CRH stimulation of corticosteroids production in melanocytes is mediated by ACTH

Andrzej Slominski,1 Blazej Zbytek,1 Andrzej Szczesniewski,2 Igor Semak,3 Jan Kaminski,1 Trevor Sweatman,4 and Jacobo Wortsman5

Departments of 1Pathology and Laboratory Medicine and 4Pharmacology, Health Science Center, University of Tennessee, Memphis, Tennessee; 2Agilent Technologies, Incorporated, Schaumburg, Illinois; 3Department of Biochemistry, Belarus State University, Minsk, Belarus; and 5Department of Medicine, Southern Illinois University, Springfield, Illinois

Submitted 29 October 2004; accepted in final form 23 November 2004

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 costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
E702 MELANOCYTES EXPRESS FUNCTIONAL HPA AXIS

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 multiwell 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-Quint (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-20/20 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 pre-treated 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).

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 < 0.05.
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). 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).

Once we verified that melanocytes in vitro do produce cortisol, we tested the effects of CRH and found significant
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 Gs-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 cortisol (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 cortisol 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 Gs-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).

ACKNOWLEDGMENTS

We thank Dr. A. Pisarchik for construction of the reporter gene plasmid containing human POMC promoter and fragment of exon 1. Real-time RT-PCR was performed on ABI Prism 7770 in the Molecular Resource Center at the University of Tennessee, Memphis, TN. The excellent administrative assistance of Christine Crawford is also acknowledged.

GRANTS

This work was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-047079 (to A. Slominski) and a training grant from the Johnson and Johnson Skin Research Center (to B. Zbytek).

REFERENCES


