Biotransformation of Estradiol in the Human Keratinocyte Cell Line HaCaT: Metabolism Kinetics and the Inhibitory Effect of Ethanol

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Purpose. The aim of our study was to investigate the kinetics of β-estradiol (E_2) metabolism in the human keratinocyte cell line HaCaT and to estimate the effect of the potential inhibitor ethanol on the biotransformation reaction.

Methods. The formation rates of estrone (E_1) in dependence on substrate concentrations were determined in HaCaT cells using tritium labelled E_2. Experiments were conducted with and without addition of dehydroepiandrosterone (DHEA) and ethanol. Possible toxic effects on the cells due to ethanol were investigated by cytotoxicity tests.

Results. The metabolism of E_2 in HaCaT cells exhibited Michaelis-Menten kinetics with K_m and V_{max} values of 3.5 μM and 216 pmol × mg^{-1} protein × h^{-1}, respectively. The reaction was inhibited by DHEA and ethanol. The alcohol showed a reversible competitive inhibition mechanism for concentrations of 4 to 8% (v/v). Lower ethanol concentrations had no effect, whereas levels ≥10% significantly decreased cell viability leading to a different inhibition mechanism.

Conclusions. The HaCaT cell line seems to be a suitable model for studying enzyme kinetics equivalent to the human skin. The concentration dependent inhibitory effect of ethanol observed in this cell line may be relevant for the transdermal E_2 application in patients.

KEY WORDS: cell culture; keratinocytes; estradiol; ethanol; metabolism; transdermal delivery.

INTRODUCTION

Transdermal application of drugs for systemic therapy was shown to have several advantages over the oral application route, such as the avoidance of liver first pass metabolism and the formation of more constant blood levels (1). The main barrier for transdermally delivered drugs is the stratum corneum, the outermost layer of human skin, consisting of dead cornified cells (1). Therefore, various approaches were studied to increase the permeability of substances across this layer, including the use of enhancers and prodrugs (2).

In recent years, however, it has been recognized that the metabolic activity of the skin needs to be taken into account in transdermal therapy (3). Although the metabolic activities in the skin are substantially lower than in the liver (4), the systemic bioavailability of some extensively metabolized drugs may be affected following transdermal application.

Remarkably high cutaneous biotransformation rates were reported for glyceryl trinitrate (5) and the steroid hormones estradiol (E_2) and testosterone (6). The ability of skin to metabolize topically administered xenobiotics was demonstrated to be inducible. On the other hand, inhibition of skin enzyme activity was reported as a consequence of simultaneous application of drugs (7) or organic solvents (8). For several enhancers, inhibitory effects on drug metabolism were reported (8), which may alter the proportion of systemically available drug and metabolite(s).

In the case of E_2, it was reported that ethanol acts as an inhibitor of the conversion of E_2 to the less active metabolite estrone (E_1) (9), catalyzed by the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD) subtype II. These findings may have practical relevance, because ethanol is used as solvent and enhancer in the commercially available membrane controlled reservoir system (MCRS) for the transdermal application of E_2.

We have recently reported that the transdermal input rate of E_2 from this patch is strictly dependent on the ethanol content in the reservoir (10) and that ethanol modulates the barrier properties of the dense membrane and the adhesive layer used in this MCRS (11).

The objective of our present investigation was to estimate the influence of the enhancer ethanol on the biotransformation of E_2 in the skin. The spontaneously transformed human keratinocyte cell line HaCaT, which exhibits a similar differentiation behavior as normal human keratinocytes (12), was chosen as an in vitro cell culture model. Cell lines represent a reproducible source of viable cells in a controlled environment. Therefore, variability of skin source with regard to different donors or anatomical body sites, which were both shown to have a strong influence on E_2 metabolism (13), can be ruled out.

We have recently shown that HaCaT cells expressed the steroid metabolizing enzymes responsible for skin biotransformation of testosterone (14). The metabolic pattern was comparable to that from excised human skin and other in vitro models, indicating that the HaCaT cell line may be suitable as a model for studying cutaneous steroid metabolism. In the case of testosterone, it was not possible to calculate kinetic parameters for the enzymes, due to the complex metabolic pathway. In contrast, the only metabolite formed after topical E_2 application was reported to be E_1, while little, if any, estriol was formed in the skin (13,15,16). Therefore, the aim of our study was twofold: Firstly, we investigated whether it is possible to study enzyme kinetics in intact HaCaT cells. In addition, the effect of a well known inhibitor of the type II 17β-HSD, dehydroepiandrosterone (DHEA) (17), on E_2 metabolism was determined. Secondly, we tried to elucidate the inhibitory effect of ethanol on the biotransformation of E_2.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM, 1.8 μM Ca^{2+}) with and without HEPES, fetal calf serum (FCS), 0.05% trypsin/0.025%, ethylenediamine tetra-acetic acid (EDTA) solution and penicillin 10000 U/ml/streptomycin 10000 μg/ml solution were obtained from Gibco BRL-Life Technologies, Paisley, U.K. PBS with and without Ca^{2+} and Mg^{2+} was prepared from double distilled water. Cell culture dishes of 10 cm diameter and 96-well multidishes were purchased from Nunc, Roskilde,

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Denmark. Twelve-well multidishes (area: 4 cm^2/well) were obtained from Costar, Cambridge, USA.

Estradiol (E_2), estrone (E_1), estriol (E_3) and dehydroepiandrosterone (DHEA) were obtained from Sigma Chemical Co., St. Louis, USA. [2,4,6,7-^{3}H]estradiol (spec. activity 75.0 Ci/mmol, radiochemical purity: 98.4%) was purchased from Amersham Life Science, Braunschweig, Germany. TLC silica gel plates (Kieselgel 60 F_{254}), acetone, chloroforme, diethylether, dimethyl sulfoxide (DMSO) and ethanol were obtained from Merck KGaA, Darmstadt, Germany.

Cell Culture

The spontaneously transformed human keratinocyte cell line HaCaT was provided by N. Fusenig, German Cancer Research Institute, Heidelberg, Germany. HaCaT cells at passages between 36 and 42 seeded at a density of 2×10^4 cells/cm^2 were maintained in DMEM supplemented with 10% FCS, 2mM glutamine and antibiotic additives (100 U/ml penicillin/100 µg/ml streptomycin) at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. The culture medium was replaced twice a week.

For passaging, cells were washed with PBS without Ca^{2+} and Mg^{2+}, preincubated with 0.05% EDTA solution and then disintegrated with a 0.05% trypsin/0.025% EDTA solution at 37°C. After counting the cells in the resulting suspension, a defined amount of keratinocytes was plated in culture dishes.

Metabolism Studies with ^{3}H-estradiol in HaCaT

HaCaT cells were seeded into 12-well plates at a density of 2×10^4 cells/cm^2. After 3 weeks medium was removed, the cells were washed and 0.5 ml of a solution of [2,4,6,7-^{3}H]-E_2 (^{3}H-E_2) together with unlabelled substrate in DMEM with HEPES (pH 7.4) was added. The concentration of E_2 in the medium ranged from 0.1 to 10 µM. If necessary, DMSO was used as a cosolvent for E_2. The final DMSO concentration did not exceed 1% (v/v). This concentration of DMSO was shown to be non-toxic for HaCaT cells (14) and had no influence on E_2 metabolism. The incubation temperature was 37°C in a humidified atmosphere. Control incubations were conducted under the same conditions in the absence of cells. The pH of the medium was constant during the experiments. At the end of each incubation period, the medium was removed and the cells were washed with 0.5 ml PBS. Cell protein was determined by the method of Lowry (18), using bovine serum albumin as the standard. Combined media and washes were extracted twice with 1 ml of diethylether. The solvent was evaporated to dryness and the residue was redissolved in 100 µl ether. The recovery of radioactivity in the organic phase was measured by scintillation counting of control incubations (TRI-CARB 2100 TR liquid scintillation analyzer, Canberra-Packard GmbH, Dreieich, Germany) and was found to be 92 ± 4% (n = 3).

The etheric solutions were applied to Silica-TLC plates, which were then developed in chloroforme-acetone (70:30). Plates were scanned for radioactivity using a linear β-scanner and the Chroma software (Berthold, Wildbad, Germany). To identify the steroids, unlabelled E_2, E_1 and E_3 were added on TLC-plates. After developing of the plates, steroids were visualized by spraying with 10% H_2SO_4 in ethanol followed by heating. The identity of the metabolite was verified using an alternative solvent system consisting of chloroforme-ethyl acetate (80:20). Steroids were quantified as a percentage of total radioactivity by integration of the peaks. The sensitivity was 50 dpm per 10 min for tritium at a resolution of 1.0 mm. The analyzer automatically subtracted the background count rate.

Inhibition Studies Using DHEA

The experimental conditions used for studying the effect of DHEA on E_2 metabolism were the same as outlined above, except that 5 or 10 µM of DHEA were added to the incubation medium.

Inhibition Studies Using Ethanol

The experimental conditions used for studying the effect of ethanol on E_2 metabolism were the same as outlined above, except that the incubation medium contained 1, 2, 4, 6, 8 or 10% (v/v) of ethanol. To avoid evaporation of the alcohol, the plates were sealed with adhesive tape for multiwell plates (Boehringer Mannheim GmbH, Mannheim, Germany).

To investigate whether the effect of ethanol is reversible, HaCaT cells were preincubated with DMEM with HEPES containing 6 or 8% (v/v) ethanol. After 1.5 h the cells were washed and incubated for another 1.5 h with ^{3}H- E_2 as described above.

Data Analysis

For the determination of apparent K_m and V_{max} values, data were analyzed with the Microcal ™ Origin 4.1 program (Microcal Software Inc., Northampton, USA). This program fits data to the Michaelis-Menten equation using nonlinear regression analysis.

Effect of Ethanol on MTT Formation in HaCaT Cells

The effect of ethanol on the mitochondrial activity was determined using a colorimetric assay (MTT) based on the reduction of a yellow tetrazolium salt to a purple formazan precipitate by mitochondrial enzymes of viable cells. HaCaT cells were seeded at a density of 2×10^4 cells/cm^2 into 96-well multidishes and cultivated under normal conditions. After 3 weeks the cells were incubated for 1.5 h in DMEM with Hepes containing ethanol from 0 to 15% (v/v) at 37°C. The cells were kept under occlusion by means of an adhesive tape to avoid evaporation of the alcohol. Afterwards the cells were washed and incubated with 200 µl medium containing 0.5 mg/ml MTT for 4 hours. The MTT solution was removed, developed formazan crystals were dissolved with 200 µl DMSO and quantitated spectrophotometrically using an ELISA reader (Titertek Plus MS 212, ICN, Eschwege, Germany) at wavelength of 570 nm and 690 nm. The MTT transformation in cells maintained in DMEM without ethanol was set as 100%.

Effect of Ethanol on Lactate Dehydrogenase (LDH) Release from HaCaT Cells

LDH is a cytosolic enzyme and its presence in the incubation medium is generally regarded as evidence for cell membrane damage.

HaCaT cells were seeded at a density of 2×10^4 cells/cm^2 into 12-well mutidishes and cultivated under normal conditions. After 3 weeks the cells were washed and incubated for 1.5 h in PBS supplemented with glucose containing ethanol from 0 to 12% (v/v) at 37°C. PBS was chosen, because DMEM interfered with the photometric assay. Recently, it was shown that the viability of HaCaT cells maintained in PBS for 1.5 h exceeds 90% (14). The plates were sealed with adhesive tape in order to prevent evaporation of the alcohol. After 1.5 h the LDH content in the samples was assayed using a test kit (DG 1340-K, Sigma, Deisenhofen, Germany), which allows the photometric determination (UV-160, Shimadzu, Kyoto, Japan) of the reduction of NAD in the presence of lactate and LDH. In preliminary experiments it was verified that concentrations up to 12% ethanol did not interfere with the test. Control experiments were performed with 0.1% Triton X-100 (ICN, Eschwege, Germany), which is known to completely lyse cell membranes.

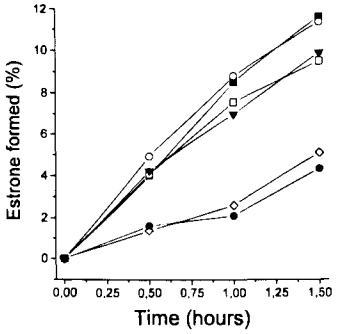

Fig. 1. Time course of the formation of estrone by HaCaT cells. Incubation with different concentrations of ^{3}H-estradiol for up to 1.5 hours. (-■-, 0.1 μM; -○-, 0.5 μM; -□-, 1 μM; -▼-, 2 μM; -◇-, 5 μM; -●-, 10μM)

RESULTS AND DISCUSSION

Metabolism of E_2 in HaCaT Cells

In this study we investigated the kinetics of E_2 metabolism in the spontaneously transformed human keratinocyte cell line HaCaT and estimated the effect of enzymatic inhibitors on the formation of E_1.

We have recently shown that HaCaT cells exhibited constant cell growth, when maintained in culture for 3 weeks (14). This reproducible cell density is seen as a necessary condition for metabolism studies, where results from experiments conducted with cells of different passages are compared.

After incubation of HaCaT cells with ^{3}H-E_2 the only metabolite detected was E_1. There was no evidence for the formation of estriol, which is in accordance to studies using human skin (13). From control incubations in the absence of keratinocytes, no E_1 was found, indicating that there was no spontaneous (non-biological) oxidation of E_2. Overall recovery of radioactivity was satisfactory, typically being about 92% in culture medium and wash.

To investigate the influence of incubation time and substrate concentration, HaCaT cells were incubated with amounts of 0.1–10 μM E_2 for up to 1.5 hours. These supraphysiological substrate concentrations were chosen, because the local E_2 concentration in the skin after transdermal application should exceed the average plasma concentrations in women. Higher substrate concentrations than 10 μM were not investigated, due to the low solubility of E_2 in the aqueous medium. From Fig. 1 it is obvious, that formation of E_1 was linear with incubation time up to 1.5 hours for all concentrations tested. Furthermore, E_2 degradation did not exceed 12% under these conditions, indicating that substrate concentrations can be judged as constant in this case. Therefore, all subsequent investigations were performed for 1.5 hours under the conditions described above.

For the metabolism of hydrophilic peptides in HaCaT cell sheets, it was reported that the diffusion of the substrate into the cells strongly affected the metabolism kinetics, resulting in enzyme kinetics, which could not be described by the Michaelis-Menten equation (19). In contrast, for lipophilic steroids including E_2 it was demonstrated that the diffusion across the membrane of animal cells does not limit interaction between steroids and cellular components. This was explained by the large permeability coefficient of E_2 of 4×10^{-4} cm × sec^{-1} found for different cell lines (20).

In Fig. 2 velocities of E_1 formation estimated over the concentration range of 0.1–10 μM E_2 are shown. Additional curves demonstrate the effect of 5 and 10 μM DHEA, an inhibitor of 17β-HSD, on the formation rate of E_1. It is obvious, that the metabolic rate versus substrate concentration plot exhibits Michaelis-Menten kinetics for the observed concentration interval. Therefore, we assumed that it depends on the polarity of the substrate, whether the metabolism kinetics are partially controlled by the transport of substances into the cells or solely by the enzyme characteristics. Our findings were confirmed by others, where the metabolism of steroid hormones, such as androstenedione and progesterone in human keratinocytes clearly exhibited Michaelis-Menten kinetics (21).

The apparent K_m value estimated from our experiments was found to be 3.51 μM, which is in the range of values found for 17β-HSD in other human tissues (1.6–11 μM) (17,22). Other studies, where the metabolism of E_2 was investigated in primary keratinocytes after incubation with 1 μM E_2 revealed a rate of E_1 formation of about 23 pmol × 10^{-6} cells × h^{-1} (23). With a typical cell number of 3.9×10^5 per cm^2 determined

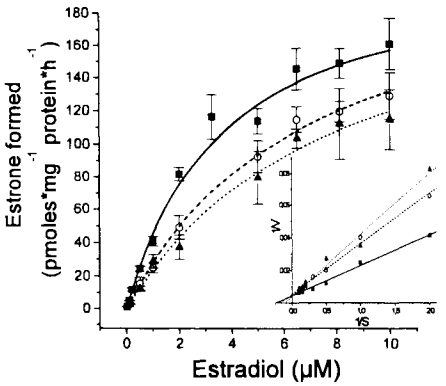

Fig. 2. Formation of estrone as a function of substrate concentration. HaCaT cells were incubated with ^{3}H-estradiol for 1.5 hours. Incubations with estradiol only (■, solid line) and with 5 μM DHEA (○, dashed line) or 10 μM DHEA (▲, dotted line) are presented. The curves were fitted with the Michaelis-Menten equation. The inset graph represents a Lineweaver-Burk plot. Each data point represents the mean and standard deviation of three determinations.

from growth curves of HaCaT cells at confluency (data not shown) and an average protein content of 0.21 mg × cm^{-2} previously estimated (14), a velocity of E_1 formation of 21.9 pmol × 10^{-6} cells × h^{-1} results for the HaCaT cell line at this E_2 concentration. Since both rates are very similar, it can be assumed that the HaCaT cells exhibit similar properties compared to normal keratinocytes with respect to their enzymatic pattern.

From the above findings, it seems that the HaCaT cell line represents a model suitable for studying kinetics of steroid metabolizing enzymes. This model system has some advantages in comparison to other in vitro methods. In contrast to excised skin, the cell line is readily available and expresses a higher reproducibility, due to the lack of inter-individual variations. In comparison to homogenates, which are frequently used for estimating enzyme kinetics, intact HaCaT cell layers exhibit the compartmentation of enzyme systems as occurring in vivo. In homogenates, enzymes are separated from their physiological environment, which may alter their characteristics (19). Furthermore, the harsh homogenization methods used may result in the destruction of enzyme activity.

Inhibition by DHEA

From Fig. 2, it can be also seen that the metabolism of E_2 was inhibited in a concentration dependent manner by DHEA. From the Lineweaver-Burk plot in Fig. 2 and the calculated K_m and V_{max} values shown in Table 1, it was derived that DHEA acted by a competitive inhibition mechanism.

The observed inhibition characteristics may also have practical consequences, because there is growing interest in the transdermal delivery of DHEA (24). The simultaneous application of E_2 and DHEA for the therapy of postmenopausal symptoms as a parenteral depot system is well established (25). Therefore, there may be a rationale for the development of transdermal systems for the combined application of both steroids, with the advantage of a reduced cutaneous first pass metabolism of E_2.

Inhibitory Effect of Ethanol

Previously, several studies have established the inhibitory action of ethanol on the biotransformation of E_2 (9) and other drug substances or endogenous substrates (26). While the ethanol concentration after alcohol ingestion rarely exceeds 0.4% (v/v) (27), there may be elevated local concentrations after topical application of ethanol. This fact was previously realized and investigated in hairless mouse skin for E_2 (9). In this study, the interpretation of results was complicated, because two effects, the diffusion and metabolism of E_2 were simultaneously

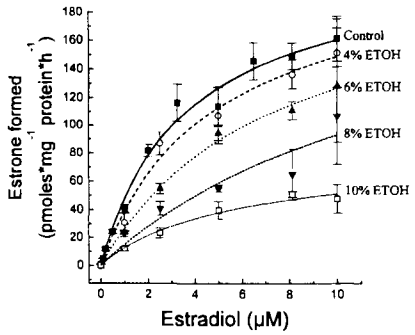

Fig. 3. Influence of ethanol on formation rates of estrone as a function of substrate concentration. HaCaT cells were incubated with ^{3}H-estradiol (0.1–10 µM) for 1.5 hours. The curves were fitted with the Michaelis-Menten equation. Each data point represents the mean and standard deviation of three determinations. (■—, 0; ○--, 4; ▲· · ·, 6; ▼---, 8; □·····, 10% (v/v) ethanol).

investigated. It is well known, that ethanol strongly enhances the transport of E_2 across the skin. For testosterone, it was reported that the relative extent of cutaneous first pass metabolism was decreased, when the amount of permeating substrate was increased, due to the potential saturation of the cutaneous drug metabolism enzymes (16). Therefore, an overestimation of the inhibitory effect of ethanol may result, if the enhanced transport rates of substrate and metabolite are assayed.

To determine the effect of ethanol on metabolism kinetics of E_2 in the metabolic active skin cells, the keratinocytes, HaCaT cells were incubated with different amounts of ethanol from 1 to 10% (v/v). The addition of 1 or 2% (v/v) ethanol had no significant effect on E_2 metabolism compared to control incubations. The in vitro rates of E_1 formation in relation to the substrate concentration in the presence of ethanol are shown in Fig. 3. With 4 up to 10% (v/v) alcohol a significant reduction of E_1 formation was detected. The apparent K_m and V_{max} values calculated from the Michaelis-Menten fit are presented in Table 2. The V_{max} values for control incubations and the reaction in presence of 4 to 8% (v/v) ethanol are similar, while the K_m values differed significantly, suggesting that ethanol acted by a competitive inhibition mechanism in HaCaT cells. This kind of mechanism was also determined for the inhibitory effect of ethanol on the isolated enzyme 17β-HSD (28) and other drug metabolizing enzymes (26). For the addition of 10% (v/v) ethanol the apparent V_{max} value was significantly lower than seen in control incubations, suggesting an altered inhibition mechanism existing for this higher alcohol concentration.

Table I. Kinetic Parameters for the Oxidation of E_2 in HaCaT Cells in the Presence of DHEA

	K_m[a] (µM)	V_{max}[a] (pmol × h^{-1} × mg^{-1} protein)
Control	3.51	215,7
5 µM DHEA	6.97	223,3
10 µM DHEA	8.10	216,4

[a] Data were calculated from Michaelis-Menten fit.

Table 2. Kinetic Parameters for the Oxidation of E_2 in HaCaT Cells in the Presence of Ethanol

Ethanol concentration	K_m[a] (µM)	V_{max}[a] (pmol × h^{-1} × mg^{-1} protein)
Control	3.51	215,7
4% (v/v)	4.57	215,6
6% (v/v)	7.25	217,6
8% (v/v)	13.37	216,2
10% (v/v)	5.25	77,8

[a] Data were calculated from Michaelis-Menten fit.

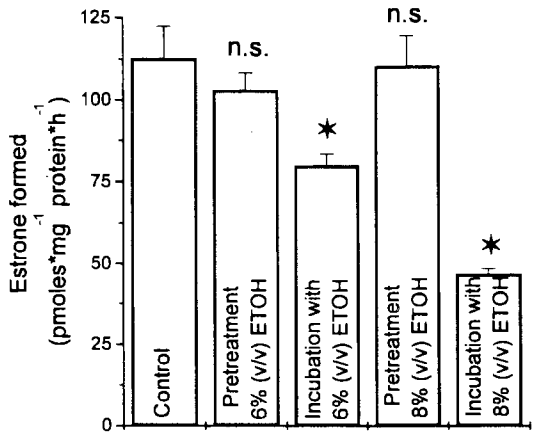

Fig. 4. Effect of ethanol pretreatment on the metabolism of E_2. HaCaT cells were incubated with 5 μM E_2 for 1.5 hours after pretreatment with 6 and 8% (v/v) ethanol. Control incubations were performed in the absence of alcohol or by simultaneous incubation with 5μM E_2 and 6 or 8% (v/v) ethanol. Results are expressed as the mean of three determinations ± SD. A two-sided independent t-test was performed related to the control incubation (n.s., not significantly different at 0.05 level; ⋆, $p < 0.01$)

To evaluate reversibility of the observed effect, HaCaT cells were preincubated with 6 and 8% (v/v) ethanol, washed and afterwards incubated with 5 μM E_2. In Fig. 4 the formation rates of control incubations, experiments after ethanol pretreatment and incubations with simultaneous application of ethanol and E_2 are presented. It can be derived, that pretreatment with ethanol had no effect on the 17β-HSD activity in HaCaT cells, although simultaneous treatment significantly suppressed the reaction. Therefore, it can be concluded, that the effect of ethanol application for 1.5 hours up to 8% (v/v) is completely reversible.

Toxicity of Ethanol

To further characterize the observed effect of ethanol on E_2 metabolism, the toxicity of the solvent was determined in HaCaT cells. Two different tests were performed to estimate the effect on different cell structures. In Fig. 5 the LDH release from HaCaT cells in dependence on ethanol contents in the incubation medium is presented. This test reflects cell membrane damages caused by foreign substances. Values are expressed as percent of total LDH content of cells. It can be seen, that the LDH release slightly increased from 4 to 8% (v/v), while at 10% and more impressively at 12% (v/v) ethanol concentration the release distinctly increased. The effect of ethanol on the mitochondrial dehydrogenases, estimated by the MTT test, is shown in Fig. 6. Again, addition of up to 8% (v/v) ethanol to the incubation medium for 1.5 hours had no significant effect on the dehydrogenase activity. Higher alcohol concentrations (≥10% v/v) decreased MTT formation to values below 20% of control experiments.

In summary, it was shown that HaCaT cells are relatively resistant to environmental ethanol. This is in accordance to another study, where the toxic effects of several enhancers on keratinocytes and fibroblasts were investigated (29).

For our incubation conditions, an elevated toxicity at ethanol concentrations ≥10% (v/v) with regard to membrane damage and mitochondrial activity was detectable. This may explain the deviation from the competitive inhibition mechanism, when 10% (v/v) ethanol was present during the incubation. In this case, a reduced cell viability and/or partially destruction of enzyme systems may result, decreasing the maximum metabolism rate (V_{max}).

However, the reduction in E_1 formation caused by ethanol contents from 4 to 8% (v/v) was due to a reversible inhibition of 17β-HSD activity and was not influenced by toxic effects of the enhancer on the cells.

CONCLUSIONS

The human keratinocyte cell line HaCaT was found to be suitable for studying the metabolism kinetics of estradiol (E_2), as well as the inhibitory effects of DHEA and ethanol on this metabolic reaction. Regarding the advantages of this readily available model system, such as a high degree of reproducibility for steroid metabolism (14) in a controlled environment and a compartmentation of enzyme systems comparable to living skin (19), the HaCaT model represents a readily available tool in the investigation of enzyme kinetics.

For the enhancer ethanol, it was observed that 4 to 8% (v/v) inhibited 17β-HSD activity in a concentration dependent

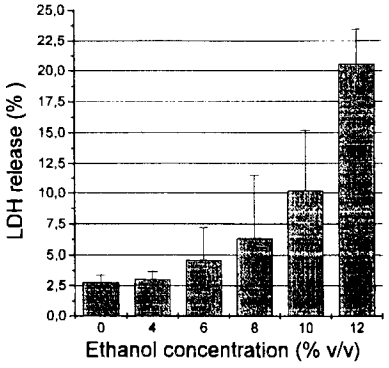

Fig. 5. LDH release from HaCaT cells after incubation with different amounts of ethanol in PBS supplemented with glucose for 1.5 hours. Values are expressed as percentage of total LDH release after incubation with 0.1% Triton. Values are mean and standard deviation of three determinations.

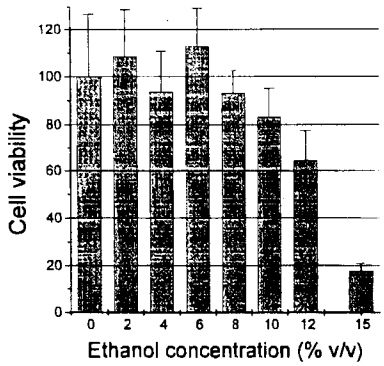

Fig. 6. MTT formation in HaCaT cells after incubation with different amounts of ethanol in DMEM + HEPES. Viability in medium without ethanol was set as 100%. Values are mean and standard deviation of eight determinations.

manner, while higher ethanol levels (≥10% v/v) irreversibly damaged the keratinocytes. However, skin cells were shown to be rather resistant to ethanol treatment, supporting the thesis that ethanol represents a relatively safe enhancer (29).

The inhibitory effect may have consequences for the use of the MCRS delivering E_2, because both substances, ethanol and E_2, are released from the patch and permeate the skin. Since it is difficult to determine the absolute alcohol concentration in the skin during the application of the patch, we are not able to clearly evaluate the influence of the solvent on 17β-HSD activity in living skin. However, from another study, where two transdermal systems for the delivery of nitroglycerin were compared, a smaller extent of metabolism was observed in an ethanol-based system compared to an ethanol-free system (30). This indicates, that the design and the composition of transdermal systems may influence the cutaneous first pass metabolism of the delivered drug.

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