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Metabolism of Safrole, a Betel Quid Component, and its Role in the Development of Oral Cancer in Taiwan

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ABSTRACT *: Chewing betel quid is associated with an increased risk of oral cancer. The betel quid chewed in Taiwan includes the inflorescence of Piper betle, which contains high concentrations of safrole (15 mg/fresh weight). Piper betle leaf is also used in betel quid; however, the concentration of safrole in betel leaf has not been documented. Chewing betel quid may contribute to safrole exposure in man (420 mm in saliva). Using a ^{32}P-postlabeling method, we have recently demonstrated the presence of stable safrole-like DNA adducts in human oral tissues following betel quid chewing. Safrole is a rodent hepatocarcinogen, and the real nature of safrole-DNA adducts in human tissues beside oral has not been elucidated. In this paper, we tested the safrole DNA adducts forming potential in human hepatic and oral derived cells by the ^{32}P-postlabeling technique. The results suggest that oral cancer derived cell OC-2 alone is not able to form safrole-DNA adduct. However, safrole DNA adducts can be detected following 1'-hydroxysafrole, a proximate safrole metabolite, treatment. In addition, pretreament of cytochrome P450 inducers also enhanced the formation of previously undetectable safrole DNA adducts. This finding couples with our previous results suggest that oral may serve as a target tissue for safrole, and safrole may be involved in oral carcinogenesis.*

Key Words *: Safrole, oral cancer, betel quid*

I. INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the fifth leading cause of male cancer mortality in Taiwan (Anonymous, 1998). Epidemiological studies demonstrated that betel quid (BQ) chewing is associated with the development of OSCC (Ko *et al.*, 1995; Lu *et al.*, 1996). The composition of BQ varies according to different geographic locations. In Taiwan, BQ is composed of areca nut, *Piper betle* inflorescence or leaf and lime paste. *Piper betle* inflorescence is added to BQ for its aromatic flavor and is only used in Taiwan and Papua New Guinea (Thomas and MacLennan, 1992). *Piper betle* inflorescence contains a high concentration (15 mg/g) of safrole (Hwang *et al.*, 1992). Consequently, chewing BQ containing *Piper betle* inflorescence may contribute to safrole exposure (420 μM in saliva during chewing) (Wang and Hwang, 1993). On the other hand, the concentration of safrole in *Piper betle* leaf has not been documented.

Safrole is a weak rodent hepatocarcinogen as classified by IARC (IARC, 1976). The carcinogenicity of safrole is mediated through 1'-hydroxysafrole formation, followed by sulfonation to an unstable sulfuric acid ester that reacts to form stable safrole-DNA adducts (Miller and Miller, 1983). These safrole-DNA adducts can be efficiently detected by the ^{32}P-postlabeling technique in rodent liver and other tissues treated with safrole (Randerath *et al.*, 1984; Reddy and Randerath, 1990; Gupta *et al.*, 1993; Daimon *et al.*, 1998). We have recently demonstrated the presence of safrole-like DNA adducts in oral cancer tissue with betel quid chewing history (Chen *et al.*, 1999). However, the real nature of safrole-DNA adducts in human beings has not been elucidated. In this communication, we will first demonstrated the presence of safrole in *Piper betle* inflorescence and leaf, then detect the presence of safrole DNA adducts in human liver and oral cancer derived cells using ^{32}P-postlabeling technique and compare the importance of safrole-

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DNA adducts and the oxidative DNA damage induced 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in rodent liver.

II. MATERIALS AND METHODS

1. Chemicals

Safrole, 2'-deoxyguanosine 3'-monophosphate (dGMP), 2'-deoxyadenosine 3'-monophosphate (dAMP), 3'-phosphoadenosine 5'-phosphosulfate (PAPS), micrococcal nuclease, spleen phosphodiesterase, nuclease P1, and potato apyrase were purchased from Sigma Chemical Co. (St. Louis, MO). RNase A and RNase T1 were from Boehringer Mannheim (Mannheim, Germany). T4 polynucleotide kinase was purchased from BioLabs Inc. (Beverly, MA). [γ-^{32}P] ATP (6000 Ci/mmol) was from NEN Life Science Products, Inc. (Boston, MA). 1'-OH-safrole was synthesized from vinyl bromide and piperonal as described previously (Borchert *et al.*, 1973).

2. Determination of Safrole by HPLC

Fresh *Piper betle* inflorescence and leaf, purchased from local market were weight and minced in 80% acetone. The homogenate were separated by centrifugation then followed by filtration. The 0.22 μm filtrates were analyzed by HPLC according to the published method (Carlson and Thompson, 1997).

3. Treatment of Cells

Human hepatoblastoma HepG2 and oral cancer OC-2 cells were grown as monolayer cultures in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, USA). Only cells in exponential growth were used for the experiments. The cells were seeded in complete medium, changed to medium without FBS immediately before drug treatment, and harvested at designated times.

4. Animals and Treatment

Male ICR mice (20~25 g) purchased from the National Animal Breeding Center was housed in a temperature and humidity controlled room with 12 hr light-dark cycles. Feed (Purina Lab Chow, USA) and tab water was provided ad libitum. Mice were given a single ip injection of safrole (0 and 250 mg/kg) and euthanized at designated times.

5. ^{32}P-Postlabeling of DNA Adducts

DNA adducts were detected by the nuclease P1-enhanced ^{32}P-postlabeling procedure as described by Reddy and Randerath (Reddy and Randerath, 1990). The [γ-^{32}P] ATP-labeled safrole-DNA adducts were resolved on polyethyleneimine (PEI)-cellulose TLC plates (Machery-Nagel, Germany) with two different buffer systems. Development condition A consists of 2.3 M sodium phosphate, pH 6.0 (D1) and 1.8 M lithium formate, 4.25 M urea, pH 3.5 (D2) (bottom to top); 0.36 M LiCl, 0.22 M Tris-HCl, 3.8 M urea, pH 8.0 (D3) and 1.7 M sodium phosphate, pH 6.0 (D4) (left to right). Safrole-DNA adducts were detected by autoradiography and quantified by scintillation counting. Adduct levels were expressed as RAL (relative adduct level) where 1 RAL represents 1 adduct per 10^8 nucleotides. To compare the adducts detected in OSCC tissues with safrole-DNA adducts in 1'-OH-safrole-treated HepG2 cells, the two DNA samples were mixed before enzymatic digestion; and using the above mentioned condition A, the resulting chromatogram was compared with maps derived from individual DNA samples. To further characterize these adducts, a different development condition was used: 1.7 M sodium phosphate, pH 6.0 (D1); 1.9 M lithium formate, 3.8 M urea, pH 3.5 (D2); 0.36 M sodium phosphate, 0.23 M Tris-HCl, pH 8.0 (D3); 1.7 M sodium phosphate, pH 6.0 (D4) [development condition B] (Reddy *et al.*, 1989).

III. RESULTS

By using HPLC equipped with UV detector, safrole has been shown in *Piper betle* inflorescence (14.9 mg safrole per gm wet weight) (Fig. 1B, 2). On the other hand, safrole was not detectable under the same condition in *Piper betle* leaf (Fig. 1C).

By using the ^{32}P-postlabeling technique, safrole-DNA adducts were detected in HepG2 cells (Fig. 2). The levels of safrole DNA adduct 1 increased from 4.6 to 10.6 per 10^8 nucleotides as the incubation time

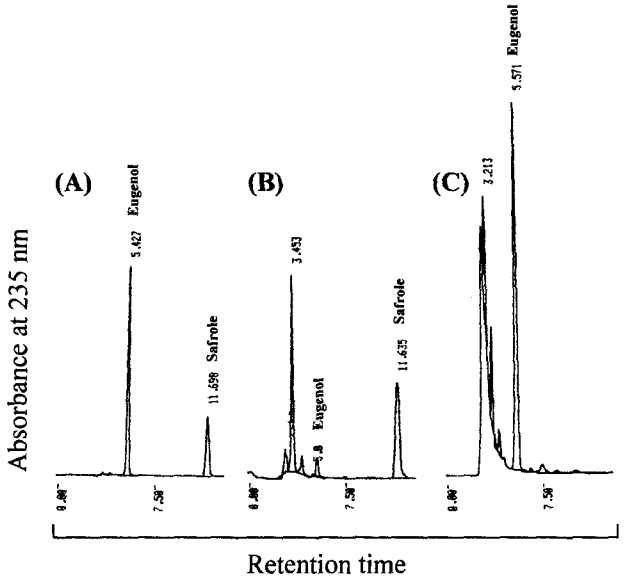

Fig. 1. The chromatography of safrole and eugenol from standard (A); *Piper betle* inflorescence (B); *Piper betle* leaf (C).

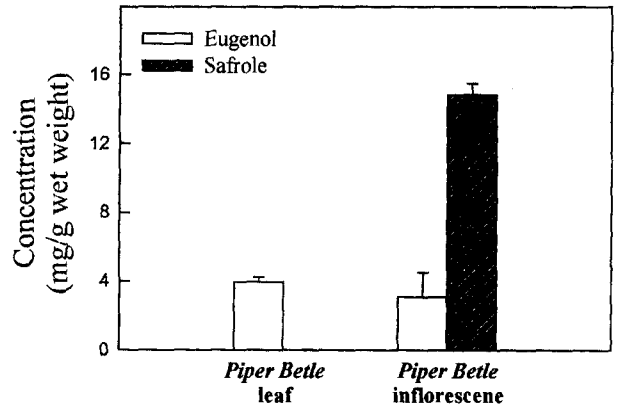

Fig. 2. The concentrations of eugenol and safrole in *Piper betle* inflorescence and *Piper betle* leaf.

for safrole (800 μM) increased from 24 to 48 hrs. Following 3-methylcholanthrene (1 μM) pretreatment for 24 hr, the previously undetected adduct 2 can also be visualized, and the level of adduct 1 was increased further to 17.3 (Table 1). In oral cancer derived OC-2 cells, safrole DNA adducts eluded detection by using the same technique following 800 μM treatment (data not shown). However, safrole-DNA adducts were detected by incubation of 400 μM 1'-hydroxysafrole, the metabolite of safrole, in OC-2 cells. Such treatment induced both adducts 1 and 2, and the levels were much higher than 800 μM safrole formed in HepG2 cells (Table 1).

Table 1. The levels of safrole-DNA adduct when HepG2 cells or mice treated with safrole or 1-hydroxysafrole for indicated times.

HepG2 cells		Safrole-DNA adduct (RAL×10^8)	
		Adduct 1	Adduct 2
Safrole 800 μM	24 hr	4.6	n.d.
Safrole 800 μM	48 hr	10.6	n.d.
3-Methylcholanthrene 1 μM	24 hr		
Safrole 800 μM	24 hr	17.3	2.9
1'-Hydroxysafrole 400 μM	24 hr	153.1±57.3	44.5±22.9
ICR mice			
Safrole 250 μg/kg	24 hr	958.7±128.5	228.3±32.3

Safrole induced significant amount of safrole-DNA adducts in rat liver 24 hrs following 250 mg/kg safrole challenge (Fig. 1 and Table 1).

IV. DISCUSSION

Piper betle inflorescence contains high concentration of safrole (14.9 mg/g wet weight), whereas safrole was not detectable in *Piper betle* leaf. (The sensitivity of the assay is 15 ng/ml.) Therefore, chewing BQ containing betle leaf may not contribute to safrole exposure.

Safrole is a documented rodent hepatocarcinogen. The hepatocarcinogenicity of safrole is mediated through 1'-hydroxysafrole formation, followed by sulfonation to an unstable sulfuric acid ester that reacts to form stable safrole-DNA adducts (Miller and Miller, 1983) (Fig. 5). In this study, we first demonstrated the formation of safrole-DNA adducts in human HepG2 cells by ^{32}P-postlabeling technique. HepG2 cells is famous for its intact phase I and II enzyme systems, and therefore is widely used as a target cells for mutagen screening (Knasmuller *et al.*, 1998). In addition, enzyme inducers are well documented to exert its effects in this cell (Knasmuller *et al.*, 1998). In this study, the effect of 3-methylcholantrene and 1'-hydroxysaforle all added to the safrole-DNA forming potential in human hepatoblastoma-derived HepG2 cells.

In Taiwan, chewing BQ containing *Piper betle* inflorescence will expose oral cavity to high concentration of safrole (420 μM in saliva). Whether oral tissue can metabolize safrole is not known. Our study using oral cancer derived OC-2 cells demonstrated that 800 μM

safrole alone is not able to form safrole-DNA adducts. However, the proximate metabolite of safrole, 1'-hydroxysafrole, induced safrole-DNA adduct 1 and 2 in OC-2 cells. In addition, pretreatment of cytochrome P4501A inducers benzo(a)pyrene and isoniazide revealed the previously undetectable adduct 1 in OC-2 cells (Fig. 4 C, 4D). These observations suggest that oral tissue has less safrole biotransformation enzymes than that in hepatic tissue, but can still form significant amount of safrole-DNA adducts under specific induction conditions. Our recent study correlated with this assumption, which showed small amount of safrole-DNA adducts, ranged from 4.0 to $9.7/10^8$ nucleotides in oral cancer tissues from oral cancer patients with known BQ chewing history (Chen *et al.*, 1999).

Safrole induced 4 DNA adducts on the autoradiogram from rodent liver (Fig. 3A). The profile and location of adduct 1 and 2 are similar to that found from Reddy and Randerath report, and has been identified as N^2 (trans-isosafrole-3'-yl) 2-deoxyguanosine and N^2-(safrole-1'-yl)2'-deoxyganosine, respectively (Phillips *et al.*, 1981). However, the real nature of these adducts need to be further confirmed.

In addition to formation of safrole DNA adducts, safrole can also be biotransformed through the cleavage of the methylenedioxy ring, and form hydroxychavicol (4-allyl-1,2-dihyhydroxybenzene) (Ioannides *et al.*, 1981). Recently, HC has been postulated to form o-quinone through 2-electron oxidation in vitro (Bolton *et al.*, 1994). This redox-active quinone has potential to induce oxidative damages (O'Brien, 1991).

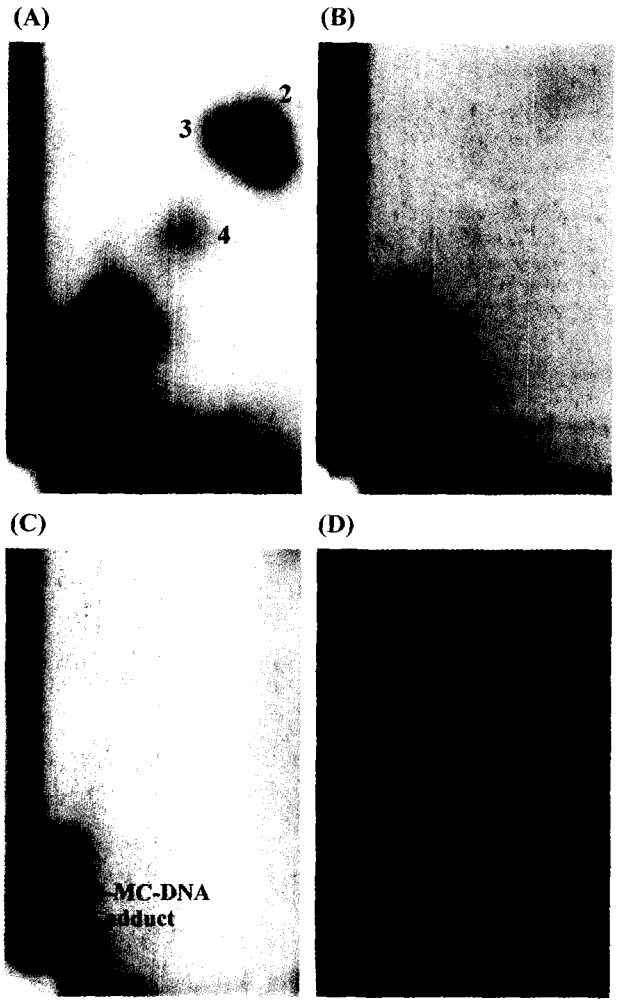

Fig. 3. Autoradiograms of polyethyleneimine (PEI)-cellulose TLC maps of ^{32}P-labeled safrole-DNA adducts. DNA from rats treated with 250 mg/kg of safrole (A); HepG2 cells treated with 800 mM of safrole (B); 1 mM of 3-methylcholanthrene (3-MC) (C); 1 mM of 3-MC pretreatment and 800 mM of safrole (D) for 24 hr. Adducts were visualized by autoradiography employing Kodak Biomas MR imaging film with intensifying screen for 24 hr at -70°C.

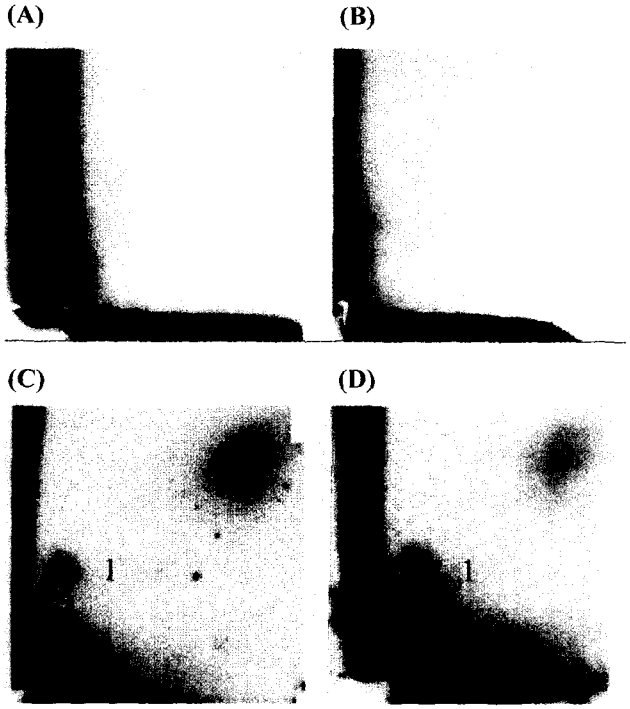

Fig. 4. Autoradiograms of PEI-cellulose TLC maps of ^{32}P-labeled safrole-DNA adducts. DNA from OC2 cells treated with DMSO (A); 400 mM of safrole (B); 10 mM of banzo(a)pyrene [B(a)P] pretreatment and 400 mM of safrole (C); 1 mM of iosniazid (INH) pretreatment and 400 mM of safrole (D) for 24 hr. Adducts were visualized by autoradiography employing Kodak Biomas MR imaging film with intensifying screen for 48 hr at -70°C.

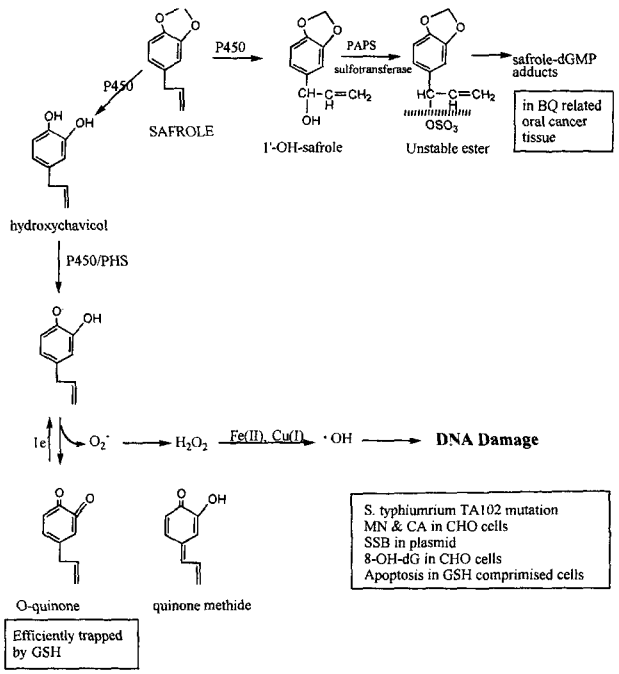

Fig. 5. Proposed metabolic scheme of safrole. Safrole is biotransformed to 1-hydroxysafrole and sulfonated to an unstable ester before stable safrole DNA adducts formation. Safrole can also form oxidative DNA related damages through redox active o-quinone formation. However, the latter part of the reaction can efficiently be trapped by GSH.

Our recent in vitro study supports this hypothesis. We have demonstrated that HC generated reactive oxygen species such as H_2O_2 and participated in the Fenton-type reaction, which in turn resulted in increased revertants in S. typhimurium TA102, and the induction of plasmid DNA strand breaks. HC also induced the formation of oxidative DNA damage, 8-OH-dG, which may be responsible for the chromosome aberration, MN formation and cytotoxicity in CHO-K1 cells (Lee-Chen *et al.*, 1996). However, we have also demonstrated that safrole alone is slightly toxic to metabolically competent HepG2 cells. The cytotoxicity and apoptosis inducing potential increased significantly ($P < 0.05$) when HepG2 cells were pretreated with buthionine sulfoximine (BSO), suggesting that endogenous GSH plays an important role in HC-induced cytotoxic potential (Chen *et al.*, 2000). This phenomenon is also reflected in the in vivo system. We have previously shown that high dose safrole (1000 mg/kg) induced 8-OH-dG levels in the target tissue of rats, which increased from $3.49/10^5$ dG (day 0) to $7.18/10^5$ dG (5th day) and rapidly repaired to basal level on the 15th day. In addition, pretreatment of BSO enhanced the safrole-induced 8-OH-dG (Liu *et al.*, 1999). This also suggests that GSH plays a protective role in safrole-induced oxidative damages.

This is in sharp contrast to the safrole-DNA adducts generated from the cytochrome P450 activation and followed by sulfation (Miller and Miller, 1983). By using the ^{32}P-postlabeling technique, this safrole-DNA adducts can be detected in mouse liver 30 and 140 days after a high dose (10 mg/mouse) administration (Randerath *et al.*, 1984; Gupta *et al.*, 1993). Furthermore, these safrole-DNA adducts can even be detected 30 days after a low dose (0.001 mg/mouse) safrole challenge (Gupta *et al.*, 1993). Therefore, the stable safrole-DNA adducts may represent a more significant initiation lesion as compared to the rapidly repaired safrole-induced 8-OH-dG.

In conclusion, our results demonstrate that safrole has potential to induce stable safrole-DNA adducts in human oral derived cells. This results couples with our previous findings suggest that safrole may be involved in the BQ related oral caner in Taiwan.

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