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Mechanism of oxidative DNA damage induced by capsaicin, a principal ingredient of hot chili pepper

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Abstract
Although capsaicin exhibits antitumor activity, carcinogenic potential has also been reported. To clarify the mechanism for expression of potential carcinogenicity of capsaicin, we examined DNA damage induced by capsaicin in the presence of metal ion and various kinds of cytochrome P450 (CYP) using ^{32}P-5′-end-labeled DNA fragments. Capsaicin induced Cu(II)-mediated DNA damage efficiently in the presence of CYP1A2 and partially in the presence of 2D6. CYP1A2-treated capsaicin caused double-base lesions at 5′-TG-3′, 5′-GC-3′ and CG of the 5′-ACG-3′ sequence complementary to codon 273, a hotspot of *p53* gene. DNA damage was inhibited by catalase and bathocuproine, a Cu(I) chelator, suggesting that reactive species derived from the reaction of H_2O_2 with Cu(I) participate in DNA damage. Formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine was significantly increased by CYP1A2-treated capsaicin in the presence of Cu(II). Therefore, we conclude that Cu(II)-mediated oxidative DNA damage by CYP-treated capsaicin seems to be relevant for the expression of its carcinogenicity.

Keywords: *Capsaicin, cytochrome P450, H_2O_2, carcinogenesis, copper*

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 performance liquid chromatography; Fpg,* Escherichia coli *formamidopyrimidine-DNA glycosylase; DTPA, diethylenetriamine-N,N,N′,N″,N‴-pentaacetic acid; $O_2^{\cdot-}$, superoxide anion radical; H_2O_2, hydrogen peroxide; DMSO, dimethyl sulfoxide; CYP, cytochrome P450; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate (oxidized form); G-6-PDH, glucose 6-phosphate dehydrogenase; G-6-P, glucose 6-phosphate; CIP, calf intestine phosphatase; SOD, superoxide dismutase*

Introduction

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), a principal pungent component of *Capsicum* fruits, is frequently consumed as spices and drugs. Capsaicin is known to have antioxidant properties [1,2]. Several studies have indicated that capsaicin possesses potent antimutagenic and anticarcinogenic activities [3,4]. In addition, capsaicin is currently considered a novel agent for use in the management of patients with arthritic pain and inflammation [5,6]. Therefore, capsaicin has been considered as one of the potential chemopreventive agents.

On the other hand, epidemiological studies have reported association between red chili pepper consumption and gastric cancer [7,8], gallbladder cancer [9,10], liver and pancreas cancer [11]. Capsaicin is the most abundant of the capsaicinoids, constituting approximately 40–60% of the total capsaicinoid content in hot pepper products [12]. It has been observed that dietary administration of semisynthetic

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powdered diet with capsaicin induces tumors of the cecum in mice [13]. Relevantly, epidemiological studies have demonstrated that some antioxidant chemopreventive agents including beta-carotene and alpha-tocopherol have carcinogenic potential [14–18]. We have evaluated the safety of antioxidants on the basis of reactivity with DNA [19]. Previously, we demonstrated that several antioxidants, such as melatonin [20], propyl gallate [21], catechins [22,23], beta-carotene [24], alpha-tocopherol [25], etc. or those metabolites caused oxidative DNA damage to cellular and isolated DNA.

In this study, to clarify mechanism for expression of potential carcinogenicity of capsaicin, we investigated DNA damage induced by capsaicin in the presence of metal ion and cytochrome P450 (CYP) using ^{32}P-5′-end-labeled DNA fragments obtained from the human *p16* and *p53* tumor suppressor genes and *c*-Ha-*ras-1* protooncogene. We also analyzed the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) using an electrochemical detector coupled to a high performance liquid chromatography (HPLC-ECD). A characteristic oxidative DNA lesion, 8-oxodG, has attracted much attention in relation to mutagenesis and carcinogenesis [26].

Materials and methods

Materials

The restriction enzymes and glucose 6-phosphate dehydrogenase (G-6-PDH) were obtained from Boehringer Mannheim GmbH (Germany). T_4 polynucleotide kinase was from New England Biolabs (Beverly, MA, USA). [γ-^{32}P]ATP (222 TBq/mmol) was from New England Nuclear (Boston, MA, USA). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and bathocuproine disulfonic acid were from Dojin Chemical Corporation (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 from Wako (Osaka, Japan). CYP isozymes from human microsomes (1A1, 1A2, 2C9 and 2D6) and CYP reductase (10.0 mg/ml protein from human microsomes) were from Gentest Corporation (Woburn, MA, USA). $CuCl_2$, ethanol, D-mannitol and sodium formate were from Nacalai Tesque (Kyoto, Japan). Calf thymus DNA, calf intestine phosphatase (CIP), superoxide dismutase (SOD, 3000 units/mg from bovine erythrocytes), catalase (45,000 units/mg from bovine liver) and capsaicin were from Sigma Chemical Corporation (St Louis, MO, USA). Nuclease P1 (400 units/mg) was from Yamasa Shoyu Corporation (Chiba, Japan). *Echerichia coli* formamidopyrimidine-DNA glycosylase (Fpg) was from Trevigen Inc. (Gaithersburg, MD, USA).

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

Two fragments containing exon 1 or 2 of the human *p16* tumor suppressor gene [27] were obtained as described previously [28]. The 5′-end-labeled 490-base pair fragment (*EcoR I*⋆ 5841–*EcoR I*⋆ 6330) containing exon 1 was further digested with *Mro I* to obtain the singly labeled 328-base pair fragment (*EcoR I*⋆ 5841–*Mro I* 6168) and the 158-base pair fragment (*Mro I* 6173–*EcoR I*⋆ 6330). The 5′-end-labeled 460-base pair fragment (*EcoR I*⋆ 9481–*EcoR I*⋆ 9940) containing exon 2 was also further digested with *BssH II* to obtain the singly labeled 309-base pair fragment (*EcoR I*⋆ 9481–*BssH II* 9789) and the 147-base pair fragment (*BssH II* 9794–*EcoR I*⋆ 9940).

DNA fragments were also obtained from the human *p53* tumor suppressor gene. The ^{32}P-5′-end-labeled 650-base pair (*Hind III*⋆ 13,972–*EcoR I*⋆ 14,621) and 460-base pair (*Hind III*⋆ 13,038–*EcoR I*⋆ 13,507) fragments were obtained as described previously [29]. The 650-base pair fragment was digested with *Apa I* to obtain the singly labeled 211-base pair (*Hind III*⋆ 13,972–*Apa I* 14,182) and the 443-base pair (*Apa I* 14,179–*EcoR I*⋆ 14,621) DNA fragments. The 460-base pair fragment was digested with *Sty I* to obtain the singly labeled 118-base pair (*Hind III*⋆ 13,038–*Sty I*13,155) and the 348-base pair (*Sty I* 13,160–*Eco* R I⋆ 13,507) fragments.

DNA fragments were prepared from the plasmid pbc NI, which carries a 6.6 kb *BamH I* chromosomal DNA segment containing the *c*-Ha-*ras-1* protooncogene. The singly labeled 261-base pair fragment (*Ava I*⋆ 1645–*Xba I* 1905), 341-base pair fragment (*XbaI* 1906–*Ava I*⋆ 2246), 98-base pair fragment (*Ava I*⋆ 2247–*Pst I* 2344), and 337-base pair fragment (*Pst I* 2345–*Ava I*⋆ 2681) were obtained as previously described [30,31]. For reference, nucleotide numbering starts with the *BamH I* site [32]. An asterisk indicates ^{32}P-labeling.

Detection of DNA damage induced by capsaicin treated with CYP isozyme

Standard reaction mixtures containing capsaicin, 0.25 nM various CYP isozyme, 0.05 nM NADPH–CYP reductase and NADPH-generating system (200 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. After preincubation, ^{32}P-5′-end-labeled DNA fragments, calf thymus DNA (20 μM/base) and 20 μM $CuCl_2$ were added to the mixtures, followed by the incubation for 1 h at 37°C. Subsequently, the DNA was treated with 1 M piperidine at 90°C for 20 min 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

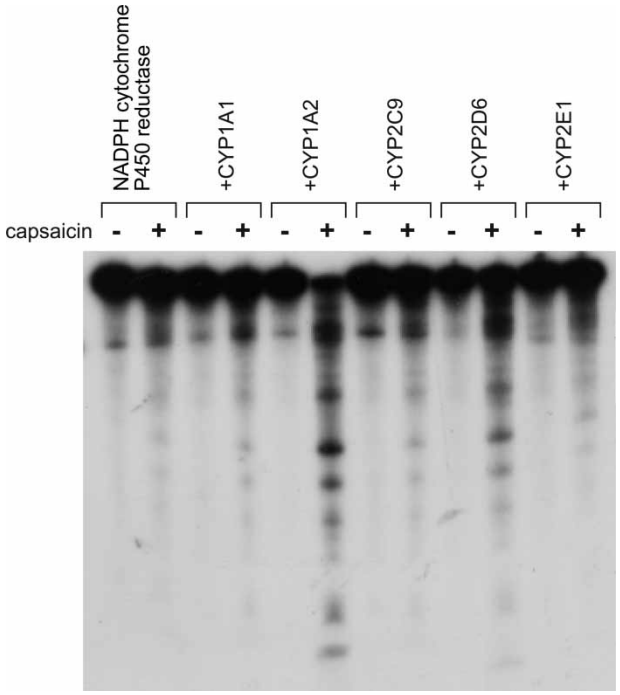

Figure 1. Autoradiogram of ^{32}P-labeled DNA fragments incubated with capsaicin and Cu(II) in the presence of various CYP isozyme. The reaction mixtures containing 500 μM capsaicin, 0.25 nM various CYP isozyme, 0.05 nM NADPH–CYP reductase and NADPH-generating system (200 μM $NADP^+$, 500 μM G-6-P, 0.07 units G-6-PDH and 500 μM $MgCl_2$) in 200 μl of 10 mM sodiumphosphate buffer (pH 7.8) containing 5 μM DTPA were preincubated for 2 h at 37°C. After preincubation, ^{32}P-5′-end-labeled 443-bp DNA fragments, calf thymus DNA (20 μM/base) and 20 μM $CuCl_2$ were added to the preincubated mixtures, followed by the incubation for 1 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. The autoradiogram was visualized by exposing an X-ray film to the gel.

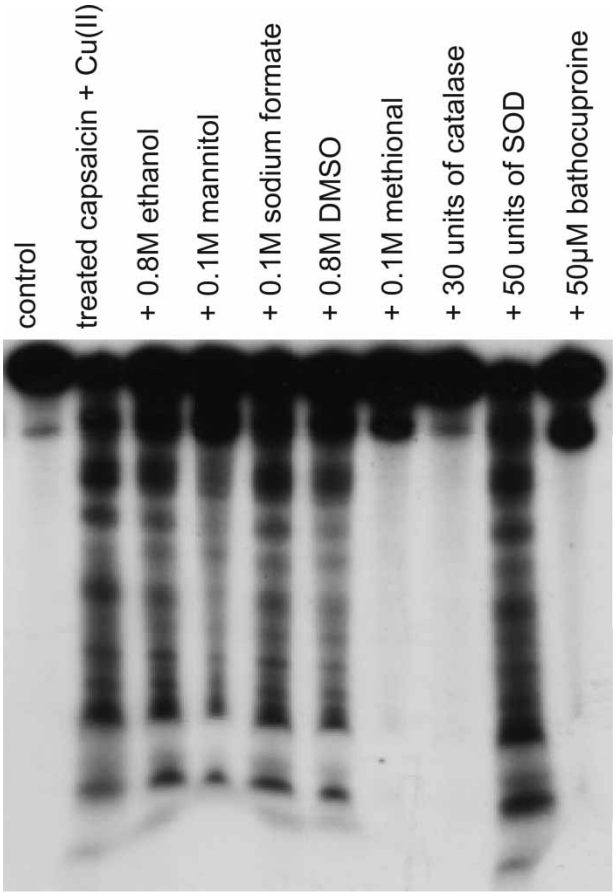

Figure 2. Effects of scavengers and bathocuproine on DNA damage induced by capsaicin treated with CYP1A2 in the presence of Cu(II). The reaction mixtures containing 500 μM capsaicin, 0.25 nM CYP1A2, 0.05 nM NADPH–CYP reductase and NADPH-generating system (200 μM $NADP^+$, 500 μM G-6-P, 0.07 units G-6-PDH and 500 μM $MgCl_2$) in 200 μl of 10 mM sodiumphosphate buffer (pH 7.8) containing 5 μM DTPA were preincubated for 2 h at 37°C. After preincubation, ^{32}P-5′-end-labeled 211-bp DNA fragments, calf thymus DNA (20 μM/base), scavengers and 20 μM $CuCl_2$ were added to the preincubated mixtures, followed by the incubation for 1 h at 37°C. Following piperidine treatment, the DNA fragments were analyzed as described in Figure 1 legend.

catalyzes the excision of 8-oxodG as well as Fapy residues [33–35]. After ethanol precipitation, the DNA fragments were electrophoresed and the autoradiogram was obtained by exposing X-ray film to the gel as described previously [30,31]. 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 [36] 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.

Analysis of 8-oxodG formation in calf thymus DNA induced by capsaicin treated with CYP1A2

The quantity of 8-oxodG was measured utilizing a modification of the method described by Kasai et al. [37]. Standard reaction mixtures containing capsaicin, 0.25 nM CYP1A2, 0.05 nM NADPH–CYP reductase and NADPH generating system (200 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. Calf thymus DNA (100 μM/base) and 20 μM $CuCl_2$ were added to the mixtures, followed by the incubation for 1 h at 37°C. After ethanol precipitation, the DNA was digested into the nucleosides with nuclease P1 and CIP, and analyzed by HPLC–ECD, as described previously [38].

Results

DNA damage induced by capsaicin treated with various CYP isozyme in the presence of metal ion

Capsaicin treated with CYP1A2 or 2D6 induced DNA damage in the presence of Cu(II), whereas

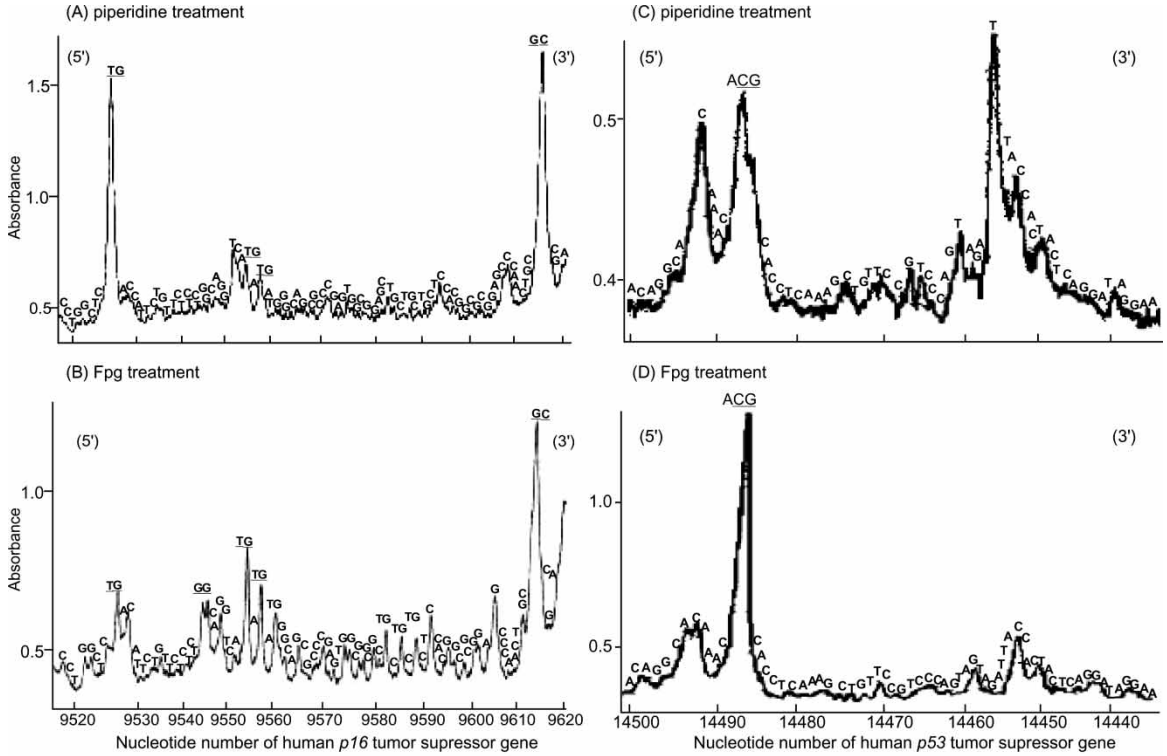

Figure 3. Site specificity of DNA damage induced by capsaicin treated with CYP1A2 in the presence of Cu(II). The reaction mixtures containing 10 μM capsaicin, 0.25 nM CYP1A2, 0.05 nM NADPH–CYP reductase and NADPH-generating system (200 μM $NADP^+$, 500 μM G-6-P, 0.07 units G-6-PDH and 500 μM $MgCl_2$) in 200 μl of 10 mM sodiumphosphate buffer (pH 7.8) containing 5 μM DTPA were preincubated for 2 h at 37°C. After preincubation, ^{32}P-5′-end-labeled 309-bp DNA fragment obtained from the *p16* tumor suppressor gene (A and B) or 443-bp DNA fragment obtained from the *p53* tumor suppressor gene (C and D), calf thymus DNA (20 μM/base) and 20 μM $CuCl_2$ were added to the preincubated mixtures, followed by the incubation for 1 h at 37°C. Following piperidine or Fpg treatment, the DNA fragments were analyzed as described in Materials and Methods. 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.

1A1-, 2C9- or 2E1-treated capsaicin did not cause DNA damage under the conditions used (Figure 1). CYP1A2-treated capsaicin induced Cu(II)-mediated DNA damage more efficiently than 2D6-treated capsaicin. Capsaicin treated with CYP1A2 induced DNA damage in a dose-dependent manner (data not shown). The DNA damage was enhanced by piperidine treatment, suggesting that CYP1A2-treated capsaicin caused not only DNA strand breakage but also base modification (data not shown). Without CYP treatment, capsaicin did not induce DNA damage (Figure 1). In addition, CYP1A2-treated capsaicin did not induce DNA damage in the presence of other metal ions, including Co(II), Ni(II), Mn(II), Mn(III), Fe(II), Fe(III) or Fe(III)EDTA (data not shown).

Effects of scavengers and a metal chelator on DNA damage induced by capsaicin treated with CYP1A2

Figure 2 shows the effects of scavengers and a metal chelator on Cu(II)-mediated DNA damage induced by capsaicin in the presence of CYP1A2. Catalase and bathocuproine, a Cu(I) chelator, inhibited DNA damage, suggesting the involvement of H_2O_2 and Cu(I). SOD and hydroxyl radical (·OH) scavengers, such as ethanol, mannitol, sodium formate and DMSO, showed little or no inhibitory effect on the DNA damages. Methional, which is capable of scavenging both ·OH and reactive oxygen species with weaker reactivity [39,40], inhibited the DNA damage.

Site specificity of DNA damage induced by capsaicin treated with CYP1A2

The patterns of DNA damage induced by CYP1A2-treated capsaicin in the presence of Cu(II) were determined by the Maxam–Gilbert procedure [36]. An autoradiogram was obtained and scanned with a laser densitometer to measure relative intensity of DNA damage in the human *p16* tumor suppressor gene (Figure 3A and B) and the human *p53* tumor suppressor gene (Figure 3C and D). CYP1A2-treated capsaicin induced DNA damage frequently at T of 5′-TG-3′ and C of 5′-GC-3′ with a piperidine treatment (Figure 3A). With Fpg treatment, the DNA cleavage occurred mainly at G-residues,

especially of 5′-TG-3′, 5′-GC-3′ and GG sequences (Figure 3B). Furthermore, CYP1A2-treated capsaicin caused piperidine labile and Fpg-sensitive lesions at CG in the 5′-ACG-3′ sequence, the complementary sequence to codon 273 (a known hotspot) in exon 8 of the *p53* gene [41,42] (Figure 3C and D). Thus, the treated capsaicin can cause double base lesions at 5′-TG-3′, 5′-GC-3′ and 5′-ACG-3′ sequences.

Formation of 8-oxodG in calf thymus DNA induced by capsaicin treated with CYP1A2 in the presence of Cu(II)

By using an HPLC-ECD, we measured 8-oxodG content in calf thymus DNA treated with capsaicin in the presence of Cu(II) and CYP1A2. CYP1A2-treated capsaicin induced an increase of 8-oxodG formation in a dose-dependent manner (Figure 4). No significant increase was observed in DNA treated with heat-inactivated CYP1A2 or NADPH–CYP reductase only in the presence of NADPH-generating system. In addition, CYP1A2-treated capsaicin did not induce 8-oxodG formation in the absence of Cu(II) (data not shown).

Discussion

The present study demonstrated that capsaicin treated with CYP1A2 efficiently induced DNA damage in the presence of Cu(II). CYP1A2-treated capsaicin caused piperidine-labile lesions at T of 5′-TG-3′ sequence and C of 5′-GC-3′ sequence. The treated capsaicin caused DNA damage mainly at G of 5′-TG-3′, 5′-GC-3′ and GG sequences in DNA fragments treated with Fpg protein, which catalyzes the excision of piperidine-resistant 8-oxodG [33–35]. These results suggest that capsaicin treated with CYP1A2 efficiently induces formation of 8-oxodG, which might lead to mutation (G:C → T:A transversion) through the misreplication of DNA [33,43,44], adjacent to piperidine-labile thymine lesions. Relevantly, it has been reported that reactive oxygen species induce double-base lesions at the 5′-TG-3′ sequence [45,46]. Interestingly, CYP1A2-treated capsaicin induced double-base lesions at CG of the 5′-ACG-3′ sequence complementary to codon 273, a well-known hotspot of the *p53* gene [41,42]. Since cluster DNA damage is difficult to repair [47], such double-base lesions appear to play an important role in capsaicin-induced carcinogenesis. Previously, we demonstrated that CYP-treated eugenol and melatonin, which have potential carcinogenicity, can cause double base lesions [20,48].

The result of site specificity of DNA damage induced by CYP1A2-treated capsaicin supports the involvement of reactive species other than ·OH because ·OH causes DNA damage at any nucleotides with little site specificity [49,50]. In order to clarify what kinds of ROS cause the site-specific DNA damage, we examined the effects of various scavengers on the DNA damage. Typical ·OH scavengers showed little or no inhibitory effect on the DNA damage, whereas methional inhibited it. Methional scavenges not only ·OH but also a variety of reactive species other than ·OH [39,40]. The inhibitory effects of bathocuproine and catalase on Cu(II)-mediated DNA damage indicate that Cu(I) and H_2O_2 have important roles in the production of the active species responsible for causing DNA damage. On the basis of these data, we propose a possible mechanism by which capsaicin induces Cu(II)-mediated DNA damage (Figure 5). Capsaicin is metabolized to demethyl capsaicin through CYP1A2-catalyzed *o*-demethylation [12]. It is reported that CYP1A2 and 2D6 can catalyze some *o*-demethylation reactions [51–53]. Demethyl capsaicin is then autoxidized into the semiquinone radical, which is further oxidized to the corresponding *o*-quinone form. Cu(II) is reduced to Cu(I) during the autoxidation, and O_2^- is concomitantly generated, followed by dismutation to H_2O_2. H_2O_2 interacts with Cu(I) to form the Cu(I)-hydroperoxo complex such as Cu(I)OOH, capable of inducing DNA damage [54]. Copper has been found in the nucleus in close association with chromosomes and DNA bases, where it has physiological functions in maintaining DNA structure [55]. Therefore, copper ions and molecular oxygen may play significant roles in the mechanism of capsaicin metabolites-mediated DNA damage *in vivo*.

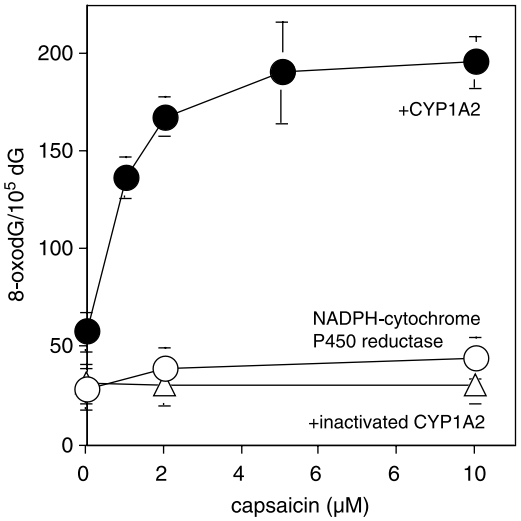

Figure 4. Formation of 8-oxodG induced by capsaicin treated with CYP1A2 in the presence of Cu(II). Standard reaction mixtures containing capsaicin, 0.25 nM CYP1A2, 0.05 nM NADPH–CYP reductase and NADPH-generating system (200 μM $NADP^+$, 500 μM G-6-P, 0.07 units G-6-PDH and 500 μM $MgCl_2$) in 400 μl of 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated for 2 h at 37°C. Calf thymus DNA (100 μM/base) and 20 μM $CuCl_2$ were added to the preincubated mixtures, followed by the incubation for 1 h at 37°C. After ethanol precipitation, the DNA was subjected to enzyme digestion and analyzed by HPLC-ECD as described under Materials and Methods. Data represent the means ± SE of four independent experiments. In a certain experiment, CYP1A2 was inactivated by 90°C for 10 min and quickly chilled before incubation.

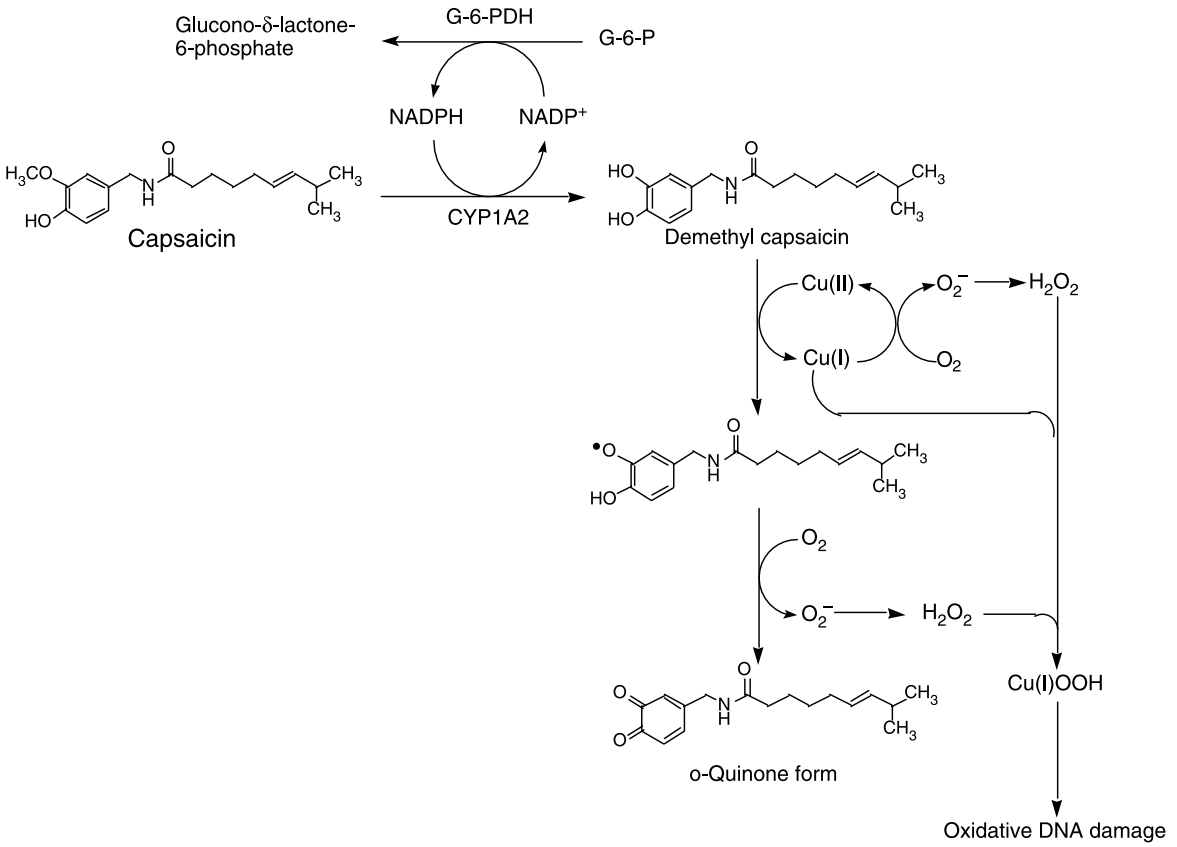

Figure 5. A possible mechanism for Cu(II)-mediated DNA damage induced by capsaicin treated with CYP1A2 in the presence of Cu(II).

Capsaicin has been variously reported as carcinogenic, co-carcinogenic and anti-carcinogenic [3]. Capsaicin plays paradoxical roles, acting as "double-edged sword." In this study, we demonstrated that CYP1A2-treated capsaicin generated H_2O_2 to induce oxidative DNA damage including 8-oxodG, which plays critical roles in carcinogenesis [54]. In addition, it is reported that CYP2D6 catalyzes not only *o*-demethylation reactions but also the *N*-dehydrogenation and ring oxygenation of capsaicin [12]. Thus, we considered that CYP2D6-treated capsaicin induced Cu(II)-mediated DNA damage less efficiently than 1A2-treated capsaicin. Demethyl capsaicin, a catechol metabolite of capsaicin, plays an important role in carcinogenic process caused by capsaicin. Relevantly, catechol has also been shown to have strong promotion activity [56,57] and induce glandular stomach tumors to rodent [58–60]. Previously, we demonstrated that oxidative DNA damage by catechol through the generation of H_2O_2 plays an important role in the carcinogenic process of catechol and benzene [61,62]. In addition, catechol-type compounds, such as carcinogenic catechol estrogens and flavonoids, also induced oxidative DNA damage through H_2O_2 generation [63–65]. On the other hand, it is reported that capsaicin induces mitochondrial dysfunction in vanilloid receptor subtype 1 (VR-1)-expressing cells, leading to apoptotic cell death [66,67]. Therefore, it is considered that various mechanisms including oxidative DNA damage induced by ROS are involved in the expression of carcinogenicity of capsaicin. Finally, further studies on safety should be required when capsaicin is used for chemo-prevention.

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