The induction of ornithine decarboxylase (ODC) activity may be an essential component of skin tumor promotion. ODC requires pyridoxal 5'-phosphate (PLP) as a cofactor. We have measured the epidermal PLP concentration and investigated its relationship to DNA synthesis and ODC activity in the hairless mouse.

The epidermal PLP concentration was approximately 1.0 μg/g. When tape-stripping was used to induce ODC activity in the epidermis the concentration of PLP was significantly elevated 4.5 h later at the time of peak ODC activity and when DNA synthesis was reduced.

Systemic treatment with the vitamin B-6 antagonist 4'-deoxypyridoxine (4-DOP) significantly reduced the epidermal PLP concentration and DNA synthesis. The ODC activity induced in the epidermis 4.5 h after tape-stripping in 4-DOP-treated mice was only 17% of that induced in untreated tape-stripped controls. In in vitro experiments it was shown that while 4-DOP does not inhibit ODC activity, a major metabolite of 4-DOP, 4-DOP-phosphate (K, 0.6 mm), does. In mixing experiments it was shown that the epidermal extracts from 4-DOP-treated mice did not contain significant amounts of ODC inhibitors. 4-DOP may inhibit ODC induction in the epidermis by depleting the PLP content.

The pyridoxine (vitamin B-6) derivative, pyridoxal 5'-phosphate (PLP), is a cofactor for several mammalian decarboxylases including the polyamine synthesis enzyme ornithine decarboxylase (ODC). ODC activity is induced in mouse skin by tumor promoters [1] and polyamine levels are elevated in proliferating tissues [2]. Current evidence indicates that the induction of ODC activity, while not necessarily specific for tumor promotion, may be an essential component of the process [3-5]. If the induction of ODC activity is essential for carcinogenesis, then inhibitors of ODC should be good candidates as antitumor agents. In this respect the irreversible, highly specific (suicide) inhibitor of ODC, alpha-difluoromethyl-ornithine, has recently been shown to inhibit tumor promotion in mouse skin [5,6].

Hepatocarcinogenesis is reduced [7,8] and the growth of hepatomas severely impaired [9] in pyridoxine deficiency. The pyridoxine antagonist 4'-deoxypyridoxine (4-DOP) inhibits tumor growth [10] and has been used in the treatment of adult lymphatic leukemia [11]. Tissue putrescine and spermidine levels are reduced in pyridoxine deficiency [12], and the PLP concentration may therefore regulate putrescine synthesis. Murray and Froscio [13] found that reduced levels of ODC activity were induced in the epidermis of pyridoxine-deficient compared to pyridoxine-replete mice after treatment with the tumor promoter tetradecanoylphorbol-13-acetate. Thus epidermal ODC activity is influenced in vivo by the pyridoxine status and this may explain in part the anticancer and antiproliferative properties of pyridoxine deficiency.

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We have been investigating the possible use of 4-DOP for regulating the supply of PLP to the epidermis to control ODC activity more directly, and to establish a new route for inhibiting
putrescine synthesis and epidermal proliferation. In the present study we have measured the PLP concentration of hairless mouse epidermis and have investigated its relationship to epidermal DNA synthesis and ODC activity. We report the ability of the pyridoxine antagonist 4-DOP to deplete epidermal PLP levels, to inhibit DNA synthesis, and to inhibit the expression of ODC activity.

MATERIALS AND METHODS

Materials

L-[1-14C]Ornithine hydrochloride (specific radioactivity 57 Ci/mol), [methyl-3H]thymidine (specific radioactivity 2 Ci/mmol), and NCS were bought from Amersham (Arlington Hts, Illinois). L-Ornithine, PLP, 4'-deoxypyridoxine, tartaric acid, dithiothreitol, bovine gamma globulin, and calf thymus DNA were bought from Sigma (St. Louis, Missouri). 4'-Deoxypyridoxine-phosphate (4'-DOP-phosphate) was bought from Calbiochem (La Jolla, California). All experiments were performed on 8 to 10-week-old skh/HR1 hairless female mice originating from the Skin Cancer Hospital (Temple University, Philadelphia, Pennsylvania).

Preparation of Mouse Epidermis

Mice were killed by cervical dislocation and the back skins removed. To separate off the epidermis each skin was immersed in a water bath at 55°C for 30 s, cooled in ice/water, blotted dry with filter paper, placed flat on a cooled glass plate, and the epidermis (0.08-0.15 g) scraped off the dermis with a scalpel blade. For ODC assay, each epidermis was homogenized in ice-cold 50 mM sodium phosphate buffer pH 7.2 (1 ml containing 0.4 mM PLP and 5 mM dithiothreitol, with a Brinkman homogenizer, at setting 6, for 15 s. The homogenates were centrifuged at 30,000 g for 30 min and the supernatants recovered and stored at -70°C until used. The stored extracts retained their activity for several months under these conditions.

To measure DNA synthesis mice were injected 1 h prior to sacrifice with 30 µCi [3H]thymidine, the mice killed and the epidermis recovered as above, and the DNA extracted and assayed after the method of Baird et al [14]. Each epidermis was homogenized in 1 ml ice-cold 0.4 M perchloric acid with a Brinkman homogenizer, at setting 6, for 30 s. After standing the homogenates on ice for 15 min, they were centrifuged at 1,500 rpm, the supernatant removed for PLP assay, and the pellets recovered and washed free of unbound [3H]thymidine by resuspending in a further 1 ml 0.4 M perchloric acid and recentrifuging, and resuspending and recentrifuging twice in absolute ethanol. The pellets were then suspended in 2 ml 0.4 M perchloric acid and the DNA hydrolyzed by heating at 90°C for 20 min in a block heater.

Tape-Stripping

Mice were injected i.p. with chloral hydrate (300 mg/kg body weight) 15 min before tape-stripping. Since ODC induction by tape-stripping is dose-dependent (unpublished observations), each mouse was stripped 10 times with Scotch Magic Tape. During tape-stripping, strips of tape about 10 cm long were placed along the back of the mice, pressed down firmly to make contact with the skin, and massaged on the skin for about 10 s before being removed by a single pull. Each piece of tape was used once only.

ODC Assay

ODC activity in epidermal samples was determined by measuring the release of 14CO2 from L-[1-14C]ornithine. Incubations were performed in 0.05 M sodium phosphate buffer pH 7.2 containing 5 mM dithiothreitol, 0.4 mM PLP, 1 mM L-ornithine, 0.5 µCi L-[1-14C]ornithine, and 0 to 0.2-ml sample in a final volume of 0.3 ml. The reaction mixtures were incubated at 37°C for 1 h in sealed tubes fitted with side arms [15]. The released 14CO2 was collected directly into scintillation vials containing 0.15 ml of NCS, attached to the side arms by holed rubber stoppers. The reactions were terminated by injecting 2 µl 1 M citric acid (0.5 ml) through a sleeve stopper in the mouth of each tube. Total protein in the epidermal extracts was determined with the BioRad protein assay (BioRad Laboratories, Richmond, California) using bovine gamma globulin as the standard. ODC activities are expressed as nmol CO2 released per mg soluble protein per hour.

DNA Synthesis

Total epidermal DNA was determined on aliquots of hydrolyzed samples by the diphenylamine reaction using calf thymus DNA as the standard. The specific radioactivity of the DNA was determined by counting 0.2-ml aliquots of each hydrolyzed sample in a scintillation counter. DNA synthesis is expressed as cpm/µg DNA.

Assay of PLP Levels

Epidermal PLP was assayed fluorometrically, after quantitative reaction with cyanide, by modification of existing laboratory methods [16,17]. The fluorescence assay was employed rather than the enzymic methods because of the possible presence of PLP antagonists in the extracts.

PLP determinations were performed directly on the supernatant obtained when epidermis was homogenized in 0.4 M perchloric acid and centrifuged at 1,500 g as for the DNA synthesis studies. Aliquots (0.3 ml) of each sample were placed in clean disposable test tubes and 2 ml of 0.5 M NaHPO4, added, followed by 0.1 ml of 0.05 M KCN in 0.5 M NaHPO4, or 0.1 ml of 0.5 M NaHPO4 only. The contents of the tubes were mixed and the tubes stood in a water bath at 50°C for 30 min. The tubes were removed, cooled, and 0.6 ml of 1.0 M L-tartaric acid added. The fluorescence was read in a Varian SF30 spectrofluorometer at an excitation wavelength of 325 nm and an emission wavelength of 425 nm. The difference in fluorescence of each extract after reaction with KCN compared to without KCN was used to calculate the PLP content by reference to a standard curve of 0-4 nmol PLP. The concentration of the PLP standard was determined exactly by spectrophotometry using an extinction coefficient of 6,700 at 286 nm, determined in 0.4 M perchloric acid. In preliminary experiments it was found that 4-DOP is not detected and does not interfere with this assay.

Statistical Analysis

The differences among the various treatment groups were compared using Student's t-test; values of p < 0.05 were considered significant.

RESULTS

Epidermal PLP Content and DNA Synthesis

The PLP concentrations and rates of DNA synthesis were measured in the epidermis from untreated, saline-treated, or tape-stripped mice. The mice were killed 4.5 h after tape-stripping, at the time of peak induction of ODC activity [18]. Separate groups of mice were treated with saline or tape-stripping, but were killed and the epidermis extracted and assayed for ODC activity. The results of these experiments are summarized in Table I. The PLP content (1.02±0.18) and DNA synthesis (67.3±7.3) of epidermis from untreated mice (n=5) was not significantly different from the saline-treated control group. In preliminary experiments it was found that the chloral hydrate anesthetic used in tape-stripping experiments, had no effect on the parameters measured.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DNA synthesis (cpm/µg)</th>
<th>PLP (µg/g)</th>
<th>ODC activity (nmol/mg/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>71.7 ± 15.8</td>
<td>.97 ± .15</td>
<td>.08 ± .05</td>
</tr>
<tr>
<td>(n = 9)</td>
<td></td>
<td>(n = 9)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>4-DOP</td>
<td>33.2 ± 5.6 a</td>
<td>.51 ± .11 a</td>
<td>not determined</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>Tape-stripped + saline</td>
<td>38.5 ± 2.6 b, c</td>
<td>1.63 ± .35 b</td>
<td>2.68 ± 1.23</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td>(n = 10)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>Tape-stripped + saline</td>
<td>35.6 ± 6.6 c</td>
<td>.63 ± .29 c</td>
<td>.47 ± .38</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td>(n = 5)</td>
<td>(n = 5)</td>
</tr>
</tbody>
</table>

* Each value represents the mean ± SD of samples from n mice.
* a Significantly lower than after saline treatment, p < 0.005.
* b Significantly higher than after saline treatment, p < 0.005.
* c Not significantly different than after 4-DOP or tape-stripping + saline treatment.
* d Significantly lower than after tape-stripping + saline treatment, p < 0.005; not significantly different from 4-DOP treatment alone.
* e ODC activity induced by tape-stripping was significantly lower after 4-DOP treatment, p < .005.
The PLP concentration found in hairless mouse epidermis was about 1 \( \mu g/g \) (4 \( \times 10^{-6} \) M) and is about 50% that reported for mouse brain and 10% of mouse liver [16] and is similar to that of mouse dermis (unpublished observation). There was a significant increase in the PLP concentration 4.5 h after tape-stripping, at a time when ODC activity was induced and when DNA synthesis was still suppressed. The experiment demonstrated that the epidermal PLP concentration may change in response to proliferative stimuli prior to the induction of DNA synthesis.

**Systemic 4-DOP and Epidermal PLP, ODC Activity, and DNA Synthesis**

Groups of mice were injected i.p. with 4-DOP (150 mg/kg in saline) or saline 24 and 4 h prior to killing, and epidermal PLP and DNA synthesis determined. The results are presented in Table I.

The epidermal PLP content and DNA synthesis were significantly reduced (\( p < .0005 \)) after 4-DOP treatment. The results are interesting in that 4-DOP treatment significantly depleted epidermal PLP and induced a corresponding inhibition of DNA synthesis. The experiment demonstrated the feasibility of manipulating epidermal PLP levels by systemic 4-DOP treatment.

Two groups of mice were injected with 4-DOP or saline as above but tape-stripped 4 h after the second injection to induce epidermal ODC activity. The mice were killed 4.5 h later and the epidermis extracted and assayed for ODC activity. The results are summarized in Table I. The ODC activity induced in the epidermis of the 4-DOP-treated mice was significantly lower at 17% of the saline-treated tape-stripped controls.

**The Effects of Epidermal Extracts from 4-DOP-Treated Mice, of 4-DOP, and of 4-DOP-Phosphate on ODC Activity**

To confirm that the reduced ODC activity induced by tape-stripping after 4-DOP treatment reflected a real decrease in the amount of ODC present and was not simply due to inhibition of ODC activity in vitro, by the presence of 4-DOP or metabolites in the extracts, mixing experiments were performed. 4-DOP is metabolized to 4-DOP-phosphate, a potent inhibitor of several PLP-requiring decarboxylases [19]. 4-DOP, 4-DOP-phosphate, and epidermal extracts from mice treated with 4-DOP prior to tape-stripping were tested as inhibitors of ODC. The source of the ODC activity used was a crude extract of epidermis from tape-stripped mice.

ODC activity (0.1 ml; total activity 1.20 nmol CO\(_2\)/h) was incubated with epidermal extract from 4-DOP-treated tape-stripped mice (0.1 ml; total activity 0.10 nmol CO\(_2\)/h) in the standard ODC assay. The total ODC activity of the mixture was 1.34 nmol CO\(_2\)/h. Since the activity of the mixture was equal to the sum of the activities of the 2 extracts, the extracts from the 4-DOP-treated mice do not contain appreciable amounts of active ODC inhibitors.

In other experiments 4-DOP concentrations up to 5 \( \times 10^{-3} \) M were found not to inhibit ODC in vitro. In contrast, 4-DOP-phosphate was a potent ODC inhibitor. The inhibition kinetics were examined in detail. Inhibition of ODC activity by 4-DOP-phosphate obeyed Michaelis-Menton kinetics of the mixed competitive/noncompetitive type with an apparent \( K_i \) of 6 \( \times 10^{-3} \) M (the same order of magnitude as the \( K_m \) for PLP, 3 \( \times 10^{-3} \) M) determined using Line-Weaver-Burke plots; at higher concentrations of 4-DOP-phosphate (> 5 mM) the plots were nonlinear, but this may be anticipated in view of the crudity of the enzyme preparation.

**DISCUSSION**

In the present study epidermal PLP concentrations have been measured in normal and tape-stripped hairless mouse epidermis. Tape-stripping removes the outer layers of the stratum corneum and induces a benign epidermal hyperplasia; DNA synthesis is initially inhibited (with a nadir at about 4 h after insult) and then increases to a peak at 24 h. Epidermal ODC activity is also induced by tape-stripping, peaking 4.5 h after insult [18]. The epidermal PLP concentration was increased 4.5 h after tape-stripping, well before the induction of DNA synthesis. The raised concentration of PLP in the epidermis when ODC is induced may facilitate putrescine synthesis.

The depletion of epidermal PLP by systemic 4-DOP treatment may indicate a tissue-specific requirement for PLP or one of its congeners by the epidermis, since PLP depletion is not seen in other tissues such as liver, kidney, and muscle [20,21]. The PLP supply to the epidermis may be particularly crucial, since even in chronic vitamin B-6 deficiency hepatic PLP levels are reduced to 55–60% of control values [12,21], which is still 5–6 times higher than in hairless mouse epidermis and more than 10 times higher than in the epidermis of 4-DOP-treated mice. Further studies are required to determine whether the epidermis has distinct metabolic or transport requirements for pyridoxine derivatives.

The expression of epidermal ODC activity was inhibited following 4-DOP administration. In view of the extremely short half-life of ODC this could represent a change in the rate of induction or degradation of ODC. Since we could not find inhibitors of ODC activity in epidermal extracts from 4-DOP-treated mice, and the induction of epidermal ODC activity was reduced in dietary vitamin B-6 deficiency [13], the primary effect on epidermal ODC activity is probably the depletion of PLP rather than a direct effect of 4-DOP or one of its metabolites.

A requirement for PLP in the expression of activity is common among PLP-requiring enzymes [22] and many PLP-requiring enzymes are stabilized by PLP [23,24]. The mechanism of stabilization is unclear but may involve induction of conformational changes in the protein structure on the binding of the cofactor. Such a conformational change is likely for ODC since PLP protects it from proteolysis by trypsin [13]. Hence, in PLP deficiency ODC may be destabilized or degraded at an enhanced rate. Pyridoxine deficiency has a marked effect on the induction of several hepatic and hematopoietic PLP-requiring enzymes [25,26], and it is possible that PLP may be required for the induction of ODC per se.

Even under conditions where ODC activity was not induced, 4-DOP treatment decreased the epidermal PLP concentration and inhibited DNA synthesis. The relationship between PLP and epidermal proliferation is evidently complex. The manipulation of epidermal PLP levels using pyridoxine antagonists is feasible and may result in the inhibition of the expression of ODC activity.

**REFERENCES**

The Relationship Between the In Vitro Activity of 3β-Hydroxysteroid Dehydrogenase Δ\(^{4-5}\)-Isomerase in Human Sebaceous Glands and Their Secretory Activity In Vivo*

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3β-Hydroxysteroid dehydrogenase Δ\(^{4-3}\)-isomerase (Δ\(^{4-3}\)-HSD) catalyzes an early step in the synthesis of testosterone from dehydroepiandrosterone (DHA). We compared enzyme activity in back skin biopsies with sebum excretion rate (SER) in 14 individuals. The rate of conversion of [7\(\alpha\)-H]DHA into \([\text{H}]^4\)-androstene-3,17-dione was measured in cryostat sections of skin and compared with the sebaceous gland content of the same biopsies. Reaction rate was proportional to the volume of sebaceous gland tissue in the sections. Enzyme activity was absent from sections without histologically identifiable sebaceous gland tissue. This suggests that the Δ\(^{3}\)-HSD is localized in sebaceous glands. SER, measured by a modified photometric technique at the biopsy site, correlated highly with sebaceous gland volume and with the rate of conversion of DHA into androstenedione in the biopsy. For each biopsy, specific activity of Δ\(^{3}\)-HSD in sebaceous glands was calculated by dividing the rate of formation of \([\text{H}]^4\)-androstene-3,17-dione by sebaceous gland volume. Specific activity of Δ\(^{3}\)-HSD did not correlate significantly with SER, suggesting that variations in concentration of Δ\(^{3}\)-HSD in sebaceous glands probably do not underlie variations in sebaceous gland activity.

The weak androgen dehydroepiandrosterone (DHA) stimulates sebaceous gland secretion in humans [1,2]. The increase