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Keratinocytes Synthesize and Activate Cortisol

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ABSTRACT

The bioavailability of circulating and/or endogenous hydrocortisone (cortisol) in epidermal cells is a key determinant in inflammatory disease and chronic wounds. It is not known, however, whether epidermal cells can regulate tissue cortisol and whether they are capable of producing endogenous glucocorticoids. In the present study, we show by microarray analysis that epidermal cells express mRNAs to all the major enzymes involved in the metabolic chain from cholesterol to cortisol, including cytocrome P450 chain, 11β-hydroxysteroid dehydrogenases (HSD11Bs), adrenocorticotropic hormone (ACTH) receptor (MC2R), and glucocorticoid receptor. The two enzymes mediating activation/deactivation of cortisone to cortisol, namely HSD11B1 and HSD11B2, were expressed at the protein level in cultured keratinocytes as well as human skin samples, as shown by Western blotting and immunohistochemistry, respectively. In functional assays, we show that keratinocytes are not only able to activate cortisone to cortisol in a HSD11B-dependent manner but also silencing of either HSD11B1 or HSD11B2 specifically modulates the bioavailability of the inactive glucocorticoid and the active steroid, respectively. A further key observation was that keratinocytes responded to stimulation with ACTH by a significant increase in the de novo synthesis of cortisol. Taken together, we provide evidence for a novel non-adrenal steroideal system in human keratinocytes. J. Cell. Biochem. 112: 1499–1505, 2011.

KEY WORDS: CORTISOL; 11β-HSD; KERATINOCYTES; GLYCYRRHETINIC ACID; SKIN

The principal glucocorticoid hormone in humans is hydrocortisone or cortisol (corticosterone in rodents) which is synthesized in the adrenal cortex from cholesterol and then secreted in response to pituitary-derived adrenocorticotropic hormone (ACTH) [Viau, 2002]. Cholesterol is processed by a chain of cytochrome P450 enzymes and transformed into cortisone, the inactive form of cortisol. The activation of cortisone occurs via 11β-Hydroxysteroid dehydrogenase (HSD) 1 (gene name HSD11B1), whereas the reverse, cortisol to cortisone, involves 11β-HSD2 [Tomlinson et al., 2004]. A schematic diagram of these changes is shown in Figure 1A.

It has long been thought that the magnitude of glucocorticoid action was determined by the circulating concentration of active steroid together with the intracellular concentration of glucocorticoid receptors (GR) in the target tissues [Odermatt and Nashev, 2010]. In the past two decades, however, the intracellular interconversion of inactive and active glucocorticoids by the 11β-hydroxysteroid dehydrogenases has emerged as a key mechanism of tissue-specific regulation of glucocorticoid action. Early studies demonstrated significant amounts of 11β-HSD activity in human placenta [Osinski, 1960], kidney [Jenkins, 1966], and liver [Bush, 1969] but more recently, it has been shown that fibroblasts and adipocytes have the potential to activate cortisone to cortisol [Hammami and Siiteri, 1991; Bujalska et al., 1997]. Unfortunately, there is very little information as to whether an endogenous steroideal system exist in human skin and what data have been reported relate to the presence of steroidogenic enzymes in dermal fibroblasts [Hammami and Siiteri, 1991] and melanocytes [Slominski et al., 2004]. Furthermore, it is not known whether epidermal keratinocytes can synthesize hydrocortisone de novo on stimulation with ACTH.

Glucocorticoids are exogenously administered to treat a large number of diseases that require anti-inflammatory activity and immunosuppression [Hengge et al., 2006; Jackson et al., 2007; Stahn and Buttgereit, 2008]. Topical and systemic preparations of corticosteroids are probably most widely used in Dermatology and Oral Medicine but the mechanisms by which they induce disease remission are unclear. Several lines of evidence suggest that corticosteroids control skin disease via actions that are not entirely associated with their immunosuppressive properties but instead, they may have direct effects on keratinocytes [Nguyen et al., 2004]. This notion suggests that epidermal cells may be functionally responsive to corticosteroids. In an attempt to characterize the biochemical mechanisms that account for the direct effects of

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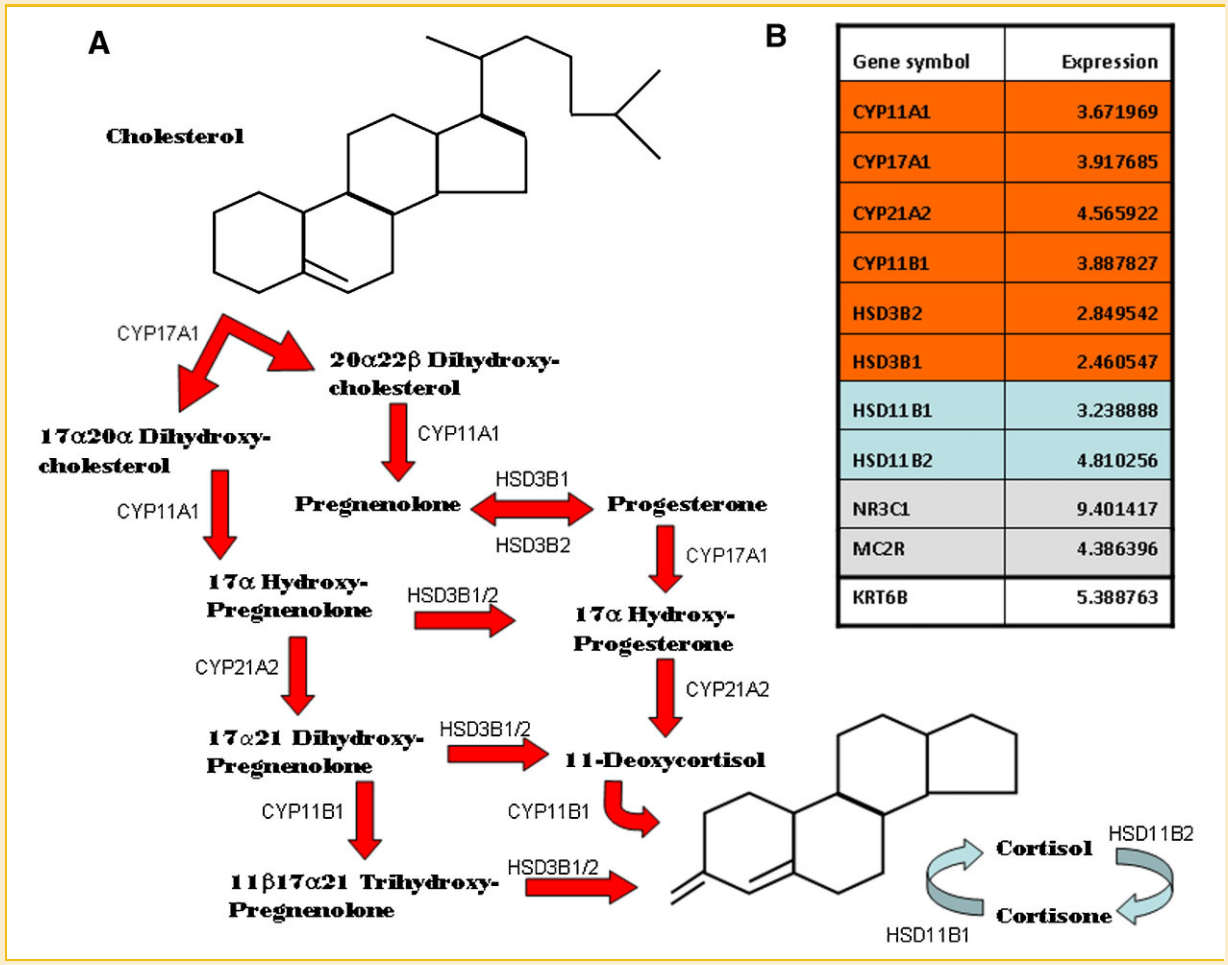

Gene symbol	Expression
CYP11A1	3.671969
CYP17A1	3.917685
CYP21A2	4.565922
CYP11B1	3.887827
HSD3B2	2.849542
HSD3B1	2.460547
HSD11B1	3.238888
HSD11B2	4.810256
NR3C1	9.401417
MC2R	4.386396
KRT6B	5.388763

Fig. 1. A: Metabolic pathway demonstrating the production of cortisol from cholesterol. The cholesterol side-chain cleavage enzyme CYP11A1 catalyzes conversion of cholesterol, a C27 compound, to the first C21 steroid pregnenolone which, in turn, is converted by a bifunctional enzyme complex to the gestagen hormone, progesterone. Pregnenolone and progesterone are the starting materials for the C21 steroids of glucocorticoids and mineralocorticoids. Cortisol, the main glucocorticoid, is then formed from 17alpha-hydroxyprogesterone with 11-deoxycortisol as an intermediate. B: Microarray analysis of mRNA transcripts in HaCat keratinocytes. In addition to the eight enzymes reported in Figure 1, levels of GR receptor (NR3C1) and ACTH receptor (MC2R) are also shown. KRT6B is reported as a control. Expression levels >2 arbitrary units are considered positive.

corticosteroids on keratinocytes, we made a serendipitous observation that epithelial-derived cell lines expressed all of the enzymes of the glucocorticoid pathway associated with the inter-conversion of cholesterol and cortisol. The subsequent observation that local cortisol levels may be altered by the 11β-HSD inhibitor glycyrrhetinic acid (GA) led us to conclude that epidermal keratinocytes possess an endogenous system that controls glucocorticoid levels. This novel observation raises the possibility that components of the system could be targeted for the treatment of pathological processes such as inflammatory skin disease, wound healing, and cancer.

MATERIALS AND METHODS

CELL CULTURES AND REAGENTS

The culture of normal human oral keratinocytes, human skin keratinocytes, and mutant c-Ha-ras-transfected HaCaT cell clone RT-3 have been described previously [Boukamp et al., 1988; Prime et al., 1990; Fusenig and Boukamp, 1998]. Oral keratinocytes were cultured on mitomycin C-treated 3T3 fibroblasts whilst the HaCaT cells and RT-3 were grown without mesenchymal support. Primary keratinocytes were cultured with GIBCO Keratinocytes Serum Free Medium (GIBCO KER-SFM) containing 0.4 ng/ml bovine pituitary extract (PBE), 0.2 ng/ml epidermal growth factor (EGF), 0.3 mM CaCl2, and Pen/Strep antibiotic solution. HaCat cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and were grown in a humidified atmosphere of 5% CO_2/air at 37°C. RT-3 were grown in media containing 400 μg/ml geneticin sulphate (G418; PAA laboratories, UK). At the time of experimentation, cells were seeded on 35-mm Petri plastic dishes and grown to confluence.

Antibodies against the residues 65–164 of human 11β-HSD1 and residues 261–405 of human 11β-HSD2, mouse IgG to alpha-tubulin,

18 β-GA, ACTH, siRNA pools specific to 11β-HSD1 and 11β-HSD2 and scrambled siRNAs (negative control) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Nitrocellulose membranes were purchased from Invitrogen, reagents for enhanced chemiluminescence and films were from Amersham Biosciences, the majority of reagents for protein extraction and cell culture were from Sigma, and keratinocyte growth medium and antibiotics/antimycotic were from Invitrogen.

RNA EXTRACTION AND MICROARRAY ANALYSIS

Total RNAs were extracted from cultured cells using Trizol™ solution (Invitrogen), according to the manufacturer's instructions. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis (virtual presence of sharp 28 and 18 S bands) [Napoli et al., 2005]. Five microgram total RNA was used as starting material for the cDNA preparation. The first and second strand cDNA syntheses were performed using the GeneChip One-Cycle cDNA Synthesis Kit (Affymetrix). Labeled cRNA was prepared using the GeneChip IVT Labeling Kit (Affymetrix) according to the manufacturer's instructions. Following the IVT reaction, the unincorporated nucleotides were removed using RNeasy columns (Qiagen) [Lanza et al., 2008]. Fifteen microgram cRNA was used for hybridization onto the Affymetrix Human Genome U133 2.0 probe array cartridge. The washing and staining procedures were performed in the Affymetrix Fluidics Station 450. The probe arrays were scanned at 560 nm using a confocal laser-scanning microscope (GeneChip Scanner 3000). The readings from the quantitative scanning were analyzed by the Affymetrix Gene Expression Analysis Software. Only genes that had the threshold >2 arbitrary detection units were included in the list of relevant genes. The complete list of oligonucleotide sequences utilized is reported in Supplementary Table I.

BIOSYNTHETIC PATHWAY OF CORTISOL

To select the enzymes involved in the biosynthetic pathway associated with the inter-conversion of cholesterol and cortisol, the KEGG pathway database (http://www.genome.jp/kegg/pathway.html) was used; he entry "hsa00140" was selected to identify the components of steroid hormone biosynthesis. Enzyme codes were as follows: 1.14.15.6 for CYP11A1; 1.14.15.4 for CYP11B1; 1.1499.9 for CYP17A1; 1.1499.10 for CYP21A2; 1.1.1.145 for HSD3B1; 5.3.3.1 for HSD3B2; 1.1.1.146 (3290) for HSD11B1; and 1.1.1.146 (3291) for HSD11B2.

WESTERN BLOTTING AND IMMUNOHISTOCHEMISTRY

Western blotting on whole cell lysates was performed according to standard procedures [Cirillo et al., 2006]. The primary antibodies to 11β-HSD1/2 were used at 1:500 and the species-specific secondary IgG was diluted at 1:5,000. Formalin-fixed, paraffin-embedded skin biopsies from individuals without skin disease were subjected to immunohistochemical analysis as described by us previously [Lanza et al., 2008]. Primary antibody was withheld from negative controls, which were incubated in diluent alone.

siRNA TRANSFECTION EXPERIMENTS

HaCaT cells were used as an in vitro model for functional assays of cortisol production in silencing experiments. The 11β-HSD1/2-directed siRNA pools and the negative control pool were transfected at a final concentration of 80 nM, according to the manufacturer's instructions. Cultured HaCaT cells were incubated overnight with the siRNAs in serum-free transfection medium (sc-36868) and then for an additional 24 h in complete medium with 10% FBS [Cirillo and Prime, 2009]. Cells were then treated as specified in the Results section. The efficiency of transfection was monitored by Western blot analysis of 11β-HSD1 and 11β-HSD2 expression.

ASSESSMENT OF CORTISOL LEVELS BY ELISA

HaCaT keratinocytes were grown to confluence and incubated with cortisone (100 nM), ACTH (10 nM), and 0.5% (w/v) 18 β-GA, according to the experimental protocol specified in the Results section. Cells were incubated with the above compounds, together or in isolation, in serum-free DMEM. The absence of cortisol in the control medium was always checked by ELISA in control experiments. The conditioned medium was collected after 24 h and cortisol levels were assessed with a Cortisol Parameter Assay Kit (R&D System) and quantified at 415 nm using the ELx808 microplate reader (BioTek Instruments, Inc., Winooski, VT).

STATISTICAL ANALYSIS

Statistical significance of the data was performed using the Student's *t*-test. Mean differences were considered to be significant when $P < 0.05$.

RESULTS

MICROARRAY ANALYSIS REVEALS THAT THE ENZYMES INVOLVED IN THE BIOSYNTHETIC PATHWAY OF CORTISOL ARE EXPRESSED IN KERATINOCYTES

Microarrays were performed with RNAs extracted from normal and malignant keratinocytes to assess basal expression. All of the key enzymes (n = 8) involved in cortisol production were detected in HaCaT cells (Fig. 1B). In addition to the cytochrome P450 chain, GR and ACTH receptors (MC2R) were found at high levels. More importantly, mRNAs for the two enzymes catalyzing activation (HSD11B1) and deactivation (HSD11B2) of cortisol were detected. The levels of mRNAs to the same molecules were also investigated in the cancer cell line RT-3 and were all positive, which suggests that the biosynthetic apparatus leading to cortisol production is retained in cancer cells. The complete Affymetrix results of the relevant molecules and ontology assessment is reported in Supplementary Table II.

Taken together, these data demonstrate that keratinocytes express the enzymes involved in cortisol biosynthesis at the level of transcription.

BOTH 11*B*-HYDROXYSTEROID DEHYDROGENASE ISOTYPES ARE EXPRESSED IN KERATINOCYTES AND EPIDERMAL TISSUES AT A PROTEIN LEVEL

Since HSD11B1 and HSD11B2 are key enzymes in cortisol activation and deactivation, we investigated their expression at a protein level

in both cultured cells and human skin tissue by Western blotting and immunohistochemistry, respectively. Both molecules were clearly detectable in primary normal keratinocytes and keratinocyte cell lines by Western blotting using standard experimental conditions (Fig. 2). Immunohistochemistry confirmed that both HSD11B1 and HSD11B2 were also present in the epidermis. The staining of HSD11B1 on skin was relatively weak and the antibody bound throughout the epidermis with equal intensity. In contrast, HSD11B2 exhibited a strong staining in the upper layers of the epidermis, especially the stratum granulosum, but not in the basal and suprabasal layers. This finding strongly suggests that HSD11B2 is associated with keratinocyte differentiation. No staining was found in tissues samples were primary antibody was omitted (negative control, not shown).

KERATINOCYTE REGULATE CORTISOL LEVELS THROUGH HSD11 ACTIVITY

To investigate whether epidermal keratinocytes metabolized cortisol, functional assays were undertaken. HaCaT monolayers were incubated with cortisone (100 nmol/L) alone or in the presence of 0.5% (w/v) 18β-GA, a HSD inhibitor that exhibits preferential activity to 11β-HSD2 [Zhang et al., 2009]. In cells exposed overnight to cortisone alone, the level of cortisol in the medium increased (Fig. 3). The results were not biased by the possible presence of cortisone in the culture medium since the manufacturer's report only a 0.2% cross reaction between cortisol and cortisone in the ELISA assay. Conversion of cortisone to cortisol was energy dependent since supernatants from cells incubated at 4°C did not show increased levels of cortisol (not shown). When pre-treated with the HSD11B2 inhibitor 18 β-GA, the concentration active cortisol in the supernatants was higher than in controls (Fig. 3A). These findings indicate that keratinocytes can convert cortisone to cortisol and modulate cortisol degradation.

In order to confirm the specific role of HSD11B1 and HSD11B2 in cortisol activation and deactivation, we employed siRNA technology. Cells silenced with HSD11B1-specific siRNAs produced significantly ($P < 0.05$) lower levels of cortisol after incubation with cortisone. By contrast, in HSD11B2-silenced cells, cortisol levels increased when compared to controls (Fig. 3B). These data demonstrate that the HSD11 isoenzymes can modulate the local concentration of cortisol in keratinocytes.

KERATINOCYTES SYNTHESIZE CORTISOL DE NOVO

We tested whether human keratinocytes were capable of producing cortisol de novo. Cells were cultured in the presence of ACTH (10 nM) and the culture medium was collected after 24 h. Cells

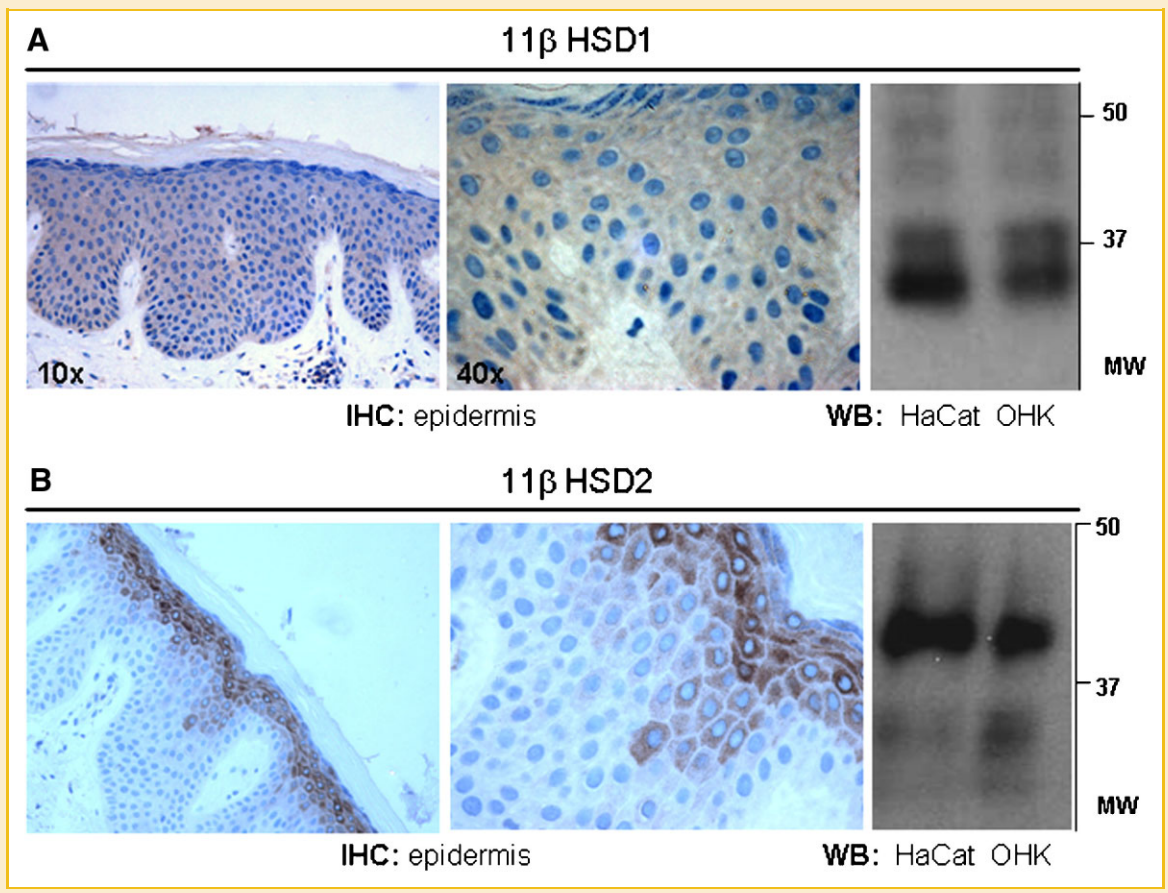

Fig. 2. 11β-HSD enzymes are expressed in keratinocytes. 11β-HSD1 (A) and 11β-HSD2 (B) were assessed in normal skin samples and cultured keratinocytes by immunohistochemistry and Western blotting, respectively. OHK, oral primary human keratinocytes. Typical bands at each sample were of the expected molecular weights and are representative of three independent experiments.

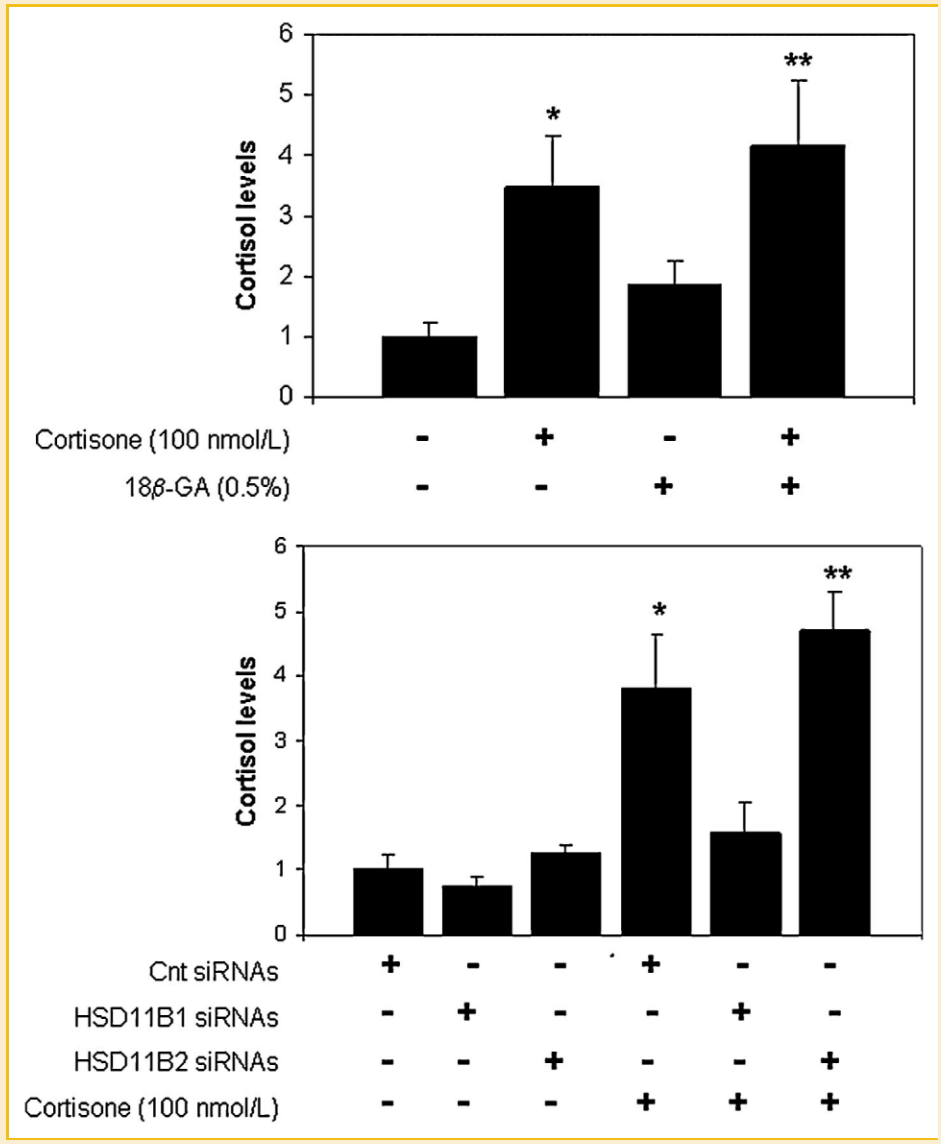

Fig. 3. 11β-HSD enzymes modulate the levels of cortisol. (A) HaCaT keratinocytes were treated for 24 h with cortisone (100 nmol/l) and/or 0.5% (w/v) 18β-glycyrrhetinic acid as reported in the graph, or left untreated. Cortisol levels were assessed from culture conditioned media. B: HaCaT cells were transfected with HSD11B-spefic siRNAs, or scrambled siRNAs as control, and then incubated with fresh medium in the presence or absence of cortisone (100 nmol/l). The levels of cortisol in the culture medium were assessed after 24 h. The results are shown as a mean of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

incubated with ACTH, but not those exposed to the vehicle alone, displayed an increase in cortisol levels (Fig. 4). The increase of cortisol in response to ACTH was dose-dependent (Supplementary Figure 1). Cortisol increased significantly both in cells treated with ACTH alone and those incubated with both ACTH and the HSD11 inhibitor 18 β-GA. Collectively, the data demonstrate that keratinocytes are not only able to activate cortisone to cortisol but also they respond to stimulation with ACTH by initiating de novo cortisol production.

DISCUSSION

In the present study, we report that keratinocytes produce and metabolize cortisol endogenously. First, we showed the presence of detectable amounts of mRNA transcripts of molecules involved in cortisol biosynthesis. Second, we confirmed the presence at the protein level of 11β-HSD1 and 11β-HSD2, enzymes that mediate the activation and deactivation of cortisol, in cultured keratinocytes and skin tissues. Third, we demonstrated that epidermal keratinocytes are able to modulate the local bioavailability of cortisol through the activity of 11β-HSD1/2 isoenzymes. And fourth, we showed that keratinocytes produce active cortisol de novo on stimulation with ACTH. To the best of our knowledge, this is the first article describing an endogenous glucocorticoid system in epidermal keratinocytes.

The expression of up to 10% of all genes in a higher organism are modulated by glucocorticoids in order for a time- and tissue-specific regulation of metabolic processes [John et al., 2009]. 11β-HSDs are specific regulators of glucocorticoid responses in tissues and act by

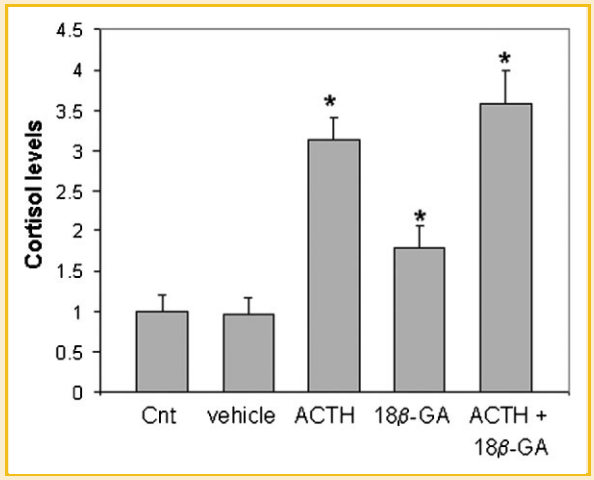

Fig. 4. ACTH induces cortisol synthesis in keratinocytes. HaCaT cells were incubated for 24 h with ACTH (10 nM), 0.5% (w/v) 18β–glycyrrhetinic acid, or both, and the cortisol levels in the supernatants were assessed by ELISA. Untreated keratinocytes (Cnt) and monolayers exposed to the vehicle only (ethanol, 10 μl) were used as controls. The mean level of cortisol in controls was arbitrarily fixed as = 1. The results are shown as a mean of four independent experiments.

the inter-conversion of biologically inactive glucocorticoids, such as cortisone and prednisone, to their active counterparts, cortisol (hydrocortisone), and prednisolone. Without these enzymes, cortisone and prednisone would be therapeutically ineffective. In human synovial tissue and bone, 11β-HSDs have been implicated in the control of synovial inflammation, the development of periarticular bone loss and the sensitivity of bone to therapeutic glucocorticoids [Hardy et al., 2008; Raza et al., 2010]. Significant expression of 11β-HSD1, but not 11β-HSD2, has been found in human adipose tissue, where inhibition of 11β-HSD1 prevents cortisone-mediated adipocyte differentiation [Bujalska et al., 1999].

Whilst it is recognized that skin can exert local endocrine functions, the ability to modulate epidermal glucocorticoid levels is usually attributed to dermal fibroblasts [Hardy et al., 2006]. An early article reported the presence of 11β-HSD in skin biopsy samples from healthy volunteers and from patients with psoriasis [Teelucksingh et al., 1990]. It was shown using skin vasoconstrictor assays that GA potentiated the action of hydrocortisone. At that time, it was thought that the only known isoform of 11β-HSD catalyzed exclusively the conversion of cortisol to the inactive steroid cortisone. Similar findings have also been reported in mice where the pharmacological inhibition of 11β-HSD potentiates the anti-inflammatory actions of glucocorticoids [Hennebold and Daynes, 1998]. These data are consistent with our own findings because we demonstrate that GA, by inhibiting 11β-HSD2, prevents the inactivation of hydrocortisone (cortisol) into cortisone. The present report, however, is the first not only to demonstrate the expression and activity of 11β-HSDs in keratinocytes but also the bi-directional modulation of cortisol levels by 11β-HSD isoforms.

In the present study, silencing of either 11β-HSD1 or 11β-HSD2 by siRNAs resulted in significant changes in the level of active cortisol in keratinocyte culture media. This finding raises the possibility that pharmacological targeting of these enzymes in epithelium by selective 11β-HSD inhibitors may have therapeutic benefit. For example, molecules blocking specifically 11β-HSD2 activity would increase the half-life of active cortisol in keratinocytes, thus potentiating its effect. This could be useful in inflammatory oral and skin disease such as psoriasis, lichen planus, and aphthous stomatitis. On the other hand, 11β-HSD1 inhibitors could be useful in chronic skin wounds in diabetic patients where glucocorticoids act by retarding/altering the wound-healing process. Deregulated levels of 11β-HSD1 mRNAs have also been found in certain types of cancer, including oropharyngeal carcinomas [Gronau et al., 2002], thus supporting the idea that the development of selective 11β-HSD inhibitors may have clinical application. Further research is needed to address these possibilities.

The other important original finding of the present study is that, in addition to expressing 11β-HSDs enzymes that metabolize glucocorticoids, we demonstrate that cultured keratinocytes can synthesize cortisol de novo after stimulation with ACTH. We believe that this is the first study to demonstrate that skin keratinocytes can act as a glucocorticoid-producing organ in culture monolayers. Previous work has focussed on the expression of 11β-HSDs enzymes or, at most, on the ability of 11β-HSDs to interconvert systemic/exogenous corticosteroids rather than examining the production of cortisol de novo. The present study extends previous observations [Slominski et al., 1995; Ito et al., 2005] and is consistent with the concept of peripheral HPA axis proposed by Slominski et al. [2007]; it also shows that the fundamental stages of steroidogenesis occur in keratinocytes and serve as a peripheral endocrine system specialized in glucocorticoid production.

In conclusion, we have described the first non-adrenal glucocorticoid-producing system in the epidermis. This novel system may have pathobiological implications in a variety of human diseases and could be potentially targeted for translational research in the near future.

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The authors wish to acknowledge that two papers relevant to this article have recently appeared online (Hannen et al., 2011; Vukelic et al., 2011). These new articles have been published during the editorial process of our JCB paper and therefore they could not be properly discussed in the present manuscript.

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