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Calcitonin Gene-Related Peptide Induces IL-8 Synthesis in Human Corneal Epithelial Cells

Mau T. Tran, Mary H. Ritchie, Robert N. Lausch, and John E. Oakes

Calcitonin gene-related peptide (CGRP), a neuropeptide with proinflammatory activities, is released from termini of corneal sensory neurons in response to pain stimuli. Because neutrophil infiltration of the clear corneal surface is a hallmark of corneal inflammation in the human eye, we determined whether CGRP can bind to human corneal epithelial cells (HCEC) and induce expression of the neutrophil chemotactic protein IL-8. It was found that HCEC specifically bound CGRP in a saturable manner with a \( K_D \) of \( 2.0 \times 10^{-9} \) M. Exposure of HCEC to CGRP induced a significant increase in intracellular cAMP levels and enhanced IL-8 synthesis nearly 4-fold. The capacity of CGRP to stimulate cAMP and IL-8 synthesis was abrogated in the presence of the CGRP receptor antagonist CGRP\(_{8-37}\). CGRP stimulation had no effect on the half-life of IL-8 mRNA while increasing IL-8 pre-mRNA synthesis >2-fold. In contrast to IL-8, CGRP did not induce monocyte chemotactic protein-1 or RANTES synthesis, nor did the neuropeptide enhance detectable increases in steady state levels of mRNA specific for these two \( \beta \)-chemokines. The results suggest that HCEC possess CGRP receptors capable of initiating a signal transduction cascade that differentially activates expression of the IL-8 gene but not the genes for monocyte chemotactic protein-1 or RANTES. The capacity of CGRP to stimulate IL-8 synthesis in HCEC suggests that sensory neurons are involved in induction of acute inflammation at the eye surface. The Journal of Immunology, 2000, 164: 4307–4312.

The human cornea is densely innervated with CGRP containing C-type sensory neurons (20, 21). Because CGRP has known proinflammatory properties, this study was initiated to investigate the possibility that CGRP plays a role in corneal inflammation by specifically binding to epithelial cells and inducing expression of proinflammatory chemokines (22). It was found that HCEC have functional receptors for CGRP and that activation of these receptors by CGRP differentially induces expression of the neutrophil chemotactic chemokine IL-8, but not the monocyte chemotactic chemokine MCP-1 or the lymphocyte chemotactic chemokine RANTES. The role that sensory neurons within the human cornea may play in induction of acute inflammation after injury to the corneal epithelium is discussed.

Materials and Methods

Preparation of HCEC

Human corneas were obtained from the National Disease Research Interchange (Philadelphia, PA). Pure cultures of HCEC were established within 48 h of enucleation as previously described (5, 23). IL-8 levels in cell cultures exposed to adrenomedullin, CGRP, or CGRP receptor antagonist (CGRP\(_{8-37}\), purity >97%) (Sigma, St. Louis, MO) were quantitated with ELISA kits purchased from R&D Systems (Minneapolis, MN). cAMP was quantitated with a cAMP enzyme immunosassay kit purchased from Stratagene (La Jolla, CA). The significance in chemokine or cAMP levels between unstimulated and stimulated cells was determined by using small sample paired \( t \) statistics. \( p < 0.05 \) was considered significant.

CGRP receptor binding assay

Confluent cultures of HCEC were exposed to 1 × 10^{-16} M to 1 × 10^{-8} M 125I-CGRP (Amersham, Arlington Heights, IL) with a specific activity of >2000 Ci/mmol in a final volume of 200 \( \mu \)l for 3 h on ice. A duplicate set of experiments was conducted in which 1 \( \mu \)M unlabeled CGRP was added.

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to the binding buffer to determine nonspecific binding. Duplicate unlabeled wells were used to count cell numbers. After incubation with label, the supernatants were removed from the cultures, and the monolayers were washed twice with 200 μl PBS and then lysed using 200 μl 0.6% SDS in 10 mM Tris-EDTA buffer for 10 min. The lysates and supernatants were mixed with a scintillation cocktail and counted in a Beckman LX500CE scintillation counter (Beckman, Fullerton, CA) to determine levels of bound and unbound ligand. Nonspecific binding was found to be <15%. Scatchard analysis of the equilibrium saturation binding data was performed with the GraphPad Prizm program (GraphPad, Harvard University, Cambridge, MA).

**Results**

**HCEC express receptors for CGRP**

The CRLR gene product has been identified as the CGRP-binding receptor (26). In preliminary experiments, it was found that a RT-PCR product complementary to the mRNA of the human CRLR protein could be amplified from HCEC RNA samples (Fig. 1, inset A). These results suggest, therefore, that HCEC express a CGRP-binding protein. Equilibrium-binding experiments with 125I-CGRP were then performed to determine whether HCEC specifically bind this neuropeptide. Analysis of the binding data showed that HCEC bound CGRP in a specific and saturable manner (Fig. 1). A Scatchard plot revealed ~500 binding sites per cell with a Kd of 2.0 × 10^-9 M (Fig. 1, inset B). This dissociation constant is consistent with Kd values for CGRP receptors found on other cell types (27–29).

**CGRP stimulates IL-8 gene expression**

To determine whether CGRP induces IL-8 gene expression in HCEC, a dose-response experiment was performed. HCEC from individual corneal donors were incubated with 10^-3 to 10^-11 M CGRP for 3 h. Media were then removed from stimulated cells and assayed for IL-8. As noted in earlier studies (5), unstimulated HCEC produced constitutive levels of IL-8 (Fig. 2A). However, exposure of HCEC to increasing concentrations of CGRP resulted in a dose-dependent increase in IL-8 synthesis resulting in significant IL-8 production in the presence of both 0.01 and 0.1 nM CGRP (p < 0.05). The C-terminal fragment of CGRP (CGRP8-37) is a specific CGRP receptor antagonist (27–30). When corneal epithelial cell cultures were exposed to CGRP in the presence of 1

**FIGURE 1.** Steady state binding of 125I-CGRP to HCEC. Confluent monolayers of cells were incubated with increasing concentrations of 125I-CGRP. Specific binding was determined by subtraction of nonspecific binding measured in the presence of 1 μM unlabeled CGRP from total binding. Inset A, ethidium bromide-stained gel containing CRLR and GAPDH RT-PCR products amplified from RNA harvested from HCEC; Inset B, Scatchard plot generated from the radioligand binding data. The results are representative of three independent experiments done with three different donors.

**FIGURE 2.** Effects of CGRP and its receptor antagonist on IL-8 synthesis. A, HCEC were cultured for 3 h with CGRP in the concentrations indicated. Culture supernatants were then collected and assayed for IL-8 by ELISA. The results are representative of the mean ± SEM from four corneal donors. B, Confluent monolayers of human epithelial cells were stimulated with either fresh medium, CGRP or CGRP plus 1 μM CGRP receptor antagonist CGRP8-37. At selected times poststimulation, supernatants were collected and assayed for IL-8 by ELISA. The results represent the mean ± SEM from three different donors.
μM CGRP8–37, the amount of IL-8 secreted was not significantly enhanced (p > 0.05) (Fig. 2B). Thus, specific inhibition of the binding of CGRP to HCEC blocked induction of IL-8 synthesis.

In addition to its interaction with CGRP, the CRLR gene product can be modified by receptor-activity-modifying proteins to interact with adrenomedullin, a neuropeptide related to CGRP (26). To determine whether adrenomedullin can also function as a stimulator of IL-8 synthesis, we compared IL-8 synthesis in HCEC stimulated with adrenomedullin with its synthesis in cells stimulated with CGRP. In contrast to CGRP, it was found that adrenomedullin had no effect on IL-8 synthesis (Fig. 3). These results suggest, therefore, that adrenomedullin does not function as a physiological inducer of IL-8 synthesis in HCEC.

**CGRP induces cAMP synthesis**

CGRP receptors are G-linked proteins that initiate signal transduction by activating adenyl cyclase to catalyze formation of the intracellular second messenger cAMP (10). To test the hypothesis that increased IL-8 production in CGRP-stimulated cells is accompanied by increased intracellular cAMP levels, cell lysates were harvested at selected times after CGRP stimulation, and intracellular concentrations of cAMP were determined by an enzyme immunoassay. CGRP stimulation nearly doubled the concentration of intracellular cAMP (Fig. 4). This increase in cAMP levels was highly significant (p < 0.05). The dependence of cAMP formation on activation of the CGRP receptor was confirmed by demonstrating that CGRP did not stimulate cAMP synthesis in the presence of the receptor antagonist CGRP8–37 (Fig. 3). These results suggest, therefore, that CGRP receptors expressed on HCEC stimulate cAMP synthesis.

**CGRP stimulates IL-8 gene expression by enhancing IL-8-specific RNA synthesis**

IL-8 gene expression in CGRP-stimulated cells could potentially be induced at either the level of IL-8-specific RNA synthesis or at the level of IL-8 mRNA stability (31–34). To determine whether increases in specific RNA synthesis contribute to the enhanced IL-8 production observed in CGRP-stimulated cells, IL-8 pre-mRNA levels were measured by RT-PCR. It was found that CGRP stimulation more than doubled the amounts of IL-8 pre-mRNA expressed as compared with levels expressed in unstimulated cells (Fig. 5). To determine whether IL-8 mRNA stability is increased in CGRP-stimulated cells, 10 μg/ml actinomycin D was added to cell cultures 1 h after the addition of CGRP. At selected times post-actinomycin D treatment, the levels of both IL-8 pre-mRNA and mRNA were measured by RT-PCR. After actinomycin D treatment, the half-life of IL-8 mRNA was <1.5 h in both CGRP-stimulated and unstimulated cells (Fig. 6). It was clear that actinomycin D inhibited IL-8 transcription in these experiments.
and MCP-1 are not up-regulated in HCEC in response to CGRP. Therefore, that in contrast to the IL-8 gene, the genes for RANTES or MCP-1 transcripts (data not shown). The cells were metabolically active in these experiments because CGRP induced the synthesis of significant levels of IL-8. These results suggest, therefore, that exposure of HCEC to CGRP had no effect on the half-life of IL-8 mRNA while increasing levels of nuclear IL-8 pre-RNA by ~2-fold. These results would be expected if CGRP up-regulated the IL-8 gene by enhancing its transcription. It was also found that CGRP readily enhanced intracellular cAMP levels in stimulated cells. The transcriptional factor AP-2 has been reported to mediate signal transduction in response to cAMP (35). Because the IL-8 promoter also possesses a consensus binding site for the transcriptional factor AP-2, this factor could be involved in linking CGRP receptors to IL-8 gene expression (36). The IL-8 promoter also possesses a consensus binding site for the transcriptional factor AP-1 (36). In some cells, AP-1 is activated by stimulation of G-linked cell surface receptors through a mitogen-activated protein kinase cascade (37). Thus, it is possible that IL-8 gene expression was activated in CGRP-treated cells through the capacity of CGRP receptors to initiate a G-linked biochemical cascade that resulted in AP-1 activation.

In our study, it was found that the concentration of CGRP responsible for stimulating maximum levels of IL-8 synthesis was ~20-fold less than the K_d of its receptor. A similar situation has been found in lymphocytes where CGRP concentrations involved in stimulating these cells is also significantly less than the K_d (16). These results suggest that only a fraction of the cell surface CGRP receptors need to be occupied for the neuropeptide to produce significant biological responses. This finding is not unique to CGRP receptors given that it has been reported that the peptide hormone insulin can also produce maximal responses in its target tissues when only a fraction of cell surface receptors are occupied (38, 39). A contributing factor in the capacity of small numbers of CGRP receptors to activate IL-8 gene expression may be that these receptors are linked to the IL-8 gene promoter through more than one signal transduction pathway, providing opportunities to dramatically amplify signals initiated by receptor occupancy (35, 37).

It was interesting that exposure of HCEC to CGRP did not enhance either MCP-1 or RANTES synthesis because it was found in earlier experiments that potent chemokine inducers such as IL-1α

**Discussion**

Even though the CRLR gene product has been identified as the CGRP receptor (26), little is known about the precise signal transduction pathways initiated by this protein. It is likely that the receptor stimulates IL-8 synthesis in HCEC by enhancing transcription of the IL-8 gene. This conclusion is supported by the fact that exposure of HCEC to CGRP had no effect on the half-life of IL-8 mRNA while increasing levels of nuclear IL-8 pre-RNA by >2-fold. These results would be expected if CGRP up-regulated the IL-8 gene by enhancing its transcription. It was also found that CGRP readily enhanced intracellular cAMP levels in stimulated cells. This indicates that CGRP stimulates IL-8 synthesis in HCEC by enhancing transcription of the IL-8 gene. This conclusion is supported by the fact that exposure of HCEC to CGRP had no effect on the half-life of IL-8 mRNA while increasing levels of nuclear IL-8 pre-RNA by >2-fold. These results would be expected if CGRP up-regulated the IL-8 gene by enhancing its transcription. It was also found that CGRP readily enhanced intracellular cAMP levels in stimulated cells. The transcriptional factor AP-2 has been reported to mediate signal transduction in response to cAMP (35). Because the IL-8 promoter also possesses a consensus binding site for the transcriptional factor AP-1 (36). In some cells, AP-1 is activated by stimulation of G-linked cell surface receptors through a mitogen-activated protein kinase cascade (37). Thus, it is possible that IL-8 gene expression was activated in CGRP-treated cells through the capacity of CGRP receptors to initiate a G-linked biochemical cascade that resulted in AP-1 activation.
and TNF-α also failed to stimulate production of these two chemokines (25). The assemblage of transcriptional factor consensus binding elements within the IL-8 promoter is considerably different from those found within promoters of the MCP-1 and RANTES genes (33, 40, 41). Therefore, we can speculate that these two chemokine genes are unresponsive to stimulation in these experiments either because one or more of the specific transcriptional factors needed to induce MCP-1 and RANTES genes are missing in HCEC or because transcriptional factors needed to up-regulate these two genes are not activated by the same stimuli responsible for IL-8 gene activation.

Recruitment of neutrophils to the eye surface is an important host defense mechanism against infectious agents that impinge on the corneal epithelium (3). IL-8 plays a crucial role in this process because it is the major chemotactic protein made by corneal epithelial cells (4, 5). Previous studies have identified IL-1α and TNF-α as major inducers of IL-8 synthesis in inflamed corneal tissues (4, 7). Data in this report suggest that release of CGRP from sensory neurons represents a third mechanism for induction of IL-8 synthesis at the eye surface. An explanation for this finding is that sensory neurons represent a third mechanism for induction of IL-8 synthesis, whether antagonists of CGRP can be used as therapeutic agents to dampen corneal inflammatory responses.

In addition, some pathogens can initiate acute inflammation without insults or infections that are not severe enough to disrupt epithelial cell membranes or to stimulate cell-mediated immune responses. In addition, some pathogens can initiate acute inflammation without inducing detectable IL-1α release (8). Under these circumstances, CGRP released from sensory neurons may be an important mechanism whereby IL-8 expression is stimulated.

In summary, the accumulation of neutrophils within the cornea can alter the structure of this sensitive tissue, leading to vision loss. IL-8 plays an important role in inducing neutrophil infiltration into the eye surface because it is the major chemotactic protein made by corneal epithelium. In this study, CGRP receptors were found on HCEC with the capacity to stimulate IL-8 synthesis. These findings represent a novel mechanism for stimulating IL-8 gene expression within this ocular tissue. Future studies need to be directed at the identification of signal transduction pathways by which CGRP activates IL-8 gene expression and to determine whether antagonists of CGRP can be used as therapeutic agents to dampen corneal inflammatory responses.

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