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Radical Oxidation of the Adenine Moiety of Nucleoside and DNA: 2-Hydroxy-2′-deoxyadenosine is a Minor Decomposition Product

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A method involving high performance liquid chromatography (HPLC) separation associated with tandem mass spectrometry (MS/MS) detection in the multiple reaction monitoring mode was set-up for the measurement of 2-hydroxy-2′-deoxyadenosine (2-OHdAdo). This modified nucleoside, arising from the radical oxidation of 2′-deoxyadenosine (dAdo), has been described in the literature as a potential biological marker of the Fenton reaction. Using the specific and sensitive HPLC-MS/MS assay, 8-oxo-7,8-dihydro-2′-deoxyadenosine, 4,6-diamino-5-formamidopyrimidine and 2-hydroxy-2′-deoxyadenosine (2-OHdAdo) were measured within 2′-deoxyadenosine and DNA solutions either exposed to γ-rays or treated under Fenton reaction conditions. It was found that the yield of 2-OHdAdo was low compared to that of 8-oxodAdo under most of the oxidative conditions studied. In particular and in contrast to previous works, the formation of 2-OHdAdo was shown to be a minor process both upon gamma irradiation and under Fenton reaction conditions. However, a significant yield of formation of 2-OHdAdo was observed either upon incubation with high concentrations of Fe^{2+} ions in the absence of hydrogen peroxide or upon γ-radiolysis of a nucleoside solution in the presence of the copper/(*o*)-phenanthroline complex.

Keywords: Oxidative DNA damage; Ionizing radiation; Fenton reaction; HPLC-MS/MS

Abbreviations: dAdo, 2′-deoxyadenosine; FapyAde, 4,6-diamino-5-formamidopyrimidine; 2-OHdAdo, 2-hydroxy-2′-deoxyadenosine; dGuo, 2′-deoxyguanosine; MRM, multiple reaction monitoring; HPLC-MS/MS, high performance liquid chromatography associated with electrospray ionization tandem mass spectrometry; [M+5] 2-OHdAdo, [6-amino-1,3,7,9-$^{15}N_5$]-2′-deoxyadenosine; mCPBA, *meta*-chloro-perbenzoic acid; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxodAdo, 8-oxo-7,8-dihydro-2′-deoxyadenosine; Cu^{+}/oPhe, $Copper^{(I)}$/(*ortho*)-phenanthroline complex; Fe^{2+}/EDTA, $Iron^{(II)}$/Ethylenediaminetetraacetic acid complex

INTRODUCTION

Oxidative damage to DNA may lead to deleterious cellular effects such as lethality, mutagenicity, carcinogenesis and aging.[1] The main classes of oxidative DNA lesions include strand breaks, DNA-proteins crosslinks, abasic sites and altered nucleobases. The latter class of damage induced by hydroxyl radicals (˙OH), one-electron oxidation, or singlet oxygen has been extensively studied using monomeric model systems and isolated DNA.[2] Oxidative degradation pathways have been proposed for the four DNA bases, on the basis of the characterization of the final products by a series of spectroscopic measurements. Kinetic and chemical features of the radical intermediates implicated in the latter reactions were obtained by time-resolved techniques.[3,4] ˙OH-mediated decomposition of 2′-deoxyadenosine (dAdo) in aqueous solution gives rise to two main modified nucleosides through the fate of the transient 8-hydroxy-7,8-dihydroaden-7-yl reducing radical. These include 8-oxo-7,8-dihydro-2′-deoxyadenosine (8-oxodAdo) and the related 4,6-diamino-5-formamidopyrimidine (FapyAde)

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derivative as a mixture of α and β furanosidic and pyranosidic isomers.[5–7] 5′,8-Cyclo-2′-deoxyadenosine has also been shown to be generated upon exposure of 2′-deoxyadenosine (dAdo)[8,9] and adenosine 5′-monophosphate aqueous solutions[10] to gamma radiation under anaerobic conditions. Deamination of dAdo into 2′-deoxyinosine has also been found to occur upon photosensitized one-electron abstraction of dAdo (Raoul and Cadet, unpublished results). A likely mechanism involves the initial formation of the oxidizing 6-aminyl radical.

2-Hydroxy-2′-deoxyadenosine (2-OHdAdo), which also exists in equilibrium with its 2-keto tautomer form,[11] and 2-hydroxy-2′-deoxyadenosine 5′-triphosphate (2-OHdATP), have been characterized as major adenine decomposition products of dAdo and its corresponding 5′-triphosphate (dATP), respectively, upon treatment by high concentrations of a Fe^{2+}/EDTA complex in aerated aqueous solution.[12,13] However, 2-OHAde was found to be produced only in small amount upon exposure of dAdo in aqueous solution to gamma radiation. In addition, using a poorly sensitive and rather unspecific HPLC/UV detection assay, 2-OHdAdo has been shown to be produced in low yield within DNA.[12] Therefore, 2-OHdATP has been proposed to be generated within cells as the result of Fenton chemistry mostly in the nucleotide pool. It should be mentioned that 2-hydroxyadenine (2-OHAde) has been first detected within DNA using the gas chromatography/mass spectrometry (GC–MS) assay.[14] Most of the data available on the formation of 2-OHdAdo involve the use of GC–MS.[15–20] However, even though the accuracy of the method for the measurement of 2-OHdAdo has not been specifically investigated, the drawbacks of the GC–MS assay in its standard version should be reminded.[21,22] In addition, some of the works were carried out without isotopically labeled internal standard,[23–25] which is a requisite for accurate GC–MS measurements.

Numerous *in vitro* studies have been designed to determine the biological properties of 2-OHdAdo. It has been found that 2-OHdATP is efficiently incorporated within the elongated DNA strand by several polymerases in front of T, C or G.[12,26,27] Translesional DNA synthesis of 2-OHAde-containing oligonucleotides and plasmids has been shown to lead to mutations in a sequence and polymerase-dependent manner.[28,29] Investigations on the repair of 2-OHAde within DNA have led to contradictory results regarding the existence of a *N*-glycosylase activity for this damage.[30–32] Finally, 2-OHdATP and 2-OHATP have been found to be good substrates for the human nucleotide pool detoxifying enzyme hMTH1.[33,34] Altogether, a large amount of information is available on the biological properties of 2-OHAde, even though its formation in cells is not clearly established. We report here the results of a detailed study of the radical-induced formation of 2-OH Ado within both DNA and dAdo solutions exposed to gamma radiation. For this purpose, a highly sensitive and specific HPLC-tandem mass spectrometry assay was designed. The possibility for 2-OHdAdo to be a marker of the Fenton reaction was also investigated by performing several oxidation experiments that involve hydrogen peroxide with either iron/EDTA or copper/(*o*)-phenanthroline at much lower concentrations than previously reported.[12] Reactions involving photosensitized electron abstraction from dAdo have also been investigated. In all cases, 2-OHAde was found to be a minor product, when compared to 8-oxoAde and FapyAde, both in isolated DNA and within free nucleoside.

EXPERIMENTAL PART

Biochemicals and Chemicals

Nuclease P1 (*Penicillium citrium*), calf spleen phosphodiesterase I and II were obtained from Sigma (St Louis, MO). Alkaline phosphatase was purchased from Roche Diagnostics (Mannheim, Germany). Calf thymus DNA was from Sigma (St Louis, MO). Ethylenediaminetetraacetic acid (EDTA) was from Interchim (Montluçon, France). Water was deionized with a Millipore-Milli-Q system (Millipore, Molsheim, France). dAdo was from Genofit (Geneva, Switzerland) and [$^{15}N_5$] dAdo was a gift from the Pr Poulsen (University of Copenhagen, Denmark)

Preparation of 2′-deoxyadenosine N-1-oxide

2′-Deoxyadenosine (3 mg) was dissolved in 1.7 ml of phosphate buffer (50 mM-pH 7). A 0.1 M ethanolic solution (1.2 ml) of *m*-chloroperbenzoic acid (*m*CPBA) was added and the resulting mixture held at 37°C for 15 h. Most of the solvent was then removed under vacuum in order to induce the precipitation of *m*CPBA. The remaining *m*CPBA was extracted with diethylether (2 × 1 ml) and the aqueous solution was evaporated to dryness. The compound was identified by UV measurements ($UV_{\lambda max} = 232$ nm) but not quantified and directly used in the second step of the synthesis.

Preparation of 2-hydroxy-2′-deoxyadenosine

The latter crude product was dissolved in 2.5 ml of water and subsequently exposed under a stream of nitrogen to a UVC source emitting mostly at 254 nm for 2 h. Thereafter, the irradiated solution was injected onto a Hypersil C18 column. Separation

was achieved using water/methanol (98:2, v/v) as the mobile phase at a flow rate of 1 ml/min. The elution of the reaction products was monitored using a differential refractometric detector. The retention time of 2-OHdAdo was 17 min. The yield (12%) was determined by monitoring the UV absorption at 247 nm of the isolated fraction ($\varepsilon_{247} = 8900$) $UV_{\lambda max}$ (292, 247, 207 nm).

200 MHz ^{1}H-NMR (D_2O), (δ, ppm): 2.55 (m, 1H, H-2″, $J_{2'',3'} = 3.2$ Hz), 2.81 (m, 1H, H-2′, $J_{2',3'} = 6.1$ Hz, $J_{2'',2'} = -14.0$ Hz), 3.83 (m, 1H, H-5″), 3.89 (m, 1H, H-5′, $J_{5',5''} = -12.6$ Hz), 4.20 (m, 1H, H-4′, $J_{4'5'} = 3.2$ Hz, $J_{4'5''} = 4.3$ Hz), 4.70 (m 1H, H-3′, $J_{3',4'} = 2.8$ Hz), 6.35 (m, 1H, H-1′, $J_{1'2'} = 7.7$ Hz, $J_{1'2''} = 6.3$ Hz), 8.06 (s,1H, H-8) 50 MHz ^{13}C-NMR (D_20), (δ, ppm): 41.6 (C-2′), 64.5 (C-5′), 74.1 (C-3′), 86.9 (C-1′), 90.1 (C-4′), 142.0 (C-8); in this experiment, the signals of the quaternary carbons were not observed because they relax too slowly.

Preparation of [6-Amino-1,3,7,9-$^{15}N_5$]-2-hydroxy-2′-deoxyadenosine

The synthesis was performed using the same procedure than for the non labeled compound. The starting material was [6-amino-1,3,7,9,-$^{15}N_5$]-2′-deoxyadenosine (600 µl of a 5.8 mM solution). The first step yielded [6-amino-1,3,7,9-$^{15}N_5$]-2′-deoxyadenosine *N*-1-oxide. Subsequent UVC irradiation led to the desired compound with an overall yield of 10%. [6-amino-1,3,7,9-$^{15}N_5$]-2-hydroxy-2′-deoxyadenosine was purified by reverse phase high performance liquid chromatography (HPLC).

γ-Irradiation of 2′-Deoxyadenosine and Isolated DNA in Aerated Aqueous Solutions

An aerated aqueous solution of either 1 mM dAdo or 0.5 mg ml^{-1} DNA with or without traces of metallic ion (0–100 µM ferrous sulfate or cuprous chloride) in the presence of metal chelators (EDTA and (*o*)-phenanthroline, respectively), was exposed to the γ-rays of a ^{60}Co source immersed in a water pool. The dose rate was 20 Gy/min as determined by poly(methylmethacrylate) dosimetry. Air was continuously bubbled through the solution during the irradiation period (2–5 min).

Oxidation of 2′-Deoxyadenosine and Isolated DNA Under Fenton Chemistry Conditions

An aerated aqueous solution of either 1 mM dAdo or 0.5 mg ml^{-1} DNA was treated with either ferrous sulfate or cuprous chloride freshly made solutions (0–100 µM) in the presence of their respective chelators EDTA or (*o*)-phenanthroline. Hydrogen peroxide was then added (0–200 µM). The reaction mixture was kept at 37°C for 1 h.

Riboflavin-mediated Photosensitization of 2′-Deoxyadenosine and Naked DNA

A saturated solution of riboflavin (28 µl) was added to 2.8 ml of an aerated aqueous solution of substrate (either 1 mM dAdo or 0.5 mg ml^{-1} DNA). The resulting mixture was then exposed to a UVA source emitting mostly at 365 nm for 1, 2, 5, 10, 15 and 30 min. Then, 200 µl of the photosensitized solution was withdrawn from each of the irradiated samples.

HPLC-MS/MS Analyses

On-line HPLC-MS/MS measurements were carried out using a 7100 Hitachi–Merck pumping system (Merck, Darmstadt, Germany). Loop injections were performed using a Sil 9A (Shimadzu, Tokyo, Japan) autosampler equipped with a 20 µl loop. Two chromatographic conditions, already described in a previous paper[35] and summarized here, were used:

System I consisted of an Uptisphere ODB (5 µm, 150 × 2 mm i.d.) column (Interchim, Montluçon, France). A gradient of solvent A (2 mM ammonium formate) and solvent B (2 mM ammonium formate that contained 10% acetonitrile), was used at a flow rate of 0.2 ml/min. Methanol was added at the outlet of the column at a flow rate of 0.2 ml/min. The measurement of normal nucleosides was performed with a L 4000 Merck–Hitachi UV detector set at 280 nm.

System II consisted of a Hypersil NH_2 (5 µm, 150 × 2 mm i.d.) column (Interchim, Montluçon, France). A mixture of acetonitrile and 10 mM ammonium formate [90/10] v/v was used as the isocratic eluent at a flow rate of 0.2 ml/min.

In both cases the output of the chromatographic system was connected without splitting to an API 3000 triple quadrupole mass spectrometer through a turboionspray source (PE-SCIEX, Thornill, Canada).

Measurement of 8-OxodAdo and 2-OHdAdo

Aliquots of the DNA solution (100 µl, 50 µg) were digested upon incubation with 0.004 U of phosphodiesterase II and 5 U of nuclease P1 stored in the appropriate buffer (300 mM sodium acetate, 1 mM $ZnSO_4$, pH 5.3) together with 10 µl of digestion buffer (200 mM succinic acid, 100 mM $CaCl_2$, pH 6) for 2 h at 37°C. Then, 0.003 U of phosphodiesterase I and 5 U of alkaline phosphatase in 10 µl of the related buffer (500 mM Tris, 1 mM EDTA pH 8.5) were added. The reaction mixture was subsequently incubated for 2 h at 37°C. Samples were then transferred into HPLC injection vials. Internal standards (25 pmol) were added and the samples were analyzed by HPLC-MS/MS using system I.

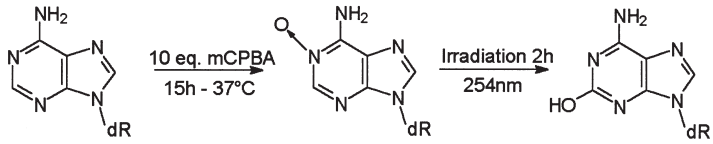

FIGURE 1 Synthesis pathway of 2-OHdAdo.

Measurement of FapyAde

Aliquots of the DNA solution (100 µl, 50 µg) were digested in the presence of 25 pmol of [4,6-diamino-$^{15}N_2$,5-formylamino-^{13}C]FapyAde. This was achieved by incubation for 2 h at 37°C in the presence of 10 U nuclease P1 suspended in 10 µl of an aqueous solution of 30 mM NH_4OAc and 0.1 mM $ZnSO_4$ at pH 5.5. After completion of the digestion, the enzyme was precipitated by addition of 50 µl of chloroform. Then, the aqueous layer was transferred into HPLC injection vials, frozen and lyophilized. The resulting mixture of nucleotides was dissolved into 60 µl of 88% formic acid. The sample was left for 20 min at room temperature. Then, formic acid was removed *in vacuo*. Prior to HPLC-MS/MS analyses using system II, 50 µl of the HPLC eluent (acetonitrile/10 mM ammonium formate [90:10] v/v) was added to each sample.

RESULTS AND DISCUSSION

Design of the HPLC-MS/MS Assay for 2-OHdAdo

The accurate HPLC-MS/MS measurement of 2-hydroxy-2′-deoxyadenosine required the synthesis of both 2-OHdAdo and its [M + 5] labeled analog. The latter compound that was prepared from [6-amino-1,3,7,9-$^{15}N_5$]-2′-deoxyadenosine ([M + 5]2-OHdAdo) was used as the internal standard.[36,37] Interestingly, $^{15}N_5$ labeled 2-OHdAdo did not exhibit any shift in the HPLC retention time with respect to the unlabeled 2-OHdAdo. Therefore, the quantitative measurement of 2-OHdAdo could be achieved using the isotopic dilution method. The modified nucleoside **3** (Fig. 1) was prepared according to the method proposed by Mouret *et al*.[38]

The synthetic 2-OHdAdo was first used to optimize the mass spectrometer parameters to obtain the highest sensitivity. The nucleoside was ionized in the positive mode by an electrospray source. The HPLC-MS/MS measurements were performed in the multiple reaction monitoring (MRM) mode. Under the latter conditions, a specific transition is used for each targeted molecule. The pseudo-molecular ion is isolated in the first quadrupole, and then fragmented in the collision cell (second quadrupole). Finally, a specific daughter fragment is isolated by the third quadrupole and used for the quantification. Thus, the MS detection in the MRM mode is highly specific and sensitive. In that respect, it may be noted that no compound exhibiting the expected mass spectroscopic features for 2-OHdAdo could be detected in N_2O saturated aqueous solution of DNA exposed to gamma rays using a LC/MS method.[39] This may be probably accounted for by the lack of calibration of the HPLC separation in the absence of standard and the very low yield of 2-OHdAdo observed in the present study as well as by GC–MS measurements.[40]

To avoid variations, an internal standard was added to the samples. As the detection was done by mass spectrometry, the internal standard was the targeted molecule that is labeled with stable isotopes, namely five ^{15}N atoms. The fragmentation spectrum of 2-OHdAdo (Fig. 2) showed that the best transition for its quantitation was 268 → 152, corresponding to the loss of the 2-deoxyribose moiety. Similarly, the detection of [M + 5] 2-OHdAdo, used as the internal standard, was achieved using the 273 → 157 transition. It should be pointed out that the fragmentation spectrum of 2-OHdAdo is the same than for the 2′-deoxyguanosine (dGuo). However, the overwhelming presence of dGuo in the DNA samples did not interfere with the detection of 2-OHdAdo because the latter compound eluted before dGuo.

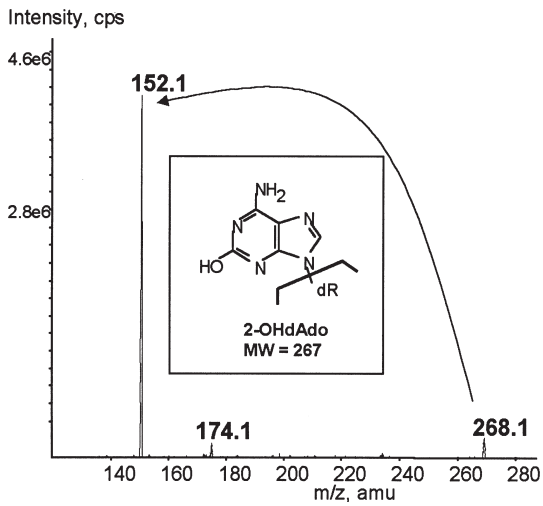

FIGURE 2 Fragmentation mass spectrum of 2-hydroxy-2′-deoxyadenosine recorded in the positive mode. The parent ion was set at $m/z = 268$ ($[M + H]^+$).

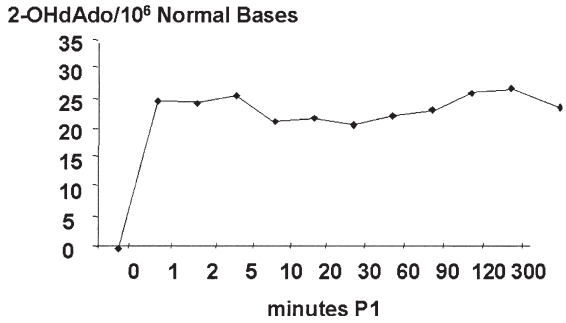

FIGURE 3 Kinetic of the nuclease P1-mediated release of 2-OHdAdo from oxidized DNA with increasing incubation times.

Importantly, accurate measurement of 2-OHdAdo requires its quantitative release from DNA. This was checked by monitoring the level of enzymatically digested 2-OHdAdo from *m*CPBA/UVC-treated DNA after increasing periods of incubation. It was shown that 2-OHdAdo was quantitatively released within the first minutes of digestion (Fig. 3).

Adenine Oxidation by High Concentration of Iron in the Presence of Oxygen

Preliminary experiments with concentrations of ferrous sulfate ranging between 0 and 5 mM were carried out. As previously reported by Kamiya *et al.*,[12] 2-OHdAdo was produced within both DNA (Fig. 4) and dAdo (Fig. 5) in aerated phosphate buffer solutions in the presence of ferrous iron. The level of 2-OHdAdo was relatively high in the dAdo solutions, whereas the yield was much lower in DNA. The comparison of the latter data with those obtained using the same ferrous salts at the same concentration in the presence of hydrogen peroxide shows striking differences. The level of 2-OHdAdo decreased whereas that of 8-oxodAdo increased. The differences between both conditions are more significant for dAdo than for DNA. Therefore, it might be concluded that the oxidative mechanism involved in Fe^{2+}/O_2 and $Fe^{2+}/H_2O_2/O_2$ oxidation systems are different. In the first case, ferrous autoxidation occurs. The rate of autoxidation reaction in the presence of phosphate buffer is linear with Fe^{2+} concentration.[41–43] The role of the iron concentration in the formation of 2-OHdAdo is clearly shown by the drastic decrease in the ratio between the yields of 2-OHdAdo and 8-oxodAdo when the Fe/EDTA concentration was reduced from 5 to 0.1 mM. The second oxidizing system ($Fe^{2+}/H_2O_2/O_2$) is known to yield hydroxyl radicals or species exhibiting similar oxidizing properties. The present results thus suggest that 2-OHdAdo is not significantly produced in the reactions involving the latter reactive oxygen species. Considering that cellular oxygen tension is much lower than in saturated aqueous solutions and that the concentration of ferrous ions is low, it may be concluded that a significant implication of the Fe^{2+}/O_2 mechanism in oxidation of cellular DNA is questionable. The formation of 2-OHdAdo was then studied under conditions of Fenton chemistry at low concentrations of ferrous and cuprous ions. A comparison was made with the degradation induced by $^{\cdot}OH$ produced by γ-rays in the presence of metal ion complexes.

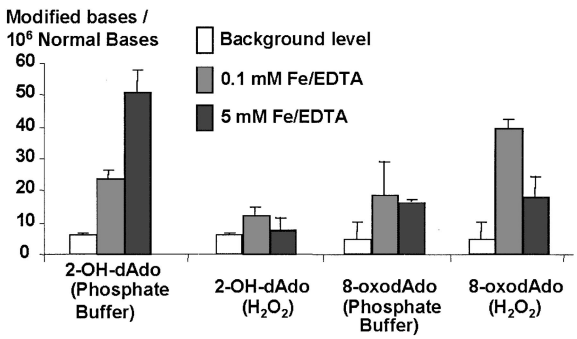

FIGURE 4 Levels of formation of 8-oxodAdo and 2-OHdAdo within DNA either exposed to high concentration of ferrous ions in phosphate buffer or treated under the Fenton reaction conditions (presence of hydrogen peroxide (50 μM) with a high concentration of ferrous ions).

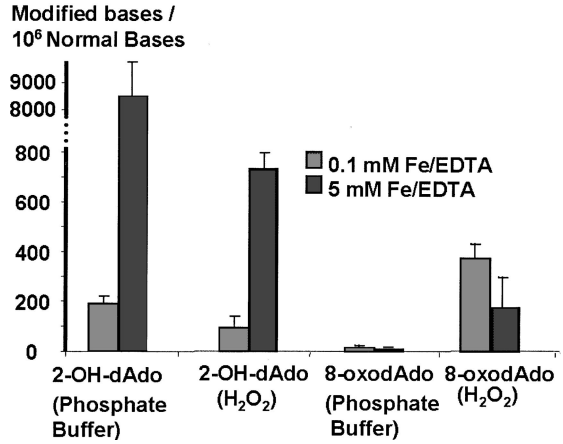

FIGURE 5 Distribution pattern of the degradation products of a 1 mM dAdo solution treated with high concentration of ferrous ions in the presence (50 μM) or not of hydrogen peroxide. The background level of 8-oxodAdo and 2-OHdAdo were 0.35 and 1.55 lesions per 10^6 normal bases, respectively.

Formation of 2-OHdAdo Upon Oxidation of a dAdo Solution in the Presence of Low Amount of Metal Ions

Fenton Reaction

The Fenton reaction carried out by the reduction of H_2O_2 with low concentrations of ferrous sulfate or cuprous chloride led to a linear formation of 2-OHdAdo with the increase in metal ion concen-

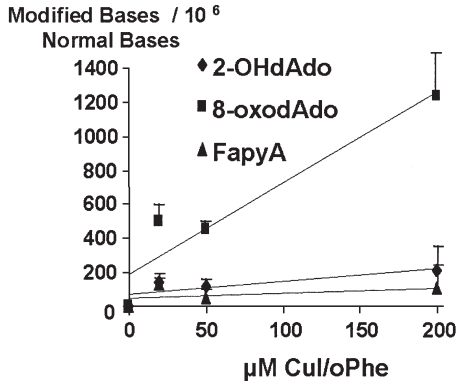

FIGURE 6 Formation of 2-OHdAdo upon exposure of dAdo to Fenton conditions (100 µM H_2O_2) with increasing amounts of CuI/oPhe in aerated aqueous solution.

tration. However, the yield of 2-OHdAdo was much lower than that of 8-oxodAdo. Indeed, in the case of the Fenton reaction with ferrous sulfate, the yield of formation of 2-OHdAdo and FapyAde was 14-fold and 8-fold lower than that of 8-oxodAdo (rate calculated between 0 and 200 µM of ferrous salt, with 100 µM H_2O_2). In the case of the Fenton reaction with cuprous chloride (Fig. 6), the yield of formation of 2-OHdAdo was 6-fold lower whereas that of FapyAde was 20 times lower than that of 8-oxodAdo. It may be inferred from this that 2-OHdAdo is not efficiently produced from dAdo in the studied range of metal ion concentration. This is in contrast to results obtained in the millimolar range.

Gamma Radiation

Similar results were obtained after exposure of dAdo in aerated aqueous solution to gamma radiation in the presence or the absence of ferrous/EDTA complex. Indeed, 8-oxodAdo was the major degradation product (Fig. 7). FapyAde was also produced but to a lower extent. Interestingly, the yield of formation of FapyAde slightly increased with increasing concentrations of Fe^{2+}. The level of

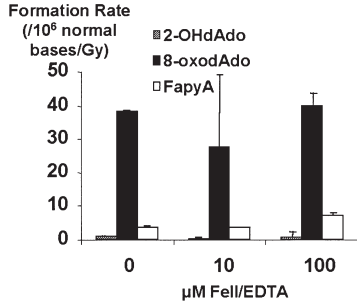

FIGURE 7 Distribution of the degradation products upon exposure to γ-rays (0–200 Gy) of dAdo solutions that contained increasing concentrations of Fe^{II}/EDTA.

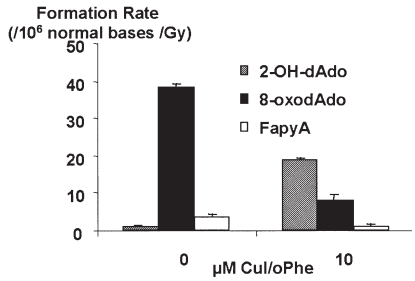

FIGURE 8 Rate of formation of 2-OHdAdo upon exposure of dAdo to γ-rays 0–200 Gy in either the presence of the absence of 10 µM CuI/oPhe.

2-OHdAdo was the lowest among the three DNA lesions studied since it represented only 3% of that of 8-oxodAdo. In addition, the rate of formation did not vary with increasing concentrations of ferrous salt.

The results were totally different when γ-radiolysis of a dAdo aqueous solution was performed in the presence of copper ion (Fig. 8). Indeed, the presence of 10 µm CuI/oPhe led to a radiolytic yield of 2-OHdAdo twice higher than that of 8-oxodAdo. Under these conditions, the yield of the latter lesion was 10-fold lower than in the absence of Cu. The difference of damage profile observed within solutions of dAdo that contained the Cu^+/oPhe complex in the presence of hydrogen peroxide on one hand, and exposed to gamma radiation on the other hand, is a puzzling result. Indeed, in both cases, hydroxyl radicals are expected to be the main reactive species. Therefore, the favored formation of 2-OHdAdo at the expense of 8-oxodAdo upon addition of Cu^+/oPhe in the irradiated solution cannot be rationalized in term of production of secondary species exhibiting different oxidizing properties with respect to $^{\cdot}OH$. A main difference between the two oxidizing systems could be the higher concentration of Cu^+ ions in the gamma irradiation experiments. Indeed, Cu^{2+} ions are reduced to Cu^+ by the superoxide ions ($k = 5 - 8 \times 10^9\ M^{-1}\ s^{-1}$)[44] produced upon the reaction of hydrated electrons[45] with molecular oxygen.[44] This does not apply as well to iron since the reaction between Fe^{3+} and $O_2^{\cdot-}$ is slower ($1.5 \times 10^8\ M^{-1}\ s^{-1}$). In contrast, under Fenton reaction conditions, the Cu^+ ions are irreversibly converted into Cu^{2+} by the reaction with hydrogen peroxide.[41,46,47] Therefore, additional experiments, involving irradiation of dAdo in the presence of free Cu^+ ions and Cu^{2+}/(*o*)-phenanthroline complex were performed. A drastic decrease in the yield of 8-oxodAdo was observed in both cases. A significant increase in the yield of 2-OHdAdo was also observed (the ratio 2-OHdAdo/8-oxodAdo varies between 0.3 and 0.5), even though to a lesser extent than upon addition of the Cu^+/oPhe complex (ratio 2-OHdAdo/8-oxodAdo = 2). It can therefore be

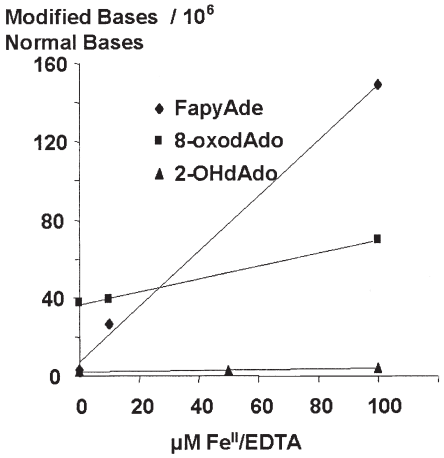

FIGURE 9 Formation of the three main degradation products of dAdo (2-OHdAdo, 8-oxodAdo and FapyAde) within DNA upon exposure to increasing concentrations of Fe^{II}/EDTA in the presence of 100 µM H_2O_2—The level of 8-oxodGuo was found to be 424, 448 and 774 lesions per 10^6 normal bases for Fe^{II}/EDTA concentrations of 0, 10 and 100 µM, respectively.

proposed that Cu^+ ions are able to react with 8-hydroxy-7,8-dihydroaden-7-yl radicals, leading to the decrease in the yield of 8-oxodAdo and the increase in that of 2-OHdAdo. It should be pointed out that reduction of the latter radical by Cu^+ atoms is not likely to occur since the yield of FapyAde did not increase upon addition of Cu^+/oPhe, the latter being less reducing than Fe^{2+}.[48] It can be added that formation of a complex between Cu^+ and dAdo may also be ruled out because the nucleoside was in large excess. Therefore, even if a quantitative complexation took place, it would not explain the 8-fold decrease in the yield of 8-oxodAdo. Alternatively, it may be proposed that the CuI/oPhe complex specifically reacts with the 8-hydroxy-7,8-dihydroaden-7-yl reducing radical to yield 2-OHdAdo. Altogether, there is a need for deeper understanding of the transient species produced in the dAdo/γ radiation/Cu^+ system.

The formation of 2-OHdAdo was poorly promoted upon exposure of dAdo samples to UVA in the presence of riboflavin. The reaction of photoexcited riboflavin with dAdo that involves a type I photosensitization mechanism leads to the formation of a dAdo radical cation through an one-electron abstraction reaction. As already observed with gamma radiation and Fenton conditions, 8-oxodAdo was the main decomposition product whereas a low 2-OHdAdo formation yield was observed.

Measurement Within Isolated DNA

Fenton Reaction

Similar experiments were performed with isolated DNA. Fenton chemistry involving Fe^{2+}/EDTA and hydrogen peroxide (Fig. 9) led to a linear formation of the three measured base lesions with increasing iron concentration. FapyAde and 8-oxodAdo were the major adenine modification products measured whereas 2-OHdAdo was produced in a hundred times lower yield than FapyAde. The latter compound corresponds to the open-ring product coming from the reduction of the neutral 8-hydroxy-7,8-dihydroaden-7-yl reducing radical. This radical is initially produced by the addition of ·OH at the 8-position of dAdo. Depending on the redox conditions, it can evolve to the reduced form (FapyAde) or the oxidized form (8-oxodAdo).[2] Since FapyAde was the major product, this indicates that the reducing effect of iron is enhanced in the Fenton reaction.

Fenton chemistry with Cu^+/oPhe led to the predominant formation of 8-oxodAdo whereas FapyAde and 2-OHdAdo were produced in a similar and much lower amount. The ratio of FapyAde and 2-OHdAdo with respect to 8-oxodAdo was respectively, 2.7 and 4.3. This demonstrates that cuprous ions exhibit a lower reducing effect than ferrous ions.

In both cases, it is interesting to mention that the major degradation products of dAdo, either 8-oxodAdo (formation rate of 0.33 per 10^6 normal bases and per µM Fe/EDTA with a level of 69 8-oxodAdo/10^6 normal bases for an oxidizing system consisting of 50 µM Fe/EDTA and 100 µM H_2O_2) or FapyAde (formation rate of 1.42 per 10^6 normal bases and per µM Fe/EDTA with a level of 148 FapyAde/10^6 normal bases for an oxidizing system consisting of 50 µM Fe/EDTA and 100 µM H_2O_2) were produced with a much lower efficiency than 8-oxoGuo (formation rate 3.55 per 10^6 normal bases and per µM Fe/EDTA with a level of 775 8-oxodGuo/10^6 normal bases for an oxidizing system consisting of 50 µM Fe/EDTA and 100 µM H_2O_2.

Gamma Irradiation

The three adenine lesions were also measured within DNA samples upon exposure to γ-rays in the presence or the absence of Fe^{2+} or Cu^+. The level of both 8-oxodAdo and FapyAde slightly increased when Fe^{2+} ions were added to the solution (Fig. 10). Indeed, the presence of iron during gamma irradiation leads to an increased production of ·OH. However, the ratio between the yields of 8-oxodAdo and FapyAde slightly decreased in the presence of additional ferrous salt. Indeed, the iron ions induce a reduction of the common radical precursor of both lesions promoting the formation of FapyAde. In contrast, the yield of 2-OHdAdo was very low (2.3% of that of 8-oxodAdo) and not affected by the presence of Fe^{2+}. The minor formation of 2-OHdAdo with respect to 8-oxodAdo has also been observed upon gamma irradiation of DNA in the absence of oxygen, on the basis of GC–MS analyses.[40]

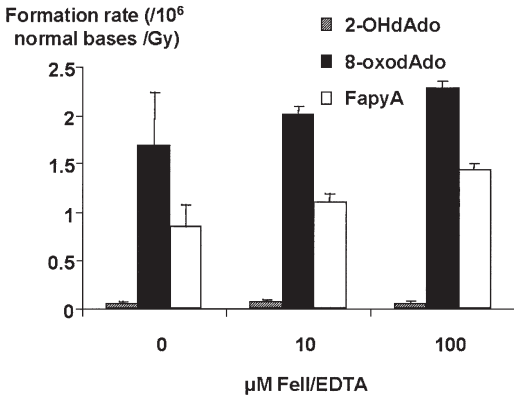

FIGURE 10 Formation rate of 2-OHdAdo upon γ irrdiation of an aerated aqueous solution of DNA (0–100 Gy) in the presence of increasing concentrations of Fe^{II}/EDTA.

The strong effect of Cu^+ on the yield of 2-OHdAdo observed with dAdo was not operating for DNA. Indeed the yield of 2-OHdAdo slightly increased upon addition of Cu^+/oPhe (Fig. 11) but remained one order of magnitude lower than that of 8-oxodAdo. This can be the result of a lower accessibility of the bases or their radicals to Cu^+/oPhe. Therefore, this would prevent the reaction proposed above for the free nucleoside. In addition, the yield of FapyAde and, to a smaller extent of 8-oxodAdo, decreased with an increasing concentration of copper. Moreover, the yield of 8-oxodAdo was higher than the yield of FapyAde because some oxidizing species produced by the reactants could be released and oxidize the transient radical. As observed upon Fenton chemistry, 8-oxodAdo, the major product in the radiation-induced degradation of dAdo, was generated in a very low yield with respect to that of 8-oxodGuo (12%).

Experiments involving riboflavin sensitization of isolated DNA to UVA radiation led to a very low yield of dAdo degradation. This is in agreement with a preferential one-electron oxidation of dGuo which has the lowest oxidation potential among DNA nucleosides.[49]

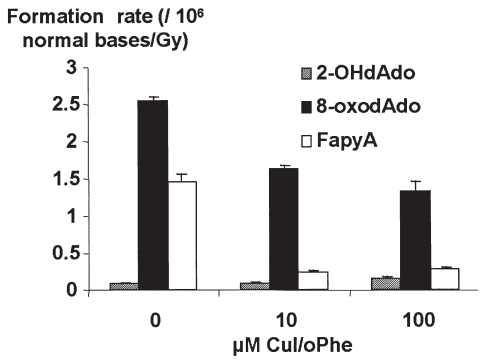

FIGURE 11 Distribution pattern of the degradation products of dAdo within DNA upon exposure to γ-rays (0–100 Gy) in the presence of increasing concentrations of Cu^I/oPhe in aerated aqueous solution.

CONCLUSION

The main conclusion of the present work is that 2-OHdAdo is a minor product of the hydroxyl radical-mediated degradation of dAdo compared to 8-oxodAdo and FapyAde. It should be reminded that, in the presence of oxygen, the yield of 8-oxodAdo is ca. 10-fold lower than that of 8-oxodGuo in DNA exposed to a series of oxidizing conditions including gamma irradiation[35] and type I photosensitization.[50] Therefore, formation of 2-OHdAdo may be regarded as a minor process. This is in agreement with pulse radiolysis studies showing that addition of $^{\cdot}OH$ mostly takes place at the C8 and C4 positions of the purine ring. It may be added that 2-OHdAdo does not appear to be either a major degradation product of dAdo by the Fenton reagent. A major exception is the formation of 2-OHdAdo with high efficiency upon treatment of dAdo by large amounts of Fe^{2+}/EDTA in the presence of oxygen. However, such high concentrations are unlikely to be biologically relevant. A puzzling result is the effect of Cu^+ ion during γ-radiolysis of the free nucleoside which leads to the formation of 2-OHdAdo in relatively high yield. However, the concentration of free Cu^+ ions in cells is low and may not significantly modify the chemistry of hydroxyl radical-mediated oxidation reactions of adenine or its derivatives. Altogether, the later observations suggest that 2-OHdAdo is at the best produced in low amounts in cells. This is partially confirmed by measurements within cellular DNA using HPLC-MS/MS. The basal level of 2-OHdAdo was so low that the lesion could not be detected within the DNA of cultured human monocytes and *Escherichia coli* cells. Based on the sensitivity of the assay and the large amount of DNA analyzed, the upper limit for the level of 2-OHdAdo may be estimated to be around 1 lesion per 10^7 normal bases (data not shown). However, it should be kept in mind that 2-OHdAdo exhibits mutagenic properties and that, if not recognized by the repair systems, this lesion may partially contribute to the mutagenicity of oxidative processes.

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