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Role of glucocorticoids and *Toxoplasma gondii* infection on murine intestinal epithelial cells

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ABSTRACT

Glucocorticoids (GCs) are stress hormones secreted in response to perceived psychological and or physiological stress. GCs have been shown to reduce tissue inflammation by down-regulating the production of inflammatory chemokines produced by epithelial cells. The protozoan parasite *Toxoplasma gondii* is known to increase cytokine, chemokine, and Toll-like receptors (TLRs) expression in parasite infected mouse intestinal epithelial cells (IECs). We sought to analyze the role of an anti-inflammatory protein, glucocorticoid-induced leucine zipper (GILZ) in MODE-K cells during infection with *T. gondii.* GILZ expression in MODE-K cells was assessed by PCR and immunoblotting after stimulation with GCs (corticosterone, CORT) or *T. gondii* infection. GILZ mRNA was constitutively expressed in MODE-K cells but not its protein product. While infection and pre-exposure to CORT decreased GILZ isoforms of 28 and 17 kD, the presence of CORT during infection increased levels of 17 kD isoform. Infected cells treated with CORT had decreased expression of chemokines (IP-10/CXCL10, MCP-1/CCL2, MIP-2/CXCL8) while their expression was increased when endogenous GILZ was removed by siRNA treatment. GILZ up-regulation during infection may serve as a mechanism to decrease epithelial cell responses and facilitate parasite replication.

1. Introduction

During acute and chronic stress (such as with severe injury and prolonged fear/anxiety), the hypothalamic–pituitary–adrenal (HPA) axis is activated elevating blood glucocorticoid (GC) levels, which induces an immunosuppressive effect such as inhibition of the inflammatory response as well as increased antibody production [1,2]. GCs (cortisol in humans and corticosterone in mice) regulate immune cell function, such as differentiation, trafficking, cytokine production, proliferation, chemokine expression, adhesion, molecule-ligand expression, and leukocyte effector function [3]. The role of GCs on the mammalian immune system is generally known to exert an anti-inflammatory effect by down regulating the production and secretion of inflammatory cytokines such as tumor necrosis factor (TNF)-α, and interleukin (IL)-1, IL-2, IL-6, and the chemokine macrophage inhibitory protein (MIP)-1, and macrophage chemotactic protein/s (MCP)-1 and 2 from epithelial cells and macrophages, dendritic cells (DCs), T cells, and through apoptosis of T cells [4,5].

The effects of GCs are mediated through the glucocorticoid receptor, which regulates the expression of GC-responsive genes [6,7]. One of the prominent GC responsive genes is the glucocorticoid-induced leucine zipper (GILZ) [8,9]. GILZ has been found to be expressed in immune tissues, T-cells, macrophages, dendritic cells, mast cells, airway epithelial cells, endothelial cells, and ovarian cancer/tumor cells, and is known to have anti-inflammatory functions [9–11]. GILZ was up-regulated by the synthetic GC dexamethasone (DEX), and exerted anti-inflammatory effects in airway cells presumably through inactivation of NF-κB and AP-1 by direct binding of GILZ to NF-κB's p65 subunit, and AP-1 respectively [12–16]. GILZ has been shown to bind cytosolic Raf-1/Ras and suppression of ERK MAPK pathway activation [15,17].

Toxoplasma gondii is an intracellular parasite found worldwide and infects most mammalian hosts [18]. Humans may acquire *T. gondii* infection orally from ingestion of oocysts in infected water, cat boxes, and from tissue cysts after consuming raw or undercooked meat or unwashed vegetables. After ingestion and release in the small intestine, parasites may quickly invade epithelial cells forming a parasitophorous vacuole [19]. Intestinal epithelial cells (IECs) are the first cells to come in contact with the invading parasite and respond to infection by secreting cytokines and chemokines that drive the ensuing inflammatory immune response [20–23]. Stress activates two major neuroendocrine systems, the hypothalamic–pituitary adrenal (HPA) axis and the sympathetic nervous system (SNS). Their activation results in the production of glucocorticoids (GC) hormones and catecholamines respectively [24]. Although both systems mediate adaptive metabolic, cardiovascular, and anti-inflammatory effects; in the context of this infection model, the role of GCs is largely unknown. We have shown that cold water stress (CWS) and nor-epinephrine (NE) in particular modulated IECs immune responses during *T. gondii* infection both in vivo and in vitro. IECs recovered from stressed mice had decreased expression of

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Toll-like receptors (TLRs) and chemokine secretion [22,23]. The aim of this study was to determine the effect of corticosterone (CORT) on GILZ in the mouse MODE-K cell line during in *T. gondii* infection. We determined gene expression levels of chemokines (MCP-1, MIP-2, IP-10), and provided an insight on the role-played by the endogenous GC responsive gene, GILZ that has been shown to be an important anti-inflammatory protein in animal models of inflammatory diseases [25].

2. Materials and methods

2.1. Cell culture

The murine intestinal epithelial cell line MODE-K was established from the duodenum of C3H/HeJ mice by K. Vidal [26], and provided to us by Dr. K. Croitoru, McMaster University, Canada. Cells were grown in T-75 flasks in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum, 10 mM HEPES, 0.4 g/L L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 0.1 mM non-essential amino acids, and 50 μM 2-mercaptoethanol, and incubated at 37 °C.

2.2. T. gondii maintenance

Human foreskin fibroblasts and MODE-K cells were infected with tachyzoites of *T. gondii* RH strain and allowed to multiply until host cells were nearing lysis. The flasks were scraped and the intracellular parasites released by forced passage through 21, 25, and 27 gauge hypodermic needles. The remaining cell debris was removed by filtration through a 3 μm filter. Parasites were counted under 40× magnification and diluted to 1×10^6 *T. gondii* tachyzoites per milliliter.

2.3. RNA isolation and conversion to cDNA by reverse transcription

Total RNA was isolated from MODE-K cells using the RNA-easy kit following the manufacturer's instructions (Quiagen, Valencia, CA). Briefly, samples were treated with DNase I at 1 μL/μg of RNA (Fermentas Inc, Glen Burnie, MD) for 30 min, and to remove contaminating genomic DNA. RNA was reverse-transcribed to cDNA following standard methods using the avian myeloblastosis virus reverse transcriptase (20 U/μL) in 20 μL reaction containing random hexamers (0.2 μg/μL), RNAse inhibitor (20 U/μL), and 10 mM dNTPs mix for 20 μL reaction (First Strand cDNA Synthesis Kit, Fermentas). RNA, 2-5 μg was used to obtain cDNA. Successful cDNA conversions were confirmed by amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by conventional PCR. The primer sequence for GAPDH is shown below and the conditions were: 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. PCR reactions were performed in a MJ minicycler. The PCR product was viewed in a 1.5% agarose gel electrophoresis staining with ethidium bromide.

2.4. Quantitative real time PCR

The PCR primers were obtained from published sequences or designed with the Roche diagnostics software (Roche Diagnostics Corp. Indianapolis, IN). The PCR products ranged in length from 90 to 300 base pairs, and used in concentrations of 250 nM in 20 μL reactions using SYBR green master mix by (SuperArray Bioscience Corp, Frederick MD). Samples were ran in 96 well PCR plates using the BioRad MyiQ real time PCR machine (BioRad Labs, Hercules, CA). Each gene of interest, (MCP-1, IP-10, MIP-2, *Toxoplasma* 529, and GILZ) was run in separate plates/runs relative to their respective prepared standards (see below). The primers for GAPDH were: forward 5′-CGTCGTGGACATGA CGTG-3′ reverse 5′-CCTGCTTCACCACCTTCTTG-3′; MCP-1/CCL2 forward 5′-CTTCTGGGCCTGCTGTTCA-3′ and reverse 5′-CCAGCCTACTCATTGGGA TCA-3′; MIP-2/CXCL2 forward 5′-TCCAGAGCTTGAGTGTGACG-3′ and reverse 5′-TTCAGGGTCAAGGCAAACTT-3′; IP-10/CXCL10 forward 5′-GCCGTCATTTTCTGCCTCAT-3′ reverse 5′-GCTTCCCTATGGCCCTCATT-3′; *Toxoplasma* p529 forward 5′-CACAGAAGGGACAGAAGT-3′ reverse 5′-TCGCCTTCATCTACAGTC-3′; TLR-9 forward 5′-TTCTCAAGACGGTG GATCGC-3′ reverse 5′-CGAGAGGGTGCTTCTCA-3′; and GILZ forward 5′-GTGGCTCTGTCCTTAGGGTGG-3′ reverse 5′-CCAGATGGGCATGTGC TTG-3′. All reactions were run in triplicate and appropriate controls in which DNase/RNase free water instead of samples or standards were included in each run.

A standard curve was generated using 10-fold serial dilutions of purified DNA product (QIA Quick Gel Extraction kit, QIAGEN, Valencia, CA) of gene of interest [23]. The relative standards varied from 10^9 to 10^1 copies of DNA and this curve was considered acceptable if a difference of 3.3 + or −0.3 cycles was demonstrated between each of the 10-fold dilutions and if the correlation coefficient was at least 0.99. Samples were heated at 95 °C for 10 min, and then subjected to 40 cycles of amplification by melting at 95 °C, 15 s, annealing at 58 °C, 1 min, and extension at 72° C for 30 s. Experimental samples and standards were run in triplicate every time. Threshold cycle numbers (Ct) were used to calculate as per the method outlined in "Relative Standard Curve Method" in user Bulletin provided by Applied Biosystems using GAPDH as the reference gene as indicated. Real-time data were collected and analyzed using Microsoft Excel program. Based on the standards the copy numbers were calculated by relative quantification.

2.5. SDS-PAGE and Western blotting

Cells were collected directly from 6-well culture plates by addition of Laemmli buffer. Samples were boiled for 3 min and resolved in a 15% separating gel. SDS-PAGE was carried out in a Bio-Rad mini Protein unit (Bio Rad Labs, Hercules, CA) for 1.5 h at 200 V. After electrophoresis, samples were transferred onto 0.2 μm PVDF membranes using a Bio-Rad transfer unit at 10 V overnight. The membranes were blocked overnight with 5% powdered milk in PBS (blocking buffer), followed by incubation in primary antibody (rabbit anti-GILZ, Santa Cruz Biotechnology) diluted 1:500 with 5% milk + PBS containing 0.1% Tween 20 (PBST) for 2 h. The secondary antibody was horse radish peroxide-conjugated goat anti-rabbit (ZYMED, San Francisco, CA) diluted at 1:30,000 in 5% fat free milk + PBST solution. After extensive washing with PBST, membranes were incubated with chemiluminescence substrate, FEMTO ECL reagent (Pierce Inc. Glen Burnie, Maryland) for 5 min, and exposed for 30 s to X-ray film. After exposure, the PVDF membranes were stripped of all previous antibodies by immersing in 0.2 M NaOH solution for 20 min under gentle rocking. The membranes were neutralized by washing 3 times for 10 min with PBST, and subsequently placed in 5% PBST-5% fat free milk over night. Membranes were reacted with anti-β-actin antibody (Santa Cruz Biotechnology Inc, Santa Cruz CA) at a 1:500 dilution. Secondary antibody, horse-radish peroxidase donkey anti-mouse antibody (Santa Cruz Biotechnology Inc.) was used at 1:10,000 dilution. Membranes were incubated with FEMTO ECL reagent as described above.

2.6. GILZ knockdown by small interfering RNA (siRNA)

Knockdown of GILZ by siRNA was undertaken using siRNA reagents from Santa Cruz Biotechnology. MODE-K cells were grown in 6-well plates in DMEM media without antibiotics. Groups were as follows: Control (CON); Dexamethasone (DEX, 10^{-7} M); *Toxoplasma* infection (TOXO); Corticosterone (CORT, 10^{-5} M); DEX plus positive siRNA (DEX-siRNA$^+$); *Toxoplasma*-CORT; *Toxoplasma* plus negative siRNA (TOXO-siRNA$^-$; *Toxoplasma*-siRNA$^+$; CORT-siRNA$^+$; *Toxoplasma*-siRNA$^-$, *Toxoplasma*-siRNA$^-$, and CORT-siRNA$^-$. Positive siRNA and negative wells were first washed with siRNA transfection medium, and overlaid with 10 μM positive and negative siRNA duplexes. At this time, *Toxoplasma* tachyzoites were added to the infected groups along with the addition of DEX and CORT. Positive and negative siRNA treated cells were allowed to incubate for 5 h, after which 1 ml normal DMEM (with antibiotics) was added to all groups, incubated for 10 h, and