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Purification and Characterization of NADPH-Cytochrome P-450 Reductase From Rat Epidermis

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Abstract NADPH-cytochrome P-450 oxidoreductase (P-450 red) transfers reducing equivalents from NADPH to cytochrome P-450 (P-450) in the monooxygenase system. Detergent solubilized proteins from the membrane fraction of neonatal rat epidermis were purified by 2',5'-ADP-agarose affinity column chromatography. The purified protein showed an apparent homogeneity on sodium dodecylsulfate-polyacrylamide gel electrophoresis and molecular weight was estimated to be 78 kDa. NADPH-cytochrome c reductase activity increased by 95-fold in the purified enzyme. Epidermal P-450 red in vitro reconstituted benzo(a)pyrene hydroxylase activity in a dose dependent manner with P-450 purified from either rat liver or epidermis. Western blot analysis demonstrated that epidermal P-450 red immunologically cross reacts to liver P-450 red. Immunohistochemical staining showed that the enzyme was predominantly localized in the epidermis. The intensity of immunohistochemical staining of rat skin sections and tissue distribution did not change in the skin treated with β-naphtoflavone, which results in a substantial increase in P-450 1A1 activity. Quantitative assessment of P-450 red in treated and untreated epidermis also showed no change. These findings indicate that constitutive P-450 red, fully capable of supporting P-450, exists in rat epidermis, and can function in metabolism of endogenous and exogenous compounds.

Key words: NADPH-cytochrome P-450 oxidoreductase, rat epidermis, reconstitution with P-450 1A1, immunohistochemistry

The microsomal monooxygenases, also known as mixed function oxidase system, are involved in the metabolism of endogenous compounds as well as activation and detoxication of hydrophobic compounds of external sources, such as therapeutic drugs, xenobiotic compounds, environmental pollutants, and chemical carcinogens [Nelson et al., 1993]. The system works in the local microenvironment of tissues through cooperative action of cytochrome P-450 (P-450) and NADPH-cytochrome P-450 oxidoreductase (P-450 red). The essential role of P-450 red is to transfer reducing equivalents from NADPH to P-450. P-450 belongs to a multigenetically coded superfamily of enzymes and according to current update [Nelson et al., 1993], 221 P-450 genes and 12 putative pseudogenes have been identified. The enzymatic components of the monooxygenase system in the liver have been extensibly investigated with respect to the catalysis and metabolism of intake drugs and endogenous compounds [Omura and Sato, 1964; Singh and Piette, 1992; Wrighton and Stevens, 1992]. However, these enzymes remain to be further studied in extrahepatic tissues.

Epidermis, covering the outer surface of the body also has been demonstrated to play a role in the defense against exogenous substances through the P-450 dependent monooxygenase system. The distribution and inducibility of

Abbreviations used: BSA, bovine serum albumin; β-NF: β-naphtoflavone; CHAPS, 3-[(3-cholamindopropyl)dimethylammonio]-1-propanesulfonate; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; P-450, cytochrome P-450; P-450 red, NADPH-cytochrome P-450 oxidoreductase; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis.

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P-450 and some monooxygenases were reported in skin [Pohl et al., 1976; Bickers et al., 1982; Mukhtar et al., 1989] and cultured keratinocytes [Jin et al., 1986; Guo et al., 1990]. While extremely low levels of P-450 and some monooxygenase activities (less than 1% of liver) are present in the epidermis of untreated, they are induced from moderate to highly significant levels by skin application of various chemicals in experimental animals and humans [Raza et al., 1992]. On the other hand epidermal P-450 red, though not appear to be rate limiting, has not been characterized. In this paper, we report purification of P-450 red to apparent homogeneity from rat epidermis and demonstrate its catalytic activity in an in vitro reconstitution system with purified epidermal and hepatic P-450. In addition, epidermal localization and changes in the tissue contents are immunologically compared in P-450 stimulated and unstimulated skin.

MATERIALS AND METHODS

Materials

NADPH, 3-[(3-cholamindopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 2′,5′-ADP-agarose, benzo(a)pyrene, cytochrome c and dilaurylphosphatidylcholine, were purchased from Sigma Chemical Co., (St. Louis, MO). β-naphtoflavone (β-NF) was obtained from Aldrich Chemical Co (Milwaukee, WI). Rabbit anti-rat liver NADPH-cytochrome P-450 reductase serum IgG was a product of Oxygene Dallas (Dallas, TX). Sprague Dawley rats were bred at UCSF Animal Care Facility and in these studies neonatal rats of mixed sex were used when they were 2–3 days old.

Purification of NADPH-Cytochrome P-450 Reductase

Skin removed from neonatal rats was attached to surgical tape and soaked in 0.24 M NH_4Cl, pH 9.0, for 10 min. The epidermis was pealed from the dermis and epidermal cells were scraped off from the tape. The cells (47 g, wet weight) were homogenized in 500 ml of buffer A (50 mM Tris-HCl, pH 7.4, containing 0.43 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 1 μM pepstatin A). The homogenate thus obtained was centrifuged at 105,000 × *g* for 60 min. The pellet was rehomogenized in 500 ml of buffer A containing 1 M KCl and centrifuged at 105,000 *g* for 60 min. The KCl-washed particulate fraction was solubilized with 20 mM CHAPS in buffer A (250 ml) for overnight at 4°C with gentle rotation of the sample. After centrifugation at 105,000 *g* for 60 min, the solubilized proteins were loaded onto a 2.8-ml column of preswolen 2′,5′-ADP-agarose and recirculated three times. The column was washed with buffer A containing 20 mM CHAPS and 0.5 M NaCl and then with buffer A containing 20 mM CHAPS. Finally, the adsorped proteins were eluted with buffer A containing 20 mM CHAPS and 10 mM NADPH and regarded purified enzyme. For comparison, the CHAPS extract also was prepared from adult rat liver by the same extraction method above described. The concentration of proteins was determined by the method of Read and Northcote [1981] using bovine serum albumin (BSA) as a standard. Purity and molecular weight of proteins in different fractions were estimated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 9% acrylamide slab gels by the method of Laemmli [1970]. Rabbit muscle phosphorelase a (92.5 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), bovine erythrocyte carbonic anhydrase (30.0 kDa) and soybean trypsin inhibitor (20.1 kDa) served as molecular markers.

Enzyme Assays

NADPH-cytochrome c reductase activity was measured as described by Williams and Kamin [1962]. The reconstitution of purified epidermal P-450 red for benzo(a)pyrene hydroxylation by purified epidermal and hepatic P-450 1A1 in the presence of dilaurylphosphatidyl choline was conducted essentially as described by Raza et al. [1992].

β-NF Induction of P-450

As previously reported by Raza et al. [1992] neonatal rats were withdrawn from their mothers and treated with a single topical application of 100 μl of 0.04% β-NF in acetone. The animals were killed 24 hr later and epidermal proteins were solubilized in CHAPS.

Western and Dot-Blot Analysis

For Western blot analysis, proteins from rat hepatic microsomal extract as well as purified epidermal P-450 red separated by SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane by the method of Towbin et al. [1979]. For dot immunoblot analysis, proteins were directly blotted on a nitrocellulose mem-

TABLE I. Purification of NADPH-Cytochrome P-450 Reductase From Rat Epidermis

Purification step	Total protein (mg)	Total activity[a] (nmol/min)	Specific activity (nmol/min/mg)	Yield (%)	Purification (fold)
Crude CHAPS extract	156.2	13,110	83.92	100	1
Purified enzyme	0.672	5,333	7,937	40.7	94.6

[a]NADPH-cytochrome c reductase activity was measured as the rate of reduction of cytochrome c at 550 nm using an extinction coefficient of 21 mM^{-1} cm^{-1} [Williams and Kamin, 1962] at 30°C in 1.0-ml reaction mixtures containing 0.3 M potassium phosphate buffer (pH 7.5).

brane using Bio-Dot Microfiltration Apparatus (Bio-Rad). The membranes were immersed in phosphate buffered saline (PBS) containing 2% BSA and 0.05% Tween 20 to block nonspecific binding sites. They were reacted with rabbit polyclonal anti-rat NADPH-cytochrome P-450 reductase IgG (1:1,000 dilution) for 1 hr at 22°C. Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG was used as the secondary antibody and immunoreactive proteins were visualized by Konica immunostain HRP kits (Konika, Tokyo).

Immunohistochemistry

Small skin pieces (about 3 ×3 × 3 mm) excised from the animals were immersed in Tissue-Tek compound (Miles, Elkhart, IN) and frozen in a mixture of acetone-carbon dioxide. They were sectioned at 4 μm thick in a cryostat, mounted on glass slides and air dried. Endogenous peroxidase activity was blocked in a methanol/3% H_2O_2 (9/1, v/v) solution, and nonspecific antibody binding was prevented with 10% normal goat serum in PBS. The sections were sequentially incubated with rabbit anti-rat NADPH-cytochrom P-450 reductase IgG (1:100 dilution) at 4°C, for overnight, biotin-labeled goat anti-rabbit IgG (1:200 dilution, Vector, Burlingame, CA) at 20°C, for 45 min and avidin-biotin-peroxidase complex (Vestain-ABC Kit, Vector, Burlingame, CA) at 20°C for 30 min. PBS was used to wash the sections after each step. The immunoreactant was stained in a saturated 3′,3′-diaminobenzidine (Sigma, St. Louis, MO) solution containing 0.005% H_2O_2. The sections were dehydrated, mounted in Permount and photographed under a Zeiss photomicroscope. Control sections were treated similarly using preimmune rabbit IgG as the primary antibody and unrelated biotin-labeled antibody as the secondary antibody.

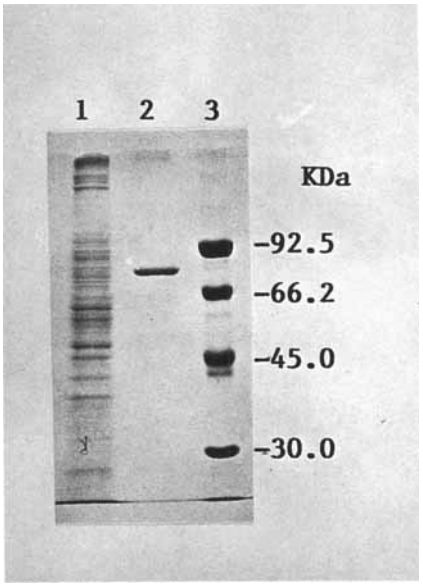

Fig. 1. Electrophoretic profiles of epidermal P-450 red before and after purification. The bands portray: **lane 1,** CHAPS extract (6.3 μg of protein); **lane 2,** purified P-450 red (1.1 μg of protein); **lane 3,** molecular weight markers.

RESULTS

Purification of P-450 Red

Cytochrome c reductase activity detected in crude CHAPS extract prepared from rat epidermis was 95-fold enriched in the eluate from 2′,5′-ADP agarose affinity column (Table I). SDS-PAGE analysis of the crude fraction showed numerous proteins of different molecular weights while the only single protein band of 78 KDa was visualized after purification (Fig. 1).

Reconstitution of Monooxygenase Activity

Catalytic activity for benzo(a)pyrene hydroxylation of purified epidermal and hepatic P-450 1A1 in the presence of different amounts of purified epidermal P-450 red is summarized in Table II. In the absence of P-450 red, insignifi-

TABLE II. In Vitro Reconstitution of Benzo(a)pyrene Hydroxylation by Purified Epidermal and Hepatic P-450 1A1 With Purified Epidermal NADPH-Cytochrome P-450 Reductase

	Benzo(a)pyrene hydroxylase activity (nmol benzo(a)pyrene metabolized/min/nmol P-450)[a]	
	Epidermal P-450 1A1	Hepatic P-450 1A1
Without P-450 red	0.6	0.9
With P-450 red		
5 pmol	3.2	4.1
20 pmol	5.9	8.2
30 pmol	8.4	12.9
50 pmol	8.9	12.8

[a]The reaction was carried out in final volume of 0.5 ml using 20 pmol of purified P-450 1A1 from both rat epidermis and liver [Raza et al., 1992] and 5, 20, 30, or 50 pmol of epidermal P-450 red in the presence of 10 μg dilaurylphosphatidylcholine. Data of a typical experiment repeated twice with similar results are shown.

cant hydroxylation of benzo(a)pyrene occurred, whereas the metabolic activity increased in a dose-dependent manner with the addition of 5 pmol to 30 pmol of epidermal P-450 red. However, increase of the reductase concentration from 30 to 50 pmol did not result in any change in hydroxylation of benzo(a)pyrene.

Immunological Detection of Epidermal P-450 Red

Cross reaction of antibody to hepatic P-450 red between hepatic enzyme and epidermal enzyme was demonstrated by Western blot analysis (Fig. 2). Both enzymes showed a single band of 78 kDa. The antibody was used to detect tissue localization of P-450 red in neonatal rat skin. The strong positive immunoreactivity was seen throughout the epidermis, but basal cells appeared to be least reactive and the strongest reaction was seen over the cornified cells. (Fig. 3A). The staining was diffuse in the cytoplasm. Dermal elements including vascular components and hair follicles were not stained. We next examined the effect in the skin application of epidermal P-450 1A1 inducer, β-NF on P-450 red activity. Immunoreactivity of skin sections from β-NF treated rats was indistinguishable from the untreated skin (Fig. 3B). In order to quantify the antigenic protein concentration in

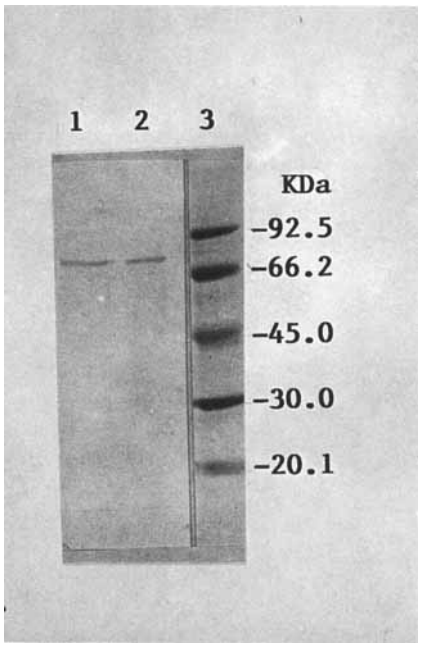

Fig. 2. Western blot analysis of purified epidermal P-450 red and hepatic extract using polyclonal antibody to rat hepatic P-450 red. Purified epidermal P-450 red (0.11 μg of protein in **lane 1**) and hepatic microsomal extract (10 μg in **lane 2**) were applied to adjacent lanes of SDS-PAGE. The resolved protein bands were transferred to nitrocellulose membrane and immunologically stained; **lane 3,** molecular weight markers.

the treated and untreated epidermis, CHAPS extracts prepared were blotted on nitrocellulose membrane at different dilutions. As shown in Figure 4, the staining density of the comparable dilutions from the two epidermal sources was identical to each other indicating that P-450 red is not induced in the epidermis by β-NF treatment.

DISCUSSION

A functionally active P-450 red was purified from CHAPS extract of rat epidermis by 2′,5′-ADP-agarose affinity chromatography. The methodology was remarkably effective on separation of an apparent single protein band of P-450 red from other epidermal proteins detected as numerous bands by SDS-PAGE without additional chromatography steps such as ion-exchange column, [Dignam and Strobel, 1977] and other columns [Yasukochi and Masters, 1976; Guengerich and Martin, 1980] being required. Molecular weight of the purified P-450 red is 78 kDa and identical to P-450 red purified from rat liver [Iyanagi and Mason, 1973], pulmonary [Guengerich, 1977], colon [Oshinsky and Strobel, 1987], and brain [Bergh and Strobel, 1992]

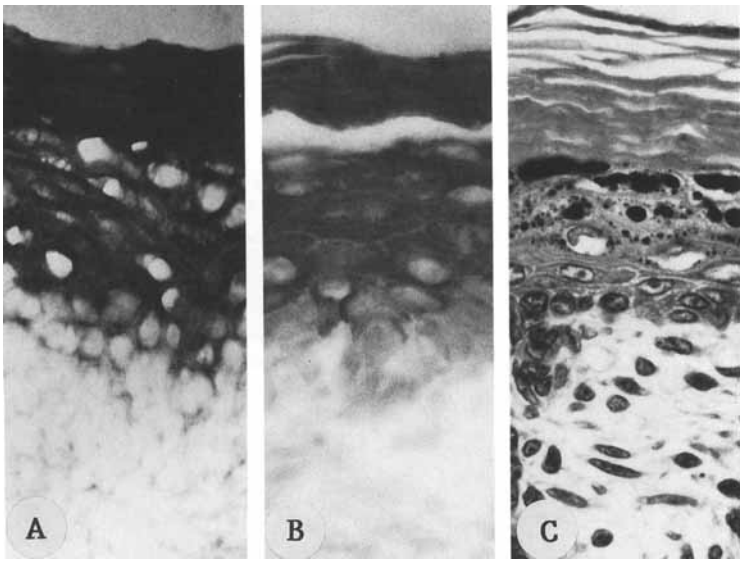

Fig. 3. Skin sections stained with rabbit anti-P-450 red. **A:** Untreated rat. **B:** Rat treated with β-NF for 24 hr. **C:** Rat skin section stained with haematoxylin and eosin for morphological identification.

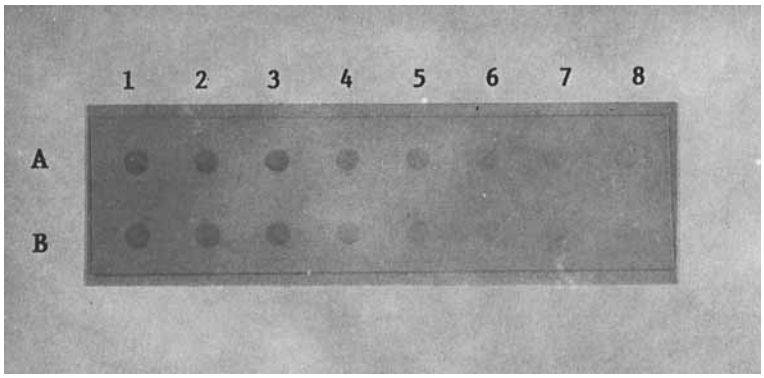

Fig. 4. Dot-immunoblot analysis of epidermal P-450 red extracted from untreated (**A**) and β-NF treated (**B**) rat. CHAPS extracts were serially 2-fold diluted and applied to nitrocellulose membrane. The amounts applied, ranging from 1.0 μg to 0.008 μg marked as 1–8, are shown on the top. The membrane was subjected to the detection procedures, as described in the text.

supporting the conclusion drawn for animal P-450 red that no isozymes exist in the specific animal source and there is no tissue-specific regulation by the enzyme [Porter et al., 1990; Shephard et al., 1992]. The purified epidermal P-450 red showed immunocross reactivity to rat hepatic reductase.

Rate of the epidermal protein recovery from the CHAPS fraction after the affinity chromatography was compatible with the value reported by Dignam and Strobel [1977] and Guengerich and Martin [1980]. However, specific activity of epidermal P-450 red calculated for catalysis of cytochrome c was considerably lower (about 8 μmol/min/mg protein) than that of hepatic enzymes (ranging 37.4 to 69.4 μmol/min/mg), although comparable with the activity of purified cerebral P-450 red [Bergh and Strobel, 1992]. In order to offer a logical explanation for the low specific activity of cerebral P-450 red, Bergh and Strobel [1992] tested the possibility of loss of FMN and/or FAD moiety in the cerebral enzyme. No conclusive explanation has been found from their experiments. The purified epidermal enzyme was able to reconstitute benzo(a)pyrene hydroxylase activity with P-450 1A1 purified from rat epidermis and liver, demonstrating that during the purification steps employed FMN

and/or FAD moeity is not lost and that the P-450 red is fully capable of stimulating P-450 monooxygenase activity at the skin surface.

Immunohistochemistry using monospecific antibody demonstrated that P-450 red localizes primarily in the epidermis of rat skin. Moreover, the immune reaction was almost spared over the basal cells, a layer of the most proximal epidermal cells which undergo proliferation. The intense reaction was seen in the cornified cells which cover the body surface indicating that epidermis contains constitutive P-450 red, even though the level of P-450 in unstimulated rodent epidermis is extremely low [Bickers et al., 1982; Khan et al., 1989]. An immunohistochemical study by Adams et al. [1991] to correlate the subregional distribution of P-450 red in the nasal cavity, also demonstrated that the epithelial cells, excepting basal cells, showed strong immunoreactivity. The enzyme localization in the nasal cavity was considered to correspond to the regions which were sensitive to inhaled noxious agents. Functions of the monooxygenase system in the nasal mucosa as well as in the airways [Serabjit-Singh et al., 1988; Ovebry et al., 1992] are expected to be essential for biotransforming extrinsic particles that impinge on it from inhaled air currents. The similar corporal situation exists in the epidermis, it being the largest entry of xenobiotics. At the same time, P-450 and P-450 red in the epidermis participates in the metabolism of endogenous steroids and other substances [Mukhtar et al., 1989]. The finding of P-450 red in the epidermis seems to suggest that a part of monooxygenase is readily functional always, and induction of only P-450 is necessary to facilitate the complete function. P-450 isozymes are stimulated in the neonatal rat epidermis by a variety of topically applied chemicals. [Kahn et al., 1989; Guo et al., 1990; Raza et al., 1992], and β-NF induces in the epidermis an enzyme which shows homology with the known sequence of hepatic P-450 1A1 [Raza et al., 1992]. Distribution of P-450 red in β-NF treated rat skin was unchanged from that in untreated animal, confirming that pre-existing P-450 red is capable of transfering electrons to P-450 1A1-like enzyme in the epidermis. Quantitative assessment of P-450 red by immunoblot analysis coincided with the histological findings, and there were no changes in the P-450 red content in the epidermis of β-NF treated rats. However, the present study does not totally rule out the possibility that epidermal P-450 red may be increased under some situations. Gonzales and Kasper [1980] reported that NADPH-cytochrome c oxidoreductase mRNA increased in rat liver after phenobarbital administration. Shephard et al. [1982, 1983] also observed that phenobarbital increased P-450 red and its mRNA, but other chemicals, such as β-NF, did not induce P-450 red. Evidence has been accumulated to consider that P-450 mixed function oxidase system plays important biological and toxicological functions in the skin. The present findings of P-450 red further support this view. The roles played by the system at the body surface, are expected to be beyond the functions in liver and brain, owing to the skin's exposure to various pharmaceutical and environmental agents.

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