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The negative effect of dexamethasone on calcium-processing gene expressions is associated with a glucocorticoid-induced calcium-absorbing disorder

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ABSTRACT

Aims: Although dexamethasone (Dex) is used widely as an anti-inflammatory and immunosuppressive drug, Dex appears to have severe side-effects, including osteoporosis. This study determined the effects of Dex on duodenal and renal expressions of the calcium-processing genes transient receptor potential cation channel, subfamily V, member 5/6 (*TRPV5*/*6*), calbindin-D9k/-D28k (*CaBP-9k*/*28k*), Na^+/Ca^{2+} exchanger 1 (*NCX1*), and plasma membrane Ca^{2+}-ATPase (*PMCA*) *1b*.

Main methods: Mice were injected subcutaneously with Dex for 1 or 5 days. The mRNA and protein expression levels of these calcium-processing genes were measured by real-time PCR and immunohistochemistry/immunoblot analysis, respectively. In addition, serum parathyroid hormone (PTH) levels were measured following Dex treatments.

Key findings: Treatment with Dex for 24 h resulted in the inductions of duodenal *TRPV6*, *CaBP-9k* and *PMCA1b* transcripts and renal *TRPV5*, *CaBP-9k*, and *NCX1* transcripts, while it reduced the transcription of renal *TRPV6*. Although the expressional changes were weak, duodenal expressions of glucocorticoid receptor (*GR*), the vitamin D receptor (*VDR*), and renal expressions of the parathyroid hormone receptor (*PTHR*) and *VDR* were increased following 24 h treatment with Dex. A five-day treatment with Dex reduced the transcriptional levels of duodenal *TRPV6* and *CaBP-9k* by 60%. Transcripts for *VDR* and *GR* in the duodenum increased marginally.

Significance: These results suggest that the expressions of *TRPV6* and *CaBP-9k* in the duodenum appear to be a major regulatory target for glucocorticoids (GCs), and may be involved in the negative regulation of calcium absorption in GC-induced osteoporosis (GIO). The transcriptional regulation of *TRPV6* and *CaBP-9k* in the duodenum seems complex given that there is an increase at 1-day treatment followed by a decrease at 5-day treatment.

Introduction

Glucocorticoids (GCs) regulate the inflammatory response by modulating the expression of pro- and anti-inflammatory cytokine genes. Therapeutic administration of GCs has many systemic side-effects, such as the inhibition of the recruitment and activity of osteoblasts or apoptosis of osteoblasts and osteocytes, which results in a significant reduction in bone formation and calcium absorption (Hurson et al. 2007; Jia et al. 2006). Potentially the most serious and debilitating condition is the GC-induced osteoporosis (GIO) (McLaughlin et al. 2002), but its precise mechanism(s) of induction is unknown. Thus, in the present study, we examined the effect of GCs on the duodenal and renal active calcium transport system to understand the mechanism of GIO.

We showed that the actions of GCs could be reversed by a specific *GR* antagonist, which suggests that the effects of GCs are mediated by the *GR* (Lee et al. 2006). Several mechanisms underlying the decline in bone formation induced by GCs have been proposed, i.e., its direct inhibitory effect on osteoblasts (Lukert and Raisz 1990; Nijenhuis et al. 2004), inhibition of the production of insulin-like growth factor-I (Adler and Rosen 1994), and increased apoptosis of osteoblasts and osteocytes (Manolagas and Weinstein 1999). GCs directly and indirectly affect bone mineralization at many levels. The effect of GCs on calcium absorption and resorption indicates that GC-induced reduction in bone formation is associated with a decrease in the mineral apposition rate (Lukert and Raisz 1990). The reduced bone mineral density is accompanied by the decrease of intestinal calcium absorption, and increases the excretion of urinary calcium ions (Suzuki et al. 1983).

Calcium transport proteins regulate calcium influx by mediating the transport of calcium from the cytosol to the blood-stream. In intestinal and renal models of calcium flux, calcium transport is carried

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out by three classes of transport proteins: the calcium entry-channel proteins of the outer membrane, cytosolic buffering or transfer proteins, and excretory pump proteins (Choi and Jeung 2008; Lee et al. 2007b). Two highly selective calcium channels at the apical side of cells, transient receptor potential cation channel, subfamily V, member 5/6 (*TRPV5*/*6*), are the major sites of calcium-ion entry. Calbindin-D9k (*CaBP-9k*) and -D28k (*CaBP-28k*) are intracellular calcium ion-binding proteins which appear to be involved in shuttling calcium ions from the apical to the basolateral membrane, where Na^+/Ca^{2+} exchanger 1 (*NCX1*) and plasma membrane Ca^{2+}-ATPase (*PMCA*) *1b* mediate calcium extrusion (van Abel et al. 2005).

While GCs have been implicated in a decrease in calcium uptake in the duodenum and an increase in the loss of calcium in the kidney, the effect of GCs on the expression of calcium-processing genes has not been determined. Thus, in the present study, we examined the transcriptional regulation of duodenal and renal calcium-processing genes by GCs in a mouse model. In this study we include the major regulating factors, such as vitamin D receptor (*VDR*), parathyroid hormone receptor (*PTHR*) and glucocorticoid receptor (*GR*), and measured their expression levels following dexamethasone (Dex) treatment in this mouse model.

Materials and methods

Experimental animals and treatments

Mature ICR male mice (12 weeks old, $n=46$, around 25 g of body weight) were obtained from Koatech (Pyeongtaek, Gyeonggido, Korea). All animals were housed in polycarbonate cages and used after acclimation to an environmentally controlled room (temperature: 23 ± 2 °C, relative humidity: 50 ± 10%, frequent ventilation and 12 h light–dark cycle).

To examine the effect of Dex on the expression of calcium-processing genes, the mice were divided into four groups. Two groups ($n=10$ per group) were injected subcutaneously with saline as a negative control or Dex (Sigma-Aldrich, St. Louis, MO; 10 mg/kg, 25 μg per a mouse), and euthanized 24 h after injection according to the previous studies (Besnard et al. 1989; Huybers et al. 2007; Iacopino and Christakos 1990; Lee et al. 2006; Rabadan-Diehl and Aguilera 1998). The other two groups ($n=13$ per group) were injected daily with saline or Dex (10 mg/kg) for 5 days, and euthanized 24 h after the final injection. The present concentration of Dex does not affect protein and mRNA synthesis of internal controls (Lee et al. 2006). All experimental procedures and animal use were approved by the Ethics Committee of the Chungbuk National University.

Real-time PCR using TaqMan™ probe

Total RNA was prepared from the duodenum and kidney using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The complementary DNAs (cDNAs) were prepared by subjecting total RNAs (1 μg) to reverse transcription using mMLV reverse transcriptase (Invitrogen Life Technologies, Inc.) and random primers (9-mer, TaKaRa Bio. Inc., Otsu, Shiga, Japan) (Lee et al. 2006, 2007b). A real-time PCR was performed in a 20 μl reaction volume containing 10 μl of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster, CA, U.S.A.), 1 μl of 20× Assays-on-Demand™ Gene Expression Assay Mix (Applied Biosystems, *TRPV6*, Mm00499069_m1; *TRPV5*, Mm01166029_m1; *CaBP-9k*, Mm00486654_m1; *CaBP-28k*, Mm00486645_m1; *NCX1*, Mm01232248_m1; *PMCA1b*, Mm00670208_m1; *VDR*, Mm00437297_m1; *PTHR*, Mm00441046_m1; *GR*, Mm00433832_m1; and *HPRT1*, Mm00446968_m1) and 2 μl of cDNA. An amplification was carried out using a 7300 Real-Time PCR System (Applied Biosystems) and the following cycle parameters: initial denaturation at 50 °C for 2 min; 90 °C for 10 min; 40 cycles of denaturation at 95 °C for 15 s; and annealing and extension at 60 °C for 1 min. The expression levels were determined using RQ software (Applied Biosystems). The expression of *TRPV6*, *TRPV5*, *CaBP-9k*, *CaBP-28k*, *NCX1*, *PMCA1b*, *VDR* and *PTHR* was normalized to that of hypoxanthine guanine phosphoribosyl transferase 1 (*HPRT1*) as an internal control gene.

Biochemical and hormone analysis

Blood was collected from post-cava, and transported to serum separator tubes, and subjected to centrifugation at 3000 rpm for 10 min. Serum calcium levels were determined using ASAN Ca-Lq Reagents (Asanpharm, Kyeonggido, Korea).

Histological analysis

The thyroid and parathyroid glands were removed *en bloc* with the trachea and fixed in 10% formalin overnight. Briefly, tissues were dehydrated and embedded in paraffin, after which serial sections (5 μm) were cut with a microtome. The sections were stained with hematoxylin and eosin (H&E). To measure the size of the parathyroid gland, sections were analyzed using the MIP image analysis program (Motic China Group Co. Ltd.).

For immunohistochemistry, the duodenum and kidney were embedded in paraffin (Lee and Jeung 2007; Tinnanooru et al. 2008). Sections (5 μm) were deparaffinizd in xyline and hydrated in a series of descending concentrations of ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase in PBS-T (PBS plus Tween-20) for 20 min, and then sections were incubated in 10% normal goat serum (NGS) for 2 h at room temperature (RT) to block nonspecific binding. Sections were then incubated with a polyclonal rabbit antibody specific to *CaBP-9k* (diluted 1: 1,000, Swant, Bellinzona, Switzerland) or *TRPV6* (diluted 1:100, Almone Labs Ltd., Jerusalem, Israel) dissolved in 10% NGS at RT for 1 h. After a washing with PBS-T, the sections were incubated with biotinylated secondary antibody (rabbit IgG; Vector Laboratories, Burlingame, CA) for 30 min at 37 °C and then incubated in ABC-Elite (Vector Laboratories, Burlingame, CA, U.S.A.) for 30 min at 37 °C. Immunoreactive proteins were visualized with diaminobenzidine (Sigma-Aldrich), and then the sections were counterstained with hematoxylin and mounted using a coverslip.

Western blot analysis

Protein was extracted using Proprep (iNtRON Bio, Sungnam, Kyeonggido, Korea), according to the manufacturer's protocol and previous studies (Dang et al. 2007; Hong et al. 2007; Lee et al. 2007a). Forty-micrograms of total protein (per lane) were resolved by SDS-PAGE (10% acrylamide) and transferred to a nitrocellulose membrane using a Trans-Blot Cell (Bio-Rad, Hercules, CA, U.S.A.). The membranes were then blocked overnight with PBS-T and 5% skim milk. The membrane was incubated in primary antibodies diluted in 5% skim milk for 90 min at RT. Primary antibodies to *CaBP-9k* (diluted 1: 500; Swant), and ß-Actin (1:500; Santa Cruz Biotechnology, CA, U.S.A.) were used. A horseradish peroxidase-conjugated anti-rabbit IgG (diluted 1: 3,000; Santa Cruz Biotechonology) was used as a secondary antibody. Immunoreactive proteins were visualized using the West-one™ Western Blot Detection System (iNtRON Bio), according to manufacturer's instructions. Signals were detected with Chemi Doc EQ (Bio-Rad) and analyzed by Quantity One program.

Data analysis

Data was analyzed by nonparametric one-way analysis of variance using the Kruskal–Wallis test, followed by Dunnett's test for multiple comparisons to vehicle (saline). Values were converted to rank for these tests. All statistical analyses were performed with SPSS for Windows Edition (SPSS, Chicago, IL). $P<0.05$ was considered statistically significant.