Hydroxyl radicals are involved in the oxidation of isolated and cellular DNA bases by 5-aminolevulinic acid

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Abstract 5-Aminolevulinic acid (ALA) is a heme precursor, pathological accumulation of which is associated with liver cancer. We show that the reactive oxygen species produced upon ALA metal-catalyzed oxidation promote the formation of several radical-induced base degradation products in isolated DNA. The distribution of modified bases is similar to that obtained upon gamma irradiation. This observation strongly suggests the involvement of hydroxyl radicals in the ALA-mediated DNA damage. Increased levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine and 5-hydroxy-2′-deoxycytidine in organ DNA of rats chronically treated with ALA were observed. This is strongly suggestive of the implication of hydroxyl radicals in the ALA-induced degradation of cellular DNA.

Key words: DNA damage; DNA lesion; 5-Aminolevulinic acid; Hydroxyl radical; Transition metal

1. Introduction

In plants and animals, 5-aminolevulinic acid (ALA) initiates the biosynthesis of porphyrin IX, a precursor of haem proteins. Under several pathological conditions, including lead poisoning [1], and inherited diseases such as acute intermittent porphyria (AIP) and tyrosinosis [2,3], ALA can accumulate in organs, mainly the liver. Noteworthy is the correlation of ALA accumulation with an increase in the occurrence of hepatic cancers [4–6], ferritin deposits in hepatocytes of AIP liver biopsy samples [7], and iron overload in liver and brain of ALA-treated rats [8]. Exogenous ALA is also to be considered since it has been used as an inducer of elevated porphyrin levels in tumor photodynamic therapy in recent years [9–11]. It has been hypothesized that ALA could induce DNA damage via the reactive oxygen species (ROS) produced during its metal-catalyzed oxidation into 4,5-dioxovaleric acid [12]. It can be mentioned that the final oxidation product of ALA, 4,5-dioxovaleric acid, is also a potential DNA damaging agent since it is able to alkylate guanine moieties [13]. Evidence for the biological relevance of ALA-mediated oxidation of DNA has been provided by the observation of an increase in the level of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) in liver DNA of rats treated with ALA [14]. In vitro, iron and copper catalyzed oxidation of ALA has been shown to release reactive intermediates able to induce strand breaks in plasmids [15,16]. Hydroxyl radicals or related oxygen reactive species are likely to be predominantly involved in the latter reactions. In order to check this hypothesis, the formation of several characteristic hydroxyl radical-mediated degradation base products in isolated DNA was investigated. As an extension of this study, it was found that the level of 8-oxodGuo in the DNA of several rat organs increased after i.p. injection of ALA. This shows that oxidation of DNA constituents occurs not only in the liver but also in the kidney and to a lesser extent in the spleen. This also applies to 5-hydroxy-2′-deoxycytidine (5-OHdCyd), the level of which was increased in the liver of ALA-treated rats. Altogether these data strongly suggest that hydroxyl radicals (OH·) and/or related species generated by the Fenton reaction are involved in the ALA-mediated oxidation of liver DNA.

2. Materials and methods

2.1. Chemicals

Calf thymus DNA (CT DNA), 5-aminolevulinic acid (ALA), ammonium formate, nuclease P1, alkaline phosphatase and 70% w/w hydrogen fluoride in pyridine (HF/Pyr) were purchased from Sigma (St. Louis, MO, USA). Analytical grade formic acid (99%) was obtained from Merck (Darmstadt, Germany). Water was purified on a Milli-Q system (Molsheim, France). 8-OxodGuo, 8-oxo-7,8-dihydro-2′-deoxyadenosine (8-oxodAdo), 5-hydroxy-2′-deoxycytidine (5-OHdCyd), 5-hydroxy-2′-deoxyuridine (5-OHdUrd), 5-hydroxycytosine (5-OHCyt), 5-hydroxyuracil (5-OHUra), 5-(hydroxymethyl)uracil (5-HMUra), 5-formyluracil (5-ForUra), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and the isotopically labeled derivatives of the five latter modified bases were synthesized following procedures reported elsewhere [17–19].

2.2. Oxidation of isolated DNA by ALA

5-Aminolevulinic acid was added to 10 ml of a 1 mg/ml aqueous solution of CT DNA in order to reach a final concentration of 2, 15 and 30 mM, respectively. The pH was adjusted to 8 by addition of 1 M sodium hydroxide. The samples were incubated at 37°C overnight. Then 1 ml of 4 M sodium chloride and 25 ml of cold ethanol (−20°C) were added. Precipitated DNA was recovered upon centrifugation at 5000×*g* for 15 min at room temperature. The resulting solid pellet was washed with 500 μl of 70% v/v ethanol and then solubilized in 10 ml of water. Each sample was split into three equal aliquots.

2.3. Animal treatment and liver DNA extraction

Male Wistar albino rats (3 months old, 250 g) were maintained in a room at 25°C under a 12:12 light/dark cycle and fed with a commercial diet ad libitum. Rats were injected i.p. every 2 days for 15 days with 0.5 ml of an isotonic solution of ALA (40 mg/kg body weight) as described by McGillion et al. [20]. The animals were sacrified by

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Abbreviations: 5-OHCyt, 5-hydroxycytosine; 5-OHdCyd, 5-hydroxy-2′-deoxycytidine; 5-OHdUrd, 5-hydroxy-2′-deoxyuridine; 5-OHUra, 5-hydroxyuracil; 5-ForUra, 5-formyluracil; 5-HMUra, 5-(hydroxymethyl)uracil; 8-oxodAdo, 8-oxo-7,8-dihydro-2′-deoxyadenosine; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; ALA, 5-aminolevulinic acid; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine

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decapitation 24 h after the last injection. The organs (liver, kidney and spleen) were excised and immediately placed in liquid nitrogen. Typically, frozen liver (200 mg) was homogenized in 1 ml of a solution containing 1 mM EDTA and 1% sodium dodecylsulfate. Then, proteinase K (0.5 mg) was added and the resulting solution was incubated for 60 min at 37°C. Subsequently, 50 μl of 1 mM Tris-HCl (pH 7.4) was added and the homogenate was successively extracted with 1 volume of phenol, 1 volume of a mixture of phenol/chloroform/isoamyl alcohol (25:24:1) and 1 volume of chloroform/isoamyl alcohol (24:1). Nucleic acids were precipitated from the aqueous solution and then dissolved in 150 μl of water. Samples were then incubated with RNase T1 (50 U/ml) and RNase A (100 μg/ml) at 37°C for 90 min. Proteins were removed with 0.5 volume of chloroform/isoamyl alcohol (24:1). Then, the resulting aqueous solution was centrifuged at room temperature for 30 min at 16 000 × *g*. DNA was recovered by precipitation. Since lower quantities of spleen and kidney samples were available, the amounts of buffer and enzymes were reduced proportionally for the extraction of DNA. 8-OxodGuo was measured in the DNA (50 μg) of the three organs by HPLC-EC. In addition, the formation of 5-OHdCyd was assessed in liver DNA (200 μg) using an HPLC-EC assay. Due to the small number of animals treated (3 in each group), only mean values and standard deviation are reported and no statistical analysis was made.

2.4. HPLC-EC detection of 8-oxodGuo, 8-oxodAdo, 5-OHdUrd and 5-OHdCyd within DNA

DNA was digested into nucleosides by successive incubation with nuclease P1 and alkaline phosphatase as previously reported [17,18,21]. Samples were analyzed by HPLC on an Inertsil ODS 2 (particle size 5 μm) octadecylsilyl silica gel column (250 × 4.6 mm I.D.) (Interchim, Montluçon, France). The isocratic eluent was a 50-mM aqueous solution of potassium phosphate (pH 5.5) for the simultaneous measurement of 5-OHdCyd and 5-OHdUrd. Methanol (9%) was added to the latter buffer when 8-oxodGuo was analyzed. Analysis of 8-oxodAdo was performed using a mixture of 5% v/v acetonitrile in 50 mM aqueous solution of potassium phosphate (pH 5.5) as the isocratic eluent. Coulometric detection was provided by a Coulochem II detector (Esa, Chelmsford, MA, USA). The potentials of the two electrodes were set at +150 and +450 mV for the simultaneous detection of 5-OHdCyd and 5-OHdUrd. Potentials of 200 and 400 mV were used for the measurement of 8-oxodGuo, whereas the latter values were raised to 300 and 450 mV for 8-oxodAdo. Elution of unmodified nucleosides was simultaneously monitored by a Waters 484 UV variable spectrophotometer set at 280 nm. All analyses were made in triplicate.

2.5. GC-MS analysis of FapyGua, 5-OHCyt, 5-OHUra, 5-HMUra and 5-ForUra

An aliquot of the DNA solution was analyzed for FapyGua content using the HF/pyridine hydrolysis procedure previously reported [17], following addition of [$^{15}N_3$]-FapyGua. A HPLC prepurification was performed and the fraction that contained FapyGua and its isotopically labeled derivative was collected and freeze-dried. The resulting residue was then silylated in 100 μl of an equal volume of *N*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and acetonitrile for 25 min at 110°C. 5-OHUra, 5-OHCyt, 5-HMUra and 5-ForUra were analyzed as previously described [18], in the presence of internal standards including [$^{15}N_2$,^{18}O]-5-OHUra, [$^{15}N_2$,^{13}C]-5-OHCyt, [$^{15}N_2$,$^{2}H_2$]-HMUra and [$^{15}N_2$,^{2}H]-5-ForUra. Following 88% formic acid hydrolysis and HPLC prepurification, the fractions containing the oxidized pyrimidines were freeze dried and their content subsequently derivatized in 100 μl of a 1:1 mixture of silylation grade acetonitrile (Pierce, Rockford, IL, USA) and *N-tert*-butyldimethylsilyl-*N*-methyl-trifluoroacetamide (MTBSTFA, Fluka, Buchs, Switzerland) for 20 min at 110°C. Samples were analyzed on a GC-MS system consisting of a HP 5890 chromatograph and an MSD 5972 mass detector used in the single ion monitoring mode. Samples (1 μl) were injected in the splitless mode onto a HP5-trace column (15 m, 0.25 mm I.D., 0.1 μM film thickness; Hewlett-Packard, Les Ulis, France). The temperature of the injector was 250°C. The column was maintained at 130°C for 1 min. Then, the temperature was increased linearly to 280°C at a rate of 10°C/min. The ions collected were [M] and [M-methyl] for BSTFA derivatized FapyGua, and [M-*tert*-butyl] for MTBSTFA treated pyrimidine bases. The respective retention times of the five silylated modified bases were the following: FapyGua: 7.0 min; 5-OHUra: 7.9 min; 5-OHCyt: 8.7 min; 5-ForUra: 6.0 min; 5-HMUra: 8.4 min. All analyses were made in triplicate.

3. Results and discussion

3.1. ALA-induced base modifications within isolated DNA

Isolated DNA samples were incubated with ALA in aerated aqueous solutions. Previous work has already shown that oxidative DNA damage, including strand breaks and 8-oxodGuo, occurred within isolated DNA in the presence of ALA [14–16]. In the latter studies, stimulatory effects of reduced transition metals on ALA-promoted DNA oxidation were observed. In addition, the presence of superoxide dismutase, catalase, a metal chelator or mannitol was found to protect DNA. Altogether, this is suggestive of the involvement of either hydroxyl radicals or related oxidizing species in the ALA-mediated DNA oxidation processes. The present study was aimed at providing additional support to the latter hypothesis by comparing the distribution of the major radical degradation products of nucleobases which were obtained upon exposure of DNA to ALA and γ-rays in aerated aqueous solution [22]. Formation of both 8-oxodGuo and FapyGua can be rationalized in terms of initial addition of $OH^{\cdot}$ to the C8 atom of the guanine moiety, followed by respective oxidation and reduction of the resulting 8-hydroxy-7,8-dihydroguanyl radical. 8-OxodAdo is produced from adenine by a similar process involving the oxidation of the transient 8-hydroxy-7,8-dihydroadenyl radical. Hydrogen abstraction from thymine by $OH^{\cdot}$ gives rise to the methyl centered radical which further reacts with O_2 to yield 5-HMUra and 5-ForUra. 5-OHCyt is produced by a consecutive addition of $OH^{\cdot}$ and a molecule of oxygen to the C5-C6 double bond of cytosine. This leads to the formation of a mixture of the *cis* and *trans* isomers of 5,6-dihydroxy-5,6-dihydrocytosine (cytosine glycols). Subsequently, cytosine glycols may be converted into either 5-OHCyt or uracil glycols through dehydration and deamination, respectively. Dehydration of uracil glycols gives rise to 5-OHUra.

The degradation products of DNA bases were measured by either HPLC-EC following enzymatic digestion [23–25] or GC-MS analysis of DNA hydrolyzed under acidic conditions [26] in the presence of isotopically labeled internal standards. For the latter assay, a prepurification step was systematically performed. This was achieved in order to prevent the artefactual formation of oxidized nucleobases during the silylation

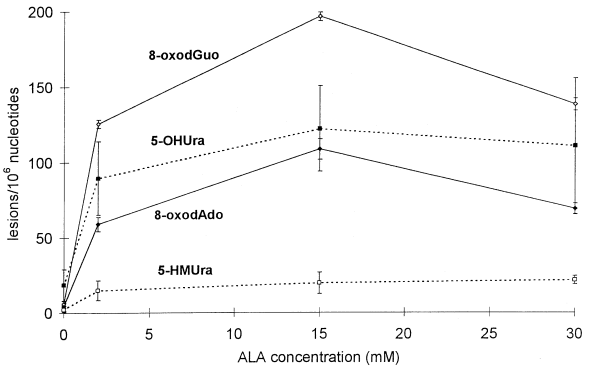

Fig. 1. Dose-response curve for the formation of oxidized DNA bases in isolated DNA exposed to ALA. Each value is the average of three determinations. The error bars represent standard deviations.

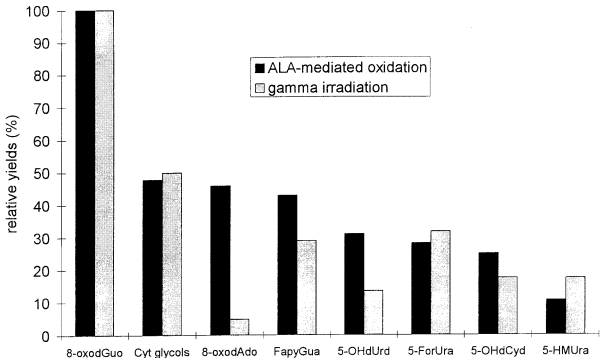

Fig. 2. Levels of formation of several modified bases in isolated DNA either treated with 2 mM ALA or exposed to γ-rays in aerated aqueous solution. The yield of 8-oxodGuo was used as reference and set at 100 arbitrary units. Data for radiation-induced decomposition of DNA were taken from previous work [17,18,21].

step which is required to make volatile the compounds of interest prior to GC analysis [18,27,28]. In addition, acidic hydrolysis of DNA was carried out under optimized conditions, in order to minimize the degradation of unstable modified bases like FapyGua [17]. The yields of 5-OHCyt and 5-OHUra as determined by GC-MS are overestimated. Indeed, cytosine and uracil glycols dehydrate under the acidic DNA hydrolysis conditions. However, an accurate determination of 5-OHdCyd and 5-OHdUrd may be achieved by HPLC-EC measurement of enzymatic hydrolysate of oxidized DNA. Interestingly, the comparison of the results obtained by the two approaches allows the estimation of the combined yield of cytosine and uracil glycols [18].

ALA-mediated oxidation of DNA was carried out without addition of metal ions such as either Fe^{2+} or Cu^{+}. This was aimed at preventing the occurrence of side reactions of DNA bases (or their transient radical species) due to the presence of reducing transition metal ions [22]. Therefore, the bulk of $OH^{\cdot}$ radicals and/or related reactive species produced upon initial oxidation of ALA can be mostly accounted for by the iron and copper present in the solution and/or bound to DNA. This may explain the shape of the dose-response curves dealing with the ALA-mediated formation of the modified bases. As illustrated in Fig. 1, a maximum yield was obtained for a 15-mM concentration in ALA. In addition, the relative increase in the level of modified bases was much higher in DNA samples treated with an ALA concentration of 2 mM than of 15 mM. Plateaus in the formation of 8-oxodGuo have already been observed in ALA-treated isolated DNA. This was the case for concentrations of ALA either within the 2–5-mM range in the presence of 10 μM Fe^{2+} [14] or above 1 mM in the presence of 20 μM Cu^{2+} [16]. This can be explained, in the absence of added transition metal ions in the incubation mixture, by the limited availability of metal bound to DNA. Therefore, the amount of hydroxyl radicals produced at highest ALA concentrations (15 and 30 mM) is not expected to be ALA concentration-dependent since ALA becomes a competitive substrate for DNA. Consequently, at high ALA concentrations, the yield of DNA damage decreases. An alternative explanation to be considered is the possibility for ALA to dimerize, leading to the formation of a dihydropyrazine derivative [16]. The relative amount of free ALA is much lower at a concentration of 30 mM than of 2 mM. Consequently, the latter conditions were used to assess the level of modified bases within isolated DNA samples (Table 1).

Table 1
Level of modified bases in isolated DNA incubated with 2 mM ALA[a]

Lesion	Untreated DNA	2 mM ALA
8-OxodGuo	5.5 ± 0.7	125 ± 3
FapyGua	18 ± 16	70 ± 6
5-OHCyt	26 ± 13	79 ± 22
5-OHUra	18.5 ± 10	90 ± 24
5-OHdCyd	5.5 ± 0.3	35 ± 10
5-OHdUrd	2.8 ± 0.5	40 ± 5
5-HMUra	2 ± 1	15 ± 6
5-ForUra	4.6 ± 2.8	38 ± 18
8-OxodAdo	4.0 ± 1.2	59 ± 4

[a]Expressed in lesions per 10^6 nucleotides, mean value ± standard deviation ($n = 3$).

A comparison of the respective yields of 8-oxodGuo, 5-OHdCyd, 5-OHdUrd, cytosine and uracil glycols as the sum, 5-HMUra and 5-ForUra in isolated DNA upon exposure to ALA and γ-rays in aerated aqueous solutions has been made for mechanistic purposes. Interestingly, it appears that the product distribution exhibits similarities irrespective of the oxidation conditions used (Fig. 2). The latter observations are indicative of the involvement of species exhibiting oxidizing properties similar to those of hydroxyl radicals in ALA treated samples. A one-electron oxidation mechanism can be ruled out since this process has been found to lead to a 10-fold higher yield of degradation of purine residues (Fpg sensitive sites) with respect to pyrimidine (endo III sensitive sites) [29]. However, the ratio between the respective yields of 8-oxodAdo and 8-oxodGuo was higher in the ALA treated samples (ratio = 2.2) than that obtained upon exposure of aerated solutions of DNA to γ-rays (ratio = 20.2) [21]. In contrast, the value of the 8-oxodAdo/8-oxodGuo ratio obtained in ALA-treated DNA was much closer to that determined upon incubation of calf thymus DNA with hydrogen peroxide (ratio = 4.2) [21]. A recent work carried out at the nucleoside level also reported drastic differences between the radiation-induced degradation of dAdo with respect to Fenton reaction mediated oxidation [30]. Interestingly, metal ions are required for the action of both ALA and hydrogen peroxide. It can therefore be proposed that highly oxidizing species released via Fenton type reactions such as ferryl ($Fe=O^{2+}$) and per-

Table 2
Level of oxidized bases in the DNA of organs of ALA-treated rats[a]

Organ	Spleen	Kidney	Liver	
Lesion	8-OxodGuo	8-OxodGuo	8-OxodGuo	5-OHdCyd
Control	7.6 ± 0.25	15.2 ± 3.0	14.2 ± 3.0	1.0 ± 0.3
Treated	14.0 ± 2.5	58.2 ± 16.2	23.2 ± 1.8	3.5 ± 1.8

[a]Expressed in lesions per 10^6 nucleotides, mean value ± standard deviation ($n = 3$).

ferryl ($Fe=O^{3+}$) ions [31], exhibit relatively different oxidizing ability toward DNA purine bases with respect to hydroxyl radicals. An alternative explanation could be a preferential binding of metals to adenine or adenine rich regions of DNA, thus leading to higher yields of adenine oxidation products with respect to the effects of radiation-induced hydroxyl radicals which are generated in the bulk of the solution. Further work is required to elucidate the peculiar behavior of adenine in metal-mediated oxidation processes.

3.2. *Oxidation of guanine and cytosine in the DNA of rat organs exposed to ALA*

Evidence for the occurrence of ALA-mediated in vivo oxidation of DNA has already been provided. This was inferred from the observation of an increase in the level of 8-oxodGuo in the liver DNA of rats chronically exposed to ALA [14]. Using a similar protocol, the latter findings were confirmed and extended to the spleen and kidney DNA of rats (Table 2). It should be remembered that 8-oxodGuo is not a good chemical probe for mechanistic studies since it may be produced by a wide variety of oxidants and processes including hydroxyl radicals, type I photosensitization and singlet oxygen [32–34]. Therefore, the level of 5-OHdCyd was also determined in the liver of rats treated with ALA. The latter measurement, which was achieved by HPLC-EC, was facilitated by the fact that relatively large amounts of DNA were extracted from the liver. A three-fold increase in 5-OHdCyd was observed in ALA-treated animals with respect to controls. Attempts were made to measure 5-OHCyt, 5-OHUra, 5-HMUra and 5-ForUra by the optimized GC-MS assay involving a HPLC prepurification. However, the sensitivity of the method (approximately 5 lesions per 10^6 normal bases in 200 μg DNA) was too low to detect the formation of any of the latter oxidized bases. Interestingly, the ratio between the increase in the level of 8-oxodGuo and that of 5-OHdCyd (ratio = 3.6) is very close to that determined in isolated DNA (ratio = 4.0). This indicates that the involvement of hydroxyl radicals or related reactive oxygen species produced via a Fenton-like reaction is likely to be involved in the in vivo oxidation reactions of DNA mediated by ALA.

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