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Calcitonin gene-related peptide inhibits chemokine production by human dermal microvascular endothelial cells

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ABSTRACT

This study examined whether the sensory neuropeptide calcitonin gene-related peptide (CGRP) inhibits release of chemokines by dermal microvascular endothelial cells. Dermal blood vessels are associated with nerves containing CGRP, suggesting that CGRP-containing nerves may regulate cutaneous inflammation through effects on vessels. We examined CGRP effects on stimulated chemokine production by a human dermal microvascular endothelial cell line (HMEC-1) and primary human dermal microvascular endothelial cells (pHDMECs). HMEC-1 cells and pHDMECs expressed mRNA for components of the CGRP and adrenomedullin receptors and CGRP inhibited LPS-induced production of the chemokines CXCL8, CCL2, and CXCL1 by both HMEC-1 cells and pHDMECs. The receptor activity-modifying protein (RAMP)1/calcitonin receptor-like receptor (CL)-specific antagonists $CGRP_{8-37}$ and BIBN4096BS, blocked this effect of CGRP in a dose-dependent manner. CGRP prevented LPS-induced IκBα degradation and NF-κB binding to the promoters of CXCL1, CXCL8 and CCL2 in HMEC-1 cells and Bay 11-7085, an inhibitor of NF-κB activation, suppressed LPS-induced production of CXCL1, CXCL8 and CCL2. Thus, the NF-κB pathway appears to be involved in CGRP-mediated suppression of chemokine production. Accordingly, CGRP treatment of LPS-stimulated HMEC-1 cells inhibited their ability to chemoattract human neutrophils and mononuclear cells. Elucidation of this pathway may suggest new avenues for therapeutic manipulation of cutaneous inflammation.

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1. Introduction

Dermal microvascular endothelial cells contribute to cutaneous inflammation through many mechanisms. Amongst these is the capacity to release chemokines that play a role in recruiting inflammatory cells (Goebeler et al., 1997; Bender et al., 2008). Hence, as endothelial-derived chemokines play a crucial role in inflammation and disease, regulation of their expression presents an approach to the management of inflammatory skin disease.

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide generated by tissue-specific alternative processing of the calcitonin gene (Amara et al., 1982; Rosenfeld et al., 1983). It is widely distributed in organs of the immune system as well as the central and peripheral nervous systems and is generally co-expressed with either somatostatin or substance P in sensory neurons (Brain and Williams, 1988; Zaidi et al., 1990; Brain, 1997). CGRP attenuates various immune responses including inhibition of the production of Th1-type cytokines, attenuation of interleukin (IL)-1β induced reactive oxygen species in alveolar epithelial type II cells (Li et al., 2006), inhibition of mitogen-stimulated T lymphocyte and thymocyte proliferation (Umeda et al., 1988; Bulloch et al., 1991, 1998), and suppression of the production of IL-2 and other cytokines by $CD4^+$ Th1 cell clones (Wang et al., 1992). CGRP can also function as a mediator of inflammation; it is a potent endogenous vasodilator and is involved in the accumulation of inflammatory cells in areas of inflammation (Li and Wang, 2006; Hartung and Toyka, 1989; Merhi et al., 1998; Benrath et al., 1995). In accord with these observations, CGRP enhances adhesion of neutrophils to endothelium in vitro (Zimmerman et al., 1992). However, in other experiments CGRP was found to inhibit inflammation not associated with adaptive immunity. For example, CGRP inhibits edema-promoting actions of inflammatory mediators (histamine, leukotrine B4, 5-hydroxytryptamine) in vivo in the hamster cheek pouch, human skin, and rat paw (Raud et al., 1991). Additionally, systemic treatment of mice with CGRP reduced blood neutrophilia induced by systemic administration of LPS and also protected against a lethal dose of LPS (Gomes et al., 2005).

With regard to adaptive immunity, CGRP-containing epidermal nerves are intimately associated with Langerhans cells (LC) in the skin and CGRP inhibits antigen presentation by LC in vitro (Hosoi et al., 1993). After intradermal administration, CGRP inhibits the

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induction of contact hypersensitivity at the injected site in mice (Asahina et al., 1995; Niizeki et al., 1997).

Given these findings and the knowledge that dermal blood vessels are associated with CGRP-containing nerves (Garcia-Caballero et al.,1989), we wished to examine whether CGRP regulates chemokine expression by dermal microvascular endothelial cells. In the present study, we investigated the effect of CGRP on the expression of the chemokines CXCL8 (interleukin-8), CCL2 (monocyte chemotactic protein-1), and CXCL1 (growth related oncogene-alpha) by activated HMEC-1 cells [a transformed human dermal microvascular endothelial cell (HDMEC) line] and pHDMECs and explored possible mechanisms of this effect. We have also shown directly that CGRP inhibits the ability of stimulated HMEC-1 cells or supernatants conditioned by these cells to chemoattract neutrophils or mononuclear cells. We have chosen these chemokines for study as previous work has demonstrated that stimulated HDMECs produce them (Bender et al., 2008; Seiffert et al., 2006) and they are known to play significant roles in cutaneous inflammation, wound healing and other cutaneous pathologies (Zaja-Milatovic and Richmond, 2008; Britschgi and Pichler, 2002; Dearman et al., 2004; Yamamoto, 2003).

This study has identified a novel mechanism by which CGRP may participate in the attenuation of inflammatory responses. There has been much attention to a putative role for stress-induced neurologic mediators playing a role in stress-induced exacerbation of inflammatory skin disorders (Pavlovic et al., 2008; Amano et al., 2008; Seiffert et al., 2006) and a possible role for stress reduction and avoidance behavior in ameliorating such disorders (Farber and Nall, 1993; Arndt et al., 2008). These findings may suggest a mechanism by which the nervous system can limit inflammation. They also may suggest new therapeutic approaches to the treatment of inflammatory skin disease.

2. Materials and methods

2.1. Reagents and peptides

αCGRP and $CGRP_{8-37}$ were purchased from Peninsula Laboratories (Bachem America, Torrance, CA). The CGRP antagonist BIBN4096BS was a kind gift from Dr. Henri Doods (Biological Research, Boehringer Ingelheim Pharma KG, Ingelheim, Germany); LPS (*E. coli* 0111:B4), 8-bromoadenosine-3′,5′-cyclic monophosphorothioate (Rp-8-Br-cAMPS), bisindolylmaleimide VIII acetate (Ro 31-7549) and Bay 11-7085 were purchased from Sigma–Aldrich, St. Louis, MO, USA; human CXCL8, CXCL1 and CCL2 ELISA kits, and Parameter™ cAMP assay kits were purchased from R&D Systems, Minneapolis, MN, USA. Rabbit polyclonal anti-mouse IκBα was purchased from Cell Signaling Technology; anti-p50 and anti-p65 rabbit polyclonal antisera were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

2.2. Media and cells

HMEC-1 cells were the kind gift of T.J. Lawley (Emory University, Atlanta, GA, USA). HMEC-1 cells were cultured in endothelial cell basal medium (Lonza, Walkersville, VA, USA) containing 10% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA, USA), 10 ng/ml epidermal growth factor (BD Biosciences, San Jose, CA, USA), 1 μg/ml hydrocortisone (Sigma–Aldrich), and 100 U/ml penicillin/100 mg/ml streptomycin (Mediatech, Manassas, VA, USA). For all experiments using HMEC-1 cells other than examination of mRNA expression for components of CGRP and AM receptors, the cells were kept in endothelial cell basal medium supplemented only with 2% FBS and penicillin/streptomycin (depleted medium) without epidermal growth factor or hydrocortisone overnight and during the experimental procedures. Primary cultures of neonatal foreskin-derived HDMEC were obtained commercially (Lonza). pHDMECs were cultured in endothelial cell EBM-2 basal medium (Lonza) supplemented with 5% FBS, hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, HEGF, gentamicin, and amphotericin B (EGM-2 MV Bulletkit, Lonza). For all experiments other than examination of mRNA expression for components of CGRP and AM receptors, pHDMECs were kept in depleted medium (EBM-2 basal medium with 5% FBS) for 6 h prior to and during the experimental procedures.

The decision to use the transformed human dermal microvascular EC line HMEC-1 (immortalized by simian virus 40 transformation) as a surrogate for HDMEC was based on its relatively flexible growth requirements, homogeneity (Ades et al., 1992; Pruckler et al., 1993) and its retention of the properties of HDMEC including cell adhesion molecule expression and cytokine production (Xu et al., 1994), providing us with a reliable source of endothelial cells.

2.3. RNA isolation and RT-PCR

Total RNA was extracted from HMEC-1 cells and pHDMECs using a total RNA extraction kit as per the manufacturer's instructions (Qiagen, Valencia, CA, USA). A genomic DNA eliminator spin column was used to remove DNA from the samples. Total RNA treated with RNase-free DNase (Qiagen) (500 ng per 20 μl reaction) was reverse-transcribed into cDNA using Superscript™ II reverse transcriptase (Invitrogen, Carisbad, CA), following the manufacturer's instructions. Specific primers were constructed for the human calcitonin receptor-like receptor (CL), CGRP-receptor component protein (CRCP), and receptor activity-modifying proteins (RAMPs) 1–3 on the basis of sequences published in GenBank: CL, 5′-TCAAGAGCCTAAGTTGCCAAA-3′ (sense) and 5′-AATCAGCACAAATTCAATGCC-3′ (anti-sense); CRCP, 5′-AACTGATCTGAAAGAGCAGCG-3′ (sense) and 5′-TCTTCTTCTGCTCAGCCTCTG-3′ (anti-sense); human RAMP1, 5′-GAGACGCTGTGGTGTGACTG-3′ (sense) and 5′-TCGGCTACTCTGGACTCCTG-3′ (anti-sense) (Swerlick and Lawley, 1993); RAMP2, 5′-GGGGGACGGTGAAGAACTAT-3′ (sense) and 5′-GTTGGCAAAGTGGATCTGGT-3′ (anti-sense) (Gupta et al., 2006) and RAMP3, 5′-TCGTGGGCTGCTACTGG-3′ (sense) and 5′-CTCACAGCAGCGTGTCG-3′ (anti-sense) (Nikitenko et al., 2001). PCR was performed by transferring 2 μl of cDNA to a PCR mixture containing 200 nM of each specific primer and platinum PCR Supermix (Invitrogen, Carlsbad, CA, USA) for a total volume of 25 μl, using a thermal cycler (Gene AMP PCR System 9700; Perkin–Elmer, Waltham, MA, USA). PCR products were resolved on a 1.5% agarose gel and visualized by ethidium bromide.

2.4. DNA sequencing

PCR products were gel isolated and DNA was extracted using a QIAquick Gel Extraction kit as per the manufacturer's instructions (Qiagen). DNA was sequenced by the Cornell University Life Sciences Core Laboratories Center.

2.5. Chemokine determinations

To measure CXCL8, CCL2 and CXCL1 secretion by HMEC-1 cells and pHDMECs, cells were plated at a concentration of 0.25×10^6 cells/well in twelve-well plates in triplicate in 2 ml of depleted medium. After overnight incubation for HMEC-1 cells or 6-h incubation for pHDMECs, cells were treated with or without 1 μg/ml LPS in the presence or absence of CGRP with CGRP added 1 h prior to LPS. Supernatants were harvested at the indicated times. CXCL8, CCL2 and CXCL1 production was quantified by

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