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Research article

Glucocorticoid action in human corneal epithelial cells establishes roles for corticosteroids in wound healing and barrier function of the eye

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ABSTRACT

Glucocorticoids play diverse roles in almost all physiological systems of the body, including both anti-inflammatory and immunosuppressive roles. Synthetic glucocorticoids are one of the most widely prescribed drugs and are used in the treatment of conditions such as autoimmune diseases, allergies, ocular disorders and certain types of cancers. In the interest of investigating glucocorticoid actions in the cornea of the eye, we established that multiple cell types in mouse corneas express functional glucocorticoid receptor (GR) with corneal epithelial cells having robust expression. To define glucocorticoid actions in a cell type-specific manner, we employed immortalized human corneal epithelial (HCE) cell line to define the glucocorticoid transcriptome and elucidated its functions in corneal epithelial cells. Over 4000 genes were significantly regulated within 6 h of dexamethasone treatment, and genes associated with cell movement, cytoskeletal remodeling and permeability were highly regulated. Real-time *in vitro* wound healing assays revealed that glucocorticoids delay wound healing by attenuating cell migration. These functional alterations were associated with cytoskeletal remodeling at the wounded edge of a scratch-wounded monolayer. However, glucocorticoid treatment improved the organization of tight-junction proteins and enhanced the epithelial barrier function. Our results demonstrate that glucocorticoids profoundly alter corneal epithelial gene expression and many of these changes likely impact both wound healing and epithelial cell barrier function.

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1. Introduction

Glucocorticoids are steroid hormones that have a critical role in regulating stress response in the body. Endogenous glucocorticoids in humans are necessary for life and they are synthesized by the adrenal cortex in a tight regulation by the hypothalamic-pituitary-adrenal axis. Both endogenous glucocorticoids and their synthetic derivatives used in patient treatment signal through their canonical receptor, the glucocorticoid receptor (gene name *NR3C1*) that belongs to the super family of nuclear receptors. Glucocorticoid actions span a wide range of cellular and systemic effects including cell cycle, cell movement, glucose homeostasis and fluid regulation. They are most known for their anti-inflammatory and immunosuppressive roles. Due to their potent immunosuppressive property, glucocorticoids have been exploited pharmacologically and they have become one of the largest selling class of drugs in the world today. The nearly ubiquitous expression of the glucocorticoid receptor suggests a role for glucocorticoid signaling in every cell type, which is further supported by studies establishing that glucocorticoid signaling is indeed cell type-specific. For example, glucocorticoids exert an anti-apoptotic role in cardiomyocytes (Ren et al., 2012) while exerting a pro-apoptotic role in lymphocytes (Gruver-Yates et al., 2014). Cell type specificity of glucocorticoid signaling diversifies the actions of glucocorticoids and therefore, there is a need to understand the role of glucocorticoids in a cell/tissue-specific manner.

The cornea is the clear part of the eye that covers the iris, pupil and the anterior chamber. By providing a physical barrier, the cornea

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protects the interior of the eye from external agents such as bacteria, viruses and debris. By refracting light through the lens and onto the retina where the light signal converts into vision, the cornea also plays an important role in maintaining vision. Synthetic glucocorticoids have been widely used to successfully treat several ocular disorders, however, the functions of glucocorticoid receptor signaling in the eye, particularly in the cornea are largely under studied. Corticosteroids are also used in transplant surgeries such as in lens transplantation and keratoplasty, to minimize graft rejection. Corticosteroids are used in treating sight-threatening conditions of the cornea such as corneal inflammation and corneal neovascularization (Chang et al., 2001). Despite the fact that glucocorticoids have numerous benefits in treating ocular conditions, some patients receiving chronic glucocorticoid treatment are susceptible to increase in intraocular pressure that could develop into steroid-induced glaucoma and eventually loss of vision (Lambiase et al., 2014). Opacification of the lens or cataract formation are also adverse events seen in sustained corticosteroid use. Glucocorticoids have also been reported to be synthesized in the human ocular surface and they have the ability to regulate corneal immune response (Xie et al., 2011a; Susarla et al., 2014; Mirabelli et al., 2014). In the cornea, glucocorticoids have been shown to regulate their circadian rhythm (Cardoso and Ferreira, 1967; Pezuk et al., 2012), inhibit blood and lymphatic vessel growth (Ellenberg et al., 2010; Steele et al., 2011), curb inflammation (Saud et al., 2012; The Loteprednol Etabonate Postoperative Inflammation Study Group 2, 1998; Bron et al., 1998; Bielory, 2008), and increase epithelial integrity under a hypoxic challenge (Kimura et al., 2011), as well as retard wound healing in rabbits (Petroutsos et al., 1982). Although it has been established that corticosteroids are effective in treating diseases of the cornea, the molecular functions in specific cell types where they occur have not been fully characterized.

In the current investigation, we establish that mouse corneas express functional GR with strong expression of GR by the corneal epithelial cells. Subsequently, we employed immortalized corneal epithelial cell line derived from human cornea to understand glucocorticoid signaling in a single cell type. Here we demonstrate that glucocorticoids can profoundly alter the gene expression profile of human corneal epithelial cells. Ingenuity pathway analysis (IPA) of the glucocorticoid transcriptome revealed that glucocorticoid signaling in corneal epithelial cells was enriched for genes involved in pathways associated with inflammatory diseases and organismal growth and development. Additionally, Ingenuity Pathway Analysis indicated that glucocorticoid signaling in corneal epithelial cells may regulate cellular functions, such as cell movement and cell growth, by altering the expression of a large cohort of genes. Since cornea is at the interface with the environment and prone to injuries, we focused our further analysis of glucocorticoid signaling in corneal epithelial cells on wound healing which included processes such as cell migration, cytoskeletal remodeling and epithelial permeability. Real time *in vitro* wound healing assays demonstrated that glucocorticoid treatment delayed wound healing of HCE cell monolayer by altering their cytoskeleton. Interestingly, the distribution of tight junction proteins and paracellular permeability in response to glucocorticoid treatment indicated that glucocorticoids enhance barrier function in corneal epithelial cells. The study presented here provides a new understanding of the diversity of glucocorticoid actions on corneal epithelial cell wound healing and barrier function.

2. Materials and methods

2.1. Animals

Wild type C57BL/6 female mice aged 2-months old purchased from Charles River Laboratories were used for all animal experiments. For dexamethasone treatment studies, mice were adrenalectomized at Charles River Laboratories to remove endogenous glucocorticoids and were rested for a week after the surgery before being shipped to the National Institute of Environmental Health Sciences (NIEHS). Upon arrival at NIEHS, the animals were rested for 7–10 days before being treated. For dexamethasone treatment experiment, each mouse was treated with vehicle in the left eye and dexamethasone in the right eye. Dexamethasone was purchased from Steraloids and was prepared in Refresh artificial tears manufactured by Allergan, Irvine, CA. For each animal, one eye received 3 μL of vehicle (Refresh artificial tears) or dexamethasone prepared at a concentration of 1 mg/ml. Six hours after the treatment, mice were euthanized by cervical dislocation and eyes were enucleated and corneas were dissected immediately and stored in RNA later (Qiagen) at 4 °C overnight. Six corneas were pooled to generate one sample of RNA, therefore, requiring 24 corneas/treatment to generate an n of 4. RNA was extracted using Trizol and chloroform and purified using RNeasy Micro kit and Dnase digested (Qiagen). For immunofluorescence studies, mice were euthanized by cervical dislocation and eyes were enucleated from euthanized animals. Eyes were fresh frozen in Optimal Cutting Temperature (O.C.T.) Compound (VWR, Pennsylvania) and six-micron sections were prepared. Sections were stained at 4 °C overnight with antibodies to glucocorticoid receptor (Cell Signaling, cat#3660, 1:300). Hoechst 33342 and Alexa Fluor 488 Phalloidin (both from Life Technologies, New York) were used to visualize nuclei and actin filaments, respectively. Z-stack images were taken using the Zeiss LSM710 and Zen 2012 software and Image J software were used to process the images.

2.2. Cell culture and treatment

A widely studied immortalized human corneal epithelial cell line (HCE) obtained from RIKEN was used (Araki-Sasaki et al., 1995). HCE cells were cultured in DMEM/F12 medium supplemented with 5% fetal bovine serum, 5 μg/ml insulin, 10 ng/ml human epidermal growth factor, 0.5% dimethyl sulfoxide and antibiotics. Anti-glucocorticoid- RU486 (mifepristone) were purchased from Steraloids. Cells were incubated in DMEM/F12 medium containing 5% charcoal stripped fetal bovine serum for 18–24 h before being treated with vehicle or dexamethasone or RU486.

2.3. RNA isolation and quantitative RT-PCR analysis

Total RNA was isolated using the RNeasy Kit (micro kit for Trizol/Chloroform extracted mouse corneal RNA and mini kit for human cells) and DNase digested using the RNase-Free DNase Kit (Qiagen) according to the manufacturer's protocol. The abundance of individual mRNAs was determined using a Taqman one-step RT-PCR method on a 7900HT sequence detection system (Applied Biosystems). Pre-developed Taqman primer probe sets for *GILZ* (Hs00608272_m1, Mm00726417_s1), *FKBP5* (Mm00487406_m1), *TNFRSF11b (Hs00900358_m1)*, *BDNF (Hs00380947_m1)*, *EREG (Hs00154995_m1)*, *NGF (Hs00171458_m1)* and *PPIB* (Hs00168719_m1, Mm00478295_m1) were purchased from Life Technologies, Grand Island, NY. Target gene expression was normalized to the housekeeping gene *PPIB*, which is not regulated by glucocorticoids.

2.4. SDS-PAGE and immunoblot analyses

Cells were washed with ice-cold phosphate buffered saline and lysed in SDS sample buffer (Life Technologies) supplemented with B-mercaptoethanol (final concentration 2.5%). Samples were sonicated on ice for 5 s and boiled for 5 min and 104° centigrade. Equal