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Mechanism of 7,12-Dimethylbenz[*a*]anthracene-Induced Immunotoxicity: Role of Metabolic Activation at the Target Organ

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ABSTRACT—The polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[*a*]anthracene (DMBA), is an immunosuppressor as well as a potent organ-specific carcinogen. To understand the organ-specific mechanism of DMBA-induced lymphoid toxicity, aryl hydrocarbon-nonresponsive mice and microsomal epoxide hydrolase (mEH)-null mice were analyzed. DMBA caused a dose-dependent decrease in spleen weights, but not the thymus weights in aryl hydrocarbon-nonresponsive mice. On the other hand, both spleen and thymus weights were decreased to less than a half in wild-type mice exposed to 30 mg/kg of DMBA. In contrast, no decrease was detected in spleen weights of mEH-null mice exposed to up to 100 mg/kg of DMBA, while thymus weights were markedly lower. Responses to the B-cell mitogen lipopolysaccharide and to T-cell mitogen phytohemagglutinin were nearly completely abolished in splenocytes isolated from wild-type mice treated with 100 mg/kg of DMBA. These responses were decreased, but maintained in splenocytes isolated from mEH-null mice treated with DMBA. Two DMBA metabolites dependent on mEH including DMBA-3,4-diol were detected in an HPLC chromatogram of spleen microsomes isolated from wild-type mice, but not those from mEH-null mice. These results suggest the involvement of mEH in splenic activation of DMBA for immunotoxicity and the difference for the DMBA-induced lymphoid toxicity between spleen and thymus.

Keywords: 7,12-Dimethylbenz[*a*]anthracene, Immunotoxicity, Microsomal epoxide hydrolase, Spleen, Null-mouse

7,12-Dimethylbenz[*a*]anthracene (DMBA), a polycyclic aromatic hydrocarbon (PAH), has been used extensively as a model carcinogen in cancer research. Recently, studies have focussed on the immunotoxicity of DMBA given to experimental animals to induce tumors in skin and mammary gland. DMBA elicits immunotoxicity in the spleen, thymus and bone marrow. It has been shown to suppress both humoral and cell-mediated immune responses in spleen and cultured splenocytes (1 – 4). Although several studies reported that the lymphotoxicity of DMBA is mediated through an apoptosis-like mechanism (5 – 8), biotransformation of DMBA in lymphoid organs to induce immunotoxicity has not been well investigated.

The aryl hydrocarbon receptor (AHR) is a ligand-dependent transcription factor that mediates activation of several genes encoding CYP1A1, CYP1B1 and interleukin-2. Studies with aryl hydrocarbon (Ah)-nonresponsive mice and AHR-null mice demonstrated the AHR-dependent mechanism for chemical-induced toxicity of DMBA, benz[*a*]pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (9 – 12). Recent reports suggest the involvement of AHR on the apoptosis of bone marrow pre-B-cell caused by DMBA and benz[*a*]pyrene (7, 8, 13). Halogenated hydrocarbons such as TCDD and polychlorinated biphenyls (PCBs) are known to cause immunotoxicity through both AHR-dependent and -independent mechanisms in several lymphoid organs (14, 15). TCDD-induced alteration of the thymus is reported to depend on AHR (10, 16). These data suggest the requirement of AHR for immunotoxicity of dioxins and PAHs in lymphoid organs, at least partially.

It was also established that PAHs such as DMBA require metabolic activation for carcinogenicity. The carcinogenic potency of PAHs correlates with splenic immunosuppresive potency (17, 18). Thus, PAHs could cause toxicities through two distinct mechanisms, AHR-mediated and meta-

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bolic activation.

Microsomal epoxide hydrolase (mEH) is an enzyme catalyzing hydrolysis of aliphatic and arene epoxides and these reactions are generally considered the detoxication pathway. The hydrolysis of epoxide derivatives of DMBA and benzo[*a*]pyrene is, however, required for a major metabolic pathway for activating carcinogens to the ultimate electrophilic derivatives. (19 – 21). In the metabolic activation of DMBA, mEH is the only enzyme that transforms DMBA-3,4-epoxide to DMBA-3,4-dihydrodiol (DMBA-3,4-diol), and then CYP1A1 or CYP1B1 oxidizes DMBA-3,4-diol to the ultimate carcinogenic form, DMBA-3,4-diol-1,2-epoxide (22 – 25). mEH is expressed not only in liver but also in several extrahepatic tissues including kidney, testis, ovary, lung, thymus and spleen (26, 27). Thus, mEH is believed to play a critical role in the multiorgan carcinogenesis of DMBA.

mEH-null mice were used to establish that mEH is necessary for DMBA-induced skin carcinogenesis (28). However, the role of mEH for DMBA-induced lymphoid immunotoxicity remains unclear. To understand the mechanisms of DMBA-induced immunotoxicity, it is critical to determine whether the target organ can produce reactive intermediates of DMBA. To this end, we have compared the susceptibility of DMBA-induced lymphoid organ toxicity among Ah-nonresponsive mice, mEH-null mice and the wild-type mice. The results obtained show an involvement of splenic mEH in DMBA-induced immunotoxicity, which is distinct from the mechanisms for DMBA-induced immunotoxicity for thymus.

MATERIALS AND METHODS

Chemicals

7,12-Dimethylbenz[*a*]anthracene (DMBA), lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (protein content<1%), phytohemagglutinin PHA-P (PHA), trichloropropene oxide (TCPO) and RPMI-1640 medium were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Penicillin-streptomycin was obtained from Gibco BRL (Santa Clara, CA, USA). [Methyl-^{3}H]-thymidine (25 Ci/mmol, 1 mCi/ml) was obtained from Amersham Pharmacia Biotech (Tokyo). DMBA-3,4-dihydrodiol (DMBA-3,4-diol) was obtained from the NCI Chemical Carcinogen Repository (Frederick, MD, USA).

Animals and treatments

Ah-nonresponsive mice (crossbreed of 129/SVJ and C57BL/6), mEH-null mice (28) and the wild-type mice provided by National Institutes of Health (Bethesda, MD, USA) were used in this study. Although the genetic background of these three strains of mice is a mix between 129/SVJ and C57BL/6, only mEH-null and the wild-type mice are Ah-responsive. mEH-null mice and the wild-type mice were derived from same parents backcrossed with C57BL/6 mice. Male mice of these strains were allowed free access to food and water and used at 7 – 10 weeks of age in all the experiments. β-Naphthoflavone was dissolved in corn oil (5 mg/ml) and the mice were treated with i.p. with β-naphthoflavone (50 mg/kg) daily for 3 days. DMBA dissolved in dimethylsulfoxide was diluted 5 times with water (5 mg/ml or 1.5 mg/ml). The mice were treated i.p. with DMBA (30 mg/kg or 100 mg/kg) daily for 5 days, and euthanized by cervical dislocation.

Preparation of lymphoid cells from spleen

Spleens were harvested and placed in a sterile phosphate-buffered saline. Cell suspensions were prepared by gentle dissociation using glass slides. Debris was separated from cells by filtration and cell suspensions were centrifuged at $250 \times g$ for 5 min. Cell pellets were resuspended in RPMI-1640 media containing 10% fetal calf serum, penicillin-streptomycin (Gibco BRL).

Mitogen-induced lymphocyte proliferation assays

Cells were seeded in a volume of 100 μl with a concentration of 3×10^6 cells/ml in 96-well plates. Mitogens were added in a volume of 10 μl at final concentrations of 25 μg/ml for LPS and 5 μg/ml for PHA, and then the cells were incubated in 5% CO_2 at 37°C for 3 days before DNA synthesis was assessed by 24-h labeling with 1 μCi /well [^{3}H]-thymidine. The cells were harvested onto filter strips using a harvester and [^{3}H]-thymidine incorporation was determined by liquid scintillation (LS6500; Beckman, Fullerton, CA, USA). For mitogen-induced lymphocyte proliferation assays after DMBA treatment in vitro, cell preparations from untreated mice were simultaneously exposed to mitogen (LPS) and DMBA.

Microsome preparation and immunoblotting

Microsomes from liver, spleen and thymus were prepared, and microsomal proteins were subjected to immunoblot analysis using anti-rat mEH antibody provided by Dr. James P. Hardwick (Northeastern Ohio University College of Medicine), anti-rat CYP1A1 antibody provided by Dr. Kiyoshi Nagata (Tohoku University) and anti-rat CYP1B1 antibody provided by Daiichi Pure Chemicals Co. (Tokyo). The anti-rat CYP1A1 antibody cross-reacts to CYP1A2 form.

DMBA metabolism

DMBA metabolites were analyzed as described previously (29, 30). DMBA was recrystallized from acetone /hexane. Incubation mixtures contained 100 μg of liver microsomes from untreated or DMBA-treated mice (mEH-null or wild-type) or 500 μg of spleen, thymus or liver

microsomes from untreated or DMBA-treated mice (mEH-null or wild-type) and DMBA (20 μM) in methanol (10 μl) in a final volume of 1.0 ml. The mixtures were preincubated at 37°C for 2 min. To initiate the reaction, an NADPH generating system was added to the reaction mixture. Incubations were carried out at 37°C under subdued lighting for 10 or 20 min, and 4 ml of ethyl acetate / acetone (2:1) was added into the reaction mixtures to terminate the reaction and to extract DMBA metabolites. Pyrene methanol was used as the internal standard. Comparing retention time with authentic compound (DMBA-3,4-diol) identified the metabolite formed.

HPLC analysis

HPLC analyses were performed with a Jasco Intelligent Model PV-980 pump and FP-920S fluorescence detector (Jasco, Tokyo). DMBA metabolites were separated with an ODS-AM column (250 × 4.6 mm) using a flow rate of 0.8 ml/min. The column was eluted with methanol / H_2O (1:1) for 10 min and followed by a 50-min linear gradient of methanol / H_2O (1:1) to methanol (100%) and 10 min holding of methanol (100%). DMBA metabolites were detected using an excitation wavelength of 268 nm and emission wavelength of 395 nm for the first 40 min, followed by 478 nm between 40 and 50 min and then 415 nm between 50 and 70 min.

Statistical analyses

Data were analyzed with one-way factorial ANOVA and post-hoc test (Fisher's PLSD test). All statistical tests were performed using the StatView-J 4.02, and a P value of less than 0.05 was considered significant.

RESULTS

Effect of DMBA treatment on lymphoid organ weights of Ah-nonresponsive, mEH-null and wild-type mice

To understand the relationship of AHR and metabolic activation in the mechanism of DMBA-induced immunotoxicity, lymphoid organs (spleen and thymus) were examined using an Ah-nonresponsive, mEH-null, and the wild-type mice. As shown in Fig. 1, induction potency of CYP1A1 and CYP1A2 in Ah-nonresponsive mice treated with β-naphthoflavone was lower than that of mEH-null and the wild-type mice treated with β-naphthoflavone. An upper band corresponding to CYP1A1 was not detected in liver microsomes from Ah-nonresponsive mice treated with β-naphthoflavone. On the other hand, expression level of mEH was not different between Ah-nonresponsive mice and the wild-type mice.

Although there was no decrease in body weights and no difference in the ratios of liver and kidney to body weights after the DMBA treatment (100 mg/kg, daily for 5 days) in all three mouse lines (data not shown), the ratios of thymus to body weights were decreased in a dose-dependent manner in both wild-type and mEH-null mice, but not Ah-nonresponsive mice (Fig. 2A). On the other hand, the

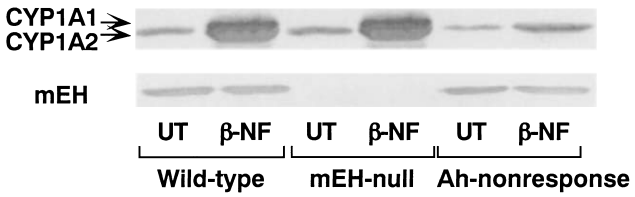


Fig. 1. Immunoblot analyses of mEH, CYP1A1 and CYP1A2 in liver of wild-type, mEH-null and Ah-nonresponsive mice. Liver microsomes were prepared from wild-type, mEH-null and Ah-nonresponsive mice (Ah-nonresponse). The amounts of microsomal protein loaded were 25 μg. The mouse mEH and CYP1A1 and CYP1A2 proteins were detected with anti-rat mEH antibody and anti-rat CYP1A1 antibody, respectively. UT, untreated mice; β-NF, 50 mg/kg β-naphthoflavone-treated mice.

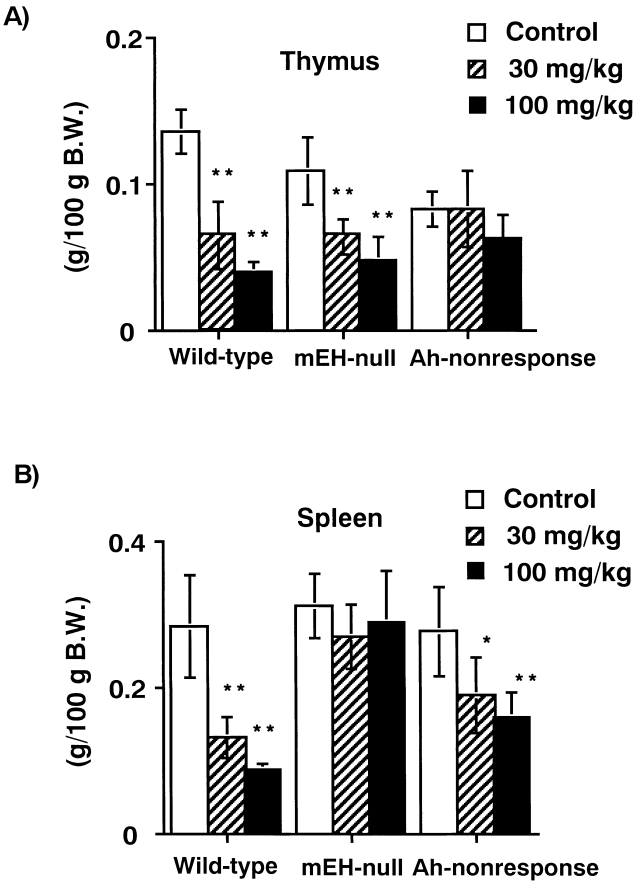


Fig. 2. Effect of DMBA treatment on lymphoid organ weights among wild-type, mEH-null and Ah-nonresponsive mice. wild-type (+/+), mEH-null (−/−) and Ah-nonresponsive mice (Ah-nonresponse) were treated i.p. with DMBA (0, 30 and 100 mg/kg) daily for 5 days. The ratios of thymus (A) or spleen (B) weight to 100 g body weight are shown. Values represent the mean ± S.D. (n = 6). Significant difference from the vehicle control (*P<0.05, **P<0.01).

ratios of spleen to body weights were decreased in a dose-dependent manner in wild-type mice and Ah-nonresponsive mice, but not in mEH-null mice (Fig. 2B).

To understand the relationship between spleen weight and spleen cellularity (splenocyte cell number per spleen), another experiment was performed in mEH-null and wild-type mice. The spleen cellularity changed concordant with spleen weights in both wild-type and mEH-null mice (Table 1).

Effect of DMBA treatment on splenic lymphocyte function

To assess the influence of DMBA treatment on splenocyte function, mitogen-induced lymphocyte proliferation was examined in DMBA-treated mice. Splenocytes prepared from mice pre-treated with DMBA (0, 30 and 100 mg/kg) were exposed to mitogens. Since splenocytes consist of both T- and B-cells, PHA or LPS which specifically activates T-cells or B-cells were used as mitogen, respectively. As shown in Fig. 3A, LPS-stimulated proliferation of B-cells was completely suppressed in wild-type mice pre-treated with 30 mg/kg of DMBA, but detected in mEH-null mice pre-treated even at 100 mg/kg of DMBA. PHA-stimulated proliferation was also completely suppressed in splenocytes from wild-type mice pre-treated with 100 mg /kg of DMBA (Fig. 3B). The activity was decreased, but detectable for splenocytes from mEH-null mice treated with 100 mg/kg of DMBA. These results indicate the difference in susceptibility between mEH-null and wild-type mice on DMBA-induced immunosuppression of splenocytes.

To assess the effect of DMBA on splenocyte function in vitro, splenocytes isolated from mice were exposed to DMBA and LPS, simultaneously. Although LPS-stimulated proliferation in both cells from wild-type and mEH-null mice was not suppressed in the presence of 10 μM DMBA, the proliferation was completely suppressed with wild-type mouse splenocytes in the presence of 20 μM of DMBA, whereas no decrease was observed in splenocytes from mEH-null mice (Fig. 4). Lymphocyte function of both mouse lines was completely suppressed by the treatment with 50 μM DMBA.

Expression of P450s and mEH in liver and spleen

To determine the site of activation of DMBA in mice, tissue distribution of CYP1A1/2, CYP1B1 and mEH was examined. Liver and spleen microsomes were prepared from mEH-null and wild-type mice with or without treat-

Table 1. Spleen weight and spleen cellularity in mEH-null and wild-type mice treated with DMBA

Strain	Treatment (mg/kg)	Body weight (g)	Spleen weight (mg)	Spleen cellularity ($\times 10^7$)
Wile-type	0	25.6 ± 3.7	73.2 ± 13.1	6.2 ± 1.6
	30	25.1 ± 3.3	36.4 ± 9.3[a]	1.6 ± 0.4[a]
	100	25.9 ± 1.6	24.9 ± 2.6[a]	0.8 ± 0.6[a]
mEH-null	0	25.1 ± 0.4	88.1 ± 19.6	10.6 ± 1.6
	30	25.3 ± 1.2	78.9 ± 9.4	9.4 ± 2.3
	100	25.6 ± 1.5	74.5 ± 16.4	9.4 ± 3.2

Values represent the mean ± S.D. (n = 4). [a]Significant difference from the vehicle control (P<0.01).

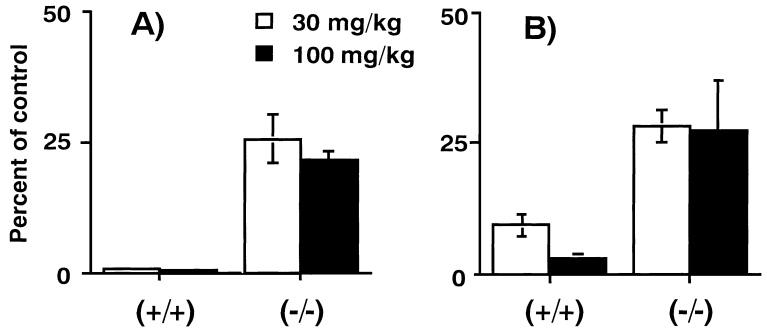


Fig. 3. Suppression of splenic lymphocyte function of DMBA-pretreated mice. wild-type mice (+/+) and mEH-null mice (−/−) were treated i.p. with DMBA (0, 30 and 100 mg/kg) daily for 5 days. Splenocytes from each mouse were treated with lipopolysaccharide (LPS) (A) or phytohemagglutinin (PHA) (B). [^{3}H]-Thymidine incorporated into the splenocyte was counted. Data are expressed as a percentage of the vehicle control for each mouse. Values represent the mean ± S.E.M. (n = 4). Control mitogen-induced [^{3}H]-thymidine incorporation in the wild-type mice was 14233 cpm (LPS) and 7264 cpm (PHA); [^{3}H]-thymidine incorporation in mEH-null mice was 9756 cpm (LPS) and 7943 cpm (PHA).

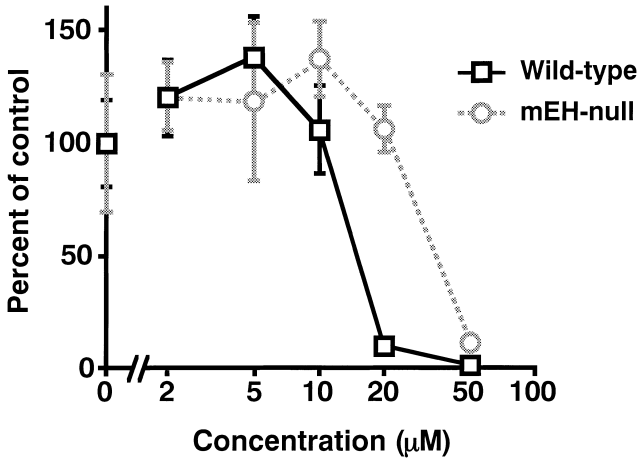


Fig. 4. Suppression of splenic lymphocyte function by in vitro DMBA-treatment. Splenocytes from wild-type and mEH-null mice were treated with both DMBA and lipopolysaccharide, and [^{3}H]-thymidine incorporation into the splenocyte was counted. Data are expressed as a percentage of the vehicle control for each mouse. Values represent the mean ± S.D. Control mitogen-induced [^{3}H]-thymidine incorporation was 13020 cpm (wild-type mice) and 7136 cpm (mEH-null mice).

ment with DMBA. mEH protein was detected in liver and spleen microsomes of wild-type mice, but not in mEH-null mice (Fig. 5). CYP1A2 protein was detected in liver microsomes from mEH-null and wild-type mice, while CYP1A1 protein was detected in liver microsomes from mice treated with DMBA, suggesting that the AHR of both mouse lines was activated by DMBA. CYP1A1 and CYP1A2 proteins were not detected in spleen microsomes from even after DMBA-treatment. CYP1B1 protein was detected in spleen microsomes from mEH-null and wild-type mice, but not in liver microsomes. No significant increase in the expression level of CYP1B1 protein was observed in spleen microsomes from DMBA-treated mice.

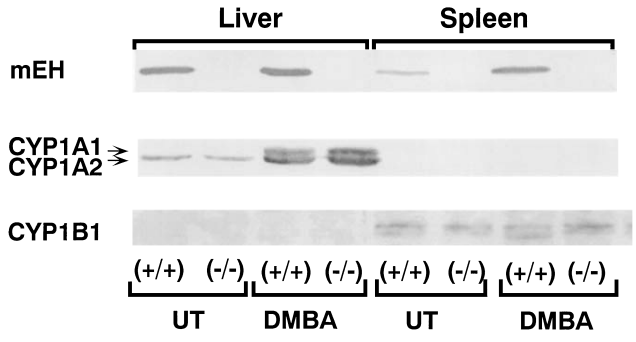


Fig. 5. Immunoblot analyses of mEH and several CYP forms in liver and spleen of wild-type and mEH-null mice. Liver microsomes were prepared from untreated (UT) and 100 mg/kg DMBA-treated (DMBA) mice. Spleen microsomes were prepared from untreated (UT) and 30 mg/kg DMBA-treated (DMBA) mice. The amounts of microsomal protein loaded were 20 μg for liver and 50 μg for spleen. The mouse mEH, CYP1A1 and CYP1A2, and CYP1B1 proteins were detected with anti-rat mEH antibody, anti-rat CYP1A1 antibody and anti-rat CYP1B1 antibody, respectively. (+/+), wild-type mice; (−/−), mEH-null mice.

DMBA metabolism in liver, spleen and thymus

To verify whether microsomal DMBA metabolism was different between mEH-null and wild-type mice, DMBA metabolites formed in liver (100 and 500 μg), spleen (500 μg) or thymus (500 μg) microsomes from DMBA-treated mice were analyzed. DMBA-3,4-diol was not detected in DMBA metabolites of 100 μg liver microsomes from DMBA-treated wild-type mice, but it was detected in those of 500 μg liver microsomes. Spleen microsomes from DMBA-treated wild-type mice were able to form DMBA-3,4-diol (Fig. 6). In contrast, no detectable DMBA-3,4-diol was formed in spleen microsomes from DMBA-treated mEH-null mice. These data indicate that spleen microsomes from mEH-null mice are unable to produce the proximate immunosuppressive metabolite of DMBA,

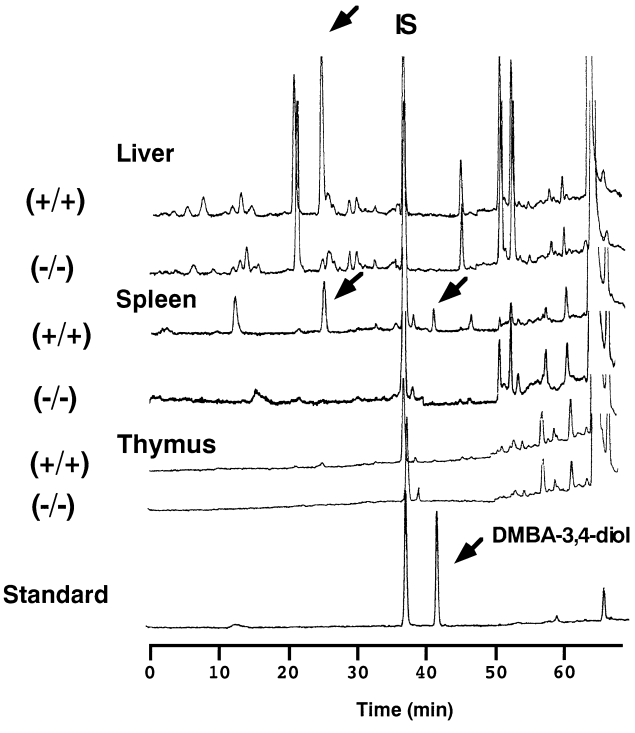


Fig. 6. HPLC profile of microsomal DMBA metabolites in liver, spleen and thymus of wild-type and mEH-null mice. The chromatograms represent metabolic profiles obtained from10 min incubations of DMBA with liver microsomes (100 μg) from100 mg/kg DMBA-treated mice and 20-min incubations of DMBA with spleen and thymus microsomes (500 μg) from 30 mg/kg DMBA-treated mice. IS represents the pyrene methanol peak as an internal standard. The arrows represent metabolite peaks that disappeared with the addition of TCPO (0.5 mM) to the reaction mixture. (+/+), wild-type mice; (−/−), mEH-null mice.

DMBA-3,4-diol (31). Two metabolites including DMBA-3,4-diol disappeared by the addition of TCPO (0.5 mM), an mEH inhibitor, to the reaction mixture of spleen microsomes from wild-type mice. We also analyzed DMBA metabolites formed with thymus microsomes from the wild-type mice. Polar DMBA metabolites containing DMBA-3,4-diol produced in spleen microsomes from the wild-type mice were not detected in thymus microsomes, suggesting low DMBA-metabolic activation in the thymus.

DISCUSSION

In the present study, mEH-null mice were found to be more resistant to splenic immunotoxicity of DMBA than the wild-type mice. Spleen weights were decreased to 30% of control in wild-type mice after the treatment with 100 mg/kg of DMBA. However, no significant decrease was observed for spleen weights of mEH-null mice treated with 100 mg/kg of DMBA (Fig. 2B). The change in spleen cellularity (cell number of splenocyte) concordant with spleen weights indicates that cell death of splenocytes occurs only in wild-type mice treated with DMBA (Table 1). Moreover, splenic lymphocyte function was suppressed more profoundly in wild-type mice than in mEH-null mice after treatment with DMBA (Fig. 3). These results suggest that splenocytes of mEH-null mice are more resistant to DMBA-induced cytotoxicity (cell death) as well as immunosuppression. The mitogen-induced lymphocyte proliferation in vitro also revealed lower susceptibility of splenocytes isolated from mEH-null mice than from wild-type mice on the immunosuppression activity of DMBA (Fig. 4). These results suggest an essential role of mEH on DMBA-induced splenocyte immunosuppression.

Western blot analyses showed that mEH is expressed in the spleen of wild-type mice, but not mEH-null mice (Fig. 5). Although CYP1A1 and CYP1A2 were not detected in splenic microsomes by immunoblot analysis, CYP1B1 was detected in splenic microsomes from mEH-null and wild-type mice. The DMBA-metabolite profiles of spleen microsomes were clearly different between mEH-null mice and wild-type mice. DMBA-3,4-diol was detected in spleen microsomes from DMBA-treated wild-type mice, but not those from mEH-null mice. Previous reports showed that DMBA-3,4-diol was one of major microsomal DMBA metabolites generated in the spleen of B6C3F1 mice treated with DMBA (32) and DMBA-3,4-diol was 65-fold more potent immunosuppressor than the parent compound DMBA using an in vitro splenic lymphocyte function assay (31). Our results are consistent with these data and provide genetic evidence that the metabolic activation of DMBA in the spleen plays a key role in the splenic immunotoxicity, thus indicating a critical role for mEH in DMBA-induced splenic immunotoxicity.

It was shown that TCDD-induced thymic immunotoxicity is dependent on an AHR-mediated mechanism (10, 14 – 16, 33, 34). Recent reports showed that AHR activation of bone marrow stromal cells was necessary for benz[*a*]pyrene- or DMBA-induced pre-B-cell apoptosis (7, 8, 13). In contrast to these reports, Heidel et al. showed that the metabolic activation of DMBA in bone marrow stromal cells is required for pre-B-cell apoptosis (35, 36). We also examined DMBA-induced lymphoid cytotoxicity using Ah-nonresponsive mice, mEH-null mice and the wild-type mice. The spleen weight was significantly decreased in a dose-dependent manner in the Ah-nonresponsive mice and the wild-type mice, but not in mEH-null mice (Fig. 2). mEH-null mice that lack the ability to metabolically activate DMBA did not show the DMBA-induced splenic cytotoxicity, suggesting that AHR is not obligatorily for DMBA-induced splenic cytotoxicity. These results are consistent with previous reports that the susceptibility of DMBA-induced splenic immunotoxicity was not significantly different between Ah-responsive mice and Ah-nonresponsive mice (3, 4). On the other hand, the metabolic activation of DMBA dependent on mEH seems not to be involved in the DMBA-induced thymic cytotoxicity. No DMBA metabolite was detected in the diol metabolite-migration regions of HPLC chromatogram from thymus microsomes. In contrast to the spleen, the thymus may be unable to produce the reactive intermediates of DMBA due to a low capacity for metabolic activation.

As shown in Fig. 3, mEH-null mice were more resistant to DMBA-induced splenic immunosuppression than the wild-type mice. However, in contrast to the splenic cytotoxicity, the splenic lymphocyte function was suppressed 72 – 78% in even mEH-null mice. These results suggest the possibility of DMBA-activation pathways that are independent of mEH for DMBA-induced splenic immunosuppression (37) and also a possibility of receptor-mediated mechanism that is independent of metabolic activation (7, 8, 13, 38, 39).

In the present study, we demonstrated a requirement for splenic mEH in DMBA-induced splenic immunotoxicity, and a difference in the mechanism of DMBA-induced imunotoxicity between spleen and thymus. These differences are consistent with the metabolic activating capacities of spleen and thymus in mice.

Acknowledgments

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