Prednicarbate Biotransformation in Human Foreskin Keratinocytes and Fibroblasts

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Purpose. Evaluation of skin layer-specific prednicarbate (PC) biotransformation, possibly explaining the improved benefit/risk ratio of this topical corticosteroid in atopic dermatitis (1,2).

Methods. Metabolism of PC in keratinocyte and fibroblast monolayers derived from human juvenile foreskin was evaluated. Drug concentration was determined by HPLC/UV-absorption. Accompanying cell viability tests (MTT-tests) were performed to exclude toxic drug effects.

Results. Keratinocytes hydrolyzed the double ester PC (2.5×10^{-6} M) at position 21 to the monoester prednisolone 17-ethylcarbonate (P17EC) which nonenzymatically transformed to prednisolone 21-ethylcarbonate (P21EC). This metabolite was enzymatically cleaved to prednisolone (PD), the main biotransformation product at 24 hours. Fibroblasts, however, showed a distinctively lower enzyme activity. Both, PC and P17EC (or rather P21EC) were hydrolyzed to a minor extent only. The biotransformation pathway, however, was the same. When P17EC was added separately, it transformed to P21EC and again was cleaved by keratinocytes to a much higher extent. Despite of the rather high glucocorticoid concentration MTT-tests proved a nondisturbed cell viability and proliferation rate.

Conclusions. Extrapolating our results to the in-vivo situation, topically applied PC may be metabolized by epidermal cells during skin penetration. A complex mixture of compounds reaches the dermis, whose fibroblasts are barely able to metabolize the steroids. Since skin atrophy is less pronounced with PC as compared to conventional halogenated glucocorticoids, less potent PC metabolites appear to be the dominant species in the dermis.

KEY WORDS: prednicarbate; topical glucocorticoids; pharmacokinetics; biotransformation; keratinocytes; fibroblasts.

INTRODUCTION

Topical corticosteroids are first-line drugs in the therapy of acute exacerbatious atopic dermatitis and contact dermatitis (3). Glucocorticoids are also used in psoriasis vulgaris and skin irritations of unknown origin. This holds true despite the side effects frequently feared by the patients. These side effects include the suppression of endogenous cortisol-levels and even more local adverse phenomena, including hypersensitivity reactions and dermal skin thinning due to the antiproliferative effect of the glucocorticoids on fibroblasts (4). The loss and degeneration of elastic and collagen fibrils–in healthy skin synthesized by fibroblasts–can result in irreversible striae formation. Modern corticoid therapy tries to avoid this serious risk (5). Prednicarbate (PC), the nonhalogenated 17-ethylcarbonate, 21-propionate double ester of prednisolone has an advantageous ratio of strong antiinflammatory and antipruritic efficacy to a reasonably low rate of local side effects. This holds true in healthy volunteers (6) as well as in patients suffering from chronic skin diseases (7,8). In in-vitro cultivated human fibroblasts, PC did not reduce chemotactic activity, whereas equipotent fluorinated compounds like betamethasone 17-valerate (BMV) or desoximetasone (9) strongly inhibited cell migration (10). Moreover in a neutral red release assay, the toxic potential of PC for keratinocytes and fibroblasts appeared significantly lower as compared to BMV (11), indicating distinct subtypes of the glucocorticoid receptor (12) or a substance-specific activation and deactivation by skin metabolism. Ex-vivo and in-vivo studies in various animal species demonstrated PC hydrolysis to the monoester prednisolone 17-ethylcarbonate (P17EC), prednisolone (PD), and several oxidation products (13,14,15). Receptor binding is low with PC and PD whereas P17EC has a high affinity towards the glucocorticoid receptor (13,16). To delineate the mechanisms of the improved benefit/risk ratio of PC the present study compared if and to what extent PC biotransformation differs between epidermis and dermis. The defined in-vitro system of keratinocyte and fibroblast monolayers was convenient in quantifying cell viability by a MTT-assay parallel to the measurement of metabolite formation.

MATERIALS AND METHODS

Materials

PC, P17EC, prednisolone 21-ethylcarbonate (P21EC), and PD were kindly donated by Hoechst AG (Frankfurt, Germany). Betamethasone (BM), trypsin, ethylenediaminetetraacetic acid (EDTA), Dulbecco's modified Eagle's medium (DMEM), glutamine, phosphate buffered saline (PBS) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA); fetal calf serum (FCS) was from GibcoBRL, Life Technologies (Eggenstein, Germany). Keratinocyte basal medium (KBM), supplements and antibiotics were obtained from Clonetics (San Diego, CA, USA). Sodium dodecyl sulfate (SDS), acetic acid, dimethyl sulfoxide (DMSO) and sodium fluoride (p.a. quality), ethanol, ethyl acetate, methanol, acetonitrile (gradient grade), chloroform (p.a. quality), and TLC plates (silica gel 60 F_{254}, 1 mm) were purchased by Merck (Darmstadt, Germany).

Cell Culture

Keratinocytes

Primary cultures of keratinocytes were obtained from human juvenile foreskin biopsies after incubating the specimens

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ABBREVIATIONS: PC, Prednicarbate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; P17EC, Prednisolone 17-ethylcarbonate; P21EC, Prednisolone 21-ethylcarbonate; PD, Prednisolone; BMV, Betamethasone 17-valerate; BM, Betamethasone; DMEM, Dulbecco's modified Eagle's medium; PBS, Phosphate buffered saline; FCS, Fetal calf serum; KBM, Keratinocyte basal medium; SDS, Sodium dodecyl sulfate; KGM, Keratinocyte growth medium; BPE, Bovine pituitary extract; HC, Hydrocortisone; FGM, Fibroblast growth medium.

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for 20 h in a 0.25% trypsin/0.1% EDTA solution at 4°C. Using forceps the keratinocytes were scraped off the tissue into ice-cold PBS, filtered through sterile gauze, centrifuged for 5 min at 1000 rpm and resuspended in keratinocyte growth medium (KGM), KBM supplemented with 30 μg/ml bovine pituitary extract (BPE), 0.5 μg/ml hydrocortisone (HC), 0.1 μg/ml human epidermal growth factor, 25.0 μg/ml amphotericin and 25.0 μg/ml gentamicin. The suspension was dispensed into tissue culture flasks and incubated at 37°C/5% CO_2.

Fibroblasts

After removing the keratinocytes, the dermis was trypsinised for another 10 min at 37°C; fibroblasts were obtained as described above for keratinocytes and cultivated in fibroblast growth medium (FGM), adding 0.2 nM/ml glutamine, 50 ng/ml amphotericin, 20 μg/ml gentamicin and 10% FCS to DMEM.

Kinetics

Glucocorticoid stock solutions (10^{-3} M) were prepared in ethanol (96%) and remained stable for 1 month when stored at 4 °C. Test medium was prepared immediately before the experiments by dilution with the respective cell medium (KGM without BPE and HC, and FGM without FCS to avoid hydrolysis by nonspecific esterases). Pooling the cells of three donors, keratinocytes of the 2^{nd}–3^{rd} and fibroblasts of the 2^{nd}–7^{th} passage, respectively, were seeded in a density of 1×10^5 cells per well into 6-well-dishes and incubated for 24 h at 37 °C/5% CO_2. Medium was replaced by 2.0 ml/well of the test medium, containing PC or P17EC in a concentration high enough for detection purposes but affecting cell proliferation only to an acceptable extent (2.5×10^{-6} M, PC 24 μg/ml, P17EC 22 μg/ml). After incubation for 0, 3, 6, 9, 12 h (in keratinocytes also for 24 h) 1 ml sodium fluoride (3 g NaF/25 ml PBS) was added to inhibit the enzymatic reaction. Enzyme inhibition was verified by monitoring PC decay over 24 h in keratinocytes treated with NaF. PC hydrolysis did not differ from its spontaneous hydrolysis rate in cell-free medium (data not shown). At the end of incubation, cells were scraped off the plate and lysed by high frequency ultrasound. Suspensions were extracted twice with 3 ml ethylacetate (vortex 1 min) and centrifuged (5 min/1000 rpm). Combined organic phases were exsiccated under nitrogen. The remainder was reconstituted in 1 ml of methanol (vortex 1 min) and again exsiccated in a conic centrifugation tube. After resuspension in 100 μl methanol and centrifugation, 20 μl were injected into HPLC-system. Stability tests of PC and P17EC in cell-free medium served as controls to correct for non-enzymatic hydrolysis.

MTT-Test

In parallel to the kinetic experiments cell viability and proliferation rate were surveyed by MTT-test, modifying the method of Pagé *et al.* (17). After glucocorticoid incubation for 0, 3, 6, 9 and 24 h, 200 μl of MTT-solution (5 mg/ml in PBS) were added to each well and the cells were incubated for another 4 h at 37 °C/5% CO_2. Cells incubated with pure growth medium served as control. The MTT-solution was removed, cells were lysed by 1 ml of lysis solution (DMSO 99.4 ml, acetic acid 0.6 ml, SDS 10 g) shaking carefully for 5 min. Formazan formation was read at 540 nm via UV-absorption. Lysis solution served as blank.

HPLC-Method

Samples were analyzed with a LaChrom HPLC system (Merck Hitachi, Darmstadt, Germany) and on-line UV-detection at 254 nm. The chromatographic column consisted of a 125 mm × 4 mm ID LiChroCART® packed with LiChrospher® 100 RP-18 (5 μm particle size). The mobile phase was a gradient of acetonitrile/water (20:80 to 100:0 v/v within 20 min), delivered at a flow rate of 1 ml/min. Betamethasone (BM) served as an internal standard. The retention times for PD, BM, P17EC, P21EC and PC were 7.8, 9.4, 11.1, 12.5, and 15.1 min, respectively [Fig. 1]. Limits of detection were 10 ng/ml for PC and PD and 20 ng/ml for P17EC and P21EC (signal-to-noise ratio 3:1). Linearity (correlation coefficients > 0.994) was observed when performing weighted linear regression ($w = 1/y^2$) of concentration ranges from the limit of quantification (100 ng/ml) to 50 μg/ml versus measured peak areas. The coefficients of variation for intra- and inter-assay precision of the quality control samples were <10% and 16%, respectively. The accuracy was within 16% of the nominal concentration (PC < 13%, P17EC and P21EC < 16%, and PD < 5%). Recovery of the steroids from the cell medium exceeded 95%.

Identification of the compounds was achieved through ^1H NMR spectroscopy following preparative thin layer chromatography (chloroform/methanol 93:7, v/v, one-dimensional, two step development).

Data Analysis

The concentration-time profiles of PC and its metabolites were analyzed using a one-compartment method. The area under the curve from time zero to the last measured time point (AUC_{0-t}) was estimated by the linear trapezoidal method; the peak concentration (C_{max}) and its time of occurence (t_{max}) for P17EC and P21EC were derived directly from the plotted data. The terminal elimination rate constant (k_{el}) and metabolite formation rate constant (k_m) were calculated by least squares

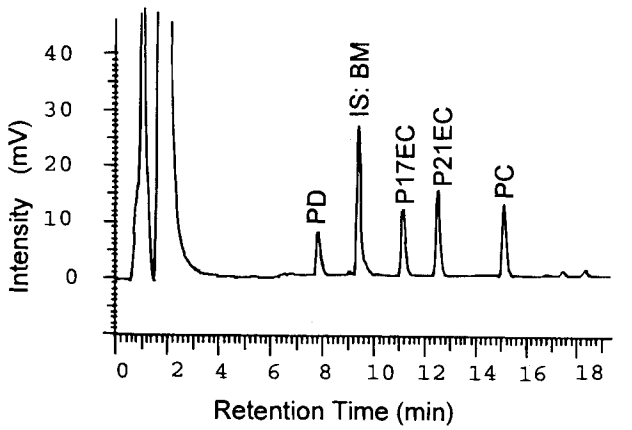

Fig. 1. Sample chromatogram for PC and its metabolites after PC incubation for 9 h in keratinocytes [Prednicarbate (PC), Prednisolone 17-ethylcarbonate (P17EC), Prednisolone 21-ethylcarbonate (P21EC), and Prednisolone (PD)].

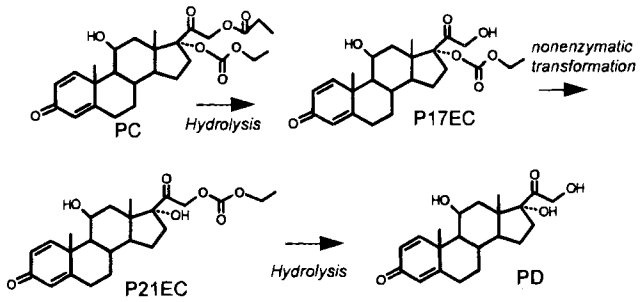

Fig. 2. Metabolic scheme for PC biotransformation [Prednicarbate (PC), Prednisolone 17-ethylcarbonate (P17EC), Prednisolone 21-ethylcarbonate (P21EC), and Prednisolone (PD)].

regression on the basis of a metabolic pathway in series [Fig. 2] using TOPFIT™ 2.0. software, estimating the single compounds independently from their respective precursors. The elimination half-life ($t_{1/2}$) was determined from ln_2/k_{el} and the formation/elimination ratio by k_m/k_{el}. Kinetic modeling was performed on the basis of mean values (n = 6).

Statistics

Arithmetic mean values and standard deviations of drug concentration and optical density (MTT-test) were determined. Significance of differences between substances was analyzed using Shapiro-Wilk-test, F-, and Student's t-test. A value of $p \leq 0.05$ was considered significant.

RESULTS

PC Metabolism

Fig. 3A shows PC biotransformation in fibroblasts surveyed for 12 h. PC concentrations declined from 21.3 ± 0.31 to 13.99 ± 0.92 µg/ml. The respective values for the cell-free system read 23.06 ± 1.12 and 18.07 ± 0.88 µg/ml. When correcting for spontaneous hydrolysis in cell-free medium, PC was cleaved by fibroblasts with a rate constant of 0.020 h^{-1} and $t_{1/2}$ of almost 35 h. Accordingly, the increase of the monoester P17EC was rather low; at the end of the experiment the concentration amounted to 1.15 ± 0.03 µg/ml only after subtraction of cell-free hydrolysis. P21EC and PD concentrations were below limits of detection.

Stability tests of PC in cell-free keratinocyte medium revealed that after 24 h only 4% of the drug cleavage was due to spontaneous hydrolysis [Fig. 3B]. Keratinocytes induced pronounced cleavage of the double ester at position 21 to

Table I. PC Kinetics in Keratinocytes

	PC	P17EC	P21EC	PD
AUC_{0-24} (µ·h·ml^{-1})	135	136	90.4	111
k_m (h^{-1})		0.220	0.082	0.10
k_{el} (h^{-1})	0.136	0.051	0.083	
$t_{1/2}$ (h)	5.1	13.6	8.4	
C_{max} (µg·ml^{-1})		7.15	4.34	
t_{max}(h)		9	13.6	

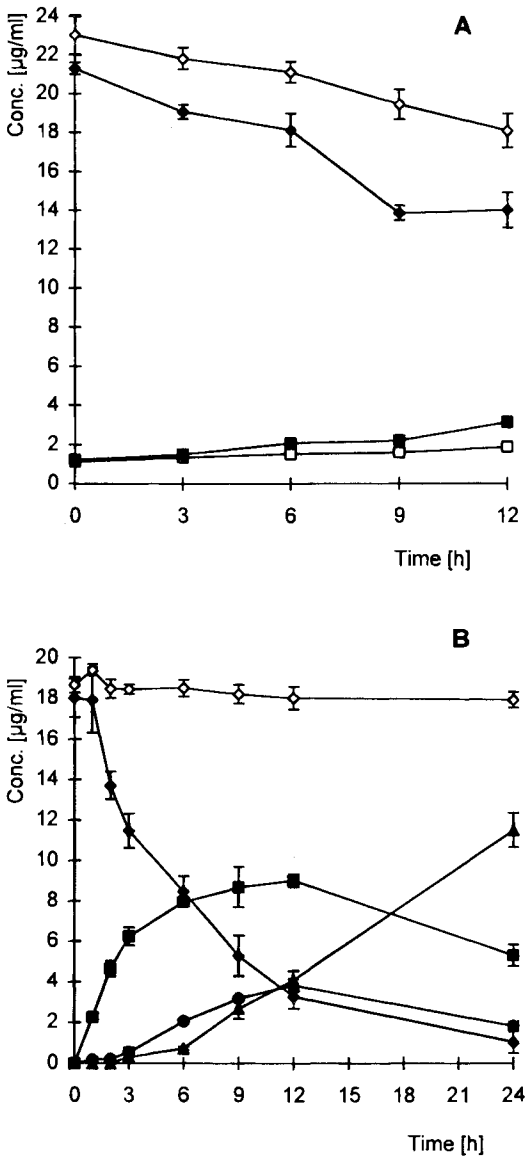

Fig. 3. PC metabolism, A: fibroblasts, B: keratinocytes. PC (◇), P17EC (□) in cell-free control system, PC (◆), P17EC (■), P21EC (●), and PD (▲) in the cell system (mean values ± SD).

P17EC. P17EC was the main metabolite during 12 h. AUC_{0-24} for P17EC exceeded the one for P21EC [Table I] and the formation/elimination ratio appeared 4.4 times as high for P17EC when compared to P21EC. Thus, the transformation to P21EC represented the rate limiting step. This observation is in agreement with our theoretical prediction that P17EC is a rather unstable compound, spontaneously rearranging its 17-ester through acylmigration to the 21 position. The final metabolite PD appeared first after 3 h. At 24 h, the free steroid alcohol was the main metabolite with a concentration of 11.53 ± 0.85 µg/ml which was obviously still rising. Chemical structures were finally proven by TLC-^{1}H NMR analysis (data not shown).

P17EC Metabolism

To evaluate acylmigration kinetics, studies were performed using P17EC as the parent compound. In fibroblasts [Fig. 4B] as

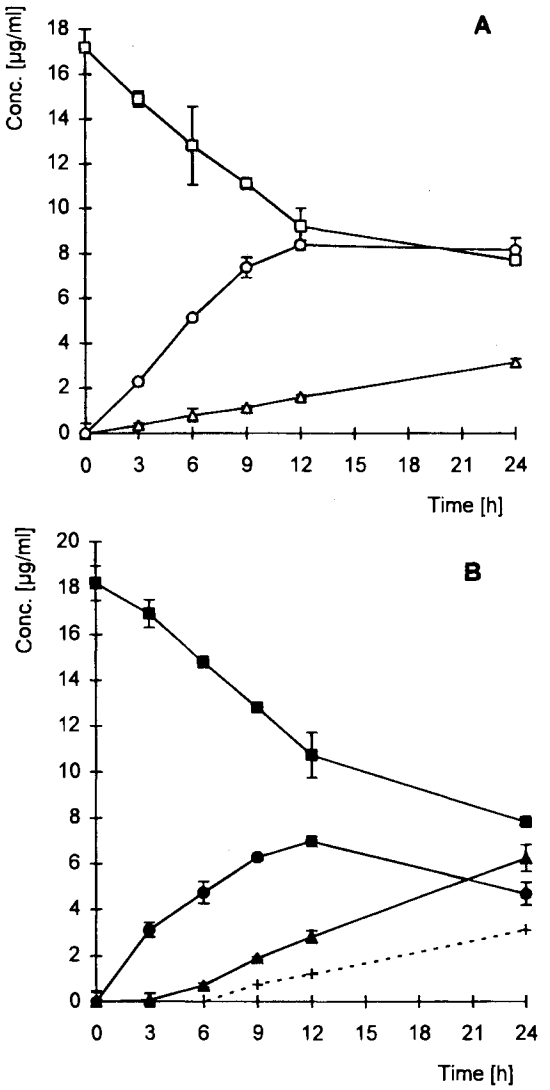

Fig. 4. P17EC metabolism. A: Cell-free control system. P17EC (□), P21EC (○), and PD (Δ). B: Fibroblasts. P17EC (■), P21EC (●), PD (▲), and PD (+) corrected for spontaneous hydrolysis (mean values ± SD).

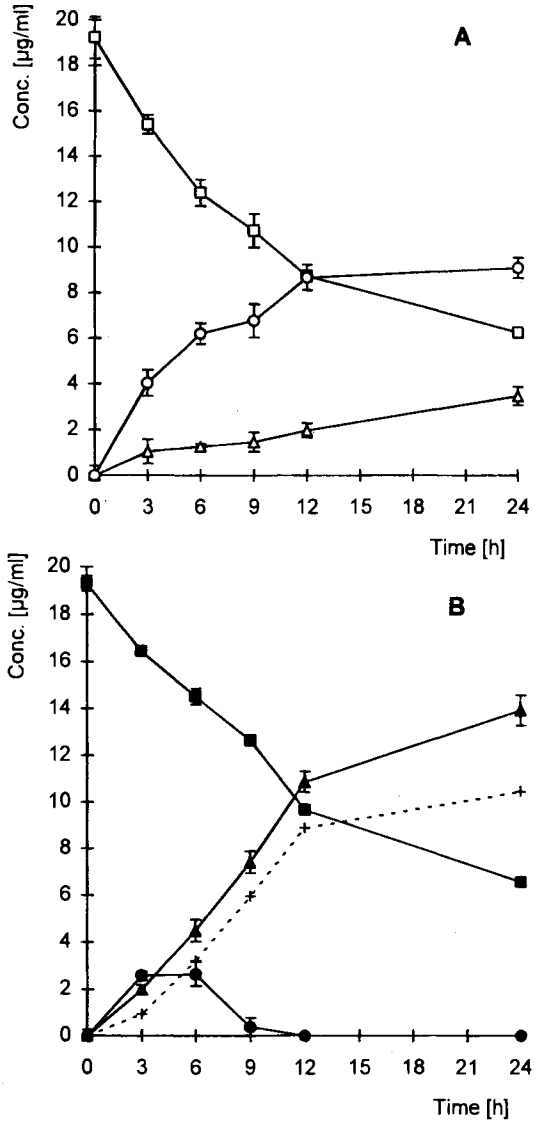

Fig. 5. P17EC metabolism. A: Cell-free control system. P17EC (□), P21EC (○), and PD (Δ). B: Keratinocytes. P17EC (■), P21EC (●), PD (▲), and PD (+) corrected for spontaneous hydrolysis (mean values ± SD).

well as in the cell-free system [Fig. 4A], P17EC concentrations decreased with identical half-lives (18.38 h and 18.67 h, respectively); the drug levels were superimposable. P21EC concentrations, however, were clearly lower in the cell system due to ester hydrolysis (AUC_{0-24} 123 $\mu g \cdot h \cdot ml^{-1}$ versus 156.1 $\mu g \cdot h \cdot ml^{-1}$). Correcting for chemical hydrolysis, fibroblasts formed PD with k_m = 0.011 h-1; at the end of the experiment the concentration of PD did not exceed 3.13 ± 0.16 µg/ml.

Moreover, P17EC transformation to P21EC occured as rapidly in the cell-free system as in cultivated keratinocytes ($t_{1/2}$ = 13.52 h and 14.58 h, respectively). As expected from the PC study, keratinocytes hydrolyzed P21EC to PD very rapidly [Fig. 5B]. In the cell-system the AUC_{0-24} for P21EC was below 17 $\mu g \cdot h \cdot ml^{-1}$ but 167.4 $\mu g \cdot h \cdot ml^{-1}$ in the control sample [Fig. 5A]. Correcting for spontaneous hydrolysis, PD concentration increased with k_m = 0.123 h^{-1} to 10.44 ± 0.25 µg/ml at 24 h [5B].

To exclude the toxic effects of PC and its metabolites on keratinocytes and fibroblasts, accompanying MTT-tests were carried out measuring the reduction of yellow MTT to blue formazan within the mitochondria (data not shown). There were no significant differences in enzyme activity between glucocorticoid-treated cells and controls ($p \leq 0.05$). Thus, enzymatic PC metabolism also appears nonsuppressed during the examination period.

DISCUSSION

Human skin is extraordinarily efficient in protecting the organism against the uncontrolled uptake of xenobiotics. The stratum corneum, serving as a penetration barrier and as a drug reservoir at the same time (18), allows penetration only for few, mainly lipophilic, compounds and must be bypassed by topical drugs for the treatment of skin disease. Glucocorticoids

are rather lipophilic compounds. PC especially, is able to penetrate into the skin very rapidly due to esterification at positions 17 and 21. PC is a moderate to potent topical corticosteroid, showing dissociation of beneficial and negative affections in clinical studies. The present study indicates PC metabolism in keratinocytes to be highly relevant in regard to the improved benefit/risk ratio. In the epidermis, mainly consisting of keratinocytes, PC is hydrolyzed rapidly to P17EC. Because of its higher receptor affinity, as compared to native PC, (13,16) P17EC may induce the antiinflammatory effect in keratinocytes. If at all, P17EC can only reach the dermis via diffusion from the epidermis. It transforms nonenzymatically–presumably through acylmigration–to P21EC, a compound almost devoid of glucocorticoid receptor binding. The ester at position 21 now is cleavable by the dermal cells, resulting in PD, the free alcohol, also showing a rather low receptor binding affinity. Thus, with PC first activation takes place in the upper skin layer, which is followed by detoxification. For a final assessment of the benefit/risk ratio (6), a model also concerning drug penetration across the dermis appears most interesting. Studies in animals and man indicate PC to have a very slow transdermal permeation; in rats low blood levels were detected 24 h after administration, whereas in human volunteers neither PC nor its metabolites could be detected in serum samples (13–15).

For the final prediction of drug toxicity and efficacy, taking together all influencing parameters of skin events, knowledge about biotransformation conditions in inflamed tissue would be important, indicating a loss of intact barrier function of the stratum corneum and a change in metabolism rate.

In the future such studies should be performed on a regular basis during drug development.

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