ERCİYES ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ
Journal of Faculty of Veterinary Medicine, Erciyes University

Araştırma Makalesi / Research Article
15(1), 6-10, 2018

The Effects of Dexamethasone on Claudin Tight Junction proteins in 18-day Embryonic Chick Intestine*

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Summary: Claudin (cldn) protein family is the main component of tight junction (TJ) strands and it is directly involved in the barrier function of TJs. In addition to the contributing to the barrier function of TJs in mature tissues, these junctions are also present and required for normal embryonic development of epithelial sheets. Glucocorticoids (GC) are essential for the maturation of numerous tissues including the intestine during embryonic development. They also have stimulatory effects on the integrity of tight junctions. In this study, the effect of a synthetic glucocorticoid, dexamethasone (DEX) on mRNA expression of tight junctional proteins was investigated in 18-day embryonic chick intestine *in vitro* using quantitative Real-Time PCR (RT-PCR) analyses. Pre-hatched chick intestine will provide a convenient model because the organization of the barrier is not completely established, and it is relatively easy to maintain its morphological integrity for at least 24 hours in an organ culture system. RT-PCR analyses revealed that both claudin-3 (cldn-3) and claudin-5 (cldn-5) tight junction gene expressions were induced in response to DEX in a dose dependent manner.

Key words: Claudin, dexamethasone, gene expression, glucocorticoids, intestine

Dexamethasonun 18 Günlük Embriyonik Civciv Bağırsağındaki Claudin Sıkı Bağlantı Proteinlerine Etkileri

Özet: Claudin (cldn) protein ailesi sıkı bağlantı yapılarının (tight junctions) temel birleşenidir ve sıkı bağlantı zincirlerinin bariyer fonksiyonuna direk etki eder. Sıkı bağlantıların gelişmiş dokulardaki bariyer fonksiyonuna katkısının yanında, bu bağlantı proteinleri, epitelin embriyonik gelişimi süresince mevcuttur ve normal gelişim için gereklidir. Glukokortikoitler bağırsak dahil birçok dokunun embriyonik gelişimi için esastır. Aynı şekilde, sıkı bağlantıların bütünleşmesine uyarıcı etkileri mevcuttur. Bu çalışmada, sentetik bir glukokortikoit olan dexamethasone (DEX)'in *in vitro* koşullarda 18 günlük embriyonik civciv bağırsağındaki sıkı bağlantı proteinlerinin mRNA ekspresyonlarına bir etkisinin olup olmadığı kantitatif Real-Time PCR (RT-PCR) kullanılarak araştırıldı. Henüz yumurtadan çıkmamış civciv bağırsağı bu çalışma için uygun bir model olmaktadır, çünkü epitelyumdaki bariyer henüz tam olarak oluşmamış ve ayrıca bu bağırsak 24 saatlik organ kültüründe morfolojik bütünlüğünü koruyabilmektedir. RT-PCR analizleri hem cldn-3 hem de cldn-5 sıkı bağlantı proteinlerinin gen ekspresyonlarının DEX'in etkisiyle doza bağlı olarak arttığını ortaya çıkartmıştır.

Anahtar kelimeler: Bağırsak, claudin, dexamethasone, gen ekspresyonu, glukokortikoidler

Introduction

In chick embryos, rapid functional differentiation of the epithelium occurs between 14th and 21st days (the week before hatching), including changes in cell shape, formation of microvilli and the terminal web, and a dramatic increase in the density of goblet cells (1,2). The organization and maturation of epithelial and endothelial tight junctions (TJs) occur during this period as well. Claudin (cldn) protein family is the main component of TJ strands and these proteins are directly involved in the barrier function of TJs (3). Additionally, the cldn protein family with its more than 20 members creates charge and size selective pores in the paracellular pathways, thus exerting a critical influence in the composition of the transported solutes in a tissue specific manner (4-6).

The stimulative effects of glucocorticoids (GCs) on the barrier function of TJs in the mammary gland, intestine, lung, kidney and cerebral endothelia were demonstrated by using transepithelial resistance (TER) and paracellular permeability studies in vitro and/or in vivo (7-10). Moreover, the enhancing effects of GCs on the expression of endothelial occludin, ZO-1 and cldn-5 were reported (7,11-13). However, not much is known about the effects of GCs on the expression of other tight junctional proteins in various epithelia, even though GCs are currently

Geliş Tarihi/Submission Date : 14.06.2016
Kabul Tarihi/Accepted Date : 28.02.2017

* This article was summarized from Dr. Özkan ÖZDEN's PhD dissertation.

used clinically for the treatment of various epithelial permeability dysfunctions. GCs induce the expression and/or reorganization of TJ proteins in the epithelial and endothelial cell layers; and accordingly, they promote the tightening of the TJ barrier (14). Abnormalities in the barrier function of TJs (increase in paracellular permeability) are seen in various pathological conditions, such as in Crohn's disease, alcoholic liver disease, celiac disease and various diarrheal syndromes (15,16). A number of studies have showed that GC treatment induces a tightening effect on cerebral endothelial TJs in vitro (1,11,17). In endothelial cells, GC treatment changes the position of occludin by its dephosphorylation, and further increases the expression of other TJ proteins to form tighter TJs (18). In cultured brain endothelial cells, 1M dexamethasone (DEX) (a synthetic glucocorticoid) treatment decreased paracellular permeability for sucrose, flurescein and dextrans of up to 20kDa, probably modulating the expression and/or organization of TJ proteins (10). GCs were suggested to support the integrity of blood -brain barrier (BBB), and they are currently used for the treatment of brain tumors. GCs are also considered a potential treatment for diabetic retinopathy, which is characterized by the loss of blood-retinal barrier (BRB) (14,16). In addition to their tightening effects on endothelial TJs, GCs also affect the strength of epithelial layers in various tissues. In this study, Real-Time PCR (RT PCR) was utilized to investigate the changes in the expression of cldn-3 and cldn-5 in response to different concentrations of DEX at different time points in 18-day pre-hatch chick intestine.

Materials and Methods

Animals Fertile broiler-type chicken fertile eggs were obtained from the Poultry Farm of North Carolina State University and incubated for 18 days in a humidified incubator at 37 ºC. ***Tissue Preparation and Intestinal Organ Cul-ture*** Five embryonic chicks were sacrificed, and the duodenal loop was immediately removed and rinsed with phosphate buffered saline (PBS), pH 7.4 to remove blood and debris. Tissue approximately 2-3 mm long pieces were taken from the duodenum loop, split opened. Three 25-mL Erlenmeyer flasks including 3 mL culture medium (Medium 199, GIBCO, USA) and streptomycin were prepared and supplemented with a synthetic glucocorticoid, (DEX) with final concentrations of 0M, 10^{-2}M, 10^{-1}M, and 1M (Figure 1). Three to five duodenal segments were placed into flasks and gassed with a mixture of 95% O_2 and 5% CO_2. Flasks including tissue pieces were incubated in a 37 ºC incubator. Cultured tissue pieces were preserved in RNAlater (Ambion, USA) solution and stored at -20 ºC.

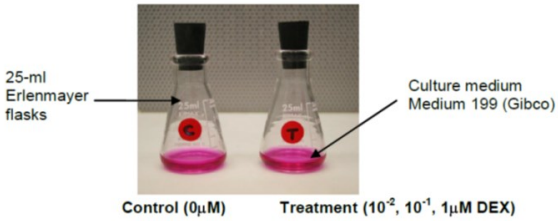

Figure 1. Intestinal organ culture was performed in 25-mL Erlenmeyer flasks containing Medium 199.

Isolation of Epithelial Cells from Intestinal Villi The isolation of epithelial cells from intestinal villi was performed as previously described (19). Briefly, tissue pieces removed from the small intestine were incubated in citrate buffer for 20 minutes at room temperature. Then, they were transferred to an EDTA-containing buffer and incubated at 37 ºC with shaking for 30 minutes. The resulting cell suspension was filtered through silk screen to remove tissue debris and centrifuged 1000 rpm for 5 minutes to obtain a pellet of epithelial cells. The cells were re-suspended in PBS buffer and utilized for RT-PCR analyses.

Total RNA Extraction and SYBR Green Real-Time PCR Analyses Organ cultured tissues were homogenized in RLT buffer (Qiagen, USA) including β-mercaptoethanol using a bead beater. Total RNA was purified with DNase according to manufacturer instructions (Ambion, USA). Reverse transcription and SYBR green RT-PCR analyses were performed. β-actin was used as an internal control. Cycle threshold (Ct) values were obtained using the Icycler Software, duplicate values of each cDNA were averaged, and relative fold changes were determined using $2^{-\Delta\Delta Ct}$ method. Experimental errors were calculated by the standard error of the mean (SEM) of normalized Ct values from each treatment group, and were indicated as error bars in the graphs. Least Square Means differences with Student's t test were performed to determine the significance between treatment groups and

P < 0.05 was accepted as statistical significance. The annealing temperatures and the expected sizes of produced amplicons for cldns, and the RT-PCR protocol were previously published (20).

Results

The expression of cldn-3 and -5 was investigated using comparative SYBR Green RT-PCR analysis. Cldn-3 expression was increased in a dose dependent manner in response to DEX (Figure 2). There was no significant time effect on cldn-3 in a given DEX dosage between 12 hours and 24 hours of exposures. At physiological levels (10^{-2}M) DEX did not induce any significant increase in cldn-3 for 12 hours. Moderate (10^{-1}M) and high levels (1M) of DEX exposure for 12 hours caused approximately 2.5 and 3.2 folds increase in cldn-3 expression, respectively (Figure 2A). DEX exposure for 24 hours caused a dose dependent increase in cldn-3 mRNA levels; however none of them were significant (Figure 2B).

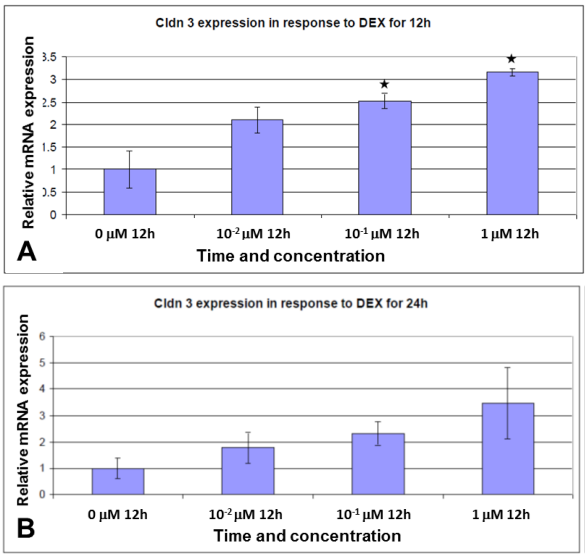

Figure 2. Relative expression of cldn-3 in response to DEX. The effects of different concentrations of DEX on cldn-3 expression for 12 hours (A), and for 24 hours (B). n = 3-4. * indicates the significant difference from 0µM concentration at P < 0.05.

Relative cldn-5 mRNA levels in response to different concentrations of DEX at different time points were also investigated (Figure 3). Cldn-5 displayed a similar pattern to cldn-3 expression. At a concentration of 10^{-2}M, DEX did not induce any significant increase in cldn-5 for 12-hour exposure (Figure 3A). However, moderate (10^{-1}M) and high (1M) doses of DEX exposure for 12 hours induced cldn-5 by approximately 3.4 and 5 fold, respectively (Figure 3A). Twenty-four hours of DEX exposure caused a dose dependent increase in cldn-5 mRNA levels; however none of them were significant (Figure 3B).

In summary, cldn-3 and cldn-5 tight junction

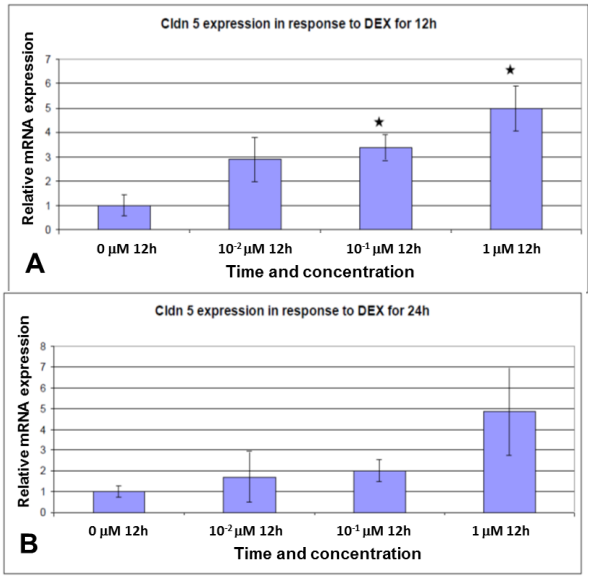

Figure 3. Relative expression of cldn-5 in response to DEX. The effects of different concentrations of DEX on cldn-5 expression for 12 hours **(A)**, and for 24 hours **(B)**. n = 3-4. * indicates the significant difference from 0µM concentration at P < 0.05.

gene expressions were induced in response to DEX in a dose dependent manner for 12 hours.

Discussion

GCs have wide range of effects in the body and modulate approximately 10% of human genes. GCs control metabolism, immune system, growth, differentiation, and development (21). GCs also play essential roles for growth differentiation, metabolism and morphogenesis of numerous mammalian and avian embryonic tissues and organs, such as the intestine, lung and central nervous system (21). GCs play important roles in functional differentiation of intestinal epithelium during the last week of chick embryonic development (19). In addition, usage of exogenous GCs is important for the treatment of various pathological conditions due to anti-inflammatory and epithelial and endothelial barrier enhancing qualities of GCs.

GCs are claimed to decrease TJ permeability significantly (shown by decreased paracellular

radiolabeled inulin flux) by suppressing myosin light chain kinase (MLCK) gene activity in Caco-2 intestinal cell lines (8). Exposure of subconfluent Madin-Darby Canine Kidney Cells (MDCK) renal epithelial cells, in which the formation of epithelial barrier is not complete, to 4M DEX for 24 hours did not affect the expression of cldn-1, ZO-1, and occludin, but it significantly increased the TER, probably by modulating the organization and relocalization of TJ proteins to the TJ areas (9). In these studies, DEX had a stronger effect on the formation of the epithelial barrier in subconfluent epithelial cells than in the confluent cells with relatively stable TJ structure (9).

GCs affect cldn-5 expression directly and indirectly in endothelial cell lines (7). Tumor necrosis factor-alpha (TNF) down-regulates the expression of cldn-5 in brain cEND and myocardial MyEND endothelial cells (7). DEX indirectly up-regulates cldn-5 expression by down-regulating the expression of TNF in these endothelial cell lines (7). Additionally, DEX can induce both the promoter activity and mRNA levels of cldn-5 directly in cEND cells but not in MyEND cell lines, which indicate the tissue specific actions of GCs. In this study, as it was hypothesized, GCs induced the expression of cldn -3 and -5 in a dose dependent manner. However, it is not known if DEX directly regulated the expression of these genes by binding their glucocorticoid response element (GRE) regions in their promoters or indirectly changed the activity of other genes such as TNF, cytokines, and glutamine synthetase.

In order to understand the effects of GCs (and other hormones, transcription factors, etc.) on cldn expression, promoter regions of cldns should be cloned and transcription factor binding sites should be identified to determine whether they include any GRE for glucocorticoid activity. However, even though murine cldn-5 includes GRE in its promoter, the regulation of cldn-5 by GCs is cell specific, possibly due to differential activity and distribution of glucocorticoid receptors (7). In addition to genomic regulation, GCs may modulate the organization of cldns at post-transcriptional level. Therefore, the changes in the distribution of cldns at tight junctional areas in response to GCs at protein level should also be examined at the protein level. DEX exposure for 24 hours also seems to induce both cldn-3 and cldn-5 expressions in a dose dependent manner although none of the doses used caused a significant change. This might stem from variation between culture conditions due to possible decline in the stability of cultured tissues or DEX over prolonged periods. Secondly, desensitization of glucocorticoid receptors or a decline in promoter activity of these genes are possible explanations.

To conclude, the characterization of a general GC action on cldn expression and regulation is very complex. GCs exert their effect in a cell specific manner because there are multiple GC receptors for their binding in different cell types. Secondly, the responsiveness of target genes might be different in different tissues and different developmental stages of an organism. Thirdly, the activity of GCs might be modulated by their interactions with other hormones in a tissue specific manner. The diverse effects of GCs have been reported between different species, and even between individuals in the same species. In addition, the expression and organization of cldns are tissue specific and a role of a specific cldn depends on the presence of other cldns or compatibility of cldns to each other.

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