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Formation of 8-Hydroxyguanine and 2,6-Diamino-4-hydroxy-5-formamidopyrimidine in DNA by Riboflavin Mediated Photosensitization

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Calf thymus DNA was photoirradiated in the presence of riboflavin. Altered bases were detected and quantified by the GC/MS-SIM method after hydrolysis and derivatization of DNA. Seven types of modified purine bases were detected in control DNA. Among them, the yields of 8-OH-Gua and FapyGua increased significantly in DNA photoirradiated with riboflavin, whereas the yields of xanthine, 8-OH-Ade, 2-OH-Ade, FapyAde and hypoxanthine were not affected. A dose dependent increase in the formation of 8-OH-Gua was observed with increasing riboflavin concentration for 30 min irradiation. On the other hand, FapyGua reached plateau at 10 μg/ml of riboflavin for 30 min irradiation. Our results indicate that guanine moiety in DNA is the most susceptible to riboflavin mediated photosensitization.

The cytotoxic, carcinogenic and mutagenic effects in cells exposed to sunlight are thought to be primarily the result of the damage inflicted on DNA. A lot of investigations have been made to elucidate resulting lesions on DNA induced by sunlight. The solar ultraviolet light induces pyrimidine dimers and pyrimidine (6-4) pyrimidone photoadducts. They are the most common products and no doubt have mutagenic and genotoxic potentials (1). With UV radiation to DNA, other monomeric base damage products have been observed recently (2). Although the yields of monomeric base damage products were much less frequent compared with pyrimidine dimers and pyrimidine (6-4) pyrimidone photoadducts (3), they are biologically important and responsible for the deleterious effects of UV radiation.

DNA lesions induced by photosensitizers are thought to proceed with either two competitive processes, characterized as type I or type II. A type I mechanism is a direct proton or electron transfer by the excited photosensitizer from the substrate to generate free radicals or cation radicals. A type II mechanism is the transfer of energy from the excited photosensitizers to ground state of oxygen to produce singlet oxygen.

8-Hydroxyguanine (8-OH-Gua) is one of the major modified bases in DNA induced by reactive oxygen species and widely used as a marker for oxidative damage to DNA. 8-OH-Gua can make a pairing with adenine at the similar frequency with cytosine resulting in G:C to T:A transversion at the next round of replication (4-7). Methylene blue plus visible light produced 8-OH-Gua in DNA (8). In this photosensitization reaction singlet oxygen was generated as a precursor and the guanine residue was selectively damaged. In addition to 8-OH-Gua, significant formation of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) was also detected (9).

Riboflavin is an endogeneous cellular photosensitizer. Little or no involvement of singlet oxygen is suggested in the riboflavin mediated photosensitization and degradation of DNA, since D_2O effect was not observed (10,11). The reaction has been suggested to proceed with a mechanism generating the guanine radical cation from subtraction of electron by the triplet excited state of riboflavin (type I). Formation of 8-OH-Gua by riboflavin mediated photosensitization was observed in DNA, however, DNA base modifications was not well characterized.

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Abbreviations used: 8-OH-Gua, 8-hydroxyguanine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH-Ade, 8-hydroxyadenine; 2-OH-Ade, 2-hydroxyadenine; FapyAde, 4,6-diamino-5-formamidopyrimidine; 8-OHdG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; $Thyd_4$, Thymine-α, α, α, 6-$^2H_4$; GC/MS-SIM, gas chromatography-mass spectrometry with selected-ion monitoring.

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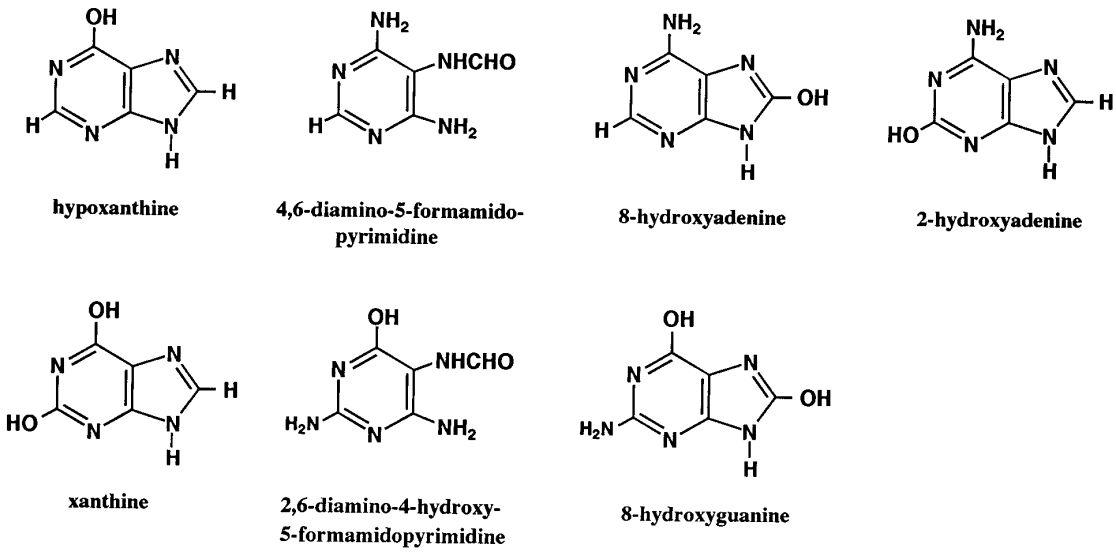

FIG. 1. Structures of modified purine bases.

GC/MS is a powerful tool for the investigation of modified bases in DNA providing us a wide knowledge about DNA damage. In this report, by using this method, we attempted to characterize purine base modifications in riboflavin mediated photosensitization of DNA in cell-free systems.

MATERIALS AND METHODS

Chemicals. Hypoxanthine, xanthine, acetonitrile, *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and formic acid were from Wako Pure Chemicals, Tokyo, Japan. Thymine-$\alpha, \alpha, \alpha, 6$-$^2H_4$ ($Thyd_4$) was from Nacalai Tesque, Kyoto, Japan. Calf thymus DNA, 4,6-diamino-5-formamidopyrimidine (FapyAde) and 8-azaadenine were from Sigma Chemical Company, St. Louis. 8-OH-Gua was from Aldrich, Milwaukee. FapyGua (12), 8-hydroxyadenine (8-OH-Ade) (13), 2-hydroxyadenine (2-OH-Ade) (14) and 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-OHdG) (15) were prepared as described.

Irradiation of DNA in the presence of riboflavin. Calf thymus DNA mixed with aqueous solution of riboflavin was irradiated with white light from 150 W tungsten lamp at a distance of 21 cm from the surface of the sample. A beaker filled with 3000 ml of water (19 cm-height) and an acrylic plate (1 cm-thickness) were placed between light source and the sample for the removal of heat and UV radiation. After the photoirradiation of DNA with riboflavin, treated DNA was collected by ethanol precipitation. Control DNA was not photoirradiated.

Hydrolysis and trimethylsilylation. To aliquots of DNA samples, 2 nmol of 8-azaadenine and 20 nmol of $Thyd_4$ were added as internal standards (16). Samples were lyophilized, and subsequently hydrolyzed with 0.5 ml of 60% formic acid in evacuated and sealed glass tubes at 130 °C for 30 min. Hydrolyzed samples were lyophilized and then trimethylsilylated with 0.1 ml of BSTFA/acetonitrile (4/1; v/v) mixture at 130 °C for 30 min under dry nitrogen in poly(tetrafluoroethylene)-capped screw vials.

GC/MS measurement. Derivatized samples were analyzed by GC/MS-selected-ion monitoring (GC/MS-SIM) with a JEOL model JMA-DX-303 mass spectrometer operated in the EI mode. GC separations were performed with a Hewlett-Packard 5890 gas chromatograph equipped with a fused silica capillary column (SPB-5, 12 m × 0.2 mm i.d.) coated with crosslinked 5% phenylmethyl silicone, film thickness 0.33 μm. The injection port, GC/MS interface and ion source were kept at 250 °C. One μl of derivatized sample was injected into the column in the splitless mode. After the initial temperature of the column was kept at 125 °C for 2 min, the column temperature was increased as follows; from 125 °C to 175 °C at 8 °C/min held 175 °C for 2 min, then from 175 °C to 220 °C at 30 °C/min held 220 °C for 1 min and finally from 220 °C to 280 °C at 40 °C/min, held 280 °C for 2 min. Helium was used as the carrier gas at an inlet pressure of 70 kPa. On the JEOL mass spectrometer, the ionization current and electron multiplier were set at 100 μA and 1.4 kV, respectively and electron multiplier was increased to 1.9 kV after the elution of thymine at 3 min. Mass spectra were obtained at 70 eV. The concentrations of modified bases in DNA were calculated from the calibration curves obtained from aliquots of the mixture of known concentrations of the modified bases and internal standards.

TABLE 1

Yield of Modified Bases (Molecules/10^5 DNA Bases) in Riboflavin Mediated Photosensitization of DNA

Base	Control[a]	Photoirradiated[b]
8-OH-Gua	10.6 ± 1.4	68.0 ± 3.5[c]
FapyGua	6.8 ± 0.5	40.3 ± 4.4[c]
Xanthine	27.8 ± 7.5	30.2 ± 2.8
8-OH-Ade	3.2 ± 0.7	3.2 ± 1.3
2-OH-Ade	1.2 ± 0.2	1.4 ± 0.4
FapyAde	1.8 ± 0.5	1.9 ± 1.2
Hypoxanthine	38.6 ± 6.4	39.2 ± 1.4

[a] Unirradiated with riboflavin.
[b] Irradiated for 30 min with riboflavin 5 μg/ml.
[c] Significant increase over control, $p < 0.05$.
Note. Values represent the mean ± standard deviation from three independent measurements.

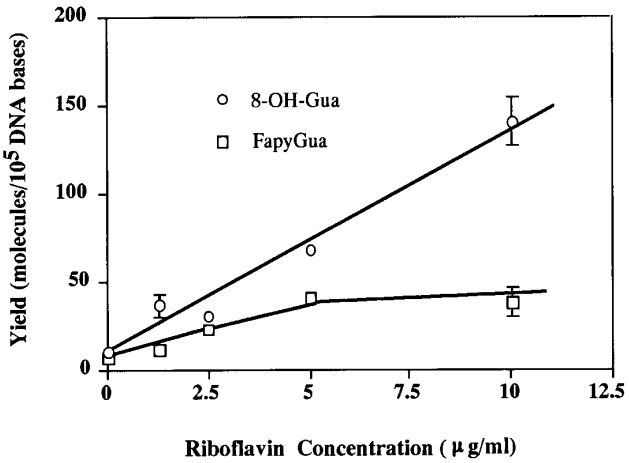

FIG. 2. Formation of 8-OH-Gua and FapyGua by varying the concentration of riboflavin. DNA was irradiated for 30 min. Data points were obtained from three independent measurements.

RESULTS AND DISCUSSION

Chemical detection and quantification of modified bases in DNA is available with GC/MS providing us a wide spectrum of DNA modified bases (17-19).

Calf thymus DNA photoirradiated in the presence of riboflavin was analyzed by means of GC/MS-SIM after hydrolysis and derivatization. Seven modified purine bases were identified and quantified in control DNA. They were 8-OH-Gua, FapyGua, xanthine, 8-OH-Ade, 2-OH-Ade, FapyAde and hypoxanthine as illustrated in Fig.1. Among them 8-OH-Gua and FapyGua increased significantly in DNA for 30 min irradiation with 5 μg/ml riboflavin. Enhanced production of other compounds was not observed. They are summarized in table 1. Formation of FapyGua by the riboflavin mediated photosensitization was not reported before although 8-OH-dG was observed in cell-free (10,11) and cellular (20) systems.

The increase of the yields of 8-OH-Gua and FapyGua was observed for 30 min irradiation as the riboflavin concentration was changed from 1.25 to 10 μg/ml which is shown in Fig.2. The ratio of the yield of 8-OH-Gua to that of FapyGua was 1.7 at 5 μg/ml of riboflavin and it increased to four at 10 μg/ml of riboflavin concentration. The yield of 8-OH-Gua increased linearly depending on riboflavin concentrations, whereas, the yield of FapyGua reached plateau at 10 μg/ml of riboflavin for 30 min irradiation. Characteristic formation of 8-OH-Gua and FapyGua implies that guanine moiety is preferentially affected by riboflavin mediated photosensitization. There are several reports that guanine is a predominant target of photosensitization reactions. Exposure of calf thymus DNA to 365 nm UV irradiation in the presence of riboflavin induced site specific cleavage of DNA at 5′ guanine of 5′-GG-3′ sequence and 8-OH-Gua formation (11). A similar photocleavage selectively at 5′-GG-3′ sequences has been observed with Ru(II) and naphtalimide derivatives (21-24).

Significant increase in 8-OH-Gua and FapyGua was also observed in DNA photoirradiated with methylene blue (9). Singlet oxygen is generated in methylene blue plus visible light (characterized as type II) and oxidative DNA damage is induced in this reaction (25). On the other hand, singlet oxygen is not involved in riboflavin mediated photosensitization (10, 11). Since the guanine base is know to have the lowest ionization potential among four DNA nucleobases (26), electron transfer from guanine moiety to triplet excited riboflavin generating guanine radical cation was predominant (type I). Further reaction of guanine radical cation may result in the formation of 8-OH-Gua upon one-electron oxidation and FapyGua upon one-electron reduction resulting in ring opening (27). We confirmed that FapyGua was not produced from 8-OH-Gua during acid hydrolysis and derivatization. For the confirmation 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-OHdG) was treated with 60% formic acid at 130 °C for 30 min in evacuated and sealed glass tubes. Hydrolyzed 8-OHdG was lyophilized and then derivatized. 8-OH-Gua was formed stoichiometrically but no FapyGua. Modified bases detected in control DNA were in good agreement with other published data using a GC/MS method (28, 29). Besides, in calf thymus DNA treated with a photosensitizer plus UV irradiation yields of 8-OHdG measured by an HPLC method and 8-OH-Gua by GC/MS-SIM were the same (30).

We found no increase in 8-OH-Ade, 2-OH-Ade, FapyAde, xanthine and hypoxanthine. If hydroxyl radicals were produced in this system, we could expect formation of 8-OH-Ade, 2-OH-Ade and FapyAde in addition to 8-OH-Gua and FapyGua. Xanthine and hypoxanthine are reported to be produced by active nitrogen species (31). Therefore it can be suggested that photoirradiation of DNA with riboflavin generated neither hydroxyl radicals nor active nitrogen species.

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