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STRAIN DIFFERENCES IN THE INDUCTION OF MONO-OXYGENASE ACTIVITY IN MOUSE SKIN BY TOPICAL CLOBETASOL PROPIONATE: EVIDENCE OF A ROLE FOR THE HR LOCUS

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Summary—The effect of the topical application of clobetasol propionate on cutaneous ethoxycoumarin O'dealkylation (EOD) has been studied in various strains of mice. Clobetasol propionate markedly increased cutaneous EOD activity in adult hairless mice only. Similar treatment of adult haired C57BL/6J mice, or adult haired DBA/2J mice had no significant effect on cutaneous EOD activity. In contrast 3 methylcholanthrene induced cutaneous EOD activity in both hairless and C57 strains to a far greater extent than in the DBA strain. EOD activity in hairless mice non-responsive to polycyclic hydrocarbons, derived by selective breeding of hairless and DBA strains was induced by clobetasol propionate to a similar extent to that observed in responsive hairless strains. Hepatic EOD activity was not induced by clobetasol propionate in any of the strains tested. Strain differences in the induction of EOD by clobetasol propionate were not related to differences in either the concentration of cytosolic glucocorticoid receptor in the skin, the dissociation constant of the cytosolic receptor, or differences in percutaneous absorption. Polycyclic hydrocarbons did not compete with triaminolone acetonide for binding to the cytosolic glucocorticoid receptor. Strain differences in the induction of EOD activity by clobetasol propionate appear therefore not to be related to strain differences in either the Ah receptor or the glucocorticoid receptor, but to be regulated by the hr locus.

INTRODUCTION

The microsomal mono-oxygenase system catalyses the oxidation of a large number of exogenous and endogenous lipophillic substrates to more polar products. The enzyme system is membrane bound and comprises a flavoprotein reductase and a family of haemproteins of differing substrate specificities, collectively called cytochrome P-450 [1]. A major feature of the mono-oxygenase system is its ability to be selectively induced by numerous drugs, carcinogens and xenobiotics [2]. There are over 300 compounds that are known to induce the microsomal mono-oxygenase system and these may be divided into six or more categories according to the profiles of enzyme activity evident after induction. Inducing agents such as the polycyclic hydrocarbons as exemplified by 3 methylcholanthrene, induce a different profile of cytochrome P-450 dependent enzyme activity from drugs such as phenobarbitone [1, 3]. Other classes of inducing agents include steroids, alcohol, isosafrole, cholestyramine and polychlorinated biphenyls which also induce distinct profiles of enzyme activity [2]. The induction of monoxygenase activity in the mouse by polycyclic hydrocarbons is known to be mediated by a stereospecific cytosolic receptor [4], a product of the regulatory genes of the murine Ah locus [5]. The study of strain differences in the inducibility of mono-oxygenase activity in the mouse has led to a detailed knowledge of the biochemistry and genetics of the induction of mono-oxygenase activity by polycyclic hydrocarbons. Strains of mice (e.g. C57BL/6J) that are "responsive" (Ah^b/Ah^b) to induction by polycyclic hydrocarbons are known to possess a cytosolic receptor that binds inducing agents and mediates the induction process [6]. Strains of mice (e.g. DBA/2J) that are "non-responsive" (Ah^d/Ah^d) to induction have no detectable cytosolic binding receptor [5]. Responsiveness to polycyclic hydrocarbons is inherited as an autosomal dominated trait with heterozygotes (Ah^b/Ah^d or Ah^d/Ah^b) possessing an active receptor [8]. The cytosolic location of the Ah receptor; the stereospecific binding of ligand and the temperature dependent translocation of the inducer–receptor complex to the nucleus [7, 8] are very similar properties to those described for the glucocorticoid steroid hormone receptors [9]. Recently we have reported that microsomal mono-oxygenase activity in hairless mouse skin is inducible by the topical application of synthetic glucocorticoids routinely used in clinical practice [10]. Induction was in proportion to therapeutic potency, and clobetasol propionate was the most potent inducer. In view of the reported similarity between mechanism of induction of mono-oxygenase activity via the Ah receptor, and enzyme induction by steroids, we have

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investigated the relationship between induction of mono-oxygenase activity by polycyclic hydrocarbons and glucocorticoids, and their respective cytosolic receptors.

EXPERIMENTAL

Animals and treatments

Adult male mice were used in all experiments. Inbred strains of hairless (Ah^b/Ah^b; hr/hr); haired C57Bl/6J (Ah^bAh^b; +/+) and haired DBA/2J (Ah^d/Ah^d; +/+) were housed in plastic cages and allowed unlimited access to a pellet and water diet. Mice homozygous for hairlessness (hr/hr) develop a normal coat of hair until aged 12–15 days, when hair loss starts, which is usually completed at the age of 18–21 days. The trait of hairlessness was bred into the non-responsive DBA strain by mating hairless and DBA strains; the F1 progeny of which were all heterozygous haired (hr/+) and responsive to polycyclic hydrocarbons (Ah^b/Ah^d). The F1 progeny were then brother–sister mated and the hairless offspring phenotyped using the zoxazolamine test (see below). The hairless non-responsive progeny (Ah^d/Ah^d; hr/hr) were then inbred for 4–6 generations before use. Similarly hairlessness was bred into the C57 strain by crossing hairless and C57 strains, inbreeding the heterozygous haired (hr/+) progeny and selecting the hairless offspring. All strains of mice were routinely phenotyped using the zoxazolamine test [11] as follows. Mice were injected i.p. with either beta naphthoflavone (80 mg/kg) or 3-methylcholanthrene (80 mg/kg) in corn oil, once daily for 2 days. 18–24 hours after the final injection zoxazolamine was administered by i.p. injection at a dose of 225 mg/kg in corn oil, and the duration of paralysis caused by zoxazolamine noted. Mice non-responsive to induction by polycyclic hydrocarbons (Ah^d/Ah^d) were paralysed for greater than 30 min, whereas those responsive to induction by polycyclic hydrocarbons (Ah^b/Ah^b or Ah^d/Ah^b) were not paralysed. This test cannot distinguish between heterozygous and homozygous responsive strains.

For the topical application of inducing agents haired mice were shaved with electric clippers and depilated with Immac® cream (facial quality) prior to the application of inducing agents. In some experiments hairless mice were similarly treated and the depillatory procedure was found to have no effect on basal or induced levels of enzyme activity. Clobetasol propionate (0.05%) was applied in a cream base to the dorsal skin as previously described [10]. We have previously shown that this dose of clobetasol propionate causes maximal induction of enzyme activity 18–24 h after treatment [10]. 3-Methylcholanthrene (2 μmol) was applied to the dorsal skin in 2 × 100 μl of acetone. Mice were killed at various intervals after treatment, as indicated in the text.

Enzyme activity

Ethoxycoumarin O'Dealkylase (EOD) activity in 9,000 *g* supernatants of whole mouse skin was determined using the method of Greenlee and Poland [12] previously described in detail [10]. Results were expressed as pmol of 7-hydroxycoumarin formed/min/g. tissue. Expression of results on a DNA basis gives essentially similar values [10].

The glucocorticoid receptor in mouse whole skin was quantified using an *in vitro* exchange assay to measure occupied and unoccupied binding sites. The conditions for the incubation of skin cytosol with triamcinolone acetonide, together with rthe validation of the charcoal–dextran assay for measuring receptor bound glucocorticoid have been described in detail elsewhere [13]. Briefly, skin was homogenised in 10 mM Tris buffer pH 7.25 containing 2 mM MgCl; 5 mM dithiothreitol; 10 mM sodium molybdate and 10% glycerol. The homogenates were centrifuged at 100,000 *g* for 1 h and 200 μl of the resulting cytosol (4.0–6.0 mg protein) were incubated at 4° for 20 h with 10–15 nM [^{3}H] triamcinolone acetonide in the presence or absence of a 100-fold molar excess of non-radioactive triamcinolone acetonide to assess non-specific binding. After incubation 110 μl of a charcoal suspension (3.75% activated charcoal; 0.375% dextran T-500; 0.1% gelatin; 10% glycerol in 10 mM Tris buffer) were added and the tubes incubated for a further 20 min at 4°C. The charcoal was pelleted by centrifugation and the radioactivity in 200 μl portions of the supernatant estimated by liquid scintillation counting using standard procedures.

RESULTS

The effects of a single topical application of clobetasol proprionate on cutaneous ethoxycoumarin O'dealkylase (EOD) in adult hairless (hr/hr), C57BL/6J (C57) and DBA/2J (DBA) mice are shown in Table 1. Topical treatment of adult hairless mice with clobetasol propionate induced cutaneous EOD activity 5–6-fold. Similar treatment of C57 and DBA mice however had no significant effect on levels of cutaneous EOD activity. This was in marked contrast to the effects of 3MC on cutaneous EOD activity where a single topical application resulted in an 8–10-fold increase in cutaneous EOD activity in hr/hr and C57 strains of mice, yet only doubled activity in the DBA strain (Table 2). Repeated applications of 0.05% clobetasol propionate twice daily for 7 days was also without effect on cutaneous EOD activity in haired DBA and C57 strains of mice, with treated values (DBA = 3.6 pmol/min/g. $n = 4$; C57 = 2.9 pmol/min/g. $n = 4$), not differing significantly from their respective controls. Similarly, repeated applications of 0.5% clobetasol propionate to haired strains of mice did not significantly induce cutaneous EOD activity. In addition, removal by cellotape

Table 1. The effects of a single application of clobetasol proprionate or a single application of 3 methylcholanthrene (3MC) on cutaneous ethoxycoumarin dealkylation

Strain	Phenotype/ Genotype	Ethoxycoumarin O'dealkylation Control	Clobetasol Propionate	3MC
DBA/2J	Non responsive Ah/Ah +/+	2.6 ± 0.3 $n = 16$	3.0 ± 0.6 $n = 12$	6.8 ± 0.7*† $n = 4$
C57BL/67J	Responsive Ah/Ah +/+	2.9 ± 0.4 $n = 20$	3.6 ± 0.4[c] $n = 17$	32.0 ± 6.7*† $n = 4$
hr/hr	Responsive Ah/Ah +/+	3.2 ± 0.5 $n = 32$	17.0 ± 2.3[c] $n = 12$	25.4 ± 1.6*† $n = 4$

Clobetasol proprionate 0.05% in a cream base, or 3MC 1 μmol in 2 × 100 μl of acetone was applied to the dorsal skin of mice and enzyme activity determined 18 h later as described in the Experimental section.
Results are shown as the mean ± SE for the number of mice indicated.
*Significantly different from control value $P < 0.01$.
†Significantly different from clobetasol propionate treated value $P < 0.01$.
[c]Significantly different from 3MC treated value $P < 0.01$.

stripping of the principal barrier to cutaneous absorption, the stratum corneum, had no effect on strain differences in induction by clobetasol propionate with differences 24 and 48 h after stripping being the same as those shown in Table 1. Injection of clobetasol propionate (1.0 mg/mouse i.p.) for 7 days did not induce hepatic EOD in either strain of mouse.

Topical application of clobetasol propionate to the heteroxygous haired (hr/+) Ah responsive (Ah^b/Ah^d) F1 progeny of (C57 × hr/hr) and (DBA × hr/hr) crosses did not induce cutaneous EOD activity, whereas topical 3MC induced EOD activity in both strains (Table 2). However clobetasol proprionate treatment of both Ah responsive (Ah^b/Ah^b or Ah^b/Ah^d) and Ah non-responsive (Ah^d/Ah^d) hairless (hr/hr) progeny resulted in a significant induction of cutaneous EOD activity (Table 2). Topical 3MC induced EOD activity in the Ah responsive strains only.

To investigate whether the inductive effect of clobetasol proprionate on cutaneous EOD activity in hairless mice was a result of the hairless genotype or a consequence of the hairless phenotype, homozygous hairless mice prior to hair loss, aged 15 days, were shaved, depilated and treated with clobetasol propionate. EOD activity was induced in these mice to a similar extent to that observed with adult hairless mice (+476%). Enzyme activity in 15-day old haired C57 and DBA strains of mice identically treated, was not induced.

The assayable glucocorticoid receptor concentrations in the skin cytosol the various strains of mice used are shown in Table 3. There were no significant differences between the various strains of mice in the concentration of glucocorticoid receptors per milligram of cytosolic protein.

In addition, dissociation constants for the cytosolic glucocorticoid receptor in the different strains of mice were not significantly different from each other

Table 2. The effects of clobetasol proprionate and 3MC on cutaneous etoxycoumarin dealkylation in haired and hairless mouse strains

Strain propionate		Ethoxycoumarin O'dealkylation pmol/min/g tissue Control	Clobetasol proprionate	3MC
F1	Haired	2.7 ± 0.7	2.9 ± 0.2	29.6 ± 6.6*†
C57/BL6 × hr/hr	responsive	$n = 4$	$n = 8$	$n = 12$
F1	Haired non	2.6 ± 0.6	3.8 ± 0.4	24.7 ± 5.9*
DBA/6J × hr/hr	responsive	$n = 4$	$n = 8$	$n = 8$
F6	Hairless non	4.3 ± 0.5	16.0 ± 1.6*‡	6.9 ± 1.1†
DBA/6J × hr/hr	responsive	$n = 8$	$n = 8$	$n = 8$
F6	Hairless	3.8 ± 0.4	14.1 ± 1.1*‡	32.9 ± 8.1*†
C57/BL6 × hr/hr	responsive	$n = 8$	$n = 8$	$n = 8$

For the determination of cutaneous EOD activity clobetasol proprionate 0.05% in a cream base or 1 μmol of 3 methylcholanthrene (3MC) in 2 × 100 μl of acetone was applied to the dorsal skin of mice and enzyme activity determined 18 h later as described in the Experimental section.
Results are shown as the mean ± SE for the number of mice indicated.
*Significantly different from control values $P < 0.01$.
†Significantly different from clobetasol proprionate treated value $P < 0.01$.
‡Significantly different from 3MC treated value $P < 0.01$.

Table 3. Characteristics of the cytosolic glucocorticoid receptor in the skin of DBA/2J C57BL/6J and hr/hr mice

Strain	Receptors fmol/mg protein	K_d mM
DBA/2J	156.0 ± 6.7	0.40 ± 0.05
C57BL/6J	162.6 ± 17.0	0.69 ± 0.36
hr/hr	188.8 ± 19.3	0.54 ± 0.13
C57/hr	187.2 ± 5.9	1.09 ± 0.28
DBA/hr	156.6 ± 6.4	0.87 ± 0.08

Results are shown as the mean ± SE for 4 mice per group. None of the values are significantly different from each other.

(Table 3). The polycyclic hydrocarbons 3 methylcholanthrene, benzanthracene and benzpyrene did not compete with triamcinolone acetonide for *in vitro* binding to the cytosolic glucocorticoid receptor, with no detectable displacement of triamcinolone acetonide binding to receptor being observed with concentrations of 3MC, benzanthracene or benzpyrene between 10 μM and 50 mM. In agreement with previous findings in rat skin [13], clobetasol propianate competed with triamcinolone acetonide for the glucocorticoid receptor.

DISCUSSION

The present results indicate that differences in neither the glucocorticoid receptor nor the Ah receptor mediate strain differences in the induction of cutaneous drug metabolising enzymes by cobetasol propionate. Whereas marked strain differences in the induction of cutaneous EOD activity by clobetasol propionate were evident (Table 1), there were no detectable corresponding differences in the concentration of glucocorticoid receptor per milligram of cytosol protein, or the dissociation constants for steroid binding to the receptor (Table 3). In addition polycyclic hydrocarbons did not compete with triamcinolone acetonide for the glucocorticoid receptor, suggesting that the glucocorticoid receptor is not active in the binding and translocation of polycyclic hydrocarbons to the nucleus. Moreover, strain differences in the presence of the Ah receptor did not correspond to differences in the extent of induction of drug metabolising enzymes by clobetasol propionate. In agreement with other workers [14] we presently find that induction of mono-oxygenase activity in the skin by polycyclic hydrocarbons is observed in the non-responsive DBA strain of mice, although the extent of induction is nevertheless far lower than that in responsive strains. Thus while cutaneous EOD activity was induced 2-fold by 3MC treatment of DBA mice (Table 1), similar treatment of hr/hr and C57 strains, both of which are Ah responsive, resulted in an 8–10-fold increase in activity. However the pattern of cutaneous enzyme induction following treatment with clobetasol propionate does not mirror that following treatment with 3MC. The Ah responsive C57 strain, possessing an active Ah receptor, did not respond with induction following treatment with clobetasol propionate. The non-responsive DBA strain, not possessing an active Ah receptor, was similarly not induced, whereas the responsive hr/hr strain was induced to a major extent following treatment with clobetasol propionate (Table 1). Furthermore, induction by clobetasol propionate was also observed in the Ah non-responsive hairless strains (Table 3). It is unlikely therefore that the Ah receptor mediates induction of the mono-oxygenase system by clobetasol propionate. In support of this conclusion Nebert and Poland [4, 16] have previously reported that glucocorticoids do not compete with 2,3,7,8 Tetrachlorodibenzo-*p*-dioxin (TCCD) for the hepatic Ah receptor. Moreover, the persistence of strain differences after removal of the stratum corneum, the principal barrier to cutaneous permeation of the drug, demonstrates that differences in induction by clobetasol propionate. However as no uptake studies have been performed differences in uptake cannot be entirely excluded.

The results do indicate however that the hairless trait is involved in the responsiveness of the skin to enzyme induction by glucocorticoids. Furthermore, the observation that induction of enzyme activity was observed in hr/hr mice prior to hair loss indicates that this is due to the hairless genotype and not a consequence of the phenotype. The hr locus has been reported to confer different susceptibilities to the toxic effects of xenobiotics in mouse skin. For example although the effects of 2,3,7,8 Tetrachlorodibenzo-*p*-dioxin (TCCD) are mediated via the Ah receptor [4], TCCD is an effective tumour promotor only in homozygous hairless Ah responsive, and not in haired responsive strains [17]. Moreover although the histopathological effects of TCCD are mediated via the Ah receptor [11], TCCD will only cause hyperplasia, hyperkeratosis and sebaceous gland metaplasia in the skin of Ah responsive mice homozygous for the hairless gene [11], and a combined role for the hr locus and the Ah locus in the control differentiation and proliferation in the skin has been proposed [11]. Thus the induction of monoxygenase activity by clobetasol propionate may be an additional response of the skin attributable to the hr locus. Moreover, our finding that the effects of the hr locus on induction are restricted to the skin, and not observed systematically in the liver, are in keeping with those of Knutson and Poland for the role of the hr locus in mediating the cutaneous toxiciy of TCCD [11]. Thus while the cutaneous toxicity of TCCD is dependent on both the Ah and the hr locus [11], systemic toxicity is not. This is in marked contrast to the effects of the Ah locus which governs induction in all tissues [8].

The precise mechanism whereby the hr locus mediates the induction of cutaneous mono-oxygenase activity by steroids is at present unknown. While the hairless trait in the mouse is thought to be due to differences in chromosome 14 [11], the induction of

mono-oxygenase activity is thought to involve at least two independent loci with a minimum of three alleles each, and chromosomes 8, 9, 17 and 19 are most suspect [8]. It is probable therefore that EOD induction is the result of the interaction of several genetic components.

Since the present results show that strain differences in the cytosolic glucocorticoid receptor are not responsible for strain differences in induction of cutaneous mono-oxygenase activity then either induction by glucocorticoids does not involve the classical cytosolic receptor or strain differences occur as a result of events subsequent to the binding of ligand to the receptor. In preliminary experiments we have found that the strain differences in EOD inducibility currently reported are correlated with the ability of clobetasol propionate to depress thymidine incorporation into dermal and epidermal DNA. We are currently investigating this with regard to dermal thinning which is a major side effect of topical steroid therapy.

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