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Oxidative DNA damage induced by a melatonin metabolite, 6-hydroxymelatonin, via a unique non-*o*-quinone type of redox cycle

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Abstract

Melatonin, an indolic pineal hormone, is produced primarily at night in mammals and is important in controlling biological rhythms. Although melatonin is known to be effective as a free radical scavenger and has an anti-cancer effect, carcinogenic properties have also been reported. In relation to its carcinogenic potential, we have examined whether 6-hydroxymelatonin, a major melatonin metabolite, can induce DNA damage in the presence of metal ion using [^{32}P]-5′-end-labeled DNA fragments obtained from genes relevant to human cancer. 6-Hydroxymelatonin induced site-specific DNA damage in the presence of Cu(II). Formamidopyrimidine-DNA glycosylase treatment induced cleavage sites mainly at G residues of the 5′-TG-3′ sequence, whereas piperidine treatment induced cleavage sites at T mainly of 5′-TG-3′. Interestingly, 6-hydroxymelatonin strongly damaged G and C of the 5′-ACG-3′ sequence complementary to codon 273 of the *p53* gene. These results suggest that 6-hydroxymelatonin can cause double-base lesions. DNA damage was inhibited by both catalase and bathocuproine, Cu(I)-specific stabilizer, suggesting that reactive species derived from the reaction of H_2O_2 with Cu(I) participate in DNA damage. Cytochrome P450 reductase efficiently enhanced 6-hydroxymelatonin-induced oxidative DNA damage and oxygen consumption, suggesting the formation of redox cycle. It is noteworthy that 6-hydroxymelatonin can efficiently induce DNA damage via non-*o*-quinone type of redox cycle. Formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), a characteristic oxidative DNA lesion, in calf thymus DNA was significantly increased by 6-hydroxymelatonin in the presence of Cu(II). Furthermore, 6-hydroxymelatonin significantly increased the formation of 8-oxodG in human leukemia cell line HL-60 but not in HP100, a hydrogen peroxide (H_2O_2)-resistant cell line derived from HL-60. The 6-hydroxymelatonin-induced 8-oxodG formation in HL-60 cells significantly decreased by the addition of bathocuproine or *o*-phenanthroline. Therefore, it is concluded that melatonin may exhibit carcinogenic potential through oxidative DNA damage by its metabolite.

Keywords: DNA damage; Melatonin; 6-Hydroxymelatonin; 8-oxodG; Copper; Hydrogen peroxide

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine), an indolic hormone, is clearly involved in the control of circadian and photoperiodic systems in mammals [1,2]. Melatonin was also discovered to be a direct free radical scavenger [3]. Beside its ability to neutralize a number of free radicals including reactive oxygen and nitrogen, it stimulates several antioxidative enzymes (superoxide dismutase, glutathione peroxidase, and glutathione reductase) [4,5]. It has been reported that melatonin has anti-cancer properties [6,7]. The growth of mammary and ovarian tumor cells was inhibited by physiological concentration of melatonin [8]. Some reports suppose that melatonin has a wide range of health benefits because of its reported anitoxidative properties [9–11]. More than 20 million Americans have taken melatonin supplement, frequently at high dose, over long periods and without any medical monitoring [12].

On the other hand, dietary administration of melatonin induced lymphomas and leukemias (and malignant tumor)

Abbreviations: 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine (and also known as 8-hydroxy-2′-deoxyguanosine); HPLC-ECD, an electrochemical detector coupled to a high-pressure liquid chromatography; DTPA, diethylenetriamine- *N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; O_2^-, superoxide anion radical; H_2O_2, hydrogen peroxide; DMSO, dimethyl sulfoxide; $NADP^+$, ß-nicotinamide adenine dinucleotide phosphate (oxidized form); CIP, calf intestine phosphatase; SOD, superoxide dismutase; CYP, cytochrome P450; G-6-PDH, glucose 6-phosphate dehydrogenase; G-6-P, glucose 6-phosphate; ROS, reactive oxygen species.

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in mice [13,14]. Lipman et al. observed lymphomas in 77.9% of male C57BL/6 mice that received melatonin in the dose of 1.9 mg/mouse with food [13]. Leukemias were detected in 70–98% of C57BL/6 mice and in 78% of CC57Br mice treated subcutaneously with melatonin in the dose of 2.5 mg/mouse twice a week during 2.5–5 months. Noteworthily, it was appeared that melatonin being given in night drinking water relatively low dose (3–3.5 mg/kg) in mice was carcinogenic as well [14]. COMET assay showed that in pharmacological doses, melatonin induced DNA damage and cleavage in hamster ovarian cells [15]. Clastogenic effect of melatonin may be involved in the mechanism of its carcinogenic effect.

Judging from these results, melatonin may have the dual function of anticarcinogenic and carcinogenic potentials. Previously, we reported that antioxidants such as vitamin A and its derivative [16], vitamin E [17], quercetin [18], *N*-acetylcysteine [19] and curcumin [20] caused oxidative damage to cellular and isolated DNA. Virtually, some of putative chemopreventive antioxidants may have potential carcinogenicity. Then, we investigated the possibility that melatonin is metabolized to an ultimate carcinogen causing DNA damage. Melatonin is rapidly metabolized in the liver to its main metabolite, 6-hydroxymelatonin, by CYP1A2 [21,22]. In this study, we have examined whether 6-hydroxymelatonin causes DNA damage in the presence of Cu(II), using [^{32}P]-5′-end-labeled DNA fragments obtained from the human *p16* and *p53* tumor suppressor genes and the c-Ha-*ras*-1 protooncogene. These genes are suitable for studying the mechanisms of carcinogenesis because they are known to be targets for chemical carcinogens [23,24]. We also analyzed the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), a DNA lesion characteristic of oxidative damage, using an electrochemical detector coupled to a high-performance liquid chromatography (HPLC-ECD).

2. Materials and methods

2.1. Materials

The restriction enzymes (*Sma*I, *Bss*HII, *Eco*RI, *Apa*I and *Sty*I) and glucose 6-phosphate dehydrogenase (G-6-PDH) were purchased from Boehringer Mannheim GmbH (Germany). The restriction enzymes (*Hin*dIII and *Xba*I) and T_4 polynucleotide kinase were obtained from New England Biolabs (Beverly, MA). [γ-^{32}P]ATP (222 TBq/mmol) was acquired from New England Nuclear (Boston, MA). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and bathocuproine disulfonic acid were procured from Dojin Chemical Co. (Kumamoto, Japan). Acrylamide, piperidine, dimethyl sulfoxide (DMSO), bisacrylamide, β-nicotinamide adenine dinucleotide phosphate (oxidized form) ($NADP^+$) and glucose 6-phosphate monosodium salt (G-6-P) were purchased from Wako (Osaka, Japan). Cytochrome P450 (CYP) reductase (10.0 mg/mL protein from human microsomes) was purchased from Gentest Corporation (Woburn, MA). $CuCl_2$, ethanol, D-mannitol and sodium formate were acquired from Nacalai Tesque (Kyoto, Japan). Calf thymus DNA, calf intestine phosphatase (CIP), bacterial alkaline phosphatase (82 units/mg from *Escherichia coli*), superoxide dismutase (SOD, 3000 units/mg from bovine erythrocytes) and catalase (45,000 units/mg from bovine liver) were obtained from Sigma Chemical Co. (St. Louis, MO). 6-Hydroxymelatonin was obtained from Aldrich Chemical Co. (Milwaukee, IL). Nuclease P_1 (400 units/mg) was purchased from Yamasa Shoyu Co. (Chiba, Japan). *E. coli* formamidopyrimidine-DNA glycosylase (Fpg) was obtained from Trevigen Inc. (Gaithersburg, MD).

2.2. Preparation of ^{32}P-5′-end-labeled DNA fragments

Exon-containing DNA fragments were obtained from the human *p53* tumor suppressor gene [25]. The 5′-end-labeled 650-base-pair fragment (*Hin*dIII*13972–*Eco*RI*14621) was obtained by dephosphorylation with calf intestine phosphatase and rephosphorylation with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The 650-base-pair fragment was further digested with *Apa*I to obtain a singly labeled 443-base-pair fragment (*Apa*I 14179–*Eco*RI*14621) and a 211-base-pair fragment (*Hin*dIII*13972–*Apa*I 14182) [26]. Exon-containing DNA fragments were also obtained from the human *p16* tumor suppressor gene [27]; these fragments were subcloned into the pGEM®-T Easy Vector (Promega Corporation) [28]. The 484-base-pair fragment (*Eco*RI*9466–*Eco*RI*9949) was further digested with *Bss*HII to obtain a singly labeled 324-base-pair fragment (*Eco*RI*9466–*Bss*HII 9789) and a 158-base-pair fragment (*Bss*HII 9794–*Eco*RI*9949). Furthermore, we prepared DNA fragments from the human c-Ha-*ras*-1 protooncogene [29], utilizing the plasmid, pbcNI. This plasmid carries a 6.6-kb *Bam*HI chromosomal DNA segment containing the c-Ha-*ras*-1 gene [30]. A singly labeled 341-base-pair fragment (*Xba*I 1906–*Ava*I* 2246), a 261-base-pair fragment (*Ava*I* 1645–*Xba*I 1905) and a 337-base-pair fragment (*Pst*I 2345–*Ava*I* 2681) were obtained as described previously [29]. Nucleotide numbering for the human c-Ha-*ras*-1 protooncogene begins with the *Bam*HI site [30]. Asterisks indicate ^{32}P-labeling.

2.3. Detection of DNA damage by 6-hydroxymelatonin in the presence of metal ion

Standard reaction mixtures (in a 1.5 mL Eppendorf microtube) containing [^{32}P]-5′-end-labeled DNA fragments, calf thymus DNA (20 μM/base), 20 μM $CuCl_2$ and 6-hydroxymelatonin in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated for 2 h at 37 °C. Subsequently, the DNA was

treated with 1 M piperidine for 20 min at 90 °C or 10 units of Fpg protein in the reaction buffer (10 mM HEPES-KOH (pH 7.4), 100 mM KCl, 10 mM EDTA and 0.1 mg/mL BSA) for 2 h at 37 °C. Fpg protein catalyzes the excision of 8-oxodG as well as Fapy residues [31–33]. After ethanol precipitation, the DNA fragments were electrophoresed and the autoradiogram was obtained by exposing X-ray film to the gel as described previously [29]. 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 [34] using a DNA-sequencing system (LKB 2010 Macrophor). 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 in calf thymus DNA by 6-hydroxymelatonin

The quantity of 8-oxodG was measured utilizing a modification of the method described by Kasai et al. [35]. Standard reaction mixtures (in a 1.5 mL Eppendorf microtube) containing indicated concentrations of 6-hydroxymalatonin and calf thymus DNA fragments (100 μM/base) in 400 μL of 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated for 2 h at 37 °C. Following ethanol precipitation, the DNA fragments were digested into the nucleosides with nuclease P_1 and calf intestine phosphate, and then analyzed by HPLC-ECD, as described previously [36]. Data represent the means ± S.D. of four independent experiments.

2.5. Measurement of oxygen consumption

Oxygen consumption by the reactions of 6-hydroxymelatonin with Cu(II) was measured using a Clarke oxygen electrode (Electronic Stirrer Model 300, Rank Brothers LTD. Bottisham Cambridge, England). The mixture contained 6-hydroxymelatonin and 4% (v/v) of ethanol in 2 mL of 10 mM sodium phosphate buffer (pH 7.8), containing 2.5 μM DTPA. Where indicated,CYP reductase was added to examine the formation of the redox cycle resulting from oxygen consumption. The reactions were started by the addition of 20 μM $CuCl_2$ into the chamber of the oxygen electrode.

2.6. Analysis of 8-oxodG formation in human cultured cells

HL-60 and HP100 cells were grown in RPMI 1640 supplemented with 6% FBS at 37 °C under 5% CO_2 in a humidified atmosphere. Catalase activity of HP100 cells was 18 times higher than that of HL-60 cells [37]. HL-60 and HP100 cells (10^6 cells/mL) were incubated with various concentrations of 6-hydroxymelatonin for 4 h. Where indicated, HL-60 cells (10^6 cells/mL) were preincubated with 0.2 mM *o*-phenanthroline or bathocuproine for 30 min, followed by incubation with 5 μM 6-hydroxymelatonin for 4 h at 37 °C. After the incubation, the medium was removed, and the cells were washed three times with cold phosphate-buffered saline (PBS). We isolated DNA from cells and digested DNA into nucleosides under complete anaerobic conditions using anaerobic incubator, which carried out the trap of the oxygen of very small quantity. The cells were suspended in 0.05 mg/mL RNase, 0.5 mg/mL proteinase K, and 500 μL of lysis buffer (Applied Biosystems), followed by incubation for 1 h at 60 °C. After ethanol precipitation, DNA was digested to the nucleosides with 8 units of nuclease P_1 and 1.2 units of bacterial alkaline phosphatase in anaerobiosis. The resulting 2′-deoxyribonucleosides mixture was injected into an HPLC apparatus (SCL-10AV, Shimadzu, Kyoto, Japan) equipped with both a UV detector (SPD-10AV, Shimadzu) and an electrochemical detector (Coulochem II, ESA, MA, USA): column, CAPCELL PAK C18 MG (0.46 × 15 cm) (Shiseido, Tokyo, Japan); eluent, 8% aqueous methanol containing 10 mM NaH_2PO_4; flow rate 1 mL/min. The molar ratio of 8-oxodG to 2′-deoxyguanosine (dG) in each DNA sample was measured based on the peak height of authentic 8-oxodG with the electrochemical detector and the UV absorbance at 254 nm of dG [36].

3. Results

3.1. Damage to ^{32}P-labeled DNA fragments by 6-hydroxymelatonin in the presence of metal ion

In the presence of Cu(II), 6-hydroxymelatonin induced DNA damage in a dose-dependent manner (Fig. 1A). 6-Hydroxymelatonin alone or melatonin could not cause DNA damage (data not shown). When cytochrome P450 (CYP) reductase was added, DNA damage was enhanced (Fig. 1A). Even without piperidine treatment, oligonucleotides were formed by 6-hydroxymelatonin in the presence of Cu(II), indicating breakage of the deoxyribose phosphate backbone (Fig. 1B, lanes 2–4). Piperidine treatment increased the formation of smaller size oligonucleotides (Fig. 1B, lanes 8–10). Since altered base is readily removed from its sugar by the piperidine treatment, it is considered that the base modification was induced by 6-hydroxymelatonin in the presence of Cu(II). Furthermore, the formation of Fpg-sensitive sites by 6-hydroxymelatonin in the presence of Cu(II) significantly increased compared with the yield of strand breaks (Fig. 1B, lanes 2–7). On the other hand, the Fpg-sensitive site formation by H_2O_2 and Fe(II) was not significantly increased compared with the yield of strand breaks (Fig. 1B, lanes 11–14). 6-Hydroxymelatonin did not induce DNA damage in the presence of other metal ions, including Co(II), Ni(II), Mn(II), Mn(III), Fe(III) and Fe(III)EDTA (data not shown).

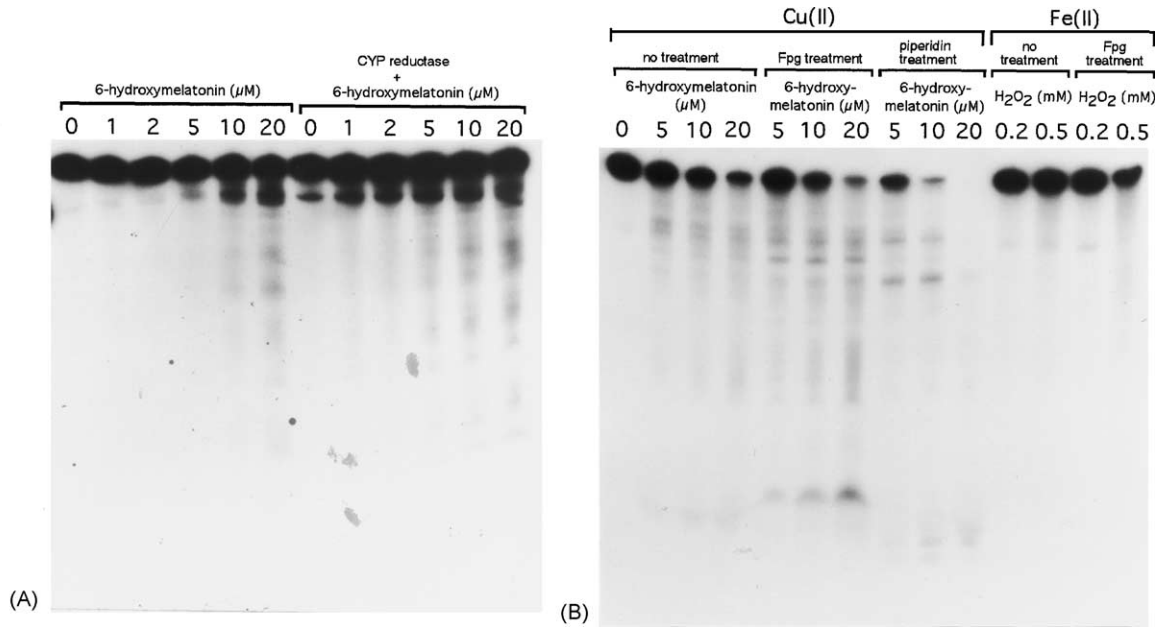

Fig. 1. Autoradiogram of ^{32}P-labeled DNA fragments incubated with 6-hydroxymelatonin plus Cu(II). (A) The reaction mixtures (in a 1.5 mL Eppendorf microtube) containing indicated concentrations of 6-hydroxymelatonin, ^{32}P-5′-end-labeled 158-base-pair DNA fragments, 20 μM/base of calf thymus DNA, 20 μM $CuCl_2$, 0.25 nM CYP450 reductase, 250 μM $NADP^+$, 500 μM G-6-P, 0.07 units G-6-PDH and 500 μM $MgCl_2$ in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated for 2 h at 37 °C. Subsequently, DNA fragments were treated with 1 M piperidine for 20 min at 90 °C, then electrophoresed on an 8% polyacrylamide/8 M urea gel. (B) The reaction mixtures (in a 1.5 mL Eppendorf microtube) containing ^{32}P-5′-end-labeled 261-base-pair DNA fragments, 20 μM/base of calf thymus DNA, 6-hydroxymelatonin plus 20 μM $CuCl_2$ or H_2O_2 plus 20 μM $FeSO_4(NH_4)_2SO_4$ in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated for 2 h at 37 °C. Subsequently, DNA fragments were treated with or without Fpg protein or with 1 M piperidine, and then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was visualized by exposing an X-ray film to the gel.

3.2. Effects of scavengers and a metal chelator on DNA damage induced by 6-hydroxymelatonin

To examine the molecular mechanism of DNA damage, we evaluated the effects of scavengers and a metal chelator on DNA damage induced by 6-hydroxymelatonin in the presence of Cu(II) (Fig. 2). Catalase and bathocuproine, a Cu(I)-specific chelator, inhibited DNA damage by 6-hydroxymelatonin, suggesting the involvement of H_2O_2 and Cu(I). Free hydroxyl radical ($^{\bullet}$OH) scavengers, such as ethanol, mannitol, sodium formate and DMSO, demonstrated little or no inhibitory effect on DNA damage by 6-hydroxymelatonin. Methional inhibited DNA damage. Methional is capable of scavenging both $^{\bullet}$OH and species with weaker reactivity [38,39].

3.3. Site specificity of DNA cleavage by 6-hydroxymelatonin

DNA cleavage sites were identified by a direct comparison of the oligonucleotide positions produced by 6-hydroxymelatonin with those produced by the Maxam–Gilbert procedure [34]. ^{32}P-5′-end-labeled DNA fragments were treated with 6-hydroxymelatonin in the presence of Cu(II). An autoradiogram was obtained and scanned with a laser densitometer to measure relative intensity of DNA cleavage in the c-Ha-*ras*-1 protooncogene (Fig. 3A) and the human *p53* tumor suppressor gene (Fig. 3B). 6-Hydroxymelatonin induced piperidine-labile sites preferentially at thymine and cytosine residues (Fig. 3). With Fpg treatment, the DNA cleavage occurred mainly at guanine and cytosine residues. 6-Hydroxymelatonin caused piperidine-labile and Fpg sensitive lesions at C and G in the 5'-ACG-3' sequence, a well-known hotspot of the *p53* gene [23,40] (Fig. 3B). From these results, it is considered that 6-hydroxymelatonin can cause DNA damage at 5′-TG-3′ and 5′-CG-3′ sequences at high frequency.

3.4. Formation of 8-oxodG in calf thymus DNA by 6-hydroxymelatonin in the presence of Cu(II)

Using an HPLC-ECD, we measured the quantity of 8-oxodG, an indicator of oxidative base damage [35,36], in calf thymus DNA following treatment with variable concentrations of 6-hydroxymelatonin in the presence of Cu(II). The level of 8-oxodG significantly increased with increasing concentrations of 6-hydroxymelatonin (Fig. 4). The addition of CYP reductase enhanced 6-hydroxymelatonin plus Cu(II)-induced 8-oxodG formation.

3.5. Oxygen consumption during Cu(II)-mediated autoxidation of 6-hydroxymelatonin and enhancement by CYP reductase

Fig. 5 shows oxygen consumption during the autoxidation of 6-hydroxymelatonin in the presence of Cu(II).

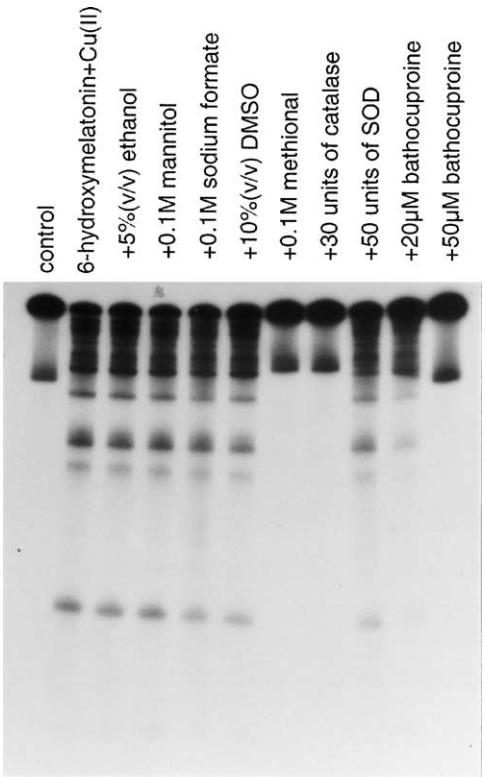

Fig. 2. Effects of scavengers and bathocuproine on the DNA damage induced by 6-hydroxymelatonin in the presence of Cu(II). Reaction mixtures contained the ^{32}P-5′-end-labeled 443-base-pair DNA fragment, 20 μM/base of calf thymus DNA, 20 μM 6-hydroxymelatonin and 20 μM $CuCl_2$ in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. Reaction mixtures were incubated for 2 h at 37 °C. DNA fragments were treated with 1 M piperidine for 20 min at 90 °C, then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was visualized by exposing an X-ray film to the gel.

When Cu(II) was added, 6-hydroxymelatonin consumed oxygen. The addition of CYP reductase increased oxygen consumption, suggesting that redox cycle was formed.

3.6. Formation of 8-oxodG in human cultured cells treated with 6-hydroxymelatonin

To investigate oxidative damage in cellular DNA, we measured the content of 8-oxodG in HL-60 and HP 100 cells treated with 6-hydroxymelatonin. Production of 8-oxodG in HL-60 cells was increased in a dose-dependent manner (Fig. 6A). The content of 8-oxodG in HL-60 cells treated with 2 μM 6-hydroxymelatonin was significantly larger in comparison with the control. However, 6-hydroxymelatonin did not increase the amount of 8-oxodG in HP100 cells (Fig. 6A). It was reported that HP100 cells were approximately 340-fold more resistant to H_2O_2 than the parent cell line, HL-60 [37]. These results suggest that generation of H_2O_2 plays a critical role in DNA damage.

To examine the possible role of endogenous cellular metal, bathocuproine or *o*-phenanthroline was added to HL-60 cells incubated with 6-hydroxymelatonin. The amounts of 8-oxodG induced by 5 μM 6-hydroxymelatonin were significantly reduced by the addition of bathocuproine or *o*-phenanthroline, confirming the involvement of metal, especially copper ion. (Fig. 6B).

4. Discussion

In this study, 6-hydroxymelatonin caused oxidative DNA damage in the presence of Cu(II). Experiments with piperidine or Fpg treatment revealed that C and G of the 5′-ACG-3′ sequence, the complementary sequence to codon 273 (a known hotspot) in exon 8 of the *p53* gene [23,40], were significantly damaged, although C was damaged to a lesser extent than the G. 6-Hydroxymelatonin also formed piperidine-labile lesions at T of the 5′-TG-3′ sequence. When DNA fragments were treated with Fpg protein, 6-hydroxymelatonin caused DNA damage mainly at G residues especially of the 5′-TG-3′ sequence. Fpg protein mainly catalyzes the excision of piperidine-resistant 8-oxodG [31] and further oxidized piperidine-labile guanine residues [41], although Fpg also mediates cleavage of uracil glycol [42], 5-hydroxycytosine and 5,6-dihydrothymine [43], in vitro. Therefore, it is reasonably considered that 6-hydroxymelatonin oxidizes the G residue of 5′-CG-3′ and 5′-TG-3′ sequences to 8-oxodG. Although, it is postulated that double-base lesions can be generated from one radical hit [44–46], further work is required to ascertain whether the putative double-base lesion with DNA is present or not.

We demonstrated that the content of 8-oxodG in HL-60 cells was increased by the 6-hydroxymelatonin treatment, whereas the content of 8-oxodG in HP100 cells was not increased. The catalase activity of HP100 cells was 18 times higher than that of HL-60 cells [37]. Therefore, it is considered that generation of H_2O_2 plays an important role in 6-hydroxymelatonin-induced 8-oxodG formation in human cultured cells. Furthermore, the formation of 8-oxodG was markedly reduced by *o*-phenanthroline or bathocuproine, suggesting that copper ion may be involved in oxidative DNA damage by 6-hydroxymelatonin in the cells.

To clarify what kinds of reactive species were involved in DNA damage by 6-hydroxymelatonin, we examined the effects of scavengers on DNA damage. The inhibitory effects of catalase and bathocuproine on 6-hydroxymelatonin-induced DNA damage suggest that both H_2O_2 and Cu(I) participate in DNA damage. Typical $^{\bullet}$OH scavengers demonstrated little or no inhibitory effect, whereas methional inhibited DNA damage. This result suggests the involvement of reactive species with a similar reactivity to $^{\bullet}$OH [38,39]. Piperidine-labile DNA lesion by 6-hydroxymelatonin with Cu(II) occurred predominantly at thymine and cytosine residues. Furthermore, the yield of Fpg-sensitive sites, such as 8-oxodG, by 6-hydroxymelatonin significantly increased compared with the yield of strand breaks. $^{\bullet}$OH induces DNA cleavage without a nucleotide bias

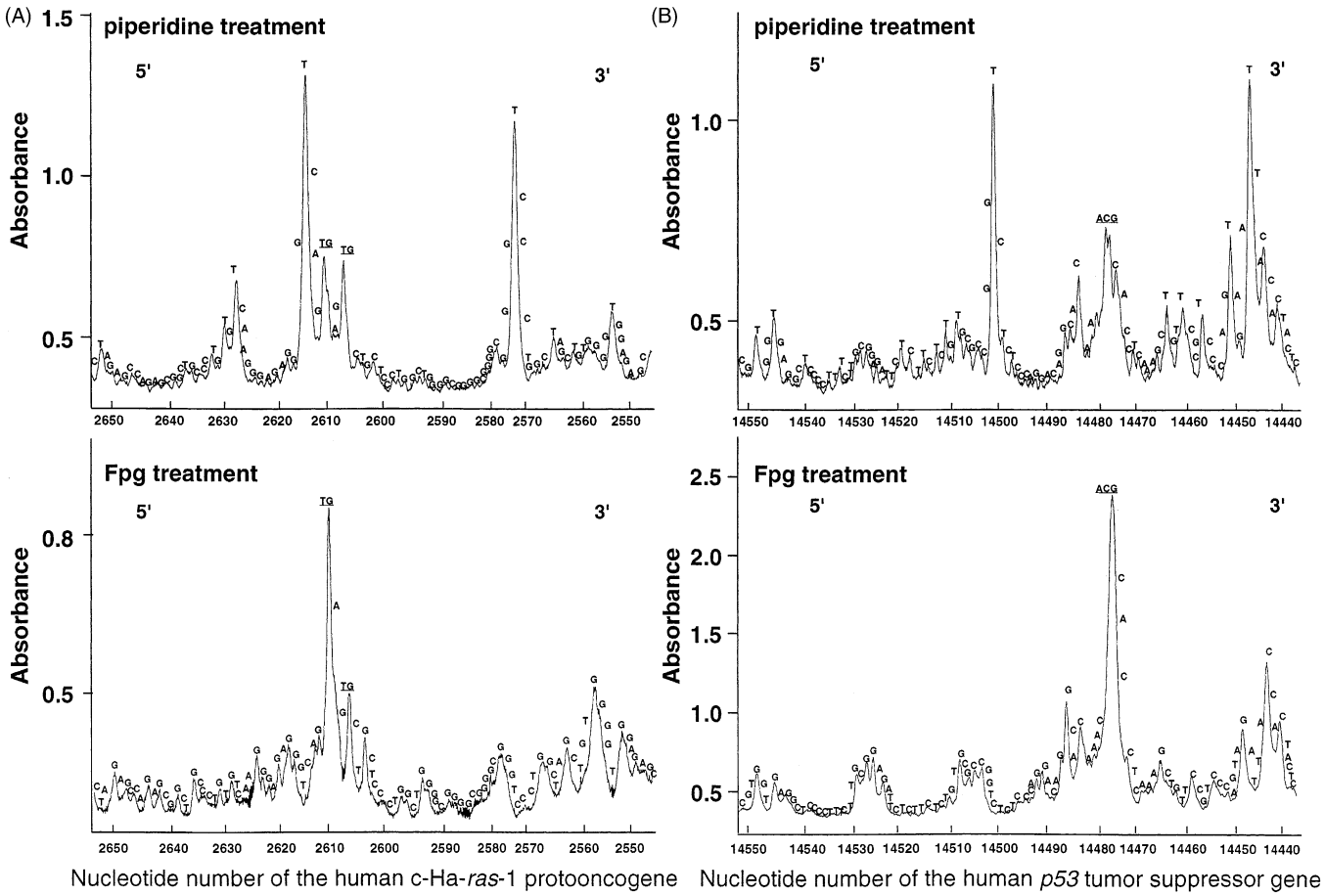

Fig. 3. Site specificity of DNA cleavage induced by 6-hydroxymelatonin in the presence of Cu(II). Reaction mixtures contained either the ^{32}P-5′-end-labeled 443-base-pair fragment (*Apa*I 14179–*Eco*RI*14621) derived from the human *p53* tumor suppressor gene (A) or the 337-base-pair fragment (*Pst*I 2345–*Ava*I* 2681) derived from the human c-Ha-*ras*-1 protooncogene (B), 20 μM/base of calf thymus DNA, 20 μM 6-hydroxymelatonin and 20 μM $CuCl_2$ in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. Reaction mixtures were incubated for 2 h at 37 °C. Following piperidine or Fpg treatment, the DNA fragments were analyzed as described in Fig. 1 legend. The relative quantities of oligonucleotides were measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL, Pharmacia Biotech). Underlined bases represent double-base lesions detected by the treatment with piperidine and Fpg protein.

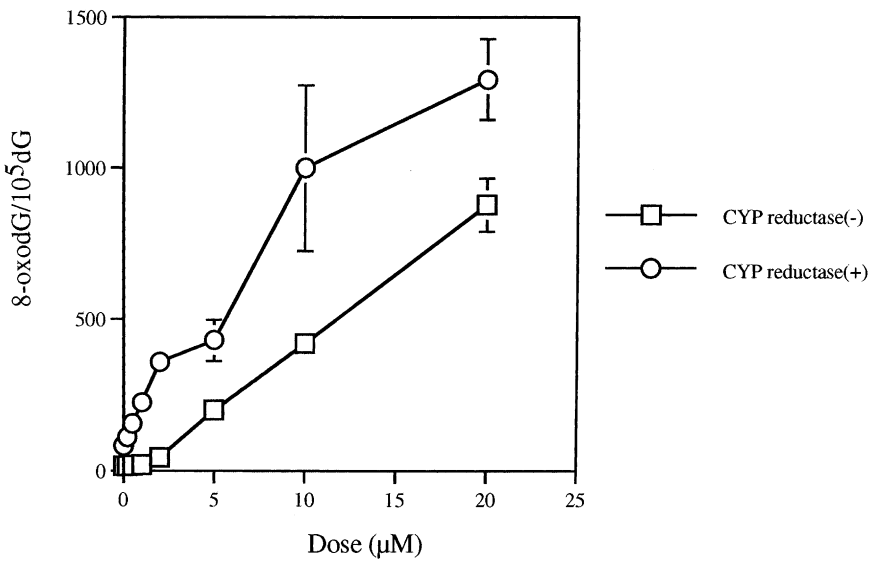

Fig. 4. Formation of 8-oxodG by 6-hydroxymelatonin in the presence of Cu(II). Standard reaction mixtures (in a 1.5 mL Eppendorf microtube) containing indicated concentrations of 6-hydroxymelatonin, 0.25 nM CYP450 reductase, 250 μM $NADP^+$, 500 μM G-6-P, 0.07 units G-6-PDH and 500 μM $MgCl_2$ in 400 μL of 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated for 2 h at 37 °C. Following an incubation, 0.2 mM DTPA was added to stop the reaction and then the DNA was precipitated in ethanol. The DNA was subjected to enzymatic digestion and analyzed by HPLC-ECD.

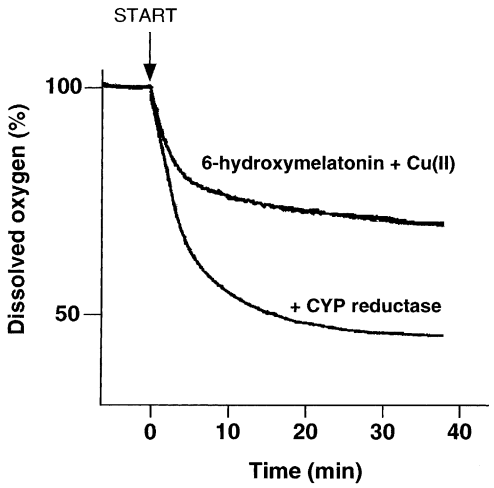

Fig. 5. Oxygen consumption by the interaction of 6-hydroxymelatonin with Cu(II). The mixture contained 6-hydroxymelatonin and 4% (v/v) of ethanol in 2 mL of 10 mM sodium phosphate buffer (pH 7.8), containing 2.5 μM DTPA at 37 °C. Where indicated, to detect redox cycle formation, 0.57 nM CYP reductase, 250 μM $NADP^+$, 500 μM G-6-P, 0.07 units G-6-PDH and 500 μM $MgCl_2$ were added. The reactions were started by the addition of 20 μM $CuCl_2$ into the chamber of the oxygen electrode (indicated by an arrow).

[47,48]. Thus, $^{\bullet}OH$ does not appear to play an important role in DNA damage by 6-hydroxymelatonin. This is supported by papers showing that the levels of 8-oxodG relative to strand breaks produced by Cu(II)/H_2O_2 were higher than that produced by Fe(II)-EDTA/H_2O_2 and γ-radiation [49,50]. Therefore, it is considered that reactive species formed from H_2O_2 and Cu(I) are involved in DNA damage by 6-hydroxymelatonin.

On the basis of this study, we propose a possible mechanism whereby melatonin induces Cu(II)-mediated DNA damage (Fig. 7). CYPs are contained in the lung, lymph nodes, liver and skin [51]. Melatonin undergoes CYP 1A2-catalyzed 6-hydroxylation to 6-hydroxymelatonin [21,22]. 6-Hydroxymelatonin undergoes Cu(II)-catalyzed autoxidation into the corresponding radical, leading to the production of the quinone methide form. Relevantly, Hadi et al. have reported that a high concentration of 5-hydroxytryptamine (serotonin), which is structurally related to 6-hydroxymelatonin, can reduce two molar equivalents of Cu(II) to Cu(I) through a reaction involving two electron oxidation of the phenol to a quinone methide [52]. During the autoxidation of 6-hydroxymelatonin, Cu(II) is reduced to Cu(I), which reacts with O_2 to generate O_2^- and subsequently H_2O_2. H_2O_2 interacts with Cu(I) to form the Cu(I)-hydroperoxo complex, capable of inducing DNA damage [53]. CYP reductase enhanced O_2 consumption, suggesting that redox cycle was formed and O_2^- generation was enhanced, leading to enhancement of oxidative DNA damage. Some quinones can undergo enzymatic and non-enzymatic redox cycling with their corresponding semiquinone radicals and generate O_2^- [54–56]. Previously, we have also reported that catechol-type compounds (quercetin, catechol, curcumin) efficiently generated reactive oxygen species (ROS) in the presence of metal ion via redox cycle [19,21,57,58]. We have demonstrated that NAD(P)H may non-enzymatically reduce *o*-quinones to catechols through two-electron reduction [57,58]. In this paper, newly we showed the non-catechol type compound (6-hydroxymelatonin) is also

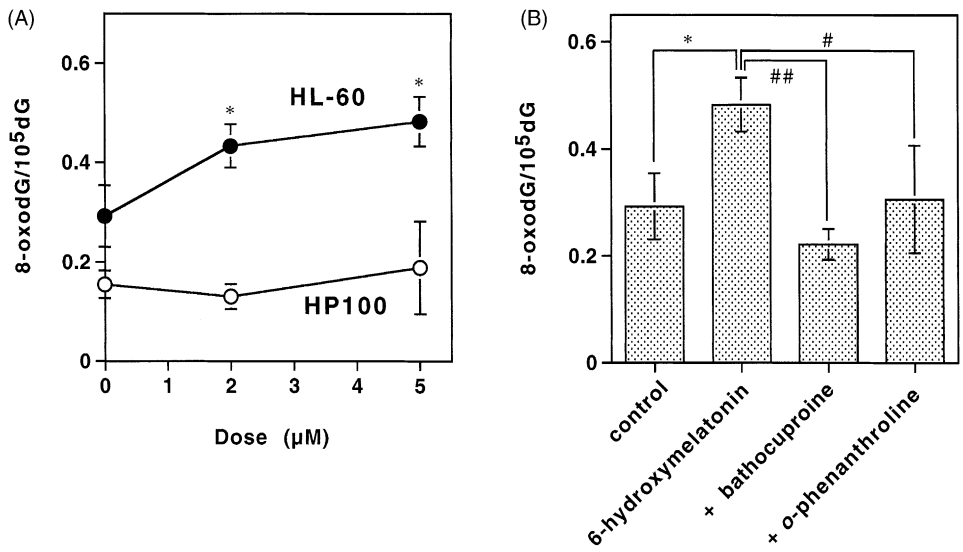

Fig. 6. Formation of 8-oxodG by 6-hydroxymelatonin in human cultured cells and the inhibitory effects of bathocuproine and *o*-phenanthroline. HL-60 (closed symbol) and HP100 (open symbol) cells (10^6 cells/mL) were incubated with various concentrations of 6-hydroxymelatonin for 4 h (A). HL-60 cells (10^6 cells/mL) were preincubated with 0.2 mM *o*-phenanthroline or bathocuproine for 30 min, followed by incubation with 5 μM 6-hydroxymelatonin for 4 h at 37 °C (B). After the incubation, DNA was extracted immediately. And then, the DNA was subjected to enzyme digestion and analyzed by HPLC-ECD as described in Materials and methods. Results are expressed as means ± S.D. of values obtained from four independent experiments. The asterisk indicates significant differences compared with the control by *t*-test ($^*P < 0.01$). $^{\#}P < 0.05$ and $^{\#\#}P < 0.001$, significantly decreased by the addition of *o*-phenanthroline or bathocuproine.

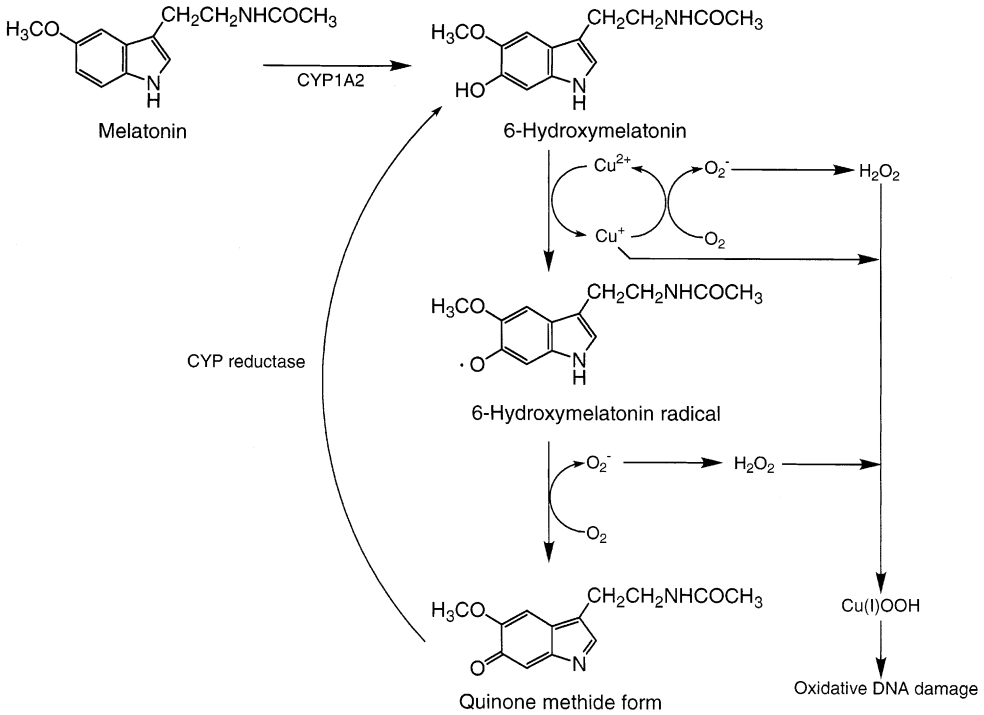

Fig. 7. A possible mechanism for Cu(II)-mediated DNA damage induced by melatonin.

autooxidated by the copper ion and found that 6-hydroxymelatonin enzymatically induced oxidative DNA damage via non-quinone type of redox cycle.

Copper exists in the mammalian cell nucleus, and may contribute to high-order chromatin structures [59]. Copper ions bind to non-histone proteins, and cause much stronger ascorbate-mediated DNA damage than iron [60]. Copper has the ability to catalyze the production of reactive oxygen species to mediate oxidative DNA damage [61–63]. Therefore, it is reasonably concluded that Cu(II) ion may participate in reactive oxygen generation under certain conditions, although mammals have evolved means of minimizing levels of free copper ions and most copper ions bind to protein carriers and transporters [64]. These studies support the finding that a melatonin metabolite caused DNA damage by generating ROS through the interaction with copper. On the other hand, several papers suggested that DNA damage was caused by H_2O_2 through an in vivo Fenton reaction [65,66]. Consequently, there still remains another possibility that Fe(II) participates in melatonin-induced DNA damage in the cells.

Melatonin and 6-hydroxymelatonin are known to be effective as free radical scavengers and have an anti-cancer effect [3–11]. On the other hand, it has been reported that long-term night administration of melatonin significantly increases malignant tumor incidence in female CBA mice [14]. In this study, we have demonstrated that a melatonin metabolite, 6-hydroxymelatonin, can cause oxidative DNA damage, probably double-base lesions at 5′-CG-3′ and 5′-TG-3′ sequences. We have also shown that G residue in 5′-CG-3′ and 5′-TG-3′ sequences was oxidized to 8-oxodG, which can cause the misreplication of DNA (G:C→T:A transversion) that might lead to mutation or cancer [67–69]. Findings of DNA damage by 6-hydroxymelatonin itself via a new type of redox cycle may provide an insight into mechanism of carcinogenesis by melatonin through ROS formation, in addition to already known types of DNA damage. Further study on safety should be required when melatonin is used for cancer prevention or nutrition supplement in humans.

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