Comparison in Metabolic Activity of Cytochrome P450 1A1 on Heterocyclic Amines between Human and Rat

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In mutagenicity and antimutagenicity tests, the toxicants have been activated to the ultimate mutagenic forms usually with rat cytochrome P450 (CYP) enzymes. An understanding is important of whether these data can be available for human. In this paper are compared the activating abilities of CYP1A1 between human and rat using recombinant yeast cells that express respective CYP1A1 and yeast NADPH−CYP−oxidoreductase simultaneously. Three different types of dietary procarcinogens, heterocyclic amines, were tested by two methods: a bioassay with *Salmonella* mutagenicity test and a chemical determination of *N*-hydroxyls as the ultimate mutagenic forms. Compared with ED_{50} values, saturation levels, and V_{max}/K_m values at an initial stage of the enzyme activity, human and rat CYP1A1 showed almost similar abilities for the metabolic activation on heterocyclic amines. The two enzymes also had the same preference for the tested procarcinogens and the same affinities to the specific inhibitors such as flavonoids.

Keywords: *CYP1A1; rat and human; heterocyclic amine; mutagenicity; N-hydroxylation*

INTRODUCTION

Mutagenicity in vitro tests such as the *Salmonella* test have provided many data in toxicity evaluation of dietary or environmental products. They have been also used for the examination of antimutagens against various carcinogens (Edenharder et al., 1993; Arimoto et al., 1993; Kanazawa et al., 1995). These tests generally use mammalian enzymes such as rat S9 fraction including cytochrome P450 (CYP) monooxygenases when metabolic activation of the toxicants is required (Ames et al., 1975; Smith et al., 1992). The CYP enzymes can activate various procarcinogens to the ultimate mutagenic forms. Therefore, it becomes important to understand the difference in the procarcinogen activations between human and rat enzymes. In environmental procarcinogens, several studies have indicated a difference between the two in terms of the CYP activity (Li et al., 1994; Doehmer et al., 1995).

We are interested in the CYP activity against food-derived procarcinogens because epidemiological studies clearly show that daily diet is closely associated with carcinogenesis (Doll and Peto, 1981). The most abundant procarcinogens in our diet are heterocyclic amines formed during the cooking process (Matsukura et al., 1981; Knasmüller et al., 1992). Humans are estimated to take in 0.4−16 μg of heterocyclic amine per day per capita (Wakabayashi et al., 1992). All heterocyclic amines are activated via the same metabolism and exhibit genotoxicity (Wakata et al., 1985; Kato and Yamazoe, 1987; Frandsen et al., 1990; Turesky et al., 1991). The primary step of metabolic activation, *N*-hydroxylation, is mediated by CYP1A family enzymes, 1A1 and 1A2 (Funae and Imaoka, 1993; Minamoto and Kanazawa, 1995; Butcher et al., 1996). Distributions of both these enzymes were demonstrated by northern blot analysis in normal human adult by Shimada et al. (1996). Liver expresses both CYP1A1 and CYP1A2, whereas other tissues, such as pancreas, thymus, prostate, small intestine, and colon, express mainly 1A1. It is also known that an environmental toxin such as dioxin induces CYP1A1 through the transformation of an aryl-hydrocarbon receptor and then the binding of a dioxin-response element upstream of the CYP1A1 gene (Gonzalez and Nebert, 1990; Lu et al., 1996). Thus, CYP1A1 is one of the major enzymes involved in dietary carcinogenesis.

To elucidate differences of CYP1A1 between human and rat, the present study used microsomes from recombinant yeast strains expressing human and rat CYP1A1 genes. The activity was compared by *Salmonella* bioassay for mutagenicity production and chemical analysis for *N*-hydroxylation of heterocyclic amines. More than 16 heterocyclic amines have been identified in grilled or broiled foods to be mutagenic and carcinogenic. They are classified according to chemical structure into three groups (Wakabayashi et al., 1992; Knitz and Felton, 1992): the indole type, such as 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2); the quinoline type, such as 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ); and the pyridine type, such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Among the three heterocyclic amines, we found that human and rat CYP1A1s had similar characters for the metabolic activation.

EXPERIMENTAL PROCEDURES

Materials. Trp-P-2 was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), and IQ and PhIP were from Funakoshi Co., Ltd. (Tokyo, Japan). For the yeast cultivation, yeast nitrogen base without amino acids was obtained from Difco Laboratory (Detroit, MI). Zymolyase was from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Nutrient broth and agar for the bacterial cultivation were from Difco Laboratory and

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Funakoshi Co., Ltd., respectively. Solvents for a high-performance liquid chromatography (HPLC) were from Nacarai Tesque (Kyoto, Japan). Flavonoids, galangin, luteolin, and quercitrin, were from Extrasynthèse S.A. Co. (Genay, France), and flavanone was from Wako Pure Chemical Industries. All other chemicals were of the highest grade available commercially.

Recombinant Yeast Strains. Yeast *Saccharomyces cerevisiae* strains AH22/pA1A1IRR and AH22/pAMR2 (Sakaki et al., 1994) were a gift from Prof. Ohkawa. The former strain expresses human CYP1A1, which was cloned from a human liver cDNA library, and the latter expresses rat CYP1A1. Both cells simultaneously express yeast NADPH−CYP−oxidoreductase, because CYP enzymes require the reductase for a high level of activity (Murakami et al., 1990). The cells were transformed with each of the CYP1A1 expression plasmids. The plasmids were constructed with pAHRR, which contains alcohol dehydrogenase promoter, terminator, and the expression unit for the gene of yeast reductase.

Preparation of the Yeast Microsomes. The recombinant yeast strains were subjected to a microsomal preparation as described previously (Kanazawa et al., 1998a). Each was cultured on a medium composed of 8% glucose, 5.4% yeast nitrogen base without amino acids, and 0.016% histidine for 3 days while the CYP contents in cells were monitored. The cells were collected by centrifugation at 8000 rpm for 5 min and successively washed with water and a 10 mM Tris-HCl buffer (pH 7.5) containing 2 M sorbitol, 0.1 mM dithiothreitol, and 0.1 mM ethylenediaminetetraacetate. The cell suspension in the Tris-HCl buffer (200 mL) was mixed with 60 mg of zymolyase and incubated at 30 °C for 1 h. The mixture was then centrifuged and washed twice with the Tris-HCl buffer. The cell precipitate was resuspended in a Tris-HCl buffer containing 0.65 M sorbitol, 0.1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetate, and 1 mM phenylmethanesulfonyl fluoride and then sonicated for 1 min twice. The microsomal fraction was obtained by centrifugation at 105000*g* for 70 min of the supernatant from the 10000*g* for 10 min spin and was resuspended in a 10 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 1 mM phenylmethane sulfonyl fluoride. The CYP content was determined by the reduced CO difference spectrum (Omura and Sato, 1964). The protein content in the microsomal fraction was measured by the method of Bradford (1976).

Mutagenicity Test. *Salmonella typhimurium* TA98 strain, being specific to the mutagenicity of all heterocyclic amines (Smith et al., 1992; Wakabayashi et al., 1992), was used, and the previous method (Kanazawa et al., 1998) was modified by an addition of preincubation with heterocyclic amines and microsomes. Briefly, the bacteria were cultured in the medium of nutrient broth overnight. Trp-P-2 in sterilized water or IQ and PhIP in dimethyl sulfoxide were diluted to various concentrations with 0.1 M sodium phosphate buffer (pH 7.4) just before use. They were preincubated with the microsomes in 0.5 mL of phosphate buffer containing 2 mM NADPH at 37 °C. The enzymatic reaction was stopped by heating at 100 °C for 20 s. After cooling, the solutions were mixed with 0.1 mL of the bacterial suspension and incubated at 37 °C for another 10 min. They were added to 2 mL of molten top agar and poured onto an agar medium of minimal glucose. After a 2-day culture, the His^+-revertant colony number was counted. Every revertant number increased by mutagens was calculated as minus spontaneous revertants (23 ± 1, $n = 612$) without mutagens. These assays were performed independently in triplicate with three plates each time.

Determination of Microsomal *N*-Hydroxylation Activity on the Heterocyclic Amines. *N*-Hydroxylation activity of the microsomes on Trp-P-2, IQ, and PhIP was measured with the respective *N*-hydroxyl products by HPLC (Shimadzu HPLC, LC-6AD). The products were determined with an electrochemical detector at +300 mV (IRICA Σ875), which was specific to *N*-hydroxyls as mentioned previously (Minamoto and Kanazawa, 1995). After the incubation of heterocyclic amines with the microsomes, a 0.5-mL aliquot of the mixture was immediately added to cold acetonitrile (0.5 mL) and centrifuged at 3500 rpm for 3 min. The resulting supernatant (10 μL) was analyzed by HPLC with an Inertsil column ODS (∅ 4.6 × 150 mm) maintaining the temperature at 40 °C. The mobile phase was a mixed solvent of 20 mM monobasic potassium phosphate (pH 4.6)/acetonitrile (80:20) for *N*-hydroxy-Trp-P-2, the same solvent (90:10) for *N*-hydroxy-IQ, and 20 mM sodium dihydrogenphosphate (pH 4.6)/acetonitrile (70:30) for *N*-hydroxy-PhIP. Every solvent contained 0.1 mM ethylenediaminetetraacetate disodium salt, and the flow rate was 1.0 mL/min. The production of these *N*-hydroxyls was estimated with the calibration curve of *N*-hydroxy-Trp-P-2 (Minamoto and Kanazawa, 1995). The determinations were carried out independently in quadruplicate on one occasion.

Table 1. Characteristics of the Microsomes from Recombinant Yeast Strains Expressing Human and Rat CYP1A1s

yeast strain	CYP content[a] (nmol/mg of protein)	ethoxycoumarin deethylase activity[b] [mol min^{-1} (mol of CYP)$^{-1}$]	reductase activity[c] [μmol min^{-1} (mg of protein)$^{-1}$]
AH22/pA1A1IRR	0.096	20.0	0.803
AH22/pAMR2	0.31	38.2	1.65

[a] CYP contents were determined with the CO difference spectrum as mentioned under Experimental Procedures. [b] The 7-ethoxycoumarin *O*-deethylase activity was evaluated with the method of Sakaki et al. (1985). With this activity, the CYP content in original yeast cell AH22/pAAH5 was calculated to be <0.075 pmol/mg of microsomal protein. [c] NADPH−CYP−oxidoreductase activity was measured from the rate of reduction of cytochrome *c* (Sakaki et al., 1994).

RESULTS

Characteristics of Microsomes from the Cells of Expressed Human and Rat CYP1A1. Microsomes from AH22/pA1A1IRR and AH22/pAMR2 cells showed a single band for CYP1A1 on Western blotting analysis with a commercial CYP1A1 antibody (data not shown). Table 1 shows the characteristics of these microsomes. They contained 0.096 and 0.31 nmol of CYP/mg of protein, respectively, and showed as much activity as *O*-deethylase against 7-ethoxycoumarin. The microsomes also had 0.803 and 1.65 μmol min^{-1} (mg of protein)$^{-1}$ of NADPH−CYP−oxidoreductase activity, respectively. Thus, both cells specifically expressed CYP1A1s and the yeast reductase. In contrast, microsomes of AH22/pAHH5, which is an original yeast cell before the recombination, had negligible activities for the above enzymes when measured with a concentration of up to 10 mg of protein. In subsequent experiments, the microsomal activities per CYP1A1 nanomole were determined.

Comparison in Mutagen Production Rate between Human and Rat Enzymes. The activity was first compared in terms of production of mutagen from heterocyclic amines by *Salmonella* test. The mutagen production depended upon the concentrations of CYPs and incubation time, as it is known that the decrease in mutagenicity is due to further metabolism of the products by the CYP enzymes (Hiramoto et al., 1988; Wild et al., 1991). We determined a suitable concentration of human and rat CYP1A1s: 0.01 nmol for 0.05 μM Trp-P-2, 0.1 μM IQ, and 2.25 μM PhIP (Figure 1). Under the fixed concentration system of heterocyclic amines, both CYP1A1s increased linearly the mutagenicity of Trp-P-2 until 4 min into the incubation with a similar activation rate. Toward IQ, the human type produced mutagenicity time dependently until 8 min into the incubation, whereas rat CYP1A1 had a weak activity.

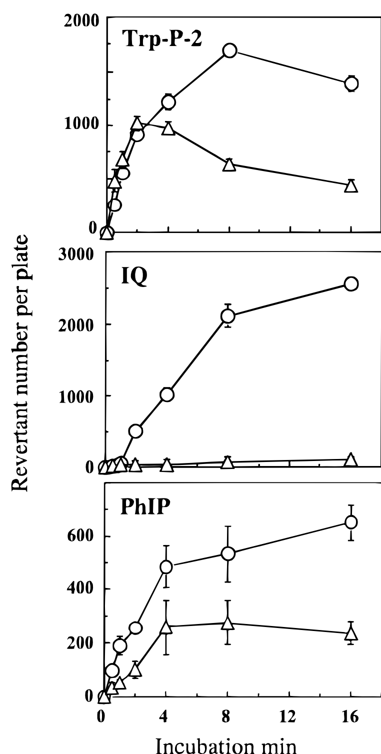

Figure 1. Mutagenicity produced by heterocyclic amines with CYP1A1s of human (○) and rat (△). The microsomes of AH22/pA1A1IRR (expressing human CYP1A1 gene) and AH22/pAMR2 (rat) cells were incubated at concentrations of 0.01 nmol of CYP1A1 with 0.05 μM Trp-P-2, 0.1 μM IQ, and 2.25 μM PhIP in 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4) containing 2 mM NADPH. The mutagenicity was evaluated by *Salmonella* test as mentioned under Experimental Procedures.

Against PhIP, both enzymes increased the mutagenicity linearly until 4 min, and the human type was the more active.

The results indicated that human and rat CYP1A1s increased the mutagenicity linearly in 2- and 1-min incubations, respectively. With these incubation times, then, the activities at the initial stage were determined kinetically using various concentrations of heterocyclic amines (Figure 2). Both CYP1A1s showed levels of activity dependent upon the concentrations of heterocyclic amines and gave polynomial fitting curves as shown in the figure. Using these curves, ED_{50} values and saturation levels for respective heterocyclic amines were determined (Table 2). On the other hand, V_{max}/K_m values are generally used to compare enzyme activities. The initial velocities within the limits increasing linearly on the concentration of heterocyclic amines were plotted with the Lineweaver–Burk method, and K_m and V_{max} values were also determined (Table 2). Human CYP1A1 exhibited close values of ED_{50} and saturation for Trp-P-2 and PhIP and values 4-fold higher for IQ than rat CYP1A1. In the V_{max}/K_m values, the activity of human type was in a similar range as that of its rat counterpart. Consequently, both CYP1A1s showed the same preference for mutagenicity: Trp-P-2 > IQ > PhIP.

Comparison in *N*-Hydroxylation Activity on Procarcinogens between Human and Rat Enzymes. The human and rat CYP1A1s were further compared as to *N*-hydroxylation of heterocyclic amines. The *N*-hydroxyl products can be determined specifically with an electrochemical detector at +300 mV, and this

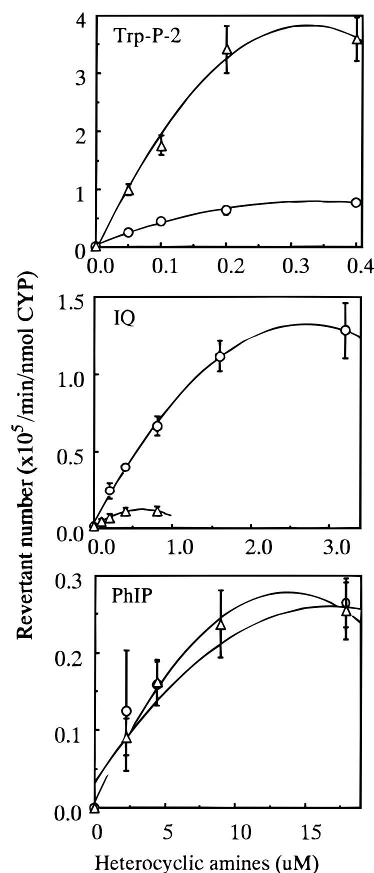

Figure 2. Mutagenicity production by human and rat CYP1A1s with the concentrations of heterocyclic amines. Human (○) and rat (△) CYP1A1s at 0.01 nmol each were incubated for 2 and 1 min, respectively, in the same system as in Figure 1. Curves are a polynomial fitting.

Table 2. Comparison in the Activity between Human and Rat CYP1A1s with Kinetic Parameters of the Mutagen Production and *N*-Hydroxylation toward Heterocyclic Amines

	human CYP1A1 on			rat CYP1A1 on		
	Trp-P-2	IQ	PhIP	Trp-P-2	IQ	PhIP
	Mutagenicity Production[a]					
ED_{50}	0.091	0.75	4.0	0.093	0.13	3.8
saturation level	0.29	2.5	15	0.26	0.64	12
K_m	0.19	1.5	3.3	0.58	1.2	28
V_{max}	1.2	1.9	0.25	12	0.38	0.96
V_{max}/K_m	6.3	1.3	0.076	21	0.32	0.034
	N-Hydroxylation[b]					
ED_{50}	3.0	48	3.9	2.8	18	3.1
saturation level	11	160	13	9.1	58	11
K_m	4.5	34	3.1	10	12	1.5
V_{max}	2.2	2.4	14	39	12	22
V_{max}/K_m	0.49	0.071	4.5	3.9	1.0	15

[a] ED_{50} and saturation level were determined with polynomial fitting curves in Figure 2, and K_m and V_{max} were determined by Lineweaver–Burk plotting as mentioned in the text. Units for ED_{50}, saturation level, and K_m: μM of heterocyclic amines; for V_{max}, revertant number [$\times 10^5$ min^{-1} (nmol of CYP1A1)$^{-1}$]. [b] Similarly, they were calculated from the formation rates of *N*-hydroxylated products determined by HPLC. Units for ED_{50}, saturation level, and K_m, μM of heterocyclic amines; for V_{max}, *N*-hydroxylated product mol min^{-1} (mol of CYP1A1)$^{-1}$].

method could determine the *N*-hydroxyls down to the level of 1 pmol, without interference from impurities (Minamoto and Kanazawa, 1995). Both CYP1A1s gave a single metabolite for Trp-P-2 at 7.0 min of retention time (data not shown). The metabolite had strong

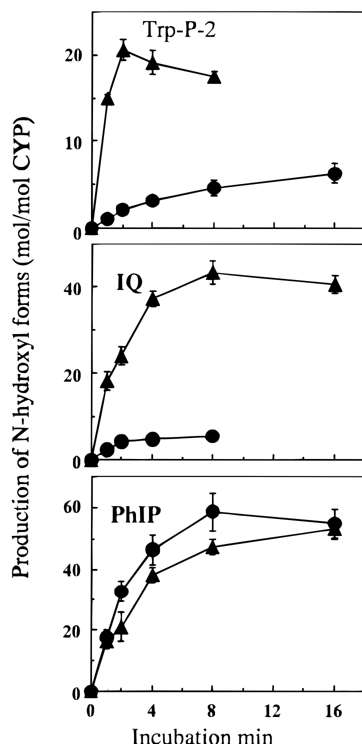

Figure 3. *N*-Hydroxylation activity of CYP1A1s of human (●) and rat (▲) on the heterocyclic amines. The microsomes of AH22/pA1A1IRR and AH22/pAMR2 cells in Figure 1 were used at concentrations of 0.03 nmol of CYP1A1 for 2.3 μM Trp-P-2, 0.02 nmol for 20 μM IQ, and 0.01 nmol for 2.7 μM PhIP in 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4) containing 2 mM NADPH. The production of the *N*-hydroxyls was determined by HPLC as mentioned under Experimental Procedures.

mutagenicity against *Salmonella* TA98. The retention time for HPLC and the mass spectrum of the metabolite with a liquid chromatography/mass spectrometer (Hitachi LC/MS M-1200H) coincided with those of a standard *N*-hydroxy-Trp-P-2 (Minamoto and Kanazawa, 1995). The CYP1A1s metabolized IQ to two products at 7.7 and 9.3 min of retention time. The former was half the latter in peak area but exhibited much stronger mutagenicity. The retention time and mass spectrum of the former peak coincided with data published for *N*-hydroxy-IQ (Yamazoe et al., 1983), and the mass spectrum of the latter suggested it to be 5-hydroxy-IQ (Vavrek and Weisburger, 1990). In PhIP, a single metabolite having strong mutagenicity appeared at 6.1 min and was identified by the retention time and mass spectrum to be *N*-hydroxy-PhIP (Frandsen et al., 1990). The *N*-hydroxylation activity was then compared between both CYP1A1s with these products, the respective single products of Trp-P-2 and PhIP, and the sum of two products of IQ after suitable conditions for the mutagenicity test had been established. Figure 3 shows that the hydroxyl productions were time-dependent when the CYP1A1s at 0.03, 0.02, and 0.01 nmol were incubated with 2.3 μM Trp-P-2, 20 μM IQ, and 2.7 μM PhIP, respectively. The human type was less active on Trp-P-2 and IQ but similarly active on PhIP compared to the rat type. The activity depending upon the concentration of heterocyclic amines was then measured with 2-min incubations (Figure 4), and ED_{50} values, saturation levels, and V_{max}/K_m were determined as shown in Figure 2. The values of ED_{50} and saturation were similar between the human and rat types for the

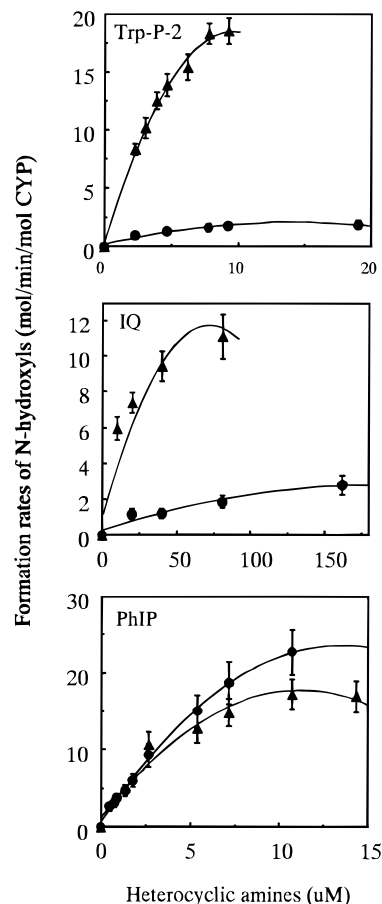

Figure 4. CYP1A1 activities with the concentrations of heterocyclic amines. Human (●) and rat (▲) CYP1A1s were used at 0.03 nmol each for Trp-P-2, 0.02 nmol for IQ, and 0.01 nmol for PhIP. After the 2 min incubation, the *N*-hydroxyls were determined as mentioned in Figure 3. Curves are a polynomial fitting.

respective heterocyclic amines (Table 2). Both types showed the same order preference: Trp-P-2 = PhIP > IQ. Comparison with the values of V_{max}/K_m showd that human CYP1A1 had 8-fold less activity on Trp-P-2 and 14-fold less activity on IQ than rat CYP1A1. However, both enzymes had the same order of preference: PhIP > Trp-P-2 > IQ.

Affinities to Inhibitors. Another interesting point of difference between the enzymes is in affinity to inhibitors. Flavonoids widespread in vegetables are found to be strong and specific inhibitors for CYP1A family enzymes (Lee et al., 1994; Siess et al., 1995; Kanazawa et al., 1998b). Table 3 shows the effects of one flavone, luteolin, two flavonols, galangin and quercetin, and flavanone on the mutagenicity produced from Trp-P-2. The inhibitory effects were evaluated with IC_{50} values for the antimutagenicity. The IC_{50} values refer to the amounts required for 50% inhibition of mutagenicity of Trp-P-2, and the smaller the value, the stronger the inhibitor. Toward both enzymes, luteolin and galangin were 1-order stronger as inhibitors than quercetin and flavanone. Comparing human and rat enzymes, luteolin was 4-fold weaker for human, galangin 2-fold weaker, and the others similar, and thus these inhibitory values are in the same range. These results indicated that human CYP1A1 was similar in character with the rat counterpart in affinity to inhibitor flavonoids.

Table 3. Inhibitory IC_{50} Values of Flavonoids on the Mutagen Production from Trp-P-2 by Human and Rat CYP1A1s

	IC_{50} (molar ratio to CYP1A1)[a] against	
inhibitor	human CYP1A1	rat CYP1A1
luteolin	6.7	1.5
galangin	6.9	13
quercetin	38	28
flavanone	44	29

[a] IC_{50} values are the amounts required for 50% inhibition of the mutagenicity of Trp-P-2. Various concentrations of flavonoids were added to the same incubation mixtures in Figure 1, and the antimutagenicity was determined by *Salmonella* test. The antimutagenicity was calculated as follows: $[(A - B) - (C - D)]/(A - B) \times 100$, where A is the number of revertants given by 0.1 nmol of Trp-P-2, B is the number of spontaneous revertants, C is the revertants by both flavonoid and Trp-P-2, and D is the revertants by each flavonoid. The IC_{50} values were determined by plotting the antimutagenicity against the log of the dosed amount of flavonoids.

DISCUSSION

This comparative study reveals that human and rat CYP1A1s have similar abilities for the metabolic activation of heterocyclic amines. In ED_{50} values and saturation levels for three heterocyclic amines, the coincidences between both the enzymes were almost found (Table 2). In the V_{max}/K_m values, mutagenicity production was in a similar range, but *N*-hydroxylation activity for human enzyme was a little different from that for rat enzyme. The latter analysis method for enzyme activity usually implicates a variation at lower concentration of substrates. Additionally, an instability of *N*-hydroxyls probably facilitates expansion of the variation. In the present comparative study, thus, the ED_{50} values and saturation levels are considered to reflect the enzyme ability better than V_{max}/K_m values.

Interestingly, both CYP1A1s had the same preference for heterocyclic amines: Trp-P-2 > IQ > PhIP for the mutagenicity and PhIP ≥ Trp-P-2 > IQ for the *N*-hydroxylation activity (Table 2). The toxicity of heterocyclic amines has been generally examined with the *Salmonella* test using rat S9 enzymes or with rat itself. A similarity found here between enzymes of human and rat in mutagenicity and preference revealed that the results for the rat enzymes were available for human.

The antimutagens against heterocyclic amines were also examined with the same system. Flavonoids in vegetables and fruits were shown to inhibit the activation of heterocyclic amines using rat CYP1A1 specifically and strongly (Lee et al., 1994; Siess et al., 1995; Kanazawa et al., 1998b). The present study found that the inhibitory IC_{50} values of flavonoids were the same for human and rat enzymes (Table 3). Flavonoids are absorbed into the human body to a level of ~50% (Hollman et al., 1995). These indicate that dietary flavonoids have a possibility to prevent human cancer caused by heterocyclic amines.

A similarity in *N*-hydroxylation activity of CYP1A1s between human and rat remains another question in the carcinogenesis study. *N*-Hydroxyls are the primary products in the activation of heterocyclic amines and are further metabolized by acetyltransferases before being conjugated with sulfotransferase and glucuronidase (Kato and Yamazoe, 1987; Davis et al., 1993). Both the *N*-hydroxyls and acetoxyls make DNA adducts (Wakata et al., 1985; Frandsen et al., 1990; Turesky et al., 1991; Snyderwine et al., 1993). To better understand the carcinogenicity of heterocyclic amines, one should focus on the difference in the activity of phase II enzymes between human and rat (Yamazoe et al., 1989; Kaderlik et al., 1994).

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