

**Pharmacokinetics and Differential Regulation of Cytochrome P450 Enzymes in  
Type 1 Allergic Mice**

Tadatoshi Tanino, Akira Komada, Koji Ueda, Toru Bando, Yukie Nojiri

Yukari Ueda and Eiichi Sakurai

Faculty of Pharmaceutical Sciences, Tokushima Bunri University, 180 Bouji Nishihama,

Yamashiro-cho, Tokushima, Tokushima 770-8514, Japan

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**Corresponding Author:**

Eiichi Sakurai, Ph.D.

Professor, Laboratory of Pharmaceutics, Faculty of Pharmaceutical Sciences,

Tokushima Bunri University

180 Bouji Nishihama, Yamashiro-cho, Tokushima, Tokushima 770-8514, Japan.

Phone +81-88-602-8468, FAX +81-88-655-3051-

E-mail: [esakurai@ph.bunri-u.ac.jp](mailto:esakurai@ph.bunri-u.ac.jp)

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**Abbreviations:**

APAP, acetaminophen;  $\alpha$ GP, alpha 1-acidic glycoprotein; BF, bufuralol; Carboxy-PTIO, (2-[4[carboxyphenyl]-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide]; CHZ, chlorzoxazone; CYP, cytochrome P450; fu, unbound fraction; 1'-OH-BF, 1-hydroxybufuralol; 6-OH-CHZ, 6-hydroxychlorzoxazone; 1'-OH-MDZ, 1'-hydroxymidazolam; 4-OH-TB, 4-hydroxytolbutamide; IMP, imipramine; IgE, immunoglobulin E; IL, interleukin; IS, internal standard; MDZ, midazolam; NO, nitric oxide; NOC7, 1-hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-1-triazene; OVA, ovalbumin; PH, phenacetin; PS7, 7 days after primary sensitization; SS7, 7 days after secondary sensitization; Th, T helper; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TB, tolbutamide.

## **Abstract**

Type 1 allergic diseases are characterized by elevated production of specific immunoglobulin E (IgE) for each antigen and have become a significant health problem worldwide. This study investigated the effect of IgE-mediated allergy on drug pharmacokinetics. To further understand differential suppression of hepatic cytochrome P450 (CYP) activity, we examined the inhibitory effect of nitric oxide (NO), a marker of allergic conditions. Seven days after primary or secondary sensitization (PS7 and SS7, respectively), hepatic CYP1A2, CYP2C, CYP2E1 and CYP3A activities were decreased to 45%-75% of the corresponding control, however, CYP2D activity was not downregulated. PS7 and SS7 did not change the expression levels of five CYP proteins. Disappearance of CYP1A2 and CYP2D substrates from the plasma was not significantly different between allergic mice and control mice. In contrast, area under the curve of a CYP1A2-mediated metabolite in PS7 and SS7 mice was reduced by 50% of control values. Total clearances of a CYP2E1 substrate in PS7 and SS7 mice were significantly decreased to 70% and 50% of the control without altering plasma protein binding, respectively. Hepatic amounts of CYP1A2 and CYP2E1 substrates were enhanced by allergic induction, being responsible for each downregulated activity. NO

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scavenger treatment completely improved the downregulated CYP activities. Therefore, our data suggest that the onset of IgE-mediated allergy alters the pharmacokinetics of major CYP-metabolic capacity-limited drugs except for CYP2D drugs. NO is highly expected to participate in regulatory mechanisms of the four CYP isoforms.

## Introduction

In many diseases, immunological responses and viruses are capable of downregulating the levels of cytochrome P450 (CYP) isozymes (Cheng and Morgan, 2001; Morgan, 2001). In the development of adjuvant-induced arthritis in rats, the production of proinflammatory cytokines downregulates mRNA levels, protein levels and activities of CYP2B1/2, CYP2C11, CYP2E1 and 3A1/2, even under acute inflammatory conditions (Sanada et al., 2011; Projean et al., 2005). Directly injected interleukin (IL)-1 $\beta$ , IL-6, tumor necrosis factor (TNF)- $\alpha$  and interferon-gamma are thought to act through mechanisms regarded as the downregulation of CYP gene expression, which leads to suppression of CYP content and activity (cytokine-dependent pathway) (Sujita et al., 1990; Ghezzi et al., 1986; Abdel-Razzak et al., 1993).

Excessive nitric oxide (NO) has been speculated to interact with the heme iron of CYP and to be responsible for depressed functioning of hepatic drug metabolism. (Hodgson and Renton, 1995; Monshouwer et al., 1996). NO has been considered as a marker of inflammatory conditions in humans. During the development of adjuvant-induced arthritis, nitric oxides (NO<sub>x</sub>) plasma levels from 24 h to 7 days after a single treatment were significantly higher in treated rats than in control rats, and peak

NO<sub>x</sub> levels occurred at 72 h post-treatment; however, Projean et al. (2005) did not focus on the inhibitory effects of NO on downregulated rat CYP activities. When testosterone is used as a CYP substrate, the inhibitory effect of NO is more pronounced in rat CYP2C11 than in rat CYP3A2 (Minamiyama et al., 1997). Since some CYP isoforms simultaneously participate in testosterone metabolism, it remains unsettled whether NO differentially regulates the microsomal CYP2C11 and CYP3A2 activities. Another investigation showed that human CYP1A1 (aryl hydrocarbon hydroxylase activity) was more sensitive to the inhibitory effect of NO than human CYP1A2 (7-ethoxyresorufin-O-dealkylase dealkylation) (Stadler et al., 1994). The findings may offer a possible mechanistic explanation of differential CYP regulation to us. However, it remains unclear whether the cytokine-dependent pathway or NO is the prevalent suppressor of hepatic CYP activity with irreversible and/or reversible inhibition. Therefore, initiation, culmination and resolution of immunological responses and species of radical gas will play more important roles in the mechanistic understanding of drug-disease interactions, but not drug-drug interactions.

Type I allergic diseases such as atopic dermatitis, asthma and allergic rhinitis, are a significant health problem, and are characterized by elevated production of the specific immunoglobulin E (IgE) for each antigen (Platts-Mills, 2001). Approximately 20% of

the world's population suffers from some kind of allergic disorder and its incidences continues to rise (Warner et al., 2006). Allergic disorders are associated with an imbalance in the expression of T helper (Th) 1 cells and Th2 cytokines towards Th2 cytokines. IL-4 is called B-cell-stimulating factor-1, and regulates the differentiation of naïve Th0 cells to develop a Th2 phenotype. Th2 cytokines (IL-4, IL-5 and IL-13) stimulate mast cells and eosinophils and increase allergen-specific IgE production, whereas Th1 cytokines (IL-12, 2, 1 $\beta$  and interferon-gamma) suppress IgE production (Broide, 2001). Subsequently, activated mast cells release proinflammatory cytokines and inflammatory mediators including histamine, leukotrienes, serotonin and prostaglandins (Zhu et al., 1999; Stassen et al., 2001). IL-4 markedly increases human CYP2E1 mRNA levels in primary culture (Abdel-Razzak et al., 1993), although IL-1 $\beta$ , IL-6 and TNF- $\alpha$  suppress the expression of rat CYP2E1 mRNA (Hakkola et al., 2003). Concerning chemical mediators, histamine is a mixed-type inhibitor of CYP1A1 in both rat liver microsomes and supersomes, and induces a significant nicotinamide adenine dinucleotide phosphate oxidation catalyzed by CYP2E1 supersomes (Davila-Borja et al., 2007). Osada et al (1994) showed that NO could participate in anaphylaxis (a type 1 allergic reaction) in the mouse; however, an attempt was not made to estimate regulated CYP functions. Little information is available in the literature on major CYP activities

and drug pharmacokinetics in type 1 allergic diseases. Therefore, we investigated hepatic CYP metabolism and drug pharmacokinetics in type 1 allergic mice induced by ovalbumin (OVA) emulsified with aluminum and inactive *Bordetella pertussis*. To further propose a possible key factor in drug-allergic disease interactions, we examined the inhibitory effect of NO, a marker of allergic conditions on the activities of major CYP isoforms.

## Materials and Methods

**Materials.** Imipramine (IMP), chlorzoxazone (CHZ), *Bordetella pertussis* inactive bacterial suspension, chloramphenicol and acetaminophen (APAP) were purchased from Nacalai Tesque Co. (Kyoto, Japan). Aluminum hydroxide gel (alhydrogel<sup>®</sup>) was obtained from InvivoGen (San Diego, CA, USA). Amitriptyline, chicken egg albumin (OVA), 2-acetamidophenol, chlorpropamide, diazepam, midazolam (MDZ), propranolol and tolbutamide (TB) were obtained from Wako Pure Chemicals (Osaka, Japan). Phenacetin (PH) was obtained from Sigma-Aldrich (St Louis, MO, USA). 1'-Hydroxy MDZ (1'-OH-MDZ) was obtained from Alsachim SAS (Illkirch, France). Bufuralol (BF), 6-hydroxy CHZ (6-OH-CHZ), 4-hydroxy TB (4-OH-TB) and 1'-hydroxy BF (1'-OH-BF) were purchased from Toronto Research

Chemicals Inc. (North York, ON, Canada).

1-Hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC7) and carboxy-PTIO (2-[4[carboxyphenyl]-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide] were obtained from Dojin Co. (Kumamoto, Japan).  $\beta$ -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium ( $\beta$ -NADP<sup>+</sup>), glucose-6-phosphate dehydrogenase (G-6-PDH) and glucose-6-phosphate (G-6-P) were obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan). All other chemicals used were of analytical grade and commercially available.

**Animals and Treatments.** Female ICR mice (Japan SLC Co., Shizuoka, Japan), weighing 15–20 g, were housed under standard conditions (light/dark cycle of 12 h; room temperature of  $23 \pm 1^\circ \text{C}$ ) and provided with food and water *ad libitum*. For the establishment of IgE-mediated allergic animals, the mice were sensitized by intraperitoneal (i.p.) injection of a mixture containing 5  $\mu\text{g}$  OVA, 2 mg aluminum hydroxide gel and *Bordetella pertussis*, inactive bacterial suspension ( $2 \times 10^9$  cells) on day 0 according to the methods described by Pauwels et al (1979) and Lebrec et al (1996). On day 7 after the primary sensitization (PS7), mice were sacrificed. On day 8 after the primary sensitization, the mice were challenged with 2.5  $\mu\text{g}$  OVA (250  $\mu\text{g}/\text{ml}$  of saline) by intravenous (i.v.) injection. At 7 days after the secondary sensitization

(SS7), the mice were sacrificed. Control mice were given single i.p. and i.v. injections of saline. All animal experiments were conducted in accordance with the Tokushima Bunri University Faculty of Pharmaceutical Sciences' Committee for the care and use of laboratory animals.

Mice were given carboxy-PTIO (0.5 mg/kg, i.p.) 30 min before the primary sensitization. Subsequently, carboxy-PTIO (0.5 mg/kg, i.p.) was injected to saline-treated mice (control) and the immunized mice once a day. The carboxy-PTIO dosage was determined by the in vivo data reported by Hirano et al (2015).

For drug disposition experiments, each bolus dose (5 mg/kg) of PH, CHZ and IMP was administered by tail vein injection to each group of control and allergic mice. Each of PH, CHZ and IMP was injected in different mice. The dosages used were determined by the pharmacokinetic data reported by Yoo et al. (1996) and Henderson et al (2014). At the appropriate times, the mice were sacrificed by decapitation, and blood was collected in heparinized tubes. Plasma was separated by centrifugation at 3000 rpm for 10 min. The liver was excised, washed, and homogenized with ice-cold 50 mM Tris-HCl (pH 7.4).

**Assay of Total Plasma IgE and Serum NO Levels.** Total plasma IgE levels were measured by enzyme-linked immunosorbent assay (ELISA) using a commercially

available mouse IgE ELISA Kit (Shibayagi, Gunma, Japan) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader.

Serum concentrations of  $\text{NO}_2^-$  were determined using a Nitrate/Nitrite Colorimetric Assay kit (Cayman Chemical Company., An Arbor, MI) according to the manufacturer's instructions. Briefly, blood was treated with negligible amount of EDTA, and was centrifuged at 10000 rpm for 10 min. The supernatants were placed into an ultrafiltration device (Millipore Co., Bedford, MA), and were centrifuged at 13,000 rpm for 20 min. Aliquots (40  $\mu\text{l}$ ) of filtrates were incubated with nitrate reductase to reduce any nitrates to nitrites. After incubation, sample were treated with Griess reagent and absorbance was measured at 540 nm.

**Assay of CHZ, PH, APAP and IMP in Plasma and Liver.** Plasma and/or liver homogenates were mixed with acetonitrile including internal standards (ISs) (chloramphenicol for CHZ, 2-acetoamidephenol for PH and APAP, and amitriptyline for IMP) available in a high-performance liquid chromatography (HPLC) assay. Sodium hydroxide 0.1 N was added to the mixture including IMP. CHZ was extracted in diethyl ether (Baek et al., 2006), and PH, APAP and IMP were transferred to ethyl acetate (Masubuchi and Horie, 2003; Narimatsu et al., 1999), and then centrifuged at 3000 rpm

for 10 min. After the organic layers were evaporated, the residues were reconstituted in mobile phases used for HPLC assay, and loaded onto each HPLC column.

**In Vivo Plasma Protein Binding.** Plasma protein binding was determined by ultrafiltration using the Centrifugal Filter Units (Millipore Co., Billerica, MA, USA). The unbound fraction ( $f_u$ ) of CHZ was determined as a ratio of the drug concentration in the ultrafiltrate to the total drug concentration in the plasma 10 min after i.v. injection of CHZ. Further treatment for HPLC analysis was described in the above CHZ assay in plasma. The amount of CHZ adsorbed onto the filter membranes was less than 0.1%.

**Enzyme Assay.** Livers from individual mice were homogenized with a Teflon homogenizer in three volumes (v/w) of 1.15% KCl (Sanada et al., 2011), and liver homogenates were centrifuged at  $9000 \times g$  for 20 min. The supernatants were further centrifuged at  $104,000 \times g$  for 60 min, and the microsomal pellet suspended in the homogenizing solution was recentrifuged at  $104,000 \times g$  for 60 min. The amount of microsomal protein was determined using a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA).

CYP isozyme-specific activities were determined by measuring the formation of APAP, 4-OH-TB, 1'-OH-BF, 6-OH-CHZ and 1'-OH-MDZ from PH (CYP1A2 substrate), TB (CYP2C substrate), BF (CYP2D substrate), CHZ (CYP2E1 substrate)

and MDZ (CYP3A substrate) (Masubuchi and Horie, 2003; Choi et al., 2014; Horie et al., 2002; Chittur and Tracy, 1997; Fujita et al., 2008). The incubation mixtures (400  $\mu$ l) contained liver microsomal protein (final protein concentration, 0.1 mg/ml for MDZ metabolism, 0.4 mg/ml for BF metabolism, 0.5 mg/ml for CHZ and TB metabolism, and 1 mg/ml for PH metabolism), an NADPH-generating system (0.2 mM NADP<sup>+</sup>, 28.6 mM MgCl<sub>2</sub>, 11.4 mM G-6-P and 20 units/ml G-6-PDH) and CYP substrate (10  $\mu$ M PH, 800  $\mu$ M TB, 2  $\mu$ M BF, 20  $\mu$ M CHZ or 10  $\mu$ M MDZ). All enzymatic reactions were initiated by the addition of the NADPH-generating system and were allowed to proceed at 37 °C for different times (4, 5 and 30 min). Further treatment for HPLC analysis of APAP and 6-OH-CHZ was as described in the above assay in plasma and liver. Concerning TB, BF and MDZ metabolism, each reaction was stopped with ice-cold acetonitrile including chlorpropamide, propranolol and diazepam available as ISs in an HPLC assay, respectively. Sodium hydroxide 0.1N was added to the mixture including weakly basic BF. Subsequently, 4-OH-TB, 1'-OH-BF and 1'-OH-MDZ were extracted in diethyl ether (Choi et al., 2014), chloroform (Hefnawy et al., 2007) and ethyl acetate (Fujita et al., 2008), respectively, and then centrifuged at 3000 rpm for 10 min. After evaporating the organic layers, the residues were reconstituted in each mobile phase used for HPLC analysis and loaded onto HPLC columns. The CYP activities were

expressed as metabolic formation rates of CYP isoform-specific substrates. Our preliminary study confirmed that the incubation time, the substrate concentration and the amount of microsomes were determined to be in the linear range for the metabolite formation rate.

To examine the direct inhibition of NO on the microsomal CYP activities, the experiments were performed according to the method reported by Minamiyama et al (1997). NOC7, a well-known NO donor, dissolved in 0.1 N NaOH was added to the microsomal incubation mixture. After 30 min of incubations at 37 °C in the absence and presence of 1 mM NOC7, CYP activities were determined by the rates of APAP formation and hydroxylation of CHZ, TB, MDZ and BF. The same volume of vehicle (final concentration, 0.025% NaOH) was added as the reference.

**Immunoblotting.** Liver microsomal proteins (5 µg for CYP3A and 10 µg for the other CYPs) were subjected to the 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred electrophoretically to Immobilon-P transfer membranes (Millipore, Bedford, MA, USA), and blocked with Blocking One (Nacalai Tesque). The membrane was incubated with mouse monoclonal anti-mouse CYP1A2 (ab22717, 1:2000 dilution; Abcam, Cambridge, UK), rabbit monoclonal anti-human CYP2C19 (ab137015, 1:2000 dilution; Abcam), rabbit monoclonal anti-human CYP2D6

(ab137426, 1:2000 dilution; Abcam), rabbit polyclonal anti-rat CYP2E1 (ab28146, 1:2000 dilution; Abcam) and rabbit polyclonal anti-human CYP3A4 (ab176310, 1:2000 dilution; Abcam). Horseradish peroxidase-conjugated anti-mouse IgG (NA931V, 1:20000 dilution; GE Healthcare, Little Chalfont, Buckinghamshire, UK) and anti-rabbit IgG (NA934, 1:20000 dilution; GE Healthcare) secondary antibodies were used for CYP1A2 and the other CYPs, respectively. The enhanced chemiluminescence method was used to visualize the protein bands. The levels were expressed as percentages with the control set as 100%.

**HPLC Assays.** HPLC analysis was performed on a system equipped with a Shimadzu SPD-10A, a UV detector, a Shimadzu LC-10A pump and a Shimadzu C-R4A chromatopac integrator. IMP and the other compounds (substrate and metabolites) were separated using a Mightysil RP-18 column (particle size 5  $\mu\text{m}$ , 4.6  $\times$  150 mm, Kanto Kagaku, Tokyo, Japan) and a COSMOSIL 5C8-AR-II column (particle size 5  $\mu\text{m}$ , 4.6  $\times$  150 mm, Nacalai Tesque Co., Kyoto, Japan), respectively.

PH and APAP were determined by the method of Masubuchi et al. (2003) with some modifications. Briefly, PH and APAP were monitored by UV detection at 245 nm. A mobile phase of methanol and 1.5% acetic acid at a ratio of 10:90 (v/v) was used at a flow rate of 1 ml/min.

BF and 1'-OH-BF were determined as reported by Barth et al. (2011) and Mankowski et al. (1999) with slight modifications. Briefly, BF and 1'-OH-BF were monitored by UV detection at 248 nm. A mobile phase of methanol and 10 mM phosphate buffer (pH 3) at a ratio of 40:60 (v/v) was used at a flow rate of 0.8 ml/min.

For IMP, the mobile phase of methanol and 20 mM phosphate buffer (pH 5.9) at a ratio of 55:45 (v/v) was used at a flow rate of 1 ml/min. IMP was monitored by UV detection at 254 nm.

4-OH-TB was determined by a minor modification of the method described by Choi et al. (2014). Briefly, TB and 4-OH-TB were monitored by UV detection at 230 nm. A mobile phase of acetonitrile and 10% sodium acetate (pH 4.3) at a ratio of 32:68 (v/v) was used at a flow rate of 1 ml/min.

6-OH-CHZ was determined as reported by Baek et al. (2006) with slight modifications. Briefly, a mobile phase consisting of acetonitrile and 0.1 M ammonium acetate at a ratio of 30:70 (v/v) was delivered at a flow rate of 0.8 ml/min. CHZ and 6-OH-CHZ were monitored by UV detection at 283 nm.

MDZ and 1'-OH-MDZ were determined by the method described by Fujita et al. (2008).

**Pharmacokinetic Data Analysis.** Plasma concentration data were analyzed by

noncompartmental analysis using WinNonlin version 2.1 (Pharsight, Mount View, CA, USA). The area under the plasma concentration-time curve from time zero to infinity ( $AUC_{0-\infty}$ ) was calculated according to the linear trapezoidal method. The elimination half-life ( $T_{1/2}$ ) was calculated using the equation:  $T_{1/2} = 0.693/\lambda$ , where  $\lambda$  (elimination rate constant) was estimated from the terminal slope of the plasma concentration versus time curve. The total body clearance ( $CL_{tot}$ ) was determined from  $Dose/AUC_{0-\infty}$ . The volume of distribution associated with the terminal phase ( $V_d$ ) was calculated as  $CL_{tot}/\lambda$ .

The IMP plasma concentration data were fitted to a two-compartment model. The kinetic parameters  $A$ ,  $\alpha$ ,  $B$  and  $\beta$  were calculated using the nonlinear least squares regression program, MULTI (Yamaoka et al, 1981).

**Statistical Analysis.** Statistical analysis was performed using a one-way analysis of variance test, and significance was assessed by employing Tukey's post-hoc test.

## Results

**Plasma Total IgE and Serum NO Levels.** Type I allergic diseases, such as atopic dermatitis and asthma, are characterized by the elevated production of an

antigen-specific IgE (Platts-Mills, 2001). As shown in Fig. 1A, an enhanced plasma total IgE concentration was observed at 3 days after the primary sensitization. Plasma total-IgE levels at 7 days after primary (PS7) or secondary sensitization (SS7) were significantly elevated to  $6357 \pm 1740$  and  $7799 \pm 2893$  ng/ml, respectively, showing 60-fold higher concentrations compared with the IgE values in the control mice. Plasma total IgE level in the SS7 mice did not differ from that in the PS7 mice. In our studies, the PS7 and SS7 mice were used as type 1 allergic mice.

Serum concentrations of nitrate plus nitrite (NOx) were used as an indicator of host NO production (Fig. 1B). Mice at 5 days after primary sensitization (PS5) showed a higher level of NOx compared with the corresponding control mice ( $p < 0.01$ ). Compared with the PS5, the PS7 and SS7 produced significantly higher NOx concentrations. However, the PS7 mice were similar in serum NOx concentration to the SS7 mice.

**Hepatic Microsomal CYP Isoform Activities.** The activities of CYP1A2, 2C, 2D, 2E1 and 3A enzymes were assessed in hepatic microsomes by using PH, TB, BF, CHZ and MDZ as specific substrates, respectively (Fig. 2). Except for the CYP1A2 activity, the activities of the four CYP isoforms were not significantly different when PS7 and SS7 mice were compared. The relative metabolic CYP1A2 activity (PH *O*-deethylation, APAP formation) in the PS7 mice was greatly decreased to  $75.6 \pm 4.8\%$ ,

and the decreased level was restored back to  $48.6 \pm 6.8\%$  inhibition in the SS7 mice. The relative CYP2C activity (TB 4-hydroxylation) was dramatically decreased to  $70.2 \pm 9.3\%$  and  $73.7 \pm 6.3\%$  by the PS7 and SS7, respectively. The decline in CYP2E1 activity (CHZ 6-hydroxylation) was  $58.2 \pm 10.9\%$  and  $56.3 \pm 8.3\%$  in the PS7 and SS7 mice, respectively. CYP3A activity (MDZ 1'-hydroxylation) was decreased by  $52.0 \pm 6.7\%$  and  $46.6 \pm 12.5\%$  by the PS7 and SS7, respectively. In contrast, the CYP2D activity (BF 1'-hydroxylation) was slightly decreased to  $17.1 \pm 10.5\%$  in PS7 mice, and was not altered by the SS7. Even at 5 days after the primary sensitization (PS5), CYP1A2, CYP2C and CYP3A activities significantly decreased. CYP 2D and CYP2E1-dependent activities were not altered (data not shown).

**Western Blot Analysis of CYP Isoforms.** The expression levels of microsomal CYP1A2, CYP2C, CYP2D, CYP2E1 and CYP3A proteins were investigated by Western blot analysis (Fig. 3). The PS7 slightly reduced the protein levels of CYP2C and CYP2E1 isoforms, resulting in an insignificant difference between the PS7 and control mice. In the SS7 mice, protein levels of five CYP enzymes were similar to those in the control and PS7 mice.

**Pharmacokinetics of PH, APAP, IMP and CHZ.** The plasma concentration profiles and pharmacokinetic parameters of PH, APAP, CHZ and IMP in PS7 and SS7

mice are shown in Fig. 4 and Table 1, respectively. The plasma levels of PH and IMP in the two allergic mice were similar to those of control mice (Figs. 4A and 4C), resulting in insignificant difference in  $CL_{tot}$ . The PS7 and SS7 did not alter the Vd and AUC values of PH. Concerning a CYP1A2-dependent PH metabolite, the plasma concentration-time profiles of APAP were significantly different between allergic and control mice (Fig. 4B). The PS7 and SS7 mice gave approximately 50% reduction in AUC value, compared with the control mice.

Plasma CHZ concentrations over the experimental periods were significantly higher in the PS7 and SS7 mice than in the control mice (Fig. 4D). The pharmacokinetic parameters calculated for PS7 mice were similar to those calculated for SS7 mice.  $CL_{tot}$  values in the allergic mice were lower than that in the control mice, and the highest AUC values were obtained in the PS7 and SS7 mice. PS7 and the SS7 did not change the Vd value of CHZ, unlike that of IMP (about 40% reduction).

**In Vivo Protein Binding of CHZ.** The plasma binding of acidic CHZ was not significantly altered in PS7 and SS7 mice, with an average  $f_u$  of  $97.0 \pm 0.9\%$  and  $96.8 \pm 1.2\%$ , respectively, compared with that of  $96.7 \pm 0.9\%$  in the control mice. These data would imply an unchanged CHZ distribution in the type 1 allergic mice. Our protein binding data obtained in the control mice were close to the in vivo data reported by Gao

et al (2013).

**Hepatic PH and CHZ Concentrations.** The time courses of hepatic PH and CHZ concentrations following intravenous injection of PH and CHZ are shown in Fig. 5. In PS7 and SS7 mice, high hepatic concentrations of parental PH were observed at the initial 5 min after the injection (Fig. 5A). Hepatic PH levels were not different between the PS7 and SS7 mice. In the control mice, hepatic PH was not detected over the experimental periods. A CYP1A2-dependent metabolite (APAP) was under the detectable limit in liver of the allergic and control mice.

Hepatic CHZ concentrations in the PS7 and SS7 mice were significantly higher than those in the control mice (Fig. 5B). Comparison of hepatic levels in the PS7 mice with those in the PS7 mice showed no significant difference over the experimental periods.

**Inhibitory activity of NO on Activities of Hepatic CYP Isoforms.** Figure 6 shows the effect of NO production on the activities of hepatic CYP isoforms. NOC7 (NO donor) pretreatment dramatically suppressed the catalytic activity of CYP2E1 and 3A isozymes with inhibition of  $88.4 \pm 3.4\%$  and  $96.9 \pm 1.8\%$ , respectively. The degree of inhibition of PH *O*-deethylation (CYP1A2 activity) and TB 4-hydroxylation (CYP2C activity) was  $50.0 \pm 4.6\%$  and  $30.6 \pm 3.4\%$ , respectively. However, NO production did

not downregulate CYP-2D-dependent BF 1'-hydroxylation activity.

To further characterize participation of NO in the downregulated CYP activity, the PS7 and SS7 mice were continuously treated with carboxy-PTIO, a NO scavenger (Fig. 7). In the PS7 mice with carboxy-PTIO treatment, CYP2C, CYP2E1 and CYP3A activities were rescued back to individual CYP levels in the control mice with saline or carboxy-PTIO. CYP1A2 activity was significantly enhanced by the carboxy-PTIO treatment, however, the carboxy-PTIO reduced the CYP1A2 activity in the saline-injected mice (control). CYP1A2 activity was not significant difference between the PS7 and control mice treated with carboxy-PTIO. We could not clarify the reasons why carboxy-PTIO downregulated only CYP1A2 activity in the saline-injected mice. Concerning the SS7 mice, continuous carboxyl-PTIO treatment (> 12 days) significantly decreased body weights of mice (data not shown).

## **Discussion**

OVA is a protein with good antigenicity and carrier activity; however, the addition of some adjuvants is necessary to reinforce immunogenicity when establishing an animal model. Aluminum adjuvants are the first choice as immune adjuvants, and can be used to induce humoral immune reactions and stimulate Th2 cells to generate a high

levels of antibodies. When aluminum adjuvants are used in allergic diseases, Al(OH)<sub>3</sub> dosage forms (powder, gel and solution) and methods provide different findings in different experiments (Xi et al., 2014). According to the method reported by Behrendt (1987), we have established herein type 1 allergic mice by treatment with a mixture of OVA, aluminum hydroxide gel and inactive *Bordetella pertussis*.

IL-4 regulates differentiation of naïve Th0 cells to develop a Th2 phenotype, and is an essential cytokine for IgE responses in both mice and humans (Coffman et al., 1988; Romagnani, 1990). A protein toxin of *Bordetella pertussis* has a potent adjuvant capacity and the ability to upregulate IgE production in mice (Mu and Sewell, 1993). Treatment with *Bordetella pertussis* and OVA produces the highest IL-4 concentration. The mice given *Bordetella pertussis* and OVA show a strong relationship between IL-4 and IgE responses. The secretion of IL-5, which activates eosinophil granulocytes from Th2 cells is not enhanced by a protein exotoxin produced by *Bordetella pertussis* (Mu et al., 1993). Serum total IgE and OVA-specific IgE levels are 5-fold and 3-fold higher in the mice immunized with *Bordetella pertussis* and OVA, respectively, than in mice at 3 days after injecting only OVA (Mu and Sewell, 1993). At 7 days, total IgE and specific IgE levels are dramatically increased. In our study, the plasma total IgE levels at 3 days after primary immunization were approximately 4-fold higher than those after the

injection of vehicle (Fig. 1). PS7 (day 7 of primary treatment) mice had a drastic increase in total plasma IgE levels, similar to the data published by Mu and Sewell (1993). At 7 days after secondary sensitization (SS7), the high IgE levels were observed (Fig.1). Therefore, we considered PS7 and SS7 mice to be a type 1 allergic disease animal model.

In this study, PS7 and SS7 significantly decreased microsomal CYP1A2, CYP2C, CYP2E1 and CYP3A activities with a variation of 50%–70% inhibition except for CYP2D activity (Fig. 2). We preliminarily found that PS7 and SS7 negligibly reduced hepatic CYP2E1 mRNA levels (data not shown). Abdel-Razzak et al. (1993) reported that IL-4 increased human CYP2E1 mRNA levels to 5-fold, but did not significantly affect human CYP1A2-mediated ethoxyresorufin-O-deethylase and CYP3A4-mediated nifedipine oxidation activities, completely differing from our metabolic activities in type 1 allergic mice. IL-1 $\beta$ , IL-6, TNF- $\alpha$  and interferon-gamma are well-known to uniformly downregulate human CYP1A2, 3A and 2C activities and their expression (Abdel-Razzak et al., 1993). The protein and mRNA expression of rat CYP2D and CYP2E1 are suppressed by IL-1 (Kurokohchi et al., 2001; Hakkola et al., 2003). At least, the PS7 did not significantly downregulate the protein levels of CYP2D and 2E1 (Fig. 3). Compared with the proinflammatory cytokines, IgE-mediated allergy may have

considerably more complex CYP regulation patterns. Some mediators of the other Th2 cytokines, chemical mediators and reactive nitrogen species may antagonize or counteract the effects of cytokines on CYP expression.

Immune-mediated diseases may change protein binding and blood flow rates, possibility leading to altered drug pharmacokinetics. We focused on the characteristic alteration of CYP1A2, CYP2D and CYP2E1 activities, and selected the CYP substrates used most often as *in vivo* probes. We studied the pharmacokinetics of metabolic capacity-limited drugs (CHZ and IMP) and a hepatic blood flow-limited drug (PH). IgE-mediated allergy restricted the disposition of CYP2E1-metabolizing CHZ from plasma (Fig. 4D). To understand the source of this altered disposition, *in vivo* protein binding and hepatic CHZ levels were examined. It is known that acidic CHZ is mainly bound to albumin (Rockich and Blouin, 1999). PS7 and the SS7 mice displayed no change in plasma protein binding and the high hepatic amounts of parent CHZ (Fig. 5B), strongly indicating the responsibility of downregulated CYP2E1 metabolism with the *in vitro* activity data. Unlike metabolic capacity-limited CHZ, the disappearance of hepatic blood-flow-limited PH (acidic CYP1A2 substrate) from plasma was not altered by PS7 and SS7 (Fig. 4A). Simultaneously, we monitored acidic APAP, a CYP1A2-specific metabolite in plasma, resulting in the low AUC values in the allergic

mice (Table 1). Hepatic APAP was below the detectable limit; however, high hepatic concentrations of parent PH were detected in allergic mice (Fig. 5A). These results indicated that the reduced AUC values of APAP would reflect CYP1A2-dependent metabolic capacity. Though we suspected an alteration of hepatic blood-flow rate and hepatic uptake in the IgE-mediated allergy with the unchanged  $V_d$ , the constant  $CL_{tot}$  and the high hepatic amounts of blood flow-limited PH, hepatic blood-flow rate and hepatic uptake did not appear to be restricted by PS7 and SS7. Mouse CYP2D unlike human CYP2D, has a limited substrate specificity and efficiently metabolizes IMP to 2-hydroxy IMP (Masubuchi et al, 1997). IMP is a weakly basic and metabolic capacity-limited drug. In this study, the extent to which IMP is bound to alpha 1-acidic glycoprotein ( $\alpha$ GP) remains unclear. Reportedly, transgenic mice with elevated  $\alpha$ GP show no significant alterations in  $CL_{tot}$  and AUC values of IMP; however, there are significant decreases in the serum unbound fraction ( $f_u$ ),  $V_d$  and  $T_{1/2}$  (Yoo et al., 1996). The altered pharmacokinetics were in good agreement with the changes in our pharmacokinetic parameters. The published findings, together with our data on CYP2D activity, allowed us to predict unchanged IMP pharmacokinetics. Practically, PS7 and SS7 did not alter the metabolic capacity-limited IMP pharmacokinetics (Fig. 4C and Table 1). Yoo et al (1996) also showed that serum IMP concentrations at the initial times

were higher in transgenic mice than in control mice. Similarly, our data showed high plasma IMP concentrations at initial times in PS7 and the SS7 mice (Fig. 4C). In liver, we found that CYP2D-metabolized 2-hydroxy IMP and parent IMP were not quantitatively different between allergic mice and control mice (data not shown). Consequently, weak basic and metabolic capacity-limited IMP pharmacokinetics depended on the metabolic capability of the CYP2D enzyme in the allergic mice.

NO inhibits the proliferation of Th1 and their production of IL-2 and interferon-gamma (Abrahamsohn and Coffman, 1995; Stenberg and Mabbott, 1996), although Th2 is not affected by NO (Taylor-Robinson et al., 1994). It can participate in the type 1 allergic reaction (anaphylaxis) (Osada et al., 1994). Patients with bronchial asthma and allergic rhinitis show an increased level of NO in exhaled air (Alving et al., 1993; Kharitonov et al., 1997). We also confirmed that the PS7 and SS7 enhanced serum NO concentrations (Fig. 1B). Minamiyama et al (1997) demonstrated that NO could interact with microsomal CYP in two ways: NO reversibly binds to the heme moiety of CYP, forming iron-nitrosyl complexes, and it irreversibly inactivates CYP through the thiol modification pathway. They also demonstrated that NO did not change the molecular size of rat microsomal CYP3A and 2C11. We confirmed that PS7 and SS7 insignificantly decreased the protein expression of microsomal CYP1A2, 2C,

2D, 2E1 and 3A enzymes (Fig. 3); however, the microsomal CYP activities were greatly reduced (Fig. 2). These results may suggest posttranslational regulation of hepatic CYP enzymes such as the inhibitory effect of NO. In this study, NO production inhibited the microsomal CYP activities in order of CYP3A, CYP2E1, CYP1A2 and CYP2C, and did not affect the functional regulation of the CYP2D enzyme (Fig. 6). These results were considerably similar to the inhibitory patterns of all five CYP enzymes shown in Fig. 2. Our in vivo study with carboxy-PTIO strongly characterized the participation of NO in the differentially downregulated CYP activities (Fig. 7). We need to clarify the existence of hepatic CYP complexed with NO in type 1 allergic mice and to clear the reason for the insensitivity of CYP2D enzyme to NO.

In conclusion, our data suggest that the onset of IgE-mediated allergic diseases would alter pharmacokinetics of the CYP1A2-, CYP2C-, CYP2E1- and CYP3A-metabolic capacity-limited drugs. NO-CYP interaction is greatly expected to participate in the regulatory mechanisms of major CYP enzymes. Our study would provide a first step of useful information to investigate the main mechanism of drug-disease interactions.

### **Authorship Contributions**

*Participated in research design:* Tanino, Sakurai

*Conducted experiments:* Tanino, Komada, Ueda K, Bando, Nojiri, Ueda Y

*Performed data analysis:* Tanino, Komada, Ueda K, Bando, Nojiri, Ueda Y

### **Conflict of Interest**

The author(s) declare(s) that they have no conflicts of interest to disclose.

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## Legends of Figures

Figure 1. Total plasma IgE and serum NO levels in type 1 allergic mice. A) ○, control mice; ●, sensitized mice. Each point represents the mean  $\pm$  S.D. of 10-15 mice. \* $p < 0.01$  compared with control mice. B) At 5 and 7 days after the primary sensitization (PS5 and PS7, respectively), mice were sacrificed. On day 8 after the primary sensitization, mice received the secondary sensitization of ovalbumin. The mice were sacrificed at 7 days after the secondary sensitization (SS7). Each point represents the mean  $\pm$  S.D. of 10 mice. \* $p < 0.01$  compared with individual control mice. \*\* $p < 0.05$  compared with PS5 mice.

Figure 2. Changes in hepatic CYP isozyme activities in IgE-mediated allergic mice. □, control mice for PS7 and SS7; ■, sensitized mice (PS7 and SS7 mice). Data are expressed as the mean  $\pm$  S.D. of 4-6 mice. \* $p < 0.01$  compared with each control mice. \*\* $p < 0.01$  compared with PS7 mice. A) APAP formed from 10  $\mu$ M PH after a 5-min incubation with microsomes (1 mg/ml); B) 4-OH-TB formed from 800  $\mu$ M TB after a 30-min incubation with microsomes (0.5 mg/ml); C) 1'-OH-BF formed from 2  $\mu$ M BF after a 5 min-incubation with

microsomes (0.4 mg/ml); D) 6-OH-CHZ formed from 20  $\mu$ M CHZ after a 5-min incubation with microsomes (0.5 mg/ml); E) 1'-OH-MDZ formed from 10  $\mu$ M MDZ after a 4-min incubation with microsomes (0.1 mg/ml).

Figure 3. Hepatic protein expression of CYP1A2, 2C, 2D, 2E1 and 3A in primary sensitized mice. The protein expression is expressed in terms of percentage of the control mice (Cont.) on day 7 after i.p. injection of saline, with the control set to 100%. All samples were loaded in triplicate.

Figure 4. Plasma concentration-time profiles of PH, APAP, IMP and CHZ after single intravenous injection. CYP probe substrates were intravenously injected at each dose of 5 mg/kg. A) PH (CYP1A2 substrate); B) APAP (CYP1A2 metabolite); C) IMP (CYP2D substrate); D) CHZ (CYP2E1 substrate).  $\circ$ , mice 7 days after i.p. injection of saline (control);  $\bullet$ , PS7 mice;  $\blacksquare$ , SS7 mice. Data are expressed as the mean  $\pm$  S.D. (n=4-6).

Figure 5. Hepatic PH and CHZ concentrations after intravenous injection. A) parent PH; B) parent CHZ. Control: mice 7 days after i.p. injection of saline.

Data are expressed as the mean  $\pm$  S.D. (n=4-6). \*p<0.01, significantly different from control mice. ND, not detectable.

Figure 6. Inhibitory effect of NO on the activities of hepatic CYP isozymes. Hepatic microsomes were preincubated with 1 mM NOC7 or vehicle for 30 min at 37°. C. Data are expressed as means  $\pm$  S.D. (n=3) of the percentage of CYP isozyme activities in the absence of NOC7. \*p<0.01, significantly different from the vehicle.

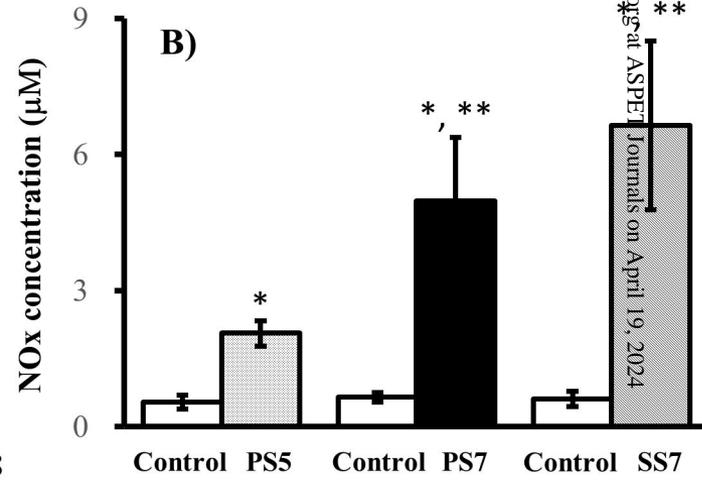
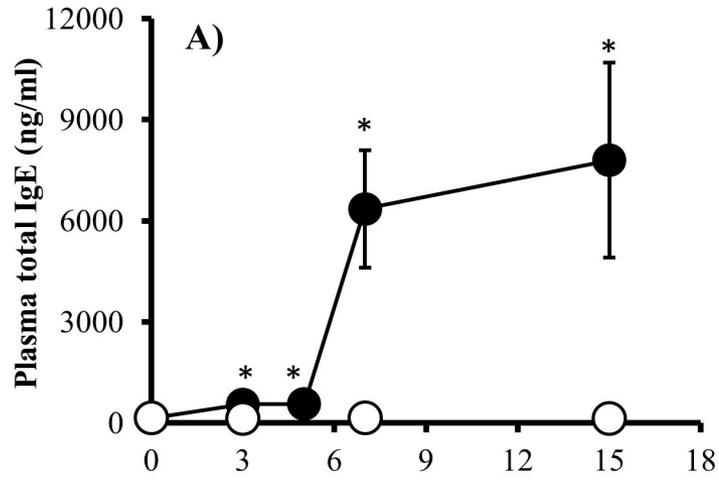
Figure 7. Changes in microsomal hepatic CYP activities in type 1 allergic mice treated with carboxy-PTIO. □, mice without carboxy-PTIO treatment; ■, mice with carboxyl-PTIO treatment. Control: 7 days after i.p. injection of saline. Data are expressed as the mean  $\pm$  S.D. (n=4). \*p<0.01, significantly different from PS7 mice without carboxy-PTIO treatment.

Table 1 Pharmacokinetic parameters after single i.v. injection of CYP probe substrates.

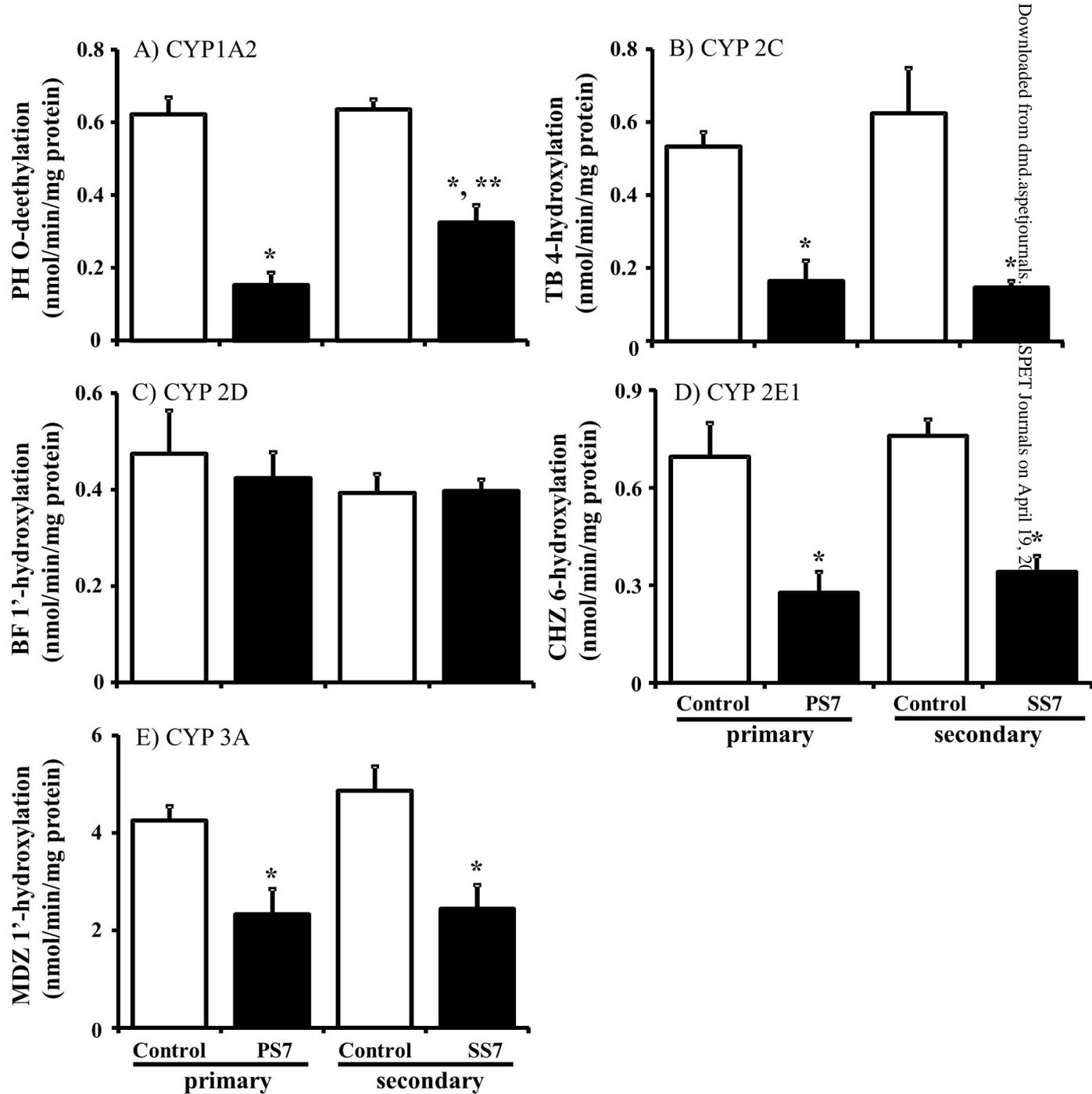
Substrate (or metabolite)	Parameters	Control	Sensitization	
			PS7	SS7
PH	$\lambda$ ( $\text{min}^{-1}$ )	$0.16 \pm 0.03$	$0.15 \pm 0.03$	$0.14 \pm 0.01$
	AUC ( $\mu\text{M} \cdot \text{min}$ )	$266.5 \pm 79.1$	$183.0 \pm 44.8$	$207.2 \pm 31.9$
	$T_{1/2}$ (min)	$4.60 \pm 1.27$	$4.91 \pm 0.81$	$4.97 \pm 0.37$
	$CL_{\text{tot}}$ (ml/min/kg)	$113.4 \pm 29.8$	$161.0 \pm 35.2$	$137.9 \pm 21.7$
	Vd (l/kg)	$0.89 \pm 0.26$	$1.19 \pm 0.41$	$0.66 \pm 0.24$
APAP	AUC ( $\mu\text{M} \cdot \text{min}$ )	$375.3 \pm 16.9$	$166.1 \pm 22.9^*$	$216.7 \pm 25.6^*$
IMP	A (nmol/ml)	$2.59 \pm 0.17$	$3.91 \pm 0.45^*$	$3.52 \pm 0.73^*$
	$\alpha$ ( $\text{min}^{-1}$ )	$0.033 \pm 0.011$	$0.055 \pm 0.007$	$0.040 \pm 0.009$
	B (nmol/ml)	$1.62 \pm 0.31$	$2.02 \pm 0.85$	$1.48 \pm 0.18$
	$\beta$ ( $\text{min}^{-1}$ )	$0.017 \pm 0.007$	$0.031 \pm 0.011$	$0.022 \pm 0.002$
	$CL_{\text{tot}}$ (ml/min/kg)	$157.9 \pm 19.5$	$189.0 \pm 6.1$	$177.9 \pm 14.8$
	AUC ( $\mu\text{M} \cdot \text{min}$ )	$101.7 \pm 13.5$	$83.7 \pm 2.7$	$89.4 \pm 7.8$
CHZ	Vd <sub>ss</sub> (l/kg) <sup>a)</sup>	$7.04 \pm 0.57$	$4.47 \pm 0.92^*$	$4.72 \pm 0.64^*$
	$\lambda$ ( $\text{min}^{-1}$ )	$0.22 \pm 0.04$	$0.14 \pm 0.01^*$	$0.11 \pm 0.01^*$
	AUC ( $\mu\text{M} \cdot \text{min}$ )	$859.9 \pm 124.5$	$1145.8 \pm 57.6^*$	$1134.6 \pm 86.9^*$
	$T_{1/2}$ (min)	$3.16 \pm 0.47$	$4.86 \pm 0.29^*$	$6.46 \pm 0.23^*$
	$CL_{\text{tot}}$ (ml/min/kg)	$35.1 \pm 5.3$	$25.8 \pm 1.3^*$	$25.9 \pm 1.8^*$
	Vd (l/kg)	$0.13 \pm 0.04$	$0.18 \pm 0.01$	$0.20 \pm 0.03$

Data are expressed as the mean (n=4-6). a) Calculated by  $V_{\text{dss}} = \text{dose} \cdot \text{AUMC}/(\text{AUC})^2$ . AUMC, the area under the first moment curve. \* p<0.05, significantly different from the control mice.

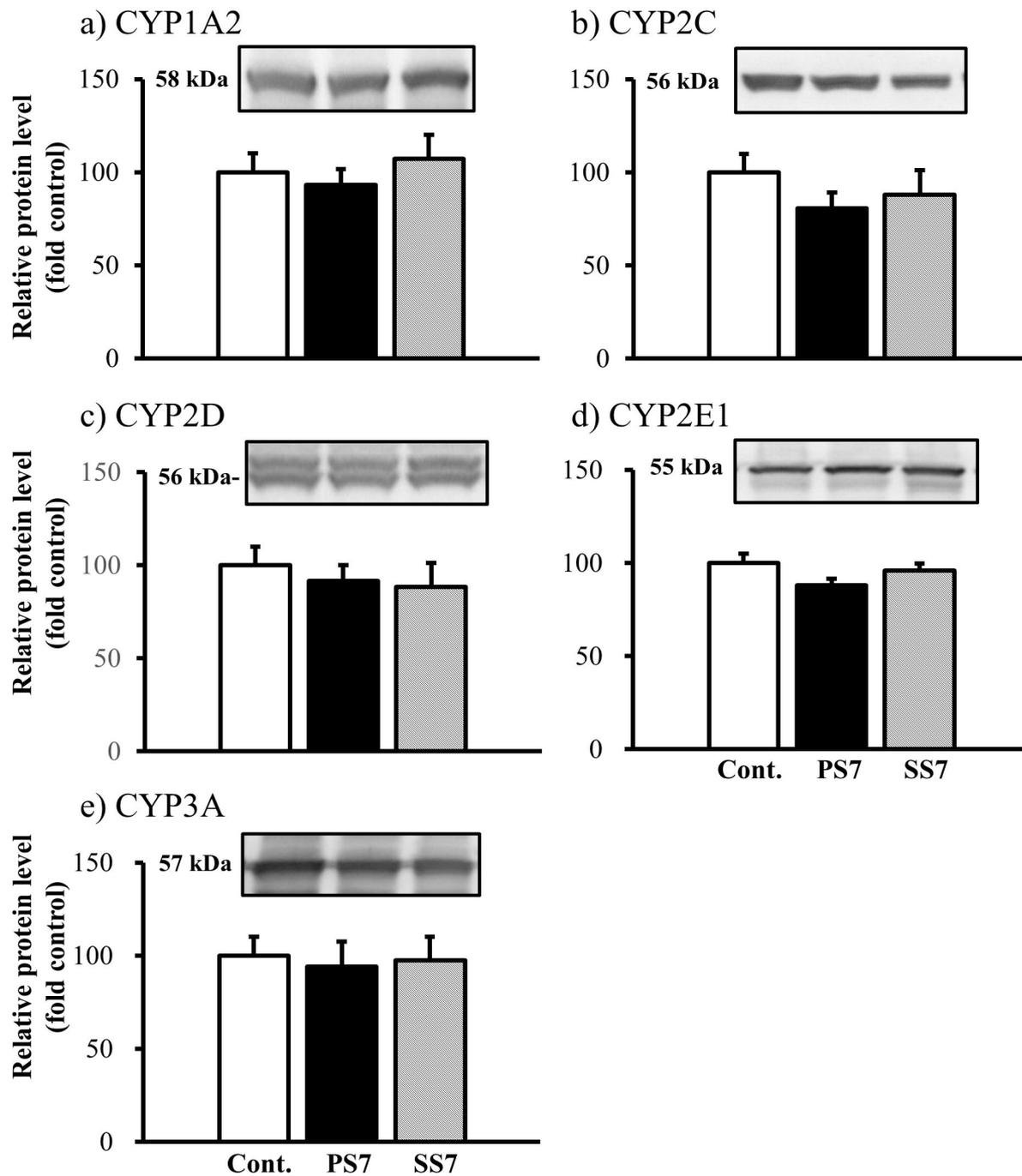
**Fig. 1**

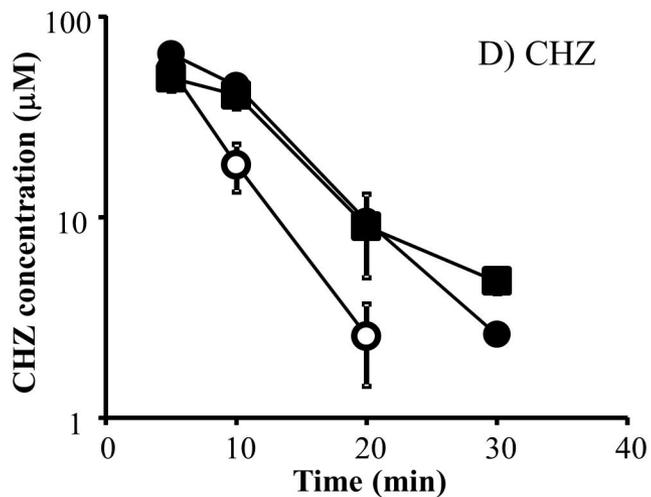
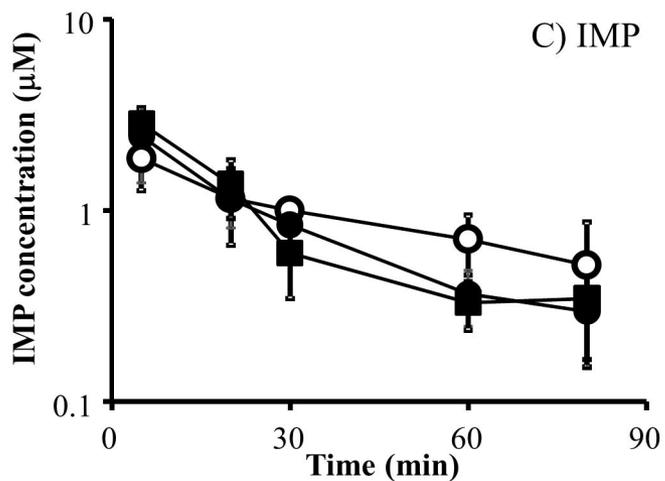
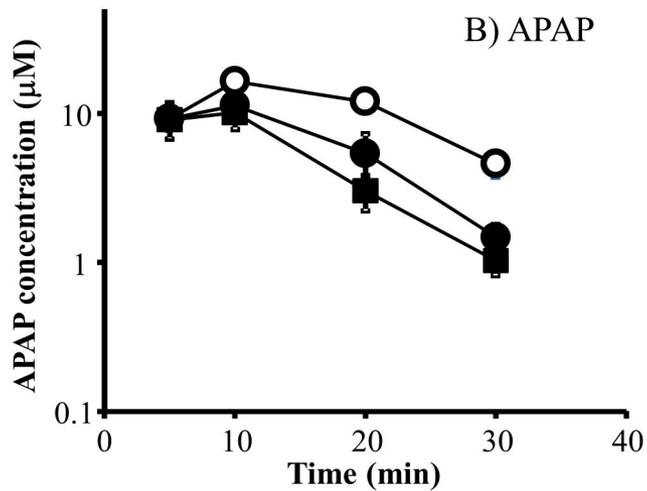
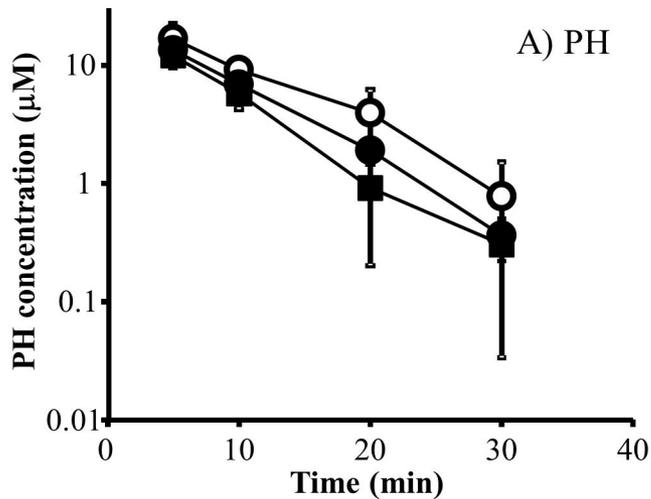


**Fig. 2**



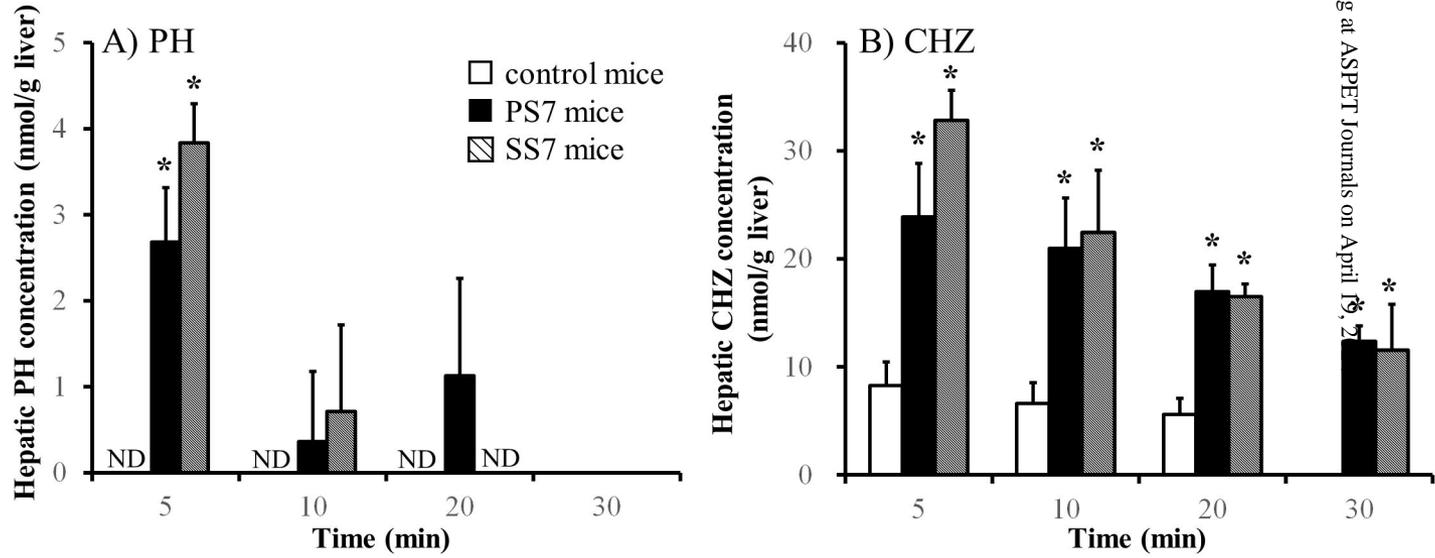
**Fig. 3**



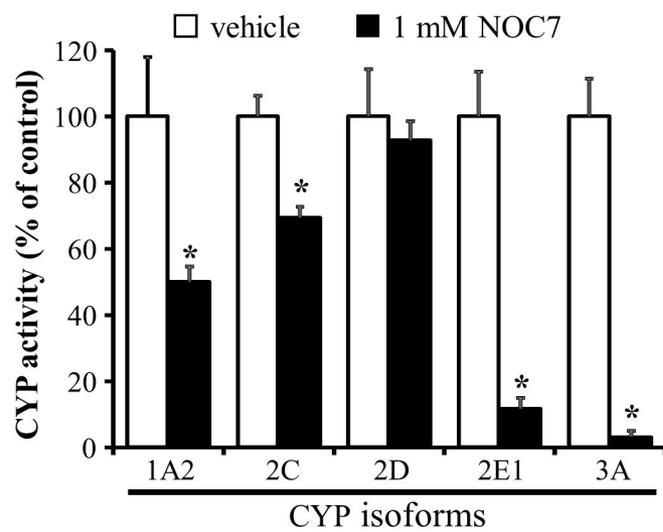


**Fig. 4**

**Fig. 5**



**Fig. 6**



**Fig. 7**

