and oxidation catalyzed by microsomes from B-lymphoblastoid cell lines expressing each CYP form were determined. Metabolism of nicardipine by the recombinant human CYPs indicated that the oxidation was mainly mediated by CYP2C8, 2D6 and 3A4 (Fig. 2). Although the debenzylation was catalyzed by essentially all CYPs examined, CYP2C8 and CYP3A4 were the major catalysts, at a low substrate condition (5 μM, Fig. 2). However, the extent of a difference in the debenzylase activity among all CYPs was relatively small at a high substrate concentration (50 μM, data not shown).

Effects of Nicardipine on the Catalytic Activities of CYP3A4 Since CYP3A4 appeared to be the major CYP form involved in the oxidation of nicardipine (Fig. 2), the inhibitory effects of nicardipine on the oxidation of triazolam, a clinically used drug metabolized by CYP3A4, was examined. Triazolam hydroxylation was competitively inhibited by co-incubation with nicardipine (Fig. 3). The K_i values for the α- and the 4-hydroxylation of triazolam are shown in Table 1.

Effects of Calcium Antagonists on the Catalytic Activities of CYP2D6 The inhibitory potencies of three Ca^{2+} antagonists, nicardipine, nifedipine or diltiazem, on bufuralol 1′-hydroxylase were compared (Fig. 4). As shown in Fig. 4, nicardipine inhibited the bufuralol 1′-hydroxylase in human liver microsomes more strongly than did nifedipine and diltiazem. CYP2D6-mediated dextromethorphan *O*-demethylation and mequitazine 3-hydroxylation (K_i, 2.1 μM) were also strongly inhibited by nicardipine. Nicardipine inhibited the CYP2D6-mediated metabolism competitively except for bufuralol 1′-hydroxylation (Fig. 5).

Effects of Nicardipine on the Catalytic Activities of Other CYPs The inhibitory potencies of nicardipine on the metabolism of prototype substrate for other CYPs were investigated. The K_i values of nicardipine for various reactions determined in human liver microsomes are summarized in Table 1. Nicardipine inhibited essentially all reactions examined in the present study, the inhibitory potency was extremely strong against CYP2C8, CYP2C19, CYP2D6 and CYP3A4.

DISCUSSION

One of the most frequent mechanisms of drug–drug inter-

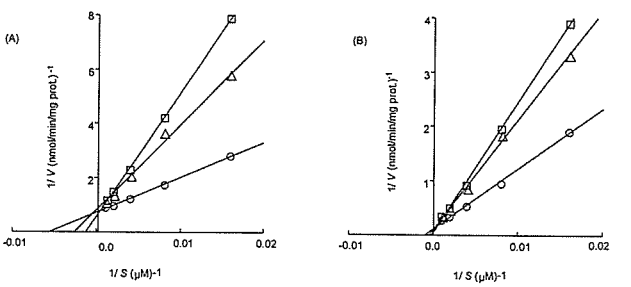

Fig. 3. Lineweaver–Burk Plots for the Effect of Nicardipine on Triazolam α-Hydroxylase (A) and 4-Hydroxylase (B) in Human Liver Microsomes

Nicardipine concentrations were 0 (○), 2.5 (△) and 5 μM (□), respectively. Each data point represents the mean value obtained from duplicate determinations.

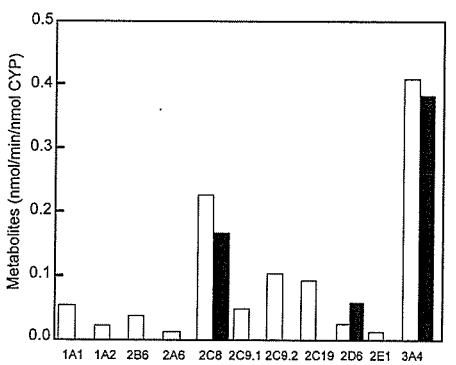

Fig. 2. Formation of Debenzylated (White Bars) and Oxidized (Black Bars) Metabolites in Microsomes from Human B-Lymphoblastoid Cells Expressing CYP Isoforms

Microsomes were preincubated in the presence of an NADPH-generating system for 5 min and then incubated with nicardipine at 5 μM. Each data point represents the mean of duplicate determinations.

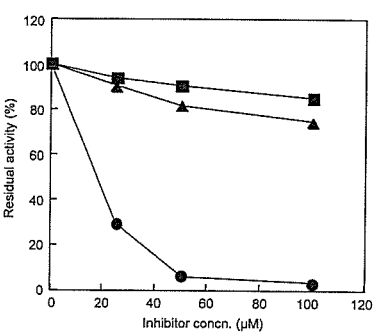

Fig. 4. Effects of Ca^{2+} Blockers on the Metabolism of Bufuralol in Human Liver Microsomes

Human liver microsomes were incubated with nicardipine (●), nifedipine (▲), or diltiazem (■). Residual activities were determined and expressed as percentage of the control value. Each data point represents the mean value obtained from duplicate determinations.

Table 1. Effect of Nicardipine on the Metabolism of Marker Substrate of CYPs in Human Liver Microsomes

Marker substrate	Reaction	K_m (μM)	V_{max} (nmol/min/mg prot.)	K_i (μM)
7-Ethoxycoumarin (1A)	*O*-Deethylation	33.3	33.3	27.0
Coumarin (2A6)	7-Hydroxylation	1.2	62.8	29.4
Paclitaxel (2C8)	6α-Hydroxylation	10.6	0.1	7.1
Diclofenac (2C9)	4′-Hydroxylation	10.1	17.9	17.3
S-Mephenitoin (2C19)	4′-Hydroxylation	31.3	0.4	1.1
Bufuralol (2D6)	1′-Hydroxylation	10.6	0.1	4.8
Dextromethorphan (2D6)	*O*-Demethylation	8.5	0.2	2.9
Midazolam (3A4)	1′-Hydroxylation	2.4	2.1	1.6
Triazolam (3A4)	α-Hydroxylation	175.4	1.4	2.1
	4-Hydroxylation	980.4	8.8	4.6

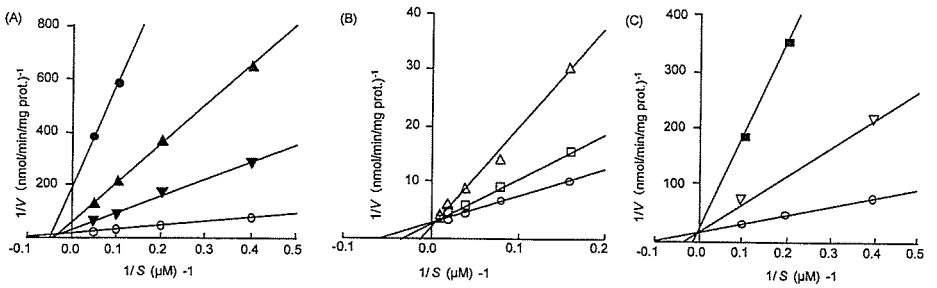

Fig. 5. Lineweaver–Burk Plots for the Effect of Nicardipine on Bufuralol 1′-Hydroxylase (A), Dextromethorphan *O*-Demethylase (B), and Mequitazine 3-Hydroxylase (C) in Human Liver Microsomes

Nicardipine concentrations were 0 (○), 2.5 (□), 5 (□), 10 (□), 12.5 (▼), 20 (■), 25 (▲), and 50 μM (●), respectively. Each data point represents the mean value obtained from duplicate determinations.

action is recognized to be a competition of several drugs on the same enzyme. Predominant binding of a drug with higher affinity to a certain enzyme results in a higher plasma concentration of a drug with lower affinity to the enzyme. Thus, to predict this type of interaction *in vivo*, the information of the *in vitro* K_i value of a reaction must be useful. Although the metabolism of nicardipine has been well characterized in experimental animals, the information on the metabolism of this drug in humans is limited. Co-administration of nicardipine at a dose of 5 mg/kg and triazolam at a dose of 0.1 mg/kg to monkeys resulted in a 1.9 times higher *AUC* value of triazolam than that after the administration of triazolam alone,[18] supporting the idea that nicardipine affected the pharmacodynamics of a certain drug metabolized by CYP3A4. In addition, inhibition on the metabolism of bufuralol and dextromethorphan was observed (Table 1). Thus, nicardipine may also interact with many clinically used drugs reported as a specific substrate for CYP2D6 such as anti-arrhythmic agents, β-blocking agents, H1 antagonists and antidepressants. Not only CYP3A4 and CYP2D6, but essentially all CYP forms investigated in the present study were also inhibited by nicardipine *in vitro* as reported by Katoh *et al.*[19] This may account for a reported *in vivo* pharmacokinetic interaction between nicardipine and other drugs such as propranolol.[20,21] Considering the mechanism of inhibition, a competitive inhibition of these CYPs was suggested in the present study (data not shown). In conclusion, nicardipine can be metabolized mainly by human CYP2C8, CYP2D6 and CYP3A4, whereas it could be a relatively potent competitive inhibitor of these CYPs. Interaction of nicardipine *in vivo* with a number of drugs metabolized by these CYPs should be worth evaluating in future studies.

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Mutagenic activation of betel quid-specific *N*-nitrosamines catalyzed by human cytochrome P450 coexpressed with NADPH-cytochrome P450 reductase in *Salmonella typhimurium* YG7108

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Abstract

Betel quid chewing is known to cause cheek cancer in a wide area covering Africa to Asia. Areca nut contained in the betel quid is believed to give rise to carcinogenic *N*-nitrosamines. In the present study, the roles of human cytochromes P450 (P450 or CYP) in the mutagenic activation of betel quid-specific *N*-nitrosamines such as 3-(*N*-nitrosomethylamino)propionitrile (NMPN), 3-(*N*-nitrosomethylamino)propionaldehyde (NMPA) and *N*-nitrosoguvacoline (NG) were examined by using genetically engineered *Salmonella typhimurium* YG7108 expressing each form of human P450 together with NADPH-P450 reductase, which had been established in our laboratory. Among typical P450s (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A13, CYP2D6 or CYP3A4) examined, CYP2A6 was the most efficient activator of NMPN, followed by CYP1A1 and CYP1B1. The mutagenic activation of NMPN by CYP2A6 was seen at the substrate concentrations of μM levels (~100 μM). The activation of NMPA was catalyzed predominantly by CYP2A13 and to lesser extents by CYP2A6, CYP1A1, CYP1A2 and CYP1B1. The activation of NMPA by CYP2A13 was detectable at the substrate concentrations of μM levels (~1 μM). NG was activated by CYP2A13 and CYP2A6, the genotoxicity of NG being much lower than that of NMPA or NMPN. Based on these data, we conclude that human CYP2A

Abbreviations: CPR, NADPH-cytochrome P450 reductase (EC 1.6.2.4, NADPH:ferrihemoprotein reductase); CYP, individual forms of cytochrome P450 (EC 1.14.14.1); NG, *N*-nitrosoguvacoline; NGC, *N*-nitrosoguvacine; NMPA, 3-(*N*-nitrosomethylamino)propionaldehyde; NMPN, 3-(*N*-nitrosomethylamino)propionitrile; P450, general term for cytochrome P450

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subfamily members play important roles in the mutagenic activation of essentially all betel quid-related *N*-nitrosamines tested in the present study.

Keywords: Areca nut; Betel leaf; 3-(*N*-Nitrosomethylamino)propionitrile; 3-(*N*-Nitrosomethylamino)propionaldehyde; *N*-Nitrosoguvacoline; Metabolic activation; Genetically engineered *S. typhimurium*

1. Introduction

Oral cancer is one of the most frequent malignant types of cancer in Southeast Asian countries such as Sri Lanka and India, accounting for ~40% of the total cancer incidence [1,2]. The high frequency of cheek cancer has been believed to be caused by the prevalent habit of chewing betel quid with tobacco and areca nut (also called as betel nut), which contains certain alkaloids such as arecoline, arecaidine, guvacoline and guvacine. Arecoline is reported to be the most abundant alkaloid in the areca nut [3]. Chemical nitrosation of arecoline leads to the formation of areca-specific *N*-nitrosamines such as 3-(*N*-nitrosomethylamino)propionitrile (NMPN)[1], NMPA, NG and NGC (Fig. 1) [3]. The formation of NMPA during betel quid chewing has not yet been proven, although it is likely the case. Salivary concentrations of NMPN, NG and NGC have been determined to be around 0.5–11.4 ng/mL, 3.5–350 ng/mL and 30.4 ng/mL, respectively [4–6]. Since NMPN, NMPA and NG were shown to be carcinogenic in rats [4,5,7,8], these betel quid-specific *N*-nitrosamines may act as an adjacency to tobacco-specific *N*-nitrosamines that are strongly implicated as an etiologic factor for oral cancer in habitual betel quid chewers.

Most *N*-nitrosamines in natural environments are generally believed to be metabolically activated to elicit their genotoxicity. The α-hydroxylation of these *N*-nitrosamines is an important activation pathway mediated mainly by P450s [9,10]. This pathway yields alkyldiazohydroxide ions which alkylate DNA bases. The alkylated bases such as O^6-methylguanine and O^4-methylthymine have been shown to cause a miscoding during lesions in *N*-nitrosamine carcinogenesis [11,12].

P450 is a heme-containing enzyme responsible for the metabolism of exogenous and endogenous compounds and consists of a number of P450 isoforms with some substrate specificity [13]. Some forms of

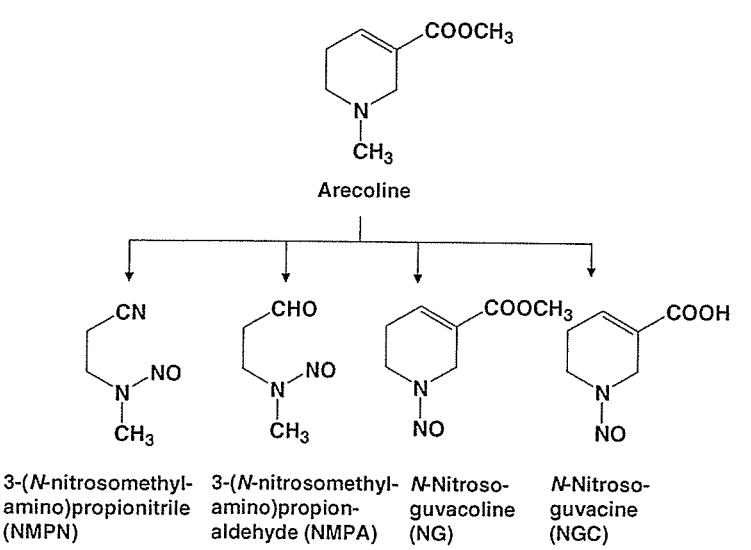

Fig. 1. Chemical structures of *N*-nitrosamines derived from arecoline.

P450 have been proven to activate promutagens to their mutagenic intermediates [14]. Thus, it is important to clarify the form of P450 involved in the activation of promutagens, since the catalytic properties and the population of P450s are sometimes considered as the determinants of the formation of reactive metabolites in the body.

A series of *S. typhimurium* YG7108 strains, each expressing a form of human P450 (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 or CYP3A5) together with human CPR were established in our laboratory [15]. The *S. typhimurium* YG7108, derived from TA1535, lacks two O^6-methylguanine-DNA methyltransferase genes, ada_{ST} and ogt_{ST}, and is highly sensitive to alkylating agents such as *N*-nitrosamines [15]. The capacities of the 11 forms of human P450s to metabolically activate tobacco-related *N*-nitrosamines have been compared using the tester strains [15,16].

In the previous studies, we found that CYP2A6 was involved in the mutagenic activation of essentially all tobacco-related *N*-nitrosamines tested [15,16]. In other studies, we discovered a deletional mutation of the *CYP2A6* gene (*CYP2A6*4*) in a Japanese population [17]. Combining these two concepts as mentioned above, we hypothesized that subjects homozygous for the *CYP2A6*4* allele have less risk for tobacco-related lung cancer. Thus, we performed a large-scale epidemiological study on the association between the *CYP2A6* genetic polymorphism and lung cancer risk [18,19]. The results were completely in accordance with our hypothesis. As mentioned above, the habit of betel quid chewing is thought to cause an oral cancer. Betel quid contains carcinogenic *N*-nitrosamines (NMPN, NMPA and NG) [3]. Our epidemiological study with Sri Lankan subjects showed that the *CYP2A6*4* allele reduced a risk for oral cancer in betel quid chewers [20], as seen in the lung cancer risk in smokers [18,19]. Although we reported previously that the metabolic activation of most *N*-nitrosamines including tobacco-related *N*-nitrosamines was catalyzed by P450s, no studies to our knowledge have been reported so far whether or not these betel quid-specific *N*-nitrosamines are activated by human P450s.

Accordingly, we intended to compare the capacity of seven forms of human P450 in the metabolic activation of three betel quid-specific *N*-nitrosamines in the *Salmonella* strains as described above [15,21]. In the present study, however, we did not perform the mutation assays with NGC, since NGC was reported to be non-carcinogenic or non-mutagenic [1,4,22]. We compared the capacities of seven or four forms of carcinogen-activating human P450 (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A13, CYP2D6 and CYP3A4) [14,16] in the mutagenic activation of three areca nut-specific *N*-nitrosamines (NMPN, NMPA and NG). We report here that human CYP2As, CYP2A6 and CYP2A13 efficiently activate betel quid-specific *N*-nitrosamines.

2. Materials and methods

2.1. Materials

NMPN, NMPA and NG were synthesized according to the methods reported previously [23]. The purity of the synthesized chemicals (>99%) was verified by thin-layer chromatography and gas chromatography using a flame ionization detector. Structures were confirmed by mass spectrometry and fourier transform nuclear magnetic resonance spectroscopy. Cytochrome *c* was purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals and solvents were of the highest grade commercially available.

2.2. Expression of P450 and CPR in S. typhimurium YG7108 cells

Seven strains of the genetically engineered *S. typhimuiurm* YG7108 cells each co-expressing a human P450 and the CPR were established previously in our laboratory [15,24]. The expression of human P450 and the CPR in the genetically engineered *S. typhimurium* YG7108 cells was performed according to the method previously described by Fujita et al. [15]. The content of P450 holo-protein in the *S. typhimurium* cells was determined by Fe^{2+} CO versus Fe^{2+} difference spectra, according to the method of Omura and Sato [25]. To determine the CPR expression levels, the activity of the CPR in sonicated bacterial cells was measured with cytochrome *c* as an electron acceptor by measuring the absorbance change at 550 nm at 20 °C according to the method of Phillips and Longdon [26]. The expression levels of P450 ranged

from 50 nmol/L culture for CYP1A1 to 240 nmol/L culture for CYP2A13. The molar ratio of the expressed CPR to P450 varied from 1.0 for CYP1A2 to 2.5 for CYP2A6.

2.3. Mutation assay

The number of *S. typhimurium* cells co-expressing human P450 with the CPR was adjusted to give (1–2) × 10^9 cells/mL by dilution with a nutrient broth. The assay was carried out as described previously in our laboratory [27]. The bacterial cells were pre-exposed to a promutagen at 37 °C for 20 min. NADPH was not added to a reaction mixture, since the mutagenic activation of promutagens was not affected by the addition of NADPH, probably because NADPH present in the viable bacterial cells was utilized as an electron donor. The plates were incubated at 37 °C for 2 days. Assays were carried out at least twice in duplicate at each concentration of a promutagen. When the variability of the values of duplicate determinations was within ± 20%, the results were adopted. Induced revertants/pmol P450 were calculated based on the increased colonies per pmol of P450 content expressed in the tester strains. Since the mutagenicity of NMPA was detected with the parental YG7108 itself, the number of mutants with the parental YG7108 at each concentration of the promutagen was subtracted from each experimental number of revertants using the genetically engineered YG7108. The spontaneous revertant number per plate ranged from 20 to 40.

3. Results

The roles of seven forms of human P450 in the mutagenic activation of betel quid-specific *N*-nitrosamines were examined by using the genetically engineered *S. typhimurium* YG7108 cells. Three betel quid-specific *N*-nitrosamines, NMPN, NMPA and NG were used as promutagens. The mutagen-producing activities of P450s from NMPN are shown in Fig. 2. CYP2A6, CYP1A1, CYP1B1 and CYP2A13 showed the capacity to activate NMPN. Among these P450s, CYP2A6 was found as the major form responsible for the activation of NMPN. The activation of NMPN was detectable at a concentration of around 100 μM.

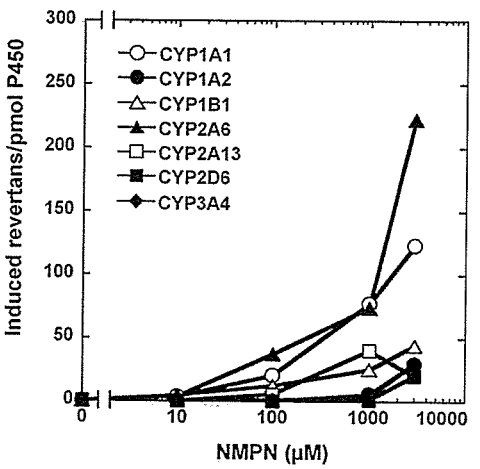

Fig. 2. Mutagenic activation of NMPN by human P450s expressed in *S. typhimurium* YG7108. Seven strains of *S. typhimurium* YG7108 each expressing a form of human P450 were preincubated with the indicated concentrations of NMPN at 37 °C for 20 min. Induced revertants/pmol P450 were calculated based on the increased colonies per pmol of P450 content expressed in the tester strains as described in Section.2 Data represent the mean of duplicate determinations.

The mutagen-producing activities of P450s from NMPA are shown in Fig. 3. NMPA was metabolically activated by human CYP2A13, CYP2A6, CYP1A1, CYP1A2 and CYP1B1. Among these P450s, CYP2A13 was found to be the most predominant form in the activation of NMPA; the activation was detectable at a concentration of around 1 μM of NMPA.

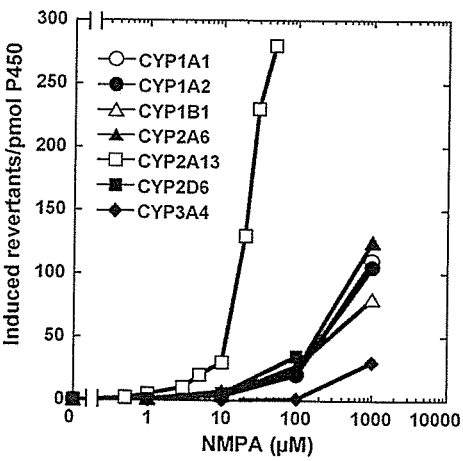

Fig. 3. Mutagenic activation of NMPA by human P450s expressed in *S. typhimurium* YG7108. See legend of Fig. 2 for details.

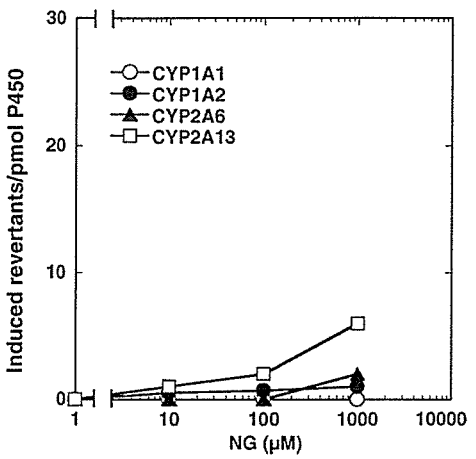

Fig. 4. Mutagenic activation of NG by human P450s expressed in *S. typhimurium* YG7108. See legend of Fig. 2 for details.

Fig. 4 shows the results of the mutagenic activation of NG by four forms of P450 (CYP1A1, CYP1B1, CYP2A6 and CYP2A13). NG was activated by CYP2A13 and CYP2A6, but much less potently. Thus, it appeared that the mutagenic potency of NG after undergoing activation by CYP2A13 or CYP2A6 was much lower than that of NMPN and NMPA.

4. Discussion

NMPN, NMPA and NG have been considered as risk factors for oral cancer in habitual betel quid chewers [3]. In fact, treatment with these *N*-nitrosamines resulted in the induction of tumors in laboratory animals [3]. The metabolic activation of these promutagens by human P450s needed to be examined to predict the cancer risk of the chemicals in humans, despite that the formation of MNPA during betel quid chewing, although likely, has not been directly proven yet [4–6]. Thus, we examined the roles of human P450s in the mutagenic activation of three carcinogenic areca nut-specific *N*-nitrosamines by using seven strains of *S. typhimurium* YG7108 cells, each expressing a form of human P450 and the CPR.

CYP2A6 showed the highest capacities for the mutagenic activation of NMPN among human P450s tested. The genetic polymorphism of *CYP2A6* has been well recognized (http://www.imm.ki.se/CYPalleles/cyp2a6.htm). NMPN has been detected in the saliva of betel quid chewers [5] and shown to be the most powerful carcinogen in rats, inducing tumors in the liver, esophagus, nasal cavity, oral cavity and tongue of rats [4,8]. Our results and these data are highly in accordance with our previous findings [20] that subjects homozygous for *CYP2A6* gene deletion showed reduced oral cancer risk in habitual chewers of betel quid. In general, the mutagen production from promutagens may be altered by the expression levels of P450 involved in the activation in addition to the capacity to activate promutagens. Since treatment with diets containing areca-nut resulted in a significant increase in hepatic levels of P450 in rodents [28], it may be possible to assume that CYP2A6 in the oral tissues is induced by habitual betel quid chewing. Although an analysis from 13 healthy subjects demonstrated that CYP2A6 was not expressed in human buccal tissues [29], CYP2A6 mRNA was expressed in oral tissues of three habitual betel quid chewers in Sri Lanka in our preliminary study (data not shown). The expression of CYP2A13 is currently under investigations. Thus, studies on the expression of CYP2A6 using biopsy specimens of oral tissues of habitual betel quid chewers in a large population are needed, with relevance to our epidemiological study [20]. In summary, mutagenic activation of NMPN by polymorphic CYP2A6 in the oral cavity would be one of the determinant factors for oral cancer risk in betel quid chewers.

NMPA was efficiently activated by human CYP2A13 in the present study. It has been reported that CYP2A13 catalyzed the α-hydroxylation (activation reaction) of the most potent tobacco-specific *N*-nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [30]. These results probably suggest that CYP2A13 might contribute to the occurrence of the oral cancer in habitual betel quid chewers via the genotoxic activation of NMPA. CYP2A13 are known to be expressed in the respiratory tracts such as lung, trachea and nasal mucosa in humans [30]. However, it has not been clarified that CYP2A13 would be expressed in the oral tissues of the habitual betel quid chewers. Several genetic polymorphisms of *CYP2A13* gene have been found in our laboratory [31] and others (http://www.imm.ki.se/CYPalleles/cyp2a13.htm), whereas it has not yet been clarified whether the genetic polymorphisms of *CYP2A13* gene are associated with the risk for the oral cancer in the habitual betel quid chewers.

Further studies on these points are now ongoing in our laboratory.

DNA methylation (and probably cyanoethylation) and 1,N^2-propanodeoxyguanosine formation in target tissues are suggested to be the mechanisms for genotoxicity of NMPN [32] and NMPA [33], respectively. Single i.v. and s.c. injection of NMPN (45 mg/kg body weight) or swabbing the oral cavity with NMPN elevated the levels of N^7-methylguanine and O^6-methylguanime in the liver, nasal mucosa, esophagus and oral tissues of F344 rats [5]. The highest levels of methylated guanines are found in the nasal cavity, independent of the route of administration, suggesting the tissue specificity for carcinogen activation. It is of interest to note in this context that CYP2A forms are abundantly expressed in the nasal cavity in rats and mice [34]. In our preliminary study, we found that rat CYP2A3 and mouse CYP2A4 and CYP2A5 showed the capacities to activate NMPN and NMPA similar to those for human CYP2As (data not shown). Taking these lines of evidence into account, CYP2A subfamily members may play important roles in the mutagenic activation of betel quid-related *N*-nitrosamines in common over animal species.

NG, the major nitrosation pruduct of arecoline, was activated by CYP2A, whereas the mutagenic potency was much lower than that of NMPN or NMPA in the present study using four strains of *S. typhimurium* YG7108 expressing human P450. NG is reported to be formed readily at around pH 7.0 chemically as the major nitrosation product of arecoline [35,36]. This *N*-nitrosamine has been reported to require metabolic activation to exert mutagenicity in *S. typhimurium* TA1535 stain [35,36]. However, NG is suggested to be a weak carcinogen according to the following evidence showing that NG is not carcinogenic when given to Sprague-Dawley rats in their drinking water [35]. Taken together with our results, we estimate that contribution of NG to the risk of the oral cancer in the habitual betel quid chewers may still be low as compared to those of NMPN and NMPA, despite its abundance in the human saliva.

In conclusion, we propose that human CYP2A subfamily members play crucial roles in the mutagenic activation of all betel quid-specific *N*-nitrosamines tested. This is probably one of the most important mechanisms responsible for the occurrence of the oral cancer in the habitual betel quid chewers.

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PHARMACOGENETICS

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High prevalence of cytochrome P_{450} *2A6*1A* alleles in a black African population of Ghana

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Abstract *Objective*: We investigated the frequencies of the functionally important variants of the *CYP2A6* gene in black African populations.
Methods: Using genomic DNA sequencing, polymerase chain reaction (PCR)–restriction fragment length polymorphism and allele-specific PCR, the allele frequencies of *CYP2A6 *1A*, **1B*, **2*, **4A*, **5*, **6*, **7*, **8*, **9*, **10* and ** 11* among 120 black Africans— including 105 Ghanaians, 12 Nigerians, 2 Ivorians and 1 Ugandan—were determined.
Results: The allele frequencies were 80.5% for *CYP2A6*1A*, 11.9% for *CYP2A6*1B*, 1.9% for *CYP2A6*4A* and 5.7% for *CYP2A6*9* in the Ghanaian subjects. No subject homozygous for the *CYP2A6*4A* allele, a whole gene deletion type of polymorphism prevalent among Orientals, was found. Furthermore, *CYP2A6* variants such as **2*, **5*, **6*, **7*, **8*, **10* and **11* were absent in these black African populations.
Conclusions: This study provides, for the first time, the results of the analysis of *CYP2A6* allele frequency in black African populations and confirms large ethnic differences in the polymorphic *CYP2A6* gene.

Keywords Genetic polymorphism · Cytochrome P_{450} · CYP2A6 · Black Africans · Ghanaians · Ethnic differences · Genotyping

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Introduction

Cytochrome P_{450} (P_{450} or CYP) 2A6 is a polymorphically expressed enzyme of potential importance for the metabolism of nicotine, a number of medicines and activation of carcinogens [1].

The large interethnic differences in allele frequency of *CYP2A6* have been extensively reported among Caucasians and Orientals [1–10]. The *CYP2A* gene cluster containing three complete genes, *CYP2A6*, *CYP2A7* and *CYP2A13*, has been intensively investigated [11]. To date, 17 allelic variants of *CYP2A6* have been identified (**1–*16* and gene duplication, URL: http://www.imm.ki.se/CYPalleles/cyp2a6.htm). However, information regarding *CYP2A6* alleles in black African populations is conspicuously absent in the literature, despite the high frequency of the whole gene deletion of *CYP2A6* in some ethnics [1, 6, 10].

We therefore investigated the *CYP2A6* locus in black African populations and compared it with previous findings in other populations to elucidate the genetic basis for individual differences of CYP2A6 in Africans.

Materials and methods

Subjects

A total of 120 apparently healthy and unrelated black African nonsmokers, including 105 Ghanaians, 12 Nigerians, 2 Ivorians and 1 Ugandan (86 males and 34 females), aged 21–71 years and currently resident in various parts of Japan, were recruited for the study. The 105 Ghanaians included 86 Akans, 8 Guans, 5 Ewes, 4 Gas, 1 Nzima and 1 Dargarti. This study was approved by the ethics committee of the Hokkaido University (Sapporo, Japan). Informed consent was obtained from each participant.

Genotyping methods

Genotyping for the *CYP2A6*1A/*1A*, *CYP2A6*1A/*1B*, *CYP2A6*1B/*1B*, *CYP2A6*1A/*4A*, *CYP2A6*1B/*4A* and *CYP2A6*4A/*4A* alleles was done as previously reported [10] with an exception that the product (1323-bp) was digested by the restriction enzyme *Fok* I after amplification by polymerase chain reaction (PCR). The *CYP2A6*1A/*1A* yielded 573-bp and 513-bp fragments and *CYP2A6*1A/*1B* yielded 661-bp, 573-bp and 513-bp fragments. The *CYP2A6*1B/*1B* yielded 661-bp and 513-bp fragments and *CYP2A6*1A/*4A* yielded 661-bp, 573-bp, 513-bp and 400-bp fragments. The *CYP2A6*1B/*4A* yielded 661-bp, 513-bp and 400-bp fragments and *CYP2A6*4A/*4A* yielded 661-bp and 400-bp fragments. Based on the new *CYP2A6* nomenclature, the *CYP2A6*1B* allele which we detected using the *Fok* I–restriction fragment length polymorphism (RFLP) represents *CYP2A6*1B1*, *CYP2A6*1B2* or *CYP2A6*1B3*. Our results were verified with genomic DNA sequencing.

The *CYP2A6*2*, *CYP2A6*5*, *CYP2A6*6*, *CYP2A6*7*, *CYP2A6*8*, *CYP2A6*9*, *CYP2A6*10* and *CYP2A6*11* alleles were determined using methods previously reported [5, 12–14].

Statistical analysis

The expected genotype frequencies were calculated using the Hardy–Weinberg equation from the allele frequencies, $p^2+2pq+q^2=1$. The χ^2 and the Fischer's Exact tests were used to compare the observed and calculated or reported genotype frequencies. P values of <0.05 were regarded as significant.

Table 1 The frequencies of *CYP2A6* genotypes in black African subjects

Genotype	Ghanaians, *n* (%)	All subjects[a], *n* (%)
**1A/*1A*	67 (63.8)	74 (61.7)
**1A/*1B*	22 (21.0)	25 (20.8)
**1A/*4A*	3 (2.9)	5 (4.2)
**1A/*9*	10 (9.5)	12 (10.0)
**1B/*1B*	1 (1.0)	1 (0.8)
**1B/*4A*	1 (1.0)	1 (0.8)
**1B/*9*	0 (0.0)	1 (0.8)
**9/*9*	1 (1.0)	1 (0.8)
Total	105 (100)	120 (100)

n number of subjects
[a]All subjects include the 105 Ghanaians, 12 Nigerians, 2 Ivorians and 1 Ugandan. The observed genotype frequencies were obtained as described in Materials and methods. The *CYP2A6* genotype frequencies observed in the Ghanaians ($\chi^2=3.116$, $P=0.87$) and all subjects ($\chi^2=1.775$, $P=0.97$) were in complete agreement with those calculated according to the Hardy-Weinberg equilibrium

Results and Discussion

Among the Ghanaian subjects, while the genotype frequencies of *CYP2A6*1A/*1A*, *CYP2A6*1A/*1B*, *CYP2A6*1A/*4A* and *CYP2A6*1A/*9* were 63.8, 21.0, 2.9 and 9.5%, respectively, the frequencies of *CYP2A6*1B/*1B*, *CYP2A6*1B/*4A* and *CYP2A6*9/*9* were 1.0% each (Table 1). The genotype frequency of the *CYP2A6*1A/*1A* was higher (63.8%) in the Ghanaian population than in Caucasian, African–Americans and Oriental populations ($P<0.0001$) [1, 6, 10, 15]. Similar results were obtained in the 120 black Africans (Table 1).

A high frequency (80.5%) of the *CYP2A6*1A* allele in the Ghanaians (Table 2) might suggest higher CYP2A6 activities of black Africans than Caucasians and Orientals. However after submitting our manuscript, various *CYP2A6*1* alleles (*CYP2A6 *1D *1E *1F*

Table 2 *CYP2A6* allele frequencies in various ethnic groups

Alleles	Ghanaians[a] (%)	Caucasians (%)	Japanese[b] (%)	Japanese (%)	Chinese (%)	Koreans (%)	Thai[c] (%)
**1A*	80.5	58.4 [1][d]	16.4	20.6 [6][d]	27.2 [1][d]	22.7 [6][d]	31.7
**1B*	11.9	30.0 [1]	25.6	27.7 [6]	34.5 [1][d]	37.1 [6]	27.1
**2*	0.0	3.0 [1]	0.0	0.0 [6]	0.0 [1]	0.0 [6]	No data
**4A*	1.9	0.5 [1]	19.8	20.1 [6]	15.1 [1]	11.0 [6]	14.2
**5*	0.0	0.0 [1]	0.0	0.0 [6]	1.0 [1]	0.5 [6]	No data
**6*	0.0	No data	0.0	0.4 [9]	No data	0.0 [6]	No data
**7*	0.0	0.0 [2]	12.6	6.5 [6]	2.2 [2]	3.6 [6]	5.0
**8*	0.0	0.0 [2]	No data	2.2 [6]	3.5 [2]	1.4 [6]	0.0
**9*	5.7	5.2 [3]	20.7	21.3 [8]	15.7 [3]	22.3 [6]	20.4
**10*	0.0	0.0 [2]	4.3	1.1 [6]	0.4 [2]	0.5 [6]	1.6
**11*	0.0	No data	0.6	0.5 [7]	No data	0.7 [7]	No data
**12*	ND	2.2 [4]	No data	No data	0.0 [4]	No data	No data
**1× 2*	ND	0.7 [2]	No data	0.0 [2]	0.4 [2]	0.2 [7]	No data

N number of alleles *ND* not determined in the present study
[a]Data from the present study ($N=210$)
[b]Data from Fujieda et al. [5] obtained in our laboratory ($N=1222$)
[c]Data from unpublished results obtained in our laboratory ($N=240$)
[d]*CYP2A6 *1A* and **1B* allele frequencies were recalculated from the original references to account for *CYP2A6*7*, **8*, **9*, **10*, **11*, **12* and **1×2* allele frequencies determined recently

1G *1H* and *1J*) were reported (URL:http://www.imm.ki.se/CYPalleles/cyp2a6.htm) with a high frequency of *CYP2A6*1G* (13.3%) in African–Americans [15]. The allele frequency of the *CYP2A6*1B* allele, carrying a gene conversion with *CYP2A7* in the 3′UTR in this Ghanaian population, was 11.9%—similar to that in African–Americans (11.2%) [15]. We did not determine the *CYP2A6*1G* allele frequency in this Ghanaian population; however, if the *CYP2A6*1G* is accounted for with results from African–Americans (13.3%), then the *CYP2A6*1A* allele frequency of the Ghanaian population (80.5%) becomes 67.2%, which is similar to that in African–Americans (66.5%) [15].

The *CYP2A6*4A* allele, which is a deletion of the whole *CYP2A6* gene, was found in only four Ghanaians (4 of 105) with an allele frequency of 1.9%; no subject was found as homozygous for the *CYP2A6*4A* allele among the 120 black Africans. The low frequency of the *CYP2A6*4A* allele was similar to that in Caucasians [1, 15] and African–Americans [15], which is consistent with the previous findings that the *CYP2A6*4A* is a major mutant allele in Orientals (7.8–22.2%) [1, 6, 10] (Table 2). The allele frequency of *CYP2A6*9*, which has a point mutation in the TATA box (−48T > G) and is one of the most common alleles among Caucasians (5.2 – 7.9%) [3, 15], African–Americans (8.0%) [15] and Orientals (15.7–22.3%) [3, 8], was found at 5.7% among the Ghanaians (Table 2). No *CYP2A6*2* was found among the Ghanaian population. The *CYP2A6*2* allele has a point mutation in exon 3 (479T > A) and has been found in African–Americans [16], suggesting that the *CYP2A6*2* allele might have originated from a Caucasian ancestry.

In summary, our genotyping results in this black African population revealed that only 15 Ghanaians (14.3%) may carry impaired CYP2A6 phenotype, predicting that a large number of Ghanaians will be extensive metabolizers with the wild-type of the *CYP2A6* gene and that these individuals might exhibit a higher capacity to metabolize drugs and carcinogens that are CYP2A6 substrates.

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環境ストレス応答と生体ホメオスターシス

―総　論―

井上　達

◎生体は，内外の環境中に漂ううたかたのようなものである．生体の外部環境が外気や水環境によることはもちろんのことであるが，内部環境についてもその主たる部分は，それら外部環境中の物質を食物もしくはその混入物として消化管内に取り込んだ状態にほかならないから，消化管腔には積々の防御機構が備わっているとはいえ対外環境の延長線上で理解されうるものである(図1)．環境と食品を同列で取り扱う所以もこの点にある．ちなみに外気を取り込む呼吸器も消化器を発生原基としているので，消化器の機能を考える際に，そうした生体内腔を貫通する外部環境としての共通性を呼吸器を含めて念頭におくことは無駄にはならない．本章における生体と内外環境物質についての生体の異物ストレス応答の各論に入る前に，環境・食品などによるレドックス制御の逸脱と，環境・食品中の成分によるそうした酸化ストレスの消去の例の概略を紹介する．

Key word：環境ストレス応答，チオレドキシン，放射線，臭素酸カリウム，食品化学発癌

各環境ストレス応答と生命系

内外の環境と生体の相互作用の中で，レドックス平衡機能に働くチオレドキシン(TRX)などの分子種は，①原核生物から真核生物まで普遍的に備わっている酸素種による傷害の防御機構としての役割から，②有酸素下でのミトコンドリアを利用した好気的生命活動レベルで発揮される諸機能，さらに，③そうしたレドックスを生体の調節機構として利用する機能系にいたるまで，それぞれ異なったレベルでの環境応答機構に役割を果たしていることが明らかになっている[1]．放射線や紫外線に対する物理・化学的で直接的に作用する強い活性酸素やラジカル生成に対する還元蛋白としての役割を①とすれば，水溶性分子として排出する代謝過程での水解酵素チトクローム P450 の転写活性化など異物代謝の制御に働く機構は②に該当し，また NF-κB のような転写因子の遺伝子発現制御を通じた免疫系のレドックス調節などは③の一例として理解される．本書では，それらレドックス機構の基礎については第１章，関連する病態については第２章に紹介されており，その破綻・修復と予防のためのメカニズムを研究して健康医学への方策を考えることを，本章の目的としてい

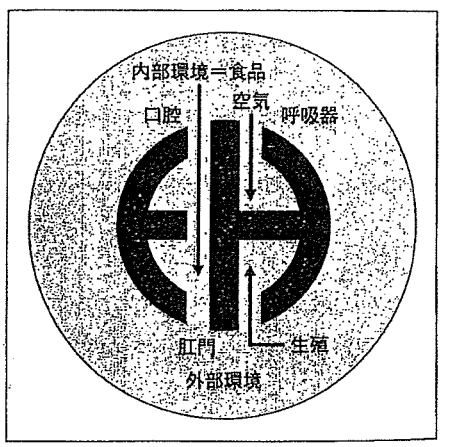

図１　生体内外環境の概念図
消化管は，外部環境の陥入組織である．食品を環境成分としてとらえる意義はこの点にある．ちなみに呼吸器は，消化器を原基として発達する組織であり同様に理解すべきものと考えられる．

Redox regulation in xenobiotic response
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る.

たとえば,先のTRX遺伝子をノックアウトしてさまざまのレベルで働いているTRXを消去すると,ホモ欠失では胚細胞は胎生致死をきたし[2],また,その過剰発現系を作製すると,種々のレベルでのレドックスの平衡状態の破綻に対して顕著な抵抗性をみることが浮かび上がってくる(本書,平林「ダイオキシンの生体影響と防御機構」の項参照)[3,4].ここでトランスジェニックマウスの作製に導入されたTRX遺伝子は,ヒトのそれなので,この分子種の機能の普遍性が窺われよう.酸化ストレスに対する還元蛋白の誘導が原核生物からヒトまでよく保存されていることからも想定されるように,抗酸化ストレス機構については,種間の相同性がよく保存されており,本章で取り扱われる種々の環境・食品などの物質の作用も,それらの種を超えて理解することが可能と考えられる.

第4章では,まず内外環境物質として生体に異物ストレス応答を迫ってくる物質が取り上げられ,生体におけるレドックス制御の逸脱の機構が紹介されており,ついでレドックス制御の積極的な維持が健康医学の中心課題にあるものとの考え方から予防的見地にたった諸研究が紹介されている.それら個別の各論に入る前に,環境・食品などによるレドックス制御の逸脱の例と,反対に環境・食品中の成分によるそうした酸化ストレスの消去を企図する例を取り上げて,総論としてのつとめを果たしたい.

環境要因によるレドックス制御からの逸脱

放射線や紫外線あるいは,種々の食品や食品添加物は,環境要因として,レドックス平衡を酸化傾向に傾け,酸化ストレス状態を引き起こすことがある.酸化ストレスが生体に対して引き起こす傷害は,第1章で解説されているようなさまざまの細胞機能の不全状態を引き起こす.それらには細胞のプログラム死,蛋白のミス・フォールディング,ミトコンドリアの機能不全,プロテアソームの機能障害などがあげられている[5]が,とりわけその直接的なDNA傷害に基づく修復エラーは発癌を引き起こす要因となる[6].レドックス制御が抗酸素種傷害防御機構のみならず,レドックスを生体の調節機構として利用する機能系に広くかかわっていることからするならば,その平衡状態の逸脱の影響も,さらに広範な生物機能の障害として理解されるはずである.しかしそれらの背景は,TRX遺伝子のノックアウトのような系で観察するときその子細が初めて明らかになるものの,通常の動物実験などによる観察ではその認識は困難であることが少なくない.裏返すならば,そうした背景にこそ,環境医学・健康医学の新しい領域としてのおもしろさが潜んでいることが理解されよう.

ここでは,電離放射線,臭素酸カリウム($KBrO_3$),ヘテロサイクリックアミンの3つの例をとりあげて,それぞれの発癌性に介在する酸化ストレスの関与の機構を簡単に紹介する.

1. 電離放射線

放射線は,その物理化学的過程で活性酸素をつくりだし,生体に酸化ストレス状態を惹起する.その詳細は,すぐれた総説[7,8]にゆずるが,放射線の発癌機構としては,DNAや染色体に直接傷害を引き起こす一次発癌機構と,細胞増殖の亢進やアポトーシスの抑制などのエピジェネティックな機構に基づく二次発癌機構との双方が考えられる.放射線が引き起こすDNA傷害の発見は古く[9],前者の主要因と考えられ,酸化ストレスによるものと理解される.しかしながら放射線の引き起こすDNA傷害は,放射線発癌や放射線白血病の成因を,それらの頻度などとの相関性においてかならずしもよく説明しない.放射線照射後の造血幹細胞の機能変化などが詳細に探求されたが,この問題は解決していない.実験的に放射線照射を行った後に骨髄移植を行うと,骨髄は波をうって幾度かのオーバーシュートを繰り返しつつ回復に向かうが最終的には完全な回復をみることはなく,たとえば造血前駆細胞のコロニー形成能を指標にみる限り80～85%程度の回復にとどまる[10].これは骨髄の造血支持細胞の放射線障害の修復不全によるものと考えられ,骨髄は,たとえば*myc*の過剰発現状態をもって幹細胞のS期分画を高目に維持し,末梢血が無処置の状態に匹敵するまでの回復を下支えしている[11].こうした骨髄の造血支持細胞の造血にあたえる持続的なストレス[10]や,放射

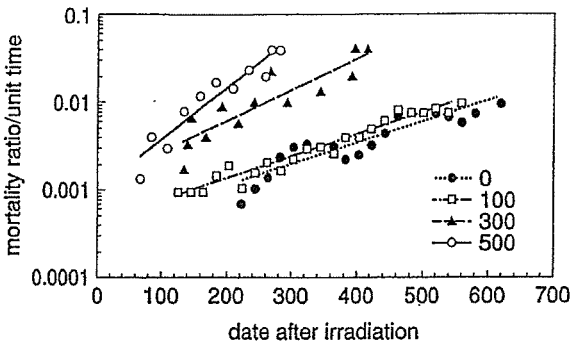

図 2　ゴンペルツ表現による照射線量に応じたマウスの死亡曲線の変化

横軸に年齢をとり，縦軸に累積死亡率を対数でとると，マウスは，指数関数的に直線的に死亡する(非照射対照群参照)．照射線量を増すごとに，死亡率は傾きが急峻化し，促進老化の形をとって早期に白血病死する(照射線量，cGy)．

線によって引き起こされる遺伝子の不安定性につながる要因が，エピジェネティックな白血病の発症機構の一翼となっているものと考えられる(図2)．図に見られるように放射線量を増すに従って死亡直線は傾きが急峻化する．反対に TRX トランスジェニックマウスによる放射線白血病の頻度低下と死亡の遅延の観察はまだできていないが，造血幹細胞などの放射線感受性や紫外線感受性が TRX トランスジェニックマウスで寛恕になることが観察されている．

2. 臭素酸カリウム($KBrO_3$)

臭素酸カリウム($KBrO_3$)は，製パンの過程でソフターとしてドー(dough)に混入させるものである．かつて黒川ら[12]は，ラットでこのものに腎腫瘍誘発性があることを見出した．実験は数次にわたって行われているが，F344 系雌雄のラットを用いた 104 週の飲水投与実験では，500, 250, 125, 60, 30, 15, および 0ppm の投与に対して，シグモイド状の発生頻度の腎細胞癌が観察され，閾値の設定が困難な結果であった[13]．実際に製パン業に使用されていたことと相まって，$KBrO_3$はサルモネラ菌に対する復帰突然変異を引き起こさないので，その発癌機構が関心を集めた．その後の研究で，これは，$KBrO_3$が放出する活性酸素が DNA 付加体，8-hydroxydeoxyguanosine を形成することによる一次発癌と，遺伝子傷害性化学物質 *N-ethyl-N*-hydroxyethylnitrosourea の前処置後の投与によって発癌性の亢進を示す二次発癌との双方の性質を有する，弱いながらも完全発癌物質であることが明らかになった[14](脚注*1)．昨今は，食品もしくは食品添加物の生体影響，発癌性のいかんに対する関心が高まっているが，この臭素酸カリのそれはひと昔前のことでもあり，歴史的に特筆される事件として記憶されることとなった．

3. ヘテロサイクリックアミン(MeIQX)による発癌

MeIQX は，魚の焼けこげから抽出された物質のひとつであり，その後高熱で焼いた肉類でも形成されることの知られている癌原物質である[15]．この MeIQX による発癌は，抗酸化物質によって抑制されることが知られている(後述)ので，酸化的 DNA 傷害に起因するものと想定されていたがその直接的証明はなかった．これに対して，あらかじめ遺伝子傷害性化学物質の diethyl nitrosamine (DEN)を投与しておいて，これに MeIQX と種々の合成抗酸化剤を併用投与し，前腫瘍性病変と考えられている胎盤型グルタチオン *S*-トランスフェラーゼ(GST-P)陽性巣を指標に抗酸化剤の効果をみた実験がある[16]．それによれば，1-*O*-hexyl-2,3,5-trimethylhydroquinone(HTHQ)などの合成抗酸化剤は，GST-P 陽性巣の抑制が顕著であったに

*1：アメリカをはじめとする各国は，このものの発癌性が軽微であったので，$KBrO_3$を製パン過程で十分加熱することによって残留量を低く抑えるなどの注意喚起をもって移行措置とし，後に使用中止とした．

もかかわらず，また同時に行った茶カテキンでは顕著なGST-P巣形成の抑制が認められたにもかかわらず，DNA付加体8-OHdGの形成の抑制は認められなかった．そしてHTHQの抑制効果は，MelQXの代謝活性化の抑制によるものであったものと結論している．MelQXの発癌性には，同時にレドックスを介したnicotinamide adenine dinucleotide(NADH)の関与を経て酸化的DNA傷害が介在するものとの報告[17]もみられるので，双方の過程が存在するものと想定され，酸化ストレスによるレドックス制御の破綻がむしろ双方の過程にかかわっているものとも考えられる．

環境制御によるレドックス制御逸脱の修復

酸化ストレスを低く抑制することによって，レドックス平衡が逸脱するのを予防する試みが健康医学の立場から活発に進められている．筆者のこれまでにかかわってきた関連する研究の中から，カロリー制限による放射線白血病の抑制，茶カテキン類やTRX過剰発現マウスを用いた実験癌の予防などについて紹介し，最後に，アリールヒドロカーボン受容体結合食品に関する松井らの研究について簡単に解説して，本章の総論としての務めを果たしたい．

1. カロリー制限

カロリー制限の歴史は古く，1935年のMcCayの実験的試みにまでさかのぼる[18]．Yuらは，ラットを用いたカロリー制限実験で，寿命の延長にリンクした腫瘍死の遅延を観察した．興味深いことに彼らはこの実験でサテライト群をもうけて経時的に屠殺し，先の腫瘍死の遅延が腫瘍発生の遅延ではなく腫瘍の成長の緩徐化にあることを明らかにした[19]．このようにカロリー制限による腫瘍死の遅延・抑制のメカニズムが，腫瘍の発生そのもの，すなわち一次発癌に影響するのか，腫瘍の増生，すなわち二次発癌としてのプロモーションに影響するのかを明らかにするため，放医研の吉田らは，興味深いデザインの実験を行った[20-22]．放射線白血病誘発のための放射線1回照射(生後8週に施行)の前と後に分けて，カロリー制限を5週齢で開始し，照射後，通常食にもどす照射前カロリー制限群(①)，反対に照射までは通常食で飼育し，照射直後から制限する照射後カロリー制限群(②)，そして，照射前後を通して生涯カロリー制限を行う群(③)の各群を設けた．①はカロリー制限により，白血病の潜在的標的細胞である造血幹細胞の数量が減ることが知られているので，いわばカロリー制限のイニシエータに対する修飾因子としての役割に注目した群と考えられるのに対して，②は，照射後にカロリー制限を行うもので，いわばカロリーというプロモータ作用を抑制した状態に注目した群と考えることができる．結果としてカロリー制限は，③の生涯カロリー制限群でもっとも白血病の発症が抑制されたことはともかくとして，②でも発症が抑制されることがわかり，イニシエータ作用の方も，プロモータ作用の方にも，抑制効果があるものと考えられた(脚注*2)．意外にも①群は，照射後，通常食に戻すことにより急速に体重は増加し，カロリー制限を行わなかった通常食群における放射線白血病頻度と有意差はみられず，抑制効果はなかったので，ここで想定したカロリーの"プロモーション効果"の方が大きいことが示される結果となった．ここでカロリー制限は，エネルギー消費の抑制として酸化の防止に役割を果たしているわけであるが，そのことを実験的に明快に示した例として，Merryの行ったマイクロアレイによる解析[23]がある．その結果によれば，組織におけるレドックス状態は，蛋白や脂質，そしてDNAの酸化産物の蓄積が顕著であることはもちろんであるが，カロリー制限などと比較して明らかになる点は，むしろ，レドックス制御による転写活性化による細胞機能により大きな差が見出されるとして，これが，加齢過程のメカニズムを解き明かすことにつながる，としている．

2. 緑茶成分

緑茶成分の抗酸化作用は，今日では知らない人がないほどであるが，緑茶のどの成分が活性酸素の吸収にどのように作用するかの詳細や，その有効量と実際の飲茶量の関係などの細かい点にはつ

*2：ところで，この③の生涯カロリー制限群の白血病頻度は抑制されたものの，この群の寿命は，最長ではなかった．発育早期の過度なカロリー制限は，健全な老年期の維持に対して負の影響を及ぼすもののようである．

きない興味がある．ちなみに緑茶は，2-nitropropane(2-NP)のような一次発癌剤[24]に対しても，pentachlorophenol(PCP)のような二次発癌剤[25-27]に対しても発癌の抑制効果が観察されている(脚注*3)．発癌用量の2-NP投与群に緑茶成分として通常の飲茶の要領で熱湯抽出した茶を飲水として飲ませた群では，顕著なトリグリセリドの低下，8-OHdG付加体の無処置群レベルまでの低下，肝臓のBrdUrd標識率の同じく無処置対照群レベルまでの低下をみている．このときの飲茶に含まれていた緑茶成分は，epigallocatechin gallate(EGCG)で822 μg/ml，総カテキン量として1,606 μg/mlであったが，その総飲水量は，1日当りEGCG換算で118 ml，カテキン換算で106 mlのお茶に相当し，通常のヒトの日常摂取量に近い値であった．発癌機構のどの部分にEGCGが関与するのかを考えてみると；これらの抗酸化成分がプレイオトロピックに発癌のさまざまな部分に関与していることがわかる[27]．

3. 実験発癌予防

魚の焼けこげから抽出された変異原物質ヘテロサイクリックアミン類と酸化ストレスの関連については先に述べた．これは，チトクローム P450 の1A2による*N*-hydroxylationと一次酵素による，*N*-acetyl transferaseによってDNA傷害物質が生成され，これがDNA-付加体の形成に関与しているものと考えられている．そこで，抗酸化物質との併用による癌予防を念頭においた精力的な実験が行われている[17]．さまざまの合成抗酸化剤のうち，この報告では，HTHQにもっとも強い癌抑制が観察されているが，興味深いことに，これらの抗酸化物質がその抗酸化作用よりも，MeIQXの代謝活性化を傷害することによって発癌抑制に作用していることがわかったという．この解釈の正否は，代謝活性化による癌原性物質の生成機構をよく研究しないとわからない点があるが，前項の茶カテキンの作用機序のところでも述べたように，抗酸化物質の癌抑制機構は，多分にプレイオトロピックに働いていることが知られており，単一のメカニズムによるとは限らない点で，研究では注意して吟味する必要がある．実験癌予防としては，TRXの過剰発現マウスによる，酸化ストレス消去モデルがある．このものは，急性の酸化ストレス障害のみならず，ベンゼンによる白血病発症を抑制し，さらに寿命の延長をももたらした(本書，平林「ダイオキシンの生体影響と防御機構」の稿参照)．

4. アリールヒドロカーボン受容体(AhR)と結合する食品

シトクローム P450 の分子種1A1の発現を引き起こす転写因子としてHoffmannらおよび藤井らのグループによってそれぞれクローニングされたAhRはダイオキシン受容体とも呼ばれ，このものはダイオキシン類(TCDD)と結合するとArntとヘテロ二量体をつくり，DNAの特定領域に結合して，CYP1A1の転写の活性化を惹起する．この応答領域はXenobiotic Response Element(XRE)といい，TRXの発現調節領域にもこのXRE配列が見出されている[28]．松田らは，このAhRが，結合する生体内天然リガンドの未知なオーファン受容体であることに注目し，これを探索していく過程で，天然の生体物質としては尿中に排泄されるインディルビンがリガンドとなることを見出し[29]，さらに食品中にも多数のAhRリガンドがあることを見出した[30]．それらの中には，緑茶，ウーロン茶，コーヒー，リンゴジュース，などの飲料中に含まれているものもある．AhRの異物代謝機能は，TRXの制御を受けることが知られており[31]，これらの食品は，なんらかの形で異物代謝に関与するものと考えられるが，それが生体にとって吉の役割を持つのか，潜在的な凶の役割を持つのか，わかっていない．

おわりに

以上，レドックス制御にかかわる生体の反応機構について通覧した．各論では，いくつかの代表的なテーマに沿って，より詳細なメカニズムについて解説される．

*3：一次発癌，二次発癌については，本稿の「環境要因によるレドックス制御からの逸脱，1．電離放射線」の項参照．

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COMPARATIVE METABOLISM OF POLYCHLORINATED BIPHENYLS AND TISSUE DISTRIBUTION OF PERSISTENT METABOLITES IN RATS, HAMSTERS, AND GUINEA PIGS

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ABSTRACT:

The present study was performed to compare the metabolite profiles of polychlorinated biphenyls (PCBs) in the liver and serum of rats, hamsters, and guinea pigs after exposure to a PCB mixture, Kanechlor 500 (100 mg/kg, i.p.). The percentage of contribution of major PCB residues in the liver 5 days after exposure indicated that nonplanar PCBs with 2,4- or 2,3,4-chlorine substitution were more abundant in the liver in the order rats (43% of total PCBs) > hamsters (20%) > guinea pigs (11%), whereas coplanar PCBs with 4-, 3,4-, or 3,4,5-chlorine substitution were predominant in guinea pigs (61%), followed by hamsters and rats (both 26%). The hepatic concentrations of methylsulfonyl metabolites ($MeSO_2$-CBs) were higher in the order guinea pigs > rats > hamsters. Whereas hamsters formed minute amounts of $MeSO_2$-CBs from 2,5-dichloro-substituted PCBs, guinea pigs formed higher levels of *meta*-$MeSO_2$-CBs derived from 2,3,6-trichloro-substituted PCBs. In contrast, the serum concentrations of phenolic PCBs were higher in the order hamsters > rats > guinea pigs. Metabolites were predominated by 4-OH-2,3,5,3′,4′-pentaCB (89% contribution) for rats, 3-OH-2,4,5,2′,4′-pentaCB (56%) for guinea pigs, and dihydroxylated metabolites (39%) for hamsters. The reduced elimination of coplanar PCBs and the specific distribution of $MeSO_2$- and phenolic PCBs may have implications for the differences in sensitivity to PCB toxicity among rats, guinea pigs, and hamsters.

Metabolism of PCBs proceeds via P450-mediated formation of arene oxide intermediates, which results in the distribution of both OH-PCBs and $MeSO_2$-CBs (Letcher et al., 2000). The ratios of formation of these metabolites are dependent on the degree of substrate chlorination (Kato et al., 1980; Haraguchi et al., 1997) as well as the metabolic capacity of the species (Safe, 1994; Koga and Yoshimura, 1996). Some OH-PCBs and $MeSO_2$-CBs are persistent and show specific retention in the blood or tissues of laboratory and wild animals (Bergman et al., 1994a,b; Oberg et al., 2002) as well as in humans (Sandau et al., 2000; Chu et al., 2003). Phenolic PCB metabolites cause alterations in thyroid hormone metabolism (Morse et al., 1996; Brouwer et al., 1998), inhibit estrogen sulfation and binding to estrogen receptors for estrogenic or antiestrogenic effects (Connor et al., 1997; Kester et al., 2002), and give rise to potentially cytotoxic dihydroxylated or quinoid PCB metabolites (Amaro et al., 1996). On the other hand, some $MeSO_2$-CBs induce expression of several P450 isozymes (Kato et al., 1997), reduce thyroid hormone levels in rats (Kato et al., 1999), and also show antiestrogenic effects (Letcher et al., 2002). These persistent metabolites may be responsible for the toxic effects of PCBs.

Guinea pigs are the most sensitive experimental animals to the toxicity of coplanar PCBs and polychlorinated dibenzo-*p*-dioxins (Kociba and Cabey, 1985), whereas hamsters are less sensitive (Olson et al., 1980; Wroblewski and Olson, 1988). These differences in sensitivity may be due to metabolic activation by the unique P450-dependent monooxygenase system and substrate specificity in these species (Koga et al., 1998). For example, in vitro studies have shown that 3,4,3′,4′-tetraCB (CYP1A1) is hydroxylated by rats or hamsters but not by guinea pigs (Koga et al., 1995), whereas 2,4,5,2′,4′,5′-hexaCB (CYP2B18) is hydroxylated by guinea pigs but hardly by rats or hamsters (Ariyoshi et al., 1997). Therefore, the different metabolic capacities of PCBs may result in the differences in distribution profiles of metabolites in animals exposed to PCBs.

In the present study, we examined the species differences in tissue distribution of PCB residues and persistent PCB metabolites among rats, hamsters, and guinea pigs 5 days after exposure to a technical PCB mixture, Kanechlor 500. This article describes the 1) comparison of the residual PCB profiles (percentage of contribution) in the liver of these three species, 2) species differences in metabolite profiles of $MeSO_2$-CBs in the liver and of OH-PCBs in serum, and 3) GC/MS characterization of unknown metabolites retained in the serum of guinea pigs.

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ABBREVIATIONS: PCB, polychlorinated biphenyl; P450, cytochrome P450; OH, hydroxyl; CB, chlorobiphenyl; OH-PCB, hydroxylated PCB; $MeSO_2$-CB, methylsulfonyl PCB; GC/MS, gas chromatography-mass spectrometry; GC/ECD, gas chromatography-electron capture detection; MC, 3-methylcholanthrene.

TABLE 1

Residual patterns of PCBs in the livers of rats, hamsters, and guinea pigs dosed with Kanechlor 500 (100 mg/kg i.p.)

The 41 major isomers of a total of 61 were quantified and values represent the means for two animals.

Type	IUPAC	Structure	Relative Composition (%) of PCBs			
			Rat	Guinea Pig	Hamster	Kanechlor 500
Group A						
4-	28	2,4,4'-	0.08	1.03	0.15	0.86
	37	3,4,4'-	N.D.	6.01	0.04	0.15
	60	2,3,4,4'-	0.09	0.57	0.10	0.10
	74	2,4,5,4'-	0.72	4.33	0.74	0.54
	81	3,4,5,4'-	N.D.	0.19	0.02	0.007
	114	2,3,4,5,4'-	0.47	2.24	0.33	0.25
3,4-	77	3,4,3',4'-	0.02	1.82	0.06	0.03
	107	2,3,5,3',4'-	N.D.	2.46	N.D.	0.62
	118	2,4,5,3',4'-	17.6	32.2	18.6	7.68
	126	3,4,5,3',4'-	0.20	0.41	0.23	0.02
	156	2,3,4,5,3',4'-	4.15	5.87	3.33	1.25
3,4,5-	157	2,3,4,3',4',5'-	0.91	1.10	0.85	0.25
	167	2,4,5,3',4',5'-	1.26	2.22	1.74	0.45
		Sum of group A	25.5	60.5	26.2	12.2
Group B						
2,4-	47	2,4,2',4'-	0.18	0.003	N.D.	0.20
	49	2,4,2',5'-	0.11	0.04	0.05	0.83
	85	2,3,4,2',4'-	1.75	0.04	0.05	0.83
	99	2,4,5,2',4'-	12.6	2.44	3.45	3.22
	137	2,3,4,5,2',4'-	1.28	0.30	0.49	0.42
	147	2,3,5,6,2',4'-	0.27	0.02	0.02	0.11
2,3,4-	87	2,3,4,2',5'-	0.73	0.34	0.62	3.92
	105	2,3,4,3',4'-	5.16	3.92	4.44	2.99
	128	2,3,4,2',3',4'-	3.05	N.D.	N.D.	2.11
	132	2,3,4,2',3',6'-	0.17	N.D.	0.08	2.34
	138	2,3,4,2',4',5'-	13.7	2.22	8.81	5.56
	170	2,3,4,5,2',3',4'-	3.01	1.72	2.13	0.96
	177	2,3,4,2',3',5',6'-	0.71	0.04	0.08	0.23
	195	2,3,4,5,6,2',3',4'-	0.21	0.02	0.04	0.05
		Sum of group B	42.9	11.1	20.3	23.8
Group C						
2,4,5-	146	2,3,5,2',4',5'-	1.32	1.18	2.41	0.62
	153	2,4,5,2',4',5'-	12.1	10.3	17.0	5.36
	180	2,3,4,5,2',4',5'-	3.79	2.11	8.68	1.34
	183	2,3,4,6,2',4',5'-	1.30	0.35	1.33	0.41
	187	2,3,5,6,2',4',5'-	0.92	0.28	1.63	0.36
	203	2,3,4,5,6,2',4',5'-	0.51	0.05	1.21	0.15
2,3,4,5-	194	2,3,4,5,2',3',4',5'-	0.57	0.4	0.57	0.19
	201	2,3,4,5,2',3',5',6'-	0.32	0.27	0.94	0.10
		Sum of group C	20.8	15	33.8	8.53
Group D						
2,5-	52	2,5,2',5'-	0.08	0.36	1.40	5.56
	95	2,3,6,2',5'-	0.13	0.18	0.73	6.52
	101	2,4,5,2',5'-	1.35	2.15	4.68	10.0
	141	2,3,4,5,2',5'-	0.20	0.08	0.59	1.13
2,3,6-	110	2,3,6,3',4'-	0.35	0.16	0.41	7.44
	149	2,3,6,2',4',5'-	0.58	0.6	0.82	2.76
		Sum of group D	2.69	3.53	8.63	33.4
		Total %	91.9	90.0	88.9	77.9

N.D., not detected (< 0.002%).

Materials and Methods

Chemicals. The standard reference compounds, methoxy-PCBs and $MeSO_2$-CBs, were synthesized as described previously (Haraguchi et al., 1987; Bergman et al., 1995). Syntheses of veratrolic PCBs will be described elsewhere. The chemical names of $MeSO_2$-CBs and OH-PCBs were simplified on the basis of the IUPAC-derived numbering system of the parent PCBs. All solvents (*n*-hexane, acetone, ethanol, and dichloromethane) were of pesticide grade (Kanto Chemical Co., Tokyo, Japan).

Animals. Male Wistar rats (b.wt. 190–200 g), male Hartley guinea pigs (280–290 g), and male Golden Syrian hamsters (80–90 g) were housed three or four per cage in the laboratory with free access to commercial chow and tap water. Animals received a single i.p. injection of 100 mg/kg Kanechlor 500 dissolved in corn oil. Control animals received an equivalent volume of vehicle alone. The animals were sacrificed by decapitation 5 days after injection, and the liver, lungs, and serum were removed and stored at −20°C until analysis.

Isolation of Metabolites. Tissue samples were treated as described previously (Haraguchi et al., 1998). Briefly, tissues were homogenized and extracted with acetone/*n*-hexane (2:1, v/v). Two internal standards, 2,3,4,5,3',4',5'-heptaCB (70 ng) and 4-methyl-3-$MeSO_2$-5,2',3',4',5'-pentaCB (24 ng), were added to each extract, and the mixtures were applied to a gel-permeation column packed with Bio-Beads S-X3 (50 g; Bio-Rad, Hercules, CA). Dichloromethane/*n*-hexane (1:1) was used as a mobile phase at a flow rate of 4 ml/min. The metabolite fraction (120–200 ml) was divided into phenolic and neutral fractions by partitioning with potassium hydroxide (0.5 M in 30% ethanol) and *n*-hexane. The potassium hydroxide phase was acidified with hydrochloric acid (2 M, 10 ml) and extracted with *n*-hexane/diethyl ether (9:1), and then methylated with diazomethane for 30 min at 4°C. The neutral fraction (*n*-hexane layer) was further subjected to silica gel column chromatography (1 g; Wakogel S-1; Wako Pure Chemicals, Osaka, Japan) by elution with 10 ml of *n*-hexane for PCBs and successively with 10 ml of dichlorometh-