



Hepatic Cytochrome P450 Activity and Nitric Oxide Production During Multiple Ovalbumin Challenges

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Abstract

Background and Objectives Mast cell-mediated allergic diseases are a significant global health problem. Nitric oxide (NO) produced by acute type 1 allergies greatly suppresses hepatic cytochrome P450 (CYP) metabolism. A recent in vitro study demonstrated that repeated FcεRI-mediated activation intrinsically modulates mast cell function. We investigated the effect of ovalbumin (OVA) challenges on CYP activity and NO production under real immune responses.

Methods After repeated sensitization with OVA once a week, serum nitrate plus nitrite (NOx) and total plasma immunoglobulin E concentrations were measured using commercially available kits. Hepatic microsomal CYP-specific activities and protein expression were determined using typical substrates and by western blot, respectively. In the liver, the levels of inducible NO synthase (iNOS), F4/80, and c-kit mRNA were determined by real-time polymerase chain reaction. Hepatic total NOS activity was measured using a colorimetric assay kit.

Results When mice received multiple OVA challenges, the 11th sensitization elevated NOx concentrations in serum and suppressed the activities of five major CYPs without altering protein expression levels. After the 7th, 11th, and 15th sensitizations, F4/80-positive Kupffer cell and hepatic c-kit-dependent mast cell mRNA levels were similar to those of the control. The 7th and 11th sensitizations increased hepatic iNOS mRNA expression to 15-fold and threefold above control levels, respectively, but did not enhance the total NOS activity in the liver.

Conclusions Multiple OVA challenges, unlike acute sensitization, greatly reduced serum NOx levels. The challenge-suppressed hepatic CYP metabolism was likely related to the increased serum NOx. Serum NOx may be an endogenous marker for CYP metabolism inhibition in type 1 allergic diseases.

Key Points

Multiple OVA challenges markedly reduced serum NOx levels.

Increased serum NOx was related to hepatic CYP metabolism inhibition.

Multiple OVA challenges did not activate Kupffer cells, mast cells, and NOS expression in the liver.

1 Introduction

Type 1 allergic diseases, such as atopic dermatitis, asthma, and allergic rhinitis, are a significant health problem. Mast cells play an important role in allergic responses (T helper 2 cell-activated immune responses) by activating the high-affinity receptor FcεRI [the immunoglobulin (Ig) E-dependent pathway] [1], and are located in connective tissues and the mucosal membranes of most organs [2]. Activated mast cells can initiate and participate in allergic and inflammatory processes through the short-term and long-term release of various cytokines, including interleukin (IL)-4, IL-5, IL-6, and IL-8 [3]. Unlike Kupffer cells, mast cells constitutively load inducible NO synthase (iNOS)-stimulating tumor necrosis factor (TNF)-α and interferon (IFN)-γ into granules that are secreted within a few minutes [4]. Hepatic drug metabolism studies have demonstrated that direct injections of IL-1β, IL-6, TNF-α, and IFN-γ act through down-regulated cytochrome P450 (CYP) gene expression, which

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suppresses the amount and activity of CYPs [5–7]. In rats with adjuvant arthritis (T helper 1 cell-activated immune responses), proinflammatory cytokines downregulate mRNA and protein levels as well as the activities of CYP2C11, CYP2E1, and CYP3A [8, 9]. The proportion of the world's population who suffer from some kind of allergic disorder is approximately 20% and rising [10], but it is unclear whether the cytokines secreted by mast cells during T helper 2 cell-activated immune responses greatly alter the pharmacokinetics of major CYP-metabolic capacity-limited drugs.

Patients with bronchial asthma and allergic rhinitis have an increased level of nitric oxide (NO) in exhaled air [11, 12]. Ovalbumin (OVA)-induced asthmatic mice show increased expression of iNOS protein in lung tissues [13]. During experimental periods of primary and secondary (short-term) sensitization with OVA, NO markedly altered the pharmacokinetics of major CYP-metabolic capacity-limited drugs without altering hepatic CYP protein levels, the protein binding of drugs, and the blood flow rate [14]. Altered pharmacokinetics are responsible for NO-derived posttranslational suppression of hepatic CYP-metabolic activities. Type 1 allergic diseases are often associated with repeated exposure to allergens (multiple allergen challenges). Unfortunately, few models for studying the effects of repeated antigen exposure and its relevance to clinical situations involving both IgE- and cell-dependent reactions have been validated. Chronic allergic responses in tissue are the result of a complex interplay between different immune cells (mast cells, eosinophils, macrophages, dendritic cells, and lymphocytes), and stromal cells. Recent evidence indicated that chronic allergic responses were especially induced by basophils through FcεRI [15, 16]. In contrast, a few researchers have suggested that mast cells play a crucial role in chronic dermatitis in mice [17, 18], but the exact changes that occur in mast cells after repeated antigen exposure are not well understood. Animal models of chronic allergic diseases would help us to understand drug pharmacokinetic alternation. Therefore, we evaluated the changes in hepatic CYP activity and protein expression in multiple-OVA-receiving mice, and further examined hepatic NO production.

2 Methods

2.1 Materials

Chlorzoxazone (CHZ), *Bordetella pertussis* inactive bacterial suspension, chloramphenicol, and acetaminophen (APAP) were purchased from Nacalai Tesque Co. (Kyoto, Japan). Aluminum hydroxide gel (alhydrogel®) was obtained from InvivoGen (San Diego, CA, USA). 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 (MDZ 1'-OH) was obtained from Alsachim SAS (Illkirch, France). Bufuralol (BF), 6-hydroxy CHZ (CHZ 6-OH), 4-hydroxy TB (TB 4-OH), and 1'-hydroxy BF (BF 1'-OH) were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). β-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium (β-NADP⁺), 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.

2.2 Animals and Treatments

Six-week-old female ICR mice (15–20 g) (Japan SLC Co., Shizuoka, Japan) were housed under standard conditions (light/dark cycle of 12 h; room temperature of 23 ± 1 °C) and provided with food and water ad libitum. The establishment of type 1 allergic mice with primary and secondary sensitization was described previously [14]. After secondary sensitization, mice intravenously received 2.5 μg OVA (250 μg/ml of saline) once a week until the 15th sensitization. The sensitized mice were sacrificed on day 7 after the primary, secondary, 7th, 11th, or 15th sensitization. Control mice were given single intraperitoneal (i.p.) and/or intravenous (i.v.) injections of saline. All animal experiments were approved by the Tokushima Bunri University Faculty of Pharmaceutical Sciences Committee for the care and use of laboratory animals.

The mice were sacrificed by decapitation, and blood was collected in heparinized tubes for the IgE assay or EGTA-treated tubes for the NO assay. Plasma and serum were separated by centrifugation at 3000 rpm for 10 min. The liver was excised and washed three times with ice-cold 50 mM Tris–HCl (pH 7.4). Further treatments performed for the high-performance liquid chromatography (HPLC) assay were described previously [14].

2.3 Assay of Total IgE and 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.

Serum concentrations of NO₂[−] were determined using a nitrate/nitrite colorimetric assay kit (Cayman Chemical Company, An Arbor, MI, USA) according to the manufacturer's instructions. The assay conditions were detailed previously [14].

2.4 CYP Enzyme Assay

Hepatic microsomes from control and treated mice were prepared as described previously [14]. The amount of microsomal protein was determined using a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA), and bovine serum albumin was used as a protein standard.

CYP isozyme-specific activities were determined by measuring the formation of APAP, TB 4-OH, BF 1'-OH, CHZ 6-OH, and MDZ 1'-OH from PH (CYP1A2 substrate), TB (CYP2C substrate), BF (CYP2D substrate), CHZ (CYP2E1 substrate), and MDZ (CYP3A substrate). The experimental conditions employed to measure CYP activity and for the HPLC analysis of the five metabolites were described previously [14].

2.5 Real-Time Polymerase Chain Reaction (PCR)

Total RNA was isolated from the liver using a High Pure RNA tissue kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The analytical conditions were detailed previously [19]. The primer sequences were as follows: mouse iNOS: forward, 5'ctttgcacggacgagac3'; reverse, 5'tcattgtactctgaggctgac3'; mouse F4/80: forward, 5'aggaggactctccaagccta3'; reverse, 5'aggcctctcagacttctgctt3'; and c-kit: forward, 5'gatctgctctgcgtctgtt3'; reverse, 5'cttgcatggtgagacg3'. Triplicate reactions per cDNA sample were prepared using FastStart Universal Probe Master (Roche Diagnostics), 500 nM forward primer, 500 nM reverse primer, 250 nM FAM-label UPL probe (#13, cat. no. 04685121001 for iNOS; #42, cat. no. 04688015001 for F4/80; #15, cat. no. 04685148001 for c-kit), 0.2 µl VIC-labeled β-actin assay, 5 µl cDNA (diluted 1:20 from stock), and DNase-free water in 20 µl of reaction mixture. Amplification and detection were performed using a StepOne Plus Real Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). Target gene expression levels were calculated after normalization to the standard housekeeping gene β-actin (Universal ProbeLibrary mouse ACTB gene assay, Roche Diagnostics). The relative expression of the gene of interest compared to β-actin expression was calculated using a standard curve of serially diluted cDNA to correct for PCR efficiency.

2.6 Western Blot Analysis of CYP Isozymes

Liver microsomal proteins (5 µg for CYP3A and 10 µg for the other CYPs) were subjected to 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), or rabbit polyclonal anti-human CYP3A4 (ab176310, 1:2000 dilution; Abcam). Horseradish peroxidase-conjugated anti-mouse IgG (NA931V, 1:20,000 dilution; GE Healthcare, Little Chalfont, Buckinghamshire, UK) and anti-rabbit IgG (NA934, 1:20,000 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 intensity changes of the CYP proteins were corrected using the intensity obtained in the internal control bands (glyceraldehyde-3-phosphate dehydrogenase, GAPDH). We selected GAPDH primary antibody (cat. no. ab181602, 1:2000 dilution; Abcam) and horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (cat. no. NA934, 1:2000 dilution; GE Healthcare). The levels were expressed as percentages, with the control set as 100%.

2.7 Hepatic NOS Activity

Hepatic microsomal total NOS activity was determined using an Ultrasensitive Colorimetric NOS Assay kit (Oxford Biomedical Research Inc., Oxford, MI, USA) according to the manufacturer's instructions.

2.8 HPLC Assay

HPLC analysis was performed on a system equipped with a Shimadzu SPD-10A UV/Vis detector, a Shimadzu LC-10A pump, and a Shimadzu C-R4A chromatopac integrator. Our recent study described the assay conditions of PH, TB, BF, CHZ, MDZ, and their main metabolites [14].

2.9 Statistical Analysis

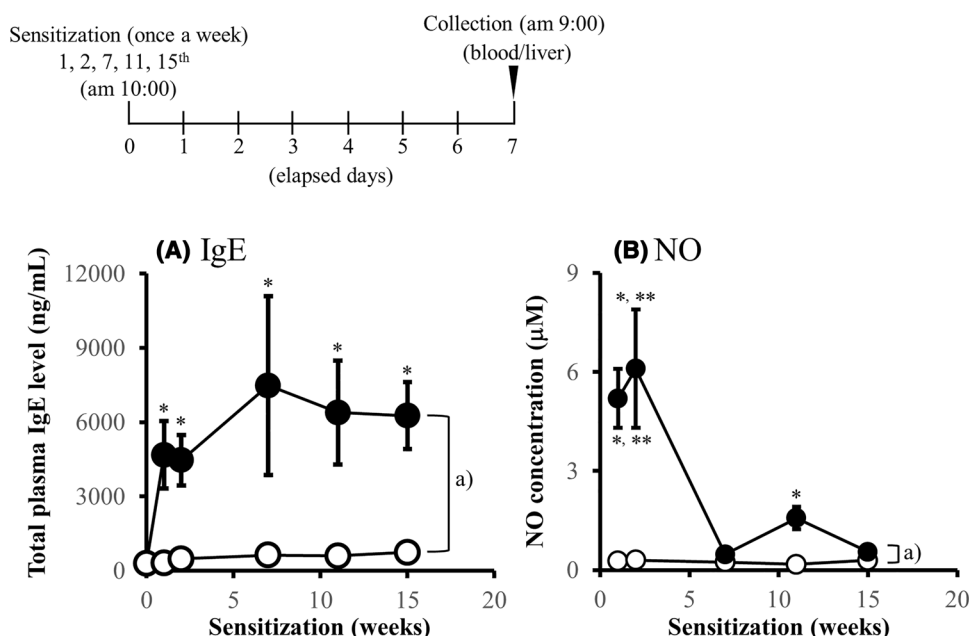
Two-way analysis of variance (ANOVA) was used to examine the behavior of IgE and NO in the two groups (the sensitized and control mice). The nonparametric Kruskal–Wallis test followed by the Steel–Dwass test was used to investigate significant differences. In all cases, $P < 0.05$ was considered to indicate statistical significance.

3 Results

3.1 Total Plasma IgE and Serum NO Levels

Type 1 allergic diseases are characterized by the elevated production of an antigen-specific IgE [20]. As shown in Fig. 1a, the 7th, 11th, and 15th sensitizations markedly

Fig. 1 Plasma IgE (**a**) and NO (**b**) levels in chronically allergic mice. Filled circles sensitized mice; unfilled circles control mice (which received saline). The mice were sensitized every week and were sacrificed at 7 days after the first, second, 7th, 11th, and 15th sensitizations. **a** $P < 0.05$, indicating a significant difference between the two groups, according to two-way ANOVA. The Kruskal–Wallis test was also used in conjunction with the Steel–Dwass test. $*P < 0.05$ compared with that of control mice. $**P < 0.05$ compared with mice upon the 11th sensitization. Data are expressed as the mean $\pm$ SD ($n = 4–6$). IgE immunoglobulin E, NO nitric oxide



elevated total plasma IgE levels, with concentrations 60-fold higher than the IgE values in control mice. The total plasma IgE concentrations were not significantly different upon comparing mice that underwent multiple challenges with those that underwent short-term (primary and secondary) sensitization. Repeatedly sensitized mice were used as type 1 allergic mice for further studies.

The serum concentration of nitrate plus nitrite (NOx) was used as an indicator of host NO production. Primary and secondary sensitization produced significantly higher NOx concentrations (Fig. 1b). Mice that received multiple OVA challenges had markedly reduced serum NOx levels after the 7th, 11th, and 15th challenges. However, compared with the 7th and 15th sensitizations, the 11th sensitization produced a significantly higher NOx concentration.

3.2 Hepatic Microsomal CYP Isoform Activities

Hepatic microsomal CYP activities after multiple OVA challenges were assessed using specific substrates (Fig. 2). After the 11th sensitization, CYP1A2, CYP2C, CYP2D, CYP2E1, and CYP3A activities were significantly inhibited by $76.1 \pm 7.0\%$, $61.8 \pm 6.1\%$, $39.5 \pm 4.6\%$, $47.9 \pm 9.0\%$, and $41.9 \pm 6.3\%$, respectively. In contrast, the 7th and 15th sensitizations did not inhibit the activities of the five CYP enzymes. The body weight of mice after multiple OVA challenges did not significantly differ from that of control mice receiving only saline (data not shown).

3.3 Western Blot Analysis of CYP Isoforms

Hepatic microsomal CYP1A2, CYP2C, CYP2D, CYP2E1, and CYP3A protein expression was investigated by western blot analysis (Fig. 3). The protein levels of the five CYP enzymes were not significantly different when multiple OVA challenges were compared with the control treatment.

3.4 Hepatic iNOS, F4/80, and c-kit mRNA Expression

In the livers of repeatedly sensitized mice, iNOS, F4/80-positive Kupffer cell, and mast cell-dependent c-kit mRNA expression levels were determined by real-time polymerase chain reaction. Hepatic iNOS mRNA expression was about 15-fold and fivefold higher at day 7 after the 7th and 11th sensitizations, respectively (Fig. 4a). In contrast, F4/80 and c-kit expression levels were around 1.0 (Fig. 4b, c).

3.5 Hepatic Total NOS Activity

We evaluated the total NOS activity in hepatic microsomes (Fig. 5). Hepatic microsomal total NOS activities after the 7th, 11th, and 15th sensitizations were quantitatively similar to those in the corresponding control mice.

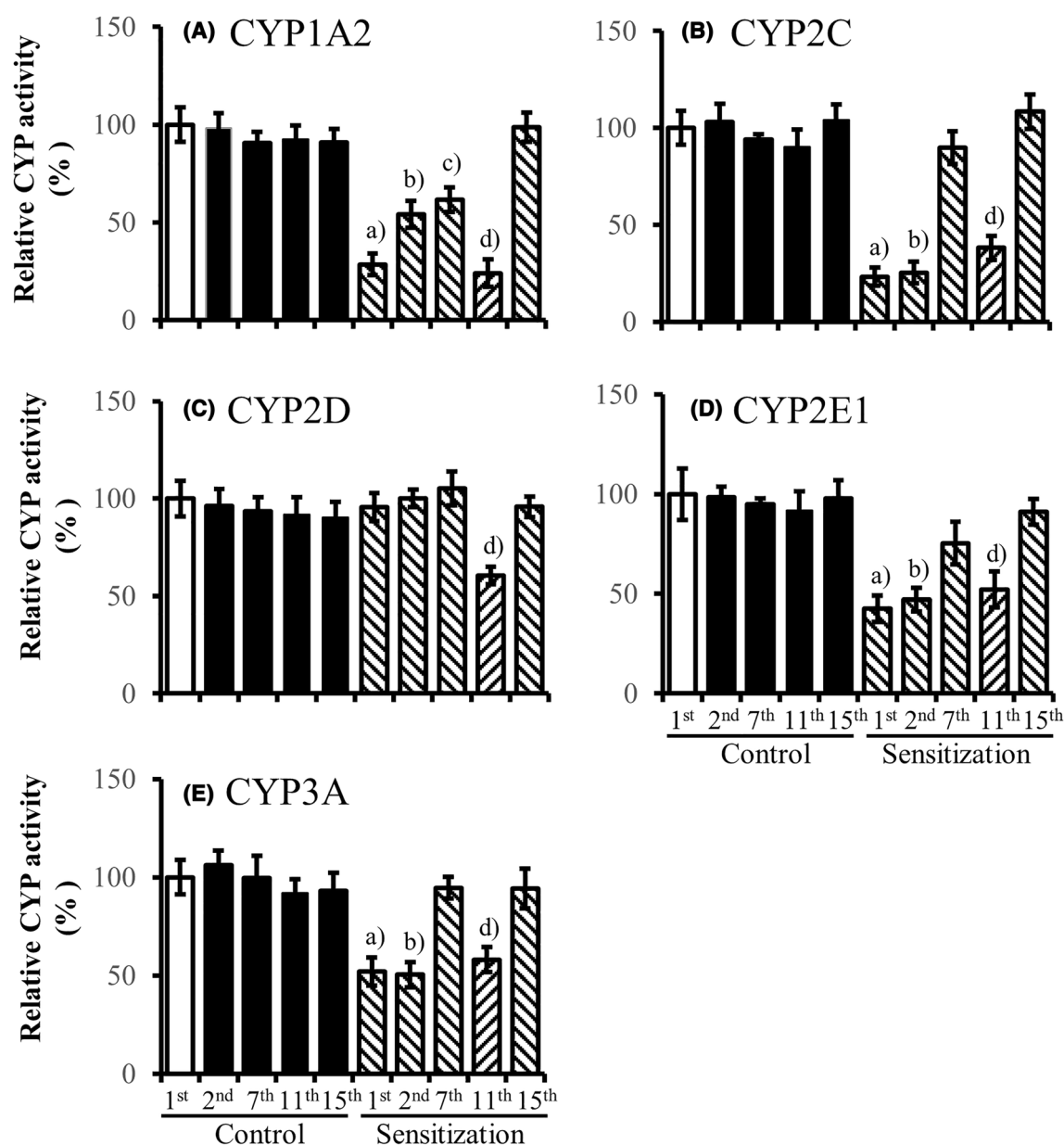


Fig. 2 Changes in hepatic microsomal CYP activities (**a** CYP1A2, **b** CYP2C, **c** CYP2D, **d** CYP2E1, and **e** CYP3A) in chronically allergic mice. Data are expressed as the mean $\pm$ SD ($n=4-6$). The values are expressed as percentages of the value for the control mice (white bar) upon primary sensitization (i.e., the control is set to 100%). The Kruskal–Wallis test was used in conjunction with the Steel–Dwass

test. **a** $P < 0.05$ compared with the control mice upon primary sensitization. **b** $P < 0.05$ compared with the control mice upon secondary sensitization. **c** $P < 0.05$ compared with the control mice upon the 7th sensitization. **d** $P < 0.05$ compared with the control mice upon the 11th sensitization. CYP cytochrome P450

4 Discussion

Repeated or chronic exposure to low doses of allergen is quite common in humans. In a pre-clinical evaluation, OVA was found to be a protein with good antigenicity and carrier activity, but the addition of some adjuvants was necessary to reinforce immunogenicity when establishing an animal model [21]. In a chronically allergic animal

model, rats that were orally exposed to OVA via drinking water or by gavage continuously for 6 weeks (total experimental period: 8 weeks) after i.p. injection of OVA with aluminum hydroxide showed significantly increased serum IgE concentrations [22]. We found that short-term type 1 allergic mice treated with OVA presented significant increases in total plasma IgE and serum NOx concentrations 3 days after primary sensitization [14]. In the present

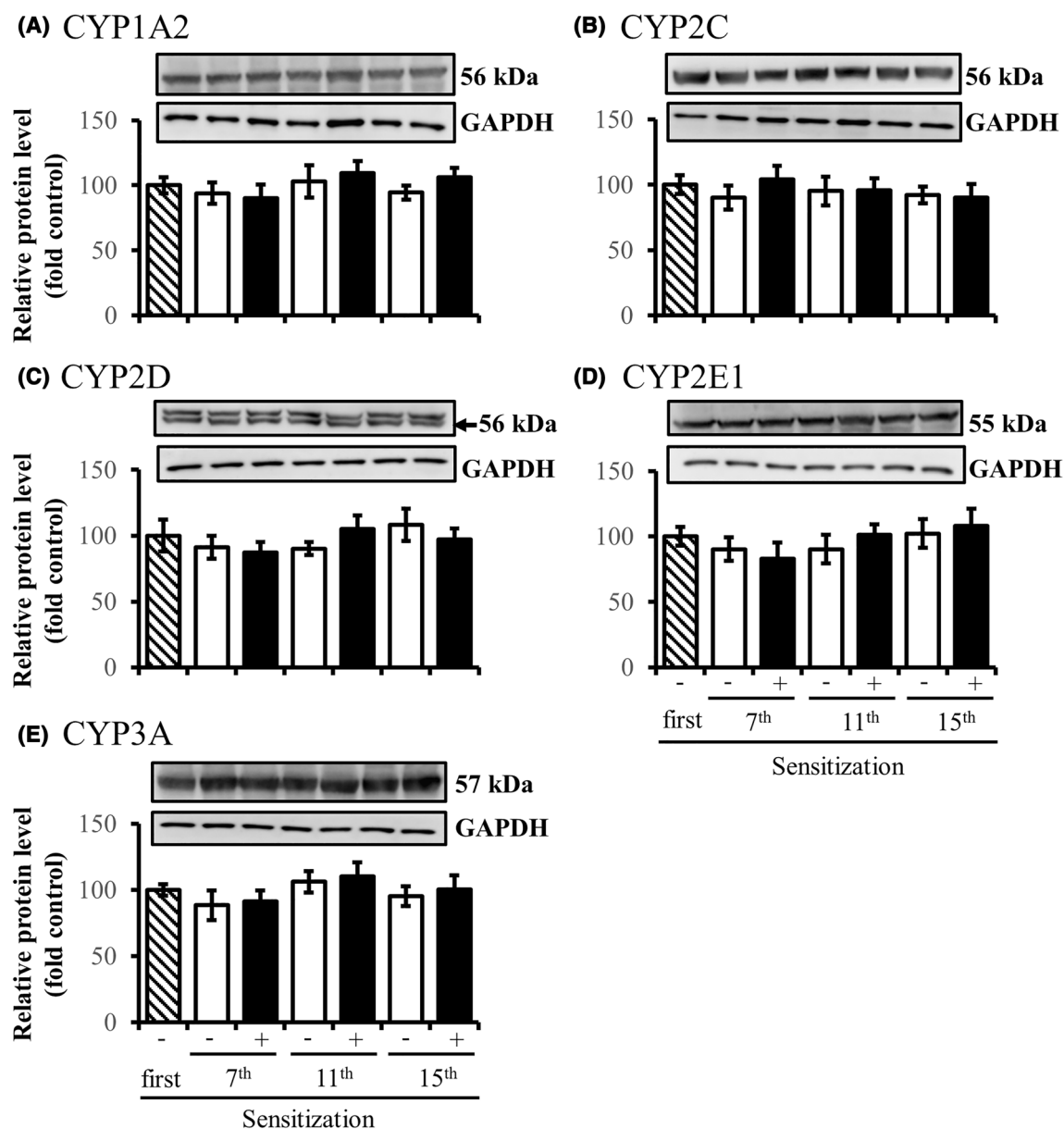


Fig. 3 Hepatic protein expression of **a** CYP1A2, **b** CYP2C, **c** CYP2D, **d** CYP2E1, and **e** CYP3A in chronically sensitized mice. The control results for sensitized mice are labeled with minus signs below the relevant bars. Protein expression is shown as a percentage

of the expression in the control mice upon primary sensitization (bars labeled first, set to 100%). All samples were loaded in triplicate. CYP cytochrome p450, GAPDH glyceraldehyde-3-phosphate dehydrogenase

experiment, mice that received multiple OVA challenges had high total plasma IgE levels (Fig. 1a). Mast cells and basophils expressing FcεRI induce immediate allergic responses through IgE-mediated crosslinking [23]. However, a few reports indicate that chronic allergic responses are induced by basophils (circulating cells) through FcεRI [15, 16]. Because basophils and mast cells secrete some iNOS-stimulating cytokines, we analyzed the effects of multiple OVA challenges on hepatic CYP metabolism and NO production.

Unlike T helper 1 cell-activated diseases, multiple OVA challenges did not alter the protein expression of CYP1A2, CYP2C, CYP2D, CYP2E1, and CYP3A isoforms (Fig. 3). The 11th sensitization significantly suppressed the activities of five CYP enzymes (Fig. 2). Previously, we found that i.v. treatment with carboxy-PTIO (a specific NO scavenger) restored short-term allergy-suppressed CYP activities to control CYP levels [14]. The NO scavenger also completely removed high serum NO. NO can post-translationally interact with CYP enzymes in two ways: NO reversibly binds

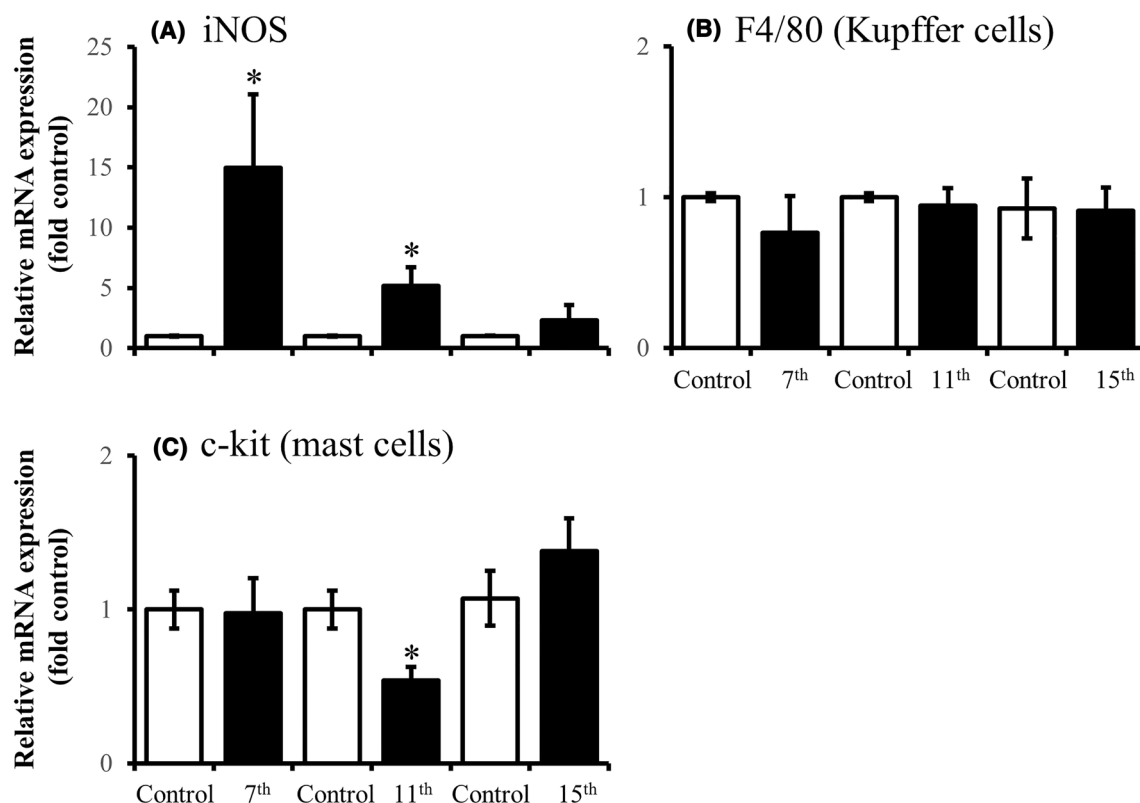


Fig. 4 Expression levels of iNOS, F4/80, and c-kit mRNA in the liver: **a** iNOS, **b** F4/80-positive Kupffer cells, and **c** c-kit (mast cells). Data were normalized to the β -actin mRNA level in the same sample. Data are expressed as the mean $\pm$ SD ($n=4-6$). The Kruskal-Wal-

lis test was used in conjunction with the Steel-Dwass test. $*P < 0.05$ (i.e., significantly different) with respect to individual control mice. *iNOS* inducible nitric oxide synthase

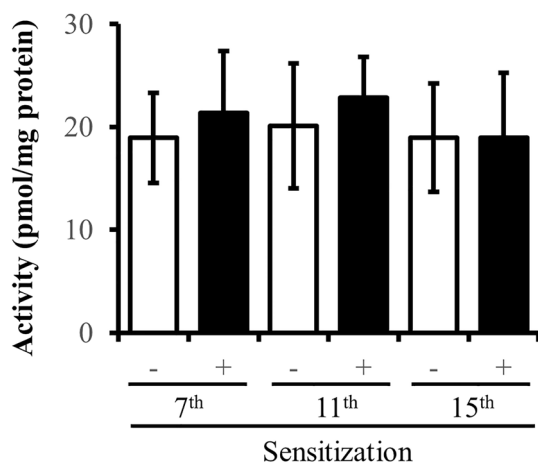


Fig. 5 Hepatic microsomal total NOS activity in chronically allergic mice. Total NOS activity was measured in hepatic microsomes (50 μ g of protein) for 20 min. Data are expressed as the mean $\pm$ SD ($n=4-6$). White bars control mice, black bars sensitized mice. *NOS* nitric oxide synthase

to the heme moiety and tyrosine residues of CYP to form iron-nitrosyl and *S*-nitrosyl complexes, and NO irreversibly inactivates CYP through the thiol modification pathway [24]. As shown in Fig. 1b, the 11th sensitization increased serum NO_x concentrations. Therefore, long-term allergy-produced NO may participate in the inhibition of hepatic CYP metabolism.

In lipopolysaccharide-stimulated animals (T helper 1 cell-activated immune responses), Kupffer cells are the main source of NO responsible for CYP inactivation [25]. In addition to Kupffer cells, hepatocytes that rapidly express iNOS are responsible for excess NO production [26, 27]. iNOS is the most important molecular source of NO in asthma [28]. Hepatic iNOS is activated via IL-1 β , IFN- γ , and TNF- α stimulation [29, 30]. In the liver, we confirmed that primary and secondary sensitization enhanced TNF- α and IFN- γ mRNA expression rather than IL-1 β mRNA expression, but induced iNOS activity and protein expression were not observed in hepatic microsomes and liver tissue (data not shown). As shown in Fig. 5, multiple OVA challenges did not enhance hepatic total NOS activity, which completely differed from the increased iNOS mRNA expression after the 7th and 11th sensitizations (Fig. 4a). We further

evaluated iNOS protein expression in hepatic 9000×g supernatant, hepatocytes, and hepatic microsomes, and found no significant difference between the mice that underwent multiple OVA challenges and the control mice (data not shown). These results suggest that the CYP metabolism-suppressing NO does not originate from cytokine-stimulated iNOS expression in the liver.

Mouse mast cells secrete excess NO via the NO pathway induced by FcεRI [31]. Except for the air exhaled by asthmatic patients [32], little information is available on chronic type 1 allergy-produced NO in the blood. Two major receptors (c-kit and FcεRI) are responsible for regulating mast cell function. The stem cell factor receptor c-kit is expressed on the surfaces of mature mast cells and in the earliest mast cell progenitors [33, 34]. Yee et al. [35] found that c-kit is a major growth and differentiation factor for murine and human mast cells. Furthermore, c-kit is expressed in a soluble form and on cell membranes [34]. Mast cells are normally found in healthy livers, but in very low numbers. After liver injury, mast cell numbers greatly increase as they infiltrate and release factors that exacerbate the fibrotic response [36]. As shown in Fig. 4c, multiple OVA challenges did not upregulate the mRNA expression of c-kit-dependent mast cells. Therefore, these results negate the possibility that hepatic CYP enzymes directly interact with hepatic mast cell-secreted NO during multiple OVA challenges.

Rats sensitized with aluminum-adsorbed OVA and inactive *Bordetella pertussis* have increased numbers of peritoneal mast cells [21]. We also found that primary and secondary sensitization produced high serum NOx levels (Fig. 1b). In our recent study, mast cell-deficient mice showed completely suppressed serum NOx concentrations on day 7 after primary sensitization and after secondary sensitization (data not shown). Multiple OVA-challenged mice had markedly reduced serum NOx levels after secondary sensitization (Fig. 1b). Plaut et al. [3] demonstrated that activated cultured mast cells can initiate and participate in allergic and inflammatory processes through the short-term and long-term release of various iNOS-stimulating cytokines, including TNF-α and IFN-γ. In multiple OVA-challenged mice, the plasma IFN-γ levels after the 7th, 11th, and 15th sensitizations were similar to that after multiple saline injections (control group) (data not shown). An in vitro study demonstrated that IgE-sensitized mast cell cultures exhibit sustained NO production [37]. This in vitro finding differs from the decreased serum NOx levels we observed under real immune responses. The recycling period of mast cells is long (hours to days) compared with that of neurons (seconds to minutes) [38, 39]. More interestingly, Suurmond et al. [40] provided the first evidence for the intrinsic modulation of mast cell function on repeated FcεRI-mediated activation. Therefore, our data raise the possibility that multiple antigen challenges, unlike short-term OVA stimulations, reduce

the frequency of OVA-dependent mast cell degranulation or mast cell-derived NO secretion. Further studies are needed to clarify the novel source(s) of the increased serum NOx together with the NO-derived CYP inhibition pattern.

5 Conclusion

Our study provides the first evidence that long-term OVA stimulation reduces serum NOx levels compared with the levels observed upon primary and secondary stimulation, but that such stimulation only temporarily inhibits CYP metabolism. Serum NO may be an endogenous marker for hepatic CYP metabolism inhibition in short-term and long-term type 1 allergic diseases.

Compliance with Ethical Standards

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Conflict of interest The authors have no conflict of interest.

Ethical approval 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.

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