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Glucocorticoids decrease the production of glucagon-like peptide-1 at the transcriptional level in intestinal L-cells

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ABSTRACT

Glucocorticoids are widely used as anti-inflammatory or immunosuppressive drugs, but often induce hyperglycemia as a side effect. Glucagon-like peptide-1 (GLP-1) is secreted from intestinal L cells and plays crucial roles in maintaining glucose homeostasis. However, the direct effects of glucocorticoids on the GLP-1 production pathway in L cells remain unclear. We investigated the effects of glucocorticoids on GLP-1 production *in vitro* and *in vivo*. In L cell lines, glucocorticoids decreased GLP-1 release and expression of the precursor, proglucagon, at protein and mRNA levels, which were inhibited by mifepristone. The administration of dexamethasone or budesonide to mice significantly decreased the mRNA expression of proglucagon in the ileum and partially decreased glucose-stimulated GLP-1 secretion. Compound A, a dissociated glucocorticoid receptor modulator, did not affect the expression of proglucagon *in vitro*. These results suggested that glucocorticoids directly reduced GLP-1 production at the transcriptional level in L cells through a glucocorticoid receptor dimerization-dependent mechanism.

1. Introduction

Steroidal anti-inflammatory drugs, glucocorticoids (GCs), are known to be the most effective anti-inflammatory or immunosuppressive drugs, and have been used in the treatment of various intractable diseases such as rheumatoid arthritis, asthma, atopic dermatitis, inflammatory bowel disease, and cancer as well as transplant rejection (Henneicke et al., 2014). Therefore, large numbers of patients have been administered GCs (Kanis et al., 2004). However, GCs frequently induce various side effects, which restrict their use (Schacke et al., 2002). An impairment in glucose homeostasis was identified as one potential side effect in many patients administered GCs. Increments in insulin resistance in insulin-targeted organs, including the liver, adipose tissues, and skeletal muscle, as well as the up-regulation of gluconeogenesis in the liver are mainly involved in GC-induced hyperglycemia (Ferris and Kahn, 2012). Osteoblasts were also recently identified as novel targets that play a role in GC-induced hyperglycemia (Brennan-Speranza et al., 2012).

GCs mainly exert their effects through intracellular glucocorticoid receptors (GRs). In the absence of a ligand, GRs exist in the cytoplasm as a multiunit complex with several proteins including 2 molecules of heat shock protein 90 (Leung and Bloom, 2003). The binding of GC causes the chaperone complex to dissociate from GR, thereby inducing its translocation to the nucleus. GRs then transactivate or transrepress the expression of various genes. Dimerized GRs have been shown to bind to GC response elements (GREs) in the promoter of GC-target genes, resulting in transactivation or transrepression in a gene-dependent manner (De Bosscher et al., 2003). On the other hand, the direct binding of GRs to negative GREs led to transrepression (Surjit et al., 2011). Moreover, GR monomers inhibit the transcriptional activity of other transcription factors, such as NF-κB and AP-1, by protein-protein interactions (De Bosscher et al., 2003). The adverse effects of GCs on glucose homeostasis may be attributed to the induction of enzymes involved in gluconeogenesis and inhibition of insulin production, which are regulated by GR dimers. Compound A (CpdA), a dissociated GR modulator, was previously shown to bind to GR strongly, but abrogated GR dimerization, resulting in dissociation between the effects of gene transactivation via the GR dimer and those of gene transrepression via the GR monomer (Robertson et al., 2010; Schacke et al., 2004). Thus, dissociated GC modulators are expected to cause less adverse effects.

Glucagon-like peptide-1 (GLP-1) is a peptide hormone that enhances the glucose-dependent release of insulin (Holst, 2007; Mojsov et al., 1987). GLP-1 is produced by prohormone convertase 1/3 (PC1/3) from proglucagon in intestinal L cells (Holst, 2007). The expression of proglucagon was reported to be induced in intestinal L cells by various factors including insulin (Yi et al., 2008), Wnt/β-catenin (Ni et al., 2003), and interleukin-6 (Ellingsgaard et al., 2011). Several transcriptional factors, such as Pax6 (Hill et al., 1999; Trinh

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et al., 2003) and TCF4 (Yi et al., 2005), have been identified as activating factors of the proglucagon gene in the gut. GPR119 wasW recently shown to be a G protein-coupled receptor linked to increases in mRNA synthesis for proglucagon through cAMP-dependent and Epac2-independent pathways (Chepurny et al., 2013). Accumulated GLP-1 is secreted in response to glucose (Reimann and Gribble, 2002) and free fatty acids (Hirasawa et al., 2005) in the diet. Moreover, various hormones and neuromodulators such as glucose-dependent insulinotropic peptide (Roberge and Brubaker, 1993), gastrin-releasing peptide (GRP) (Persson et al., 2000; Roberge et al., 1996), and muscarinic agonists (Abello et al., 1994) are known to contribute to the secretion of GLP-1. The pancreas is one of the important target organs of GLP-1. In addition to enhancing glucose-stimulated insulin secretion (Mojsov et al., 1987), GLP-1 prevented apoptotic death in β-cells and stimulated β-cell proliferation (Li et al., 2003; Wang et al., 2004).

Several reports raised the possibility that GCs regulate the production of GLP-1. In the hindbrain of the rat, proglucagon mRNA decreased after exposure to acute stressors or subcutaneous injection of corticosterone, a GC secreted by stressors (Zhang et al., 2009). GCs reduced the jejunal mRNA of proglucagon in rats in which 50% of the middle small intestine was removed (Thiesen et al., 2002). However, it currently remains unknown whether GCs directly affect the production of GLP-1 in intestinal L cells or intact gut.

In the present study, we investigated the effects of GCs on the production of GLP-1 using GLUTag and STC-1, intestinal L cells lines, and intact mice. We demonstrated that GCs reduced GLP-1 production by reducing the expression of proglucagon at the mRNA level via GR dimerization-mediated mechanisms.

2. Materials and methods

2.1. Materials

Dexamethasone (Dex), hydrocortisone (Hyd), prednisolone (Pre), budesonide (Bud), and KR62436 were purchased from Sigma-Aldrich (St. Louis, MO). Mifepristone (Mif) was purchased from Bayer AG (Leverkusen, Germany). Forskolin and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Diprotin A was purchased from Peptide Institute (Osaka, Japan). CpdA was purchased from Enzo Life Sciences (Farmingdale, NY).

2.2. Cell culture and drug treatments

The GLUTag and STC-1 cell, lines of mouse intestinal L cells were kindly provided from Dr. Drucker, Mount Sinai Hospital SLRI, Canada, and Dr. Hanahan, Swiss Federal Institute of Technology Lausanne, Switzerland, respectively. GLUTag cells were cultured in Dulbecco's modified Eagle's medium with 1.0 g/l glucose (DMEM, Nissui Seiyaku, Tokyo, Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Biowest, Miami, FL), 18 μg/ml penicillin G potassium (Meiji Seika, Tokyo, Japan), and 50 μg/ml streptomycin sulfate (Meiji Seika). STC-1 cells were cultured in DMEM with 4.5 g/l glucose supplemented with 15% (v/v) heat-inactivated horse serum (HS, Invitrogen, Carlsbad, CA) and 2.5% (v/v) heat-inactivated FBS. Cells were cultured at 37 °C in a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO_2. All studies were conducted using 80–90% confluent cells in 12- or 24-well plates, which were co-stimulated with GR agonists and Mif for 1–4 days and medium was replaced at day 2. Final concentrations of Dex, Hyd, Pre, Bud, and Mif were 1.25–20 nM, 12.5–200 nM, 2.5–40 nM, 0.0625–1 nM, and 100 nM, respectively. These all were dissolved in ethanol.

2.3. GLP-1 secretion in vitro

The *in vitro* GLP-1 secretion study was performed according to the methods reported previously with slight modifications (Hayashi et al., 2014). Cells were washed with 500 μl of glucose-free Krebs–Ringer medium containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 22 mM $NaHCO_3$, 0.1 mM diprotin A, and 0.5% (w/v) BSA, and then treated with 10 μM forskolin, 20 μM IBMX, and 25 mM glucose in 500 μl of Krebs–Ringer for 2 h at 37 °C, 5% CO_2. After the incubation, the medium was collected and centrifuged to remove any floating cells. GLP-1 in the supernatant was assayed by an enzyme-linked immunosorbent assay (ELISA) as described below.

2.4. GLP-1 ELISA

Active GLP-1 levels were measured by ELISA according to the manufacturer's instructions (Shibayagi, Gunma, Japan).

2.5. Western blotting

Western blotting was performed as described previously (Hirasawa et al., 2009; Sato et al., 2013). The antibodies used as the primary antibodies were mouse anti-proglucagon antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit PC1/3 antibody (Abcam, Cambridge, UK), and goat anti-actin antibody (Santa Cruz Biotechnology). Those used as the secondary antibodies were horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology, Beverly, MA), horseradish peroxidase-conjugated anti-mouse IgG (Cell Signaling Technology), and horseradish peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology).

2.6. Measurement of PC1/3 activity

PC1/3 activity was measured as previously reported (Hayashi et al., 2014; Imaizumi et al., 2000; Kato et al., 1998). Briefly, cell lysates (20 μg of protein) in buffer containing 25 mM PIPES, 120 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$-$6H_2O$, 40 mM NaOH, and 1 mM $CaCl_2$ (pH 7.2, PIPES-NaOH buffer) were added to the reaction mixture. The reaction mixture contained PIPES-NaOH buffer, 0.1% (w/v) BSA, and 0.4 mM Boc-Arg-Val-Arg-Arg-MCA (Peptide Institute, Osaka, Japan) in a total reaction volume of 200 μl. The reaction was performed at 37 °C for 30 min, and stopped by adding 800 μl of 1 M acetate buffer (pH 4.8). The fluorescence intensity of the solution was read at an emission of 460 nm with excitation at 380 nm with a fluorescence spectrophotometer F-2000 (Hitachi, Ibaragi, Japan).

2.7. Quantitative real-time PCR

Total RNA was extracted from cultured cells or tissues using RNAiso plus (Takara, Shiga, Japan) according to the manufacturer's instructions. cDNA was synthesized from total RNA by reverse transcription using the PrimeScript™ RT master mix (Takara) according to the manufacturer's instructions. Real-time PCR was performed using SYBR® *premix Ex Taq*™ II (Takara) in a Thermal cycler dice® real time system (TP800, Takara). As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were quantified in parallel with the target genes. The primers used for real-time PCR were (forward) 5'-TTCGCAGCTCAGGCTCACA-3' and (reverse) 5'-GCTGCCTTGCACCAGCATTA-3' for mouse proglucagon mRNA, (forward) 5'-CATGGCCAGACCAGAGTACA-3' and (reverse) 5'-CCAAGCCCCTTTTGTGGTTC-3' for mouse family with sequence similarity 107, member A (Fam107a), and (forward) 5'-TGTGTCCGTCGTGGATCTGA-3' and (reverse) 5'-TTGCTGTTGAAGTC GCAGGAG-3' for mouse GAPDH. Normalization and fold changes