



Discrimination of haptens from prohaptens using the metabolically deficient $Cpr^{low/low}$ mouse

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ABSTRACT

The murine local lymph node assay (LLNA) is a validated, well accepted method for identification of chemical contact allergens. Both direct acting haptens and prohaptens (requiring metabolic activation) can be identified, but not differentiated by this assay. This study was used to assess the utility of a pan microsomal metabolic deficient mouse to distinguish between direct acting haptens and prohaptens in the LLNA. Hapten and prohapten induced cell proliferation was compared in C57BL/6J (B6) wild type (WT) versus homozygous (HO) knockout mice with a hypomorphic NADPH-Cytochrome P450 Reductase (CPR) gene (termed $Cpr^{low/low}$) resulting in low CPR enzyme activity. Mice were dosed with known prohaptens; benzo(a)pyrene (BaP), carvone oxime (COx) and paracetamol (PCM) and haptens; oxazolone (OX), 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (EtOX), and N-acetylbenzoquinoneimine (NABQI) in this study. Skin microsomes from the WT, HO and heterozygous (HT) $Cpr^{low/low}$ mice were compared and evaluated for CPR activity. Lymphocyte proliferative responses to BaP, COx and PCM were significantly abrogated by 36.4%, 45.2% and 50.8%, respectively; in $Cpr^{low/low}$ knock out (KO) mice versus WT mice; while the lymphocyte proliferative responses to the direct acting haptens OX, EtOX and NABQI were comparable. CPR activity, determined as Units/mg protein, was determined to be significantly lower in the $Cpr^{low/low}$ mice compared to the WT. Results of the present study suggest potential utility of the $Cpr^{low/low}$ mice in the LLNA to differentiate prohaptens from direct acting haptens.

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Introduction

Allergic contact dermatitis (ACD) is a common occupational and environmental health problem which accounts for 20% of all skin disorders in the USA. ACD is an immunological response by sensitized individuals to immunogens that result from the haptenation of cutaneous proteins with reactive chemicals (haptens). Many chemical allergens are haptens which possess electrophilic moieties enabling them to react directly with nucleophilic amino acids such as lysine and cysteine on proteins to form covalent bonds. Some contact allergens, however, have no or low chemical reactivity and are thus referred to as prehaptenes or prohaptens. Prehaptenes and prohaptens need to be activated either chemically or metabolically to become protein reactive (haptens). Smith and Hotchkiss estimated that approximately one third of all skin sensitizers are either prehaptenes or prohaptens (Smith Pease et al., 2003). Metabolic activation of many prohaptens including many other xenobiotics is generally oxidative in nature and cytochrome P450 (CYP) isoforms are believed to play a major role in the metabolism (Guengerich, 2001). The discovery and

profiling of metabolic activity in the skin, particularly CYP mediated metabolism (Ahmad and Mukhtar, 2004), in recent years have generated interest in the skin as an extrahepatic metabolizing tissue. Expression, and at times activity, of CYP isoforms in the skin has been reported even though full characterization of many metabolizing cutaneous enzymes has yet to be achieved (Bergstrom et al., 2007; Merk, 2009; Ott et al., 2009). CYP isoforms such as CYP1A1, 1B1, 2B6, 2E1 and 3A have been shown to be active in the skin as demonstrated with the bioactivation of prohaptens carvone oxime, cinnamyl alcohol and isoeugenol (Bergstrom et al., 2007; Ott et al., 2009). Cutaneous CYP activity has also been demonstrated by 7-ethoxyresorufin-O-deethylase, 7-pentoxeresorufin-O-deethylase, para-nitrophenol hydroxylase and erythromycin-N-demethylase assays (Baron et al., 2001). The otherwise benign procarcinogens benzo(a)pyrene (BaP) and 7,12-dimethylbenz(a)anthracene (DMBA) have been shown to be biotransformed into diol epoxides (Bickers et al., 1984; Bigger et al., 1983) in the skin with the positive response of BaP and DMBA in the local lymph node assay (LLNA) being attributed to these epoxides (Ashby et al., 1993).

With regard to skin sensitization, the LLNA is recognized and widely used for identification and evaluation of haptens, pre- and prohaptens. The positive response observed with many prohaptens in the LLNA buttresses evidence of the skin being metabolically active

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even though the assay is unable to distinguish haptens from prohapten. While the inability to separate prohapten from haptens has not been an issue with LLNA, the advent of alternative non-animal based skin sensitization screening methods has drawn the subject of prohapten versus haptens into focus. As reviewed by Jowsey et al., 2006 many alternative tests have been proposed based on criteria such as nucleophile/peptide reactivity (*in chemico*), activation of biomarkers on cells (*in vitro*) and computer algorithms (*in silico*) with promising results. However, unlike the LLNA, alternative tests have so far failed to detect reactivity of prohapten as the systems utilized lack metabolic activity. Some improvements of existing *in vitro* (Bergstrom et al., 2007; Chipinda et al., in press) as well as *in chemico* (Gerberick et al., 2009) methods to capacitate them to test for prohapten have been reported but it is evident that further studies are required in order to understand fully the role of CYP isoforms. As efforts to replace the LLNA with these alternative assays gain traction it is imperative that there be reliable assays that are capable of testing prohapten.

The LLNA, as a validated model, can be modified and utilized to distinguish direct acting haptens from prohapten. The current study presents a mouse model on which the LLNA was used to differentiate haptens from prohapten. In this model the role of cytochrome P450 reductase (CPR), as an obligate electron donor to all CYP isoforms, has been exploited where disrupting the CPR gene in mice has been shown to inactivate CYP activity in numerous organs (Gu et al., 2003; Henderson et al., 2003; Wu et al., 2005). CYP activity requires electrons which are shunted through CPR from the cofactor nicotinamide adenine dinucleotide phosphate reduced (NADPH). Mice with low CPR activity in the skin as a result of a hypomorphic NADPH-CPR gene (designated *Cpr* low allele) (Wu et al., 2005) were bred and exposed to haptens and prohapten in the standard LLNA as a way of measuring the bioactivating ability of cutaneous CYP isoforms. Transgenic mice with a hypomorphic CPR gene were produced when the *Cpr* gene in these mice was disrupted by the insertion of a neomycin-resistance gene in intron 15 (Wu et al., 2005). Lymph node cell proliferative responses to previously reported prohapten COx, BaP and PCM were compared to direct acting haptens in both the knock out (KO) and wild type (WT) mice. Homozygous mice were found to possess the lowest extrahepatic tissue CPR protein levels and hence were chosen for LLNA studies (Wu et al., 2005). Microsomal fractions from whole skin of KO and WT mice were also assessed for CPR activity and comparisons were drawn between skin CPR activity and the LLNA results.

Materials and methods

Chemicals. Benzo(a)pyrene (BaP), paracetamol (PCM), oxazolone (OX), 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (EtOX), N-acetylbenzoquinoneimine (NABQI), potassium chloride (KCl), magnesium chloride (MgCl₂), Tris HCl, monobasic potassium hydrogen phosphate (KH₂PO₄), dibasic potassium hydrogen phosphate (K₂HPO₄), phosphate buffered saline (PBS) and the NADPH-cytochrome P450 reductase kit were acquired from Sigma Aldrich (St Louis, MO). DNeasy Blood & Tissue kit was from Qiagen (Valencia, CA). Advantage 2 PCR kit was from Clontech (Mountain View, CA) and oligonucleotide primers were synthesized by Sigma Genosys (St Louis, MO). Scal enzyme kit for DNA digestion was from Fermentas (Glen Burnie, MD). Agarose gel, ethidium bromide and 10X Triton buffer were from Invitrogen (Carlsbad, CA). Tritiated thymidine (³H-TdR, specific activity 20 µCi) was from Dupont NEN (Waltham, MA) and scintillation fluid was from Perkin Elmer (Waltham, MA). Carvone oxime ((5R)-5-isopropenyl-2-methy-2-cyclohexene-1-one oxime) (COx) was synthesized according to Nilsson et al. (2005). Briefly, 1 g carvone ((5R)-5-isopropenyl-2-methycyclohex-2-en-1-one was reacted with 0.5 g of hydroxylamine hydrochloride (NH₂OH.HCl) in 5 mL methanol and left to stand for a week after which 6 mL of water was added with mixing. Suction filtration of the formed crystals with

washing (1:1 methanol/water) afforded pure COx crystals which were air dried.

Animals. Male and female C57BL/6J (B6) mice were purchased from Taconic (Hudson, NY). Breeding pairs used to establish a colony of the knockout mice (*Cpr*^{low/low}) were donated from the Wadsworth Center (Albany, NY). Animals were 6–8 weeks old upon arrival and allowed to acclimate for a minimum of 10 days. Animals were housed in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International-accredited animal facility at NIOSH, Morgantown, WV. Animals were housed under controlled environmental conditions in HEPA-filtered ventilated polycarbonate cages on autoclaved hardwood Beta-chip bedding and provided Teklad 7913 food and autoclaved tap water *ad libitum*. All animal procedures were reviewed and approved by the NIOSH Animal Care and Use Committee. The animals were monitored and were free of parasites, mycoplasmas, CAR *Bacillus* and viral pathogens other than noroviruses. Helicobacters and noroviruses were identified in this colony.

Experimental procedures

Genotyping of KO mice

Extraction of DNA from tail snips of 4 week old pups was performed using the DNeasy Blood and Tissue Kit from Qiagen (Valencia, CA). Genotyping on the DNA extracts, PCR, DNA restriction analysis and gel electrophoresis were performed according to the protocols provided by Dr. Xinxin Ding from Wadsworth Center, NY and as published (Wu et al., 2005). In brief, the DNA extracts were analyzed by PCR using an Eppendorf Mastercycler ep system (Hauppauge, NY) under the following conditions: 30 s at 94 °C, followed by 36 cycles of 30 s at 94 °C, 30 s at 65 °C, and 1 min at 72 °C, and lastly held 7 min at 72 °C. Scal restriction digestion of the PCR product was performed at 37 °C for 90 min. Analysis of digested DNA by gel electrophoresis was performed using 1.5% agarose gel. Pups with doublet bands at 300 base pairs were homozygous (HO) (*Cpr*^{low/low}) whereas pups with doublet bands at 300 base pairs and a single band at 600 base pair were heterozygous (HE) (*Cpr*^{low}). *Cpr*^{low/low} pups were chosen for LLNA studies.

Enzymatic activities of NADPH-cytochrome P450 reductase (CPR)

Female and male mice were grouped (n ≥ 3) into 3 groups of homozygous, heterozygous and WT and euthanized via CO₂ inhalation. Whole mouse skin pellets were collected and placed on ice after removal of all subcutaneous fatty and connective tissues. Microsomal isolation was prepared as described previously (Oldfield, 1990). Homogenization was performed at 4 °C with a Laboratory Blender Model 7010 (New Hartford, CT) and a Dremel Biospec Tissue Tearor (Racine, WI) as 25% (w/v) in 50 mM TRIS/0.15 M KCl (pH 7.4). The homogenate was filtered through one thickness of gauze to remove the residual fur and centrifuged at 1000 ×g for 10 min. The resultant supernatant in each case was sequentially centrifuged at 3000 ×g for 20 min, 10,000 ×g for 20 min, 15,000 ×g for 20 min and 105,000 ×g for 75 min. The 105,000 ×g pellets were dissolved in the homogenizing buffer and aliquoted. Protein concentration was determined using the Bradford assay (Bradford, 1976) with bovine serum albumin as standard. All continuous spectrophotometric assays for CPR activity were performed using standard 1 cm disposable cuvettes in a total reaction volume of 500 µL. Cytochrome c reduction activity was measured as previously described (Guengerich et al., 2009) using 40 µM of cytochrome c from horse heart and the CPR assay kit from Sigma Aldrich (St Louis, MO). The stock solution of cytochrome c was prepared fresh in 300 mM potassium phosphate (pH 7.8) and diluted for each experiment to make a final concentration of 36 µM. Increased absorbance at the wavelength of 550 nm was followed after addition

of 200 μM NADPH. Measurement of A_{550} during the first 5 min was used to determine the rate of reduction of cytochrome c. Kinetic analysis was carried out with microsomal cytochrome c prepared from skin collected from HO and HE B6 KO mice as well as WT B6 mice. After evaluating the protein concentration in each microsomal pellet the volume of protein introduced in each assay was calculated so that all assays had the same protein concentrations. Cytochrome c reduction rates were calculated using $\epsilon_{550} = 21.1 \text{ mM}^{-1}\text{cm}^{-1}$ for reduced cytochrome c (Guengerich et al., 2009). CPR activity was determined as Units/mg microsomal protein.

Local lymph node assay

To determine the allergenic potency of test chemicals the LLNA was performed according to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) standard method (Haneke et al., 2001). Four independent studies were conducted that included one preliminary study to examine sex difference in response to oxazolone and 3 independent experiments each of which contained solvent controls, assay of a direct acting hapten and assay of a prohaptens in both KO and wild-type mice. After grouping mice into homogenous groups ($n = 5$) based on their weight, mice were dosed with 25 μL /ear of a test chemical (BaP; (1.25%), COx; (10%), PCM; (0.1%), OX; (0.02%), EtOX; (0.02%), and NABQI; (0.1%) in acetone olive oil (AOO; 4:1). The control group was dosed with AOO. The vehicle and test chemicals were applied on the dorsum of both ears. Two hundred μL of 20 μCi ^3H -TdR in 0.01 M PBS was injected into the tail vein on day 6, and after 5 h the mice were euthanized via CO_2 inhalation. Left and right draining auricular lymph nodes were excised and pooled for each animal. Single cell suspensions were made and following overnight protein precipitation using 5% TCA, samples were counted on a Packard Tri-Carb 2500TR (Meriden, CT) liquid scintillation analyzer with background subtraction. Stimulation indices (SI) were calculated by dividing the mean disintegrations per minute (DPM) per test group by the mean DPM for the vehicle control group.

Statistical analysis

Evaluation of data for whole animal experiments was conducted with SigmaStat 3 from Systat Software, Inc (San Jose, CA). Data were analyzed by one-way ANOVA followed by a post hoc Bonferroni test. WT versus $\text{Cpr}^{\text{low/low}}$ SI values were analyzed using a Student *T*-test. Data were considered significant at $p < 0.05$.

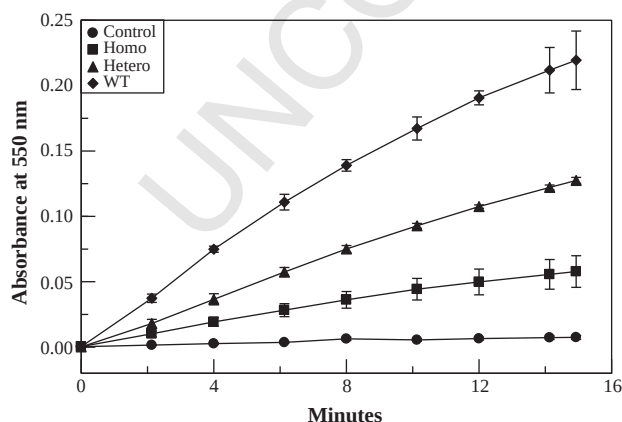


Fig. 1. Microsomal CPR mediated reduction of cytochrome c. The reduction of cytochrome c with microsomal CPR prepared from whole skin of WT, HE and HO mice ($n \geq 3$ /group). NADPH was included as the cofactor and the linear part of the curves was used to calculate CPR activity per gram of protein. Control experiments compromised of everything but the protein. Activity was in the order $\text{WT} > \text{HE} > \text{HO}$.

Table 1

Protein concentrations of microsomal proteins and the corresponding CPR activities derived from reduction of cytochrome c in the presence of NADPH.

Sample	Microsomal protein (mg/mL) (n = 3)	CPR activity (Units/mg)	
WT liver	17.5 $\pm$ 0.47	2.17 $\pm$ 0.21	t1.4
HO liver	16.5 $\pm$ 0.73	0.39 $\pm$ 0.18	t1.5
WT skin	1.56 $\pm$ 0.18	0.0222 $\pm$ 0.0003	t1.6
HO skin	1.72 $\pm$ 0.31	0.0045 $\pm$ 0.0002	t1.7
HE skin	1.52 $\pm$ 0.14	0.0110 $\pm$ 0.0004	t1.8

Results

CPR activity

Fig. 1 outlines reduction of cytochrome c for the pooled microsomes from which CPR activity per gram was derived. No difference was noted in the concentration of microsomal protein isolated from the skin of WT, HE and HO mice. Control experiments lacking only the microsomes showed no CPR activity. The activity per mg microsomal protein was decreased by about 50.4% for the HE microsomes and 79.7% for the HO microsomes as compared to the WT mice. The activity observed with HO microsomes was still significantly above the negative control. The skin microsomal protein concentration and the observed CPR activities in Table 1 are almost 100-fold less compared to liver CPR activity. The HE/HO CPR activity ratios observed with the skin microsomes agreed with the HE/HO CPR expression ratios that have been previously reported for other extrahepatic tissues, but not for the skin from $\text{Cpr}^{\text{low/low}}$ mice (Wu et al., 2005).

LLNA: allergenicity assessment of prohaptens

No overt irritation, as indicated by ear swelling and redness, was observed following application of the previously reported (Bergstrom et al., 2007; Gerberick et al., 2005) highest soluble concentrations of the test chemicals (1.25% for BaP, 0.02% OX, 0.02% EtOX and 0.1% PCM, 0.1% NABQI and 10% COx) in AOO (4:1). Exposure of the animals to these chemicals did not induce systemic toxicity as assessed by change in body weight, behavior and clinical signs of illness. The SI values for both the haptens and prohaptens are shown in Figs. 2–3. The dosing of both WT and KO animal groups with the direct acting haptens resulted in SI values presented in Fig. 2 of OX (16.3 ± 1.4 WT; 13.0 ± 1.9 KO); EtOX (4.6 ± 0.5 WT; 5.0 ± 0.4 KO); NABQI (6.0 ± 1.2

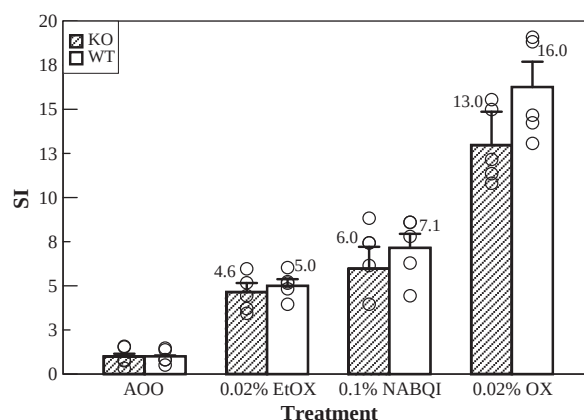


Fig. 2. Lymph node cell proliferation measured as stimulation index ($\text{SI} \pm \text{SEM}$) for direct acting haptens. WT and HO mice ($n = 5$ /group) were dosed with direct acting haptens OX, NABQI and EtOX in the standard LLNA. Control groups (WT and HO) were dosed with AOO. There were no differences in lymphocyte proliferative responses between WT and HO mice groups for the haptens.

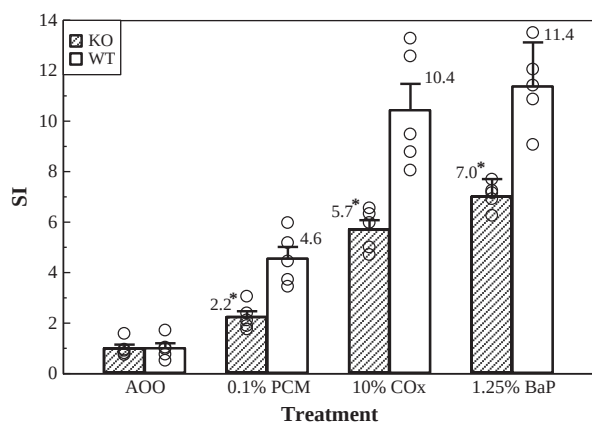


Fig. 3. Lymph node cell proliferation measured as stimulation index (SI $\pm$ SEM) for prohapten. WT and HO mice ($n = 5$ /group) were dosed with prohapten PCM, COx and BaP. Significant decreases (* $p < 0.05$) in SI values were observed for HO mice when compared with WT mice for the three prohapten indicating decreased metabolic activation of the prohapten.

the LLNA even though the distinction between a hapten and a prohapten cannot be made using normal (WT) mice in this assay. It was hypothesized in the present study that use of mice with compromised metabolic capacity in LLNA would enable the differentiation between haptens and prohapten.

We initially attempted to disrupt microsomal metabolism in the skin pharmacologically in B6 mice using diphenyliodonium chloride (DIC) and lipoic acid (LA), which are known *in vitro* inhibitors of CPR. These were either applied to ears of mice in appropriate vehicles with a 1 h wait prior to dosing with prohapten or applied together as a mixture with prohapten. Significant pharmacological inhibition of CPR and hence CYP isoforms (data not shown) was not observed. Using non specific CYP inhibitors like proadifen chloride (PA) and ketoconazole (KZ) in the same manner also failed to produce *in vivo* inhibition of prohapten activation as assessed by the LLNA (data not shown). Multiple vehicles and intradermal applications were employed without successful alteration of LLNA response to prohapten.

A significant reduction in microsomal CPR activity was observed from the skin of HO and HE KO mice as compared to the WT, with WT>HE>HO. The HE CPR activity (0.011 U/g) was 2.4 $\times$ than that of the HO (0.0045 U/g) which is in good agreement with reported CPR protein levels from both hepatic and other non-hepatic tissues previously reported (Wu et al., 2005). Some residual CPR activity (approximately 20%) was observed in HO as compared to WT. Residual cutaneous CPR or potentially, a non-CPR enzyme may be catalyzing the reduction of cytochrome c.

The prohapten utilized in the LLNA have been well characterized and serve as appropriate test chemicals to evaluate the utility of the $Cpr^{low/low}$ model. They are substrates of the CYP 1A1 (BaP) and CYP2E1 (PCM; COx) isoforms that have been shown to exist at significant mRNA expression levels as well as functionally in the skin (Bergstrom et al., 2007). The $Cpr^{low/low}$ model used herein (with a global suppression of CPR activity) for the differentiation of direct acting from prohapten is faster, economical and hence attractive compared with using knockout (KO)/transgenic mice with specific CYP isoforms deleted or inactivated. Different prohapten are substrates of different CYPs and thus multiple specific KO/transgenic mice would be required to investigate various prohapten, notwithstanding the fact that the specific CYP-prohapten combinations would need to be identified, *a priori*, to be able to match them with the specific KO/transgenic mice. Targeted reduction of CPR activity has the net effect of inactivating all CYPs. Alternative electron donors such as NADPH-cytochrome b_5 (CBR) reductase (Guengerich et al., 2009), may allow a low CYP activity to proceed, but do not use NADPH efficiently. Established LLNA doses for the hapten and prohapten test chemicals and the previous documentation of the B6 strain for use in the LLNA (Woolhiser et al., 2000) were taken advantage of in the present study allowing for a reduction of the total number of mice required to evaluate this model.

Limitations of this mouse model include the fact that dermal NADPH reductase activity is not totally abrogated. For the microsomes prepared from HO mice, residual reduction of cytochrome c was evidence that reductase activity was severely reduced but not totally absent. The possibility of contributory effects from extraneous reductases, such as cytochrome b reductase (CBR), is unlikely since the inclusion of potassium cyanide (KCN) which effectively inhibits known non-CPR reductases neither increased nor decreased observed CPR activity. Potential metabolic activation of some prohapten in the LLNA may be mediated by other metabolic enzyme systems, such as peroxidases, myeloperoxidases and cyclooxygenases that have been detected in the skin (Gibbs et al., 2007). Compensatory increases in total CYP content in the liver and kidney have been noted in $Cpr^{low/low}$ mice and while not assessed may also be true in the skin. It is thus possible that metabolic activation of prohapten could have been CYP mediated in the presence alternative (to CPR) redox partners to the CYPs. The observed decreases (36–50%) in LLNA responses may not be

KO; 7.1 ± 0.8 WT) which were not significantly different ($p \geq 0.05$) between the WT and KO mice. Prohapten elicited responses resulting in the SI values for BaP of 11.4 ± 1.7 for WT and 7.0 ± 0.7 for HO (Fig. 3); PCM of 4.6 ± 0.5 for WT and 2.2 ± 0.2 for HO (Fig. 3); and Cox of 10.4 ± 1.0 for WT and 5.7 ± 0.4 for HO (Fig. 3) which were significantly different when the WT response was compared with the HO mice response ($p \leq 0.05$). The lymphocyte proliferative responses to the prohapten BaP, COx and PCM were 36.4%, 45.2% and 50.8% lower, respectively; in $Cpr^{low/low}$ HO mice versus WT mice. Comparison of the sensitizing potential of the known prohapten sensitizers clearly indicate that metabolic activation of the prohapten was compromised in the KO mice even though there was still significant metabolic prohapten activation. Potential gender differences were assessed using male and female HO $Cpr^{low/low}$ mice that were dosed with 1.25% BaP or 0.02% EtOX in AOO. Gender related differences in the lymphocyte proliferation responses were not observed (data not shown).

Discussion

Metabolic activation is an important consideration for the development of alternative non-animal based assays for the prediction of contact allergens to avoid false negative assignments. The metabolic capability of the skin has not been fully characterized (Gibbs et al., 2007), making it difficult to incorporate metabolic activation in these predictive assays. Herein, the widely accepted and validated LLNA was utilized to distinguish between haptens and prohapten. The use of transgenic mice whose CPR gene was disrupted by insertion of a neomycin-resistance gene in the CPR intron 15 (Wu et al., 2005) is based on the assumption that most, if not all, of the metabolic activity in the skin is mediated by CYP isoforms. CPR, which is a microsomal flavoprotein, is the obligate redox partner of all microsomal CYPs and incapacitating it has the effect of inactivating all CYP isoforms. As an example, liver-specific *Cpr* deletion abolished liver microsomal CYP activity in HO mice (Henderson et al., 2003). In particular, this mouse model with global suppression of *Cpr* expression has been used for studying the involvement of extrahepatic tissue CYPs in drug metabolism (Wu et al., 2005). This model has also enabled the inference of extrahepatic tissue contributions to systemic metabolism and local drug toxicity. A 74%–95% decrease in CPR expression in all tissues such as olfactory mucosa, adrenal gland, brain, testis, ovary, lung, kidney, liver, and heart was reported in this mouse model (Wu et al., 2005). Both haptens and prohapten elicit positive cell proliferative responses in

quantitatively sufficient to distinguish prohaptens that are metabolized to weak haptens/allergens.

It has to be noted that the Cpr^{low/low} source colony mice were positive, serologically and by faecal PCR, for norovirus. This virus has only recently been identified to be widespread in laboratory mice and does not produce clinical signs of illness. While potential immunological alterations due to colonization by norovirus are possible, comparable direct acting hapten LLNA SIs were observed between WT and the Cpr^{low/low} mice. The LLNA SIs obtained are also consistent with those previously reported for the direct acting haptens. A recent review discussing the occurrence and effects of the norovirus found no norovirus related overt influence on animal experimental results in non-immunocompromised mice (Henderson, 2008).

In summary, a transgenic mouse model with globally decreased CPR expression was assessed for use in the LLNA and found to allow differentiation of haptens and prohaptens that are CYP substrates. This mouse model may potentially be utilized in the identification of prohaptens that require CYP mediated bioactivation and thereby contribute to a more robust understanding and identification of skin sensitizers that are prohaptens. Prohaptent identification is important in providing information necessary for *in vitro*, *in chemico* and *in silico* modeling, as well as for potential use in identifying appropriate chemical non- or less allergenic substitutes for a product or process.

Conflict of interest statement

The authors declare no conflict of interest.

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The findings and conclusion in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health. These studies were made possible with a gift of breeding pairs of KO mice from Dr. Xinxin Ding (Wadsworth Center, New York State Department of Health).

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