Title Page:

Metabolic Activation of Heterocyclic Amines and Expression of Xenobiotic-Metabolizing Enzymes in the Gastrointestinal Tract of Rats

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ABSTRACT:

Heterocyclic amines (HCAs) get entry into human body mainly through ingestion of pan-fried meats cooked at high temperatures. Exposure of the gastrointestinal tract (GIT) to ingested xenobiotics prior to delivery to the liver may lead to metabolic activation, which may explain the high incidence of GIT carcinogenesis. Therefore, the current study investigated the mutagenic activation of two heterocyclic amines, 2-aminoanthracene (2-AA) and 3-amino-1-methyl-5*H*-prydo[4,3-*b*]indole (Trp-P-2), in the GIT of rats. Additionally, the constitutive mRNA expression profiles of xenobiotic-metabolizing enzymes (XMEs) in the GIT of rats were examined. Metabolic activation of 2-AA was detected in all GIT tissues except the duodenum and rectum, and it was detected at high levels in the ileum and cecum. Furthermore, we revealed high metabolic activation of 2-AA and Trp-P-2 in the jejunum. The mRNA expression of phase I and II enzymes in rat GIT corresponded with their mutagenic activation ability. In conclusion, our results suggest that different expression levels of XME among GIT tissues may contribute to the tissue-specific differences in metabolic activation of xenobiotics such as heterocyclic amines in rats.

Keywords: Gastrointestinal Tract, Xenobiotic-Metabolizing Enzymes, Heterocyclic Amines

Practical Application:

This study declares mutagenic activation of two heterocyclic amines namely 2-aminoanthracene (2-AA) and 3-amino-1-methyl-5*H*-prydo[4,3-*b*]indole (Trp-P-2), in the gastrointestinal tract (GIT) of rats. Additionally, results obtained in this study suggest that GIT tissue-specific expression of xenobiotic metabolizing enzymes may contribute to the tissue-specific mutagenesis/carcinogenesis.

Introduction

Heterocyclic amines' (HCAs) mutagens are formed during cooking of food, particularly meat, at high temperatures. Ingestion of contaminated foods is considered the major route of exposure to xenobiotics such as polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (Darwish and others 2010; Maté and others 2010; Ho and others 2014). Xenobiotic metabolism takes place predominantly in the liver although extra-hepatic tissues, including the gastrointestinal tract (GIT), have capacity for numerous phase I and II metabolic reactions. In this regard, special attention has been paid to understand the contribution of the GIT to the metabolic fate of xenobiotics, particularly HCAs.

Cytochrome P450 (CYP) is a class of phase I enzymes that comprises 28 families in mammals. The CYP superfamily represents the most important group of xenobiotic-metabolizing enzymes (XMEs) that activate promutagens. Particularly, the CYP1A subfamily catalyzes the metabolic activation of PAHs and HCAs (Nebert 1991; Ma and others 2007).

HCAs, including 2-aminoanthracene (2-AA) and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), are first metabolized by CYP1A through N-hydroxylation in microsomes. These metabolites have the ability to bind directly to DNA. However, the most active metabolites of HCAs are produced by sulfotransferase (SULT) or *N*-acetyltransferase (NAT) reactions mainly in the cytosol. These active metabolites are further conjugated by glutathione-*S*-transferase (GST) or UDP-glucuronosyl-transferase (UGT) in phase II detoxification pathways (Turesky 2007; Alaejos and others 2008; Takiguchi and others 2010) (Figures 1 and 2).

CYP1A1 and several phase II enzymes are upregulated by exposure to ligands of the aryl hydrocarbon receptor (Ghosh and others 2001). These enzymes are also regulated by NF-E2 p45-related factor 2 via the antioxidant response element in their promoter regions. Induction of these enzymes could enhance the risk of metabolic activation and promote DNA damage and cancer.

HCAs have been encountered in many short and long-term mutagenic and carcinogenic effects on animals and humans. For instance, rodents fed diets containing high levels of HCAs developed tumers in the stomach, colon, rectum, breast, prostate and skin (Sugmura and others 1996). Chronic ingestion of mutagenic substances (e.g., 2-amino-3-methylimidazo[4,5-*f*]quinoline) in cooked meat has been shown to cause DNA adduct and tumor formation in the colorectum of rats (Turesky and others 1996). Furthermore, epidemiological meta-analysis suggested a relationship between meat consumption level and risk of colorectal cancer in humans (Larsson and Wolk 2006).

We previously revealed that high levels of CYP1A1 are expressed in rat tongue tissue, and that this enzyme is involved in the metabolic activation of HCAs (Takiguchi and others 2010). Thus, it could be hypothesized that there is a strong relation between oral exposure to mutagens, xenobiotic-metabolizing ability in each GIT tissue, and cancer risk. However, there is limited information about the detailed profiles of XMEs and their metabolic activation in GIT tissues. Therefore, the current study compared the metabolic activation ability of HCAs among these tissues. Moreover, the possible metabolic activation mechanisms of HCAs in the GIT of the rat were investigated.

Materials and Methods

Chemicals

All of the test reagents used were of reagent grade, including those described below. TRI reagent was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Oligo(dT) primer, reverse transcriptase buffer, and ReverTra Ace were purchased from Toyobo (Osaka, Japan). The S9 cofactor was purchased from Oriental Yeast (Tokyo, Japan). All other reagents were of analytical grade or of the highest quality available; they were purchased from Wako Pure Chemical Industries (Tokyo, Japan).

Animals

All experiments using animals were performed under supervision and with the approval of the Institutional Animal Care and Use Committee of Hokkaido University (approved experimental No. 9128/10-0067). Wistar rats (male, seven weeks old, n = 5) were obtained from Japan SLC Inc. (Hamamatsu, Japan). They were housed in plastic cages with a 12 h/12 h light/dark cycle at 22 ± 1°C, and given laboratory chow and tap water *ad libitum*. All rats were sacrificed after one week using CO_2, after which the liver, tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, and rectum were immediately collected and frozen in liquid nitrogen. All tissues were kept at −80°C until use.

Preparation of S9 Fraction

S9 fractions were prepared using five individual samples as pool. Samples were prepared as pool because of the amount of available protein material in some samples like tongue was very small. Tissues were minced and homogenized in 10 volumes of ice-cold 0.1 M potassium phosphate buffer (KPB, pH 7.4, 1 mM EDTA, 1 mM DTT, 1 μM pepstatin, 20%

glycerol), using a Teflon homogenizer. Homogenized samples were centrifuged at 9,000 × *g* at 4°C for 20 min. Supernatants, which are the S9 fractions, were collected and frozen in liquid nitrogen and kept at −80°C until use. S9 fractions contain both microsomal and cytosolic fractions. Using S9 fraction instead of either microsomal or cytosolic fractions separate ensures working of all bio-activating and detoxification enzymes at the same time. Accordingly, this gives a fair judgement on the mutagenicity of the tested HCAs. Protein concentrations of the S9 fractions were determined by using the BCA protein assay kit (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's instructions.

Umu mutagenicity assay

The mutagenic assay (umu test) in the S9 fractions (4 mg protein/mL) were performed through the method of Oda and others (1995). The *O*-acetyltransferase over-expressing tester strain *Salmonella typhimurium* NM2009 was used. A commercial umu AT test kit (Protein Purify, Isesaki, Japan) was used according to the manufacturer's instructions. 2-AA (3 ng) was used as the mutagen. β-Galactosidase activity was determined by measuring the absorbance at 650 nm using a microplate reader (Labsystems Multiskan Ms-UV, Dainippon Sumitomo Pharma, Osaka, Japan). Each experiment was repeated at least three times at different times.

Ames mutagenicity assay

The Ames test was performed using the *S. typhimurium* strain TA98 and the S9 fractions (4 mg protein/mL) according to our previous modifications (Darwish and others 2010) of the preincubation method of Ames and others (1975). In brief, 500 µL of the S9 fraction containing the complete NADPH-generating system and NADH, as well as 100 µL of an overnight culture of TA98 and coenzymes (30 mM GSH and 15 mM UDPGA, or 0.1 M potassium

phosphate buffer as a control) were added to the test tubes. Afterward, 100 µL of the mutagen Trp-P-2 (200 ng/mL) or deionized sterilized ultrapure water (negative control) was added, and the tubes were incubated for 20 min at 37°C in a rotary water bath. The reaction was stopped by the addition of 2 mL of top agar, and the contents were poured onto a minimum glucose medium plate. The plates were incubated for 48 h at 37°C, and the number of revertant colonies was counted manually. Each experiment was repeated at least three times at different times.

Total RNA Isolation, cDNA Synthesis, and Real-Time PCR

Total RNA isolation and cDNA synthesis were carried out according to our previous protocol (Takiguchi and others 2010). Quantitative real-time PCR for rat mRNA levels was performed using TaqMan Gene Expression Assays (Applied Biosystems, CA, U.S.A.) and measured on a StepOnePlus™ real-time PCR system (Applied Biosystems). The primer and probe sets for each specific gene were as follows: Rn00756113_AH (UGT1A6), Rn02769895_g1 (SULT1C2), and pre-developed TaqMan® assay reagents (β-actin). Oligo@Sigma dual labeled probe sets for microsomal epoxide hydrolase (mEH) (Sigma-Aldrich) were as follows: forward primer, 5'-cccactactgactgaccccaa-3'; reverse primer, 5'-caagaaaggtttaaattcggtggc-3'; and probe, (tcccacggtctgagtgacgagcacg). The reaction was performed for 40 cycles according to the following program: initial activation at 95°C for 20 sec, denaturation at 95°C for 1 sec, annealing and extension at 60°C for 20 sec. The measurements of these genes were performed in duplicate and repeated three times from each individual sample. The expression of each gene was normalized to the expression of β-actin, and was calculated relative to that of the liver by using the comparative threshold cycle (Ct) method.

In addition, the following specific primers were used to measure the expression of *CYP1A1* and *NAT* genes in the SYBR Green quantitative PCR. CYP1A1: forward primer, 5'-tttgtcccattcaccatccc-3'; reverse, 5'-gtctccccaatgcactttcg-3'. NAT1: forward, 5'-ccaaacatggcgaactcgt-3'; reverse, 5'-gaagatacaggtcattagttgatcaatattg-3'. NAT2: forward, 5'-gtgcctaaacatggtgatcgatt-3'; reverse, 5'-acatggtcagaagtatgtccttgtc-3'. β-actin: forward, 5'-ggaatggcaagaccaag-3'; reverse, 5'-agggtattgcgagcagatgg-3'. To measure the expression levels of these genes, SYBR Green Real-Time PCR Master Mix (Finnzyme, Espoo, Finland) was used. The conditions of the PCR reaction were as follows: the initial cycle was 50°C for 2 min and 95°C for 10 min, and then 45 cycles were performed at 95°C for 15 sec and 60°C for 1 min. β-Actin was used for normalization in the comparative Ct method. There was no difference in the PCR efficiency among primer sets.

Statistical Analyses

Results of mRNA expression and mutagenic assays (umu test and Ames test) are expressed as the mean ± standard deviation (SD). Statistical significance was assessed using the Tukey's honestly significant difference (HSD) test. Differences of $P < 0.05$ were considered to be statistically significant. The statistical package JMP v. 9.0 (SAS Institute Inc., NC, U.S.A.) was used for statistical analyses.

Results and Discussion

Metabolic activation ability in the rat GIT was investigated using umu and Ames mutagenicity assays. Interestingly, all GIT tissues exhibited metabolic activation in the presence of 2-AA, except for the duodenum and rectum. In contrast, all tissues showed variable degrees of mutagenic activation abilities toward Trp-P-2. The jejunum, ileum, and cecum showed the highest mutagenic abilities in comparison with other tissues (Figure 3A, B). This result corresponds to findings of Takayama and others (1989), Paulsen and others (1999), which demonstrate that metabolic activation of HCAs cause mutagenicity in the stomach, small intestine, and colon of rats. Our *in vitro* mutagenic assays (umu and Ames tests) revealed equal opportunities for genotoxicity after HCAs exposure and subsequently cancer incidence in different tissues. In agreement with our findings, Dingley and others (1999) recorded a comparable amount of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) DNA adducts in both the ileum and colon in humans. However, the *in vitro* mutagenic assays like the umu and Ames test are different from *in vivo* assays, since they do not include detoxification enzymes, such as UGT, GST, and SULT. Considering this point, we identified a need to clarify the causes involved in the tissue differences for cancer risk. Thus, investigation of the profile of XMEs in the GIT of rats may explain that point. In particular, there is limited information about the expression profile of XMEs in the GIT of rats. Additionally, CYP1A2 has been reported to be involved in the metabolic activation (N-hydroxylation) of 2-AA (Jemnitz and others 2004). However, no expression of CYP1A2 in the GIT of rats has been detected (Hernandez-Martinez and others 2007). Tissue distribution of XME expression was investigated in order to explain the tissue-dependent differences in their mutagenic activation. We observed different

expression profiles of XME mRNA, such as CYP1A1, mEH, NAT1, NAT2, SULT1C2 and UGT1A6. In agreement with the high mutagenic activation ability in the jejunum, there were also higher expression levels of CYP1A1 observed in the jejunum and ileum (Figure 4A). This finding goes in line with Blanchard and others (2000), who found that these tissues are frequent sites for leiomyoma formation. Since the CYP1A family is involved in the metabolic activation of mutagenic substances, such as PAHs and HCAs, high expression levels of CYP1A1 could have an important role in tumor formation in these tissues and could thus explain the high mutagenic ability of the two tested HCAs in the jejunum. Similar to CYP1A1, mEH plays an important role in the metabolic activation of benzo[a]pyrene (B[a]P) (Kim and others 1998). However, in the current study, there were no clear relationships in the tissue distribution of mRNA expression between mEH and CYP1A1. CYP1A1 was highly expressed in the small intestine, whereas mEH was highly expressed in the upper GIT (Figure 4B). Interestingly, both CYP1A1 and mEH mRNA expressions were high in the esophagus. Relationships between smoking and risk of esophageal cancer have been reported (Gammon and others 1997). Cigarette smoke contains B[a]P, which is metabolically activated by these enzymes (Kaiserman and Rickert 1992). Furthermore, it was reported that several enzymes, such as CYP1A and mEH, could contribute to esophageal cancer (Murray and others 1994). Taking together these previous findings and our results, it is possible that the esophagus has high capacity for metabolic activation of PAHs.

NAT1 mRNA expression levels in the cecum, colon, and rectum were higher compared with those in other tissues (Figure 5A). NAT2 mRNA expression levels in the cecum (29.5-fold), colon (10.4), and rectum (9.3) were higher than those in the liver (Figure 5B). NAT is also important for the detoxification and excretion of carcinogenic chemicals, such as 4-aminobiphenyl (Hein

and others 2006). However, HCAs can be metabolically activated by NAT and SULT (Alaejos and others 2008). Our results show that NAT was highly expressed in the large intestine. Therefore, the metabolic activation ability of HCAs in this tissue was high. Since PhIP has been reported to be a cancer-inducing chemical in the large intestine (Hagiwara and others 2001), the high sensitivity of HCAs in the large intestine could be related to high expression levels of NAT.

SULT is important as a detoxification enzyme and contributes to sulfoconjugation of endogenous substances, such as steroid hormones and amines (Berger and others 2011). The stomach showed the highest mRNA expression of SULT1C2, and it was 1.9 times higher than that in the liver (Figure 6A). Comparison of UGT1A6 mRNA levels among tissues in the GIT revealed that mRNA levels in the esophagus and stomach were seven times higher than those in the duodenum, jejunum, ileum, and cecum (Figure 6B). UGT is known to have a role in glucuronic acid conjugation and detoxification of many different kinds of intermediate metabolites in mammals (Williams and others 2004). UGT also contributes to the detoxification of PAHs and HCAs (Zheng and others 2002; Turesky 2007). In the present study, we revealed low expression level of UGT1A6 in the small intestine. These results suggest that the detoxification ability of the intestinal tract is low compared with the stomach and esophagus for mutagenic substances. Unlikely, CYP1A1, which is involved in the metabolic activation of the mutagenic substances such as PAHs and HCAs, is highly expressed in the intestine compared with the stomach. This finding highlights the contribution of XMEs expression in the mutagenesis and carcinogenesis among the GIT. Future studies are still needed to further evaluate other HCAs such as quinolines and quinoxalines.

Conclusion

The results of this study declares mutagenic activation of 2-AA and Trp-P-2 along the rat GIT. Additionally, different expression profiles of XME among GIT tissues may contribute in the differences in metabolic activation of xenobiotics, such as HCAs, in the rat.

Acknowledgments

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Author Contributions

W. S. Darwish conducted part of the experiments, drafted the manuscript and interpreted the results. S. M. Nakayama drafted the manuscript, interpreted the results and performed statistical analysis. Y. Itotani conducted part of the experiments. M. Ohno collected the test data. Y. Ikenaka designed the study and interpreted the results. M. Ishizuka designed the study, supervised the work and interpreted the results.

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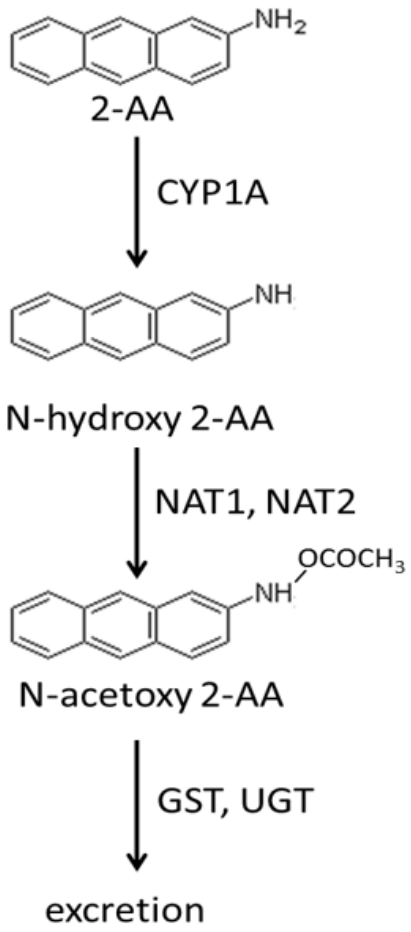

Figure 1. Metabolic pathway of 2-AA.

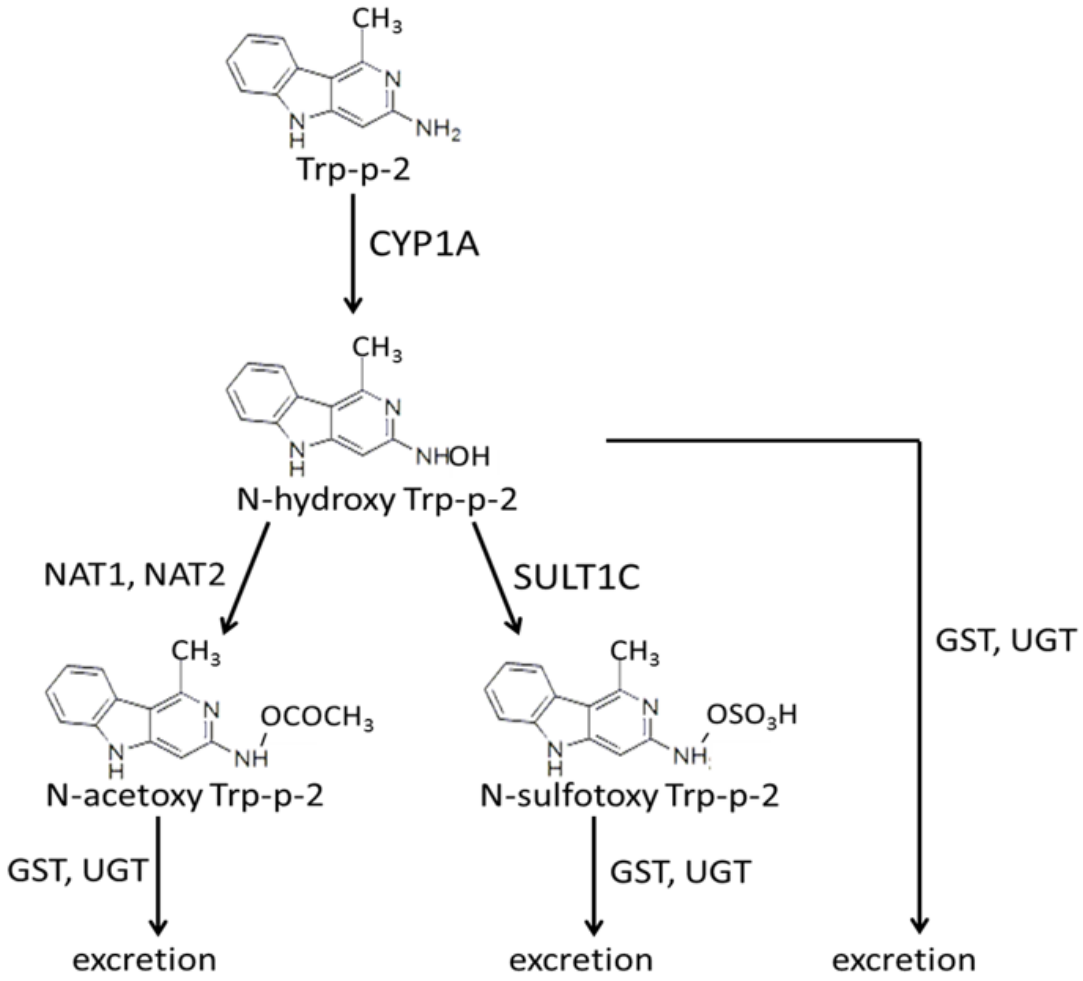

Figure 2. Metabolic pathway of Trp-P-2.

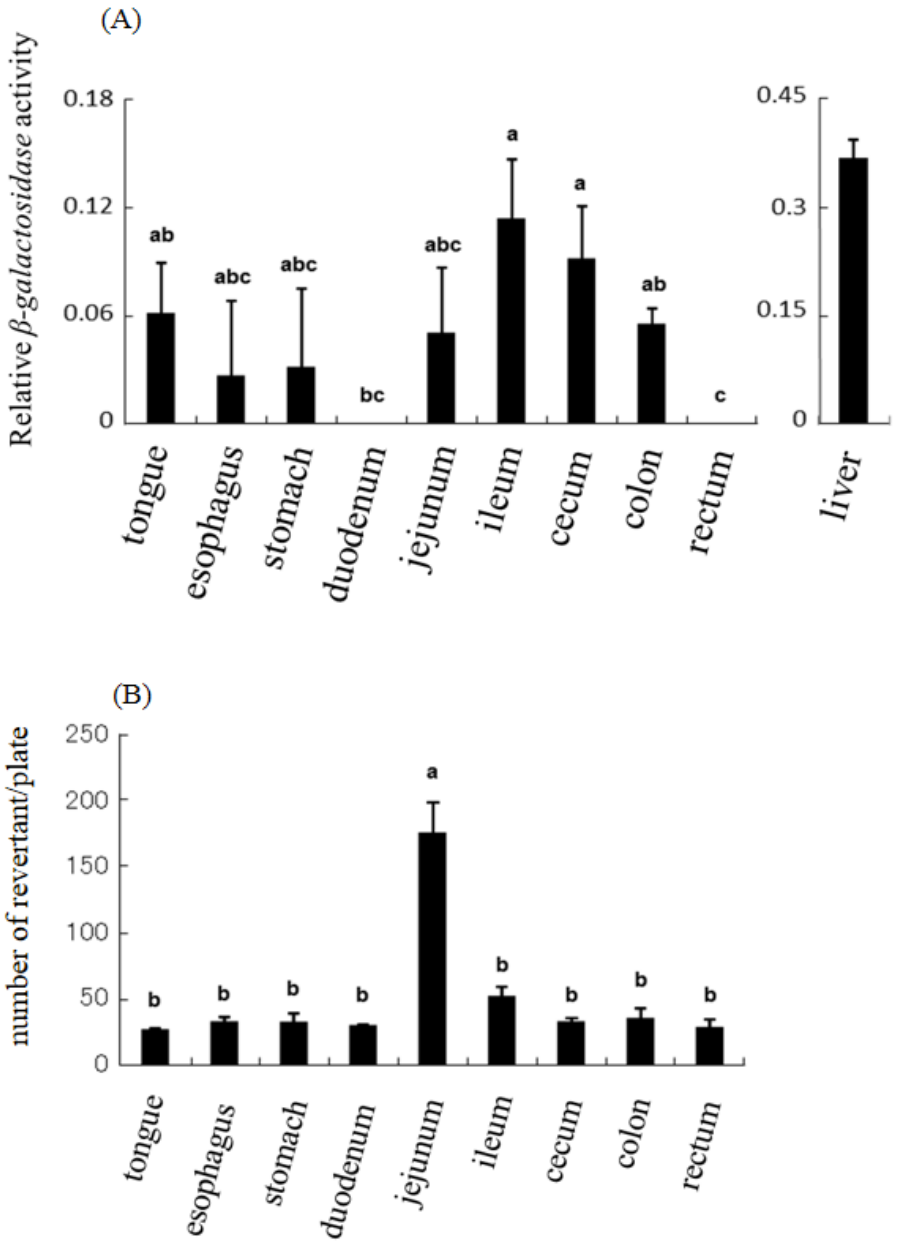

Figure 3. Metabolic activation of HCAs in various rat tissues.

(A) The S9 fractions in various tissues of rats in the umu test used to detect 2-AA metabolic activation. Data are presented as the mean ± SD (n=5). Identical letters are not significantly different from each other, as determined by the Tukey's HSD test ($P < 0.05$). (B) Metabolic activation of Trp-P-2 was detected by the Ames test using the S9 fractions of various tissue samples from rats. Data are presented as mean ± SD (n=5). Identical letters are not significantly different from each other, as determined by the Tukey's HSD test ($P < 0.05$).

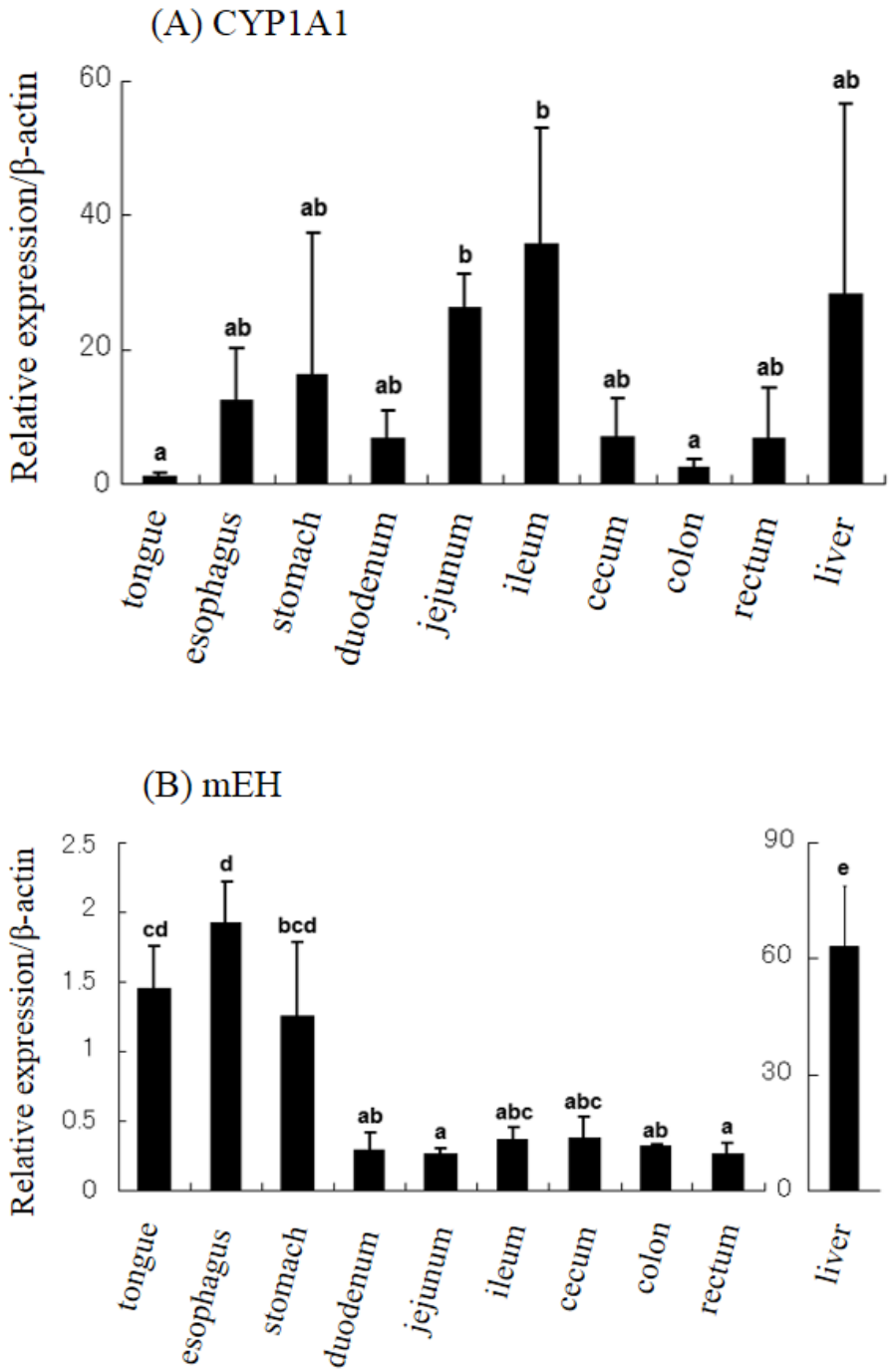

Figure 4. mRNA expression of phase I enzymes in various tissues of rats.

Total RNA was extracted from the liver, tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, and rectum in control rats, and then converted to cDNA. The expression levels of CYP1A1 (A) and mEH (B) in various tissues were determined by real-time PCR. Quantitative PCR was performed in duplicate and repeated three times. Data are presented as the mean ± SD (n=5). Identical letters are not significantly different from each other, as determined by the Tukey's HSD test ($P < 0.05$).

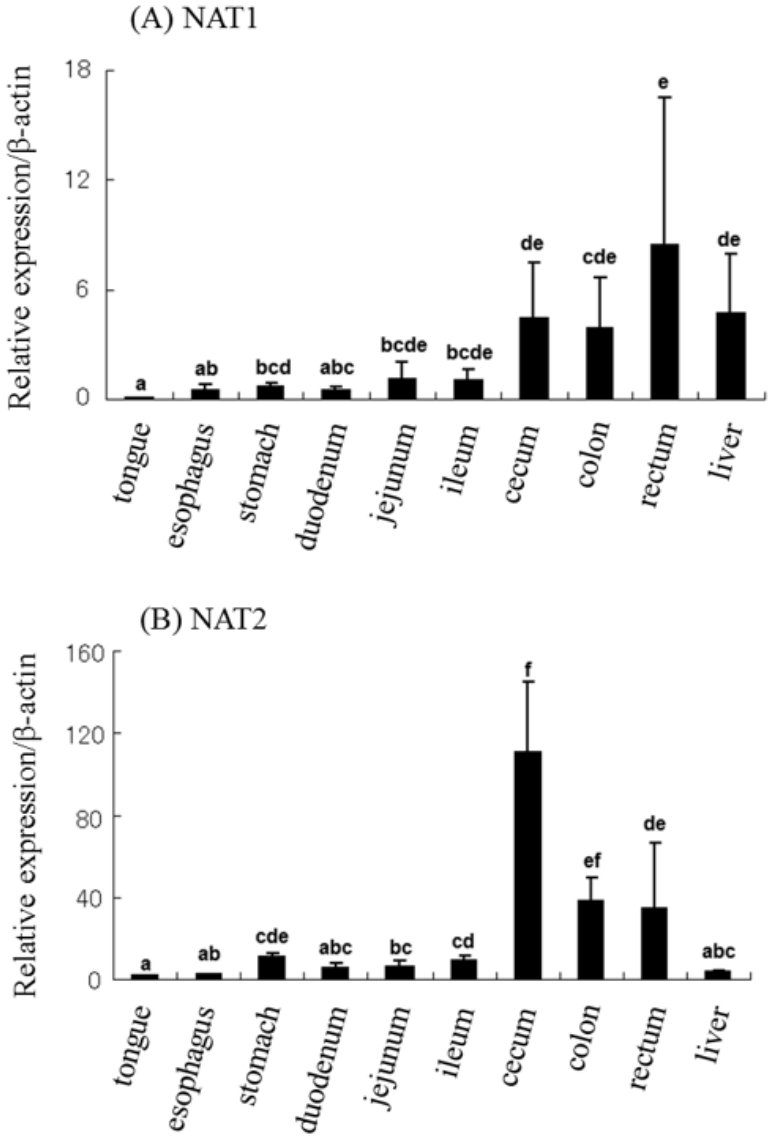

Figure 5. mRNA expression of NATs in various tissues of rats.

Total RNA was extracted from the liver, tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, and rectum in control rats, and then converted to cDNA. The expression levels of NAT1 (A) and NAT2 (B) in various tissues were determined by real-time PCR. Quantitative PCR was performed in duplicate and repeated three times. Data are presented as mean ± SD (n=5). Identical letters are not significantly different from each other, as determined by the Tukey's HSD test ($P < 0.05$).

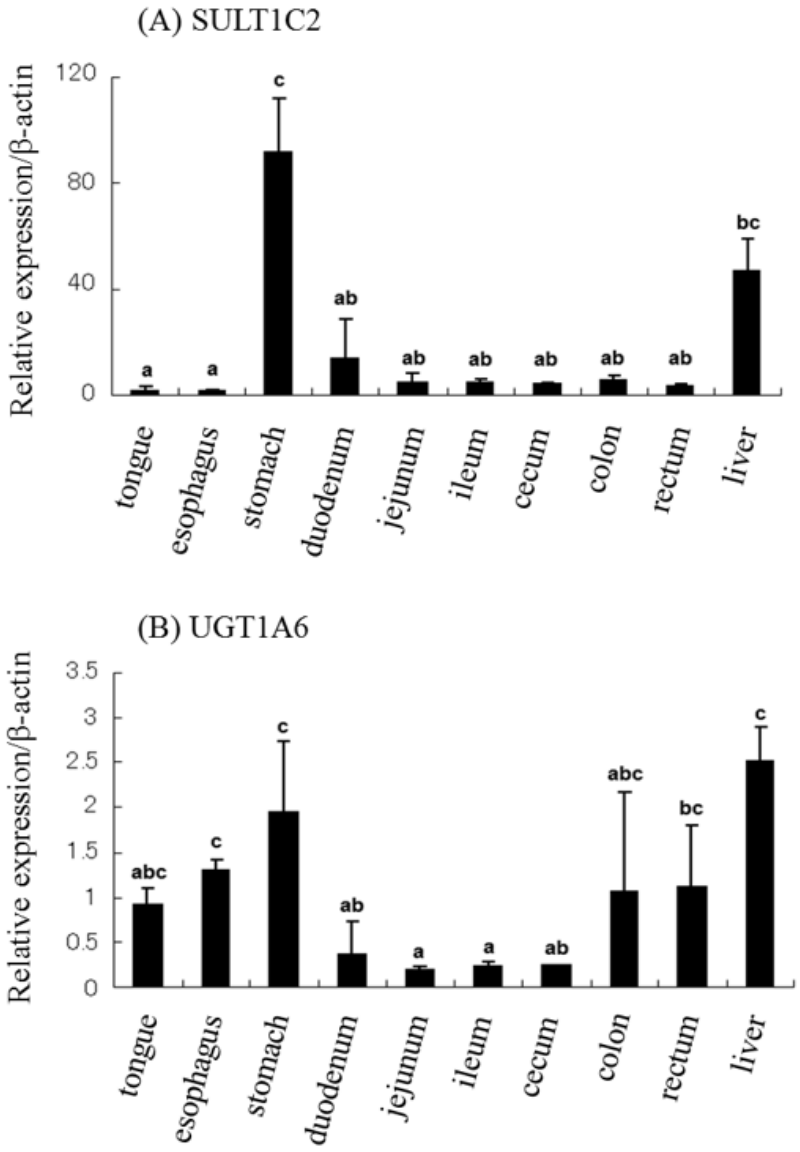

Figure 6. mRNA expression of phase II enzymes in various tissues of rats.

Total RNA was extracted from the liver, tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, and rectum in control rats, and then converted to cDNA. The expression levels of SULT1C2 (A) and UGT1A6 (B) in various tissues were determined by real-time PCR. Quantitative PCR was performed in duplicate and repeated three times. Data are presented as the mean ± SD (n=5). Identical letters are not significantly different from each other, as determined by the Tukey's HSD test ($P < 0.05$).