CRH stimulation of corticosteroids production in melanocytes is mediated by ACTH

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The skin, because of its location at the interface between external environment and internal milieu, acts to preserve body homeostasis. This is a complete function that includes the barrier-forming properties of the epidermis, the secretory activity of adnexal structures, and activities of the local immune and pigmentary systems, as well as vascular and mesenchymal components of the dermis. Being continuously exposed to acute transfers of solar, thermal, and mechanical energy, the skin requires instant responses for the restoration of its structural and functional integrity. Thus precise stress-response coordination is an additional cutaneous function that appears to be served by locally expressed neuroendocrine activities (17, 19, 20).

We have proposed that, because of the cutaneous expression of HPA mediators of the stress response and the similar functional relevance of the two structures, it was possible that the operational drives were organized in similar regulatory order (12, 17, 19, 20). This is based on the intrinsic capabilities of the skin to actually produce CRH peptides and POMC as well as express the corresponding receptors (6, 14, 15, 17, 19, 20, 24). The skin is also a powerful steroidogenic tissue, as it expresses genes and enzymes involved in the production of steroids and can also transform cholesterol to pregnenolone or progesterone to deoxycorticosterone and corticosterone (2, 9–11, 21, 23, 24). Cutaneous expression of these neuroendocrine elements is subject to regulation by environmental factors (17, 19, 20).

We therefore evaluated directly whether the cutaneous expression of endocrine mediators represents random usage or whether it is a full organizational duplication of the HPA axis (12, 17). The latter would assume that information flow in this cutaneous axis is structured hierarchically; thus CRH-mediated activation of CRH-R1 would be followed by POMC expression with production of ACTH, which would then stimulate cortisol/corticosterone production. Because α-melanocyte-stimulating hormone (α-MSH) is not a functional component of the sustained stress response, processing of ACTH to this peptide was not studied. In the current investigation, we used an experimental model of cultured normal epidermal melanocytes (expressing exclusively CRH-R1; Ref. 14), because pigmentary reaction is a classical response to the environmental stress represented by UV solar light exposure (13, 16, 17, 19, 20).

THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS is activated by stress-sensing central circuits to produce and release corticotropin-releasing hormone (CRH). This, in turn, stimulate anterior pituitary CRH receptor type-1 (CRH-R1) (3, 5), which enhances the production and secretion of the anterior pituitary-derived proopiomelanocortin (POMC) peptide ACTH (22). Upon its release into systemic circulation, ACTH activates the MC2 receptors (MC2-R) of the adrenal gland to induce steroid production and secretion, most prominently corticosterone (rodents) and cortisol (humans). These steroids are powerful anti-inflammatory factors that counteract the stressors and buffer tissue damage (3). The same steroids act to terminate the stress response by attenuating CRH and POMC peptide production.

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obtained after hormone treatment. Cells were incubated with or without 100 nM CRH during 24 h of incubation. Ethidium bromide staining shows PCR products in inset promoter activity (mRNA production (open bars, control; solid bars, 100 nM CRH) and POMC gene expression in the melanocytes.

Materials and Methods

Cell culture. Normal human epidermal melanocytes from moderately pigmented skin (Cascade Biologics, Portland, OR) and immortalized PIG-I human melanocytes were cultured as described (14). Cells were seeded into multwell plates, incubated for 24 h in EpiLife medium with EpiLife Defined Growth Supplement free of serum and pituitary extract (Cascade Biologics). Afterward, the cells were washed three times with PBS and then incubated in supplement-free EpiLife medium with or without CRH (American Peptide, Sunnyvale, CA), ACTH, forskolin, or IBMX (0.1 mM) or progesterone (1 μM) (Sigma, St. Louis, MO) at indicated concentrations (see figures) (14). After 12 h, CRH or ACTH was added again, and at 24 h, media were collected for steroid assays and cells for protein or RNA extractions. ACTH production. ACTH(1–39) concentrations in cell extracts (diluted with water 1:4) and in supernatants were measured with two-site ELISA (Alpco Diagnostics, Windham, NH). It uses affinity-purified goat polyclonal antibody to human ACTH(34–39) and a mouse monoclonal antibody to the midregion and NH2 terminus of human ACTH(1–24). In accordance with the manufacturer’s instructions, the intra-assay coefficient of variation was ≤4.2%, sensitivity was 0.46 pg/ml, and cross-reactivity with β-endorphin was <0.01% and with α-MSH <5.65%. ACTH concentration in cell extracts was normalized to total protein content (quantified with bicinchoninic acid (BCA) reagent; Pierce Biotechnology, Rockford, IL).

Cortisol and corticosterone determination. Cortisol and corticosterone levels in supernatants were measured with ELISA. Corticosterone and cortisol ELISA kits were purchased from R&D Systems (Minneapolis, MN; the majority of assays) or from Bio-Quant (San Diego, CA; to measure the effect of progesterone concentration). To exclude cross-reactivity interference on the assay values, cortisol levels were measured in media incubated with serial dilutions of progesterone in multiwell plates; these values were then subtracted from values obtained from the melanocyte-conditioned media. Steroid concentrations in cell extracts were normalized to total protein content (quantified with BCA reagent, Pierce Biotechnology).

cAMP assays. cAMP concentration was measured by a cAMP functional assay kit (Packard BioScience, Meriden, CT) as described previously (7). Briefly, serial dilutions of CRH and urocortin peptides were added to the supplement-free culture media containing 0.5 mM IBMX, and the cells were incubated with the ligand for 1 h at 37°C and 5% CO2 in the incubator. The signal in cell extracts was measured by the Fusion-α instrument (Packard BioScience). cAMP concentration was recalculated from the standard curve according to the manufacturer’s protocol (Packard BioScience).

POMC promoter activity. Luciferase reporter gene plasmid containing POMC promoter region and fragment of the exon 1 (from −394 to +365 bp) was constructed, and transient transfections were performed as described previously (7). Transfection media were changed at 24 h, and cells were treated as above. Relative reporter gene response was calculated as described (7). Briefly, luciferase and Renilla luciferase signals were recorded with a TD-2020 luminometer (Turner Designs, Sunnyvale, CA), and then, after subtraction of background, the resulting POMC-specific signal was divided by the Renilla signal (signal proportional to the number of transfected cells). The values obtained were divided by the mean value of control (untreated) cells.

POMC gene silencing and CRH-R1 specificity control. Cells were transfected with POMC short interfering RNA (siRNA) and antisense oligonucleotides according to the manufacturer’s instructions (Santa Cruz Biotechnology), using a reported sequence of POMC antisense and scrambled oligonucleotides (4). Gene silencing efficiency was determined with POMC real-time RT-PCR and ACTH ELISA. ME-
dium and cell collection were as above. Antalarmin (10⁻⁵ M, CRH-R1 antagonist; Sigma) was added 1 h before CRH.

Real-time RT-PCR. Reverse transcription of RNA samples pretreated with DNase I was performed with SuperScript First-Strand Synthesis System (Invitrogen Life Technologies, Carlsbad, CA). The primers were designed with ABI Primer Express, with sequences as follows: POMC (Genebank accession no. NM_000939) forward CTACGGCGGTTTCATGACCT, reverse CCCTCACTCGCCCTTTG; 18S rRNA (Genebank accession no. X03205) forward TTCGGAACTGAGGCCATGAT, reverse TTTCGCTCTGGTCCGTCTTG. The reaction was performed with Sybr Green PCR Master Mix; data were collected on an ABI Prism 7700 and analyzed on Sequence Detector 1.9.1 (ABI, Foster City, CA). POMC amounts were related to 18S rRNA by the comparative critical threshold method.

Liquid chromatography-mass spectrometry analysis with auto mass spectrometry-mass spectrometry. Melanocytes were washed three times with PBS and then incubated in supplement-free EpiLife medium supplemented with 1 μM progesterone and 100 nM CRH for 24 h. Collected media were passed through GMF 0.45-μm pore-size filters (Whatman, Clifton, NJ). Steroids were extracted twice with methylene chloride at a ratio of 1:1. The methylene chloride layers were combined and dried under a stream of nitrogen. The dry samples were sent shipped on dry ice for liquid chromatography-mass spectrometry (LC/MS) analyses at Agilent Technologies (Schaumburg, IL).

![Fig. 3. Identification of cortisol by liquid chromatography-mass spectrometry without mass-spectrometry-mass-spectrometry (LC/MS²). LC/MS of cortisol standard (A) shows [M + H]⁺ ion at mass-to-charge ratio (m/z) = 363 with retention time of 11 min. The same [M + H]⁺ ion (m/z = 363, real mass 362) (B) with identical retention time (11 min) is present in extracts from melanocyte media. MS/MS analysis of experimental sample (D) yielded the same fragment ions at m/z = 345, m/z = 327, and m/z = 309 (resulting from the loss of 1, 2, and 3 molecules of water, respectively) as those of the cortisol standard (C).](http://ajpendo.physiology.org/)

![Fig. 4. CRH stimulates cortisol production. Cells were incubated with 100 nM CRH for 24 h. CRH-induced cortisol production is a secondary event, as it is attenuated by POMC gene silencing with antisense oligonucleotides or with short interfering RNA (siRNA) (cells transfected with gene silencers 24 h before CRH treatment); it is also specific (inhibited by antalarmin, added 1 h before CRH). Inset: effect of increasing concentrations of CRH. Differences between control and CRH treatment: *P < 0.05 and **P < 0.01. Differences between CRH treatment and CRH plus inhibitors: ***P < 0.01.](http://ajpendo.physiology.org/)
solvent B mode (70% linear gradient (55–70%) from 55 to 40 min; isocratic mode (55%) from 40 to 50 min). The capillary was maintained at 3500 V, smart parameters setting, trap drive (48.2), and with ionization temperature (325°C), nebulizer gas flow (7 l/min), and HV was used as the nebulizing gas. The MS parameters were as follows: linear increase of [M+H]+ from 50 to 400, and nitrogen with a scan mass-to-charge ratio (m/z) 363 with a retention time of 11 min, corresponding to the characteristics of the cortisol standard (Fig. 3). The residues were dissolved in methanol containing 0.1% formic acid and analyzed by LC/MS/MS using an Agilent 1100 LC/MSD-Trap-XCT system (Agilent Technologies, Palo Alto, CA). The samples were separated on an 1100 LC capillary equipped with a Zorbax SBC18 column (1 x 50 mm, 3.5-μm particle size) coupled to the LC-MSD-Trap-XCT system. Separation was performed at a flow rate of 75 μl/min by mobile phase consisting of solvent A (water with 0.1% formic acid) and solvent B (35% methanol with 0.1% formic acid) with the following program: linear increase of solvent B concentration to 45% from 0 to 10 min, followed by isocratic mode (45% solvent B) from 10 to 15 min; linear gradient (45–55% solvent B) from 15 to 25 min; isocratic mode (55% solvent B) from 25 to 30 min; linear gradient (55–70% solvent B) from 30 to 40 min; and isocratic mode (70% solvent B) from 40 to 50 min.

The MS operated in electron spray ionization-positive ion mode (ESI+) and analyzed POMC mRNA expression in a time-dependent manner (Fig. 1A). POMC increase (2-fold) became statistically significant at 1–6 h of incubation to reach a maximum 14-fold increase at 24 h. Addition of CRH at concentrations as low as 1 nM stimulated POMC promoter activity 28-fold (Fig. 1A, inset), with accompanied increased production of ACTH (Fig. 1B). Because activation of CRH-R1 is coupled to production of cAMP (Fig. 1B, inset), the stimulated POMC expression and ACTH production were likely connected to cAMP-dependent pathways. The same pathway also underlies the response to CRH in the pituitary during systemic stress (5, 22).

The skin is known to express the genes involved in the synthesis of adrenal corticosteroids as well as their enzymatically active protein products, including P450scc, adrenodoxin, adrenodoxin reductase, and P450c17 (9, 21, 23). Moreover, actual functional activity for these enzymes has been clearly demonstrated in cell extracts and cultured skin cells (10, 11, 23). Consistent with these findings, metabolism of progesterone to deoxycorticosterone and corticosterone has been documented in cultured malignant melanocytes (11, 17), although not in keratinocytes (18). Therefore, following the pathway of adrenal steroidogenesis, we tested normal melanocytes for basal production of cortisol. Addition of progesterone (1 μM) stimulated significantly (5.6-fold) cortisol production; the effect was further enhanced by the presence of IBMX (0.1 mM) (12.5-fold increase) in the media (Fig. 2). Analysis by LC/MS did demonstrate then the presence of a [M+H]+ ion at m/z = 363 with a retention time of 11 min, corresponding to the characteristics of the cortisol standard (Fig. 3). The final confirmation of the product identity as cortisol was obtained with MS/MS analysis, which yielded the same fragment ions at m/z = 345 ([M+H]+-H2O), m/z = 327 ([M+H]+-2H2O), and m/z = 309 ([M+H]+-3H2O) as those of the cortisol standard (Fig. 3).
peptide stimulation of cortisol production in a dose-dependent manner (Fig. 4, inset). The CRH effect was specific (abolished by the CRH-R1 antagonist antalarmin) and dependent on POMC expression, e.g., POMC siRNA or POMC antisense oligos completely abolished CRH stimulation (Fig. 4). As would have been expected from these findings, ACTH itself induced production of cortisol in a dose-dependent manner, demonstrating unequivocally that CRH-induced cortisol synthesis acts through an indirect mechanism, requiring mediation by ACTH (Fig. 5). This functional sequence is likely operated by G<sub>S</sub>-dependent pathways, since forskolin directly stimulated cortisol synthesis (Fig. 5, inset) and melanocytes are known to express the MC2-R gene (9).

Because we had found that malignant melanocytes synthesize corticosterone (11, 17), we also tested the effect of CRH and ACTH on production of this steroid. As a first step, we analyzed conditioned media from melanocytes for the presence of corticosterone by ELISA, which showed lower production of corticosterone than cortisol (Fig. 6). Similar to cortisol production, CRH also stimulated corticosterone production in a dose-dependent manner, effects that were abolished by POMC gene silencing (Fig. 6A), indicating again ACTH mediation. Moreover, ACTH stimulated melanocyte corticosterone production in a dose-dependent manner (Fig. 6B), pointing to mediation by a G<sub>S</sub>-dependent pathway, similar to cortisol.

Thus our findings in normal human epidermal melanocytes have uncovered a CRH-based signaling system configured as a functional equivalent of the HPA (possible evolutionary continuum), e.g., CRH through interaction with CRH-R1 activates production of POMC-derived peptides; among those is ACTH, which in turn directly stimulates local corticosteroidogenesis. Hypothalamic CRH is the main coordinator of the central response to stress (1, 3), but CRH is also produced by human epidermal keratinocytes and melanocytes (20), and UVB radiation stimulates CRH production in melanocytes (8). This suggests not only an epidermal stress-response system but also that the system would be structured hierarchically along the same algorithm as in the HPA axis. In contrast with the predominant endocrine mechanism for CRH HPA action in the skin, CRH could potentially function through auto-, para-, or intracrine modes of action. This complex response system would be susceptible to local regulation, since, similar to the pituitary and brain, the skin has preserved cytokines and growth factors as biological modifiers of CRH- and POMC-related peptide function (17, 19, 20). Taken together, our findings strongly suggest evolutionary conservation at the central and peripheral levels of the stress-response signals. Moreover, the common ectodermal origin of the brain and epidermis raises the important question of whether the peripheral CRH-signaling system is an evolutionary duplication of its central homolog or whether it has ancestral origin.

To conclude, the stress-response HPA system has both condensed (peripheral) and expanded (central) versions that follow similar functional sequential activation and differ only in the space allocated to each step.

NOTE ADDED IN PROOF

Independently, the team from Dr. Paus’ laboratory has found that CRH stimulates ACTH and cortisol production in human hair follicles maintained in organ culture in vitro (Ito N, Ito T, Kromminga A, Bettermann A, Takigawa M, Kees F, Straub RH, and Paus R. Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal (HPA) axis and synthesize cortisol. FASEB J. In press).

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REFERENCES


