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Epidermal growth factor receptor transactivation by the cannabinoid receptor (CB1) and transient receptor potential vanilloid 1 (TRPV1) induces differential responses in corneal epithelial cells

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ABSTRACT

Corneal epithelial injury induces release of endogenous metabolites that are cannabinoid receptor 1 (CB1) and transient receptor potential vanilloid 1 (TRPV1) agonists. We determined the functional contributions by CB1 and TRPV1 activation to eliciting responses underlying wound healing in human corneal epithelial cells (HCEC). Both the selective CB1 and TRPV1 agonists (i.e., WIN55,212-2 [WIN] and capsaicin [CAP], respectively) induced EGFR phosphorylation whereas either inhibition of its tyrosine kinase activity with AG1478 or functional blockage eliminated this response. Furthermore, EGFR transactivation was abolished by inhibitors of proteolytic release of heparin bound EGF (HB-EGF). CB1-induced Ca^{2+} transients were reduced during exposure to either the CB1 antagonist, AM251 or AG1478. Both CAP and WIN induced transient increases in Erk1/2, p38, JNK1/2 MAPK and Akt/PI-3K phosphorylation status resulting in cell proliferation and migration increases which mirrored those elicited by EGF. Neither EGF nor WIN induced any increases in IL-6 and IL-8 release. On the other hand, CAP-induced 3- and 6-fold increases, which were fully attenuated during exposure to CPZ, but AG1478 only suppressed them by 21%. The mixed CB1 and TRPV1 antagonist, AM251, enhanced the CAP-induced rise in IL-8 release to a higher level than that elicited by CAP alone. In conclusion, CB1 and TRPV1 activation induces increases in HCEC proliferation and migration through EGFR transactivation leading to global MAPK and Akt/PI-3K pathway stimulation. On the other hand, the TRPV1-mediated increases in IL-6 and IL-8 release are elicited through both EGFR dependent and EGFR-independent signaling pathways.

1. Introduction

Corneal transparency maintenance depends on continuous renewal of its outermost epithelial layer. This process replaces the uppermost terminally differentiated layers that are being sloughed off into the tears. Their replacement assures preservation of epithelial integrity and its smooth optical properties. In addition, epithelial renewal preserves other needed functions for visual clarity that include: 1) tight junction barrier intactness, which protects the cornea from becoming translucent due to tissue swelling caused by exposure to environmental stresses such as pathogens and anisosmotic challenges (Lu et al., 2001); 2) innate immune responsiveness, which detects the presence of pathogens and provides signals that activate the corneal defense system (Zhang et al., 2005); 3) aldehyde dehydrogenase expression, which protects this layer against UV- and 4-hydroxynonenal-induced cellular damage (Pappa et al., 2005).

As epithelial turnover is modulated by a host of cytokines, extensive effort is devoted to identifying cognate receptor-linked cell signaling drug targets for hastening this process subsequent to corneal injury (Reinach and Pokorny, 2008). These studies employ injury-induced *in vitro* epithelial and *in vivo* wound healing models to determine which receptor-linked cell signaling pathways mediate control of cell proliferation, migration, differentiation, inflammation and apoptosis. In addition to improving our understanding of corneal biology, these studies have identified potential novel drug targets to lessen corneal scarring and inflammation that can persist subsequent to wound closure. These complications may be severe enough to prevent restoration of corneal transparency and visual function (Saika et al., 2007). In human corneal epithelial cells (HCEC), epidermal growth factor receptors (EGFRs) contribute to mediating corneal epithelial renewal. EGFR activation by EGF results in stimulation of cell proliferation and migration through activation of: a) the three member pathways of the

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mitogen-activated protein kinase (MAPK) signaling cassette (i.e., ERK, p38 and JNK); b) the phosphoinositide 3-kinase (PI3-K)/Akt/GSK-3 pathway; c) adenylate cyclase and phospholipase C (PLC)-induced Ca^{2+} signaling as well as phospholipase D (PLD)-mediated phosphatidic acid formation (Islam and Akhtar, 2000; Kang et al., 2000, 2001; Mazie et al., 2006; Wang et al., 2006, 2009; Yang et al., 2005, 2001; Yin and Yu, 2009; Zhang and Akhtar, 1998). EGFR activation also can occur through transactivation by other receptors and mediators (Block and Klarlund, 2008; Lyu et al., 2006; Spix et al., 2007; Xu et al., 2006, 2007). In this process, agonists other than EGF activate their cognate receptors, which leads to matrix metalloproteinase activation and scission of EGF from membrane bound heparin. Therefore, the EGFR-linked cell signaling pathways serve as a conduit for eliciting tissue responses to a number of different mediators besides EGF.

Members of the transient receptor potential (TRP) protein superfamily are polymodal in that they are activated by numerous different stimuli. In the corneal epithelium, some members of the vanilloid (V) TRP subfamily were identified. In HCEC, there is functional expression of TRPV1, 3 and 4 (Pan et al., 2008; Yamada et al., 2010; Zhang et al., 2007). TRPV1 is a nonselective ion channel which is activated by injury-induced endogenous mediators such as endocannabinoids, endovanilloids, declines in pH, elevated temperature and hypertonicity as well as capsaicin, which is present in red pepper extracts. Capsaicin (CAP) is a selective TRPV1 agonist and in HCEC induces increases in the release of proinflammatory cytokine mediators, such as interleukin (IL)-6 and the chemoattractant, IL-8. MAPK activation is a contributor to their increases (Zhang et al., 2007). These rises induced by CAP have physiological relevance since TRPV1 activation by injury in a mouse corneal wound healing model contributes to the development of severe inflammation that persists subsequent to wound closure. Evidence of its role stems from our finding that in homozygous $TRPV1^{(-/-)}$ knockout mice the wound healing response to injury is more favorable. This is apparent since inflammation and scarring are less severe at the time of wound closure (Okada et al., 2008). Even though EGFR-linked pathways are activated by CAP, it is not known if EGFR transactivation contributes to the development of inflammation and scarring.

The cannabinoid receptor subtype 1 (CB1) modulates, through the GTP binding protein (G_i), a number of important physiological processes in different tissues including neurotransmitter release, pain and analgesia, energy homeostasis regulation, and control of immune cell function (Graham et al., 2009; Howlett, 2005; Kress and Kuner, 2009; Pertwee, 2006; Stephens, 2009). CB1 activation by cannabinoids has immunosuppressive effects, which have beneficial effects in the treatment of autoimmune disorders. These results suggest that the cannabinoid system has various roles in disease pathologies and provides potential therapeutic targets. A functional role for CB1 in the human corneal epithelium has not yet been described even though CB1 expression was detected in the corneas of isolated human eyes (Straiker et al., 1999). In some other tissues, TRPV1 and CB1 are coexpressed and functionally interact with one another. Such is the case in the colonic epithelium, in neuronal enriched mesencephalic cultures, primary sensory neurons and myometrial smooth muscle cells (Brighton et al., 2009; Kim et al., 2008; Mahmud et al., 2009; Sibaev et al., 2006). The coexpression of TRPV1 and CB1 in the corneal epithelium prompted us to probe for a functional interaction between them in HCEC.

We show in HCEC that there is a functional interaction between TRPV1 and CB1. Together they mediate increases in cell proliferation and migration through EGFR transactivation and MAPK/Akt-linked signaling. On the other hand, other EGFR independent TRPV1-linked pathway(s) contribute to mediating TRPV1 stimulation of IL-6 and IL-8 release. In contrast, CB1 activation counters TRPV1-induced increases in IL-8. It is conceivable in a clinical setting that drugs targeted to activate CB1 receptors may be effective in reducing TRPV1-induced inflammation caused by corneal injury.

2. Methods

2.1. Materials

The following chemicals were purchased from Sigma–Aldrich (St. Louis, MO): WIN55,212-2 (WIN), anandamide (AEA), capsazepine (CPZ), CAP, AM251, AG1478, GM6001, CRM197, EGF, bovine insulin, gentamicin and TrypLE™ Express Stable Trypsin-Like Enzyme. Dulbecco's modified Eagle's medium (DMEM)/F12 medium fetal bovine serum (FBS) and fura-2 acetoxymethyl ester were purchased from Invitrogen (Carlsbad, CA). Anti-CB1, anti-EGFR, phospho-EGFR, anti-Erk1/2, phospho-Erk1/2, phospho-Akt, phospho-GSK, phospho-p38; goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP antibody, anti (H196) actin, anti-Erk1/2, anti-p38, and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-EGFR neutralizing clone, LA1, was purchased from Millipore Corporate (Billerica, MA).

2.2. Cell culture

SV40-adenovirus immortalized HCEC were obtained as a generous gift from Dr. Kaoru Araki-Sasaki. The cells were cultured at 37 °C in an incubator with 5% CO_2 and 95% ambient air in DMEM/F12 medium, supplemented with 6% FBS, 5 ng/ml EGF and 5 μg/ml insulin. Cell cycle arrest was achieved by culturing cells in serum-free and EGF-free DMEM/F12 medium for 24 h before experimentation.

2.3. Single cell fluorescence imaging

Cells grown on 40-mm circular coverslips (Bioptechs Inc, Butler, PA) were loaded with 2 μM fura-2 AM at room temperature for 30 min and then washed with NaCl Ringer's solution containing (in mM): NaCl (141), KCl (4.2), $CaCl_2$ (0.8), KH_2PO_4 (2), $MgCl_2$ (1), glucose (5.5), and HEPES (10) with osmolarity 300 mOsm and pH 7.4. Cells were continuously superfused at 34 °C in a Focht Closed System 2 (FCS2) perfusion chamber with temperature control (Bioptechs Inc, Butler, PA) and placed on the stage of an inverted microscope (Nikon Diaphot 200). Cells were then alternately illuminated at 340 and 380 nm, and emission was monitored every 5 s at 510 nm using a Roper Scientific CCD camera. Each field of interest contained 15–20 cells. Changes in intracellular Ca^{2+} levels, $[Ca^{2+}]_i$, were analyzed using Ratio Tool software (Isee Imaging, Durham, NC). The *n* values provided indicate the number of experiments per data point.

2.4. Western blot analysis

HCEC were gently washed twice in cold phosphate-buffered saline (PBS) and harvested in 0.5 ml cell lysis buffer. Cell lysates were centrifuged and supernatants were collected for measuring proteins with a bichinchoninic acid assay (BCA) protein assay kit (Pierce Biotechnology, Chicago, IL). Twenty to 50 μg of denatured protein was electrophoresed on 10% polyacrylamide sodium dodecylsulphate (SDS) minigels and polyvinylidene difluoride (PVDF) membranes were blocked with nonfat dry milk. The blots were exposed to the appropriate primary antibody overnight at 4 °C and then exposed to an appropriate secondary antibody (e.g., anti-rabbit, anti-goat or anti-mouse) HRP labeled IgG for 1 h at