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Corticosterone Regulates the Expression of Neuropeptide Y and Reelin in MLO-Y4 Cells

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Osteocytes that have a dendritic appearance are widely believed to form a complex cellular network system and play crucial roles in mechanotransduction as a principal bone mechanosensor, which is the basis of their neuronal-like biology, as previously reported. Neuropeptide Y (NPY) and reelin mRNA, which are brain-specific neurogenic markers, have been identified in osteocytes. However, changes in the production of NPY and reelin in response to specific biochemical stimulation are unknown. In this study, we investigated the *in vitro* effect of corticosterone, one of the endogenous glucocorticoids, on the expression of NPY and reelin in the MLO-Y4 osteocyte cell line. Cells were treated with corticosterone at different concentrations (10^{-9} M-10^{-5} M) for 1, 3, 6, 12 and 24 h. As revealed, corticosterone reduced the MLO-Y4 cell viability and proliferation in a dose- and time-dependent manner based on an MTT assay and a Vi-CELL analyzer. The cells were then incubated with corticosterone (10^{-6} μM), and the NPY and reelin expression levels were detected at 1, 3, 6, 12 and 24 h using real-time PCR and Western blot analysis. These results demonstrated that at the gene and the protein levels, corticosterone significantly upregulated the NPY and reelin expression in a time-dependent manner. The application of a glucocorticoid receptor antagonist, RU486, reversed the reduced cell viability and the increased expression of NPY and reelin that were caused by corticosterone. To the best of our knowledge, this is the first report to verify that corticosterone regulates the NPY and reelin expression in osteocytes.

INTRODUCTION

The popular theory concerning osteocytes, which are the most abundant cells in an adult skeleton (Lanyon, 1993), is that osteocytes function as mechanosensors directing bone remodeling and regulators modifying mineralization and mineral metabolism (Bonewald, 2006). Moreover, the dendritic morphology and mechanotransduction of osteocytes and the amazing osteocytic network (Bonewald and Johnson, 2008; Papachroni et al., 2009; Rho et al., 2004) also define their neuronal-like biology (Marotti, 2000). Evidence of the expression of neurogenic

markers and several neurotransmitter-related receptors (Paic et al., 2009; Westbroek et al., 2001) suggested that osteocytes serve as a mechanosensor or a neuronal-like regulator of bone mass. It provides a fresh perspective for investigating the mechanisms through which osteocytes recognize various stimuli and coordinate the activities of osteoblasts and osteoclasts.

Recently, two brain neurogenic markers, namely neuropeptide Y (NPY) and reelin, have been identified as having higher mRNA levels in osteocytes than osteoblasts (Paic et al., 2009). NPY, a 36-amino acid peptide neurotransmitter found in the brain and the autonomic nervous system, acts as a traditional neuronal regulator of energy homeostasis (Chronwall et al., 1985). NPY is a potential modulator of bone remodeling. It exerts hypothalamic actions on the bone and adipose tissues and locally affects osteoblasts and adipocytes (Karsenty, 2000; Zengin et al., 2010). Recent studies have demonstrated NPY expression in osteocytes and an inhibitory effect of NPY on the osteoblast activity (Igwe et al., 2009; Paic et al., 2009). These results indicate that osteocytic NPY could be a potential mediator for the functions of osteocytes and osteoblasts. Due to the load-responsive nature of local NPY expression (Igwe et al., 2009), exploring the expression dynamics of osteocytes in response to bone catabolic or anabolic stimuli is therefore necessary.

Reelin, a large secreted extracellular matrix (ECM) glycoprotein (D'Arcangelo et al., 1995), plays a pivotal role in brain development (Dulabon et al., 2000) and the adult brain (Niu et al., 2008). Peripheral reelin mRNA is found in various tissues and cells including the adult mammalian blood, liver, eyes, odontoblasts, osteoblasts, and osteocytes (Kobold et al., 2002; Maurin et al., 2004; Pulido et al., 2007; Smalheiser et al., 2000). The physiological significance of reelin in bone cells is unknown, but earlier studies have suggested a site-specific expression in limb and skull bones/cells and a possible link between reelin and abnormal bone remodeling in the otic capsule (Rawlinson et al., 2009; Schrauwen et al., 2009). Notably, in osteoblast-like cells, the expression of reelin can be dramatically downregulated by certain biological materials and BMP-2 (van der Zande et al., 2010). This research article will provide more evidence for the gene and protein expression of reelin in osteocytes.

In both nervous and non-nervous tissues and cells, NPY and reelin are sensitive to dexamethasone and corticosterone ex-

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posure, which can induce bone loss systemically and/or locally (Akabayashi et al., 1994; Lussier et al., 2011; Weinstein et al., 1998). An exogenous glucocorticoid (GC), dexamethasone, has been widely used for the treatment of inflammatory and autoimmune diseases (O'Brien et al., 2004). Corticosterone in rodents and cortisol in humans, which are endogenous GCs, are produced in response to the acute and chronic stresses (Djordjevi et al., 2003). Compared with dexamethasone, the effects of endogenous corticosterone/cortisol on bone cells are closer to their activities *in vivo*. In this study, we have explored the effects of corticosterone (CORT) on the expression of NPY and reelin in osteocytes by using the MLO-Y4 cell line as an example.

MATERIALS AND METHODS

Chemicals
Corticosterone (Sigma, USA) was dissolved in fetal bovine serum (Invitrogen, USA) to a concentration of 1 mM and stored at -20°C. The diluted samples were freshly made at a concentration range of 10^{-9}-10^{-5} M. The glucocorticoid receptor (GR) antagonist RU486 (Sigma, USA) was dissolved in ethanol to a concentration of 10 mM, and a working solution was freshly prepared at a concentration of 10 μM.

Cell culture
The MLO-Y4 cells were generously given by Prof. Lynda F. Bonewald from the Department of Oral Biology, University of Missouri in Kansas City, USA. The cells were cultured in collagen-coated (rat tail collagen type I; 0.15 mg/ml in 0.02 N Acetic acid) flasks in an α-modified essential medium (α-MEM; Gibco, USA) supplemented with 5% fetal bovine serum and 5% calf serum (HyClone, USA) at 37°C in a humi-dified atmosphere with 5% CO_2, as previously described (Chen et al., 2010). The medium was replaced every two days.

Immunofluorescence
For immunostaining of the osteocytes *in vitro*, the cells were grown on glass cover slips, washed with phosphate-buffered saline (PBS; pH 7.4), fixed for 20 min in 4% paraformaldehyde, and washed and blocked with a 5% goat serum at 37°C for 20 min. The cells were subsequently stained with a mouse anti-reelin (1:150; Chemicon; MAB5364) or a rabbit anti-NPY (1:150; Santa Cruz; sc-28943) antibody, which was followed by a DyLight™ 594-conjugated anti-mouse or a DyLight™ 488-conjugated anti-rabbit IgG (1:100; Zhongshan Golden Bridge; ZF-0413/ZF-0411) for immunofluorescence detection. Additionally, the cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Photographs were taken using a fluorescence microscope (Olympus, Japan).

Cell viability assay
MLO-Y4 cells were seeded in 96-well plates, and their cell growth was arrested by a 12-h incubation in serum-free medium. The cells were then treated with CORT at the indicated concentrations (0, 10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M and 10^{-5}M) in α-MEM supplemented with 0.5% BSA for 0, 1, 3, 6, 12 and 24 h. To detect the effects of glucocorticoid receptors, RU486 was added 2 h prior to the addition of 1 μM CORT. The MTT assay data were measured by an absorbance at 490 nm using a microplate reader.

The MLO-Y4 cells were plated to a density of 3×10^5 cells/well in 6-well plates for confluency. The cells were then treated with CORT and/or RU486 as described above. Subsequently, they were harvested using 0.5% trypsin and three PBS washes.

The cells were suspended in 1 ml of fresh culture medium and transferred to a Vi-CELL™ Cell Viability Analyzer (Beckman, USA), which was utilized to determine the number of cells that which have absorbed the trypan blue dye and which have not. The Vi-CELL will analyze up to 100 images for a determination of cellular viability. Each experiment was repeated at least three times.

Real-time PCR
The arrested cells were treated with 1 μM of CORT alone for 0, 1, 3, 6, 12 and 24 h in α-MEM supplemented with 0.5% BSA. In the experiments in which RU486 was used, the cells were treated with 0.1% ethanol alone (the vehicle), 10 μM RU486 alone (the RU486 group), or 10 μM RU486 followed by 1 μM CORT (the RU+ CS groups) for 1, 3, 6, 12 and 24 h. Total RNA (1 μg) was isolated from the cells using TRIzol Reagent (Invitrogen, USA) and reverse transcribed into cDNA using a PrimeScript® RT Reagent Kit (DRR037A; Takara, Japan) according to the manufacturers' instructions. The cDNA synthesis was performed for 15 min at 37°C and 5 s at 85°C. The PCR amplification was performed using SYBR® Premix RX Taq™ II (DRR081A; Takara, Japan) at an initial melting temperature of 95°C for 30 s, which was followed by 40 cycles of 95°C for 5 s, 60°C for 31 s, and 72°C for 31s, and then a dissociation step was performed in the ABI PRISM® 7300 Fast Real-Time PCR System. The primer sets (mouse) used are as follows: reelin, forward 5′-ACC TGA CGC CCA CTG AGA ACT-3′; and reverse, 5′-CGG GTA AGC ACT GAG GGA CTA A-3′; NPY, forward 5′-GTA ACA AGC GAA TGG GGC TGT-3′; and reverse 5′-GTA GTG TCG CAG AGC GGA GTA GT-3′; DMP1, forward 5′-AGA GGG TAG AGG AAT CGC-3′; and reverse 5′-TGA CTT TCT TCT GAT GAC TCA CT-3′; GAPDH, forward 5′-GAC ATC AAG AAG GTG GTG AAGC-3′; and reverse 5′-GAA GGT GGA AGA GTG GGA GTT-3′. The fold change was calculated as follows: $2^{-\Delta\Delta Ct}$, in which, $\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$, $\Delta Ct = Ct_{targetgene} - Ct_{GAPDH}$.

Western blot analysis
Following any treatments, the MLO-Y4 cells were washed three times with ice-cold PBS, lysed with a lysis buffer [50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 0.5% Nonidet P-40, 0.5% Na deoxycholate, and 0.1% SDS supplemented with 0.5% (v/v) leupeptin, 0.1% (v/v) aprotinin and 0.5% (v/v) 100 μM phenylmethylsulfonyl fluoride], and then centrifuged at 14,000 rpm at 4°C for 15 min at 4°C. The protein content was quantified using a BCA Protein Assay Kit (Pierce, USA). Equal amounts of protein were separated using 6%, 10% and 12% SDS-polyacrylamide gel electrophoresis for reelin, β-actin and NPY, respectively. The proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dry milk in a TBS buffer containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The membranes were subsequently incubated overnight with an anti-reelin monoclonal antibody G10 (1:200), an anti-NPY polyclonal antibody (1:200), or an anti-β-actin antibody (1:500) diluted in TBST at 4°C. After removing the unbound primary antibodies by performing three 10-minute washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:8000) diluted in TBST for 1 h at 37°C and were washed with three 10-minute washes with TBST. Chemiluminescence (CL) detection was performed to detect the antibody-associated protein bands using X-ray photographic films. A densitometric analysis of the immunoreactivity of the proteins was conducted using a Bio-Rad Image Analysis System.
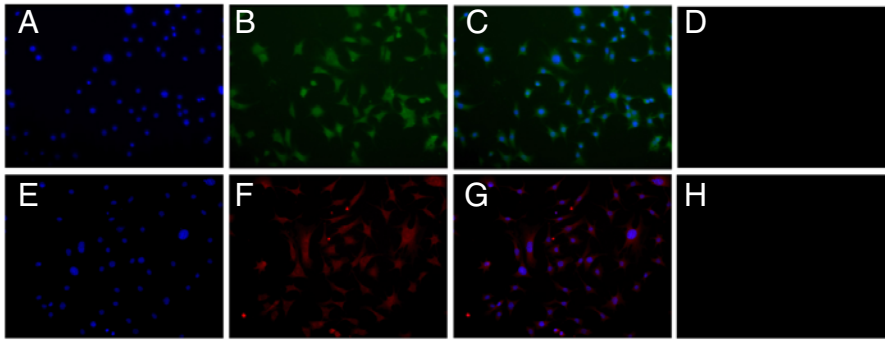
Fig. 1. Analysis of NPY (A-D) and reelin (E-H) expression in the MLO-Y4 cells by immunofluorescence. (A, E): Nuclear staining of the MLO-Y4 cells using DAPI (200×). (B, F): Immunofluorescence labelling of NPY (B) and reelin (F) performed with anti-NPY antibody and anti-reelin antibody respectively in MLO-Y4 cells (200×). (C, G): Nuclear staining mergered with the NPY (C) or reelin (G) immunostaining (200×). (D, H): Control staining of the MLO-Y4 cells without the primary antibody (200×).
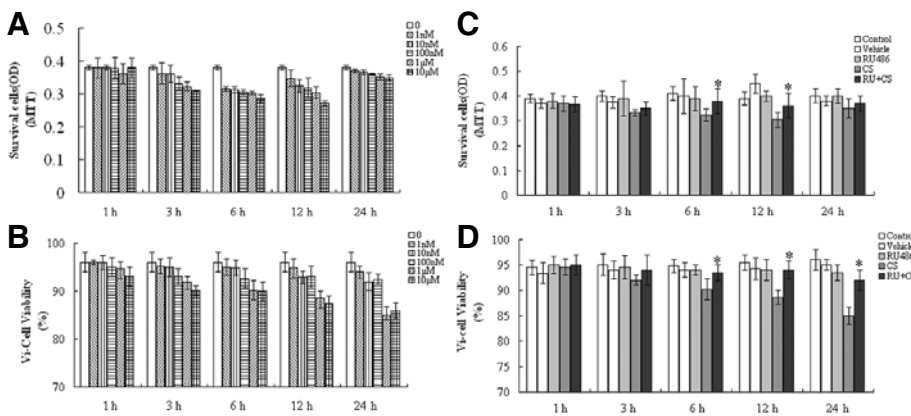

Fig. 2. A reduction in MLO-Y4 cell viability by corticosterone. (A, B) The number and proportion of viable cells were detected using an MTT assay and a Vi-Cell™ cell viability analyzer, respectively. (C, D) Pretreatment with RU486 reversed the inhibitory effectts by CORT (1 μM) on the cell viability. All data shown are the means ± SD from triplicate tests (*$p < 0.05$, RU486 plus CORT vs. CORT-treated groups). CS = corticosterone, RU + CS = RU486 plus CORT.

Statistics

The results were expressed as the means ± standard deviation. The differences between the groups were tested by a one-way ANOVA using SPSS software. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Expression of reelin and NPY in MLO-Y4 cells

NPY immunoreactivity, which was detected with a rabbit polyclonal anti-NPY antibody by immunofluorescence (IF), was present in the MLO-Y4 cells with moderate staining in the cell bodies and reduced staining in the cell dendrites (Figs. 1A-1D). The reelin identification using IF also showed a low to medium staining in the MLO-Y4 cell bodies and a weaken staining in some of cell dendrites (Figs. 1E-1H).

Reduction of MLO-Y4 cell viability by CORT

The MLO-Y4 cells were treated with various CORT concentrations (10^{-9}-10^{-5} M) for 0, 1, 3, 6, 12 and 24 h after growth arrest using a serum-free medium, and then the cell viability was determined using an MTT assay and a Vi-Cell automated analyzer. The CORT exposure reduced the expected number and propotion of viable cells in a time- and dose-dependent manner compared with the control samples (Figs. 2A and 2B). This inhibitory effect was more obvious after the CORT applications of 10^{-6} M and 10^{-5} M for 3, 6 and 12 h. There was a rebound in the OD values at 24 h of CORT treatment in the MTT assay (Fig. 2A), which most likely suggests the recovery of the metabolic activity of the cells. To investigate whether the GR was

involved in these inhibitory effects, RU 486 was added 2 h prior to the addition of 1 μM CORT. RU486 reversed the reduced viability that was caused by CORT ($p < 0.05$, Figs. 2C and 2D). Ethanol (0.1%) or RU486 alone did not affect the viability of the MLO-Y4 cells.

CORT upregulated the NPY and reelin mRNA expression through GR

Following the CORT/RU486 treatment of the MLO-Y4 cells, the gene expression of dentin matrix acidic phosphoprotein 1 (DMP1) was detected using real-time PCR. These results demonstrated that the DMP1 expression was significantly increased in a time-dependent manner, especially at 3 and 6 h following the CORT treatment compared to the control (greater than 5-fold; $p < 0.05$; Fig. 3A). This increased effect, however, was completely inhibited by the RU486 pretreatment (*$p < 0.05$, Fig. 3A). The positive effect of CORT on the NPY mRNA expression was noted after 1 h, which peaked at 6 h and then gradually decreased, but it was still superior to the control level ($p < 0.05$, Fig. 3B). The addition of RU486 decreased the increased expression of NPY that was caused by the CORT treatment (Fig. 3B). The reelin expression was induced at 1 h, returned to the basal level at 3 h and then was re-induced 6 h later ($p < 0.05$; Fig. 3C). The upregulation of reelin was completely inhibited by the pretreatment with RU486 ($p < 0.05$; Fig. 3C). These results suggested that CORT promoted the NPY and reelin mRNA expression in a GR-dependent way.

CORT increased NPY and reelin protein expression by GR

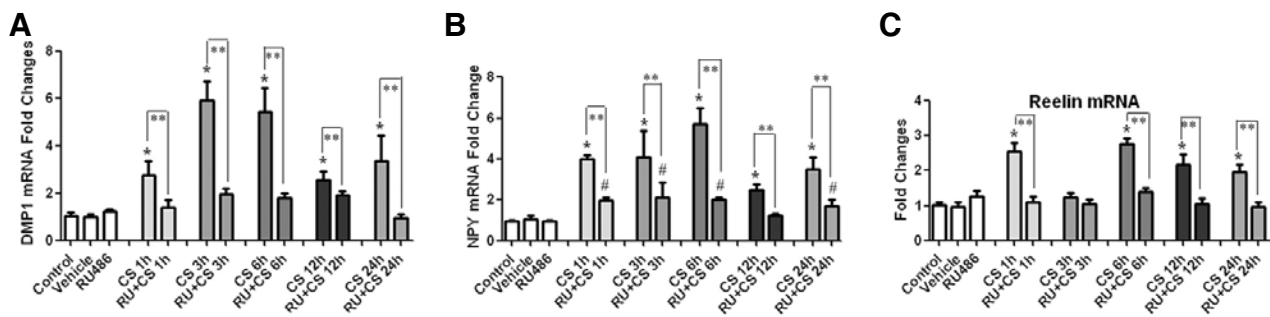Western blot analysis was performed to determine protein ex-

Fig. 3. CORT-induced gene expression in the MLO-Y4 cells. (A) The expression of DMP1 was significantly increased in a time-dependent manner, which was abrogated by RU486. (B) CORT upregulated the expression of NPY mRNA, which was partially inhibited by pretreatment with RU486. (C) The reelin gene expression was induced by the application of CORT and reversed by the RU486 pretreatment. The results of the real-time PCR analysis with GAPDH as an endogenous control are shown as the means ± SD ($n = 3$; *$p < 0.05$ vs. the control; #$p < 0.05$ vs. the vehicle or RU486-treated group; **$p < 0.05$, RU486 plus CORT vs. the corresponding CORT-treated groups). CS = corticosterone, RU + CS = RU486 plus CORT.
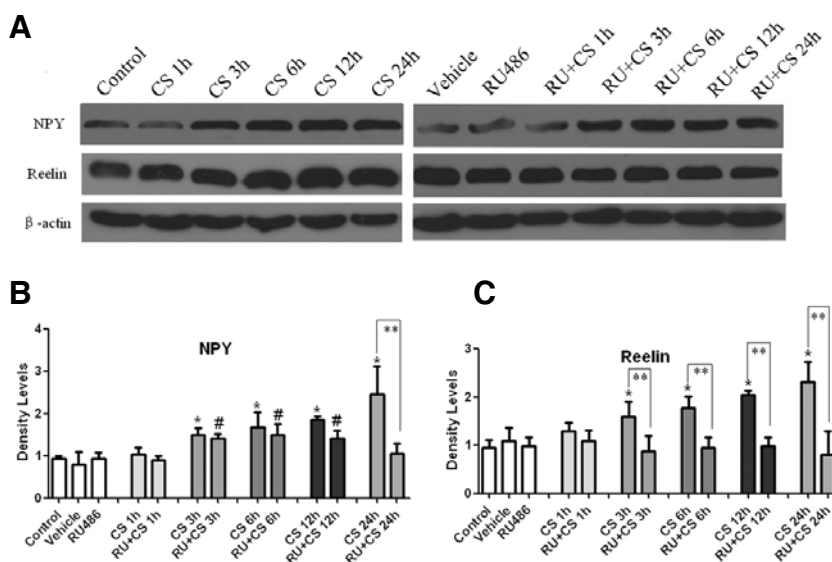

Fig. 4. CORT induced NPY and reelin protein production in the MLO-Y4 cells. The band intensities (A) were quantified with densitometry (B, C), which showed the expression of both proteins remained at the basal level at 1 h, but significantly increased from 3 to 24 h. RU486 partially reversed the CORT-induced NPY expression and abrogated the increased reelin expression. The results shown are the means ± SD ($n = 3$; *$p < 0.05$ vs. the control; #$p < 0.05$ vs. the vehicle or RU486-treated group; **$p < 0.05$, RU486 plus CORT vs. the corresponding CORT-treated groups). CS = corticosterone, RU + CS = RU486 plus CORT.

pression of NPY and reelin in the MLO-Y4 cells following the treatments (Fig. 4A). A quantitative analysis of the protein-positive bands indicated that significantly increased levels of NPY were present with 3, 6, 12 and 24 h of CORT treatment (approximately 1.5-, 1.7-, 1.9- and 2.5-fold, respectively; $p < 0.05$, Fig. 4B). The increased NPY expression at 3, 6 and 12 h was weakened by the pretreatment with RU486 but remained superior to the vehicle or RU486-treated group ($p < 0.05$; Fig. 4B). The application of RU486 abrogated the CORT-induced NPY expression at 24 h ($p < 0.05$; Fig. 4B).

For reelin, only a single band was detected at ~388 kDa in the MLO-Y4 cells (Fig. 4A), which was significantly enhanced after 3 h of CORT treatment in a time-dependent manner compared with the control ($p < 0.05$; Fig. 4C). RU486, however, abrogated these positive effects of CORT on the reelin expression. It implied that CORT upregulated the protein expression of reelin in a GR-dependent manner.

DISCUSSION

Osteocytes constitute more than 90-95% of the cells in an adult

skeleton (Lanyon, 1993). Previous studies, which reported on the physical morphology and biological functions (Bonewald and Johnson, 2008, Papachroni et al., 2009), have implied the presence of an osteocytic neuronal-like biology. More interestingly, the expression of several neurogenic markers has been found in osteocytes. NPY and reelin, two neural tissue-related genes, have been shown to have high mRNA levels in osteocytes (Paic et al., 2009). In the present paper, we provide the first evidence that the gene and protein expression of reelin and NPY can be regulated by corticosterone, a catabolic factor for bone, in the MLO-Y4 cells.

As shown in Fig. 1, the expression of NPY was predominantly present in the cytoplasm surrounding the nuclei of the MLO-Y4 cells. The faint green fluorescence was shown in some of cell processes connected to the adjacent cells (Figs. 1B and 1C). NPY was also secreted into the culture medium by osteocytes, which was confirmed by an ELISA kit (unpublished data). Given its inhibitory effect on the osteoblast activity *in vitro* (Igwe et al., 2009), NPY may be a non-negligible biological factor between osteocytes and osteoblasts *in vivo*. The reelin immunoreactivity was detected with a low to medium staining in

the MLO-Y4 cell bodies (Figs. 1E-1H). A faint reelin immuno-staining was also detected in the typical dendrites of the MLO-Y4 cells (Figs. 1F and 1G). It has been well documented that reelin stimulates dendrite outgrowth and regulates the migration of neuroblasts in the nerve system (Niu et al., 2008). However, it remains unknown whether reelin contributes to the dendritic morphology of osteocytes.

CORT is a stress hormone and can mediate NPY and reelin production in the nervous system both *in vivo* and *in vitro* (Gross et al., 2010; Husum and Mathé, 2002). In the present study, the effectiveness of the CORT treatment in MLO-Y4 cells could be verified by the expression of DMP1, an ECM protein produced by osteocytes. Consistent with previous reports that dexamethasone can increase the mRNA level of DMP1 (Mikami et al., 2008), this study also demonstrated that the DMP1 gene expression could be significantly enhanced by corticosterone in osteocytes through GR.

Through an intervention, we have shown that the mRNA and protein levels of NPY were upregulated by CORT in osteocytes (Figs. 3 and 4), which corresponds with previous reports (McKibbin et al., 1992; Shimizu et al., 2008). A positive correlation between NPY and CORT was also found in certain feeding paradigms (Wang et al., 1998). In the present study, the addition of RU486 partially reversed the upregulation of NPY that was caused by CORT. This indicated that the GR mediated the CORT-induced NPY expression. GR is a widely expressed member of the nuclear receptor superfamily, which is fundamentally required for CORT-mediated actions. Corticosteroids modulate gene transcription *via* either the binding of GR homodimers to GC-responsive elements (GREs) present in the promoters of hormone-responsive genes or the protein-protein interactions of GR monomers with transcription factors such as NF-κB, AP-1, IRF-3, and STAT5 (Reichardt and Schütz, 1998). There are several transcription factor-binding sites in the mouse NPY gene promoter, including AP-1, AP-2 and Sp1, which can interact with GR to regulate gene transcription (Titolo et al., 2008). Although it has been demonstrated that glucocorticoid-responsive elements (GREs) are present in the far upstream region of the rat NPY gene (Misaki et al., 1992), there have been no reports about GREs in the mouse NPY gene. Therefore, we speculate that the binding of transcription factors to the NPY promoter is involved in the positive effects of CORT on the NPY gene expression.

CORT administration increased the reelin mRNA and protein levels in the MLO-Y4 cells ($p < 0.05$; Figs. 3 and 4), which is partially consistent with previous reports (Gross et al., 2010; Kobold et al., 2002). A negative regulation of CORT on reelin has also been noticed in murine hippocampi (Lussier et al., 2009; 2011). The application of RU486 abrogated the CORT-increased reelin expression, suggesting the presence of GR signaling cascades. The mouse reelin promoter has been shown to contain potential binding sites for Sp1 and AP-2 (Royaux et al., 1997). Whether the reelin promoter contains GREs is presently unknown. Therefore, the exact mechanism leading to the corticosterone-mediated induction of the NPY and reelin genes remains undefined.

It is interesting that CORT increased the NPY and reelin gene expression (Figs. 3B and 3C) while the cell viability was reduced in the present study (Fig. 2). Based upon the mechanisms of GR actions, which have been documented previously (Necela and Cidlowski, 2004), CORT appears to decrease the MLO-Y4 cell viability through a complex process of signaling cascades that began with GR-mediated changes in gene expression through transactivation and transrepression. Thus, the question arises of whether there is any possible casual relation-

ship between the cell viability and NPY/reelin gene expression. In addition, CORT most likely repressed transcription factors and cytokines that were required for the survival of MLO-Y4 cells via the repression of NF-κB and AP-1 signaling and the regulation of antiapoptotic and proapoptotic Bcl-2 members (Necela and Cidlowski, 2004). Therefore, the underlying mechanism of the CORT-reduced cell viability is an interesting topic.

The role of NPY as one of major regulators of bone homeostasis is mainly manifested through a central control via the hypothalamic Y2 receptor and a peripheral pathway via the local Y1 receptor (Allison et al., 2006; Teixeira et al., 2009). Combined with the results of this experiment, it is plausible that an increase in the serum corticosterone concentration induces the NPY expression systemically and locally *in vivo*. Therefore, the local NPY pathway is expected to provide potential therapeutic targets for the treatment of glucocorticoid-induced osteoporosis. Little is known about the effects of reelin; therefore, *in vitro* odontoblast studies in which a colocalization of the nerve fibers with reelin was observed can be instructive (Maurin et al., 2004). The nerve fibers enter the bone with blood vessels and then branch to form rings around the osteoblasts and osteocytes (Sherman, 1963). Direct neurite-osteoblastic and neurite-osteoclastic cell communications have been identified by *in vitro* co-culture systems (Obata et al., 2007; Suga et al., 2010). Being approximately 10 times and 100 times greater than osteoblasts and osteoclasts respectively, osteocytes are more likely to contact or communicate with nerve terminals, and reelin serves as one possible mediator.

Taken together, the expression of NPY and reelin appear to be a common feature between the nervous system and bones. Increased serum corticosterone in mice or cortisol in humans can alter the expression of NPY and reelin in the nervous system (Akabayashi et al., 1994; Gross et al., 2010; Husum and Mathé, 2002; Lussier et al., 2011) and possibly in bone. Although the exact role of reelin still remains unclear, the significance of NPY in bone remodeling has been recognized. The above findings may not only contribute to the development of potential therapies for bone diseases, but also may enhance the understanding of osteocytic neuronal-like biology and the connection between the nervous and skeletal systems.

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