purification system (Elga, High Wycome, Bucks, UK) was used for all purposes. Ham's F12 supplemented medium was purchased from Gibco (Grand Island, New York, USA) and Alamar blue was from Alamar Biosciences (Sacramento, CA, USA).

All other chemicals were of the highest quality available from Sigma Chemical Co. (St. Louis, Missouri, USA), or from the Aldrich Chemical Co. (Milwaukee, Wisconsin, USA).

Sample Preparation and Assays. The culturing of the human bronchial epithelial cell line HBE 1 [45,46], the AlamarBlue assay (a measure of cell viability based on mitochondrial dehydrogenase activity [47]), DNA isolation and assessment of RNA contamination [48], analysis of oxidative DNA base damage [20] and measurement of DNA strand breaks [49,50] were carried out as previously described [11].

Exposure of 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 [51]), 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 solution (100 μM) was added to cell plates containing 3-5 million cells and incubations of 5, 15, 30, 45 and 60 minutes at 37°C followed. After incubation the H_2O_2 was immediately removed and cells were washed twice with filtered PBS.

RESULTS

The use of gas chromatography-mass spectroscopy (GC-MS) allowed measurement of a wide range of oxidized products, from all four DNA bases. As observed previously [11],

exposure of human bronchial epithelial cells to H_2O_2 (100 μM) led to extensive rises in oxidized bases derived from both pyrimidine (Figure 1A and 1B) and purine (Figure 1C) bases. Large increases in 8-OH-guanine, FAPy-adenine, FAPy-guanine, thymine glycol, 5-OH-hydantoin and 5-OH,Me-hydantoin were observed at the five separate time periods. Small changes in 5-OH-uracil, 5-OH,Me-uracil and 8-OH-adenine were also observed. Levels of 8-OH-guanine and the imidazole ring opened products FAPy-adenine and FAPy-guanine were at a maximum around 15-35 mins but fell steeply in cells exposed for longer incubation times (Figure 1C). In contrast, the levels of 5-OH-hydantoin and 5-OH,Me-hydantoin also reached a maximum at 20 minutes but did not fall at longer incubation times (Figure 1A). Levels of thymine glycol continued to increase over the time-course of the experiment (Figure 1B).

Exposure of cells to H_2O_2 also led to DNA strand breakage (Figure 1D) which continued to increase with time. However, strand breakage appeared to occur by a two phase process which was rapid initially but slowed at later incubation times. The level of H_2O_2 (100 μM) used had little effect on cell viability measured just before DNA extraction (97% viable relative to untreated control cells), however, further incubation of cells for 24 hours led to a modest decrease in cell viability (89% viable compared to untreated control cells).

DISCUSSION

Reactive oxygen species such as H_2O_2 and $O_2^{\bullet-}$ are produced during normal cell metabolism [1,2]. The fact that each mammalian cell sustains an average of 10^3 ''oxidative hits'' upon DNA per day [8] suggests that mechanisms of repair need to be highly efficient and accurate if they are to keep the fidelity of the original DNA code intact. Unrepaired changes in the chemical structure of the DNA bases and DNA strand breakage are known to lead to blocks in replication, miscoding and ultimately mutagenesis [39,40].

When human respiratory tract epithelial cells were incubated with H_2O_2 (100 μM) for up to 60 minutes, extensive oxidative base modification was observed which was accompanied by DNA strand breakage. 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 plus that associated with cell culture. The overall pattern of damage is suggestive of $OH^{\bullet}$ attack upon DNA, since, of the various reactive oxygen species, only $OH^{\bullet}$ is known to produce such a wide range of base modifications [6,52]. The largest increases were observed in 8-OH-guanine and in the imidazole ring opened products FAPy-adenine and FAPy-guanine (Figure 1C). However, levels of 5-OH-hydantoin, 5-OH,Me-hydantoin and thymine glycol also showed sharp rises (Table 1; Figure 1A and B), although from a lower baseline level. The initial increase (0-20 mins) in modified base products in DNA of cells exposed to H_2O_2 was followed by a reduction in the rate of generation or, in some cases of purine damage, a decrease between 30 and 60 minutes.

Early damage to cellular DNA may result from the inability of normal basal rates of DNA repair to deal with large increases in modified base products. However, after the initial oxidative insult, mechanisms of repair may be enhanced in order to rectify the damage [37,38]. Damaged DNA bases are generally removed from the DNA helix by glycosylases which hydrolyse the N-glycosylic bonds between a damaged base and the deoxyribose sugar to release the base and produce an unmodified abasic site (AP site). The AP site is then incised by the β-lyase activity of AP endonucleases (endonuclease III or IV) which cleave the phosphodiester bond immediately 5′ to the AP site. The lyase reaction also produces 3′-hydroxyl termini that can be used by DNA polymerase I to complete repair [40]. Our data suggest that there is a lag period in the activation of this DNA repair system allowing damage to accumulate rapidly after the oxidative insult. However, rapid decreases in FAPy-adenine, FAPy-guanine and 8-OH-guanine at longer incubation times might suggest increased activity in specific DNA repair systems, i.e., the efficiency of repair varies between different lesions. The FPG-protein is

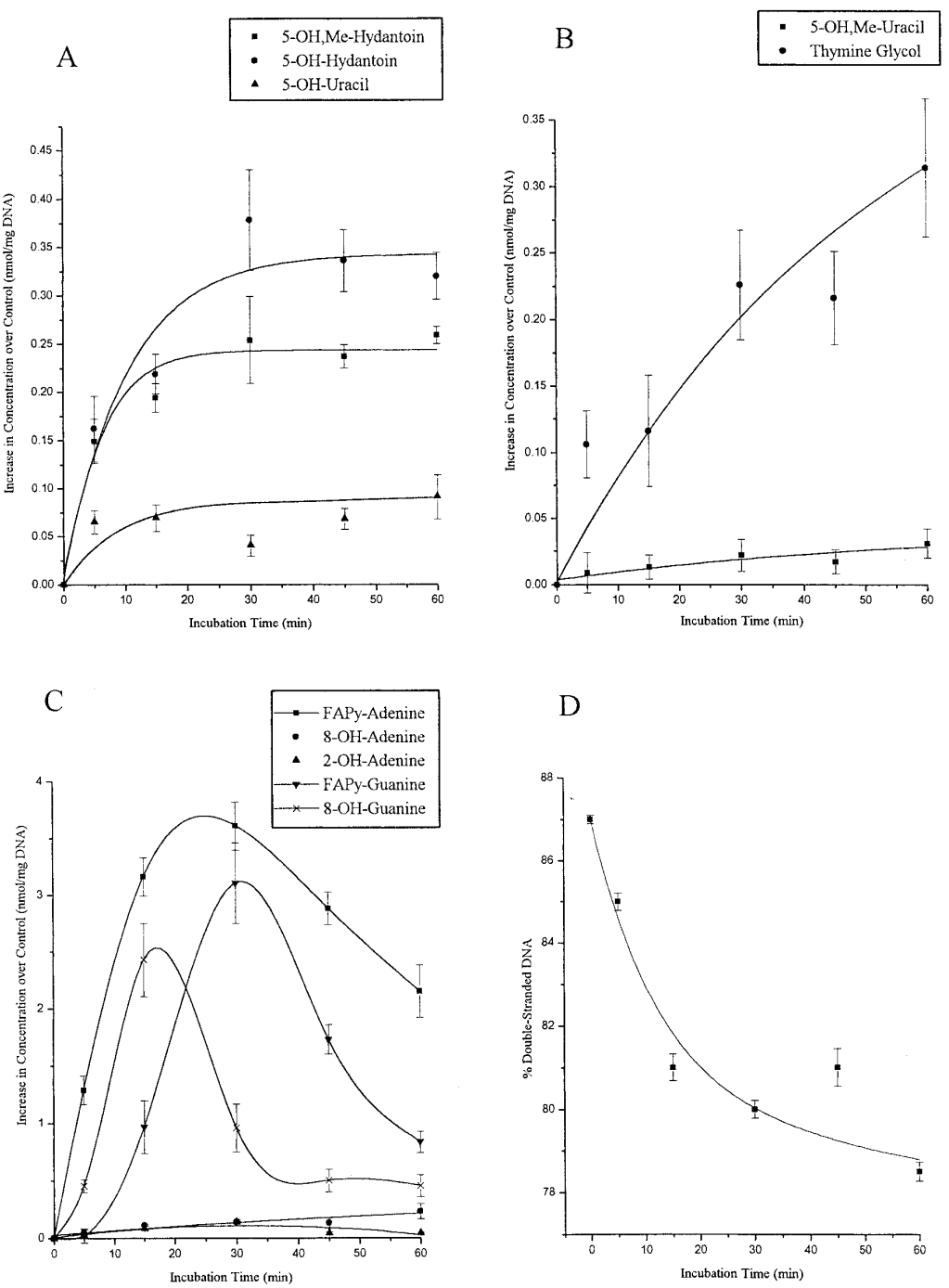

FIG. 1. Effect of H_2O_2 (100 μM) on the generation of oxidized base products and DNA strand breakage in HBE cells exposed to H_2O_2 for various incubation times. Measurement of DNA base modification was performed using GC-MS-SIM (see Materials and Methods) and data points are mean ± SD, n=4. (A) The change in 5-OH,Me-hydantoin, 5-OH-hydantoin, and 5-OH-uracil concentration over untreated cells. (B) The change in 5-OH,Me-uracil and thymine glycol concentration over untreated cells. (C) The change in FAPy-adenine, 8-OH-adenine, 2-OH-adenine, FAPy-guanine, and 8-OH-guanine concentration over untreated cells. (D) Effect of H_2O_2 (100 μM) on DNA strand breakage.

TABLE 1
Levels of Oxidized Base Products in DNA from Unexposed Cells

Modified pyrimidine base product	Concentration in control cells (nmol/mg DNA)	Modified purine base product	Concentration in control cells (nmol/mg DNA)
5-OH,Me-Hydantoin	0.275 ± 0.101	FAPy-Adenine	0.225 ± 0.012
5-OH-Hydantoin	0.392 ± 0.015	8-OH-Adenine	0.070 ± 0.005
5-OH-Uracil	0.102 ± 0.008	2-OH-Adenine	0.051 ± 0.009
5-OH,Me-Uracil	0.009 ± 0.003	FAPy-Guanine	0.518 ± 0.021
5-OH-Cytosine	0.161 ± 0.005	8-OH-Guanine	0.443 ± 0.095
Thymine glycol	0.144 ± 0.012		

Note. Measurement was by GC-MS-SIM analysis. Data points are mean ± SD, $n = 4$.

known to remove the ring-opened purine products FAPy-adenine and FAPy-guanine from DNA under oxidative stress [40], but has a broad substrate specificity and also removes hydroxylated purines, such as 8-OH-guanine. Activation of endonuclease III, another DNA glycosylase, would result in the removal of damaged bases such as 5-OH-hydantoin, 5-OH,Me-hydantoin and thymine glycol from DNA by a two step cleavage mechanism involving initial removal of the damaged base, followed by cleavage of the resulting AP site by the AP endonuclease function. Our data indicate that the activity of this enzyme may be elevated in the cells exposed to H_2O_2 for long incubation times as 5-OH-hydantoin and 5-OH,Me-hydantoin plateau at about 25 minutes. By contrast thymine glycol continued to increase over the time course of the experiment suggesting delayed repair of this lesion.

In contrast to the decrease in some modified DNA bases, DNA strand breakage was observed to increase between 20 to 60 minutes although the rate of increase was slower than over the first 20 minutes (Figure 1D). There appeared to be two phases in strand break formation. Over the first 15-20 minutes strand breakage is likely to result from oxidative damage to DNA, however, the rate of strand breakage decreases after this point and could result from the repair of DNA. The mechanism of oxidised base repair involves DNA strand breakage due to the β-lyase action of endonuclease enzymes before repair by DNA polymerase I joins the strand again. If polymerase I is unable to complete repair at a fast enough rate strand breakage would be expected to be observed.

Repair of purine damage, possibly by the FPG-protein system, occurs relatively quickly after damage to DNA by H_2O_2 but repair of pyrimidine base damage seems to occur later. Furthermore, DNA strand breakage detected in cells treated with oxidants such as H_2O_2, may be due in part, to the repair of oxidatively modified bases by glycosylase/endonuclease systems. Therefore, care must be taken in interpreting DNA strand breakage data in cells which have been exposed to oxidative stress capable of causing base modification.

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