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Characterization of xenobiotic metabolizing enzymes of a reconstructed human epidermal model from adult hair follicles

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ABSTRACT

In this study, a comprehensive characterization of xenobiotic metabolizing enzymes (XMEs) based on gene expression and enzyme functionality was made in a reconstructed skin epidermal model derived from the outer root sheath (ORS) of hair follicles (ORS-RHE). The ORS-RHE model XME gene profile was consistent with native human skin. Cytochromes P450 (CYPs) consistently reported to be detected in native human skin were also present at the gene level in the ORS-RHE model. The highest Phase I XME gene expression levels were observed for alcohol/aldehyde dehydrogenases and (carboxyl) esterases. The model was responsive to the CYP inducers, 3-methylcholanthrene (3-MC) and β-naphthoflavone (βNF) after topical and systemic applications, evident at the gene and enzyme activity level. Phase II XME levels were generally higher than those of Phase I XMEs, the highest levels were GSTs and transferases, including NAT1. The presence of functional CYPs, UGTs and SULTs was confirmed by incubating the models with 7-ethoxycoumarin, testosterone, benzo(a)pyrene and 3-MC, all of which were rapidly metabolized within 24 h after topical application. The extent of metabolism was dependent on saturable and non-saturable metabolism by the XMEs and on the residence time within the model.
In conclusion, the ORS-RHE model expresses a number of Phase I and II XMEs, some of which may be induced by AhR ligands. Functional XME activities were also demonstrated using systemic or topical application routes, supporting their use in cutaneous metabolism studies. Such a reproducible model will be of interest when evaluating the cutaneous metabolism and potential toxicity of innovative dermo-cosmetic ingredients.
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1. Introduction

The outer root sheath-derived reconstructed human epidermal (ORS-RHE) model is a three-dimensional human skin equivalent model engineered from adult undifferentiated keratinocytes from ORS cells of hair follicles. This model was developed in order to establish a cost-effective and reproducible in vitro human epidermal model that could be customized according to specific assay needs (Guiraud et al., 2014). The use of ORS derived keratinocytes affords a number of advantages over other RHE models, including the ease of the rapid and non-invasive sample collection, the ability to select a particular donor (gender, age, healthy or diseased) and, moreover, the selection of adult donors, as opposed to neonatal donors that are commonly used in commercial models. The ORS-RHE model was shown to be a good and reproducible alternative to native human epidermis, such that the architecture, general stratification and localization of differentiation markers were similar to those of native epidermis (Guiraud et al., 2014). This ORS-RHE model has been shown to be a good alternative to human skin for studying the effects of UV radiation (e.g. bipyrimidine photoproduct production, repair and induction of apoptosis) and investigating the sunscreen genoprotection (Bacqueville et al., 2015). Here, our intention was to extend the use of the ORS-RHE model to metabolism studies in the same way as previous reports using RHE models, such as SkinEthic, Phenion® Full-Thickness skin model and EpiDerm™,

Abbreviations: ADH, alcohol dehydrogenases; ALDH, aldehyde dehydrogenases; AhR, aryl hydrocarbon receptor; PI-APCI, APCI source in the positive mode; B(a)P, benzo(a)pyrene; βNF, β-naphthoflavone; CES1/2, carboxylesterase 1 and 2; COMT, catechol-*O*-methyltransferase; CYP, cytochrome P450; EPHX, epoxide hydrolase; ESD, esterase D; NI-ESI, ESI source in the negative mode; 7-EC, 7-ethoxycoumarin; EROD, ethoxyresorufin *O*-deethylase; FMO, flavin monooxidase; GST, glutathione S-transferase; HRMS, High Resolution Mass Spectrometry; HSD17B10, hydroxysteroid (17-β dehydrogenase 10); HC, hydroxycoumarin; ACSL1, long-chain-fatty-acid-CoA ligase 1; 3-MC, 3-methylcholanthrene; NAA20, N(alpha)-acetyltransferase 20; NAT, *N*-acetyl transferase; OECD, Organization for Economic Cooperation and Development; ORS-RHE, outer root sheath-derived reconstructed human epidermis; PTGS2, prostaglandin-endoperoxide synthase; RT-qPCR, real time reverse transcription PCR; SAT1, spermine N1-acetyltransferase 1; SULT, sulfotransferase; UCHL3, ubiquitin carboxyl-terminal esterase L3; UGT, UDP glucuronosyltransferase; XMEs, xenobiotic metabolizing enzymes.

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that support their applicability to this purpose by characterizing the functional activities of several Phase I and II xenobiotic metabolizing enzymes (XMEs) in these models (Eilstein et al., 2015; Jäckh et al., 2011).

The rate of percutaneous absorption of some compounds is known to be influenced by skin metabolism (Lockley et al., 2004; Jacques et al., 2010a), and as compounds penetrate the skin, they may be subject to metabolism, which can detoxify them to more water-soluble metabolites or bioactivate them to reactive metabolites (Bronaugh and Stewart, 1985; Oesch et al., 2007, 2014). Therefore, in order to help interpret toxicity endpoints, it is important to characterize the metabolic capacity of the skin models used in bioassays. In this study, we aimed to characterize as precisely as possible the metabolic capabilities of the ORS-RHE model To this end, we first investigated the overall basal XME gene expression profile of the ORS-RHE model using RT-qPCR analysis of >90 Phase I and 70 Phase II XME genes. Since the most relevant route of exposure to the skin is topical, it is important to show that RHE models can be used to investigate topically applied chemicals. This is also of importance since the metabolic capacity of such models could be altered during the course of topical treatment with a test compound (Hewitt et al., 2013). Therefore, we measured the effect of two known AhR ligands, namely β-naphthoflavone (βNF) and 3-methylcholanthrene (3-MC), on XME gene expression after systemic (added to the culture medium, located below the model) and topical (added to the upper surface of the model) application. The expression of a XME gene does not necessarily indicate that the corresponding XME is functional. In addition, we confirmed induction effects by incubating control and inducer-treated skin models with the CYP1A1/2 and CYP1B1 selective substrate, ethoxyresorufin.

In addition to analyzing the XME profile of the ORS-RHE model, we complemented this by investigating functional XME activities by measuring the metabolism of four test chemicals with varying metabolic pathway complexities. The two endpoints (gene expression and functional metabolism) were compared using similar timepoints (24 h). Additional timepoints (48 and 72 h) were added for functional metabolic studies, to capture the kinetics of metabolism. The test chemicals were 7-ethoxycoumarin (7-EC), testosterone, B(a)P, and 3-MC. 7-EC represents a chemical with a simple metabolite profile involving both Phase I and II sequential pathways (De Kanter et al., 1999). The skin is known to be able to metabolize steroids (Haag et al., 2012); therefore, we selected testosterone as a model of an endogenous steroid and a well-established diagnostic substrate for CYP activities. This substrate can also be metabolized through reductive pathways. Since the reductive capacity of the skin has previously been reported to be good (van Eijl et al., 2012) testosterone represents an ideal substrate to confirm the functional oxido-reductive capacity of the model. Testosterone is also specified in the Organization for Economic Cooperation and Development (OECD) adopted guideline 28 (OECD, 2004a) and a corresponding technical guidance document (OECD, 2004b) to describe methods assessing drug absorption as one of the model compounds to develop skin alternative models. B(a)P is a known genotoxic compound which toxicity relies on a CYP450-dependent bioactivation, into ultimate DNA reactive metabolites (Conney et al., 1994). B(a)P has been also shown to induce EROD activities in the epidermal model, Epiderm™, 24 h after topical application (Götz et al., 2012), suggesting it induces its own metabolism and that this model responds to CYP1 induction. B(a)P is also detoxified by GSTs, SULTs and UGTs, so it is important to understand the balance of metabolism of such a genotoxic compound in human skin, especially to help interpret negative outcomes in genotoxicity assays using skin epidermal models (Aardema et al., 2013). 3-MC is a direct genotoxin and is metabolized via Phase I and II pathways. It was used to investigate the inducibility of XMEs in ORS-RHEs and therefore it was of interest to measure its metabolism alongside its induction effects.

These studies aimed to provide a comprehensive characterization of the XMEs present in the novel ORS-RHE model by analyzing both gene expression profiles (PCR) and functional activities (EROD activity and test chemical metabolism). The suitability of the model for compound metabolism assays was evaluated, as well as the application of test compounds by the systemic and topical routes.

2. Materials and methods

2.1. Chemicals

7-ethoxy[3-^{14}C]-coumarin, [U-^{14}C]-bisphenol A, [5,6-^{14}C] benzo(a)pyrene [^{14}C-B(a)P], [4,7-^{14}C]-testosterone with specific activities of 2, 2.074, 2 and 2.03 GBq/mmol, respectively, were purchased from Amersham Biosciences (Buckinghamshire, UK). 3-Methylcholanthrene-[^{3}H] was purchased from Moravek Biochemicals (Brea, United States). Unlabeled 7-EC, B(a)P, 3-MC and testosterone, purchased from Sigma-Aldrich (St Quentin Fallavier, France) had a purity >98.5%.

Benzo(a)pyrene-r-7,t-8,t-9,t-10-tetrahydrotetrol, benzo(a)pyrene-cis-7,8-dihydrodiol, benzo(a)pyrene-cis-4,5-dihydrodiol, benzo(a)pyrene-1,6-dione, benzo(a)pyrene-3,6-dione, benzo(a)pyrene-7,8-dione, 8-hydroxybenzo(a)pyrene, 9-hydroxybenzo(a)pyrene, 3-hydroxybenzo(a)pyrene, 7-hydroxybenzo(a)pyrene were obtained from the National Cancer Institute Chemical Carcinogen Reference Standards Repository (Kansas City, MO, USA) and were used as reference compounds.

Testosterone reference metabolites were from Sigma-Aldrich (St Quentin Fallavier, France): 2β-hydroxytestosterone, 6β-hydroxytestosterone, 7α-hydroxytestosterone, 11β-hydroxytestosterone, 16α-hydroxytestosterone, 11-ketotestosterone, 1-dehydrotestosterone, 4-androstene-3,17-dione, 4-androsten-11β-ol-3,17-dione, 4-androsten-16α-ol-3,17-dione, epitestosterone, androsterone, epiandrosterone, 5β-androstan-3α-ol-17-one, 5β-androstan-3β-ol-17-one, 5α-androstan-3,17-dione and 5β-androstan-3,17-dione.

Other chemicals and solvents (analytical grade) were purchased from the following sources: ammonium acetate, sodium acetate, phosphate buffer (0.01 M) and sodium hydroxide, from Sigma-Aldrich; acetonitrile and ethyl acetate from Scharlau chemie S.A. (Barcelona, Spain); ethanol and acetic acid from Merck (Briare-Le-Canal, France). Ultrapure water from a Milli-Q system (Millipore, Saint Quentin en Yvelines, France) was used for HPLC mobile phase preparations. Dulbecco's Modified Eagle Medium (DMEM) and L-glutamine were obtained from Gibco™ (Cergy Pontoise, France). The antibiotics (streptomycin/penicillin, gentamycin) and the antifongic (fungizone) used in the culture media were purchased from Sigma-Aldrich.

2.2. Preparation of reconstituted human epidermis

Human epidermis was reconstructed using ORS keratinocytes isolated from hair follicles obtained from three healthy Caucasian volunteers who had given their informed consent. The tissues were engineered in-house according to the protocol described by Guiraud et al. (2014). Fig. 1A provides an overview of the culture of the ORS-RHE tissues and the structure of the tissues at the time of incubation (histology). Briefly, plucked hair follicles were explanted on a microporous membrane of a cell culture insert (Costar, Corning, New York, US) with post-mitotic human dermal fibroblasts sub-lethally treated with mitomycin C on the underside. Follicles were submerged in the medium after 6 days and then cultured for a further 2 to 3 weeks before ORS cell harvesting and long-term storage. The growth medium (0.5 mL and 1 mL per incubation for EROD activity and qPCR/metabolism assays, respectively) contained DMEM/Ham's F12 (3:1) supplemented with 10% fetal calf serum, epidermal growth factor, hydrocortisone, adenine, triiodothyronine, insulin, amphotericin and antibiotics and was refreshed three times a week. For tissue reconstruction, the ORS cells were then plated at a density of 0.3×10^6 cells/cm^2 on cell culture inserts, again with post-mitotic human dermal fibroblasts as described previously. After 2–3 days, the keratinocytes reached confluence, at which time the cultures were lifted to the air–medium interface in order to

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