Methionine Enkephalin Suppresses Osteocyte Apoptosis Induced by Compressive Force through Regulation of Nuclear Translocation of NFATc1

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ABSTRACT
Mechanical stress stimulates bone remodeling, which occurs through bone formation and resorption, resulting in bone adaptation in response to the mechanical stress. Osteocytes perceive mechanical stress loaded to bones and promote bone remodeling through various cellular processes. Osteocyte apoptosis is considered a cellular process to induce bone resorption during mechanical stress-induced bone remodeling, but the underlying molecular mechanisms are not fully understood. Recent studies have demonstrated that neuropeptides play crucial roles in bone metabolism. The neuropeptide, methionine enkephalin (MENK) regulates apoptosis positively and negatively depending on cell type, but the role of MENK in osteocyte apoptosis, followed by bone resorption, in response to mechanical stress is still unknown. Here, we examined the roles and mechanisms of MENK in osteocyte apoptosis induced by compressive force. We loaded compressive force to mouse parietal bones, resulting in a reduction of MENK expression in osteocytes. A neutralizing connective tissue growth factor (CTGF) antibody inhibited the compressive force-induced reduction of MENK. An increase in osteocyte apoptosis in the compressive force-loaded parietal bones was inhibited by MENK administration. Nuclear translocation of NFATc1 in osteocytes in the parietal bones was enhanced by compressive force. INCA-6, which inhibits NFAT translocation into nuclei, suppressed the increase in osteocyte apoptosis in the compressive force-loaded parietal bones. MENK overexpressing MLO-Y4 cells showed increased expression of apoptosis-related genes. MENK administration reduced the nuclear translocation of NFATc1 in osteocytes in the compressive force-loaded parietal bones. Moreover, MENK suppressed Ca2+ influx and calcineurin and calmodulin expression, which are known to induce the nuclear translocation of NFAT in MLO-Y4 cells. In summary, this study shows that osteocytes expressed MENK, whereas the MENK expression was suppressed by compressive force via CTGF signaling. MENK downregulated nuclear translocation of NFATc1 probably by suppressing Ca2+ signaling in osteocytes and consequently inhibiting compressive force-induced osteocyte apoptosis, followed by bone resorption. © 2020 The Authors. JBMR Plus published by Wiley Periodicals, Inc. on behalf of American Society for Bone and Mineral Research.

KEY WORDS: APOPTOSIS; MECHANICAL STRESS; METHIONINE ENKEPHALIN; NFATc1; OSTEOCYTE

Introduction
Vertebrate skeletons are influenced by various kinds of mechanical stress during daily activities. Mechanical stress stimulates bone remodeling, which occurs through bone formation and resorption, resulting in bone adaptation to the mechanical stress.1 Osteocytes are the most abundant cells in bones and are embedded in bone matrices. Osteocytes have their dendritic processes through canaliculi to form an intercellular communication network via gap junctions.2,3 Osteocytes perceive mechanical stress as mechanosensors, transform into biochemical signals, and regulate bone...
remodeling by transmitting signals related to bone formation and resorption.\textsuperscript{(14,5)}

Osteocytes express receptor activator of nuclear factor kappa B ligand (RANKL), an essential factor for osteoclastogenesis.\textsuperscript{(6)} Therefore, osteocytes are considered critical for osteoclastogenesis and bone resorption.\textsuperscript{(7,8)} Importantly, not only living osteocytes, but osteocytess undergoing apoptosis also promote bone resorption.\textsuperscript{(9–11)} We previously reported that compressive force induces osteocyte apoptosis in the alveolar bones during experimental tooth movement in mice and in cell culture of chick calvaria-derived osteocytes.\textsuperscript{(12,13)} Our data suggest that osteocyte apoptosis occurs in response to compressive force and induces bone resorption. As to the mechanism of the compressive force-induced osteocyte apoptosis, we revealed that connective tissue growth factor (CTGF) is a molecule that facilitates osteocyte apoptosis.\textsuperscript{(13)} However, the underlying molecular mechanisms of the compressive force-induced osteocyte apoptosis are not fully understood.

Recent studies have demonstrated that neuropeptides play crucial roles in bone metabolism by regulating osteoblast and osteoclast function.\textsuperscript{(14–16)} The neuropeptide methionine enkephalin (MENK) is an endogenous opioid encoded by the proenkephalin (PENK) gene. It binds mainly to the delta opioid receptor (DOR) among three opioid receptors: mu, delta, and kappa.\textsuperscript{(17)} It is well known that MENK is expressed in nervous tissue, including the brain and spinal cord. MENK is also expressed in non-nervous tissue, such as bone, heart, pancreas, and kidneys.\textsuperscript{(18–21)} Its analgesic property is a major function of MENK; moreover, MENK has other functions, including anti-inflammatory effects and regulation of embryonic organ development.\textsuperscript{(22,23)} On the other hand, long-term administration of opioids to alleviate cancer pain reduces bone mass, suggesting that opioids have a close relationship with bone remodeling.\textsuperscript{(24)} MENK is expressed in osteoblasts and mesenchymal stem cells, which are precursors of osteoblasts.\textsuperscript{(21,25)} In addition, MENK inhibits osteoblast differentiation.\textsuperscript{(26)} These findings suggest the inhibitory effect of MENK on bone formation regulating osteoblast differentiation during bone remodeling. However, the role of MENK in bone resorption is still unknown.

McTavish and colleagues reported that PENK enhances apoptosis of human embryonic kidney (HEK) 293 cells and various human tumor cell lines.\textsuperscript{(27)} Conversely, activation of DOR suppresses apoptosis of neurons and hepatocytes.\textsuperscript{(28,29)} These studies suggest that MENK regulates apoptosis positively and negatively depending on cell type. This notion led us to hypothesize that MENK has a novel role in bone resorption by regulating osteocyte apoptosis in response to compressive force. Thus, we first analyzed the expression of MENK in osteocytes and its effect on apoptosis using the mouse model of compressive force loading to cranial bones that we have already established.\textsuperscript{(30)} Furthermore, we analyzed the molecular mechanisms of MENK in osteocyte apoptosis induced by compressive force. Nuclear factor of activated T cells (NFAT) induces apoptosis in various types of cells, such as pulmonary artery smooth muscle cells, neurons, and glomerular mesangial cells.\textsuperscript{(31–33)} Lu and colleagues reported that NFATc2 activation by MENK significantly induces apoptosis of rat C6 glioma cells.\textsuperscript{(34)} Therefore, we focused on NFAT to elucidate the MENK-mediated mechanism in osteocyte apoptosis.

**Material and Methods**

Application of compressive force to mouse parietal bones

Animal experiments were performed in accordance with the regulations for animal experiments and related activities at Tohoku University. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Tohoku University Environmental and Safety Committee. Mice were housed in groups of up to four animals in a cage and kept at 20°C to 26°C with 12-hour light/dark cycles. They had *ad libitum* access to fresh chow and water.

Six-week-old male Institute of Cancer Research (ICR) mice (Clea Japan, Tokyo, Japan) were anesthetized by i.p. injection of medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg). A skin incision was made to expose the parietal bones. Two holes were made equidistant from a sagittal suture at the anteroposterior middle of the parietal bones using a round bur attached to a dental drill. The distance between the two holes was 5 mm. A spring that loads 0.2 N of compressive force onto the parietal bones was made by bending a 0.016-inch diameter orthodontic beta–titanium wire (Fig. 1A). The spring was set within the holes in the parietal bones (Fig. 1B), fully covered by skin, and the incision was closed by suturing. In the control group, identical surgical procedures were performed without spring installation.

Either 170 μL of 1-mg/mL MENK (Sigma-Aldrich, St. Louis, MO, USA), 100 μL of 25-μM INCA-6 (Cayman Chemical, Ann Arbor, MI, USA), 100 μL of 25-μM INCA-6 (Cayman Chemical, Ann Arbor, MI, USA), or 100 μL of vehicle was administered. To examine the effects on apoptosis, we used two different concentrations of tetracycline (2.5 and 5 μg/mL). Mice were housed in groups of up to four animals in a cage and kept at 20°C to 26°C with 12-hour light/dark cycles. They had *ad libitum* access to fresh chow and water.

**Fig 1** Application of compressive force to mouse parietal bones. (A) A spring for compressive force loading. Scale bar = 2 mm. (B) A schematic diagram of a compressive spring placed on mouse parietal bones. To apply 0.2 N of compressive force to the parietal bones, a compression spring was set within holes drilled in the right and left parietal bones of 6-week-old mice. Arrows indicate the direction of compressive force on the parietal bones. (C) For histomorphometric analysis, five lines dividing the thickness of the parietal bones into six equal parts were drawn from dorsal to ventral sides on images of stained sections. The four square fields (30 × 30 μm) on the first and fifth lines, in contact with the edges of the right and left parietal bones adjacent to the sagittal suture, were set-up in each specimen as the ROI. Scale bar = 100 μm.
USA), or 100 μL of 10-μg/mL neutralizing antihuman CTGF antibody (PeproTech, Rocky Hill, NJ, USA) were s.c. injected into the parietal bone area. For the negative control, the same volume of PBS or rabbit IgG (Sigma-Aldrich) was injected. A compression spring was installed on parietal bones 2 hours after the MENK and INCA-6 injection, or 6 hours after the CTGF antibody injection. Each injection was given again after surgery. During all of the experiments, mice were carefully monitored and no adverse events were observed.

Histological analysis

After application of the 6-hour compressive force, mice were perfused fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) under inhalation anesthesia with isoflurane. Calvariae, including the parietal bones, were dissected and immersed in 4% PFA at 4°C overnight. The specimens were decalcified with 20% ethylene-diaminetetraacetic acid (pH 7.4) at 4°C for 7 days. The specimens were then embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin (Sigma-Aldrich) and eosin (Wako, Osaka, Japan).

Immunohistochemical analysis

Deparaffinized sections were immersed in 0.01 M citrate solution and microwaved for 2 min, then incubated at room temperature (RT) for 30 min. The sections were treated with 10% donkey serum (Sigma-Aldrich) in PBS at RT for 1 hour and incubated with primary antibodies against MENK (1:40; ImmunoStar, New Richmond, WI, USA), XOR (1:100; Abcam, Cambridge, UK), NFATC1 (1:40, Abcam), or NFATC3 (1:50; ProteinTech, Chicago, IL, USA) at RT for 2 hours or at 4°C overnight. In the negative controls, the primary antibodies were replaced by rabbit IgG (Sigma-Aldrich). After washing with 0.3% TritonX-100 (Nacalai Tesque, Kyoto, Japan) in PBS, the sections were incubated with donkey antirabbit-IgG Alexa Fluor 568 (1:500; Invitrogen, Carlsbad, CA, USA) at RT for 1 hour. 4,6-diamidino-2-phenylindole (DAPI; 1:1000; SeraCare, Milford, MA, USA) was used for nuclei detection. Fluorescent signals were visualized using a confocal laser scanning microscope system (C2si; Nikon, Tokyo, Japan). To quantify the fluorescent-positive osteocytes, the fluorescent images were analyzed using ImageJ software (NIH, Bethesda, MD, USA; https://imagej.nih.gov/ij/); thresholds of 40 and 255 were used in red channel images. We determined the thresholds by superimposing binary images on the corresponding fluorescent images and confirming overlaps of the signals on the binary images and on the fluorescent images, according to our previous report. In our analysis of nuclear translocation of NFAT, fluorescent NFAT signals that contained nuclei of osteocytes were considered positive.

For the detection of active caspase-3, deparaffinized sections were treated with 3% H2O2 in methanol and incubated overnight at 4°C with rabbit antihuman cleaved caspase-3 (1:100; Cell Signaling Technology, Danvers, MA, USA) in Carn Get Signal Immunostain Solution B (Toyobo, Osaka, Japan). After incubation with a peroxidase-conjugated secondary antibody (Histofine Simple Stain Mouse MAX-PO; Nichirei Bioscience, Tokyo, Japan) at RT for 30 min, the signals were visualized with 3,3-diaminobenzi-dine tetrahydrochloride (DAB; Nichirei, Tokyo, Japan). The images obtained from caspase-3 immunohistochemistry were analyzed using ImageJ (NIH); thresholds of 0 and 200 were used to quantify the caspase-3-positive osteocytes.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining

Apoptotic cell death was detected using the Apoptosis in situ Detection Kit (Wako Laboratories, Osaka, Japan) according to the manufacturer’s protocol. Deparaffinized sections were incubated in prewarmed protease solution at 37°C for 10 min. For labeling 3’-terminal of DNA, TdT reaction solution was pipetted onto the sections and incubated at RT for 2 hours. Endogenous peroxidase was inactivated using 3% H2O2 for 5 min. Peroxidase-conjugated antibody solution was pipetted onto the sections at 37°C for 10 min. Apoptotic nuclei were then visualized by DAB (Nichirei) at RT for 8 min. Hematoxylin was used for counterstaining. The images obtained from TUNEL staining were analyzed using ImageJ imaging software (NIH). Thresholds of 0 and 160 were used to quantify the TUNEL-positive osteocytes. TUNEL signals overlapping with nuclei of osteocytes were considered positive.

Histomorphometric analysis

Five lines, which divide the thickness of the parietal bones adjacent to the sagittal suture into six equal parts, were drawn from the dorsal to the ventral side on images of stained sections. The four square fields (30 × 30 μm) on the first and fifth lines, in contact with edges of the right and left parietal bones adjacent to the sagittal suture, were set up as the ROI to quantitatively evaluate TUNEL-positive osteocytes, expression of MENK, DOR, and caspase-3 in osteocytes, and nuclear translocation of NFATc1 and NFATc3 in osteocytes (Fig. 1C). The number of osteocytes and the number of TUNEL- or immunohistochemically positive osteocytes in four ROIs in a section were used for calculation of ratio of positive osteocytes.

Cell culture

MLO-Y4 cells were cultured in α modified essential medium (Wako) supplemented with 5% fetal bovine serum (FBS; GE Healthcare, Buckinghamshire, UK), 5% bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 100-U/mL penicillin, and 100-μg/mL streptomycin (Thermo Fisher Scientific) on culture plates coated with 0.3-μg/mL type I collagen (Nitta Gelatin, Osaka, Japan). For a MENK administration experiment, cells were seeded onto coated 12-well plates at 1 × 10^4 cells/cm^2, and MENK was administered after 24 hours. Total mRNA was isolated from cells after 48 hours.

To load compressive force onto MLO-Y4 cells, the cells were seeded at a density of 1.4 × 10^6 cells/cm^2 on silicone chambers (size 32 × 32 mm; Strex, Osaka, Japan) coated with 0.01% poly-D-lysine (Trevigen, Gaithersburg, MD, USA), and 0.3-μg/mL type I collagen and cultured for 2 days. Compressive force was generated by 18% constriction of the chambers using a Strex cell stretch system (Strex); it was loaded onto MLO-Y4 cells for 6 hours. In the control groups, MLO-Y4 cells were cultured on the stretch chambers without compressive force loading. Cells were harvested to isolate total RNA using RNeasy Kit (Qiagen, Venlo, The Netherlands).

Construction of lentiviral vector

The mouse NFATc1 open-reading frame was amplified by PCR and cloned into the Xhol and Xbai sites of the pLVSIN-IRESV1/2ZsGreen1 lentiviral vector (Takara Bio, Shiga, Japan). Viral particles were produced in 293 T Lenti-X cell line (Clontech Laboratories, Palo Alto, CA, USA) using Lentiviral High Titer Packaging Mix.
RNA isolation from parietal bones

RNA isolation from the parietal mouse bones was performed according to our previous report. Mice were sacrificed by cervical dislocation; the parietal bones were quickly dissected. After removing the periosteum and the dura mater, 0.5 mm of parietal bones on either side were isolated and minced with scissors. The specimens were immersed in TRIzol Reagent (Thermo Fisher Scientific) and centrifuged. Total mRNA was then isolated using the RNAeasy Kit.

Real-time PCR

cDNA was synthesized from 0.4 μg of total mRNA using a Prime-Script RT Reagent Kit (Takara Bio). Real-time PCR was performed using a Thermal Cycler Dice Real Time System (Takara Bio). The reaction volume was 25 μL, which contained 2 μL of cDNA, 12.5 μL of TB Green Premix Ex Taq II (Takara Bio), and 0.4 μM of sense and antisense primers. The primer sequences are listed in Table 1. The reactions consisted of 40 cycles of 5 s at 95°C and 30 s at 60°C. Relative expression of mRNA was normalized to that of hypoxanthine-guanine phosphoribosyltransferase (HPRT) and analyzed by the ΔΔCT method.

Western blot analysis

Whole-cell proteins from MLO-Y4 cells were prepared using Cell Lytic M Lysis Reagent (Sigma-Aldrich) containing Protease Inhibitor Cocktail (Sigma-Aldrich). The protein samples were boiled in sodium dodecyl sulfate (SDS) sample buffer, separated by SDS polyacrylamide gel electrophoresis, then transferred to polyvinylidene fluoride membranes. The membranes were treated with blocking buffer and incubated overnight at 4°C. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies, and developed with a chemiluminescence detection reagent. Chemiluminescent signals were acquired using the Fusion FX Imaging System (Vilber Lourmat, Marne La Vallée, France).

Statistical analysis

All data are presented as mean ± SD. Data were statistically analyzed by Student’s t test to compare differences between two groups. For more than two groups, we used an ANOVA, followed by the Tukey–Kramer test. p Values less than 0.05 were considered statistically significant.

Results

Compressive force reduced expression of MENK in osteocytes

Compressive force of 0.2 N loading onto the mouse parietal bones for 6 hours narrowed the suture width between the edges of the right and left parietal bones adjacent to the sagittal suture (Fig. 2A). Histological analysis showed no obvious morphological change of parietal bones and signs of an acute inflammatory response, such as neutrophil infiltration or edema (Fig. 2A). The relative increase in intracellular calcium was assessed using Meta Fluor Software (Molecular Devices, Tokyo, Japan).

Table 1. Primer for Real-Time PCR

| Gene name | Forward primer (5’-3’) | Reverse primer (3’-5’) |
|-----------|------------------------|----------------------|
| PENK      | ATGCAGCTACCGCTGTTTC    | CTTGGCTAGCAGTGAGCTCTCA |
| DOR       | CAGACAGCAGACTCTGAAGCA  | ACCGCGCTGGGTAGCTCTAA  |
| NFATc1    | TGGAGTAGGAAGCGAAGACTG  | GCAAGCATAAAGACTGAGGAG |
| NFATc2    | AGGGCTTTTAGGACTGGTGC   | TGAAGTCGAGATGTGTCTCACAG |
| NFATc3    | GAGTGCTCTAAATGAGACAC  | GAAGACACATGCTACTGAATAC |
| NFATc4    | CCCCACAGCAGACTCTCCT    | TAAAGGATCTCTAAAGCAGG |
| Caspase-3 | CTGCCGGAGATCTGCTGGA   | ATCGTCCCCTAGCTGCTCATAAGT |
| Caspase-8 | CAGAGGAGAAGGAGGAGGAGGAG | GAGAGGACATGCTACTGAAGGAG |
| Caspase-9 | TCCCTGCTCCTTCTCCAAAGTTC | TGAAGATCGGCTCTAGATTG |
| Calmodulin| GTCGTGAACTCTTGTTGAT    | TACCCAGCTCTAGCTACAGTT |
| Calcinurin| GTTACGGGTATCTACTGTGT   | CTGAGGGCTGTAGATGATG |
| CalcinurinB| ACCCTCAGATGCTATG     | GGAATCGAGCTACAGTGACTC |
| HPRT      | AGGCGATGGCCACAGGACTA   | TTGTTGAGATGTCGCCCTTGACTA |

DOR = delta opioid receptor; HPRT = hypoxanthine-guanine phosphoribosyltransferase; PENK = proenkephalin.
The number of MENK-positive osteocytes and the total number of osteocytes were 7.0 ± 1.7 and 9.8 ± 2.5, and 3.0 ± 1.4 and 8.5 ± 1.7 in the nonloaded and loaded groups, respectively. In contrast, there was no significant difference in the expression of DOR between the nonloaded and the loaded groups (Fig. 2D,E). The number of DOR-positive osteocytes and the total number of osteocytes were 7.0 ± 0.8 and 10.0 ± 1.4, and 7.5 ± 0.6 and 11.3 ± 1.0 in the nonloaded and loaded groups, respectively. The negative controls of MENK and DOR immunohistochemistries showed no apparent immunoreactivity in osteocytes (Fig. 2B,D). Consistent with in vivo data, compressive force downregulated expression of PENK mRNA, which encodes MENK, but did not significantly affect DOR mRNA expression in MLO-Y4 cells (Fig. 2F).

A neutralizing CTGF antibody inhibited the decrease in MENK expression in the compressive force-loaded osteocytes

We previously reported that expression of CTGF in osteocytes is upregulated by a compressive force of 0.2 N loading for 6 hours in the same compressive force-loaded mouse model as in the present study. To examine whether the increased CTGF influences the expression of MENK and DOR in the compressive force-loaded osteocytes, we applied a neutralizing CTGF antibody to the parietal bones prior to compressive force loading. No obvious histological change was observed by administration of the neutralizing CTGF antibody in both the nonloaded and the loaded groups (Fig. 3A). In the nonloaded groups, MENK-positive osteocyte ratios without and with the neutralizing CTGF antibody were 74.1 ± 8.9% and 69.7 ± 3.5%, respectively; there was no significant difference between these groups (Fig. 3B,C). In the loaded groups, MENK-positive osteocyte ratio without the neutralizing CTGF antibody was 32.2 ± 7.4%; it was significantly lower than the nonloaded groups (Fig. 3B,C). The ratio in the loaded group with the neutralizing CTGF antibody was 71.8 ± 4.6%; it was significantly higher than the loaded group without the neutralizing CTGF antibody and was comparable to the nonloaded groups (Fig. 3B,C). The number of MENK-positive osteocytes and the total number of osteocytes were 8.8 ± 1.5 and 12.0 ± 2.9, and 7.5 ± 1.0 and 10.8 ± 1.3 in the nonloaded groups without and with the neutralizing CTGF antibody, respectively, and 2.8 ± 1.0 and 8.5 ± 1.7, and 6.3 ± 1.5 and 8.8 ± 2.4 in the loaded groups without and with the neutralizing CTGF antibody, respectively. DOR-positive osteocyte ratios in the nonloaded groups without and with the neutralizing CTGF antibody were 69.6 ± 3.9% and 66.8 ± 5.1%, respectively (Fig. 3D,E). The ratios in the loaded groups without and with the neutralizing CTGF antibody were 70.1 ± 4.8% and 65.4 ± 6.5%, respectively (Fig. 3D,E). There was no significant difference in DOR expression between any of the groups. The number of DOR-positive osteocytes and the total number of osteocytes were 6.8 ± 1.0 and 9.8 ± 1.7, and 8.0 ± 0.8 and 12.0 ± 1.2 in the nonloaded groups without and with the neutralizing CTGF antibody, respectively, and 8.3 ± 1.3 and 11.8 ± 1.3, and 7.3 ± 1.0 and 11.3 ± 2.4 in the loaded groups without and with the neutralizing CTGF antibody, respectively.

MENK suppressed osteocyte apoptosis induced by compressive force loading

Our previous study showed that the increased CTGF by compressive force loading induces osteocyte apoptosis in mouse parietal bones. To evaluate the effect of MENK on the osteocyte apoptosis induced by compressive force, exogenous MENK was s.c. injected into the parietal bone area prior to compressive force loading. There was no obvious histological change caused by MENK administration in the nonloaded and the loaded

Fig 2: Compressive force reduced expression of methionine enkephalin (MENK) in osteocytes. (A) Histological analysis of the parietal bones at 6 hours after the application of compressive force was performed by H&E staining. A magnified picture of the ROI is shown in the lower right corner of each picture. Scale bar = 100 μm, and 10 μm in insets. The expression of MENK (B,C) and the delta opioid receptor (DOR) (D,E) in the parietal bones was analyzed by immunohistochemistry (B,D), and the ratio of positive osteocytes (C,E) was calculated (four animals per group). Arrowheads indicate immunohistochemically positive osteocytes. Scale bar = 10 μm. Comp = compressive force loading. **P < 0.01. (F) Compressive force was loaded onto MLO-Y4 cells for 6 hours and expression of proenkephalin (PENK) and DOR was analyzed by real-time PCR. Comp = Compressive force loading. *P < 0.05; n = 3.
groups (Fig. 4A). TUNEL-positive osteocyte ratios without and with MENK in the nonloaded groups were 16.8 ± 5.1% and 16.4 ± 4.2%, respectively (Fig. 4B,C). In the loaded group without MENK, TUNEL-positive osteocyte ratio significantly increased to 35.2 ± 6.2% compared with the nonloaded groups (Fig. 4B,C). On the other hand, the MENK administration in the loaded group significantly decreased TUNEL-positive osteocyte ratio to 17.9 ± 8.9% compared with the loaded group without MENK, resulting in the comparable TUNEL-positive osteocyte ratio to the nonloaded groups (Fig. 4B,C). The number of TUNEL-positive osteocytes and the total number of osteocytes were 1.5 ± 0.6 and 8.8 ± 1.0, and 1.5 ± 0.6 and 9.0 ± 2.2 in the nonloaded groups without and with MENK, respectively, and 3.8 ± 0.5 and 10.8 ± 1.0, and 1.8 ± 1.0 and 9.8 ± 1.9 in the loaded groups.
without and with MENK, respectively. We also examined expression of active caspase-3 in osteocytes. In the nonloaded groups, caspase-3-positive osteocyte ratios without and with MENK were 13.3 ± 3.2% and 10.5 ± 2.8%, respectively (Fig. 4D,E). In the loaded groups, caspase-3-positive osteocyte ratio without MENK was 23.5 ± 5.6%; it was significantly higher than the nonloaded groups (Fig. 4D,E). The ratio in the loaded group with MENK was 10.9 ± 3.0%; it was significantly lower than the loaded group without MENK and comparable to the nonloaded groups (Fig. 4D,E). The number of caspase-3-positive osteocytes and the total number of osteocytes were 1.5 ± 0.6 and 11.0 ± 1.9, and 1.3 ± 0.5 and 11.8 ± 2.1 in the nonloaded groups without and with MENK, respectively, and 2.3 ± 0.5 and 9.8 ± 1.7, and 1.3 ± 0.5 and 11.3 ± 1.3 in the loaded groups without and with MENK.

**Fig 4** Methionine enkephalin (MENK) suppressed osteocyte apoptosis induced by compression force loading. (A) Histological analysis of the parietal bones at 6 hours after application of compressive force was performed by H&E staining. MENK was injected s.c. into the parietal bone area prior to application of compressive force. A magnified picture of the ROI is shown in the lower right corner of each picture. Scale bar = 100 μm, and 10 μm in insets. (B) TUNEL staining and subsequent counterstaining with hematoxylin of the parietal bones were performed. All four ROIs in a section in each group are shown. Arrowheads indicate TUNEL-positive osteocytes. Scale bar = 10 μm. (C) TUNEL-positive ratio of osteocytes was calculated (four animals per group). (D) Caspase-3 immunohistochemistry and subsequent counterstaining with hematoxylin of the parietal bones were performed. All four ROIs in a section in each group are shown. Arrowheads indicate caspase-3-positive osteocytes. Scale bar = 10 μm. (E) Caspase-3-positive ratio of osteocytes was calculated (four animals per group). Comp = compressive force loading. *P < 0.05, **P < 0.01.
MENK, respectively. The negative control of caspase-3 immunohistochemistry showed no apparent immunopositivity in osteocytes (Fig. 4D).

Compressive force induced nuclear translocation of NFATc1 in osteocytes

We next examined expression of NFATs in the mouse parietal bones and MLO-Y4 cells. Fig. 5A shows NFATc1 and NFATc3 mRNAs were highly expressed in these tissues and cells, whereas expression levels of NFATc2 and NFATc4 mRNAs were quite low. We further analyzed nuclear translocation of NFATc1 and NFATc3 of osteocytes in the compressive force-loaded parietal bones. The ratio of NFATc1-positive nuclei was 30.0 ± 6.0% in the nonloaded group, whereas the ratio was significantly increased to 65.2 ± 8.0% in the loaded group (Fig. 5B,C). The number of NFATc1-positive nuclei and the total number of nuclei were 3.3 ± 0.5 and 11.0 ± 1.6, and 6.0 ± 0.8 and 9.3 ± 1.3 in the nonloaded and loaded groups, respectively. In contrast, there was no significant difference in the ratio of NFATc3-positive nuclei between the nonloaded and the loaded groups (Fig. 5D, E). The number of NFATc3-positive nuclei and the total number of nuclei were 3.0 ± 0.8 and 10.5 ± 1.3, and 2.8 ± 1.0 and 10.3 ± 1.5 in the nonloaded and loaded groups, respectively. The negative controls of NFATc1 and NFATc3 immunohistochemistries showed no apparent immunopositivity in osteocytes (Fig. 5B,D).

The induction of osteocyte apoptosis by compressive force loading is dependent on NFATc1

To examine whether the nuclear translocation of NFATc1 induced by compressive force loading stimulates osteocyte apoptosis; INCA-6, an inhibitor of nuclear translocation of NFATs, was applied to the parietal bones prior to compressive force loading. No obvious histological changes were observed after INCA-6 administration in the nonloaded and the loaded groups (Fig. 6A). In the compressive force-loaded group, TUNEL-positive osteocyte ratio was 37.0 ± 3.5% without INCA-6 administration, whereas INCA-6 significantly decreased the ratio to 18.0 ± 5.7% (Fig. 6B,C). The number of TUNEL-positive osteocytes and the total number of osteocytes were 3.8 ± 1.3 and 10.0 ± 2.7, and 1.5 ± 0.6 and 8.3 ± 1.3 without and with INCA-6 administration, respectively. Under the compressive force-loaded condition, caspase-3-positive osteocyte ratio was 22.9 ± 5.8% without INCA-6 administration, whereas INCA-6 significantly decreased the ratio to 9.2 ± 1.1% (Fig. 6D,E). The number of caspase-3-positive osteocytes and the total number of osteocytes were 2.3 ± 0.5 and 10.0 ± 1.6, and 1.0 ± 0 and 11.0 ± 1.4 without and with INCA-6 administration, respectively. To determine a direct effect of NFATc1 on osteocyte apoptosis, we examined expression of apoptosis-related genes in NFATc1-overexpressing MLO-Y4 cells (Fig. 6F). Expression levels of caspase-3, caspase-8, and caspase-9 mRNAs in MLO-Y4 cells were significantly increased by overexpression of NFATc1 (Fig. 6G). Moreover, overexpression of NFATc1 upregulated cleaved caspase-3 protein expression in MLO-Y4 cells (Fig. 6H).

MENK inhibited nuclear translocation of NFATc1 in the compressive force-loaded osteocytes

Next, we examined whether MENK regulates nuclear translocation of NFATc1 and NFATc3. In the nonloaded groups, ratios of NFATc1-positive nuclei without and with MENK were 26.7 ± 4.9% and 30.6 ± 6.6%, respectively (Fig. 7A,B). In the loaded group without MENK, the ratio of NFATc1-positive nuclei was 65.6 ± 7.4%, a
significantly higher ratio than the nonloaded groups (Fig. 7A,B). In contrast, the ratio in the loaded group with MENK was 30.3 ± 5.0%; it was significantly lower than the loaded group without MENK and was comparable to the nonloaded groups (Fig. 7A,B). The number of NFATc1-positive nuclei and the total number of nuclei were 3.0 ± 0.5 and 10.8 ± 1.0 in the nonloaded groups without and with MENK, respectively, and 6.8 ± 1.0 and 10.5 ± 2.7, and 3.5 ± 1.0 and 11.5 ± 2.1 in the loaded groups without and with MENK, respectively. Ratios of NFATc3-positive nuclei in the nonloaded groups without and with MENK were 29.6 ± 3.0% and 29.6 ± 3.0%, respectively (Fig. 7C,D). The ratios in the loaded groups without and with MENK were 26.0 ± 8.8% and 28.9 ± 4.7%, respectively (Fig. 7C,D). There was no significant difference between any of the groups. The number
Fig 7  Methionine enkephalin (MENK) inhibited nuclear translocation of NFATc1 in the compressive force-loaded osteocytes. Nuclear translocation of NFATc1 (A,B) and NFATc3 (C,D) in the parietal bones at 6 hours after application of compressive force was analyzed by immunohistochemistry (A,C), and the ratio of positive nuclei in osteocytes (B,D) was calculated (four animals per group). MENK was injected s.c. into the parietal bone area prior to the application of compressive force. Arrowheads indicate the nuclear translocation of NFAT in osteocytes. Scale bar = 10 μm. **P < 0.01. (E) Representative images of Fura-2 AM-loaded MLO-Y4 cells before ionomycin stimulation and at the time of peak fluorescence intensity after ionomycin stimulation. MLO-Y4 cells were treated with MENK for 24 hours before Ca²⁺ imaging. The color scale represents relative fluorescence intensity. Scale bar = 50 μm. (F) Fluorescence intensity before ionomycin treatment was regarded as 1 and the relative increase in Ca²⁺ was measured. Comp = compressive force loading. *P < 0.05; n = 3. (G) MLO-Y4 cells were treated with MENK for 24 hours, and the expression levels of calcineurin A, calcineurin B, and calmodulin mRNA in each group at day 2 culture were analyzed by real-time PCR. *P < 0.05, **P < 0.01; n = 4.
of NFATc3-positive nuclei and the total number of nuclei were 
3.5 ± 0.6 and 11.8 ± 1.0, 3.5 ± 0.6, and 11.8 ± 1.0 in the non-
loaded groups without and with MENK, respectively, 3.0 ± 1.4 
and 11.3 ± 1.7, and 3.0 ± 0.8 and 10.3 ± 1.3 in the loaded groups 
without and with MENK, respectively. These data indicated that 
MENK inhibited the nuclear translocation of NFATc1 in osteocytes 
induced by compressive force loading.

To further investigate the mechanisms by which MENK regulates 
nuclear translocation of NFATc1, we focused on Ca2+ signaling. 
Real-time Ca2+ imaging using the Ca2+ indicator dye Fura-2 AM 
showed that a Ca2+ ionophore ionomycin elevated the fluores-
cence intensity of intercellular Ca2+ in MLO-Y4 cells without MENK 
treatment (Fig. 7E,F). The elevation of fluorescence intensity by 
ionomycin was decreased by MENK treatment in a dose-dependent 
manner; higher concentration of MENK showed significant reduc-
tion of the relative increase in fluorescence compared with the 
MENK-nontreated control (Fig. 7E,F). Moreover, the expression of 
Ca2+ signaling mediators, calcineurin A, calcineurin B, and calmod-
ulin in MLO-Y4 cells were assessed. Expression levels of these 
mRNAs were significantly decreased by MENK treatment in a dose-dependent manner (Fig. 7G).

Discussion

It is apparent that neuropeptides play important roles in bone 
metabolism, and the identification of neuropeptides that pro-
mote bone remodeling is a critical issue in bone biology.\textsuperscript{14–16} It is generally thought that neuropeptides are produced by nerve 
cells, but recent studies indicate that bone cells are also pro-
ducers of neuropeptides. For instance, neuropeptide Y is pro-
duced by osteocytes and facilitates bone remodeling.\textsuperscript{57} In the 
present study, we demonstrated, for the first time, that osteo-
cytes produce an endogenous opioid MENK in the mouse parietal 
bones. In addition, we found the expression of DOR, a major 
receptor of MENK, in osteocytes. Osteocytes reside in lacunae 
within the mineralized bone matrix and have their dendritic pro-
cesses through tiny tunnels called canaliculi, forming the osteo-
cyte lacunocanalicular network via gap junctions.\textsuperscript{2,3,38,39} These 
findings suggest that MENK functions in an autocrine and/or 
paracrine manner in the osteocyte lacunocanalicular network.

Osteocytes are key players of mechanotransduction in bones; 
that is, they convert external mechanical stress into biochemical 
signaling.\textsuperscript{5,38} CTGF is known as a mechanoresponsive factor; its 
expression is upregulated by mechanical stress.\textsuperscript{12,13} It binds to 
candidate receptors, such as integrin, lipoprotein receptor-related 
protein-1, and tyrosine kinase receptor A; its signaling facilitates 
various biological processes, including proliferation, differentia-
tion, and substrate production.\textsuperscript{40–43} We previously reported that 
increased expression of CTGF in osteocytes by compressive force 
duces osteocyte apoptosis in vivo and in vitro.\textsuperscript{12,13} In the pre-
sent study, we loaded compressive force to the mouse parietal 
bones, resulting in a reduction of MENK expression in osteocytes. 
A neutralizing CTGF antibody inhibited the compressive force-
induced reduction of MENK. Furthermore, we showed that an 
increase in osteocyte apoptosis in the compressive force-loaded 
parietal bones was inhibited by MENK administration. These 
results suggest that osteocyte apoptosis in response to compres-
sive force is induced through suppression of MENK expression 
by CTGF signaling. Osteocyte apoptosis is regulated positively 
by glucocorticoids and oxidative stress-related factors and nega-
tively regulated by PTH, estrogen, and androgen.\textsuperscript{54} In addition 
to these factors, neuropeptides, including MENK, can be 
considered novel regulators of osteocyte apoptosis according to 
the present study. Aging, loss of mechanical stress under a condi-
tion of weightlessness, and glucocorticoid-induced bone disease 
cause excessive osteocyte apoptosis and subsequent bone loss.\textsuperscript{45–47} Our finding about the inhibitory effect of MENK on oste-
cyte apoptosis may contribute to the development of treat-
ments for abnormal bone loss caused by osteocyte apoptosis.

Because NFAT has a close relationship with apoptosis in various 
types of cells, we examined the role of NFAT in compressive force-
induced osteocyte apoptosis. Our data showed that NFATc1 and 
NFATc3 mRNA were highly expressed in bone tissues and osteo-
cytes. Nuclear translocation of NFATc1 in the osteocytes in the parietal 
bones was enhanced by compressive force, whereas that of 
NFATc3 was not. These results indicated that translocation of 
NFATc1, among NFATs, into nuclei was increased in response to 
the compressive force in osteocytes. INCA-6, which inhibits NFAT 
translocation into nuclei, suppressed the increase in osteocyte 
apoptosis in the compressive force-loaded parietal bones. Moreover, 
overexpression of NFATc1 induced expression of apoptosis-related 
genes and cleaved caspase-3 protein in MLO-Y4 cells. Our findings 
suggest that osteocyte apoptosis induced by compressive force is 
advanced through induction of NFATc1 nuclear translocation.

Furthermore, we analyzed the relationship between MENK and 
NFATc1 in the molecular regulation of osteocytes apoptosis. Our 
data showed that MENK administration reduced nuclear translo-
cation of NFATc1 in osteocytes in vivo, suggesting that the 
decreased MENK expression promotes nuclear translocation of 
NFATc1 and following osteocyte apoptosis during osteocyte 
response to compressive force. It is known that the Ca2+ signaling 
pathway is a major mechanism of nuclear translocation of NFAT. 
That is, Ca2+ influx into cytoplasm leads to calmodulin-dependent 
activation of calcineurin, followed by NFAT dephosphorylation 
and nuclear translocation.\textsuperscript{46} Importantly, the Ca2+/calcineurin/ 
NFAT pathway promotes apoptosis in various cells such as muscle 
cells, megakaryocytes, and mesangial cells.\textsuperscript{33,49,50} In the present 
study, we found that MENK suppressed Ca2+ influx and expression 
of calcineurin and calmodulin in osteocytes, indicating that MENK 
is a negative regulator of Ca2+ signaling. Taken together, MENK 
would negatively regulate osteocyte apoptosis through the sup-
pression of the Ca2+/calcineurin/NFATc1 pathway.

Ca2+ influx is an initial biochemical event of mechanical stress-
loaded osteocytes; it stimulates osteocyte functions, such as gene 
expression and intercellular communication.\textsuperscript{51} Therefore, inhibi-
tion of Ca2+ influx by MENK may be an important mechanism to 
regulate mechanical stress-induced bone remodeling by regulat-
ing not only apoptosis, but other functions of osteocytes.

Conclusions

The present study revealed that osteocytes expressed MENK, 
whereas the MENK expression was suppressed by compressive 
force via CTGF signaling. Moreover, MENK inhibited compressive 
force-induced osteocyte apoptosis by downregulating nuclear 
translocation of NFATc1. MENK is considered to regulate nuclear 
translocation of NFATc1 by suppressing Ca2+ signaling in osteo-
cytes. These results suggest that osteocyte-produced MENK is a 
competent neuropeptide that regulates bone remodeling driven 
by mechanical stress. Our findings about novel roles of MENK 
in osteocytes not only contribute to understanding the mechanisms 
of bone remodeling regulated by neuropeptides, but are also pos-
sibly applicable to the development of treatments for pathological 
bone loss.
Disclosures

All authors state that they have no conflicts of interest.

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