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Inhibitory Effect of Chlorophyllin on Mutagenicity of Electrophilic 6-sulfooxymethylbenzo[a]pyrene

Young-Sik Cho, Sun-Taek Hong, Woo-hee Chung and **An-Sik Chung**

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea

The inhibitory effect of chlorophyllin was investigated against mutagenicity of 6-sulfooxymethylbenzo[a]pyrene (SMBP) in *S. Typhimurium* TA100 and in Chinese hamster lung fibroblast (V79) cells. Chlorophyllin (CHL) was quite effective in reducing mutagenicity for SMBP in dose-dependent manner up to 50 nmol in *S. Typhimurium* TA100 and 12.5 μM in V79 cells. A similar but less effective prevention of CHL was indicated in the mammalian and bacterial assays with 6-hydroxymethylbenzo[a]pyrene (HMBP), the proximate precursor of SMBP. Intracellular accumulation of SMBP was decreased by CHL in a dose-dependent manner and the treatment with 10 μM CHL reduced the accumulation of SMBP to almost 40% of control value. A dramatic decrease in DNA adducts were also obtained by the treatment of CHL in dose- dependent manner. CHL at a 50 μM concentration reduced the DNA-SMBP adducts to 16% of control level with SMBP-treated group. SMBP may be more stable in aqueous phase in the presence of CHL than in the absence of CHL. Furthermore, CHL decreased the amount of eluted HMBP to 50% of control value in HPLC profiles. These results suggest that the chemopreventive effect of CHL against the mutagenicity of SMBP are related to the complex formation between CHL and SMBP. The hydroxysteroid sulfotransferase responsible for activation of HMBP to SMBP was suppressed by the increased concentration of CHL. Besides adducts formation, CHL may have an effect on production of SMBP and detoxifying systems.

Key Words: Chemoprevention, Chlorophyllin, 6-sulfooxymethylbenzo[a]pyrene, Mutagenicity

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INTRODUCTION

There has been a great interest in the identification of dietary constituents which can prevent cancer caused by chemical carcinogenesis. One of such dietary constituents is chlorophyll, which is abundant in green vegetables. Antimutagenic activities associated with certain vegetable extracts have been closely related to chlorophyll contents in bacterial system[1-3]. Furthermore, chlorophyll and its water soluble derivative chlorophyllin (CHL) have been demonstrated to reduce or prevent mutagenicity of several chemicals such as benzo[a]pyrene, 2-aminoanthracene, 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-3-methylimidazo[4,5-*f*] quinoline (IQ), aflatoxin B_1 and some heterocyclic amines in cooked foods[4-7]. It has also been reported that CHL is more effective in reducing the mutagenicity than other antioxidants such as β-carotene, ascorbic acid and α-tocopherol[8]. CHL has been practically used to treat a number of human conditions without any apparent adverse effects. It has been proved to be effective in not only reducing body, fecal and urinary odors of geriatric patients[9] but also accelerating wound healing[10]. The compound has also been utilized for the management of the calcium oxalate stone disease[11].

Besides versatile chemopreventive activity against many structurally diverse promutagens and procarcinogens, CHL is also effective in suppressing activities of certain direct-acting mutagens, such as reactive epoxides of aflatoxin B_1[12], styrene[13], benzo[a]pyrene[14], and vinyl carbamate[15]. Furthermore, CHL has shown its dramatic chemopreventive effect against SMBP, an ultimate electrophilic carcinogen, in V79 cell system.

In the present study, we investigated not only the potential antimutagenic activity of CHL but also the mechanism of its chemopreventive activity against SMBP which is an ultimate electrophilic and carcinogenic metabolite of HMBP. SMBP bears a good leaving group, viz, sulfate group, and hence generates a highly reactive carbonium ion capable of interacting with DNA and other macromolecules.

MATERIALS AND METHODS

1) Cells and chemicals

Dulbecco's modified Eagle's medium (DME), 6-thioguanine (6-TG), and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St.Louis, MO). CHL was purchased from Aldrich Chemical Co. (Milwaukee, WI). SMBP and HMBP were prepared as described previously[16]. Fetal bovine serum was purchased from Gibco BRL (Grand Island, NY). Other reagents used were of analytical grade.

2) Bacterial mutagenicity assays

The procedure for mutagenicity test using bacteria has been described in detail[17,18]. The studies of mutagenicity were performed based on the modification of the standard Ames method[19]. A relatively high number ($3 \sim 4\times10^9$) of bacteria were used to increase the sensitivity of assays. HMBP (7.2 nmol) was incubated at 37°C for 60 min in a final volume of 1.1 ml of 0.1 M KH_2PO_4-Na_2HPO_4 buffer (pH 7.4) containing various concentrations of CHL in the presence of S105 prepared before and 3'-phosphoadenosine-5'-phosphosulfate (PAPS)-generating system (5 mM ATP, 5 mM sodium sulphate, 3 mM magnesium chloride and 0.1 mM EDTA). SMBP (1 nmol) dissolved in DMSO was incubated without the cytosolic fraction and the PAPS-generating system. After incubation, the mixtures were diluted with soft agar, poured onto a hard agar plate, and further incubated for 48 hr to allow the growth of His^+ revertant colonies.

3) Mammalian cytotoxicity assays

Chinese hamster lung fibroblast (V79) cells (supplied from the Korea Research Institute of Chemical Technology, Taejeon, Republic of Korea) were grown in monolayer culture in DME medium containing 5% heat inactivated (56°C for 30 min) fetal bovine serum at 37℃ in an incubator humidified to 95~100% in an atmosphere of 5% CO_2 in air. Subculture was performed by use of 0.05% trypsin solution (1:250; Gibco, Grand Island, NY) for cell detachment, and cell numbers were determined by use of a haemacytometer. Cytotoxicity was determined by the plating efficiency and the cell growth assay. For determining the cloning efficiency, SMBP was incubated with semiconfluent V79 cells in the presence of CHL for 1 hour, followed by reseeding 200~2000 cells in 60×10 mm dishes. Inhibitory effect of CHL on the cytotoxicity of SMBP was evaluated by counting the colony on the 7th day after seeding. For the determination of cell growth, V79 cells (5×10^5) were then inoculated into 60 mm tissue culture dishes and allowed to attach. After 6 hr, the original medium was removed, washed, replaced with medium containing various concentrations of CHL and 2.5 μM of SMBP. The incubation conditions were for 1hr at 37°C. Cells were then allowed to grow for 48 hr and counted with haemacytometer.

4) Mammalian mutation assays

Mutations at hypoxanthine:guanine phosphoribosyltrans-

ferase (HGPRT) loci were measured by the resistance to 6-thioguanine (6-TG). The mutagenicity tests were carried out as described previously with some modifications[20~22]. Exponentially growing cells were exposed to HMBP (90 μM) or SMBP (2.5 μM) for 1 hr in the presence or absence of CHL, followed by subculture. For selecting the 6-TG resistants, 0.2 mM of 6-TG was added after the cells were subcultured 3 times every other day. Cloning efficiency was determined by counting the number of colonies 7~8 days after seeding 200~2000 cells per petri dish while the cells were subcultured to select mutants. Mutagenicity for 6-TG resistance was expressed as mutants/10^6 survivors, and was corrected for the cloning efficiency.

5) Determination of intracellular accumulation of SMBP

Cultured V79 cells were exposed to [^{3}H-CH_2]-SMBP (3 μM; 12.0 mCi/mmol) in medium containing various concentrations of CHL (0, 2.5, 5, 10 and 25 μM) for 30 min. SMBP-treated V79 cells were washed with PBS and harvested after treatment with trypsin. Collected V79 cells were lysed with buffer containing 0.5% SDS. Aliquots of the lysates were taken to evaluate SMBP accumulation which was measured by radioactivity.

6) Covalent binding of SMBP and HMBP to DNA

For the determination of intracellular covalent binding of SMBP to DNA, confluent V79 cells were subjected to the treatment with SMBP (12.5 mM) for 1 hr at 37°C. The cells were harvested by centrifugation at 300 g for 10 min and then treated with lysis buffer (Tris buffer containing 0.5% SDS) for 2 hr at 37°C. The lysates were extracted with phenol. DNA was precipitated by 2 vol of ethanol. DNA pellets were rinsed with a series of organic solvent. The adducts were detected and quantitated by their fluorescence at 418 nm (excitation at 360 nm) based on the calibration curve obtained with HMBP and various amounts of calf thymus DNA.

For the determination of covalent binding of HMBP to calf thymus DNA, the incubation was performed at 37°C for 90 min in a final volume of 500 μl containing 0.1 M Tris-HCl (pH 7.4), 500 μg calf thymus DNA, 10 nmol HMBP, 50 μl of liver cytosol (~50 mg/ml) and PAPS-generating system[23]. The cytosolic fraction and PAPS-generating system were omitted from the mixture when SMBP (1 nmol) dissolved in 10 μl of DMSO was used instead of HMBP. These incubation mixtures were then tested together with various concentrations of CHL.

7) Assay of hydroxysteroid sulfotransferase

Standard assay of the hydroxysteroid sulfotransferase was performed toward dehydroepiandrosterone (DHEA) according to the method mentioned previously, using PAPS-generating system instead of PAPS[23]. Enzymatic reactions were started by the addition of 50 nmol DHEA to incubation mixture containing various doses of CHL, rat liver cytosol and PAPS-generating system and then were continued for 1 hr at 37°C. The sulfate formed was extracted as hydrophobic methylene blue complexes with chloroform and was measured by absorbance at 651 nm.

8) Analysis of complex formation between CHL and SMBP with HPLC and solvent extraction methods

The HPLC system utilized was equipped with Waters a model 510 pump and a model 486 tunable absorbance detector. The separation of SMBP products was performed using 4.6×250 mm Ultremex 5C18 column kept at room temperature with a 15 min linear gradient of 80% methanol in water to 100% methanol. SMBP (0.1 mM) was treated to incubation mixture containing CHL (1 or 5 mM). For analysis of profiles formed from SMBP the eluents were monitored by absorbance of its chromophore at 298 nm.

Solvent extraction experiments were performed by following precedures. Aliquot (20 μl) of [^{3}H-CH_2]SMBP (12.0 mCi/mmol) was applied to 500 μl of the reaction mixture containing various concentrations of CHL, and the reaction was continued for 30 min at 37°C. Reaction was terminated by chilling, and then reactants were partitioned with benzene 4 times to separate two phases. Partition extent of SMBP into each phase was determined by the scintillating counter.

RESULTS

As an initial approach to assess the possible protective effect of CHL against SMBP, the Ames-His^+ reversion test was performed using *S. typhimurium* strain TA100. Fig. 1 illustrates the dose-dependent inhibition of HMBP- and SMBP-induced mutagenicity by CHL. SMBP was a strong direct mutagen toward TA100, which has been originally developed to detect base-pair substitution types. CHL inhibited remarkably mutagenic activity of SMBP in a dose-related manner up to 50 nmol. The mutations induced by HMBP were also inhibited by CHL, but the inhibition was less effective compared to the inhibition of SMBP- induced mutations.

The protective activity of CHL against SMBP-induced cytotoxicity was confirmed in the mammalian cytotoxicity assays. SMBP was highly cytotoxic toward Chinese hamster V79 cells. The attenuation of SMBP-induced cytotoxicity

by CHL was evaluated in terms of plating efficiency and cell growth. Recovery of the survival rate was closely related to the CHL concentrations within the range used (12.5 μM). The relative plating efficiency at 2.5 μM SMBP was 28.5% of untreated- control level and its cytotoxic effect was completely suppressed by CHL at concentrations above 12.5 μM. The cell growth was inhibited up to 28.2% at the highest dose of SMBP and the growth inhibition induced by SMBP was abolished by CHL in a concentration-dependent manner (Fig. 2).

Both HMBP and SMBP induced mutations at the target gene which encodes HGPRT in V79 cells (Fig. 3). SMBP at 2.5 μM and HMBP at 90 μM induced 240 mutants/10^6 and 110 mutants/10^6, respectively. The decrease in the mutation frequency of SMBP by CHL was inversely related with the plating efficiency in the concentrations ranging from 2.5 to 25 μM. The Inhibitory effects of CHL against mutagenicity of HMBP were less dramatic as compared with those observed for SMBP. A similar response was also seen for HMBP in the Ames assay as described previously.

Inhibitory effect of CHL on intracellular accumulation of SMBP in V79 cells is shown in Fig. 4. SMBP was accumulated progressively and reached plateau in just 30 min (data not shown). Intracellular accumulation of SMBP was also decreased by CHL in a dose-dependent manner and SMBP accumulation was reduced to almost 40% by the treatment with 10 μM CHL.

The dose-response of CHL against DNA adducts formed from SMBP-treated V79 cells is shown in Fig. 5. When SMBP was coincubated with CHL, a significant decrease in DNA adducts was obtained. CHL at a 50 μM concentration reduced the DNA-SMBP adducts to 16% of the level observed in the SMBP-treated group.

Furthermore, CHL inhibited SMBP- or HMBP- mediated adduct formation in the calf thymus DNA (Fig. 6). The DNA adducts produced by SMBP and HMBP were decreased to 80% and 40%, respectively, by the treatment of 50 nmol CHL. CHL was more effective on HMBP- mediated DNA adduct than on that of SMBP. The results suggest that CHL inactivates the hydroxysteroid sulfotransferase pertaining to the activation of HMBP. In fact, sulfotransferase responsible for HMBP activation was suppressed in a dose-dependent manner by CHL (Fig. 7).

The differential partitioning of SMBP hydrolysates into organic phase is shown in Fig. 8. [^{3}H-CH^2]SMBP in incubation mixture containing CHL is not readily extracted into organic phase. As the concentration of CHL increased, SMBP in organic phase decreased while a considerable amount of SMBP still remained in aqueous phase. This result indicates that SMBP is more stable with CHL than without CHL since half life of SMBP is very short in aqueous solution. This result may come from the complex formation between CHL and SMBP. The products formed from CHL and SMBP were also shown by HPLC profiles (Fig. 9). The peaks appeared at 3 and 10.5 min were identified as CHL and HMBP, respectively. CHL did not interfere with the detection of HMBP profile, which can be easily identified. Addition of 1 mM CHL decreased the amount of eluted HMBP to 50% of control value level and treatment with 5 mM CHL decreased more than that of 1 mM CHL. This result also suggests that SMBP is more stable due to complex formation with CHL.

DISCUSSION

In the present study, CHL was found to be highly effective in decreasing the mutagenicity of SMBP in both *S. typhimurium* TA100 and in the V79 cells culture system. The antimutagenicity of CHL against SMBP suggests that it functions directly toward metagen. A similar type of dose-response for CHL was shown in the mammalian and bacterial mutagenicity assays with HMBP but the response was weak and less consistent. In the presence of CHL, there was an enhancement of cell survival and a reduction of mutagenicity induced by both hydrocarbons. In comparison, approximately 5-fold higher concentrations of CHL were required to eliminate completely the mutagenicity of SMBP. CHL decreased the cytotoxicity and mutagenicity of SMBP in the V79 cells only when CHL was incubated with SMBP at the same time.

The mechanisms of the antimutagenic action of CHL toward many different types of carcinogens remains unclear yet, but several postulations have been suggested. One of the most possible mechanisms has been ascribed to its ability to form complexes with aromatic hydrocarbon carcinogens or mutagens. The inhibitory effect of CHL on DNA-carcinogen adduct formation has been extensively investigated by Dashwood and his associates[12,24-26]. It is known that the antimutagenic activity of CHL toward IQ is caused by diminished IQ-DNA binding[26]. The other studies have shown that protection by CHL against genotoxicity of Trp-P-2 in *Drosophila* might be ascribed to complex formation between CHL and the heterocyclic amine, thereby reducing the bioavailability of the carcinogen[27]. CHL was also reported to interact directly with ultimate electrophilic mutagenic epoxides such as styrene oxide[13], aflatoxin B_1-8,9-epoxide[12], benzo[a]pyrene-7,8-dihydrodiol -9,10-epoxide[14], and vinyl carbamate epoxide[15]. In our studies, the reaction products of CHL and SMBP were analyzed indirectly by measuring the hydrolysis product, HMBP, using reverse-phase HPLC and

solvent extraction methods. It has been also demonstrated that PAH can form complexes with CHL, chlorophyll and phthalocyanine trisulfate[27-29]. Each compound resembles structurely each other and has a pyrrole ring with large π system. The major complex formation may be derived from π-π or face to face hydrophobic interaction between the planar aromatic surface of these molecules and the pyrrole ring structure.

It has been suggested that some compounds are related to the ability to suppress the mutagen activation system or to induce the drug detoxification materials[30,31]. It has been demonstrated that exposure of CHL decreases activities of aminopyrine N-demethylase and aniline hydroxylase and lowers content of cytochrome P-450 in liver microsomes of rats. In our study, CHL was quite effective to reduce the activity of hydroxysteroid sulfotransferase which is essential to produce SMBP from parent compound, HMBP. Other study has shown that CHL significantly elevates glutathione S- transferase and SH level in the liver and skin tissue of rats[32]. The above observations suggest that CHL is also related to detoxifying systems and to specific reduction of hydroxysteroid sulfotransferase activity.

The detection of DNA adducts in animal cells exposed to a particular carcinogen has been used as a surrogate for the genotoxicity assays. It has been shown that the level of DNA adduct is closely related to mutagenicity of a given carcinogen[33,34]. Therefore, we examined the effect of CHL on the intracellular accumulation and DNA adducts of SMBP in the V79 cells. When SMBP was coincubated with CHL in the medium, dose-dependent decrease in DNA adduct was observed with increased concentration of CHL. CHL at 50 μM concentration reduced DNA adducts more than 80% of control group. However, the uptake of SMBP in the cells was reduced to almost 40% of control by the treatment of 10 μM CHL. The reduced uptake of SMBP consequently decreased DNA adducts, but the reduction of DNA adducts were more dramatic than that of the uptake of SMBP in the V79 cells by the treatment of CHL. The inhibition of the hydroxysteroid sulfotransferase activity and the detoxification of SMBP by GST and SH group may be involved in the additional role in antimutagenicity of CHL.

Further studies are required to examine the antimutagenic and anticarcinogenic mechanism of CHL in terms of complex formation, metabolizing enzymes, detoxifying enzymes and antioxidant activity *in vivo* by animal studies.

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Fig. 1. Effect of CHL on the mutagenicities of HMBP and SMBP in *Salmonella typhimurium* TA100. Assays were performed at the indicated amounts of HMBP or SMBP as described in Materials and Methods. Data are means±SD for triplicate plates.

Fig. 2. Inhibitory effect of CHL against SMBP-mediated cytotoxicity in cultured Chinese hamster V79 cells. The absolute plating efficiency of the cell in the absence of SMBP was 80%. SMBP (2.5 μM) was incubated with CHL-preincubated V79 cells. Data are means±SD for triplicate plates.

Fig. 3. Inhibitory effect of CHL on mutagenesis induced by SMBP (A) and HMBP (B). Treatments of SMBP and HMBP were 90 μM and 2.5 μM, respectively. Mutagenicity was measured by the resistance to 6-TG which was expressed as mutants/10^6 survivors. Data are means±SD for triplicate plates.

Fig. 4. Inhibition of intracellular accumulation of [^{3}H-CH_2]-SMBP in V79 cells by CHL. Exponentially growing V79 cells were incubated for 30 min with SMBP (3 μM; 12 mCi/mmol) and various concentrations of CHL. After incubation, aliquots of the cell suspension were taken and the amount of radioactivity in the cells was measured. Each point represents the mean value of two determinations.

Fig. 5. Inhibitions of cellular DNA adduct levels in V79 cells treated with 12.5 μM of SMBP by CHL. DNA was isolated from SMBP-treated cells as described under Materials and Methods.

Fig. 6. Effect of CHL on covalent binding of SMBP and HMBP to calf thymus DNA. SMBP (1 nmol) or HMBP (10 nmol) was transferred to incubation mixture containing various CHL and DNA, and the reaction was continued for 20 minutes at 37℃. DNA was isolated and washed as described in Materials and Methods. SMBP was incubated without any spikes whereas HMBP was active in the presence of rat liver cytosol and PAPS-generating system.

Fig. 7. Effect of CHL on hydroxysteroid sulfotransferase activity. The activities were measured by the determination of enzymatic formation of sulfate ester for DHEA as described in Materials and Methods.

Fig. 8. Differential partition of SMBP into either organic or aqueous phase as a function of CHL dose. Reaction mixtures of [^{3}H-CH_2]SMBP and various concentrations of CHL were incubated for 20 minutes and then were subjected to extraction with benzene to separate phase.

Fig. 9. HPLC analysis of products formed from SMBP in aqueous solution in the absence (A) or presence of 1 mM (B) or 5 mM (C) CHL. Chromatography was performed as described in Materials and Methods.