

Oxidative DNA damage by hyperglycemia-related aldehydes and its marked enhancement by hydrogen peroxide

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Abstract Increased risks of cancers and oxidative DNA damage have been observed in diabetic patients. Many endogenous aldehydes such as 3-deoxyglucosone and glyceraldehyde (GA) increase under hyperglycemic conditions. We showed that these aldehydes induced Cu(II)-mediated DNA damage, including 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) formation. GA had the strongest ability to damage DNA, and addition of low concentrations of H₂O₂ markedly enhanced the DNA damage. GA significantly increased 8-oxodG formation in human cultured cells (HL-60), and H₂O₂ enhanced it. We conclude that oxidative DNA damage by hyperglycemia-related aldehydes, especially GA, and marked enhancement of DNA damage by H₂O₂ may participate in diabetes-associated carcinogenesis.

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Key words: Diabetes mellitus; Hyperglycemia; Oxidative DNA damage; Glyceraldehyde; 3-Deoxyglucosone

1. Introduction

Diabetes mellitus is now one of the main threats to human health worldwide [1]. Epidemiological studies have shown that diabetic patients are at higher risk of developing cancers of the liver, biliary tract, pancreas, colon, endometrium, and urinary tract [2–5]. Notably, several reports showed oxidative DNA damage in peripheral blood lymphocytes from diabetic patients [6,7]. Accumulation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), a characteristic lesion of oxidative DNA damage, was observed in mitochondrial DNA from kidney of diabetic rats and rapidly normalized by insulin treatment, suggesting the involvement of hyperglycemia in oxidative DNA damage [8].

Many endogenous aldehydes such as methylglyoxal (MG), 3-deoxyglucosone (3-DG), and glyceraldehyde (GA) increase under hyperglycemic conditions [9–11]. Glucose is converted to fructose via the polyol pathway, resulting in the increase of GA and glyceraldehyde 3-phosphate (GA3P) during hyper-

glycemia. 3-DG, MG and GA are formed from glucose degradation [12]. 3-DG and MG were identified as intermediates to form advanced glycated end-products (AGEs). Hyperglycemia induces generation of reactive oxygen species [13] and also attenuates antioxidant status [14,15], resulting in oxidative stress.

To clarify the mechanism of diabetes-associated carcinogenesis, we investigated oxidative DNA damage induced by hyperglycemia-related endogenous aldehydes, MG, 3-DG, GA and GA3P, using ³²P-5'-end-labeled DNA fragments obtained from the human *p53* tumor suppressor gene. We analyzed the formation of 8-oxodG in both isolated DNA from calf thymus and cellular DNA, using high performance liquid chromatography coupled with an electrochemical detector (HPLC-ECD).

2. Materials and methods

2.1. Materials

Restriction enzymes (*Eco*RI, *Apa*I and *Xba*I) and alkaline phosphatase from calf intestine were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Restriction enzymes (*Hind*III and *Ava*I) and T₄ polynucleotide kinase were obtained from New England Biolabs (Beverly, MA, USA). [³²P]ATP (222 TBq/mmol) was from New England Nuclear (Boston, MA, USA). 3-DG was purchased from Dojin Chemicals (Kumamoto, Japan). MG, GA, GA3P, calf thymus DNA, superoxide dismutase (SOD), bacterial alkaline phosphatase and catalase were purchased from Sigma Chemicals (St. Louis, MO, USA). Nuclease P₁ was purchased from Yamasa Shoyu (Chiba, Japan). *Escherichia coli* formamidopyrimidine-DNA glycosylase (Fpg) was obtained from Trevigen (Gaithersburg, MD, USA). Glucose oxidase was from Toyobo (Osaka, Japan). Lysis buffer for DNA extraction (model 340A) was purchased from Applied Biosystems (Foster City, CA, USA).

2.2. Preparation of ³²P-5'-end-labeled DNA fragments obtained from the *p53* gene

DNA fragments were obtained from the human *p53* tumor suppressor gene [16]. A ³²P-5'-end-labeled 443-bp fragment (*Apa*I 14179–*Eco*RI* 14621) was obtained according to the method described previously [17]. The asterisk indicates ³²P labeling.

2.3. Detection of DNA damage by aldehydes

The standard reaction mixture in a microtube (1.5-ml Eppendorf) consisted of an aldehyde, CuCl₂, the ³²P-5'-end-labeled DNA fragments and calf thymus DNA in 200 µl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 µM diethylenetriamine-*N,N,N',N'*-pentaacetic acid (DTPA), followed by incubation at 37°C for 2 h. Subsequently, the DNA was treated with 1 M piperidine at 90°C for 20 min or 10 U of Fpg protein in the buffer (10 mM HEPES–KOH (pH 7.4), 100 mM KCl, 10 mM EDTA and 0.1 mg/ml bovine serum albumin) at 37°C for 2 h. After ethanol precipitation, DNA was denatured, and the single-stranded DNA was electrophoresed. The autoradiogram was obtained by exposing X-ray film to the gel

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Abbreviations: 3-DG, 3-deoxyglucosone; GA, glyceraldehyde; GA3P, glyceraldehyde 3-phosphate; MG, methylglyoxal; AGE, advanced glycated end-product; 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine (also known as 8-hydroxy-2'-deoxyguanosine); DTPA, diethylenetriamine-*N,N,N',N'*-pentaacetic acid; HPLC-ECD, high performance liquid chromatography coupled with an electrochemical detector; SOD, superoxide dismutase

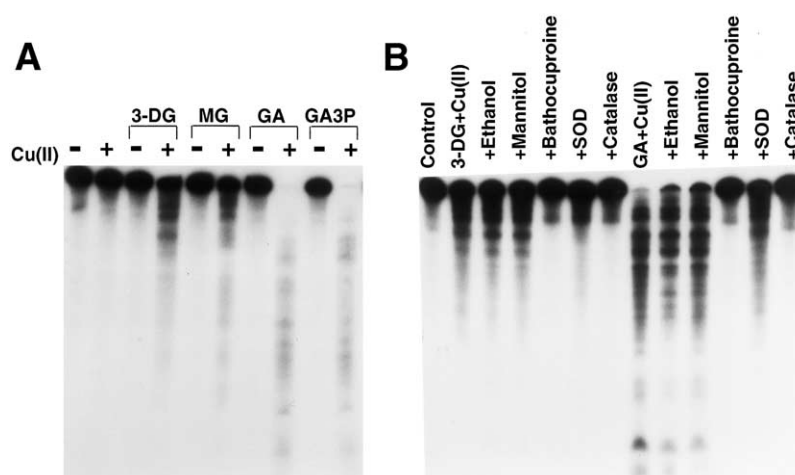


Fig. 1. Cu(II)-mediated DNA damage induced by endogenous aldehydes, and effects of scavengers. A: Reaction mixtures contained the ^{32}P -5'-end-labeled 443-bp DNA fragment, 20 μM /base of calf thymus DNA, 1 mM aldehydes and 20 μM CuCl_2 in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. B: Reaction mixtures contained the ^{32}P -5'-end-labeled 443-bp DNA fragment, 20 μM /base of calf thymus DNA, 1 mM 3-DG or 0.5 mM GA, 20 μM CuCl_2 , and the indicated scavenger (5% (v/v) ethanol, 0.1 M mannitol, 50 μM bathocuproine, 30 U of SOD, 50 U of catalase) in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation at 37°C for 2 h, DNA fragments were treated with 1 M piperidine for 20 min at 90°C and then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was visualized by exposing an X-ray film to the gel.

as described previously [18]. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam–Gilbert procedure [19] using a DNA sequencing system (LKB 2010 Macro-Phor). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

2.4. Analysis of 8-oxodG formation by aldehydes

DNA (100 μM /base) from calf thymus was incubated at 37°C for 2 h with an aldehyde and CuCl_2 in 400 μl of 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After ethanol precipitation, DNA was enzymatically digested to the nucleosides with nuclease P_1 and calf intestine phosphatase and analyzed by the HPLC-ECD, as described previously [20].

2.5. Measurement of 8-oxodG in human cultured cells

Human leukemia HL-60 cells were grown in RPMI 1640 supplemented with 6% fetal bovine serum at 37°C under 5% CO_2 in a humidified atmosphere. Cells ($1 \times 10^6/\text{ml}$) were treated with the indi-

cated concentrations of GA for 4 h. Where indicated, 0.002 U/ml glucose oxidase was added together with GA. Glucose oxidase catalyzes the formation of hydrogen peroxide (H_2O_2) from glucose contained in the medium [21]. After the incubation, the medium was removed, and the cells were washed three times with phosphate-buffered saline. The cells were treated with 0.05 mg/ml RNase A, 0.5 mg/ml proteinase K, and 500 μl of lysis buffer for DNA extraction, and incubated at 60°C for 1 h under anaerobic conditions. After ethanol precipitation, DNA was digested to nucleosides with nuclease P_1 and bacterial alkaline phosphatase and analyzed for its 8-oxodG content by HPLC-ECD, as described previously [20,22]. For statistical analysis of the data, Student's *t*-test was used at a significance level of $P < 0.05$.

3. Results

3.1. Damage to ^{32}P -labeled DNA fragments by aldehydes in the presence of Cu(II), and effects of scavengers

Oligonucleotides were detected on the autoradiogram as a

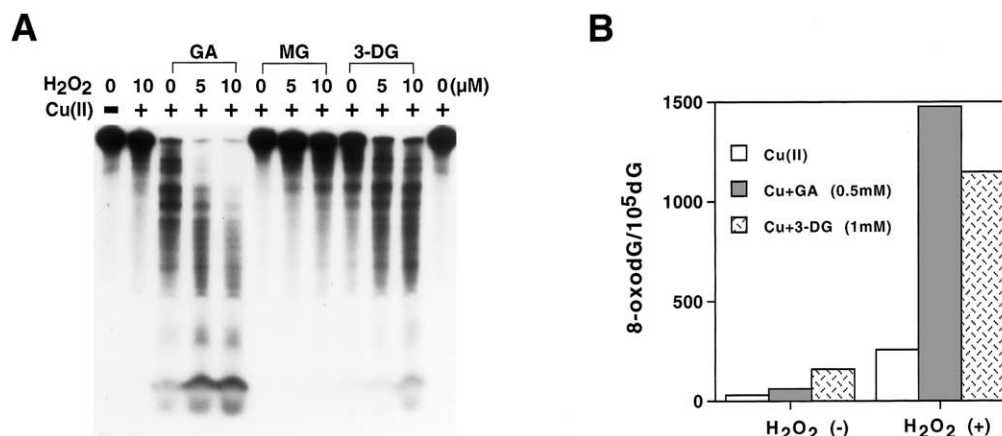


Fig. 2. Enhancement of aldehyde-induced DNA damage and 8-oxodG formation by the addition of H_2O_2 . A: Reaction mixtures contained the ^{32}P -5'-end-labeled 443-bp DNA fragment, 20 μM /base of calf thymus DNA, the indicated concentration of H_2O_2 , aldehyde (0.5 mM GA, 1 mM MG or 1 mM 3-DG) and 20 μM CuCl_2 in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation at 37°C for 2 h, DNA fragments were treated with 1 M piperidine for 20 min at 90°C and then analyzed as described in the legend to Fig. 1. B: Reaction mixtures contained calf thymus DNA (100 μM /base), 0.5 mM GA or 1 mM 3-DG, 10 μM H_2O_2 and 20 μM CuCl_2 in 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation at 37°C for 2 h, DNA was enzymatically digested to nucleosides and analyzed by HPLC-ECD.

result of DNA cleavage. 3-DG, MG, GA or GA3P caused Cu(II)-mediated DNA damage in a dose-dependent manner (data not shown). The intensity of DNA damage by GA or GA3P was greater than that by 3-DG or MG (Fig. 1A). Oligonucleotides with no treatment suggest the breakage of the deoxyribose phosphate backbone by active species. The increased amount of oligonucleotides with piperidine treatment (data not shown) suggests abasic sites and several base modifications [18]. Typical free hydroxyl radical scavengers such as ethanol and mannitol did not inhibit DNA damage (Fig. 1B). EDTA inhibited DNA damage (data not shown).

Bathocuproine, a Cu(I)-specific chelator, and catalase were found to reduce the amount of DNA damage by 3-DG and GA, suggesting the involvement of Cu(II), Cu(I) and H_2O_2 . SOD had a slight inhibitory effect on the DNA damage. Similar effects of scavengers and bathocuproine were observed with GA3P and MG (data not shown).

3.2. Enhancement of oxidative DNA damage induced by aldehydes in the presence of H_2O_2

H_2O_2 enhanced GA/Cu(II)-induced DNA damage (Fig. 2A). In the case of 3-DG, a similar effect was observed. The

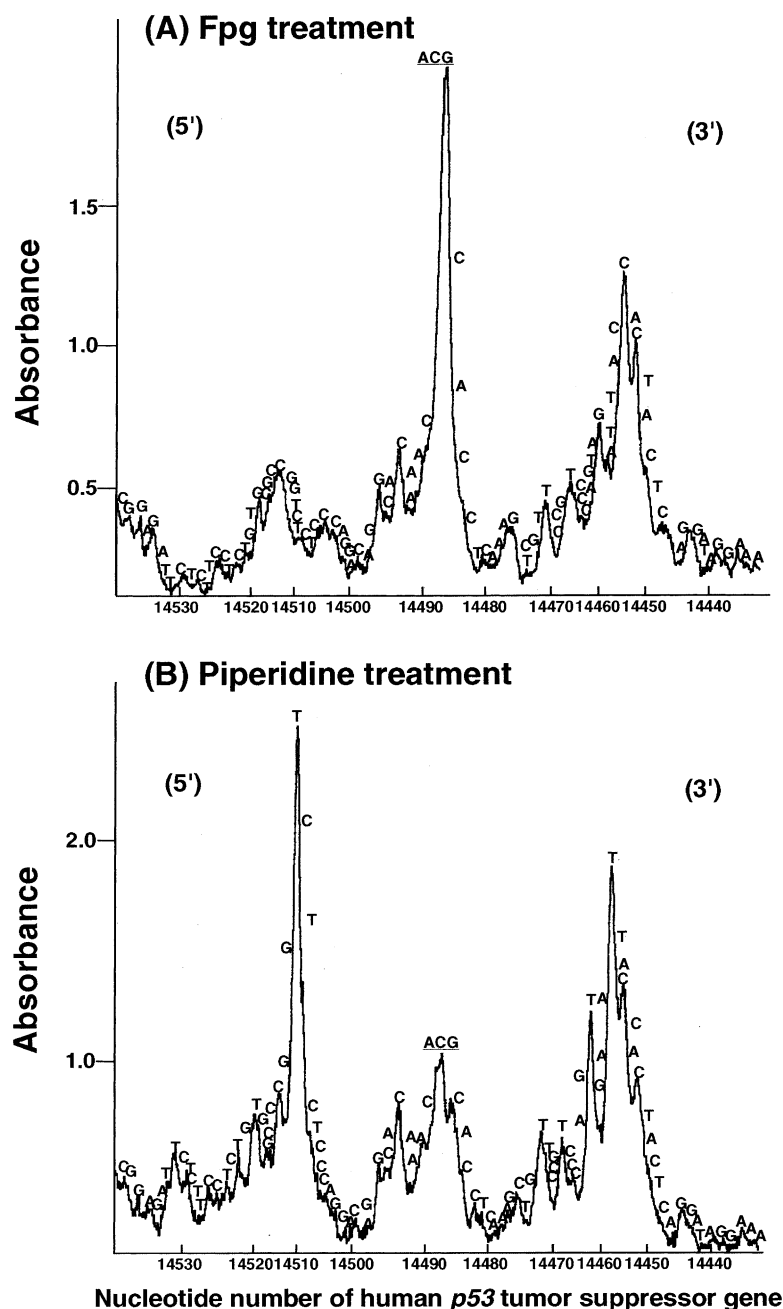


Fig. 3. Site specificity of DNA cleavage induced by GA in the presence of Cu(II). Reaction mixtures contained the ^{32}P -5'-end-labeled 443-bp fragment, 20 μM /base of calf thymus DNA, 0.5 mM GA and 20 μM $CuCl_2$ in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. Reaction mixtures were then incubated at 37°C for 2 h. Following Fpg protein (A) and piperidine (B) treatment, the DNA fragments were analyzed. The relative quantities of oligonucleotides were measured by scanning the autoradiogram with a laser densitometer. The horizontal axis shows the nucleotide number of the human *p53* tumor suppressor gene and underscoring shows the complementary sequence to codon 273 (nucleotides 14486–14488).

endogenous aldehydes themselves induced 8-oxodG formation in calf thymus DNA in the presence of Cu(II) (Fig. 2B, H₂O₂ (–)). A low concentration of H₂O₂ (10 μM) markedly enhanced the formation of 8-oxodG by GA and 3-DG (Fig. 2B, H₂O₂ (+)). The formation of 8-oxodG was approximately 20-fold increased by the addition of H₂O₂ to GA.

3.3. Site specificity of DNA damage by GA

An autoradiogram was obtained and scanned with a laser densitometer to measure the relative intensities of DNA cleavage products from the human *p53* tumor suppressor gene (Fig. 3). GA preferentially induced Fpg-catalyzed cleavage sites at guanine and cytosine residues in the presence of Cu(II) (Fig. 3A). Fpg protein catalyzes the excision of piperidine-resistant 8-oxodG and other oxidized piperidine-labile guanine residues [23]. Fpg also mediates cleavage of oxidized products of cytosine such as 5-hydroxycytosine [24]. With piperidine treatment, GA-induced DNA cleavage occurred mainly at thymine and cytosine residues (Fig. 3B). The guanine residue of the ACG sequence complementary to codon 273, a well-known hotspot [25] of the *p53* gene, was significantly cleaved with Fpg treatment (Fig. 3A). Piperidine treatment cleaved the cytosine residue of the ACG sequence of the *p53* gene (Fig. 3B). Study of the time course of the DNA cleavage pattern revealed that DNA damage at cytosine and guanine of the sequence appeared to increase in a time-dependent manner (data not shown). Similar results were obtained with other aldehydes (data not shown). Addition of H₂O₂ enhanced DNA damage by these aldehydes but did not change the site specificity (data not shown).

3.4. Formation of 8-oxodG in cells treated with GA and effects of H₂O₂

Fig. 4 shows the effect of GA and/or H₂O₂ on 8-oxodG formation in HL-60 cells. High-dose GA induced significant increases of 8-oxodG formation (Fig. 4A). An H₂O₂-generating system using glucose oxidase or 1 mM GA alone induced a slight increase of 8-oxodG formation, but there was no significant difference compared with the control under the condition used. When low-dose GA was added together with glucose oxidase, the formation of 8-oxodG increased significantly compared with the control ($P < 0.001$), and GA ($P < 0.05$) or glucose oxidase ($P < 0.05$) alone (Fig. 4B).

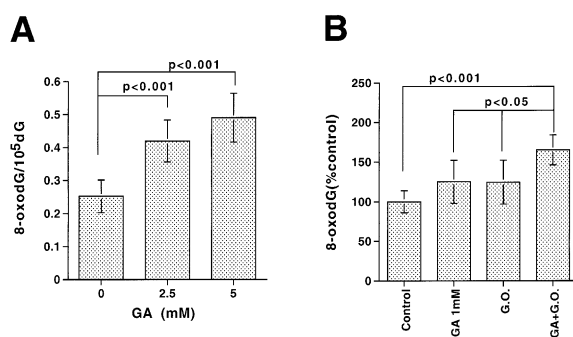


Fig. 4. Formation of 8-oxodG in HL-60 cells treated with GA and/or glucose oxidase. HL-60 cells (1.0×10^6 /ml) were incubated with the indicated concentrations of GA and/or 0.002 U/ml glucose oxidase (G.O.) for 4 h. DNA was analyzed by HPLC-ECD as described in Section 2. Results are expressed as means and S.D. of values obtained from five to eight independent experiments.

4. Discussion

Diabetic patients are at higher risk of developing cancers [2–5] and oxidative DNA damage occurs in their peripheral blood lymphocytes [6,7]. The involvement of hyperglycemia in oxidative DNA damage has been supported by experiments with diabetic rats [8]. We investigated the mechanism of oxidative DNA damage induced by hyperglycemia-related endogenous aldehydes in relation to diabetes-associated carcinogenesis, and found that these aldehydes caused DNA damage (GA, GA3P > 3-DG > MG) including 8-oxodG formation in the presence of Cu(II). Piperidine and Fpg treatment detected GA-induced cytosine and guanine damage of the ACG sequence complementary to codon 273, a well-known hotspot [25] of the *p53* gene. Since the present method based on the Maxam–Gilbert procedure cannot show straightforwardly double-base damage on the same DNA molecule, further work is required to ascertain whether the putative double-base lesion of DNA is present or not. Relevantly, it is postulated that double-base lesions can be generated from one radical hit that leads through a secondary reaction to a tandem base modification at pyrimidine and the adjacent guanine residues [26–28]. Furthermore, studies on the site-specific DNA damage induced by GA may provide an insight into assignment of reactive species, complexes of copper ions and H₂O₂ [18,29].

We showed that a low concentration of H₂O₂ (10 μM) markedly enhanced the formation of 8-oxodG by the aldehydes. In human cultured cells, 8-oxodG formation increased by treatment with high-dose GA corresponding to the concentration in the hyperglycemic condition. Simultaneous generation of H₂O₂ by the addition of glucose oxidase significantly enhanced GA-induced 8-oxodG formation. Endogenous metabolites of sugars, such as GA, GA3P, 3-DG and MG, are present in increased concentrations in diabetes [10–12]. High glucose levels stimulate production of H₂O₂ through several pathways [30,31]. Therefore, it is noteworthy in relation to diabetes-associated carcinogenesis to find that GA significantly induced 8-oxodG formation and H₂O₂ enhanced it both in a cell-free system and in cultured human cells. 8-OxodG is a prominent indicator of oxidative stress and has been well-characterized as a premutagenic lesion in mammalian cells [32]. Considerable evidence has linked oxidative damage to cancer [33]. The increase of 8-oxodG formation by GA and the enhancement by H₂O₂ may participate in carcinogenesis. Besides oxidative DNA damage, other types of DNA damage such as DNA–protein cross-links, and adducts to proteins due to AGEs [34,35] can contribute to diabetes-associated cancers. However, diabetic patients may have a risk of carcinogenesis by oxidative DNA damage due to endogenous aldehydes, especially GA, in relation to hyperglycemia.

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