Metabolic activation of heterocyclic amines and expression of CYP1A1 in the tongue

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Abstract

Xenobiotic metabolism in oral tissues, especially in the tongue, has never been reported. In the present study, the metabolic activation / detoxification ability of promutagens in the tongue and the expression levels of related enzymes were investigated.

Quantitative PCR analysis of rat tongue demonstrated constitutive mRNA expression of numerous drug-metabolizing enzymes. In particular, we detected mRNA, protein expression and enzymatic activity of cytochrome P450 (CYP) 1A1 in the tongue tissue. Metabolic activation of promutagens in the tongue was estimated using benzo[a]pyrene or heterocyclic amines (HCAs), found in cooked meat and tobacco products. Metabolic activation levels of HCAs in the tongue were comparable to those in the liver. In contrast, the expression levels of glutathione S-transferase (GST) and UDP-glucuronosyltransferase (UGT) in the tongue were considerably lower compared with those in the liver and, as a result, the mutagenic activity in the tongue was not decreased by GST- or UGT-dependent conjugation.

Treatment of rats with sudan III, a typical inducer of CYP1A1, resulted in markedly increased CYP1A1 mRNA, protein expressions, CYP1A-dependent enzymatic and mutagenic activities. In addition, CYP1A1 mRNA expression in carcinoma cells (SAS) was induced by sudan III exposure.

In conclusion, mutagenic activation of xenobiotics and an increased risk of cancer in the tongue were observed in this study. Furthermore, ingestion of drug-metabolizing enzyme inducers has the potential to increase the metabolic activation in the tongue tissue and increase the risk of biomolecular attack by promutagens.

Key Word: tongue, CYP1A1, heterocyclic amines, metabolic activation, mutagenicity

Introduction

Oral cavity squamous cancer is one of the most serious oral cancers, with 300,000 cases every year worldwide. The high prevalence and death rate (The survival rate is less than 50% within five years) is similar to malignant melanoma (Lippman and Hong, 2001). Ninety percent of oral cavity cancers reported in the world have been suggested to be caused by smoking, and this cancer occurs frequently (Jordan and Daley, 1997). The incidence of the oral cavity squamous cancer is 4-7 times higher in smokers than in nonsmokers (Ko et al., 1995). In addition, alcohol drinking and chewing tobacco or smoking increase the incidence of oral cavity squamous cancer to 19 times and 123 times, respectively (Ko et al., 1995). The incidence of the oral cavity cancer is remarkably high in India where there is a culture of chewing tobacco. In fact, the incidence of this cancer in males comprises 19% of all cancers, and is third most common cause of cancer in woman (Parkin et al., 1997). In Japan, the incidence of this cancer is about 2% of all cancers, and tongue is the most common target of oral cancer (Matsuda et al., 2008). Oral cavity squamous cancers are observed not only in humans but also in dogs and cats. In particular, oral cavity cancer is responsible for 3-12% of cat cancers, and this malignancy is the fourth highest causes among cancer in this animal species (Patnaik et al., 1975; Dorn and Priester, 1976; Stebbins et al., 1989).

The CYP superfamily is the most important drug metabolizing enzyme group that metabolically activates promutagens. Particularly, the CYP1A subfamily catalyzes the metabolic activation of polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (HCAs) (Nebert, 1991). Numerous polymorphisms of the CYP1A1 gene were reported to alter the risk of lung and colon cancers in humans (Sreelekha et al., 2001; McLemore et al., 1990; Sivaraman et al., 1994; Nakachi et al., 1991). However there are few reports concerning the oral cavity, the tongue in

particular (Lechevrel et al., 1999; Duell et al., 1996; Ahmad et al., 1996).

Benzo[a]pyrene (B[a]P), a typical PAH, is a promutagen that is contained in smoke from cigarettes and the exhaust gas of cars. CYP and microsomal epoxide hydrolase (mEH) cooperatively contribute to the metabolic activation of B[a]P (Figure 1). The final metabolite of B[a]P after the third reaction in phase II metabolism is 7,8-dihydrodiol-9,10-epoxide, which is the most active compound that attacks numerous biomolecules including DNA (Kim et al., 1998; Shimada et al., 2002).

Meanwhile, HCAs are also found in cooked meat and fish, as well as the smoke of cigarettes. Two of the typical HCAs are 3-amino-1-metyl-5H-pyrido[4,3-b]indole (Trp-P-2) or 2-amino-1-metyl-6-phenylimidazo[4,5-b]-pyridine (PhIP), and more than 20 HCAs are detected in cooked foods. HCAs are first metabolized by CYP1A as N-hydroxylation in microsomes. These metabolites possess the ability to bind DNA directly. However, the most active metabolites of HCAs are produced by sulfotransferase (SULT) or N-acetyltransferase (NAT) reactions mainly in the cytosol. Such active metabolites are further conjugated by glutathione-S-transferase (GST) or UDP-glucuronosyl-transferase (UGT), as detoxification pathways (Alaejos et al., 2008; Turesky, 2007) (Figure 2).

CYP1A1 and several phase II enzymes are up-regulated by exposure to ligands of the aryl hydrocarbon receptor (AhR) (Ghosh et al., 2001). Sudan III is also a typical inducer of CYP1A1 (Nahla et al., 2008). These enzymes are also regulated by NF-E2 p45-related factor 2 (Nrf2) via the antioxidant response element (ARE) in their promoter regions.

Expressions and activities of drug-metabolizing enzymes in the oral cavity are almost previously-unreported, and the metabolic pathways of activation of promutagens are unknown in the tongue tissue. In this study, phase I and phase II drug metabolizing enzymes of the tongue were investigated, and their ability to metabolically activate and detoxify promutagens was

studied.

Materials and a method

Chemicals

The S9 cofactor, glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PDH) and β-nicotinamide-adenine dinucleotide phosphate reduced form (NADPH) were purchased from Oriental yeast (Tokyo, Japan). Sudan III, bovine serum albumin (BSA) and 3'-phosphoadenosine 5'-phosphosulfate (PAPS) were obtained from Sigma Chemicals Co (MO, USA). Reduced glutathione (GSH) and UDP-glucuronic acid trisodium salt (UDPGA) were from Wako Pure Chemical Industry Ltd. (Osaka, Japan). Resorufin and ethoxyresorufin were purchased from Merck Ltd. (Tokyo, Japan). Anti-rat CYP1A1 antibody from goat and anti-goat IgG were purchased from Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan). Histofine Simple Stain MAX-PO and Simple Stain DAB (diaminobenzidine tetrahydrochloride) were obtained from Nichirei (Tokyo, Japan). Tissue-Tek OCT compound was from Sakura Finetechnical Co. Ltd (Tokyo, Japan).

Animals

Wistar rats (7-weeks-old) were obtained from Japan SLC Inc. (Hamamatsu, Japan). All experiments using animals were performed under the supervision and with the approval of the Institutional Animal Care and Use Committee of Hokkaido University. They were housed in plastic cages at 22 ± 1°C with a 12 h /12 h light / dark cycle and given laboratory chow and tap water ad libitum. After 1 week, six rats were orally administered corn oil (3 ml/kg body weight) or sudan III at dose of 40 mg/kg body weight for three days. We dropped and applied corn oil or sudan III on rat tongue before oral injections. All rats were sacrificed on the fourth day using CO_2, and tongue, liver, kidney, spleen, lungs, heart, brain, testis, skeletal muscle were

immediately collected and frozen in liquid nitrogen. All tissues were kept at -80°C until use. For the immunohistochemistry examination, liver and tongue samples were immediately embedded in OCT compound, frozen on dry ice, and kept at -80 °C until the assay.

Real-time PCR

Total RNA was isolated from 50 mg liver using TRI Reagent from the Sigma Chemical Co. Briefly, tissue samples were homogenized in 1 ml TRI Reagent. Then, 0.2 ml chloroform was added to the sample. The mixture was then shaken for 30 sec, followed by centrifugation at 4°C and 12,000 x g for 20 min. Supernatant layers were transferred to a new set of tubes, and an equal volume of isopropanol was added to the samples, before shaking for 15 sec and centrifuging at 4°C and 12,000 x g for 15 min. RNA pellets were washed with 70% ethanol. RNA was dissolved in deionized sterilized ultra-pure water. Total RNA concentration and quality were checked using a Nanodrop ND-1000 spectrophotometer (DYMO, Stamford, USA). The RNA quality was estimated by the 260/280 and 260/230 nm absorbance ratios and was confirmed by denaturing agarose gel electrophoresis.

The cDNA was synthesized as follows. A mixture of 5 µg of total RNA and 0.5 ng of oligo dT primer in a total volume of 24 µl of sterilized ultra-pure water was incubated at 70°C for 10 min and then removed from the thermal cycler and to the volume was increased to 40 µl with a mixture of 8 µl (5X) RT-buffer (Toyobo Co., Ltd, Osaka, Japan), 2 ml 10 mM dNTP, 2 µl water, and 2 µl reverse transcriptase (Toyobo Co., Ltd). The mixture was then re-incubated in the thermal cycler at 30°C for 10 min, at 42°C for 1 hour and at 90°C for 10 min to prepare the cDNA.

Quantitative real-time PCR for rat mRNA levels were performed using TaqMan Gene Expression Assays (Applied Biosystems, CA, USA) and measured by StepOne™ Real-Time

PCR System (Applied Biosystems). The primer and probe sets for each specific gene were as follows: Rn00487218_m1 (CYP1A1), Rn02786833_m1 (CYP2B2), Rn01475871_m1 (CYP2S1), Rn00756461_m1 (CYP3A2), Rn01644438_g1 (CYP20A1), Rn00565750_m1 (AhR), Rn00756113_AH (UGT1A6), Rn00755117_A1 (GSTA1), Rn01415093_m1 (mEH), Rn00566528_m1 (NQO1), Rn00581955_m1 (SULT1C1), Rn02769895_g1 (SULT1C2), and Rn99999916_s1 (GAPDH). The reaction was performed for 40 cycles: 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 specific enzyme and receptor genes and GAPDH were performed in duplicate and repeated three times. The expression of each gene was normalized to the expression of GAPDH and was calculated relative to that of control using the comparative threshold cycle (Ct) method.

In addition, we used the following specific primers to measure the expression of Nrf2 and NAT genes in SYBR Green quantitative PCR. Nrf2: forward primer 5'- tgcccctggaagtgtcaaa -3', reverse 5'- ggctgtactgtatccccagaaga -3'. NAT1: forward 5'- ccaaacatggcgaactcgt -3', reverse 5'- gaagatacaggtcattagttgatcaatattg -3'. NAT2: forward 5 '- gtgcctaaacatggtgatcgatt -3', reverse 5'- acatggtcagaagtatgtccttgtc -3'. Cyclophilin, forward 5'- cttcgacatcacggctgatgg -3', reverse 5'-caggacctgtatgcttcagg -3'. To measure the expression levels of Nrf2, NAT, and cyclophilin genes, we used SYBR Green real-time PCR Master Mix (Finnzyme, Espoo, Finland). The conditions of the PCR reaction was 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. Cyclophilin was used for the normalization in the comparative Ct method. We confirmed that there was no difference in the PCR efficiency between Nrf2 or NAT and cyclophilin primer sets.

Preparation for the enzyme fraction

Two tongue samples were pooled as one sample (n=3). Liver samples from three animals were used individually. Enzymatic fractions from tongue and livers were prepared according to the method of Omura and Sato (1964), with slight modifications. Tissues were minced and homogenized in 3 volumes of an ice-cold 1.15% potassium chloride solution with a Teflon homogenizer. Homogenized samples were centrifuged at 9,000 x g at 4°C for 20 min. The supernatant fraction was centrifuged at 105,000 x g at 4°C for 70 min to attain a mitochondrial-free microsomal pellet. The washed microsomes were then suspended in 0.1 M potassium phosphate buffer (KPB), pH 7.4, and divided into 1.5-ml tubes to be snap-frozen in liquid nitrogen and kept (at -80°C) until use. Microsomal protein concentrations were determined according the method of Lowry et al. using Bovine serum albumin as a standard (Lowry *et al.*, 1951).

Western blotting

Aliquots of 12 µg of microsomal protein from treated and control rats were applied to 10% sodium dodecyl-sulfate (SDS) polyacrylamide gels and separated by electrophoresis using a Protean 2 mini 1-D cell (BioRad, Hercules, CA, USA). Western blot analysis was performed according to previously reported method (Laemmli, 1970). Proteins were transferred electrophoretically to nitrocellulose membranes, blocked in 5% skimmed milk in phosphate-buffered saline (PBS) containing 1% Tween 20 for 2 hours at room temperature, and probed with the polyclonal goat anti-rat CYP1A1 antibody in PBS containing 1% Tween 20 on a shaker for 2 hours at room temperature. The anti-rat CYP1A1 antibody was also cross reacted with CYP1A2. Horseradish peroxidase-labeled anti-goat IgG was used as the secondary antibody. Immunoreactive protein bands were revealed colorimetrically by oxidation of 0.025% 3,3-diaminobenzidine tetrahydrochloride with 0.0075% hydrogen peroxide and catalyzed by

peroxidase in 50 mM Tris-HCl (pH 7.6). Intensities of the immunoreactive bands were analyzed densitometrically using the public domain Scion Image software (Scion Corp., Frederick, MD).

Ethoxyresorufin O-deethylase activity

Ethoxyresorufin O-deethylase (EROD) activity was determined by a fluorescence intensity assay using the general principles described by Crespi et al. (1997). The fluorescence of resorufin was detected at an excitation wavelength of 530 nm and emission wavelength of 590 nm. Reactions were performed in a 1 mL solution/tube containing 10 mM G6P, 10 mM $MgCl_2$, 500 µg microsomal protein and 10 µM ethoxyresorufin in 100 mM potassium phosphate buffer at pH 7.4. The tubes were preincubated in a dark room for 5 min at 37°C, and the reaction was started by adding 20 µL of a mixture of 50 mM NADPH and 200 U G6PDH and was stopped with 4 mL of ice cold methanol. This experiment was repeated twice.

Mutagenic assay

The Ames test was performed using liver S9 fractions (20 mg protein/mL) from control and sudan III-treated rats according to the preincubation method of Ames (1975) using the *Salmonella typhimurium* strain TA98 with minor modifications as follows. Five hundred microliters of the liver S9 fraction containing the complete NADPH generating system and NADH, 100 µL of an overnight culture of TA98 and coenzymes (30 mM GSH, 15 mM UDPGA, both of them, or 0.1 M potassium phosphate buffer as a control) were added to the test tubes. Then, 50 µL of B[a]P (5 µg) as the mutagen or DMSO as a negative control was added, and the tubes were incubated for 25 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 hours at 37°C, and the number of revertant colonies was

counted manually. We repeated each experiment at least twice.

Cell culture and determination of mRNA expression

Human hepatoma HepG2 cells and tongue carcinoma SAS cells (RIKEN Cell Bank, Tsukuba, Japan) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, and antibiotics (100 U/ml penicillin, 100 ug/ml streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2 in air. Cells were seeded at 70% confluence on collagen-coated (HepG2) and non-coated (SAS) 6-well plates. The medium was replaced with medium containing 0 or 50 μM of sudan III or 0, 5, 50 or 200μM of butylated hydroxytoluene (BHT) dissolved in DMSO. The final concentration of DMSO was 0.1%. After exposure of the cells to sudan III for 12 hours and BHT for 24 hours, the medium was removed, and the cells were washed twice with PBS. Total RNA was extracted using TRI reagent (Sigma-Aldrich) according to the manufacturer's instruction manual. RNA (0.1 μg) was reverse-transcribed to cDNA using the RT enzyme. Using this cDNA as a template, CYP1A1, NQO1 and GAPDH mRNA were assayed using TaqMan Gene Expression Assays and StepOne Real-Time PCR System. The specific primers and TaqMan probe used in this assay were as follows: Hs00168547_m1 (CYP1A1), Hs00153120_m1 (NQO1), Hs99999905_m1 (GAPDH).

A 20 μl aliquot of the TaqMan Universal PCR Master Mix containing an appropriate amount of the template cDNA, 3 μM of primers and 2 μM of TaqMan probe was prepared, and real-time PCR was performed by 40 cycles of incubation at 95ºC for 15 sec, followed by incubation at 60ºC for 1 min. The comparative Ct method was used to estimate each specific gene expression using GAPDH as an internal standard.

Immunohistochemistry

The resected tongue and liver specimens were obtained from cryo-embedding samples in OCT Tissue Tek. The immunohistochemical studies were performed on 10 μm thick cryostat sections. The sections were mounted onto glass slides coated with aminopropyltriethoxysilane (Matsunami Glass Ind., Ltd., Japan). The samples were then stored at -80 ºC until use.

The sections were fixed in 100% acetone for 10 minutes at room temperature and the endogenous peroxidase activity was quenched by incubating slides in 3% hydrogen peroxide in absolute methanol for 10-15 minutes at room temperature. Slides were washed three times with fresh PBS, each lasting 5 minutes. Sections were incubated for two hours at room temperature with normal goat serum (negative control) and with polyclonal goat anti-rat CYP1A1 antibody, washed in PBS for 5 minutes, incubated 30 minutes with Histofine Simple Stain MAX-PO, washed for 5 minutes in PBS, and incubated 5 minutes in Simple Stain DAB. Slides were washed with distilled water and then were mounted with permanent mounting media after dehydrating the slides in a graded series of alcohol and cleaning in xylene. Then, slides were mounted with permanent mounting media and the glass slide was covered by a cover slide and observed microscopically.

Statistical analyses

Results of enzymatic activities, mRNA expression, protein expression, and Ames assays are expressed as mean ± standard error. Statistical significance was assessed using Student's t-test for two groups or Tukey HSD test for multiple comparisons. Differences of $p<0.05$ were considered to be statistically significant. The statistical package, JMP ver. 7.0 (SAS Institute Inc., NC, USA), was used for the statistics analyses.

Results

Expressions of mRNA of drug-metabolizing enzymes and AhR in the tongue

We compared expression levels of mRNA for drug metabolizing enzymes in the tongue with those in various organs of liver, skeletal muscle, heart, brain, testis, spleen, lungs, and kidney as references. We detected mRNA of CYP1A1, CYP2B2, CYP2S1, CYP3A2, CYP20A1 and AhR in the tongue tissue of non treated control rats (Figure 3). Then, we focused on AhR regulated CYP enzymes, CYP1A1 and CYP2S1. Figure 4 shows the alterations of expression levels of these mRNAs in various tissues of control and sudan III-treated rats. Especially, sudan III treated rats showed 33.5 fold higher expression levels of CYP1A1 in the tongue than those in control rats. In the skeletal muscle, heart, lung, kidney and liver, CYP1A1 was also induced by treatment with sudan III. CYP2S1 and AhR mRNA levels were slightly increased in kidney, but we did not find any significant difference in the tongue after treatment with sudan III.

Expressions of CYP1A1 and CYP1A2 and EROD activity in the tongue

In the tongue of control rats a low but significant expression of CYP1A1 and CYP1A2 apoproteins was observed (Figure 5A). Sudan III injections induced CYP1A1 expression levels by 5.4 fold, and CYP1A2 by 9.3 fold in the tongue tissue. Tongue from control rats possessed a resorufin intensity of 21 pmol/min/mg protein (Figure 5B). After treatment with sudan III, EROD activity was 11.7 fold higher in treated tongues than those of control rats.

Metabolic activation of benzo[a]pyrene in the tongue

Tongue showed a markedly small number of revertant colonies using the S9 fraction in the Ames test (Figure 6A). In sudan III injected rats, no significant induction was observed in the

Ames test using the S9 fraction of tongue and B[a]P. Then, we measured the mEH mRNA expression level in the tongue of control rats, and we found that the mEH expression was very low at 1/230 of the expression in the liver of control rats (Figure 6B).

Metabolic activation of heterocyclic amines in the tongue

We used Trp-P-2 as a typical HCA for the mutagenic Ames assay. In this assay, we added co-factors of conjugating enzymes: PAPS for SULT, GSH for GST and UDPGA for UGT. We also use microsomal or S9 fractions to detect cytosolic phase II enzymes such as NAT1 and NAT2.

Figure 7 showed a 7.9 fold increase of metabolic activation of Trp-P-2 in the tongue S9 fractions compared with the activation in microsomes. In sudan III treated animals, not only liver but also tongue showed a significant induction of revertant colonies of TA98 (9 to 12 fold). We did not find any significant effects of PAPS on metabolic activation of Trp-P-2 in the tongue S9 fractions. Actually, results of the Ames test showed that the addition of the cofactor, SULT, did not increase the number of revertant colonies. Furthermore, in the tongue S9 fractions, cofactors of GST, UGT, GSH and UDPGA, also did not decrease the mutagenicity of activated Trp-P-2. In contrast, liver S9 fractions of control rats showed drastic changes in cofactors after metabolic activation of Trp-P-2. These phenomena were also observed in liver S9 fractions from sudan III treated rats.

Then, we measured mRNA expression levels of NAT1, NAT2, SULT1C1, SULT1C2, GSTA1, and UGT1A6, which contribute to the metabolic activation or detoxification of Trp-P-2, as shown as Figure 8 and Figure 9. Interestingly, we found high expression levels of NAT1 and NAT2 in the tongue tissue (Figure 8). The expression levels were the same as or higher than expression levels found in the liver. In contrast, the expression levels of SULT1C1 and

SULT1C2 were very low in the tongue compared with those in the liver. Figure 9 shows GSTA1 and UGT1A6 expressions, which were increased in sudan III-treated rat liver. However, there was no alteration in expressions of GSTA1 and UGT1A6 in the tongue tissue.

mRNA expression levels of NQO1 and Nrf2

The mRNA expression level of Nrf2 in the tongue is similar to that of liver as shown in Figure 10. Sudan III did not affect expression Nrf2 in the tongue. NQO1 is regulated by Nrf2. In control rats, NQO1 expression levels in the tongue were almost same as those in the liver, and sudan III induced NQO1 expression in the tongue (5.8 fold).

Effects of sudan III and BHT on CYYP1A1 and NQO1 mRNA expressions in a tongue cell line

We examined transcriptional activities of AhR and Nrf2 mRNAs in hepatoma (HepG2) and tongue carcinoma cells (SAS). Figure 11 and Figure 12 showed alterations in CYP1A1 and NQO1 gene expression after the exposure to the AhR or Nrf2 ligand, respectively. We exposed SAS and HepG2 cells to 50 μM sudan III or 5, 50, 200 μM BHT. BHT is a ligand of Nrf2. We found a significant elevation of CYP1A1 mRNA by 3.3-fold in SAS cells and 26.1-fold in HepG2 cells (Figure 11). BHT treatment induced NQO1 mRNA in SAS cells in a dose dependent manner; however we did not observe any significant alteration of NQO1 expression in HepG2 cells until a concentration of 200 μM (Figure 12).

Distribution of CYP1A1 in the tongue tissue

We used an anti-rat CYP1A1 antibody to detect the CYP1A1 protein expression pattern in resected tongue (Figure 13). Tongue epithelia consist of stratified squamous epithelium cells, and submucosal tissue does not exist in this tissue. The lamina propria of the mucous membrane

is directly connected with lingual aponeurosis. We detected CYP1A1 expression in the tongue epithelium, lingual aponeurosis and perivascular cells in control rats. Sudan III treatment increased CYP1A1 expression levels in epithelium, lingual aponeurosis and perivascular cells, especially in the muscle layer.

Discussion

Induction of expression levels of drug metabolizing enzymes resulted in not only an acceleration of the detoxification of toxic compounds, but also an increase of metabolic activation of xenobiotics and increased the risk of DNA damage. In the processes of metabolism and excretion of toxic substance, the balance of the metabolic activation ability and detoxification ability is very important. In this study, we examined the expression levels of various drug metabolizing enzymes in the tongue, and determined their abilities to metabolically activate promutagens, as the major factors that cause oral cavity cancer.

Expressions of drug metabolizing enzymes have been reported in the liver, lung and kidney tissues (Murray and Burke, 1995; Nishimura *et al.*, 2003). However, little has been known about drug metabolizing enzymes in the tongue, the organ where ingested substances first contact the body. In this study, we investigated and found the expression of phase I enzymes CYP1A1, CYP2B2, CYP2S1, CYP3A2, CYP20A1, NQO1, and mEH, and phase II enzymes GSTA1, UGT1A6, NAT1, NAT2,, SUT1C1, and SULT1C2. CYP1A1, CYP2B1, CYP2S1 and CYP3A2 are cytochrome P450 enzymes that are known to cause metabolic activation of promutagens and procarcinogens. CYP20A1 has been recently identified and is reported to be continuously expressed in ubiquitous organs. mEH cooperatively works with CYP1As and is a key enzyme for metabolic activation of PAHs such as B[a]P. Usually phase II enzymes contribute to the detoxification pathway of mutagens, however SULT and NAT metabolically activate HCA compounds.

In addition, we detected mRNA of both AhR and Nrf2, which regulated the expressions of the above mentioned drug-metabolizing enzymes in the tongue tissue. Complexes of AhR and its ligand transfer into the nucleus, and then bind to the AhR nuclear translocator (ARNT).

AhR-ARNT complexes bind to the xenobiotic responsive element (XRE) of DNA and transcriptionally activate the CYP1A1 gene. In addition to CYP1As and CYP1B1, there are XREs in the enhancer regions of NQO1, UGT1A6 and GSTA1. Nrf2 binds to Kelch ECH associating protein 1 (Keap1) and remains in cytoplasm. Nrf2 is activated by antioxidant compounds such as tert-butylhydroquinone (tBHQ) and BHT (Hayes et al., 2000; Kensler et al., 2007). In rats, there are XRE and ARE consensus sequences in the upper region of NQO1, which is different in humans (Köhle and Bock et al., 2007).

In this study, with a focus on CYP1A1, it was confirmed that there were CYP1A1 and CYP1A2 proteins and CYP1A1-dependent enzymatic activity in tongue microsomes. Yang et al. (2003) reported that they detected the metabolic activities of 4-nitrophenol, 1-phenylethanol, caffeine and 7-ethoxy-coumarin in rabbit tongue, but not in the tongue of the rat. However, the typical CYP1A1-dependent enzymatic reaction, EROD activity in the tongue was indicated in this study.

Metabolic activation of B[a]P in the tongue was very low compared with that of liver in the Ames mutagenicity assay, although the EROD activity was detected in tongue microsomes. Low expressions of CYP1A and mEH may result in the low level of metabolic activation of B[a]P in the tongue tissue.

In contrast, surprisingly, high metabolic activation of typical HCA, Trp-P-2, was found in the tongue tissue by the Ames assay. SULT expressions were very low and the addition of PAPS, a-cofactor for SULT, did not affect the increase of metabolic activation of Trp-P-2 in the tongue. The high ability of metabolic activity in the tongue may have been due to the high expression of NAT enzymes. Actually, the expression levels of NAT1 and NAT2 mRNAs were same as or higher than those of liver. In addition, phase II detoxification enzymes such as GST and UGT did not affect the decrease in mutagenicity of Trp-P-2 in the tongue, unlike the results from the

Ames assay using liver S9 fraction. The detoxification ability was markedly lower in the tongue compared with that in the liver. Previously, 4-nitroquinoline 1-oxide 4-NQO, B[a]P, and 7,12-dimethylbenzanthracene (DMBA) were reported to be metabolically activated in the tongue tissue (von Pressentin *et al.*, 1999), and the current findings support this phenomenon.

In this study, the details of CYP1A expression in the tongue tissue were examined by immunohistochemistry. Interestingly, stratified squamous epithelia, especially filiform papilla, was the most highly expressed area of CYP1A1 in the tongue of non-treated rats. Chi et al. (2009) reported an elevation of CYP1A1 expression in human stratified squamous epithelium carcinoma cells after exposure to B[a]P. The results from this study indicate that metabolic activation of promutagens by CYP1A may occur in the tongue and coincides with the previous study that reported an increase in tongue cancer incidence after coating 4-NQO on tongue (Srinivasan *et al.* 2008). In this study, an increase of CYP1A1 expression around blood vessels in the tongues of sudan III treated rats may be induced after the intestinal absorption of sudan III. Yang and Raner (2005) reported catechin treatment induced expression levels of CYP1A1, CYP1A2, CYP2C, CYP2D6, CYP2E1 and CYP4F3 in tongue carcinoma cells. Combined with previous studies, the current results could indicate the possibility that xenobiotics may be partially absorbed on tongue and metabolically activated by phase I enzymes such as CYP1A1 and phase II NAT enzymes directly in the tongue tissue, and CYP1A1 expression levels were altered by an intake of inducer chemicals. As an AhR ligand, sudan III treatment induced the levels of expression of CYP1A1 and CYP1A1-dependent enzymatic activity. The results of immunohistochemistry also showed that AhR ligands could induce CTP1A1 in tongue cells.

Using SAS and HepG2 cells, we revealed that tongue carcinoma cells possessed a higher sensitivity to Nrf2 activators compared with that of HepG2 cells. NQO1 expression level was induced by BHT exposure in SAS cells in a dose-dependent manner. In humans, not only AhR

but also Nrf2 should be considered as major factors for oral cancer risk. There is no XRE in the promoter region of human NQO1. However, AREs exist in the upper region of the human NQO1 gene, and Nrf2 regulates expression of NQO1 gene. NQO1 catalyzes a 2 electron reduction of quinine, however, it has been also reported that NQO1 metabolically activates 2-amino-9H-pyrido-[2,3-b]indole (AAC), 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-1), 4-NQO, mitomycin C and diaziquone. NQO1 expression in the tongue may be an important determinant for oral cancer risk.

In summary, this study showed that the metabolic activation of promutagens may occur directly in the tongue, e.g., stratified squamous epithelium cells. AhR and Nrf2 ligands have been suggested to induce CYP1A1 and NQO1 expression in the tongue tissue. Interestingly, mRNA of NAT enzymes, which contribute to HCAs activation, was highly expressed in the tongue tissue. In contrast, tongue possessed low activities of detoxification enzymes such as GST and UGT. It is reported that metabolism of nitrosamine by CYP2A6 is one of the major factor to cause oral cancer (Topcu et al. 2002). In this study, we additionally showed CYP1A and NAT enzymes also possessed the possibility to contribute to oral cancers risk. Generally, xenobiotics do not remain in the oral area for a long time. However, in consideration of the continuous expression of CYP1A and NAT, which contribute to the activation of toxic substances and the low ability of detoxification, further study on drug-metabolizing enzymes in the tongue is needed to determine the risk factors of oral cancer.

Funding

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Figure legends

Figure 1. Pathway of metabolic activation of benzo[a]pyrene.

Figure 2. Metabolism of Trp-P-2.

Figure 3. Cytochrome P450 enzymes and AhR mRNAs expression in various tissues of control rats

Total RNAs were extracted from tongue, liver, kidney, spleen, lungs, heart, brain, testis, skeletal muscle, and converted to cDNA. Three samples were used for the determination. The mRNA expression levels of CYP1A1, CYP2B2, CYP2S1, CYP3A2, CYP20A1 and AhR in various tissues were determined by real-time PCR. Quantitative PCR was performed in duplicate and repeated three times. Data were analyzed using samples from three rats and presented as Mean ± SE (n=3). Identical letters are not significantly different from each other by Tukey-HSD ($p < 0.05$).

Figure 4. Comparison of the mRNA expression of CYP1A1, CYP2S1 and AhR in various tissues from control and sudan III treated rats

Induction of CYP1A1, CYP2S1 and AhR mRNA expression levels were detected in the tongue, liver, kidney, spleen, lungs, heart, brain, testis, and skeletal muscle samples. Six rats were orally injected with sudan III at a dose of 40 mg/kg body weight for three days. Six control animals were injected with corn oil. Randomly, three of six samples in each group were used for the quantitative real-time PCR. Open columns show the enzyme fraction from controls and black bars show samples from sudan III-treated rats. Data were presented as Mean ± SE (n=3), and

the Student-t test was used for statistical analyses. Asterisks represent significant difference between control and sudan III treated animals. *$p<0.05$, **$p<0.01$, *** $p<0.001$.

Figure 5. Protein expression levels of CYP1A1 and CYP1A2, and CYP1A1-dependent enzymatic activities

A) Western blotting analyses were performed using microsomes of tongue and liver from control and sudan III-treated rats. An anti-rat CYP1A1 antibody was used for detection of proteins. B) Ethoxyresorufin O-deethylase activity was measured using the microsomal fraction of tongue and liver samples. Open columns show the enzyme fraction from controls and black bars show samples from sudan III-treated rats. Data were presented as Mean ± SE (n=3). Asterisks represent a significant difference between control and sudan III treated animals. **$p<0.01$, *** $p<0.001$, ****$p<0.0001$.

Figure 6. B[a]P metabolic activation and expression of microsomal epoxide hydrolase in the tongue and liver

A) The S9 fraction of tongue and liver samples were used for the Ames test to detect for B[a]P metabolic activation. Tongue and liver S9 fractions were prepared from control and sudan III treated rats. B) The expression levels of mRNA of mEH were determined in the tongue, liver, kidney, spleen, lungs, heart, brain, testis, and skeletal muscle samples from control and sudan III treated rats. Open columns show the enzyme fraction from controls and black bars show samples from sudan III-treated rats. Data were presented as Mean ± SE (n=3). Student t-test was used for the statistical analysis, and asterisks show significant difference from control animal tissue. **$p<0.01$.

Figure 7. Effects of phase II enzymes on the metabolic activation of Trp-P-2 in the tongue and liver

Metabolic activation of Trp-P-2 was detected by the Ames test using microsomal and S9 fractions of tongue and liver samples from control and sudan III-treated rats. To determine the effects of phase II enzymes on metabolic activation of Trp-P-2, cofactors of phase II enzymes were added to each reaction mixture. A: microsomes, B: S9 fraction, C: S9 + 30 μg/plate PAPS, D: S9 + PAPS + 30 mM GSH + 15 mM UDPGA. Open columns show the enzyme fraction from controls and black bars show samples from sudan III-treated rats. Data were presented as Mean ± SE (n=3). Lower-panel shows the names of working enzymes in each reaction. The data sets of control tongue, control liver, sudan III-treated tongue or sudan III-treated liver, respectively, were analyzed by Tukey-HSD. Identical letters are not significantly different from each other in each group ($p < 0.05$).

Figure 8. Expression levels of mRNA of N-acetyltransferases and sulfotransferases in various tissues

Expression of mRNA of N-acetyltransferases (NAT1, NAT2) and sulfotransferases (SULT1C1, SULT1C2) was measured in the tongue, liver, kidney, spleen, lungs, heart, brain, testis, and skeletal muscle samples from control and sudan III treated rats. The expression of NAT genes were normalized using cyclophilin expression, and the SULT gene amounts were estimated by GAPDH. Open columns show the enzyme fraction from controls and black bars show samples from sudan III-treated rats. Data were presented as Mean ± SE (n=3). Student t-test was used as the statistical analysis, and asterisks show significant difference from control animal tissue. *$p < 0.05$, ** $p < 0.01$.

Figure 9. Expression levels of GST and UGT1A6 mRNAs in various tissues

Expression levels of detoxification phase II enzymes in various tissues were quantified by real-time PCR. Total RNA from tongue, liver, kidney, spleen, lungs, heart, brain, testis, and skeletal muscle samples from control and sudan III-treated rats were extracted and used for quantitative PCR reactions. Open column show the enzyme fraction from controls and black bars show samples from sudan III-treated rats. Data were presented as Mean ± SE (n=3). Student t-test was used as the statistical analysis, and asterisks show a significant difference from control animal tissue. *$p<0.01$.

Figure 10. Expression levels of mRNA of Nrf2 and NQO1 in various tissues

Expression levels of Nrf2 and NQO1 in the tongue, liver, kidney, spleen, lungs, heart, brain, testis, and skeletal muscle samples from control and sudan III-treated rats were quantified by real-time PCR. Open column shows the enzyme fraction from controls and black bars show samples from sudan III-treated rats. Data were presented as Mean ± SE (n=3). Student t-test was used as the statistical analysis, and asterisks show the significant difference from control animal tissue. *$p<0.05$, **$p<0.01$.

Figure 11. Effects of AhR ligand on alterations of CYP1A1 mRNA in SAS and HepG2 cell lines

Tongue carcinoma cell line (SAS) and hepatocyte cancer cell line (HepG2) were used to determine effects of sudan III on the expression level of CYP1A1. Cells were exposed to 50 μM of sudan III for 12 hours and total RNAs were extracted to measure the expression of CYP1A1 by real-time PCR. Reproducibility of the results was confirmed in three individual experiments. Data were presented as Mean ± SE (n=3). Identical letters are not significantly different from each other in same cell line by Tukey-HSD ($p< 0.05$).

Figure 12. Effects of an Nrf2 activator on alteration of NQO1 mRNA in SAS and HepG2 cell lines

The effects of the Nrf2 activator, BHT, on the expression of NQO1 were measured in SAS and HepG2 cells. Cell were exposed to 5, 50, or 200 μM BHT for 24 hours and total RNAs were extracted. NQO1 mRNA expression was measured by real-time PCR. Reproducibility of the results was confirmed in three individual experiments. Data were presented as Mean ± SE (n=3). Identical letters are not significantly different from each other in SAS or HepG2 cells, respectively. Tukey-HSD was used for the statistical analyses ($p < 0.05$).

Figure 13. Distribution of CYP1A1 protein in the tongue tissues

Immunohistochemistry examination was performed for CYP1A1 expression in cryo-sectioned tongue. An anti-rat CYP1A1 antibody was used for the detection. A: negative control using preimmune goat IgG. B: CYP1A1 in the tongue from a control rat. C: CYP1A1 in the tongue from a sudan III-treated rat. 1: dorsum of the tongue with filiform papillae, original magnification x400. 2: dorsum of the tongue with filiform papillae, x800. 3: perivascular area, x800. Marks indicate epithelium (E), lamina propria of the mucous membrane (LP), aponeurosis linguae (A), and muscle layer (M).

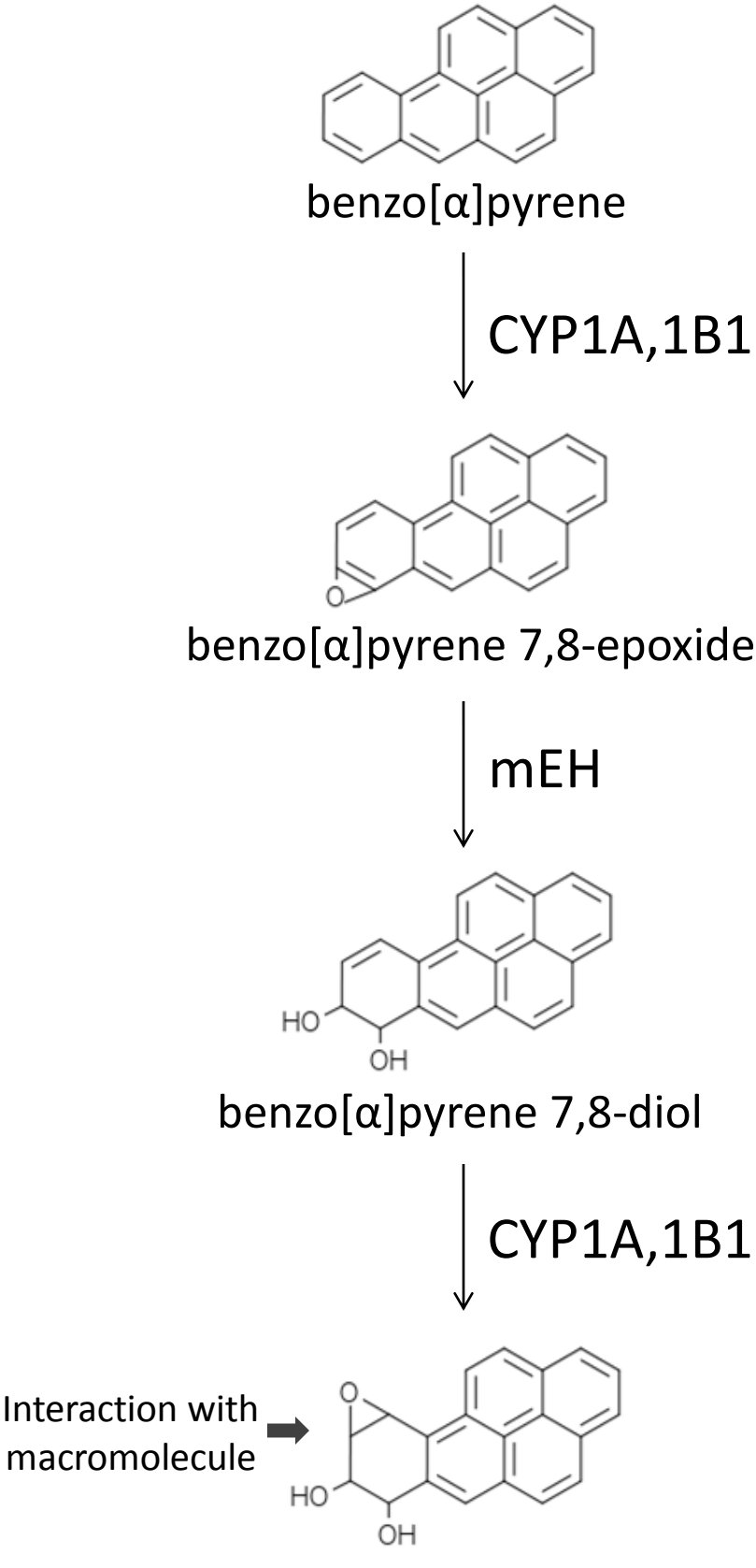
benzo[α]pyrene
CYP1A,1B1
O
benzo[α]pyrene 7,8-epoxide
mEH
HO
OH
benzo[α]pyrene 7,8-diol
CYP1A,1B1
Interaction with
macromolecule
O
HO
OH
benzo[α]pyrene 7,8-diol-
9,10-epoxide

Figure 1

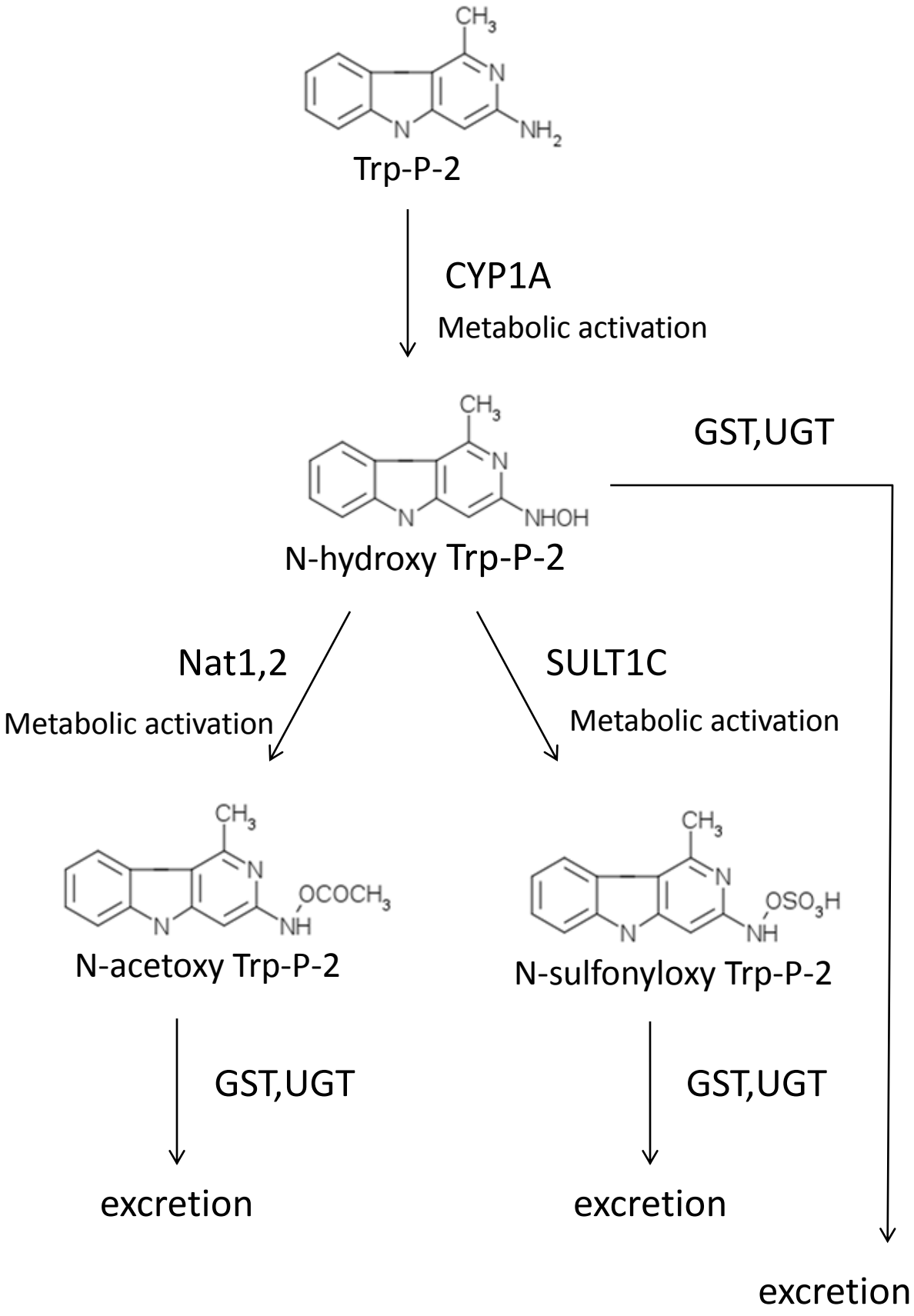
Trp-P-2
CYP1A
Metabolic activation
N-hydroxy Trp-P-2
GST,UGT
Nat1,2
Metabolic activation
SULT1C
Metabolic activation
N-acetoxy Trp-P-2
N-sulfonyloxy Trp-P-2
GST,UGT
GST,UGT
excretion
excretion
excretion

Figure 2

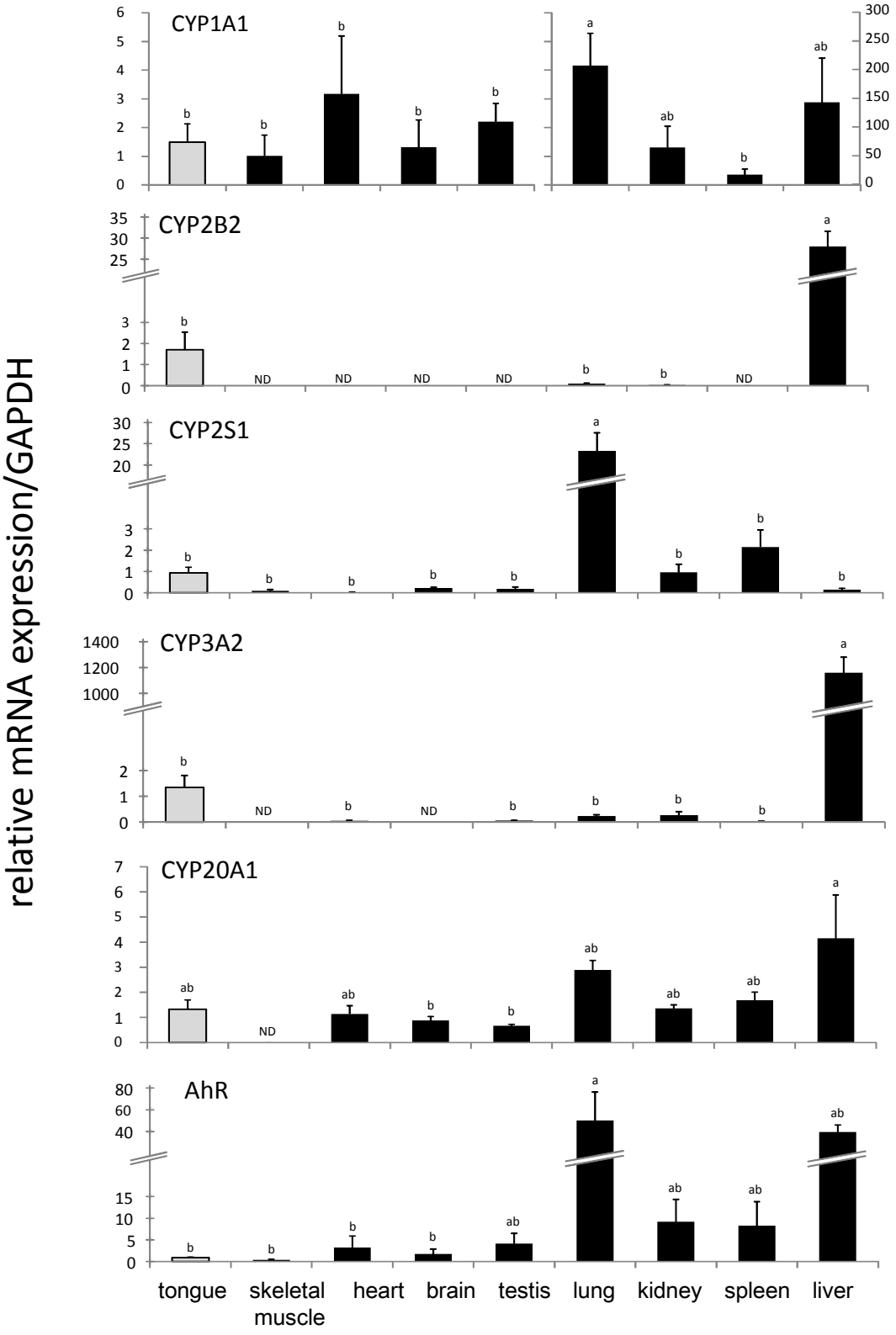
relative mRNA expression/GAPDH
CYP1A1
CYP2B2
CYP2S1
CYP3A2
CYP20A1
AhR
ND
tongue
skeletal muscle
heart
brain
testis
lung
kidney
spleen
liver

Figure 3

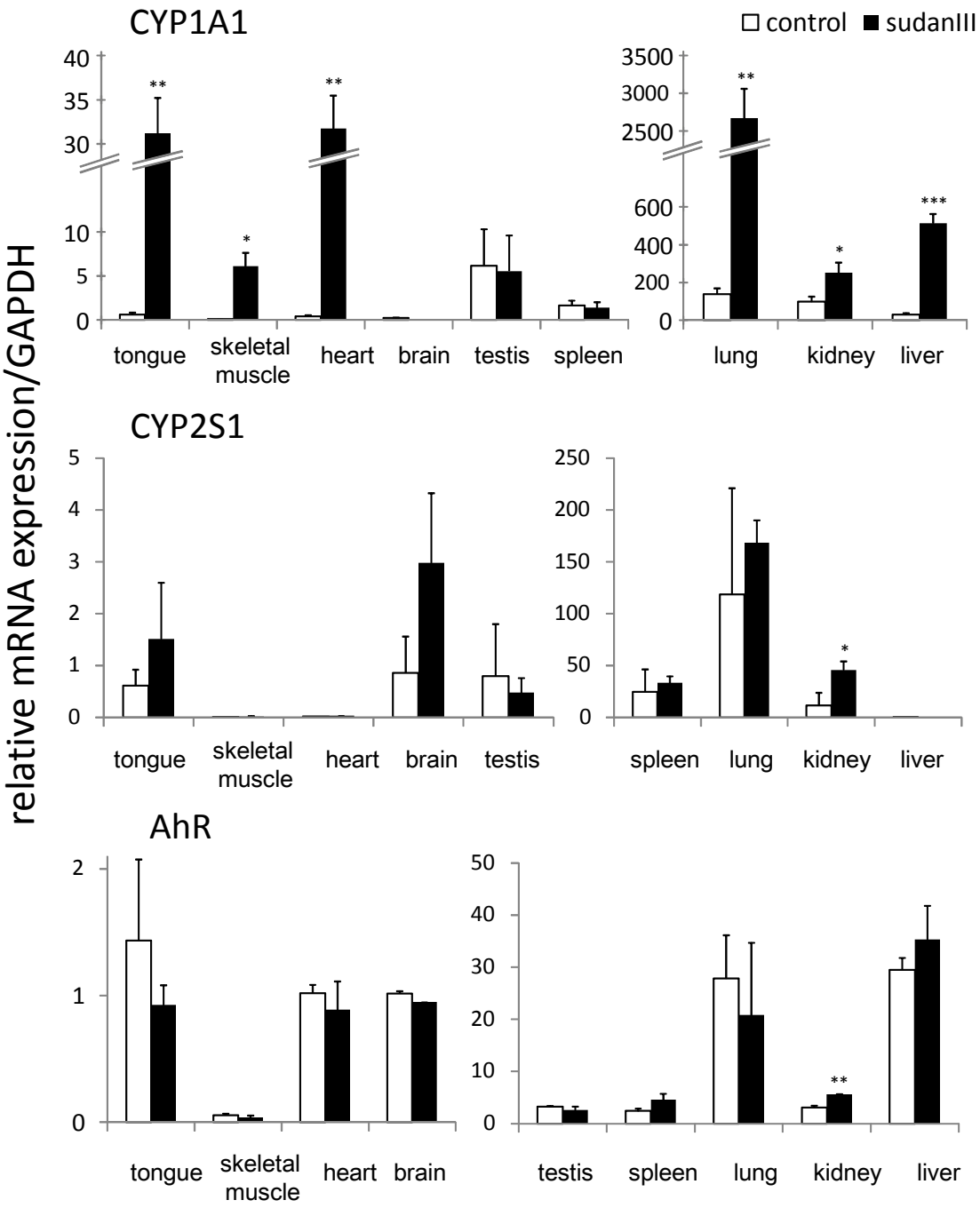
CYP1A1
control
sudanIII
relative mRNA expression/GAPDH
40
35
30
10
5
0
3500
3000
2500
600
400
200
0
**
*
***
tongue
skeletal muscle
heart
brain
testis
spleen
lung
kidney
liver
CYP2S1
5
4
3
2
1
0
250
200
150
100
50
AhR
2
1
50
40
30
20
10

Figure 4

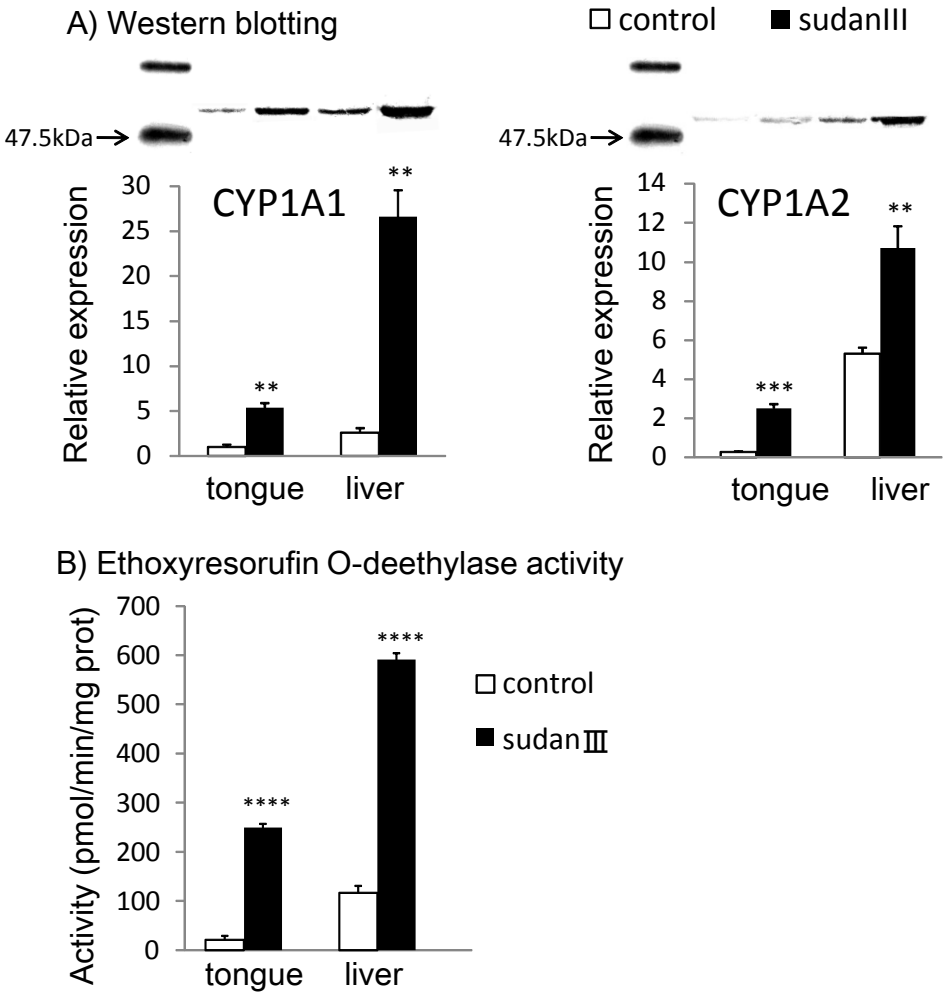
A) Western blotting
control
sudanIII
47.5kDa
47.5kDa
CYP1A1
CYP1A2
Relative expression
Relative expression
tongue
liver
tongue
liver
B) Ethoxyresorufin O-deethylase activity
Activity (pmol/min/mg prot)
control
sudanⅢ
tongue
liver

Figure 5

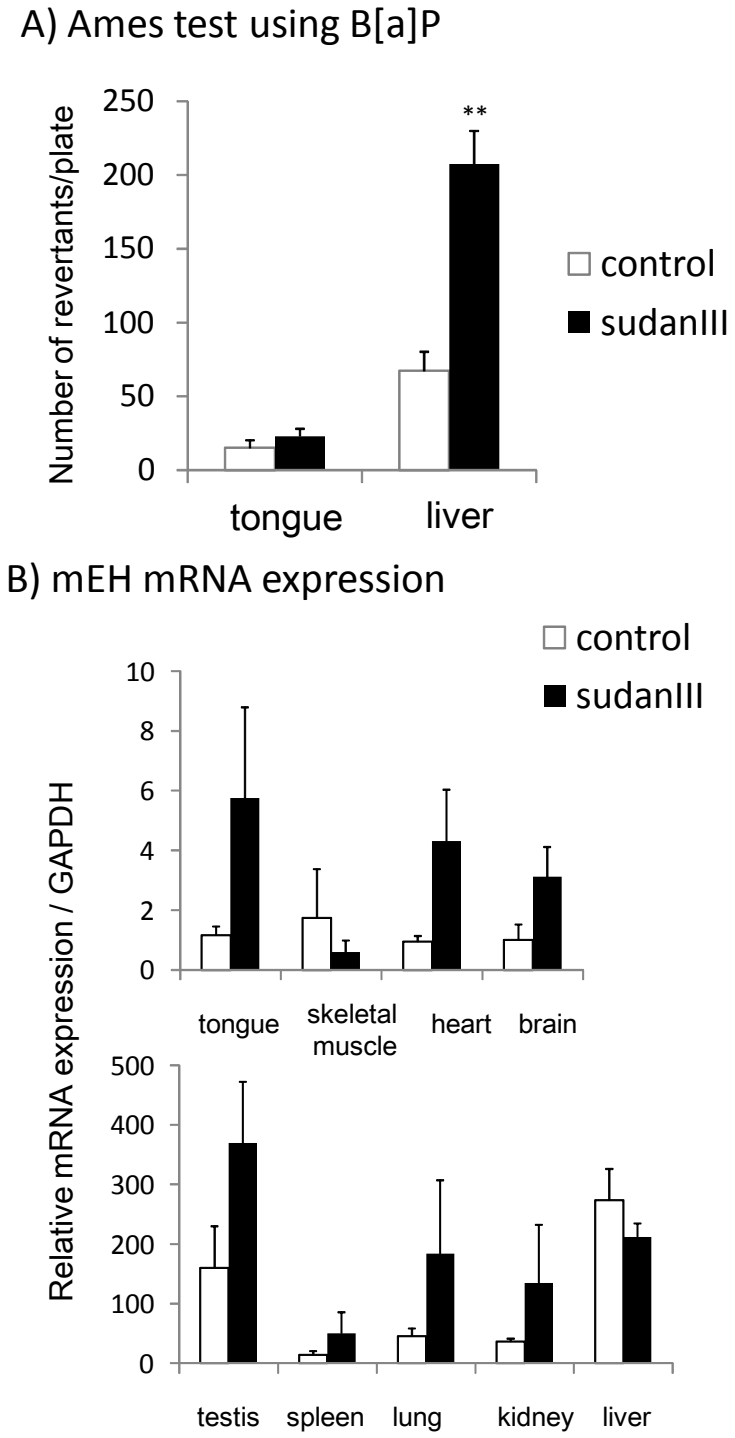
A) Ames test using B[a]P
Number of revertants/plate
250
200
150
100
50
0
**
control
sudanIII
tongue
liver
B) mEH mRNA expression
control
sudanIII
Relative mRNA expression / GAPDH
10
8
6
4
2
0
tongue
skeletal muscle
heart
brain
500
400
300
200
100
0
testis
spleen
lung
kidney
liver

Figure 6

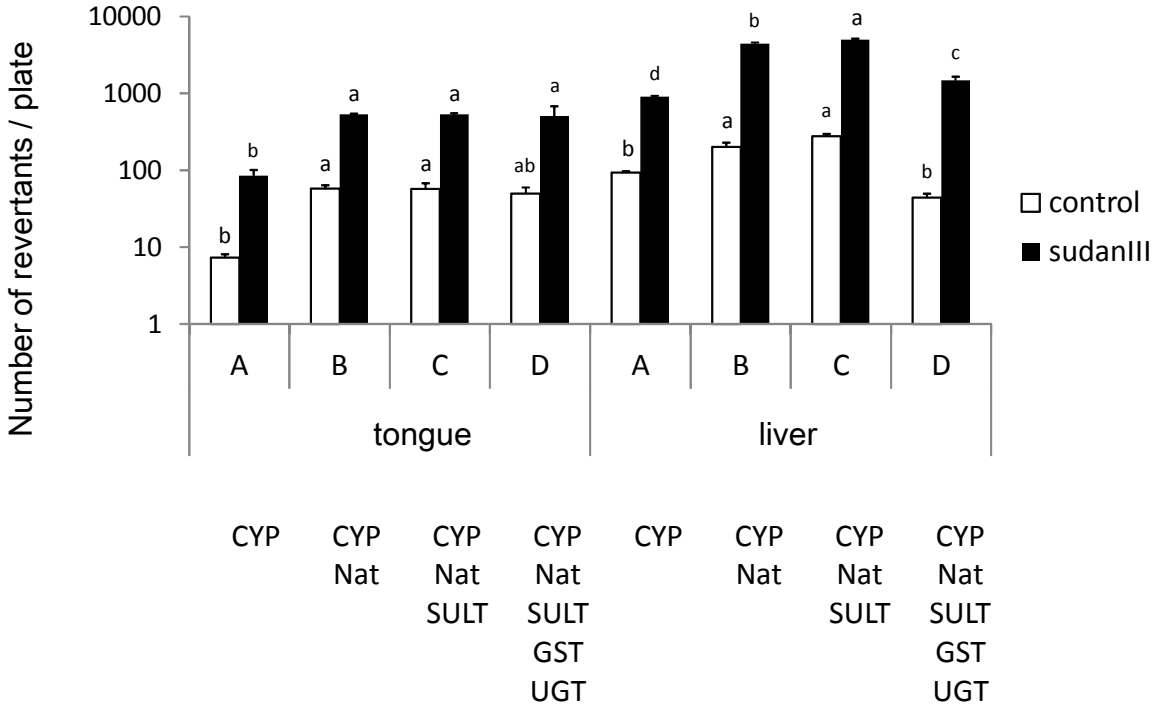
Number of revertants / plate
10000
1000
100
10
1
control
sudanIII
A
B
C
D
tongue
liver
CYP
CYP Nat
CYP Nat SULT
CYP Nat SULT GST UGT

Figure 7

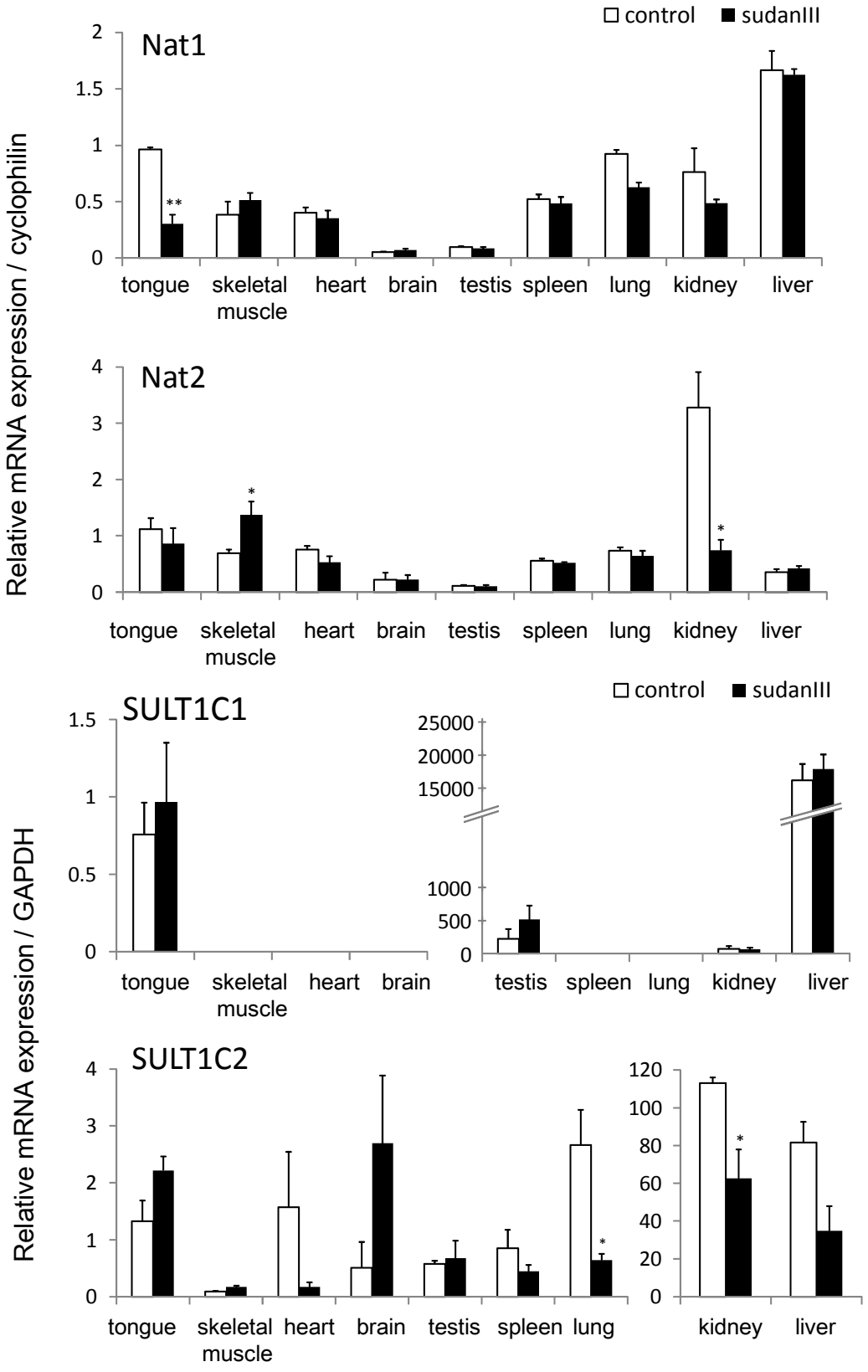
control
sudanIII
Nat1
Relative mRNA expression / cyclophilin
2
1.5
1
0.5
0
**
tongue
skeletal muscle
heart
brain
testis
spleen
lung
kidney
liver
Nat2
4
3
2
1
0
*
*
tongue
skeletal muscle
heart
brain
testis
spleen
lung
kidney
liver
control
sudanIII
SULT1C1
Relative mRNA expression / GAPDH
1.5
1
0.5
0
25000
20000
15000
1000
500
0
tongue
skeletal muscle
heart
brain
testis
spleen
lung
kidney
liver
SULT1C2
4
3
2
1
0
120
100
80
60
40
20
0
*
*
tongue
skeletal muscle
heart
brain
testis
spleen
lung
kidney
liver

Figure 8

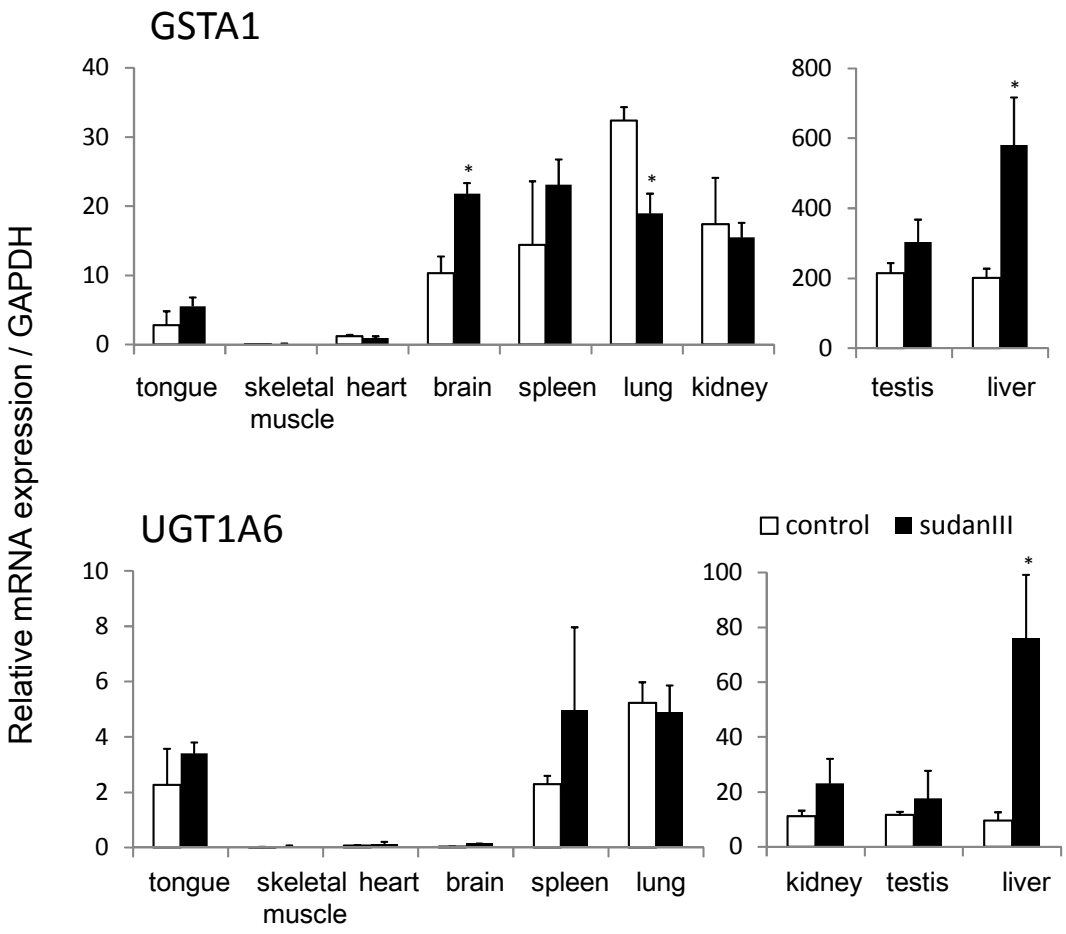
GSTA1
Relative mRNA expression / GAPDH
40
30
20
10
0
800
600
400
200
0
*
*
*
tongue
skeletal muscle
heart
brain
spleen
lung
kidney
testis
liver
UGT1A6
control
sudanIII
10
8
6
4
2
0
100
80
60
40
20
0
*
tongue
skeletal muscle
heart
brain
spleen
lung
kidney
testis
liver

Figure 9

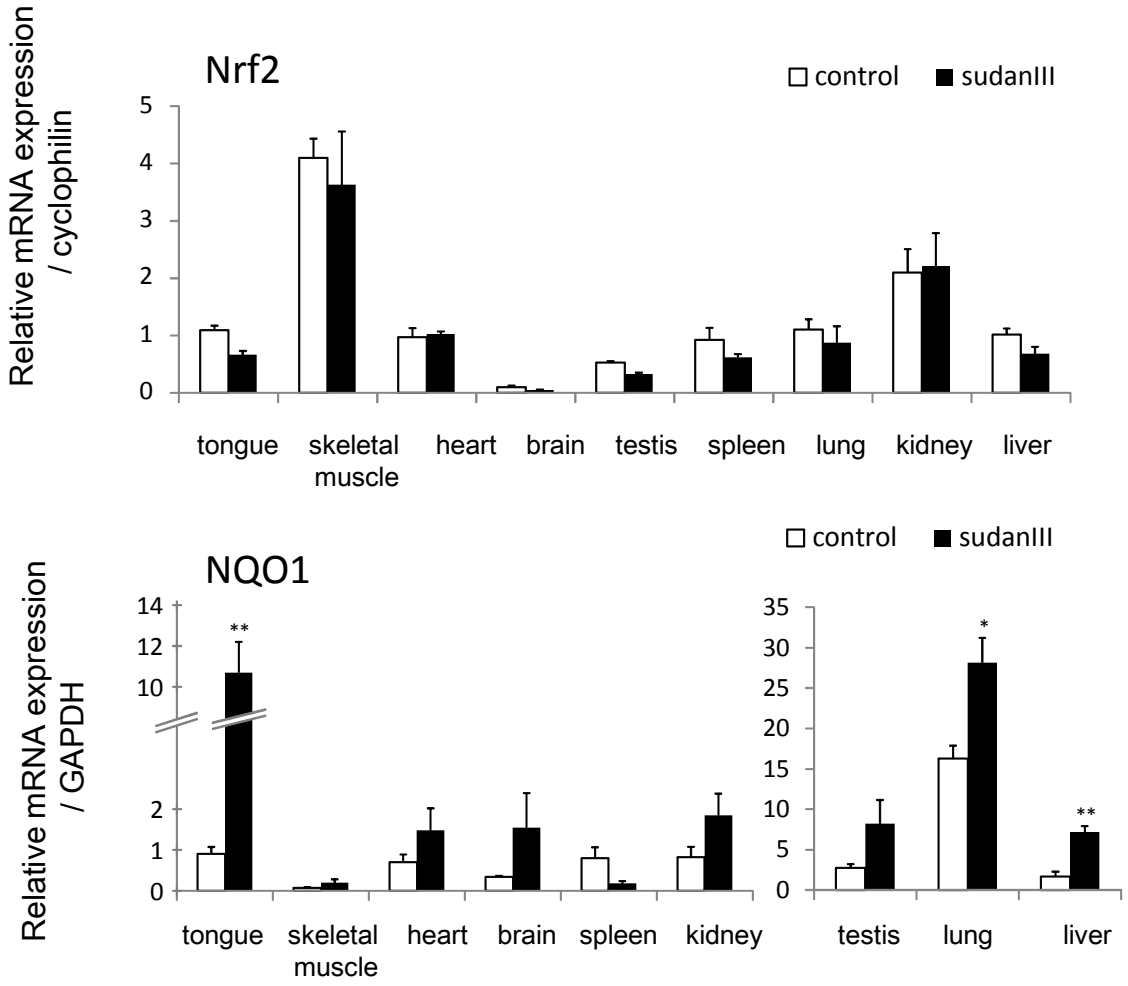
Nrf2
control
sudanIII
Relative mRNA expression / cyclophilin
5
4
3
2
1
0
tongue
skeletal muscle
heart
brain
testis
spleen
lung
kidney
liver
NQO1
control
sudanIII
Relative mRNA expression / GAPDH
14
12
10
2
1
0
**
35
30
25
20
15
10
5
0
*
**
tongue
skeletal muscle
heart
brain
spleen
kidney
testis
lung
liver

Figure 10

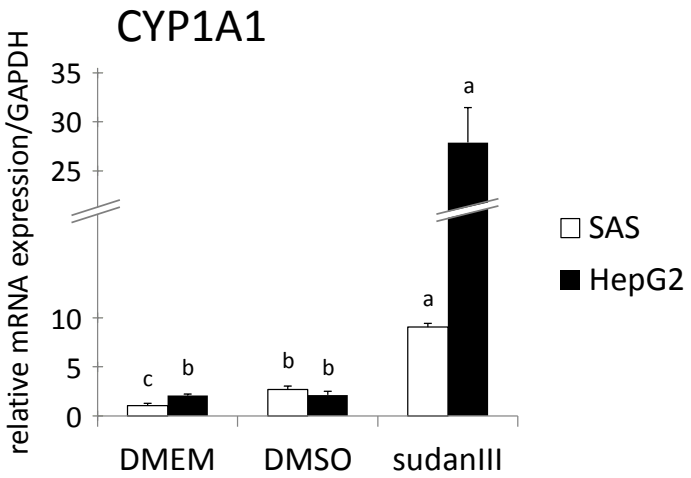
CYP1A1
relative mRNA expression/GAPDH
35
30
25
10
5
0
c
b
b
b
a
a
DMEM
DMSO
sudanIII
SAS
HepG2

Figure 11

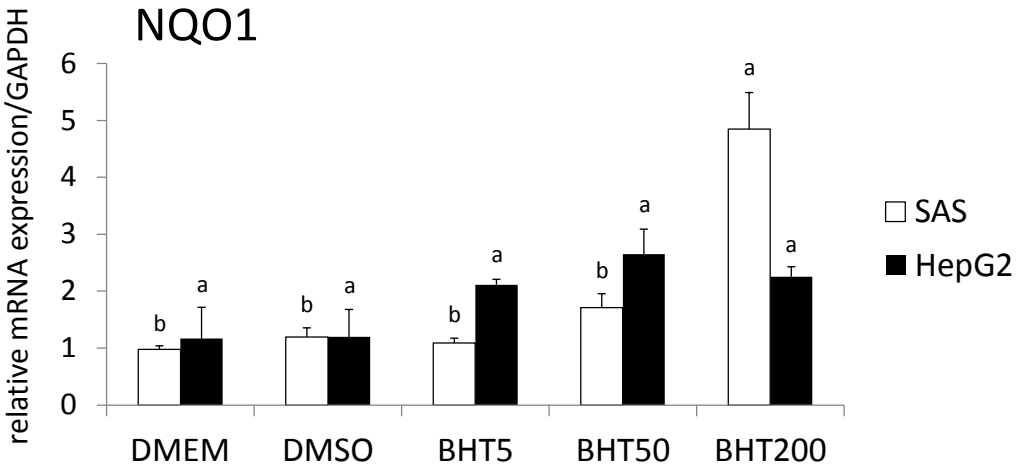
NQO1
relative mRNA expression/GAPDH
6
5
4
3
2
1
0
b
a
b
a
b
a
b
a
a
a
DMEM
DMSO
BHT5
BHT50
BHT200
SAS
HepG2

Figure 12

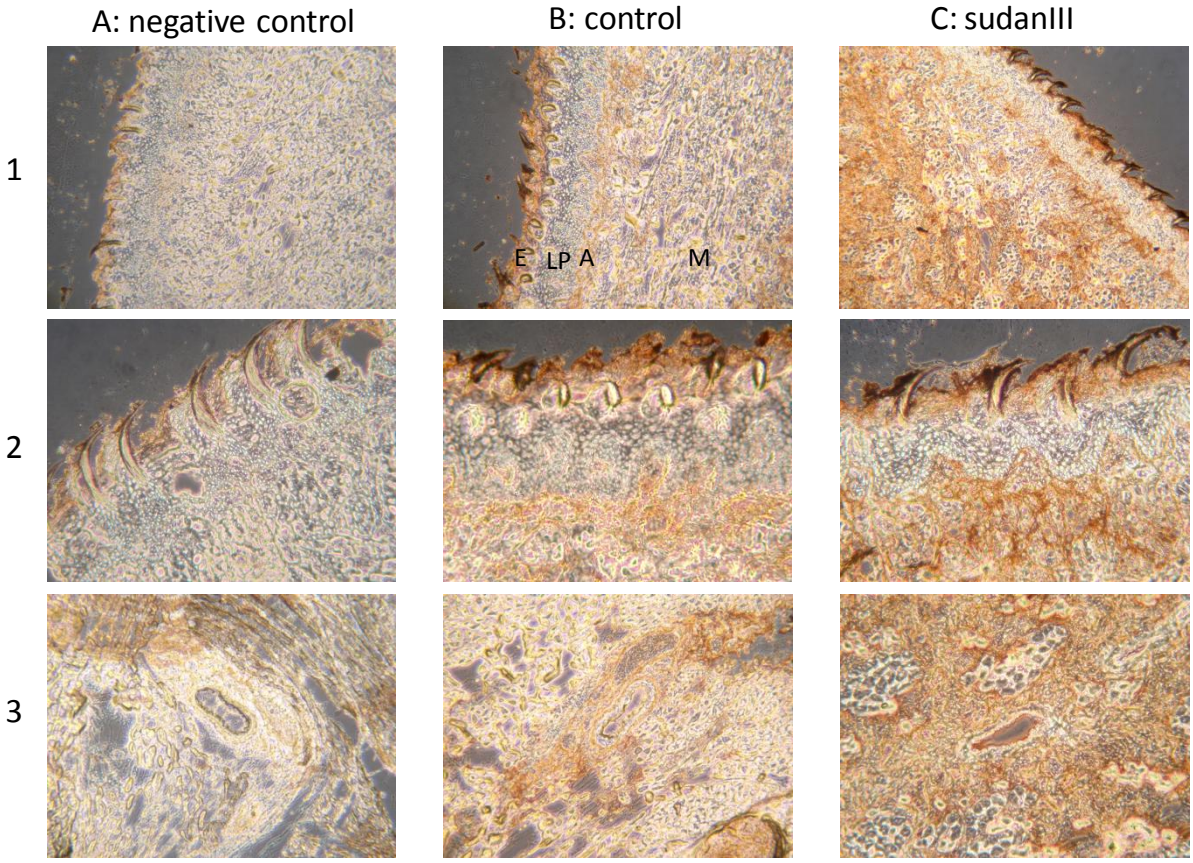

Figure 13