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Repair of products of oxidative DNA base damage in human cells

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ABSTRACT

Oxidative DNA damage is the most frequent type of damage encountered by aerobic cells and may play an important role in biological processes such as mutagenesis, carcinogenesis and aging in humans. Oxidative damage generates a myriad of modifications in DNA. We investigated the cellular repair of DNA base damage products in DNA of cultured human lymphoblast cells, which were exposed to oxidative stress by H_2O_2. This DNA-damaging agent is known to cause base modifications in genomic DNA of mammalian cells [Dizdaroglu, M., Nackerdien, Z., Chao, B.-C., Gajewski, E. and Rao, G. (1991) *Arch. Biochem. Biophys.* 285, 388–390]. Following treatment with H_2O_2, the culture medium was freed from H_2O_2 and cells were incubated for time periods ranging from 10 min to 6 h. DNA was isolated from control cells, hydrogen peroxide-treated cells and cells incubated after H_2O_2 exposure. DNA samples were analyzed by gas chromatography/isotope-dilution mass spectrometry. Eleven modified bases were identified and quantified. The results showed a significant formation of these DNA base products upon H_2O_2-treatment of cells. Subsequent incubation of cells caused a time-dependent excision of these products from cellular DNA. The cell viability did not change significantly by various treatments. There were distinct differences between the kinetics of excision of individual products. The observed excisions were attributed to DNA repair in cells. The rate of repair of purine lesions was slower than that of pyrimidine lesions. Most of the identified products are known to possess various premutagenic properties. The results of this work may contribute to the understanding of the cellular repair of oxidative DNA damage in human and other mammalian cells.

INTRODUCTION

Oxygen-derived species including free radicals are formed in living cells by normal metabolism and by exogenous sources (reviewed in 1). Of free radicals, the hydroxyl radical (˙OH) is the most reactive toward biological molecules and generates a multitude of modifications in DNA such as base damage, sugar damage and DNA–protein crosslinks (reviewed in 2,3). This type of DNA damage, also called oxidative DNA damage, has been implicated in biological processes such as mutagenesis, carcinogenesis and aging (1).

Oxidative DNA damage may be repaired in cells by a variety of repair enzymes. In both bacteria and mammalian cells, a multitude of repair enzymes have been discovered, which possess multiple activities toward products of oxidative DNA damage (reviewed in 4–6). DNA base products are repaired by both base-excision and nucleotide-excision repair, but predominantly by the former. There are 20 or so major products resulting from reactions of free radicals with four heterocyclic bases in DNA (2,3). In the past, *in vitro* studies have determined the specificities of the repair enzymes for most of these DNA base products. In particular, two well known *Escherichia coli* enzymes Nth and Fpg proteins (endonuclease III and formamidopyrimidine–DNA glycosylase, respectively) account for the excision of most modified bases from DNA (4,5). Eukaryotic counterparts of these enzymes exist in other organisms. Nucleotide-excision repair systems also act on oxidative DNA damage (4,6).

Little is known about the repair of individual products of oxidative DNA base damage and their repair kinetics in mammalian cells. We present here a study of the repair of oxidative DNA base damage in human cells. The objective was to investigate the cellular repair of individual DNA base modifications that are formed in human cells upon exposure to oxidative stress. Hydrogen peroxide was chosen as the agent that causes oxidative stress because it can cross the cellular membranes, reach the nucleus and cause damage to nuclear DNA by generating ˙OH in close proximity to DNA (1). Hydrogen peroxide is also relevant in terms of endogenous oxidative stress because it is continuously produced in aerobic cells (reviewed in 7). A previous work has shown the formation of typical ˙OH-induced products from all four DNA bases in mammalian cells upon treatment with H_2O_2 (8). In the present work, the formation and subsequent time-dependent removal of modified DNA bases in cells were determined by using the technique of gas chromatography/isotope-dilution mass spectrometry (GC/IDMS). This technique permits precise identification and quantification of modified DNA bases in cells (3,9).

MATERIALS AND METHODS

Materials

RPMI-1640 medium, Hanks' balanced salt solution, fetal bovine serum (heat inactivated), L-glutamine, penicillin–streptomycin

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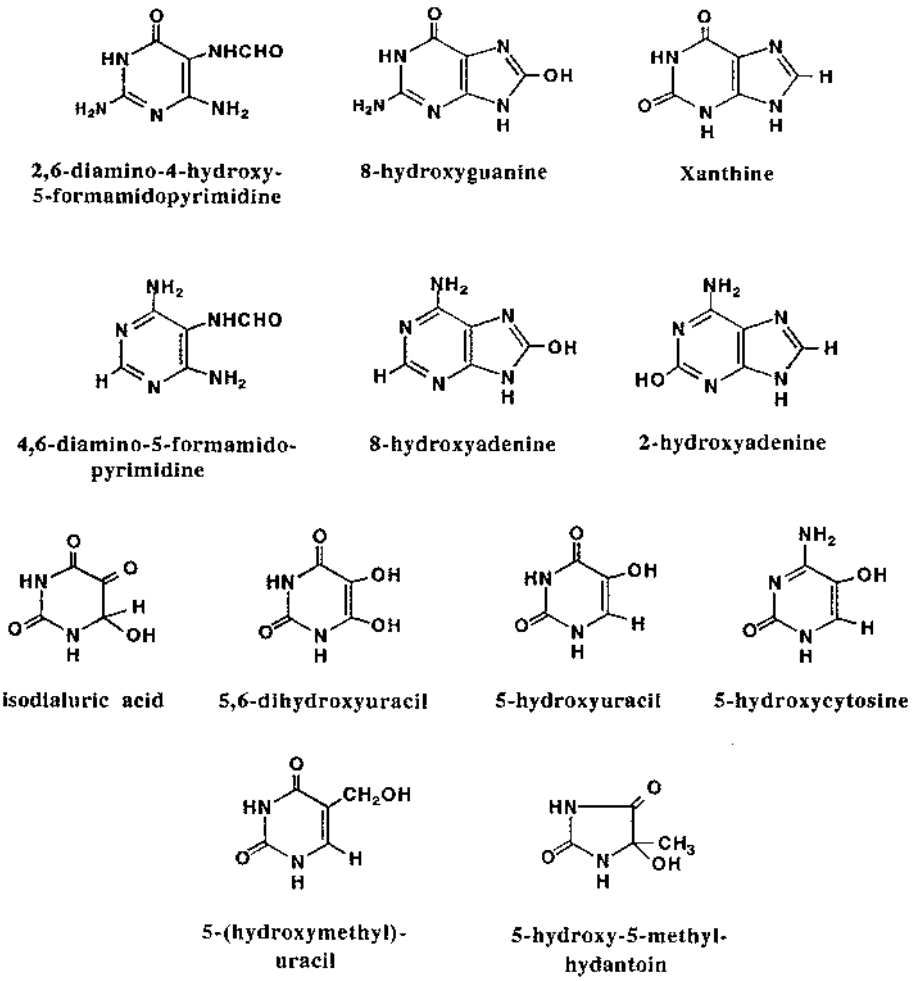

Figure 1. Structures of DNA base products measured in this work.

solution, sodium bicarbonate solution (7.5%) were purchased from Sigma Chemical Company. DNA NOW reagent was obtained from PGC Scientific. Modified DNA bases, their stable isotope-labeled analogues, and materials for GC/IDMS were obtained as described previously (9).

Cell culture and treatment of cells with hydrogen peroxide

Human lymphoblast cells were used. A sample of these cells (GM03798A) was purchased from NIGMS Human Genetic Mutant Cells Repository, Coriel Institute for Medical Research. Cells were grown in suspension in a culture medium consisting of RPMI-1640 modified medium supplemented with 15% fetal bovine serum, sodium bicarbonate (0.2%), L-glutamine (2 mM), penicillin (10 U/ml) and streptomycin (1 µg/ml) at 37°C under an atmosphere of 5% CO_2 mixed with room air. When cells reached a density of ~5×10^7 cells/ml, they were divided into three 25 ml aliquots and transferred into 50 ml tissue culture flasks. This was done for each data point. Cell viability was determined by the trypan blue exclusion test.

The cells were centrifuged at 800 *g* for 5 min, washed once with 25 ml of Hanks' balanced salt solution and then centrifuged. The solution was removed and the cells were resuspended in 25 ml of Hanks' balanced salt solution. H_2O_2 was added to the flasks at concentrations of 1–5 mM. Flasks were placed under an atmosphere of 5% CO_2 mixed with room air and kept at 37°C for 60 min. Control cells were treated in the same manner except for H_2O_2 treatment. For repair studies, cells were centrifuged at 800 *g* for 5 min, washed once with RPMI-1640 medium with fetal bovine serum and resuspended in fresh RPMI-1640 medium with fetal bovine serum. Aliquots of cells were incubated at 37°C for time periods of 10 min–6 h.

Isolation of DNA from cells

Aliquots (1 ml) of cell suspensions containing ~5×10^7 cells were centrifuged. An aliquot (1 ml) of DNA NOW reagent was added to each cell pellet. The pellet was homogenized by repetitive pipetting. Subsequently, 0.2 ml chloroform (kept at –20°C) was added and samples were shaken by hand for 20 s, kept on ice for 5 min and centrifuged. The aqueous phase was transferred to a clean tube and 2 vol of cold isopropanol were added. Samples were kept on ice for 1 h. DNA precipitate was removed with a glass rod and washed twice with cold ethanol (70%) and air dried.

Hydrolysis, derivatization and GC/IDMS

DNA samples were dissolved in 150 µl of 10 mM phosphate buffer (pH 7.4), and the concentration of DNA was determined

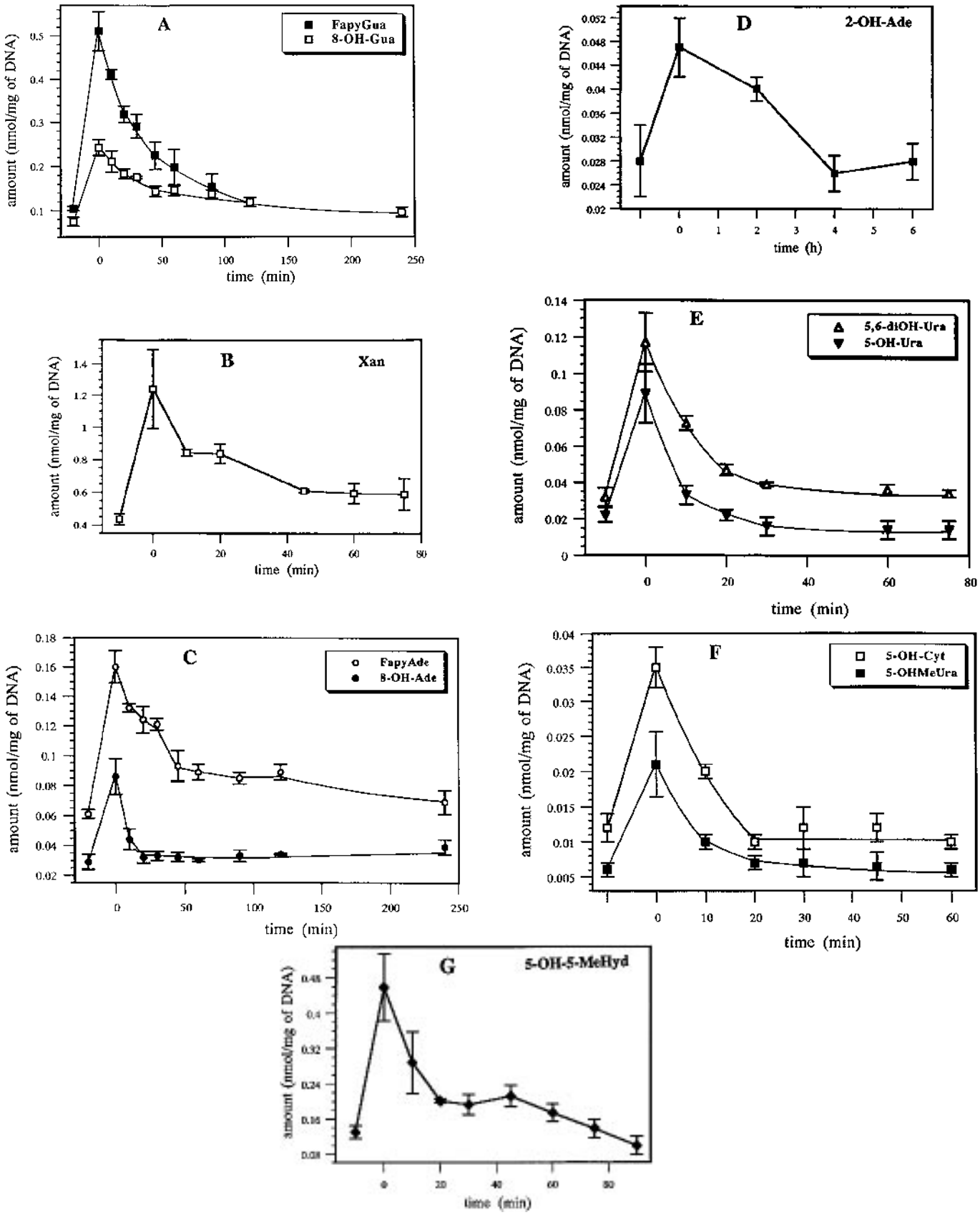

Figure 2. Kinetics of excision of products from cellular DNA. The amounts before and at time zero correspond to the control amounts and amounts found in cellular DNA following H_2O_2 treatment of cells, respectively. Data points represent the mean± standard deviation from measurement of these products in DNA samples, which were isolated from three to six independently treated batches of cells. One nmol of a modified base/mg of DNA corresponds to~32 modified bases/10^5 DNA bases.

by the absorbance at 260 nm (absorbance of 1 = 50 µg of DNA/ml). Aliquots of stable isotope-labeled analogues of modified DNA bases were added as internal standards to 50 µg aliquots of DNA samples (9). Samples were dried in a SpeedVac under vacuum and then hydrolyzed with 0.5 ml of 60% formic acid in evacuated and sealed tubes at 140°C for 30 min. The hydrolyzates were lyophilized in vials. For derivatization, a mixture (0.1 ml) of nitrogen-bubbled bis(trimethylsilyl)trifluoroacetamide (containing 1% trimethylchlorosilane) and acetonitrile (4:1, v/v) was added to the vials. They were sealed under nitrogen with Teflon-coated septa and heated at 120°C for 30 min. Analyses of derivatized samples were performed by GC/IDMS with selected-ion monitoring (SIM) (9,10). For this purpose, 2 µl of derivatized samples were injected without further treatment into the injection port of the gas chromatograph. The split mode of injection with a split ratio of 20:1 was used.

RESULTS

In order to study the cellular repair of DNA base products, their levels in cells must be elevated significantly over the background levels upon treatment of cells with the damaging agent. Furthermore, the cell viability after the treatment must remain at the same level as that of untreated control cells. For these reasons, we investigated first the formation of DNA base products in cells as a function of the concentration of H_2O_2. At the same time, the cell viability was determined. At H_2O_2 concentrations of 1–5 mM in the culture medium, a significant extent of modification of all four DNA bases was observed. The cell viability did not change significantly upon treatment of cells with ≤ 5 mM H_2O_2 (data not shown). Further experiments were undertaken using 5 mM H_2O_2.

Using the GC/IDMS-SIM, the following DNA base products were identified and quantified in cellular DNA: 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 8-hydroxyguanine (8-OH-Gua), xanthine (Xan), 4,6-diamino-5-formamidopyrimidine (FapyAde), 8-hydroxyadenine (8-OH-Ade), 2-hydroxyadenine (2-OH-Ade), isodialuric acid, 5-hydroxyuracil (5-OH-Ura), 5-hydroxycytosine (5-OH-Cyt), 5-(hydroxymethyl)uracil (5-OH-MeUra) and 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd). Of these compounds, the uracil derivatives are products of cytosine modification in DNA, except for 5-OHMeUra, which is a product of thymine modification (2,3). Isodialuric acid was detected as 5,6-dihydroxyuracil (5,6-diOH-Ura) because it enolizes during derivatization (11). Figure 1 illustrates the structures of these products. Upon treatment of cells with 5 mM H_2O_2, the product levels increased above control levels by 3–5-fold with no significant loss of cell viability (Fig. 2).

Having observed significant formation of DNA base products, we studied their excision from cellular DNA upon incubation of cells for different time intervals. Following H_2O_2 treatment, cells were washed with the medium without H_2O_2, suspended in fresh medium and then incubated at 37°C for 10 min–6 h. DNA was isolated from cells and analyzed. The results are illustrated in Figure 2.

There were three guanine-derived products, namely FapyGua, 8-OH-Gua and Xan, among those measured in cells. FapyGua and 8-OH-Gua were formed significantly in cellular DNA upon treatment of cells with H_2O_2 (Fig. 2A). Their levels increased by ~5- and 3-fold over background levels, respectively. An ~50% reduction in their levels was observed after 30–40 min of incubation. Afterwards, the rates of removal became slower. The level of FapyGua reached the background level at 2 h of incubation

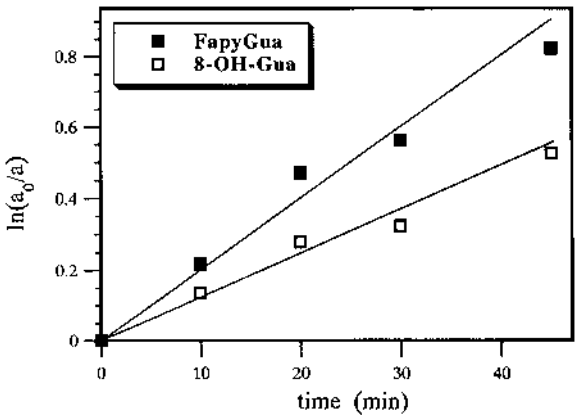

Figure 3. Plots of the logarithm of the ratio of the initial amounts (a_0) to the remaining amounts (a) of FapyGua and 8-OH-Gua in cellular DNA as a function of the incubation time. The data in Figure 2A were used for these plots. The initial amounts are those at time 0.

and remained constant thereafter up to 6 h (the last data point not shown in Fig. 2A). The excision of 8-OH-Gua required >2 h. The kinetic results in Figure 3 were analyzed to see which order of reaction applies to the excisions of FapyGua and 8-OH-Gua from cellular DNA. The logarithm of the ratio of the initial amount (a_0) to the amount (a) at a given incubation time was plotted against the incubation time (12). The plots yielded linear relationships up to 45 min of incubation (Fig. 3). This revealed that the excisions of FapyGua and 8-OH-Gua followed first-order kinetics within this time period. First-order rate constants and half-lives were calculated using the initial amounts and the amounts at incubation times from 10 to 45 min. The means (± standard deviation) of these kinetic constants are given in Table 1. The treatment of cells with H_2O_2 caused an ~3-fold increase in the level of Xan over its background level (Fig. 2B). Upon incubation, this product was excised from cellular DNA in 45 min with its level almost reduced to the background level (see also Table 1).

Table 1. Rate constants and half-lives for excision of base products from cellular DNA

Product	Rate constant[a] (min^{-1})	Half-life[b] (min)
FapyGua	0.0206 ± 0.0022	34 ± 3.5
8-OH-Gua	0.0127 ± 0.0012	55.2 ± 5.5
Xanthine	0.0182 ± 0.0005[c]	38.1 ± 1.1
FapyAde	0.0114 ± 0.0014	62.2 ± 8.8
8-OH-Ade	0.0582 ± 0.0080	12.2 ± 1.8
5-OH-Cyt	0.0593 ± 0.0033	11.7 ± 0.7
5-OH-Ura	0.0850 ± 0.0145	8.5 ± 1.5
Isodialuric acid	0.0463 ± 0.0007	15 ± 0.2
5-OH-5-MeHyd	0.0438 ± 0.0026	15.9 ± 0.9
5-OHMeUra	0.0645 ± 0.0095	11 ± 1.6

[a]Rate constant = $\ln(a_0/a)$/time (12).
[b]Half-life = 0.693/rate constant (12).
[c]The rate constant was calculated using the data points at 20 and 45 min of incubation (see Fig. 2B).

Three adenine-derived products FapyAde, 8-OH-Ade and 2-OH-Ade, were measured in cells. The levels of FapyAde and 8-OH-Ade increased by ~3-fold over their control levels by H_2O_2-treatment of cells (Fig. 2C). Over 50% of FapyAde was removed from cellular

DNA within 45 min of incubation. Afterwards, the rate of excision of this product became considerably slower. The background level was reached after ~4 h. 8-OH-Ade was rapidly excised from cellular DNA, with its level reaching the background level within 20 min of incubation. The excisions followed first-order kinetics up to 45 min with rate constants and half-lives given in Table 1. The H_2O_2-treatment of cells caused an ~2-fold increase in the level of 2-OH-Ade. The background level was reached after 4 h of incubation. No rate constant could be given in this case because the data points obtained at 2 and 4 h of incubation did not conform with any order of reaction.

Three cytosine-derived and two thymine-derived products were detected and quantified. The levels of 5,6-diOH-Ura, 5-OH-Ura, 5-OH-Cyt, 5-OHMeUra and 5-OH-5-MeHyd increased 3–5-fold over their background levels when cells were treated with H_2O_2 (Figs 2E, F and G). Incubation times for complete excision of these products ranged from 10 to 60 min. First-order rate constants were calculated using the initial amounts and the amounts at incubation times of 10 and 20 min (Table 1).

DISCUSSION

Typical ˙OH-induced DNA base products were formed in human cells upon oxidative stress by H_2O_2 with no significant loss of cell viability. No loss of cell viability upon oxidative stress was a prerequisite for the subsequent study of the repair of DNA damage. The salient feature of this work is the evidence that these modified bases were efficiently excised from cellular DNA, but with distinct differences between the kinetics of excision for individual products. Since the cell viability did not change upon various treatments, this observed excision of modified bases was attributed to actions of DNA repair systems in cells. In general, the rate of repair of purine-derived lesions was slower than that of pyrimidine-derived lesions. The kinetics of repair of guanine-derived lesions FapyGua, 8-OH-Gua and Xan were different. FapyGua was removed from cellular DNA within 2 h, whereas the repair of 8-OH-Gua lasted almost 4 h. The first-order rate constant for FapyGua was ~60% higher than that for 8-OH-Gua in the first 45 min of repair. The slower repair kinetics of 8-OH-Gua is in agreement with recent results on the repair of 8-OH-Gua in a different human cell line (13) and in liver DNA of mice (14). Xan was removed from cellular DNA within 45 min with a first-order rate constant similar to that for the excision of FapyGua.

FapyGua and 8-OH-Gua in DNA are substrates for the DNA repair enzyme Fpg protein of *E.coli* (4,15,16). Mammalian cells also possess activities that remove formamidopyrimidine lesions from DNA (reviewed in 17). There is evidence for the existence in human and other mammalian cells of DNA glycosylase and endonuclease activities for removal of 8-OH-Gua (18–20). The observed excision of FapyGua and 8-OH-Gua from DNA in human cells in this work may be due to the activity of such DNA repair enzymes. No repair enzyme specific for Xan has been described. Considering the wide substrate range of human excinuclease (21), these and other products may also be repaired by the nucleotide-excision repair system.

Of the adenine-derived products, the repair of FapyAde was slow, lasting >2 h, whereas the repair of 8-OH-Ade was complete within 20 min. The rate of excision of FapyAde within the first 45 min of repair was similar to that of 8-OH-Gua, but ~60 and 300% slower than those of FapyGua and 8-OH-Ade, respectively. FapyAde is efficiently excised from DNA by *E.coli* Fpg protein (16,22), and also by *E.coli* T4 endonuclease V (23). In contrast with 8-OH-Gua, 8-OH-Ade is a poor substrate for Fpg protein (16). The rate of repair of 2-OH-Ade was rather slow. After 2 h of repair, the level of this product was reduced by 50% only, with repair completed after 4 h. No enzymatic activity has thus far been described for 2-OH-Ade excision.

Of three cytosine-derived lesions detected, the rate of repair of 5-OH-Ura was approximately twice as fast as that of 5-OH-Cyt and isodialuric acid, reaching completion within 10 min. The repair of 5-OH-Cyt and isodialuric acid were complete in 20–30 min. The first-order rate constants for excision of 5-OH-Cyt and isodialuric acid were similar. The thymine-derived lesion, 5-OHMeUra, was also excised quickly from cellular DNA. On the other hand, 5-OH-5-MeHyd was removed at a slower rate. 5-OH-Ura, 5-OH-Cyt and 5-OH-5MeHyd are substrates for *E.coli* Nth protein (24–26), whereas *E.coli* and human uracil DNA *N*-glycosylases possess activities for isodialuric acid and 5-OH-Ura (11,26,27). A DNA *N*-glycosylase that excises 5-OHMeUra from DNA was detected in mammalian cells (4). Possible eukaryotic counterparts to *E.coli* Nth protein exist in other organisms (reviewed in 17). Human DNA *N*-glycosylases and human excinuclease repair system may be involved in the repair of cytosine- and thymine-derived lesions in human cells observed in this work.

Some of the DNA base lesions, of which repair was studied in this work, have been shown to possess premutagenic properties. In this respect, 8-OH-Gua is the most investigated lesion and has been shown to cause GC→TA transversions (28–31). FapyGua may lead to GC→CG transversions (32). The repair of these two guanine-derived lesions in human cells required up to 4 h. Considering also the extent of their formation, these two products may contribute significantly to the mutagenic effects of oxidative DNA damage. In support of this view, H_2O_2 has been shown to induce GC→TA and GC→CG transversions in the supF gene of *E.coli* (33) and in the same system after passage through a mammalian host (34). A recent work has shown that 8-OH-Ade also possesses premutagenic properties (35). Likewise, 2-OH-Ade may be potentially premutagenic in cells because it pairs with adenine and guanine (36). Our data indicate that cells may be able to repair 8-OH-Ade with a faster rate than its guanine-derived analog, whereas 2-OH-Ade repair may last as long as that of 8-OH-Gua. There is no information on the base-pairing characteristics of the other prominent adenine-derived lesion FapyAde (5) and of the guanine-derived lesion Xan.

Of the pyrimidine-derived lesions, 5-OH-Cyt and 5-OH-Ura have been shown to be potentially premutagenic lesions leading to GC→AT transitions and GC→CG transversions (37). 5-OH-Cyt appears to be more mutagenic than any other product of oxidative DNA damage (38). In mammalian cells, H_2O_2 predominantly produces GC→AT transitions followed by GC→CG and GC→TA transversions (34). The first two types of mutations may indicate the role of cytosine-derived lesions in mutagenesis induced by oxidative DNA damage. The present data show that 5-OH-Cyt and 5-OH-Ura may be repaired rapidly in human cells. The other prominent cytosine-derived lesion isodialuric acid has not been investigated for its biological effects. 5-OHMeUra codes as thymine and its observed mutagenicity has been attributed to incorporative mutagenesis (39,40). There is no information on possible mutagenic consequences of 5-OH-5-MeHyd (5). The contribution of these products to H_2O_2-induced mutagenesis is not known.

In conclusion, the results show the formation of a myriad of modified bases in cellular DNA and their subsequent cellular repair

as a function of time. This is the first systematic study on the repair of oxidative damage-induced products of all four DNA bases in a human cell line. These products substantially differ from one another in terms of their kinetics of excision from cellular DNA. The results of this work may contribute to the understanding of cellular repair of oxidative DNA damage in terms of individual DNA base products. The approach used may be applicable to studies of repair of oxidative DNA base damage in other mammalian cells and of possible differences in repair capacity between cell lines for oxidative DNA damage.

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