Multiple Cytochrome P450 Isozymes in Murine Skin: Induction of P450 1A, 2B, 2E, and 3A by Dexamethasone

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Cytochrome P450s (P450s) are a supergene family of enzymes responsible for the metabolism of a wide range of endogenous and foreign compounds. P450 isozymes possess overlapping substrate specificity. Systemic administration of dexamethasone, a widely used topical agent in dermatologic practice, to animals is known to result in the induction of multiple P450 isozymes in liver. In this study the effect of topical application of dexamethasone to mice on P450-dependent monooxygenase activities, expression of P450 isozymes, and P450 mRNA levels in skin was assessed. The treatment of mice with dexamethasone resulted in significant induction of 7-ethoxyresorufin O-deethylase (2.3 times), 7-pentoxyresorufin O-depentylase (19.2 times), para-nitrophenol hydroxylase (7.5 times), and erythromycin N-demethylase (2.2 times) activities; the monooxygenases catalyzed preferentially by P450 isozymes 1A1, 2B1, 2E1, and 3A, respectively. Immunoblot analysis of cutaneous microsomes, employing antibodies directed against purified P450s 1A1/2, 2B1/2, 2E1, and 3A, showed that dexamethasone treatment results in an increased immunoreactivity (1.8–13.9 times). In immunohistochemical staining of skin with antibody against P4502B1/2, topical application of dexamethasone resulted in an increased reactivity towards microsomal protein in the suprabasal layer of the epidermis and with the cells of the hair follicles. Whereas constitutive expression of mRNAs for CYP1A1 and CYP2E1 was evident in murine skin, any change in the levels of these mRNAs following treatment with dexamethasone was not apparent. The results of our study indicate that the application of dexamethasone to murine skin results in the induction of several families of P450 isozymes, suggesting that murine skin contains multiple inducible P450 isozymes capable of participating in the metabolism of a wide range of xenobiotics and endogenous compounds. *Key words: cytochrome P450, dexamethasone, monooxygenase activities. J Invest Dermatol 102:970–975, 1994*

The cytochrome P450 (P450) isozymes belong to a multigene superfamily of enzymes responsible for the metabolism of a wide range of exogenous compounds such as drugs, carcinogens, and environmental chemicals, as well as of endogenous substances such as fatty acids, prostaglandins, vitamin A, vitamin D, and steroids ([1–3] and references therein). Many of these P450s are products of different genes that encode proteins with distinct molecular weights, immunologic reactivities, and substrate specifiicities [1,4–6]. Of 36 P450 gene families described so far, 12 families exist in all mammals examined [7], and their inducibility, substrate turnover rates, and immunoreactivity with specific antibodies have been studied extensively in extracutaneous tissues [1,7]. Much less, however, is known about P450s in cutaneous tissue [1,4].

Skin is a major organ that functions as an interface between foreign compounds such as solvents, drugs, environmental carcinogens, etc., and the body. Our laboratory has attempted to define the role of the P450 system in rodent and human skin with respect to the metabolism of chemical carcinogens and physiologically important endogenous compounds ([1,4,8–11] and references therein). Most studies on cutaneous P450, however, have focused on P4501A1 and its role in chemical carcinogenesis [12]. The biochemical characteristics of P4501A1 isozyme and the inducibility following exposure to several polyaromatic hydrocarbons, β-naphthoflavone (βNF), glucocorticoids, etc. of monooxygenases, preferentially catalyzed by this isozyme, have been demonstrated in both human and rodent skin and in human hair follicles [13–17]. Studies employing polymerase chain reaction (PCR) and Northern blot analysis have recently shown that treatment of neonatal rat skin and normal human epidermal keratinocytes in culture with βNF or benz(a)anthracene results in an increased expression of P4501A1 at the mRNA level [18].

Glucocorticoids, which are widely used in clinical dermatology as topical antiinflammatory drugs, are known to induce several P450s, most notably P4503A, in liver *via* transcriptional activation and mRNA stabilization [19,20]. Earlier we suggested that multiple P450s exist in rodent and human skin ([1,4,10,16] and references therein). In this study, by assessing the effect of topical application of dexamethasone on mice during P450-dependent monooxygenase activities, and the expression of P450 isozymes and mRNA levels, we provide evidence that multiple inducible P450s are present in murine skin.

MATERIALS AND METHODS

Chemicals 7-ethoxyresorufin and 7-pentoxyresorufin were purchased from Pierce Chemical Co., Rockford, IL. All reagents for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Biorad Laboratories, Richmond, CA. Nitrocellulose membrane was

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Abbreviations: AHH, aryl hydrocarbon hydroxylase; βNF, β-naphthoflavone; CCT, crude coal tar; EMDM, erythromycin N-demethylase; EROD, 7-ethoxyresorufin O-deethylase; P450, cytochrome P450; pNPH, para-nitrophenol hydroxylase; PROD, 7-pentoxyresorufin O-deethylase.

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Table I. Induction of Cutaneous Monooxygenase Activities by Topical Application of Dexamethasone or Conventional Inducers of P450s

Treatments[a]	Monooxygenase Activities[b]			
	EROD[c]	PROD[c]	pNPH[d]	EMDM[e]
Control	20.6 ± 1.9	1.7 ± 0.2	0.04 ± 0.01	1.1 ± 0.1
Dexamethasone	47.1 ± 3.2 (2.3)[f]	32.6 ± 2.6 (19.2)	0.30 ± 0.03 (7.5)	2.4 ± 0.2 (2.2)
CCT	407.9 ± 36.2 (19.8)	ND[g]	ND	ND
Phenobarbital	ND	10.2 ± 0.9 (6.0)	ND	ND
Ethanol	ND	ND	0.07 ± 0.02 (1.7)	ND
CSA	ND	ND	ND	2.3 ± 0.2 (2.1)

[a] Female NMRI mice were treated topically with dexamethasone or other specific inducers as detailed in *Materials and Methods.* Twenty-four hours after the last treatment, animals were killed and skin microsomes prepared utilizing the treated area of the skin.
[b] Monooxygenase activities were determined in microsomal suspensions as detailed in *Materials and Methods.* Each assay was conducted in duplicate, and specific activity data shown are mean ± SEM of four values; two skin samples were pooled for each determination.
[c] pmol resorufin/min/mg microsomal protein.
[d] nmol 4-nitro catechol/min/mg microsomal protein.
[e] nmol formaldehyde/min/mg microsomal protein.
[f] Fold-induction compared to controls.
[g] Not done.

from Schleicher and Schuell, Keene, NH. The primary antibodies employed for Western blot analysis were either monoclonal prepared against rat liver microsomal P4501A1 (clone 1-7-1, a kind gift from Drs. H. V. Gelboin and S. S. Park, National Cancer Institute, Bethesda, MD) or rabbit polyclonal prepared against rat liver microsomal P4502B1 (002B1-P-RT), P4502E1 (002E1-P-RT), and P4503A (003A0-P-RT), and were obtained from Oxygene, Dallas, TX. Monoclonal antibody (MoAb) 1-7-1, in addition to P4501A1, also recognizes P4501A2 [21]. Secondary antibody and other reagents for immunoblotting were obtained from Kirkegaard & Perry, Gaithersburg, MD. Vectastain avidin-biofin peroxidase complex (ABC) kit for immunohistochemistry was purchased from Vector Laboratories, Inc., Burlington, CA. All the reagents employed for Northern blot analysis were from Promeaga Corp., Madison, WI. Other chemicals and reagents used were of highest purity commercially available.

Animals and Treatments Female NMRI mice (~22 g) were obtained from Tieranstalt Winkelmann, Borchen, Germany, and were treated on their backs and sides either with 0.5 ml dexamethasone emulsion (0.1% in water), 0.5 ml cyclosporin A (CSA; 0.1% in water), 0.5 ml crude coal tar (CCT), or 1 ml EtOH for two consecutive days. Following these treatments, animals were housed in individual cages to avoid any oral absorption of topically applied agents. Twenty-four hours after the last treatment, the animals were killed by cervical dislocation, and the treated areas of the skin and liver were removed and immediately placed in ice-cold 0.15 M KCl.

Preparation of Microsomal Fraction and Enzyme Assays Microsomal fractions from cutaneous tissues were prepared as decribed earlier [22]. In brief, skin samples, after removing the subcutaneous fat and muscle, were homogenized in 0.1 M phosphate buffer, pH 7.4, at 4°C using a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY) as described earlier [22]. Skin homogenates thus obtained were centrifuged at 9000 × *g* for 20 min at 4°C, followed by centrifugation of the 9000 × *g* supernatant at 100,000 × *g* for 60 min at 4°C. Liver microsomes were prepared as detailed elsewhere [23]. Skin and liver microsomal fractions were suspended in 0.1 M phosphate buffer, pH 7.4, containing 10 mM dithiothreitol, 10 mM ethylenediaminetetraacetic acid (EDTA), and 20% glycerol. A part of the microsomal suspension was frozen at −170°C under liquid nitrogen for Western blot analysis, and the remaining was used on the day of preparation for the determination of enzyme activities. Protein was estimated in all the samples by the method of Bradford [24] using bovine serum albumin (BSA) as a standard.

7-ethoxyresorufin O-deethylase (EROD) activity was determined according to slight modification of the procedure of Pohl and Fouts [25] as described earlier [23]. 7-pentoxyresorufin O-dependentylase activity (PROD) was determined according to the procedure of Lubet *et al* [26] with some modifications. In brief, identical steps were followed as in the case of EROD, except that 7-pentoxyresorufin was used as the substrate. Para-nitrophenol hydroxylase (pNPH) activity was determined by measuring the hydroxylation of para-nitrophenol to 4-nitrocatechol according to the method of Reinke and Moyer [27] as described by Koop [28]. Erythromycin N-demethylase (EMDM) activity was determined by measuring the formation of formaldehyde, generated by the demethylation of erythromycin, employing Nash reagent [29].

Western Blot Analysis The microsomal suspensions were denaturated by heating in boiling water for 10 min after the addition of 2 × SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, containing 20% glycerol, 10% 2-mercaptoethanol, 0.1% bromophenol blue, and 5% SDS), and samples were subjected to SDS-PAGE on 12.5% precasted polyacrylamide mini gels (Schleicher and Schuell), as described by Laemmli [30]. The resolved proteins were transferred onto a nitrocellulose membrane as described by Towbin *et al* [31]. After the transfer, the gels were stained to ensure equal protein loading and their subsequent uniform transfer onto the membrane. The nonspecific sites were blocked by incubating the membrane at room temperature for 4 h in 5% nonfat dairy milk powder in PBS. The membrane was then incubated at room temperature overnight with desired antibody (50 μl antibody solution diluted with 10 ml PBS). The membranes were washed vigorously with PBS containing 0.05% Tween-20 (PBST), followed by PBS, and then incubated at room temperature for 6 h with alkaline phosphatase-conjugated secondary antibody to mouse or rabbit immunoglobulin (Ig)G (goat). Visualization of P450 isozymes at membrane was achieved by color development using a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as the chromogenic substrates. The densities of immunoreactive bands were measured by a scanner (Epson GT 6000) with GEL Image software (Pharmacia, Freiburg, Germany).

Immunohistochemistry Dorsal skin of untreated or dexamethasone-treated mouse was removed, fixed in buffered 10% formalin, processed in paraffin wax, and 5-μm cryosections were cut. All sections were deparaffin-

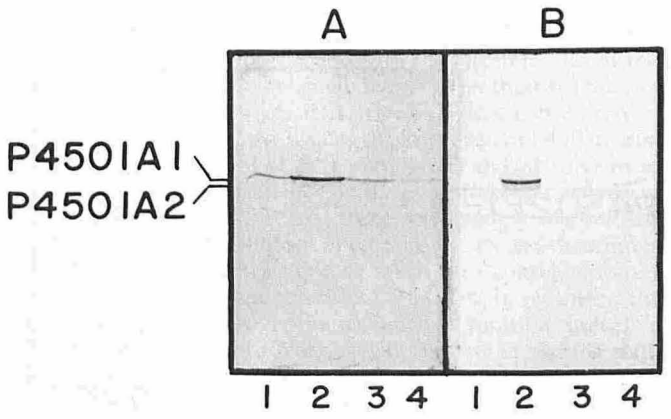

Figure 1. Induction of P4501A1/2 isozyme in skin *(A)* and liver *(B)* by dexamethasone. The treatment protocol was the same as described in *Materials and Methods.* The microsomal proteins were resolved on SDS-PAGE, transferred to membrane, and immunoblotted with monoclonal antibody 1-7-1, as described in *Materials and Methods.* In both skin and liver microsomes, 20 μg protein was loaded per *lane. Lanes 1, 2,* and *3,* microsomes prepared from, respectively, ethanol-, CCT-, and dexamethasone-treated mice; *lane 4,* untreated controls. The position of P4501A1/2 is based on molecular weight markers not shown here.

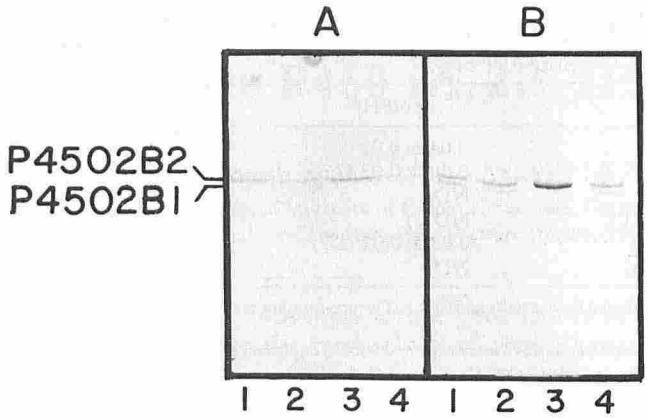

Figure 2. Induction of P4502B1/2 isozyme in skin *(A)* and liver *(B)* by dexamethasone. The treatment protocol was the same as described in *Materials and Methods.* The microsomal proteins were resolved on SDS-PAGE, transferred to membrane, and immunoblotted with polyclonal antibody directed against rat liver P4502B1, as described in *Materials and Methods.* In both skin and liver microsomes, 20 μg protein was loaded per *lane. Lanes 1, 2, and 3,* microsomes prepared from, respectively, ethanol-, CCT-, and dexamethasone-treated mice; *lane 4,* untreated controls. The position of P4502B1/2 is based on molecular weight markers.

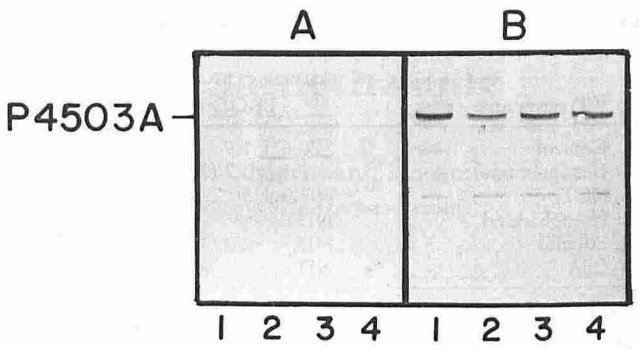

Figure 4. Induction of P4503A isozyme in skin *(A)* and liver *(B)* by dexamethasone. The treatment protocol was the same as described in *Materials and Methods.* The microsomal proteins were resolved on SDS-PAGE, transferred to membrane, and immunoblotted with polyclonal antibody directed against rat liver P4503A, as described in *Materials and Methods.* In both skin and liver microsomes, 20 μg protein was loaded per *lane. Lanes 1, 2, and 3,* microsomes prepared from, respectively, ethanol-, CCT-, and dexamethasone-treated mice; *lane 4,* untreated controls. The position of P4503A is based on molecular weight markers.

ized and rehydrated. The endogenous peroxidase activity was blocked by immersing the slides in 30% (w/v) hydrogen peroxide/methanol (1:2, v/v). The slides were then washed with phosphate-buffered saline (PBS) and incubated with Vectastain blocking solution for 30 min at 25°C in a humidity chamber. The blocking solution was replaced with the primary rabbit polyclonal antibody against rat liver microsomal P4502B1 isozyme (diluted 1:100 in PBS containing 0.1% BSA). The procedure employed for the visualization of antibody-reacting regions was the Vectastain avidin-biotin peroxidase method with diaminobenzidine as peroxidase substrate, essentially as described in the vendor's protocol.

RESULTS

Effect of Topical Application of Dexamethasone on Cutaneous Monooxygenase Activities To assess the effect of topical application of dexamethasone on cutaneous P450, first we assessed the monooxygenase activities preferentially catalyzed by known P450s [1,4,6,7]. For comparison, we also assessed the effect of topical application of known inducers of different monooxygenase activities in skin. As shown by the data in Table I, 0.1% dexamethasone treatment for 2 d resulted in highly significant inductions, though of varying degrees, of EROD (2.3 times), PROD (19.2 times), pNPH (7.5 times), and EMDM (2.2 times) activities, the monooxygenases catalyzed preferentially by P4501A1, P4502B1/2, P4502E1, and P4503A, respectively. Topical application of CCT, phenobarbital, ethanol, and CSA, the known inducer of P450 isozymes 1A1, 2B1/2, 2E1 and 3A, respectively, also showed 19.8, 6.0, 1.7, and 2.1 times induction in cutaneous EROD, PROD, pNPH, and EMDM activities (Table I). Similar effects of topical application of dexamethasone on the induction of different monooxygenase activities in the skin were also observed in SENCAR mice (data not shown).

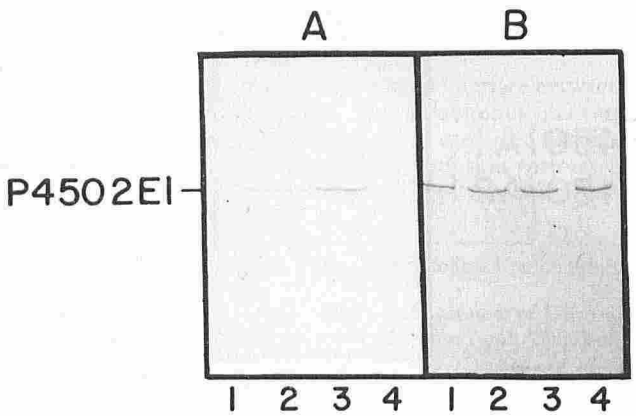

Figure 3. Induction of P4502E1 isozyme in skin *(A)* and liver *(B)* by dexamethasone. The treatment protocol was the same as described in *Materials and Methods.* The microsomal proteins were resolved on SDS-PAGE, transferred to membrane, and immunoblotted with polyclonal antibody directed against rat liver P4502E1, as described in *Materials and Methods.* In both skin and liver microsomes, 20 μg protein was loaded per *lane. Lanes 1, 2, and 3,* microsomes prepared from, respectively, ethanol-, CCT-, and dexamethasone-treated mice; *lane 4,* untreated controls. The position of P4502E1 is based on molecular weight markers.

Effect of Topical Application of Dexamethasone on Cutaneous P450 Isozymes As shown in Fig 1, compared to controls, topical application of dexamethasone resulted in significant increase (2.2 times induction over control in densitometric analysis of the bands) in the level of P4501A1 in skin (Fig 1*A*), whereas no change in P4501A1 was evident in liver (Fig 1*B*) microsomes. A far more pronounced increase in the P4501A1 level was observed by CCT treatment in the case of both skin and liver (Fig 1*A,B*), whereas ethanol treatment showed an increase in P4501A1 in skin but not liver (Fig 1*A*).

Topical application of dexamethasone also resulted in a significant increase (1.9 times induction over the control in densitometric analysis of the bands) in the level of P4502B1 in skin microsomes (Fig 2*A*). No change, however, in the level of P4502B1 was evident by the topical application of either CCT or ethanol (Fig 2*A*). When liver microsomes, prepared from the mice topically applied with dexamethasone, CCT, or ethanol, were analyzed for P4502B1/2 isozyme, a very strong band for P4502B2 isozyme was evident only in the case of the dexamethasone-treated group (Fig 2*B*), showing a highly significant increase in this P450 isozyme following dexamethasone treatment of mice. No change in either P4502B1 or P4502B2 isozymes in liver, however, was observed by skin application with CCT or ethanol (Fig 2*B*).

As evident in Fig 3*A*, compared to control, topical application of dexamethasone also showed a highly significant increase in P4502E1 isozyme in skin microsomes. Unlike P4501A1 and P4502B1 isozymes, an approximately 14 times increase in cutaneous P4502E1 isozyme (based on densitometric analysis of the bands obtained in the cases of control and dexamethasone-treated skin microsomes) was observed following treatment of mice with dexamethasone (Fig 3*A*). Topical application of ethanol, a known in-

ducer of P4502E1 ([6,7,32] and references therein), and CCT, also showed a significant increase in the expression of P4502E1 isozyme; however, the level of induction was far less than that observed with dexamethasone (Fig 3*A*). No detectable increase in the P4502E1 isozyme was evident in liver microsomes obtained from either of the treated animals (Fig 3*B*).

Western blot analysis for the detection of P4503A also showed similar patterns (Fig 4). In this case, based on densitometric analysis, dexamethasone treatment of mice showed a 1.7 times increase in P4503A isozyme in skin microsomes (Fig 4*A*). Whereas comparable results were obtained with CCT and ethanol in the case of skin microsomes, no detectable change in P4503A isozyme was evident with liver microsomes prepared from either of the treated group of mice, compared to controls (Fig 4*A,B*). It is important to mention here that whereas for EROD and EMDM activities the Western blot analysis data for P4501A1 and P4503A isozymes were corroborative (Figs 1,2), there was a big difference in the catalytic activity data and P450 isozyme induction for PROD and pNPH activities (Table I) and P4502B1/2 and P4502E1 isozymes, respectively (Figs 3,4).

Immunohistochemical Localization of P4502B1/2 in the Skin of Control and Dexamethasone-Treated Mice Because following dexamethasone treatment, the maximum increase (19.2 times) occurred in PROD activity, and because PROD is preferentially catalyzed by P4502B isozymes, for immunohistochemical studies we selected this P450 isozyme. As shown in Fig 5, in the case of control skin (Fig 5*A*), antibody raised against P4502B1/2 isozyme showed reactivity in the suprabasal layer of the epidermis and with the cells of the hair follicle. Similar to catalytic activity and Western blot analysis data, topical application of dexamethasone resulted in a significant increase in the immunoreactivity towards P4502B1/2 isozyme in these compartments of the skin (Fig 5*B*).

DISCUSSION

Skin is the primary body site in direct contact with the environment, and, therefore, is a route through which many chemicals gain entry into the body. The introduction of chemicals into the body *via* skin occurs through both the passive contact of the skin with the environment and by direct contact of chemicals on the body, *viz* drug therapy of skin diseases and cosmetics [33]. In this context, P450-dependent metabolism, in skin, of xenobiotics such as drugs, carcinogens, solvents, and other toxicants present in the environment is an important biotransformation pathway for these agents to be converted to their water-soluble metabolites, which could then be excreted from the body as such or following conjugation pathways ([1,4,12] and references therein). Ironically, the P450-mediated process also generates chemically reactive metabolites of xenobiotics, which could bind covalently to cellular macromolecules, more precisely to DNA, leading to cell toxicity, mutagenicity, and often cancer induction [1,4,12].

As reviewed recently by Mukhtar and Agarwal [4], P450 is also known to bioactivate different substances in the skin. Topical or systemic treatment of rodents with numerous xenobiotics has been shown to result in the induction of monooxygenase activities in skin [1,4]. Several studies have also shown that such an increase in enzyme activity is associated with the induction of specific P450 isozymes in skin, which in turn is also specific to the type of xenobiotic exposure of skin [1,4]. Earlier we showed that topical application of 3-methylcholanthrene to rat results in a significant increase in epidermal P4501A1, with a concurrent increase in monooxygenase activities [10]. Similar results were also observed in both epidermis and liver following exposure of rat skin to CCT [34]. The induction of P4501A1 in human skin and in keratinocytes is also reported following their exposure to different xenobiotics ([1,4] and references therein). Taken together, these studies suggested that, in skin, exposure to a xenobiotic results in the induction of P4501A1. More recently, while assessing the metabolic capacity of reconstituted epidermis from the outer root sheath cells of human hair follicle, a suggestion was made for the presence of P4502B1 in reconstituted epidermis [35]. In the present study, we extend these observations by demonstrating that dexamethasone topically applied to mice results in the induction of P4501A1, P4502B1, P4502E1, and P4503A in skin.

It is known that systemic administration of dexamethasone induces multiple P450 isozymes in liver; several studies have shown significant increases in P4502B and P4503A isozymes and their dependent monooxygenase activities, as well as mRNA, in liver following treatment with dexamethasone ([19,20] and references therein). Our data indicating that the application of dexamethasone to murine skin results in the induction of several subfamilies of P450 isozymes in skin also support previous findings, and suggest that murine skin contains multiple inducible P450 isozymes capable of participating in the metabolism of drugs, including glucocorticoids. With Northern blot analysis, we also assessed whether the increase in P450 isozymes in skin by topical application of dexamethasone also results in an increase in the amounts of mRNA for these forms of P450s. Based on the known sequences for mouse cDNAs [36,37], the mRNA levels for CYP1A1 and CYP2E1 were determined in the skin of control and dexamethasone-treated NMRI mice. For comparison, we also used total RNA isolated from the skin of CCT- or ethanol-treated mice. Whereas mRNA coding for CYP1A1 and CYP2E1 were detectable in both control and dexamethasone-treated skin, no increase in the levels of mRNA for these two P450 genes was observed by the topical application of dexamethasone (data not shown). However, compared to controls, a 1.7 and 1.8 times increase in mRNA for CYP1A1 and CYP2E1 by topical application of CCT and ethanol, respectively, was evident in these experiments (data not shown). With our results of enhanced enzyme activities and protein expression but unchanged mRNA expression after dexamethasone treatment, it seems possible that the effect of dexamethasone observed in the present study is due to protein stabilization. At least in liver, it has been shown that dexamethasone acts mainly *via* protein stabilization [19,20]. Our data showing a 14 times increase in P4502E1 isozyme compared to a sevenfold increase in pNPH activity by dexamethasone were also in accord with published findings showing P4502E1 induction in extracutaneous tissues by protein stabilization [32,38]. Besides, Northern blot analysis shows the constitutive expression of CYP1A1 and CYP2E1 in murine skin.

Although the levels of P450s and their dependent monooxygenase activities are low in skin compared to those in liver [1,4], it is important to emphasize here that only a very small amount of topically applied drug is usually absorbed through the skin barrier stratum corneum, which is not the case with the systemic administration of drugs and their metabolism in liver. Under these conditions, the corticosteroids, which are active in a very low concentration, may, therefore, be influenced even by the low levels of P450 in skin. Moreover, the metabolism of different carriers and/or solvents in skin might have further influence on the pharmacologic activity of topically applied drugs. Our data, therefore, strongly suggest that the P450-dependent metabolism of corticosteroids and their interaction with P450 isozymes must be taken into consideration to explain their pharmacologic activities in the skin. In summary, the results of this study clearly demonstrate that multiple inducible P450 isozymes are constitutively present at least in murine skin, and that they may play an important role in the metabolism of topically applied drugs. These findings may help to develop new strategies for the topical application of such drugs in dermatopharmacology.

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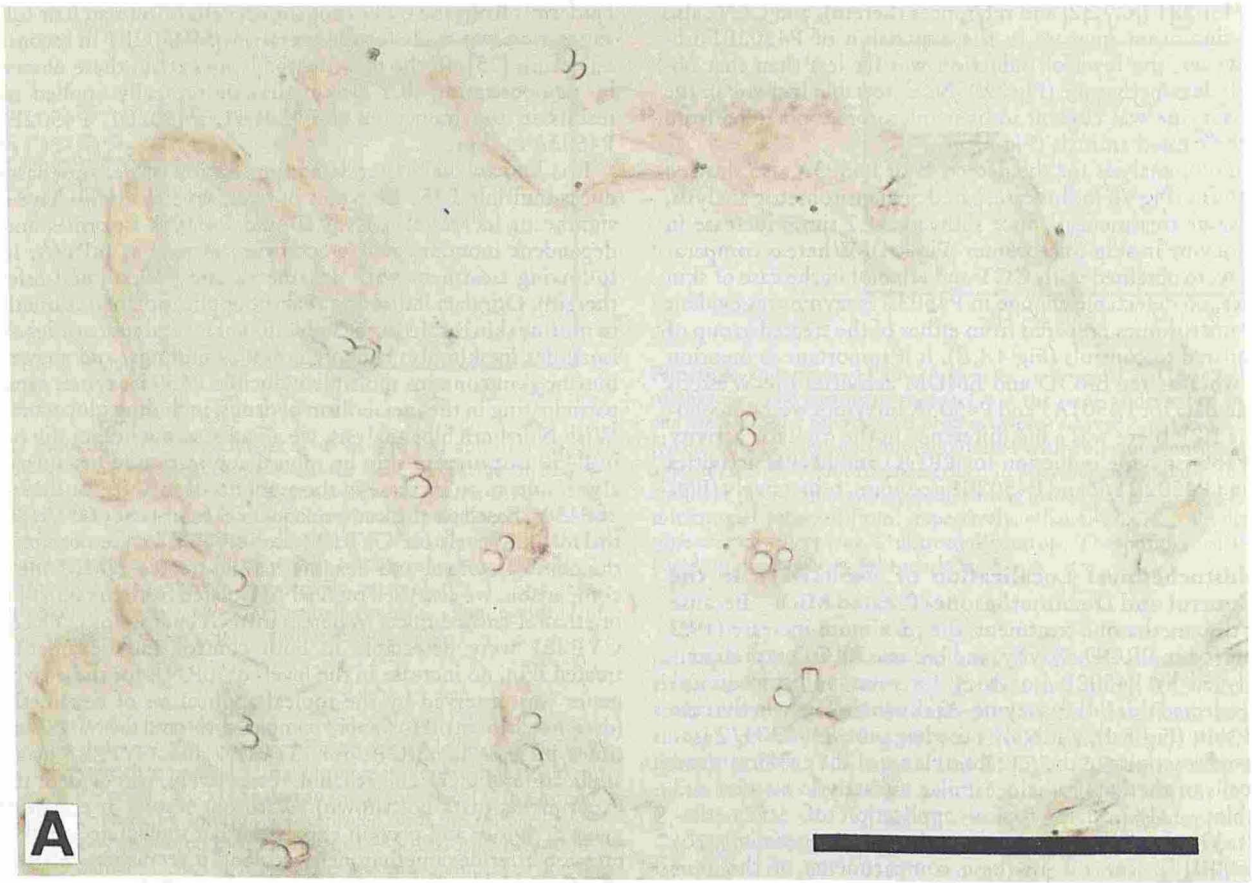
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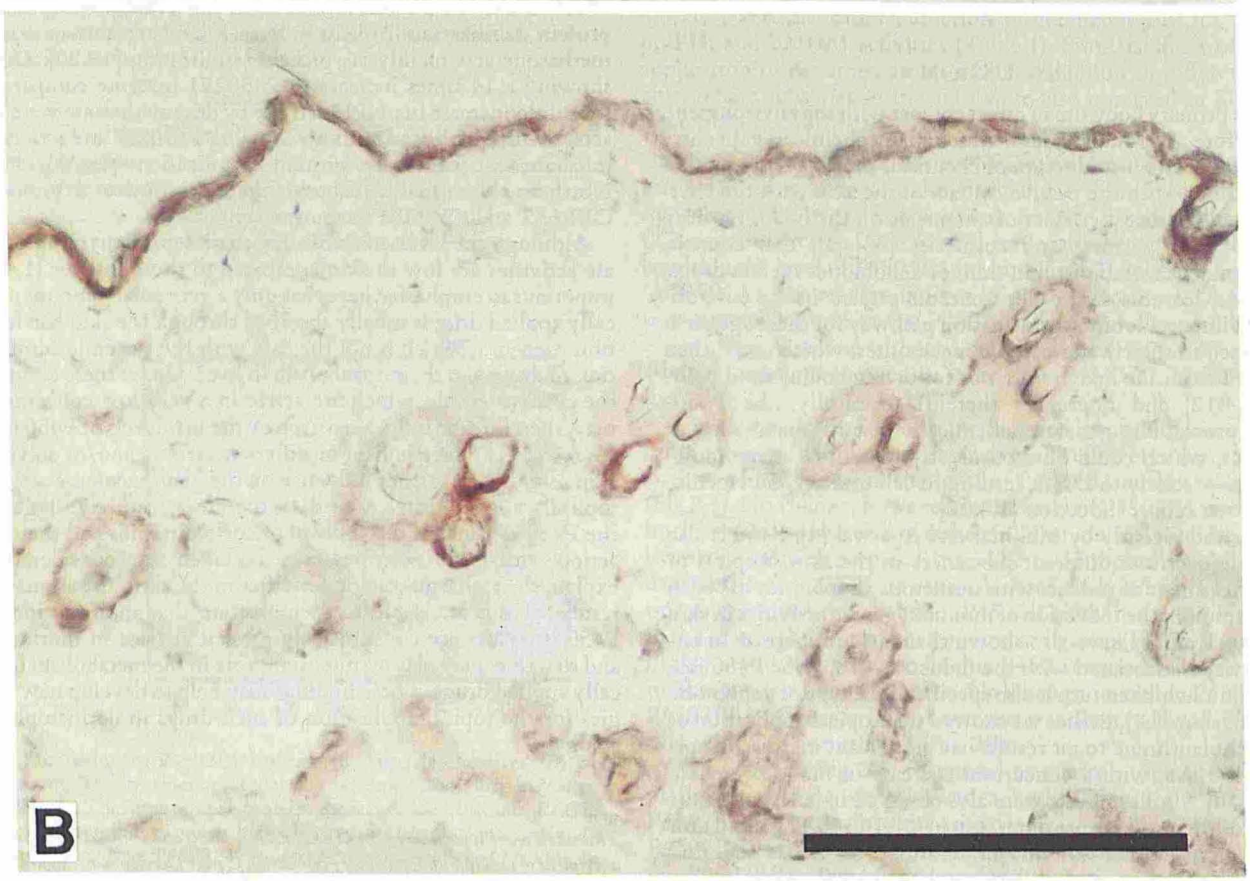
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Figure 5. *In situ* immunohistochemical localization of P4502B1 in control *(A)* and dexamethasone-treated *(B)* NMRI mouse skin. Paraffin-embedded sections were deparaffinized, incubated with 1 : 100 dilution of antibody to P4502B1, and the color was visualized employing Vectastain ABC kit, as described in *Materials and Methods.*

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