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Full Length Research Paper

The Role of Glycyrrhizin in Modulating Secretory Component Expression in Caco-2 Cells

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The aim of this study was to investigate whether glycyrrhizin, with steroid hormone-like effects, can upregulate expression of secretory component (SC) in human colonic epithelial Caco-2 and to explore its underlying mechanism. Cultures of Caco-2 were exposed to glycyrrhizin. Free SC in culture supernatants, SC protein, and SC mRNA expression were measured by enzyme-linked immunosorbent assay (ELISA), western blot, real-time PCR, respectively. Cultures of Caco- 2 were exposed to RU486, a glucocorticoid receptor (GR) antagonist, combined with glycyrrhizin or dexamethasone (DEX), SC protein and SC mRNA expression were examined. Glycyrrhizin dose-dependently upregulated free SC in culture supernatants, SC mRNA and protein expression of SC ($p<0.05$). RU486 could inhibit DEX effects on SC expression ($p<0.05$), but did not inhibit glycyrrhizin effects on SC expression ($p>0.05$). The present study indicates that glycyrrhizin has a glucocorticoid-like upregulated effect on SC production in Caco-2 cells. It is likely that this effect is different from the mechanism of glucocorticoids.

Key words: Licorice, glucocorticoid, polymeric Ig receptor, glucocorticoid receptor.

INTRODUCTION

The mucosal immune system provides the first line of defense against the entrance of a multitude of ingested and inhaled microorganisms (Tjärnlund et al., 2006). Secretory component (SC), a key antibody in mucosal immune defenses, is the extracellular cleaved ectodomain of the polymeric immunoglobulin receptor (pIgR) that is responsible for the transcytosis of newly synthesized immunoglobulin A (IgA) (Crottet et al., 1998). SC plays a protective role in preventing the proteolytic degradation of polymeric IgA, enhancing the mucosal immunity provided by IgA at these sites; Free SC is important for the enhancement of immune responses. For example, degranulation of eosinophils caused by binding of secretory immunoglobulin A S-IgA or SC is mediated through the 15.000 MW SC receptor expressed in eosinophils (Motegi et al., 1998; Lamkhioued et al., 1995). Degranulation of IL- 3-primed basophils is also mediated by SC (Iikura et al., 1998). Free SC is also a key defense against enteropathogenic *Escherichia coli* (Araújo et al., 2001), and can inhibit *Salmonella typhimurium* adhesion to HeLa cells (Bessler et al., 2006). Increasing evidence indicates that high SC expression is important in limiting the inflammatory response to bacterial and viral products, via the anti-inflammatory function of SC (Davids et al., 2006; Giugliano et al., 1995; Dallas et al., 1998).

Glycyrrhizin is the major active component extracted from licorice (*Glycyrrhiza glabra* L.) roots and with a structure of 20 -carboxy-11-oxo-30-norolean-12-en-3 -yl-2-O- -D-glucopyranuronosyl- -D-glu-copyranosiduronic acid (Tjärnlund et al., 2006). So far, glycyrrhizin has been used for numerous medical purposes. In Japan, it has been used clinically for more than 20 years for the treatment of patients with chronic hepatitis (Yoshida et al., 2010). To date, various immunomodulatory properties, including the inhibition of inflammatory responses (Kai et al., 2003; Genovese et al., 2009), augmentation of NK cell activities (Miyaji et al., 2002), and induction of various cytokines from immunocompetent cells (Zhang et al., 1993; Utsunomiya et al., 1997; Dai et al., 2001), have been attributed to glycyrrhizin. The antioxidant anti-inflammatory, antiviral, anticarcinogenic, and

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glucocorticoid-like effects of glycyrrhizin have been described (Hidaka et al., 2007; Ikeda et al., 2008).

So far, *in vitro* and animal studies have demonstrated that glucocorticoids have a direct stimulatory effect on SC production (Takei et al., 2008; Yoh et al., 2002) . We hypothesized that glycyrrhizin would induce SC production. To address this hypothesis, the present study evaluated SC synthesis in human colonic epithelial Caco-2 by glycyrrhizin, and explored the possible underlying mechanism of the effect.

MATERIALS AND METHODS

Caco-2 cells were derived from the American Type Culture Collection (Manassas, Virginia, USA) and cultured on 25-mm plastic Petri dishes at 37°C under air plus 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM; GiBCO, nitrogen Co, Carlsbad, USA) supplemented with 20% fetal calf serum (FCS, HyClone Laboratories, Logan, UT, USA), 2 mM L-glutamine, 0.1% pyruvate sodium, 1% nonessential amino acids, 100 IU/ml each of streptomycin and penicillin, and 3.7 g/L sodium hydrogen carbonate. Cells were cultured in FCS-free DMEM containing the required amount of glycyrrhizin (Minofayan Pharmaceutical Co, Ltd, Japan). Moreover cells were cultured in FCS-free DMEM containing RU486 (Sigma, St. Louis, MO, USA) in combination with glycyrrhizin or dexamethasone (DEX; Sigma, St. Louis, MO, USA).

Measurement of secretory component

The supernatant SC level was quantified by using enzyme-linked immunosorbent assay (ELISA) method as described previously (Liu et al., 2007). The absorbance of each solution was determined at a wavelength of 450 nm.

Western blot analysis of Caco-2 cell proteins

Solubilized protein obtained from Caco-2 cells was denatured with sodium dodecylsulfate and -mercaptoethanol at 100°C for 5 min, and 60 g of the resulting total protein was separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The blots were blocked with Tris-buffer (pH 7.5) containing 5% skim milk and probed with polyclonal goat anti-human SC antibody (1:1000 dilution, Zymed, San Diego, USA), followed by peroxidase-conjugated secondary antibodies, then probed with rabbit anti-human -actin antibody (1:1000 dilution, Santa Cruz, California, USA), followed by peroxidase-conjugated secondary antibodies, detected with the ECL Plus chemiluminescence detection system (Pierce, Rockford, USA), and quantified by densitometry.

Analysis of secretory component mRNA expression

Cellular RNA was extracted from intestinal tissue using the RNAse Mini Kit (Takara Biotechnology Corp, Japan). The quality of extracted RNA was determined by agarose gel electrophoresis and cDNA synthesized using 100 ng of RNA. The concentrations of individual RNA transcripts were quantified by real-time polymerase chain reaction (PCR). To control variations in the reactions, all PCRs were normalized against GAPDH expression. The primers used were:

SC-F: 5'-ACAGGGTTTCGCCATGTTGAC-3';
SC-R: 5'-AGGCTCAGTGGCTCATGCCTA-3';
GAPDH-F: 5'-GCACCGTCAAGGCTGAGAAC-3';
GAPDH-R: 5'- TGGTGAAGACGCCAGTGGA-3'.

Primers and fluorescent probes were purchased from Takara. The PCR conditions comprised a preliminary cycle of 95°C for 10 s, followed by 45 cycles of 95°C for 5 s and 57°C for 20 s, followed by 57°C for 1 min and 95°C for 5 s. The results are given as a ratio of specific mRNA/GAPDH. The RNA concentration of untreated Caco-2 cells was set at 1 for comparison with treated Caco-2 cells.

Influence of glucocorticoid receptor inhibitors on the effects of glycyrrhizin

Cells were fed in serum free culture medium supplemented with RU486 (50 nM) combined with glycyrrhizin (20 µg/ml) or DEX (100 nM) for 24 h. After cells were harvested, protein and RNA were prepared. SC protein expression measured by western blot was normalized to -actin, meanwhile SC mRNA expression detected by real-time PCR was normalized to GAPDH mRNA.

Statistical analysis

Software SPSS 11.0 set was employed for statistical analysis of the data, with each parameter expressed as the mean ± standard error of the mean (SE), and compared using one-way ANOVA, followed by a Turkey test. Statistical significance was considered at $p<0.05$.

RESULTS

ELISA of SC in Caco-2 cells incubated with glycyrrhizin

SC level almost could not be detected in Caco-2 cells incubated without glycyrrhizin, but SC increased in culture supernatants of Caco- 2 cells incubated with glycyrrhizin in a dose-dependent manner (Figure 1).

SC expression in Caco-2 cells by western blotting

Western blotting was used to evaluate SC expression in Caco-2 cells. The increased SC expression was found in glycyrrhizin treated groups in comparison with control. The optical density of the glycyrrhizin treated groups and control group was measured and expressed graphically. Dose-dependent enhancement of SC protein expression observed in glycyrrhizin treated cells was incubated for 24 h with glycyrrhizin at various concentrations (n=3, $^{*}p<0.01$ vs. control) (Figure 2).

Quantitative real-time PCR analysis of SC mRNA in Caco-2 cells

At the transcriptional level, we used real-time PCR to evaluate SC mRNA expression in Caco- 2 cells. Likewise, the expression of SC mRNA showed a dose dependent upregulation with glycyrrhizin treatment (n=3, $^{*}p<0.01$ vs. control) (Figure 3).

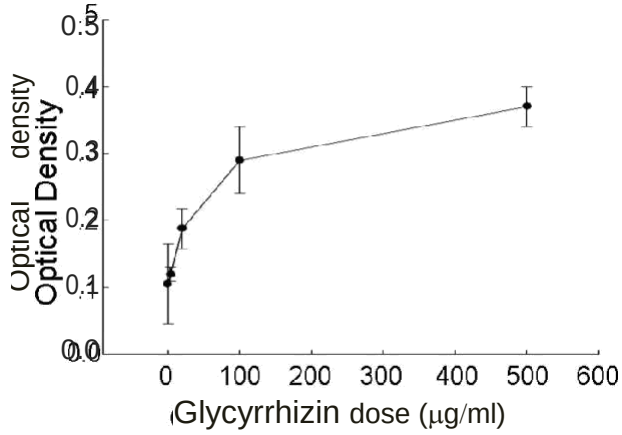

Figure 1. The relation of secretory component secretion in Caco-2 cells to dose of glycyrrhizin.

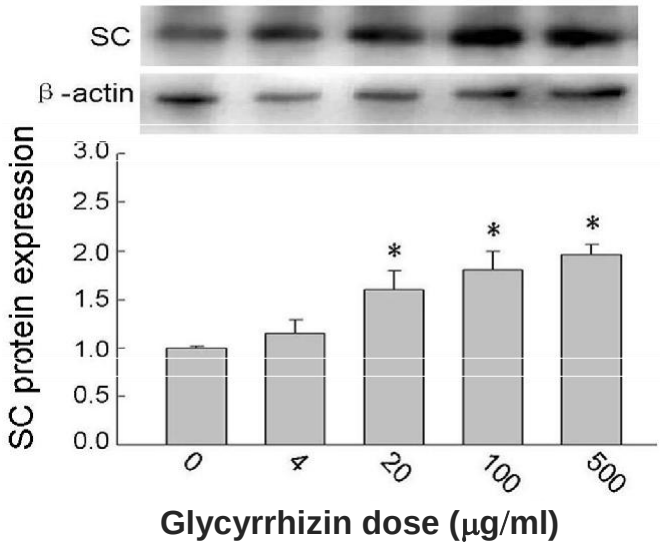

Figure 2. Western blotting for detection of secretory component expression. cells treated with serial-diluted glycyrrhizin (0, 4, 20, 100, and 500 g/ml) for 24 h; SC protein expression had a consistent association with increasing glycyrrhizin dose (n = 3, $^{*}p < 0.05$ vs. control).

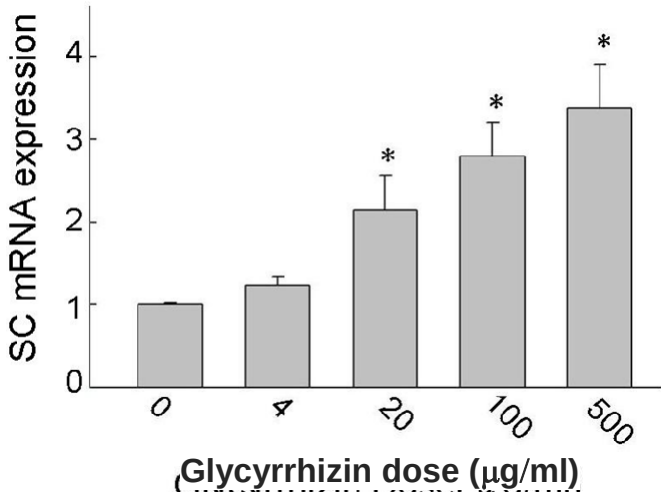

Figure 3. Real-time PCR analysis of SC mRNA. Dose-dependent enhancement of SC mRNA expression observed in glycyrrhizin treated cells, incubated for 24 h with glycyrrhizin at various concentrations (n = 3, *$p < 0.01$ vs control).

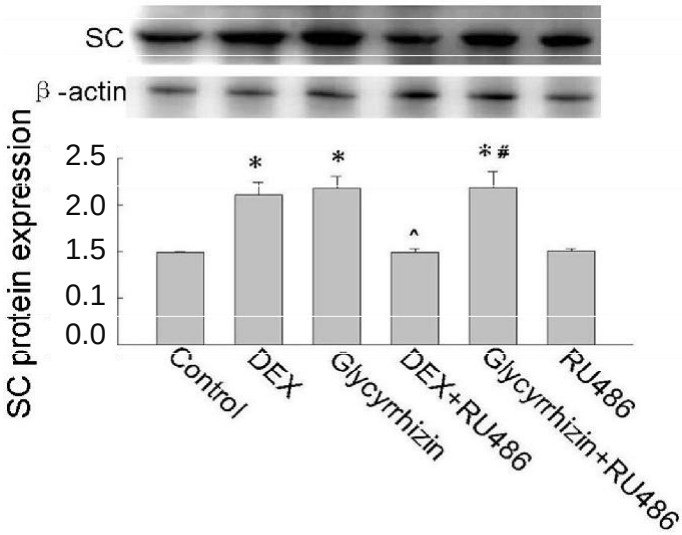

Figure 4. Influence of GR inhibitors on glycyrrhizin effects. DEX and glycyrrhizin notably upregulated the expression of SC protein in Caco- 2 cells (* compared with control group, $p < 0.05$). RU486 could inhibit DEX effects on SC protein expression (^compared with DEX group, $p < 0.05$), but did not inhibit glycyrrhizin effects on SC protein expression (# compared with glycyrrhizin group, $p > 0.05$).

Influence of glucocorticoid receptors inhibitors on glycyrrhizin effects

Considering the structural similarity between glycyrrhizin and glucocorticoids, it might be conjecturable that the glucocorticoid-like effects of glycyrrhizin might be mediated through glucocorticoid receptors (GRs) (Takei et al., 2008). The influences of a glucocorticoid receptor (GR) antagonist on glycyrrhizin effects were tested through SC protein and SC mRNA assays. DEX and glycyrrhizin notably upregulated the expression of SC protein and SC mRNA in Caco-2 cells ($p<0.05$, Figures 4 and 5), RU486 could significantly inhibit DEX effects on SC protein and SC mRNA expression (DEX combined RU486 treated group vs. no inhibitor group, $p<0.05$) (Figures 4 and 5), but RU486 did not inhibit glycyrrhizin effects on SC protein and SC mRNA expression (glycyrrhizin combined RU486 treated group vs. no inhibitor group, $p>0.05$, Figures 4 and 5).

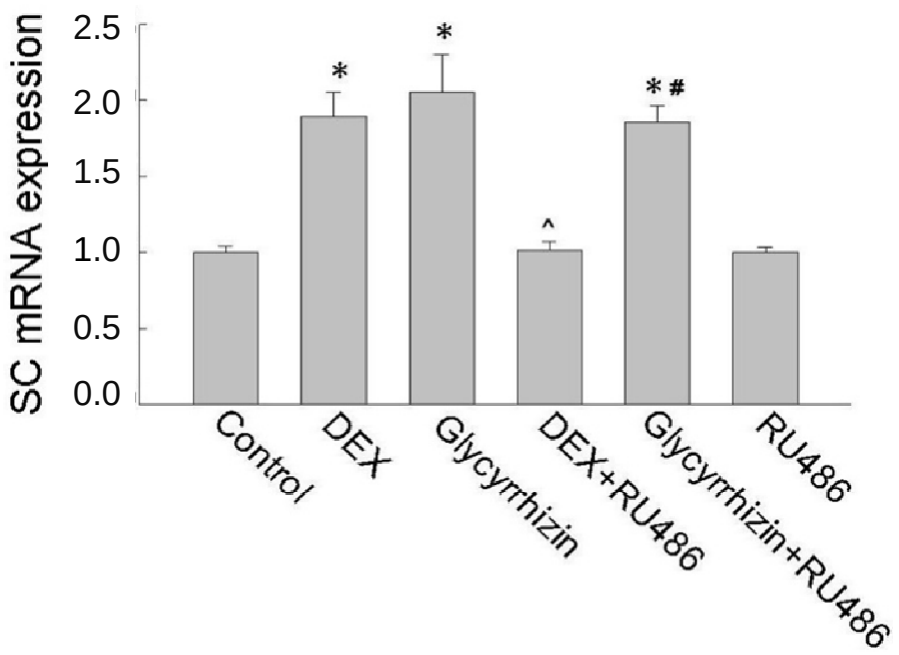

Figure 5. Influence of GR inhibitors on glycyrrhizin effects. DEX and glycyrrhizin notably upregulated the expression of SC mRNA in Caco-2 cells (* compared with control group, $p < 0.05$). RU486 could inhibit DEX effects on SC mRNA expression (^ compared with DEX group, $p < 0.05$), but did not inhibit glycyrrhizin effects on SC mRNA expression (# compared with glycyrrhizin group, $p > 0.05$).

DISCUSSION

Secretory antibodys (SAbs), particularly secretory immunoglobulin (SIgA), is the most typical feature of mucosal immune system in mammals, which can fix bacteria (Kudsk et al., 2002), viruses (Mantis et al., 2004) and other toxic molecules, preventing them adhere to mucosal surfaces. SIgA can exclude the infectious factors that had penetrated the epithelial cell layer. It shows an inverse relationship with bacterial overgrowth, bacterial translocation and intestinal permeability (Langkamg-Henken et al., 1995), so SIgA plays an important role in the anti-infection (Mayer, 1997a, b).

Polymeric immunoglobulin receptor (PIgR), also known as membrane SC, is a secreted glycoprotein expressed on basal surface of intestine and crypt epithelial cells (Brandtzaeg et al., 1984; Johansen et al., 1999), which transports dimeric IgA (dIgA) into external secretions as SIgA. It has been estimated that pIgR (SC)-mediated transport of IgA by intestinal epithelium cells results in the daily delivery of 3 g of secretory IgA into the intestine in an average adult (Kaetzel, 2005). Free SC is important for the enhancement of immune responses. Free SC can bind to several bacterial proteins, such as colonization factor antigen (Oliveira et al., 2001), *Clostridium difficile* toxin A (Dallas and Rolfe, 1998), and *Streptococcus pneumoniae* surface protein C (PspC) (Hammerschmidt et al., 2000). High expression of SC is considered to play an important role in limiting the inflammatory response (Davids et al., 2006; Giugliano et al., 1995; Dallas et al., 1998).

These findings indicated that glycyrrhizin could upregulate the expression of SC (Figures 1 to 3), which plays an important role in limiting acute inflammation. Through GRs, glucocorticoids translocate into the cell nucleus, where they bind to responsive elements present in the regulatory region of glucocorticoid- target genes (Longui, 2007; Nipapan et al., 2010). Due to the structural similarity between glycyrrhizin and glucocorticoids, glucocorticoid- like effects of glycyrrhizin may be mediated through GRs. In this study, in order to study whether glycyrrhizin regulated by glucocorticoid receptor expression in SC, we used glucocorticoid receptor antagonist RU486 combined with DEX or glycyrrhizin treated Caco-2 cells to observe changes in SC proteins and SC mRNA. In the present study, Add DEX treatment or glycyrrhizin treatment alone groups SC mRNA and SC protein expression were notably upregulated compared with the control group ($p<0.05$, Figures 4 and 5), and RU486, a glucocorticoid receptor antagonist, could significantly inhibit DEX effects on SC protein and SC mRNA expression, the differences were statistically significant ($p<0.05$, Figures 4 and 5), but the study also found that in SC, there was no significant difference in protein expression between glycyrrhizin combined RU486 treatment group and glycyrrhizin alone treatment group

(Figure 4); further analysis on the mRNA level in SC mRNA expression also, there was no significant difference between glycyrrhizin combined RU486 treatment group and glycyrrhizin alone treatment group (Figure 5). These findings indicated that glycyrrhizin had a glucocorticoid- like effect on SC production via a mechanism that differs from that of glucocorticoids. Previous, Takei et al. (2008) used RU486 combined with glycyrrhizin treatment of lung epithelial cell line A549 to observed whether glycyrrhizin was the same as the glucocorticoid inhibition of glucocorticoid receptor on IL-8 expression; they also found that glucocorticoid receptor antagonist could not block the expression of the IL-8 inhibitory effect by glycyrrhizin, suggesting that although glycyrrhizin has glucocorticoid-like effect, but it seems that the way glycyrrhizin plays on may not be the same as that of glucocorticoids. The pathway by which glycyrrhizin acted on SC gene production requires further study.

In conclusion, the results of this study indicate that glycyrrhizin has a glucocorticoid- like upregulated effect on SC gene expression in Caco-2 cells, but it seems that the way glycyrrhizin plays on may not be the same as that of glucocorticoids.

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