Published in final edited form as:
J Invest Dermatol. 2007 March ; 127(3): 730–732.

CRH Mediates Inflammation Induced by Lipopolysaccharide in Human Adult Epidermal Keratinocytes

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TO THE EDITOR

Corticotropin-releasing hormone (CRH), a 41-amino-acid peptide, is a main regulator of the systemic response that suppresses immune response on a central level (Chrousos and Gold, 1992). In the peripheral sites, including the epidermis, CRH acts as potent proinflammatory agent (Karalis *et al*., 1991; Theoharides *et al*., 2004; Slominski *et al*., 2006a, b). In normal keratinocytes, CRH activates the master regulator of inflammation NF-κB (Zbytek *et al*., 2004) and stimulates the expression of ICAM-1 and HLA-DR (Quevedo *et al*., 2001).

Epidermal keratinocytes take part in inflammatory responses initiated by various stressors (Barker *et al*., 1991). Induction of production of inflammatory cytokines in normal keratinocytes by bacteria occurs via toll-like receptor (TLR)-2 and TLR-4 (Nagy *et al*., 2005). Therefore, there is a need for the functional study of the link between bacteria, CRH, and inflammatory cytokines in human keratinocytes.

Normal human epidermal adult keratinocytes (purchased from Cascade Biologics, Portland, OR) were incubated with CRH, lipopolysaccharide (LPS; TLR-4 agonist, Sigma, St Louis, MO) or PAM3CSK4 (TLR-2 agonist, EMC microcollections, Tuebingen, Germany) in EpiLife medium with epilife defined growth supplement (EDGS) supplement and antibiotics (Cascade Biologics, Portland, OR). Thereafter, supernatants were collected and RNA extracted from the cells. Supernatants were concentrated on C18 SEP-COL-UMNS (Peninsula Laboratories, San Carlos, CA) and CRH was measured with ELISA (Phoenix Pharmaceuticals, Belmont, CA). CRH mRNA and 18SrRNA amounts were quantitated with real-time PCR using Taqman reagents (Applied Biosystems, Foster City, CA) as described previously (Zbytek *et al*., 2006). Cytokine mRNAs were quantitated by the following ABI reagents: Hs00174128_m1, Hs00174097_m1, Hs00174131_m1, and Hs00174086_m1. Small interfering RNA (siRNA) (CRH-R1-specific and scrambled, Ambion, Austin, TX) was transfected into the cells as described (Slominski *et al*., 2005). The medical ethical committee of University of Tennessee approved all described studies.

LPS (TLR-4 agonist) but not PAM3CSK4 (TLR-2 agonist) stimulated the expression of CRH mRNA (Figure 1a and b). The effect was most significant at 1 hour at 1,000 ng/ml being 5.4-fold higher than in control. LPS also stimulated the release of the peptide to the supernatant (Figure 1c). At 24 hours, LPS at 1,000 ng/ml stimulated it 14.9- fold. As regards the physiological significance of effect of LPS on CRH production, it was previously shown that cells that initiate stress response in the organism (hypothalamic neurons) produce CRH upon

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CONFLICT OF INTEREST
The authors state no conflict of interest.

LPS stimulation (Chrousos and Gold, 1992; Wei *et al*., 2002). We provide data showing that similar phenomenon takes place in the epidermal keratinocytes.

Having determined that LPS has stimulated production and release of CRH, we decided to test the effects of CRH on the production of selected cytokines that play a role in the regulation of the mode of immune response (T-helper cell 1 *versus* T-helper cell 2). The cytokines tested were tumor necrosis factor-α (TNF-α), IL-1β, IL-6, and IL-10. CRH stimulated the expression of IL-1β, IL-6, and TNF-α mRNAs, but did not stimulate the expression of IL-10 mRNA (whose amount was below the limit of detection) (Figure 2a–c). The most significant stimulatory effects, reached at 1 hour, were 12.9-fold (TNF-α), 8.5-fold (IL-1β), and 7.3-fold (IL-6). Hitherto, CRH stimulated the production of inflammatory and T-helper cell 1- (TNF-α) rather than T-helper cell 2- (IL-10) upregulating cytokines. This effect of CRH is consistent with its previously reported proinflammatory (Theoharides *et al*., 2004) and selective T-helper cell 1-enhancing effects (Benou *et al*., 2005).

To further document a role of CRH-CRH- R1 signaling in the response of keratinocytes to bacteria, we transfected keratinocytes with CRH-R1 siRNA or scrambled (control) siRNA. After 48 hours, the cells were stimulated with LPS, and then, after 1 hour, levels of cytokine mRNA measured. CRH-R1 siRNA-transfected cells responded with diminished production of TNF-α (by 80%), IL-1β (by 84%), and IL-6 (by 84%) as compared to cells transfected with control siRNA (insets in Figure 2a–c). This indicates that enhanced production of inflammatory cytokines is dependent on CRH-R1 expression.

Locally released CRH may play a role in inflammatory and immunemediated skin diseases such as psoriasis, alopecia areata or acne (O'Kane *et al*., 2006). We have previously reported that CRH stimulates epidermal keratinocyte differentiation (Zbytek and Slominski, 2005), which exclusively express CRH-R1 (Slominski *et al*., 2006b). Zouboulis *et al*. (2002) reported that CRH induces synthesis of sebaceous lipids. UV was shown to upregulate CRH release (Zbytek *et al*., 2006). This report shows that CRH may serve as a local autocrine mediator that amplifies inflammatory responses to bacterial antigens and hence may be an important target for the treatment of several skin conditions.

Acknowledgements

The work was supported by National Institutes of Health (Grant No. AR047079 to A.S.) and Johnson and Johnson Skin Research Training Grant (B.Z., A.S. as mentor). Real-time reverse transcription- PCR was performed on ABI Prism 7770 in the Molecular Resource Center, University of Tennessee, Memphis, TN. This work was performed in Memphis, TN.

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Abbreviations

CRH
: corticotropin-releasing hormone

LPS
: lipopolysaccharide

siRNA
: small interfering RNA

TLR
: toll-like receptor

TNF-α
: tumor necrosis factor-α

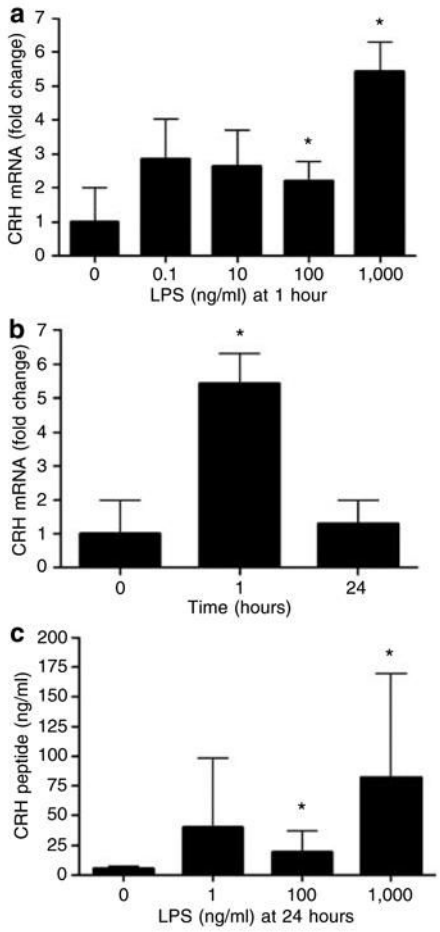

Figure 1. LPS stimulates CRH in normal human adult epidermal keratinocytes

Cells were incubated (**a**) with LPS (0–1,000 ng/ml) for 1 hour and (**b**) with LPS (1,000 ng/ml) for 1–24 hours. Cells were lysed, total RNA extracted, and real-time PCRs (*n*=3) performed for CRH. (**c**) Cells were incubated with LPS (0–1,000 ng/ml). After 24 hours, the supernatants were collected, concentrated on C18 SEP-COLUMNS, and CRH determined by ELISA (*n*=4). Data are presented as mean ± SEM. **P*< 0.05 between untreated and LPS-treated cells.

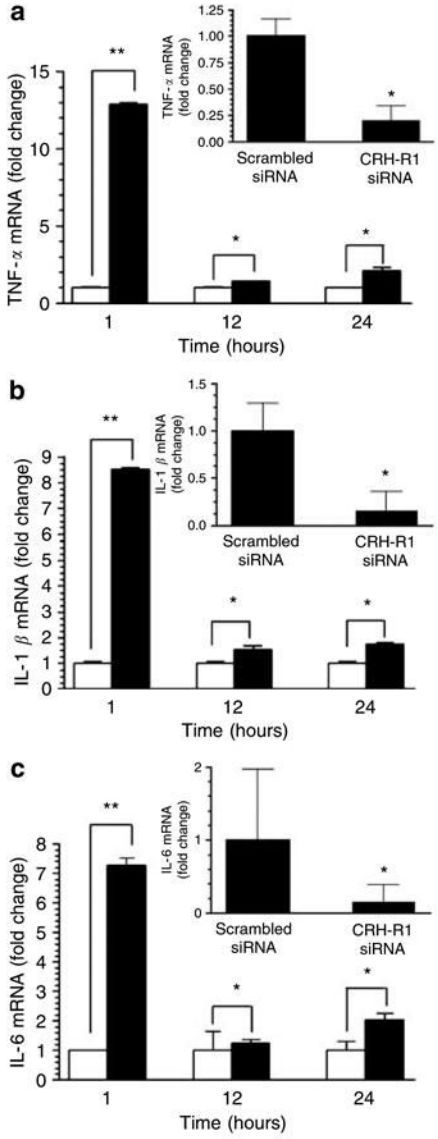

Figure 2. CRH and CRH-R1 participate in the LPS-mediated stimulation of inflammatory cytokine mRNA expression in normal human adult epidermal keratinocytes

Main panels: Keratinocytes were incubated with or without 100 nM CRH for 1–24 hours. Insets: Keratinocytes were transfected with either scrambled (control) or CRH-R1-specific siRNA. Forty-eight hours after transfection, media were changed and cells stimulated with LPS (1,000 ng/ml) for 1 hour. After incubations, cells were lysed, total RNA extracted, and real-time PCRs performed for (a) TNF-α, (b) IL-1β, and (c) IL-6. Data are presented as mean ± SEM (n=3). *P<0.05, **P<0.005 between untreated and CRH-treated cells or cells transfected with scrambled or CRH-R1 siRNA (insets).