ANESTHESIOLOGY

Endoplasmic Reticulum Stress Contributes to Nociception *via* Neuroinflammation in a Murine Bone Cancer Pain Model

Yanting Mao, Ph.D., Chenchen Wang, Ph.D., Xinyu Tian, M.D., Yulin Huang, Ph.D., Ying Zhang, M.D., Hao Wu, M.D., Shuai Yang, Ph.D., Ke Xu, M.D., Yue Liu, Ph.D., Wei Zhang, Ph.D., Xiaoping Gu, M.D., Ph.D., Zhengliang Ma, M.D., Ph.D.

ANESTHESIOLOGY 2020; 132:357–72

EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- The mechanisms supporting bone cancer pain are incompletely understood
- Stress of the endoplasmic reticulum has been implicated in supporting pain in some chronic pain states

What This Article Tells Us That Is New

- Using a murine model of bone cancer pain, it was observed that tumor growth was associated with the spinal production of inflammatory mediators and increased expression of endoplasmic reticulum stress markers
- The pharmacologic inhibition of endoplasmic reticulum stress reduced pain-related behaviors and the production of inflammatory mediators in spinal tissue

ABSTRACT

Background: Prolonged endoplasmic reticulum stress has been identified in various diseases. Inflammatory mediators, which have been shown to induce endoplasmic reticulum stress in several studies, have been suggested to serve as the important modulators in pain development. In this study, the authors hypothesized that the endoplasmic reticulum stress triggered by inflammatory mediators contributed to pain development.

Methods: The authors used a male mouse model of bone cancer pain. The control mice were intrathecally injected with tumor necrosis factor-α (TNF-α) and lipopolysaccharide, the bone cancer pain mice were intrathecally injected with the endoplasmic reticulum stress inhibitors 4-PBA and GSK2606414. The nociceptive behaviors, endoplasmic reticulum stress markers, and inflammatory mediators were assessed.

Results: Increased expression of the p-RNA-dependent protein kinase-like endoplasmic reticulum kinase and p-eukaryotic initiation factor 2α were found in the spinal neurons during bone cancer pain, along with upregulation of inflammatory mediators (TNF-α, interleukin 1β, and interleukin 6). Intrathecal administration of TNF-α or lipopolysaccharide increased the expression of endoplasmic reticulum stress markers in control mice. Inhibition of endoplasmic reticulum stress by intrathecal administration of 4-PBA (baseline *vs.* 3 h: 0.34 ± 0.16 g *vs.* 1.65 ± 0.40 g in paw withdrawal mechanical threshold, 8.00 ± 1.20 times per 2 min *vs.* 0.88 ± 0.64 times per 2 min in number of spontaneous flinches, $P < 0.001$, n = 8) or GSK2606414 (baseline *vs.* 3 h: 0.37 ± 0.08 g *vs.* 1.38 ± 0.11 g in paw withdrawal mechanical threshold, 8.00 ± 0.93 times per 2 min *vs.* 3.25 ± 1.04 times per 2 min in number of spontaneous flinches, $P < 0.001$, n = 8) showed time- and dose-dependent antinociception. Meanwhile, decreased expression of inflammatory mediators (TNF-α, interleukin 1β, and interleukin 6), as well as decreased activation of astrocytes in the spinal cord, were found after 4-PBA or GSK2606414 treatment.

Conclusions: Inhibition of inflammatory mediator–triggered endoplasmic reticulum stress in spinal neurons attenuates bone cancer pain *via* modulation of neuroinflammation, which suggests new approaches to pain relief.

(ANESTHESIOLOGY 2020; 132:357–72)

Bone cancer pain is a severe complication of metastatic or advanced malignancy, which is characterized by allodynia and hyperalgesia.[1] Bone cancer pain can compromise the patient's quality of life and impose a heavy burden on society.[2] To date, the mechanism underlying bone cancer pain has not been completely understood. As a result, existing pharmacologic agents cannot relieve pain efficiently and satisfactorily.[3,4]

As a protective mechanism, endoplasmic reticulum stress is responsible for folding and trafficking of proteins under a series of stress conditions.[5] Endoplasmic reticulum stress has been indicated to be involved in numerous neurodegenerative diseases.[6–8] Activation of the unfolded protein response by accumulation of unfolded proteins in endoplasmic reticulum initiates three endoplasmic reticulum stress pathways, which are mediated by RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α, inositol-requiring enzyme 1, and the activating transcription factor 6.[9] Endoplasmic reticulum stress has been proposed to be involved in pain development. All pathways of endoplasmic reticulum

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org).

Submitted for publication March 1, 2019. Accepted for publication September 26, 2019. Published online first on December 9, 2019. From the Department of Anaesthesiology, Affiliated Drum Tower Hospital of Medical School of Nanjing University, Nanjing, Jiangsu Province, China.

<zdoi;. DOI: 10.1097/ALN.0000000000003078>

stress have been found to be activated in the peripheral nervous system in neuropathic and inflammatory pain.[10,11] Activated RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α in astrocytes and activated activating transcription factor 6 in neurons were found in the central nervous system in neuropathic pain.[12] However, the role of endoplasmic reticulum stress in the development of bone cancer pain has still not been fully elucidated.

Various models have been proposed to describe the contribution of inflammation to pain. The production of TNF-α and interleukin 1β promotes the development of allodynia in peripheral nerve injury[13] and spinal cord injury.[14] Neuroinflammation drives widespread chronic pain *via* central sensitization.[15] Our previous studies also confirmed the upregulation of TNF-α, interleukin 1β, and interleukin 6 in the spinal cord during bone cancer pain.[16] Several studies have indicated that inflammatory mediators serve as the inducers of endoplasmic reticulum stress. TNF-α stimulation of synovial fibroblasts increased the expression of endoplasmic reticulum stress markers.[17] Similarly, the increased levels of inflammatory mediators TNF-α and interleukin 1β in rheumatoid arthritis resulted in an increase in endoplasmic reticulum stress in dendritic cells, fibroblast-like synoviocytes, T cells, and B cells.[18] On the basis of these findings, we assumed that the production of inflammatory mediators in the spinal cord during bone cancer pain serves as a trigger for endoplasmic reticulum stress.

Endoplasmic reticulum stress affects various cell signaling processes, including apoptosis[19] and inflammation. The cross-link between inflammation and endoplasmic reticulum stress has been proposed in recent studies. Three unfolded protein response signaling pathways are related to the production of inflammatory mediators *via* the activation of the transcription factor nuclear factor-κB, one of the key modulators in inflammatory gene transcription.[20] Inhibition of RNA-dependent protein kinase-like endoplasmic reticulum kinase reduced the expression of interleukin 6, chemokine ligand 2, and chemokine ligand 20 in astrocytes.[21] Additionaly, endoplasmic reticulum stress increased the production of interleukin 1β, interleukin 6, and interleukin 23 in response to lipopolysaccharide.[22] However, previous studies have not determined whether neuroinflammation could be modulated by endoplasmic reticulum stress in bone cancer pain.

In this study, we tested the hypothesis that endoplasmic reticulum stress could be triggered by the production of inflammatory mediators in the spinal cord and would exacerbate the development of mouse bone cancer pain. Inhibition of endoplasmic reticulum stress by the inhibitors 4-PBA and GSK2606414 downregulated the neuroinflammation in the spinal cord and improved the nociceptive behaviors in bone cancer pain mice.

Materials and Methods

Animals

Male C3H/HeN mice (20–25 g, 5 weeks of age; Vital River Experimental Animal Corporation of Beijing, China) were used. Mice were housed with free access to water and food and in a 12-h light–dark cycle. All experiments were carried out between 9:00 AM and 5:00 PM. All experiments were performed in strict accordance with the guidelines and approved by the Animal Care and Use Committee of the Medical School of Nanjing University (Nanjing, China).

Bone Cancer Pain Model

NCTC 2472 osteolytic sarcoma cells were cultured in NCTC 135 medium (Sigma, USA) with 10% horse serum (Gibco, USA) and maintained in a 5% CO_2 atmosphere at 37°C (Thermo Forma, USA).

The establishment of the bone cancer pain model was described previously by °Schwei *et al.*[23] Animals were anesthetized intraperitoneally with pentobarbital sodium at a dose of 50 mg/kg. Right knee arthrotomy was performed after general anesthesia. Next, 20 μl of α–minimum essential medium (Thermo Fisher Scientific, USA) containing 2×10^5 osteolytic sarcoma cells was injected into the intramedullary space of the right femur, whereas the sham group received injections of 20 μl of α–minimum essential medium alone. After sealing the drill hole with bone wax, the wound was closed with 4-0 silk sutures (Ethicon, USA). Animals recovered from anesthesia on a heated blanket.

Primary Spinal Neuron Culture

Pregnant B6 mice were anesthetized with isoflurane, and the fetuses were removed on embryonic day 14. A microscope was used to remove the meninges and blood vessels of the embryonic spinal cord. After digestion with 0.05% trypsinase at 37°C for 20 min, spinal tissues were dissociated softly 10 times. The supernatant was sieved with a 70-μm cell strainer (Falcon, USA) and centrifuged for 5 min at 1,000 rpm. The cells were resuspended in Dulbecco's Modified Eagle Medium (Biological Industries, USA) containing 10% fetal bovine serum (Gibco), 2 mM glutamine (Sigma), 25 mM glucose, and 1% penicillin/streptomycin (Gibco) and plated onto poly-L-lysine (Sigma, USA)–coated six-well plates at the density of $1 \times 10^6/cm^2$. The medium was completely changed to neurobasal medium (Gibco) containing 2% B27 (Gibco), 2 mM glutamine, and 10-μl/ml penicillin/streptomycin 4 h later. Cells were cultured at 37°C in a 5% CO_2 atmosphere (Thermo Forma). One-half medium change was performed on day 2, and cells were allowed to grow for 7 to 9 days.

Drug Treatment

In *in vivo* experiments, recombinant murine TNF-α (Peprotech, USA), lipopolysaccharides (lipopolysaccharide, Sigma), 4-phenylbutyrate (4-PBA, Sigma), and GSK2606414 (1-[5-(4-Amino-7-methyl-7H-pyrrolo[2,3-d]pyrimidin-5-y)-2,3-dihydro-1H-indol-1-yl]-2-[3-(trifluoromethyl) phenyl]ethanone; Tocris, United Kingdom) were dissolved in normal saline before administration. TNF-α (5 ng/5 μl) and lipopolysaccharide (100 ng/5μl) were intrathecally

injected in control mice. On the basis of previous studies, 4-PBA was intraperitoneally (1 mg/100 μl) and intrathecally (40 μg/5 μl, 80 μg/5 μl, 120 μg/5 μl) administered to bone cancer pain mice on day 14 after the operation. Meanwhile, GSK2606414 was intrathecally administered to bone cancer pain mice on day 14 after the operation at a dose of 50 μg/5 μl, 100 μg/5 μl, and 200 μg/5 μl on the basis of a previous study. The intrathecal injection was performed as previously described by Hylden and Wilcox.[24]

In *in vitro* experiments, TNF-α and lipopolysaccharide were dissolved in culture media at a dosage of 100 nM. Primary spinal neurons were treated with TNF-α and lipopolysaccharide for 12 h and 24 h, respectively.

Nociceptive Behavior Test

Mechanical allodynia and spontaneous pain in mice were tested before operation (day 0) as well as 4, 7, 10, 14, 21, and 28 days after operation in each group, 0, 1, 3, 6 h after administration of recombinant murine TNF-α, lipopolysaccharide, and vehicle, and 0, 1, 2, 3, 4, 5, 7, 10 h after administration of 4-PBA, GSK2606414, and vehicle. Experimenters of all behavioral tests were blinded to the group assignment data.

Paw Withdrawal Mechanical Threshold. Paw withdrawal mechanical thresholds of the right hind paw were measured using von Frey filaments (0.16, 0.4, 0.6, 1.0, 1.4, 2.0 g; Stoelting, USA) and the up-down method as previously reported.[25] Mice were placed in transparent plexiglass compartments with a wire mesh bottom for a 30-min acclimatization period, after which the von Frey filaments were stuck upright to the plantar surface and the lowest filament stimulus strength that caused paw flinching or withdrawal was recorded.

Number of Spontaneous Flinches. Mice were placed in transparent plexiglass compartments with a wire mesh bottom and acclimatized for a 30-min acclimatization period, which was followed by observation of the number of flinching episodes of the right hind paw more than 2 min. Each mouse was tested five times.

Western Blotting

After deeply anesthetizing the mice with pentobarbital (50 mg/kg, intraperitoneally), mice were euthanized at days 0, 4, 7, 10, 14, 21, and 28 after operation and 3 h after administration. The spinal tissues were removed and stored at -80°C for further study. Samples of spinal tissue or cells were homogenized in Radio Immunoprecipitation Assay Lysis Buffer (10 μl/mg for tissue, Beyotime Biotechnology, China) with phenylmethyl sulfonyl fluoride and rested on ice for 30 min. Next, the samples were centrifuged at 12,000 rpm, 4°C for 20 min and the supernatant was collected. The concentration of each protein sample was tested by the Bicinchonininc Acid Protein Assay Kit. Protein samples were subjected to sodium dodecylsulphate - polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane. The membranes were incubated with primary antibodies for binding immunoglobulin protein (BIP; 1:1,000, CST, USA), RNA-dependent protein kinase-like endoplasmic reticulum kinase (1:200, Santa Cruz, USA), p-RNA-dependent protein kinase–like endoplasmic reticulum kinase (1:200, Santa Cruz), eukaryotic initiation factor 2α (1:200, Santa Cruz), p-eukaryotic initiation factor 2α (1:200, Santa Cruz), inositol-requiring enzyme 1α (1:200, Santa Cruz), p-inositol-requiring enzyme 1α (1:1000, Abcam, USA), activating transcription factor 6α (1:200, Santa Cruz), TNF-α (1:200, Santa Cruz), interleukin 1β (1:1,000, Abcam), interleukin 6 (1:1,000, Abcam), and β-actin (1:4,000, Abcam). After incubation with the secondary antibody (1:10,000, Millipore, USA) and electro chemi luminescence solution, images were captured and analyzed using a cooled charge coupled device system (Tanon, China).

RNA Isolation and Quantitative Polymerase Chain Reaction

Total RNA was isolated from mouse spinal cord by Trizol reagent (Invitrogen, USA), and messenger RNA was reverse transcribed into cDNA by using the HiScript II First Strand cDNA Synthesis Kit (Vazyme, China). All reverse transcription reactions were performed with cDNA and ChamQ Universal SYBR qPCR Master Mix (Vazyme, China) and run in an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, USA). The following primers were used in this study: TNF-α (F): CGAGTGACAAGCCTGTAGCCC, TNF-α (R): GTCTTTGAGATCCATGCCGTTG; interleukin 1β (F): TCAGGCAGGCAGTATCACTCA, interleukin 1β (R): GGAAGGTCCACGGGAAAGAC; interleukin 6 (F): ACA ACCACGGCCTTCCCTAC, interleukin 6 (R): TCTCATTTCCACGATTTCCCAG; GAPDH (F): GGAAAGCTGTGGCGTGAT, and GAPDH (R): AAGGTGGAAGAATGGGAGTT.

Immunofluorescence

After general anesthesia described above, the animals were transcardially perfused with normal saline and 4% paraformaldehyde at day 14 after operation and 3 h after intrathecal administration. The lumbosacral enlargements were removed and fixed in 4% paraformaldehyde for 6 h, and then dehydrated in 30% sucrose for 48 to 72 h at 4°C. Cells were fixed with methanol. After freezing with the optimal cutting temperature compound, tissues were cut into 20-μm sections by using a freezing microtome. After washing with phosphate buffered saline, the sections and cells were blocked with 10% goat serum containing 0.3% Triton and incubated with primary antibodies for BIP, p-RNA-dependent protein kinase–like endoplasmic reticulum kinase, p-eukaryotic initiation factor 2α, GFAP (mouse, 1:100, Cell Signaling Tech, USA), ionized calcium binding adapter molecule 1 (mouse, 1:300, Abcam), neuronal nuclei (mouse, 1:1,000, Abcam), and GAD65 plus GAD67 (rabbit, 1:100, Abcam) separately overnight at 4°C. After washing with phosphate buffered saline, sections were incubated with Alexa 488-conjugated goat

anti-rabbit (1:3,000, ThermoFisher) and Alexa 594-conjugated goat anti-mouse (1:3,000, ThermoFisher) secondary antibodies. Next, the sections were transferred on slides and incubated with DAPI (Abcam). Images were captured using a laser-scanning confocal microscope (Olympus, Japan).

Hematoxylin and Eosin Staining

The femur tissue was fixed with 10% paraformaldehyde and decalcified in EDTA decalcification solution for 1 to 2 weeks. After dehydration, the tissue was embedded in paraffin and sliced into 5-μm sections. After staining with hematoxylin and eosin reagents, images were captured using a laser-scanning confocal microscope (Olympus).

Electron Microscopic Examination

After anesthetization, mice were perfused with normal saline and 2.5% glutaraldehyde. The lumbosacral enlargements were removed and kept in 2.5% glutaraldehyde at 4°C Cells were collected 12 h after TNF-α stimulation or 24 h after lipopolysaccharide stimulation and fixed in 2.5% glutaraldehyde at 4°C. Samples were fixed in 1% osmium tetroxide and dehydrated in graded ethanol. After embedding in epoxy resin, the samples were cut into 60-mm sections and stained with uranyl acetate and lead citrate. Images were captured using Hitachi 7100 electron microscopy.

Statistical Analysis

The number of animals used in each study was based on our previous experience with this design.[26] No *a priori* statistical power calculation was used to guide sample size. In response to peer review, sample sizes were increased to six in the Western blotting, quantitative polymerase chain reaction, and immunofluorescence. We randomized animals to the different treatment groups and blinded the experimenter to the drug treatment to reduce selection and observation bias. None of the variables had missing data, and no outliers appeared in our study. Statistical analysis was performed using SPSS 22.0 (IBM Corporation, USA). Data are presented as mean ± SD. Results from the behavioral study were analyzed using two-way repeated measurements ANOVA followed by *post hoc* tests (Bonferroni test) to assess differences at each time point between groups and one-way ANOVA followed by Bonferroni test to assess differences over time within groups. Results from western blotting and quantitative polymerase chain reaction were analyzed using one-way ANOVA followed by Bonferroni test or independent *t* tests for between-group comparisons. Normal distribution assumption was analyzed using Q-Q plots. All tests were two-tailed, and $P < 0.05$ was considered as the level of significance.

Results

Pain Hypersensitivity in Bone Cancer Pain Mice

Intrafemur implantation of NCTC 2472 sarcoma cells was performed to establish mouse bone cancer pain model. Changes in nociceptive behaviors were measured by determining the paw withdrawal mechanical threshold (fig. 1A) and number of spontaneous flinches (fig. 1B) on days 0 (baseline), 4, 7, 10, 14, 21, and 28 after the operation. The baseline paw withdrawal mechanical threshold and number of spontaneous flinches did not differ significantly between sham group and tumor group ($P > 0.05$, n = 8, respectively). A statistically significant reduction in paw withdrawal mechanical threshold and a statistically significant increase in number of spontaneous flinches were found on day 4 after the operation in comparison with the baseline both in the sham group (baseline *vs.* day 4: $P = 0.003$ in paw withdrawal mechanical threshold; $P < 0.001$ in number of spontaneous flinches; n = 8) and the tumor group (baseline *vs.* day 4: $P = 0.002$ in paw withdrawal mechanical threshold; $P < 0.001$ in number of spontaneous flinches; n = 8). The acute hyperalgesia may have resulted from the surgery since the nociceptive behaviors recovered from day 7 in the sham group. In the tumor group, the paw withdrawal mechanical threshold decreased from day 10 ($P = 0.003$ on day 10; $P = 0.001$ on day 14; $P < 0.001$ on day 21; $P = 0.001$ on day 28; n = 8), whereas the number of spontaneous flinches persistently increased for 28 days ($P < 0.001$, n = 8, respectively). The paw withdrawal mechanical threshold and number of spontaneous flinches both showed statistically significant differences compared with those in the sham group on days 10, 14, 21, and 28 after the operation ($P < 0.001$, n = 8, respectively). Specific descriptive statistics (mean ± SD) of behavior tests were shown in Supplemental Digital Content, table 1 and table 2 (http://links.lww.com/ALN/C138). Hematoxylin and eosin staining of the femur slices showed infiltration of tumor cells in the marrow cavity, discontinuous bone trabeculae, and destruction of bone cortex in bone cancer pain mice on day 14 after the operation (fig. 1C).

Increased Expression of the RNA-dependent Protein Kinase-Like Endoplasmic Reticulum Kinase–Eukaryotic Initiation Factor 2α Pathway of Endoplasmic Reticulum Stress in Bone Cancer Pain Mice

The role of endoplasmic reticulum stress in inflammatory pain and neuropathic pain has been proposed. To determine whether endoplasmic reticulum stress was involved in the mouse bone cancer pain model, several endoplasmic reticulum stress-associated proteins were examined (fig. 2A). As the marker of initiation of endoplasmic reticulum stress, the expression level of BIP (GRP76) was elevated on days 10 ($P < 0.001$, n = 6), 14 ($P < 0.001$, n = 6), 21 ($P < 0.001$, n = 6), and 28 ($P = 0.029$, n = 6) in the tumor group compared with baseline (day 0; fig. 2B). The phosphorylation of RNA-dependent protein kinase-like endoplasmic reticulum kinase (fig. 2D) and eukaryotic initiation factor 2α (fig. 2C) increased briefly on day 4 ($P < 0.001$, n = 6, respectively) and persistently from day 14 after the operation in the tumor group ($P < 0.001$, n = 6, respectively). The phosphorylation of inositol-requiring

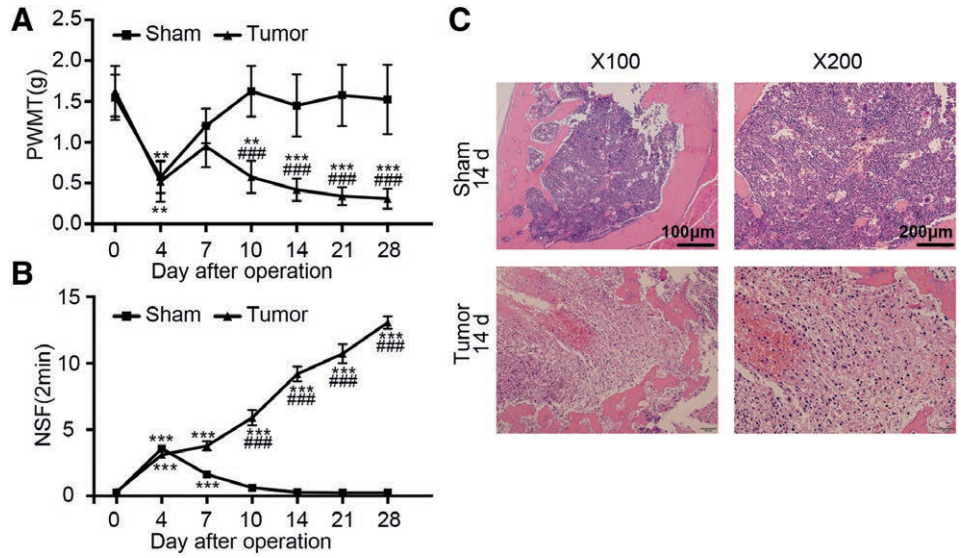

Fig. 1. Intrafemur implantation of NCTC 2476 cells induced mechanical hypersensitivity in the ipsilateral hind paw. (*A* and *B*) The paw withdrawal mechanical threshold (PWMT) and number of spontaneous flinches (NSF) were measured on days 0, 4, 7, 10, 14, 21, and 28 after operation in the sham and tumor groups. One-way ANOVA with Bonferroni *post hoc* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with day 0; two-way repeated-measures ANOVA with Bonferroni *post hoc* test, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the sham group at each point; n = 8 per group. (*C*) Photomicrographs (×100, ×200) of femur marrow cavity stained with hematoxylin and eosin of the sham and tumor group on day 14 after the operation. Data are expressed as mean ± SD.

enzyme 1α (fig. 2F) increased only on day 28 after the operation in the tumor group ($P < 0.001$, n = 6). No differences were found in activating transcription factor 6α expression during the postoperative period (fig. 2E, $P > 0.05$, n = 6, respectively). In the sham group, the expression of endoplasmic reticulum stress markers showed no statistically significant differences during the postoperative period (fig. S1, $P > 0.05$, n = 6, respectively). Because the increased expression level of p-RNA-dependent protein kinase–like endoplasmic reticulum kinase and p-eukaryotic initiation factor 2α were maintained from day 14 to day 28, whereas the expression of p-inositol-requiring enzyme 1α increased just on day 28, we considered the RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α pathway to be a key modulator in mouse bone cancer pain model. Next, electron microscopy was used to evaluate ultrastructural changes in endoplasmic reticulum on day 14 after the operation (fig. 2G). The cisternae of endoplasmic reticulum swelled obviously in the tumor group, compared with the normally narrow endoplasmic reticulum cisternae in the sham group. Also, there were more dark particles in the tumor group, which might be accumulated vesicles for degradation, and further study was required. These data suggest that activation of endoplasmic reticulum stress in mouse bone cancer pain model mainly depends on the RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α pathway.

Upregulation of Endoplasmic Reticulum Stress Markers in the Dorsal Horn Neurons

We next assessed the cellular localization of p-RNA-dependent protein kinase–like endoplasmic reticulum kinase and p-eukaryotic initiation factor 2α expression. Double immunofluorescence staining was performed. The results showed substantial p-RNA-dependent protein kinase–like endoplasmic reticulum kinase and p-eukaryotic initiation factor 2α expression in the spinal dorsal horn, which was mostly colocalized with neuronal nuclei (a neuronal marker; fig. 3, A and B). To further classify the endoplasmic reticulum stress neurons, we performed double-labeling with BIP and GAD (the marker of γ-aminobutyric acid–mediated [GABAergic] interneurons). The results showed that BIP was localized in the GABAergic interneurons (fig. 3C). We also examined the expression of p-RNA-dependent protein kinase–like endoplasmic reticulum kinase and p-eukaryotic initiation factor 2α in astrocytes and microglia. Partial colocalization with glial fibrillary acidic protein (GFAP; an astrocyte marker) in the spinal cord was found (Supplemental Digital Content, figs. S2A and S2B, http://links.lww.com/ALN/C138). No colocalization with ionized calcium binding adapter molecule 1 (a microglial marker) was found

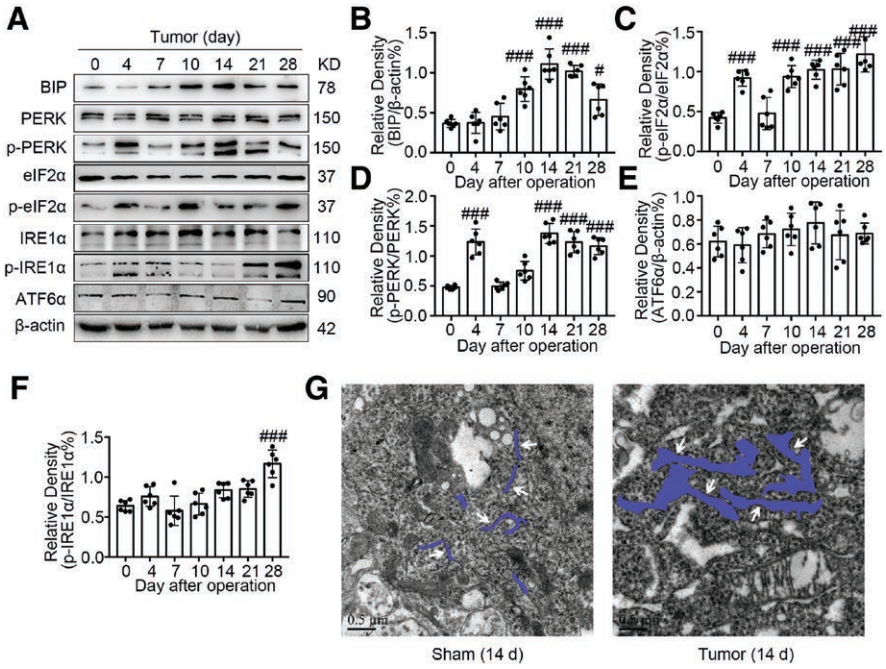

Fig. 2. The elevated endoplasmic reticulum stress in bone cancer pain mice. (*A*) The level of endoplasmic reticulum stress-related proteins in the tumor group on postoperative days. (*B*, *C*, *D*, *E*, and *F*) Quantification of endoplasmic reticulum stress–related proteins in the spinal cord in tumor group mice. One-way ANOVA with Bonferroni *post hoc* test, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with day 0; n = 6 per group. (*G*) Electron microscopic analysis of neurons in the spinal cord showed some swollen endoplasmic reticulum cisternae in the tumor group compared with the normally narrow endoplasmic reticulum cisternae on day 14 after the operation; Scale bar, 0.5 μm. Data are expressed as mean ± SD.

(Supplemental Digital Content, figs. S2C and S2D, http://links.lww.com/ALN/C138). These results indicate that the RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α pathway was activated in dorsal horn neurons in mouse bone cancer pain model.

Increased Levels of the Inflammatory Mediators TNF-α, Interleukin 1β, and Interleukin 6 in the Spinal Cord during the Development of Bone Cancer Pain

The expression of inflammatory mediators was tested by quantitative polymerase chain reaction and Western blotting. The results of quantitative polymerase chain reaction showed increased expression of TNF-α, interleukin 1β, and interleukin 6 from day 14 after the operation in the tumor group (fig. 4A). The increased expression of TNF-α was 50-fold on day 14 compared with the basal value ($P < 0.001$, n = 6), whereas sevenfold higher expression of interleukin 1β ($P < 0.001$, n = 6) and sixfold higher expression of interleukin 6 ($P < 0.001$, n = 6) were noted on day 28 after the operation. Similarly, Western blotting showed increased expression of TNF-α ($P < 0.001$, n = 6; fig. 4C), interleukin 1β ($P < 0.001$, n = 6; fig. 4D), and interleukin 6 ($P = 0.002$ on day 10, $P < 0.001$ on day 21, $P < 0.001$ on day 28, n = 6; fig. 4E) since day 14 after the operation in the tumor group (fig. 4B).

Stimulation of Primary Spinal Neurons with TNF-α and Lipopolysaccharide Upregulated Endoplasmic Reticulum Stress–related Proteins

Considering the upregulation of TNF-α, interleukin 1β, and interleukin 6 reported above, we next explored whether these inflammatory mediators could activate endoplasmic reticulum stress. Studies have indicated the increased expression of interleukin 1β and interleukin 6 after lipopolysaccharide stimulation in various cells.[27,28] Therefore, we treated primary spinal neurons with TNF-α (100 nM) and lipopolysaccharide (100 nM) to further verify the endoplasmic reticulum stress caused by inflammation. The results of immunostaining showed that the expression of p-RNA-dependent protein kinase–like endoplasmic reticulum kinase ($P < 0.001$ in the TNF-α group, $P = 0.001$ in the lipopolysaccharide group, n = 6; fig. 4, F and G) and p-eukaryotic initiation factor 2α ($P < 0.001$ in the TNF-α group, $P = 0.04$ in the lipopolysaccharide group, n = 6; fig. 4, H and I) was increased in primary spinal neurons treated with TNF-α and lipopolysaccharide in comparison with the vehicle-treated neurons. Additionally, we examined

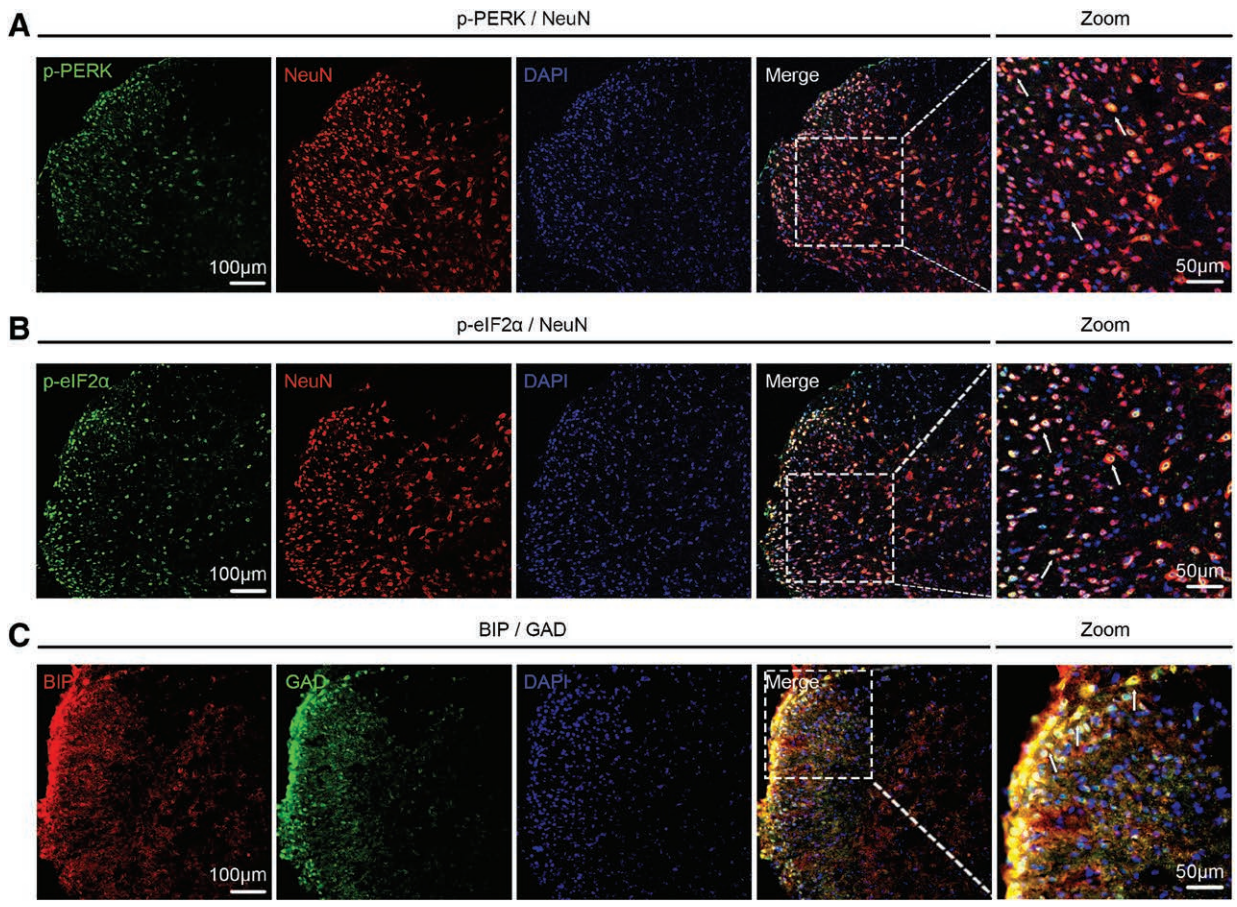

Fig. 3. Colocalization of p-PERK/p-eukaryotic initiation factor 2α with neuronal nuclei (NeuN) and BIP with GABAergic interneurons in the dorsal horn of bone cancer pain mice. (*A*) Double immunostaining with p-RNA-dependent protein kinase–like endoplasmic reticulum kinase (*green*) and neuron marker NeuN (*red*); (*B*) Double immunostaining with p-eukaryotic initiation factor 2α (*green*) and neuron marker NeuN (*red*); (*C*) Double immunostaining with BIP (*red*) and GABAergic interneurons marker GAD (*green*); Scale bar, 100 μm.

the endoplasmic reticulum stress in the primary cortical neurons after TNF-α and lipopolysaccharide stimulation. Western blotting and electron microscopy showed the evaluated endoplasmic reticulum stress in neurons treated with TNF-α and lipopolysaccharide (Supplemental Digital Content, fig. S3, http://links.lww.com/ALN/C138). These results suggest that endoplasmic reticulum stress might be triggered by upregulation of inflammatory mediators in mouse bone cancer pain model.

Intrathecal Administration of TNF-α and Lipopolysaccharide Induced Nociceptive Behaviors and Increased the Expression of Endoplasmic Reticulum Stress Markers in Control Mice

We next explored whether intrathecal administration of TNF-α (5 ng) or lipopolysaccharide (100 ng) could trigger endoplasmic reticulum stress in the spinal cord in control mice. Decreased paw withdrawal mechanical threshold ($P < 0.001$, n = 8, respectively) and increased number of spontaneous flinches ($P < 0.001$, n = 8, respectively) were found at 1 h and 3 h after injection both in TNF-α– and lipopolysaccharide-treated mice in comparison with baseline. Expression levels of the endoplasmic reticulum stress markers BIP, p-RNA-dependent protein kinase–like endoplasmic reticulum kinase, p-eukaryotic initiation factor 2α, and p-inositol-requiring enzyme 1α were increased in both TNF-α– and lipopolysaccharide-treated mice in comparison with the vehicle-treated mice at 1 h after injection ($P < 0.001$, n = 6, respectively). These results further demonstrated that inflammatory mediators might be potent inducers of endoplasmic reticulum stress in pain development.

Intrathecal Administration of 4-PBA or GSK2606414 Attenuated Nociception in Bone Cancer Pain Model

Because the findings above showed that increased expression of inflammatory mediators in lipopolysaccharide-stimulated

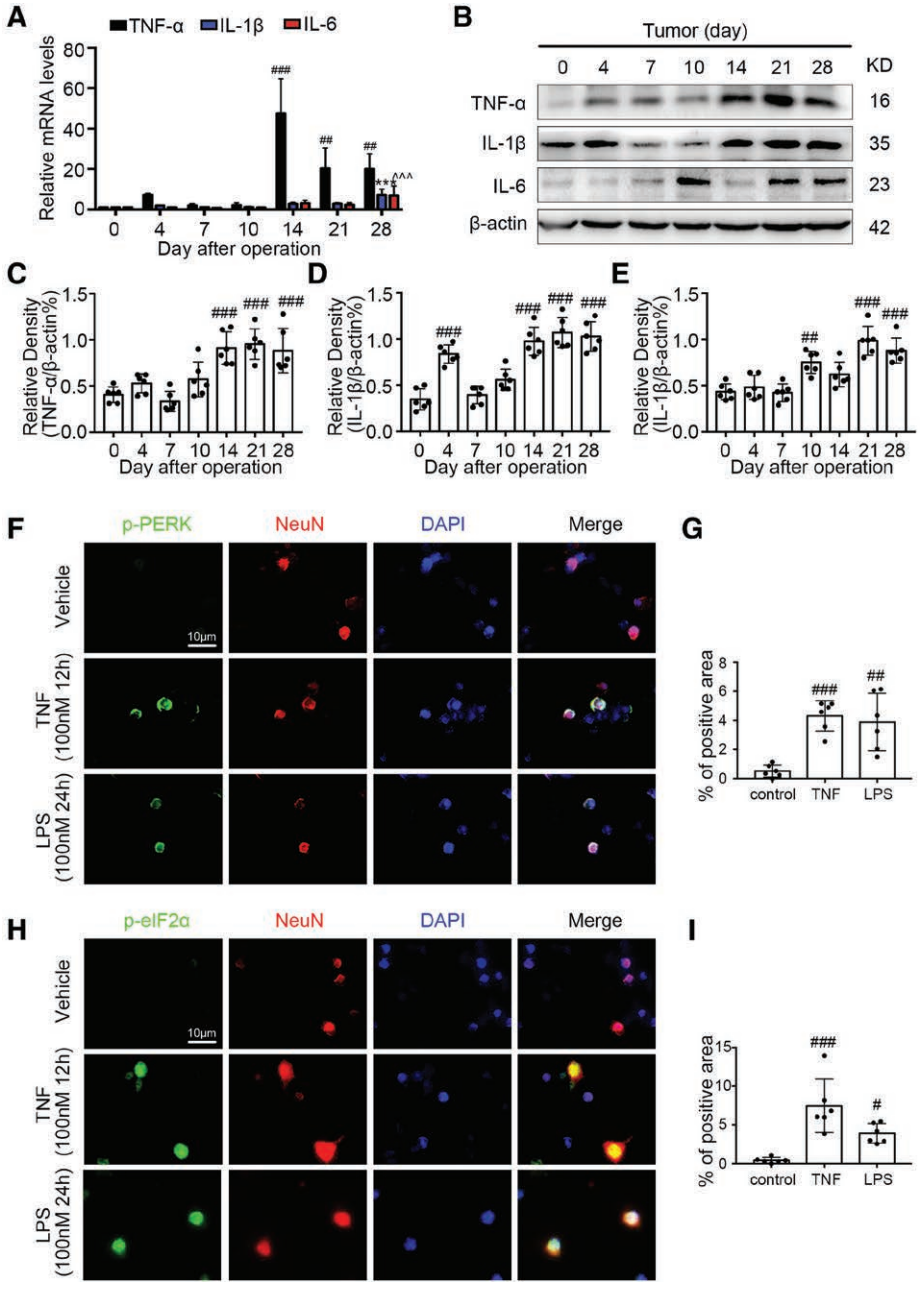

Fig. 4. Upregulations of tumor necrosis factor-α (TNF-α), interleukin (IL) 1β, and IL-6 triggered endoplasmic reticulum stress. (*A*) mRNA levels of TNF-α, IL-1β, and IL-6 in the spinal cord in tumor group mice; One-way ANOVA with Bonferroni *post hoc* test, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the expression of TNF-α on day 0; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the expression of IL-1β on day 0; $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$ compared with the expression of IL-6 on day 0; n = 6 per group. (*B*) Representative blots of TNF-α, IL-1β, and IL-6 in the spinal cord in tumor group mice. (*C*, *D*, and *E*) Quantification of TNF-α, IL-1β, and IL-6 in the spinal cords in tumor group mice. One-way ANOVA with Bonferroni *post hoc* test, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with day 0; n = 6 per group. (*F*) Double immunostaining with p-RNA-dependent protein kinase–like endoplasmic reticulum kinase (*green*) and neuronal nuclei (NeuN; *red*) in primary spinal neurons stimulated by TNF-α (100 nM) and lipopolysaccharide (LPS; 100 nM), respectively; scale bar, 10 μm. (*G*) Quantification of p-RNA-dependent protein kinase–like endoplasmic reticulum kinase positive area; one-way ANOVA with Bonferroni *post hoc* test, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with vehicle-treated primary spinal neurons. (*H*) Double immunostaining with p-eukaryotic initiation factor 2α (*green*) and NeuN (*red*) in primary spinal neurons stimulated by TNF-α (100 nM) and LPS (100 nM), respectively; scale bar, 10 μm. (*I*) Quantification of p-eukaryotic initiation factor 2α positive area; one-way ANOVA with Bonferroni *post hoc* test, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with vehicle-treated primary spinal neurons. Data are expressed as mean ± SD.

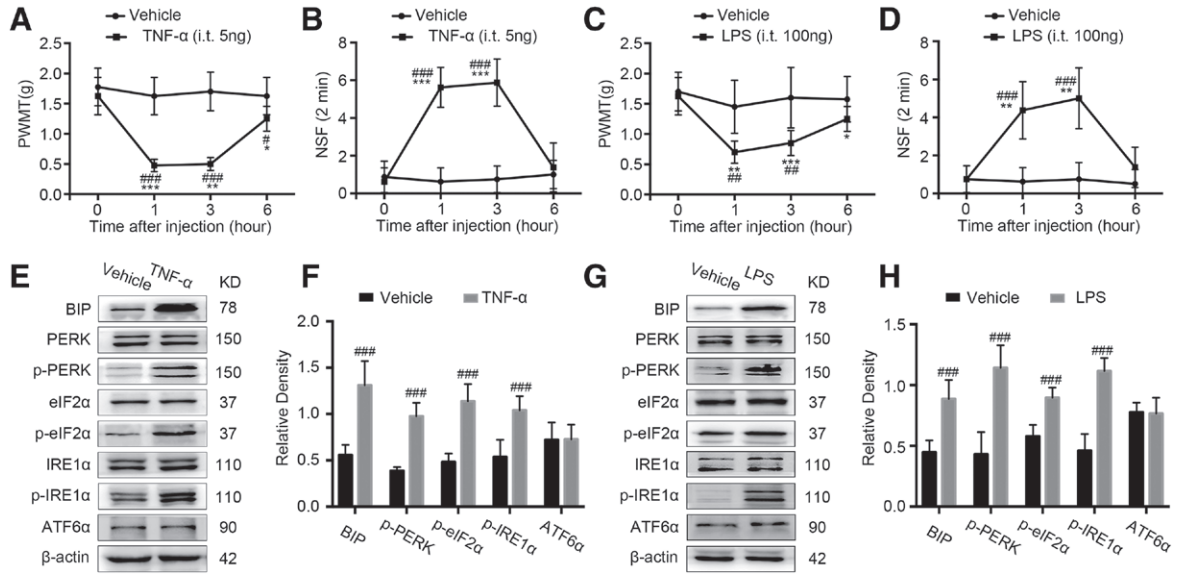

Fig. 5. Intrathecal injection of tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS) increased the expression of endoplasmic reticulum stress markers and induced nociceptive behavior in control mice. Paw withdrawal mechanical threshold (*A* and *C*) and the number of spontaneous flinches (*B* and *D*) were tested before administration (0 h) and at 1, 3, and 6 h after TNF-α, LPS, and vehicle injection. One-way ANOVA with Bonferroni *post hoc* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with 0 h. Two-way repeated-measures ANOVA with Bonferroni *post hoc* test, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the vehicle-treated tumor group mice at each point; n = 8 per group. (*E* and *G*) Increased expression of endoplasmic reticulum stress markers 1 h after TNF-α and LPS injection compared with vehicle injection respectively. (*F* and *H*) Quantification of endoplasmic reticulum stress markers in the spinal cords in different groups, independent *t* test, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with vehicle-treated mice; n = 6 per group. Data are expressed as mean ± SD.

primary neurons resulted in activation of the RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α pathway of endoplasmic reticulum stress, we aimed to determine whether the pathway was involved in bone cancer pain model. For this assessment, we used the endoplasmic reticulum stress inhibitor 4-PBA (a small molecule proposed to facilitate the correct folding of nascent proteins). Tumor-bearing mice were administered 4-PBA by intraperitoneal injection (1 mg/100 μl) and intrathecal injection (40 μg/5 μl, 80 μg/5 μl, 120 μg/5 μl) on day 14 after the operation. The expression of BIP, p-RNA-dependent protein kinase–like endoplasmic reticulum kinase, p-eukaryotic initiation factor 2α, p-inositol-requiring enzyme 1α, and activating transcription factor 6α were decreased by 4-PBA, which represented inhibition of endoplasmic reticulum stress (Supplemental Digital Content, fig. S4, http://links.lww.com/ALN/C138). The findings showed a statistically significant increase in paw withdrawal mechanical threshold and decrease in number of spontaneous flinches in the bone cancer pain plus 4-PBA group (40 μg/5 μl; paw withdrawal mechanical threshold: $P = 0.031$ at 1 h; $P = 0.001$ at 2 h; $P = 0.002$ at 3 h; $P < 0.001$ at 4 h; $P = 0.001$ at 5 h; $P = 0.041$ at 6 h; number of spontaneous flinches: $P < 0.001$ at 1 h; $P < 0.001$ at 2 h; $P < 0.001$ at 3 h; $P < 0.001$ at 4 h; $P = 0.003$ at 5 h; n = 8) and bone cancer pain plus 4-PBA group (80 μg/5 μl; paw withdrawal mechanical threshold: $P = 0.002$ at 1 h; $P = 0.001$ at 2 h; $P = 0.002$ at 3 h; $P = 0.003$ at 4 h; $P = 0.004$ at 5 h; $P = 0.006$ at 6 h; number of spontaneous flinches: $P = 0.001$ at 1 h; $P < 0.001$ at 2 h; $P < 0.001$ at 3 h; $P < 0.001$ at 4 h; $P < 0.001$ at 5 h; n = 8, respectively) that appeared at 1 h after administration and persisted for 6 h, in comparison with the baseline. Meanwhile, these mice showed statistically significant differences in comparison with bone cancer pain plus vehicle mice. However, there was no change in paw withdrawal mechanical threshold and number of spontaneous flinches in the bone cancer pain plus 4-PBA (120 μg/5 μl) mice (fig. 6, A and B). Moreover, the effect of intrathecal analgesia was better than that of intraperitoneal injection (paw withdrawal mechanical threshold in bone cancer pain plus 4-PBA (intraperitoneal 1 mg) group *vs.* bone cancer pain plus 4-PBA (intrathecal 40 μg) group *vs.* bone cancer pain plus 4-PBA (intrathecal 80 μg) group: 0.90 ± 0.283 *vs.* 1.28 ± 0.354 *vs.* 1.65 ± 0.396 at 3 h; number of spontaneous flinches in bone cancer pain plus 4-PBA (intraperitoneal 1 mg) group *vs.* bone cancer pain plus 4-PBA (intrathecal 40 μg) group *vs.* bone cancer pain plus 4-PBA (intrathecal 80 μg) group: 5.00 ± 0.756 *vs.* 3.50 ± 0.926 *vs.* 0.88 ± 0.641 at 3 h; n = 8). Specific descriptive statistics (mean ± SD) of behavior tests were shown in Supplemental Digital Content, table 3 and table 4 (http://links.lww.com/ALN/C138).

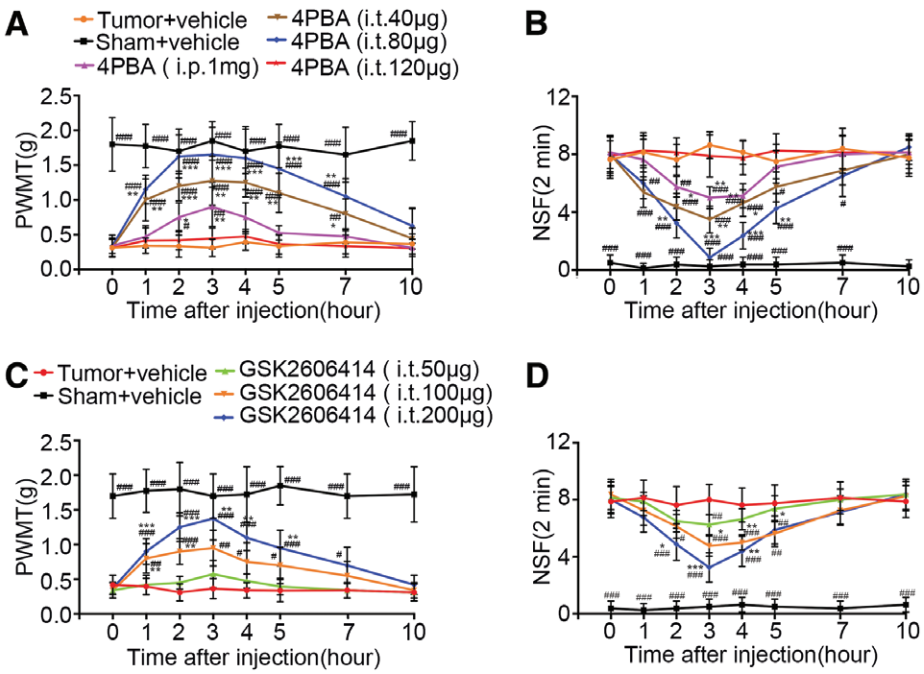

Fig. 6. Inhibition of endoplasmic reticulum stress by 4-PBA and inhibition of RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α by GSK2606414 attenuated nociception in bone cancer pain model. Paw withdrawal mechanical threshold (PWMT; *A* and *C*) and the number of spontaneous flinches (NSF; *B* and *D*) were tested before administration (0 h) and at 1, 2, 3, 4, 5, 7, and 10 h after 4-PBA, GSK2606414, and vehicle administration. One-way ANOVA with Bonferroni *post hoc* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with 0 h. Two-way repeated-measures ANOVA with Bonferroni *post hoc* test, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the vehicle-treated tumor group mice at each point; n = 8 per group. Data are expressed as mean ± SD.

Next, we administered GSK2606414 intrathecally to selectively inhibit the activation of the RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α pathway and further verify the role of the RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α pathway in bone cancer pain model. The expression of p-RNA-dependent protein kinase–like endoplasmic reticulum kinase and p-eukaryotic initiation factor 2α decreased, whereas no change was noted in p-inositol-requiring enzyme 1α and activating transcription factor 6α expression (Supplemental Digital Content, fig. S4, http://links.lww.com/ALN/C138). Similar to the findings obtained with 4-PBA treatment, time- and dose- dependent antinociception occurred after GSK2606414 administration (fig. 6, C and D). The paw withdrawal mechanical threshold and number of spontaneous flinches in the bone cancer pain plus GSK2606414 (100 μg/5 μl; paw withdrawal mechanical threshold: $P = 0.004$ at 1 h; $P = 0.001$ at 2 h; $P = 0.016$ at 3 h; number of spontaneous flinches: $P = 0.01$ at 3 h; 5.00 ± 0.19, $P = 0.004$ at 4 h; $P = 0.048$ at 5 h; n = 8) and bone cancer pain plus GSK2606414 (200 μg/5 μl; paw withdrawal mechanical threshold: $P < 0.001$ at 1 h; $P < 0.001$ at 2 h; $P = 0.004$ at 3 h; $P = 0.002$ at 4 h; $P = 0.004$ at 5 h; number of spontaneous flinches: $P = 0.038$ at 2 h; $P < 0.001$ at 3 h; $P = 0.007$ at 4 h; n = 8) group improved after administration. Specific descriptive statistics (mean ± SD) of behavior tests were shown in Supplemental Digital Content, tables 5 and 6 (http://links.lww.com/ALN/C138).

Intrathecal Administration of 4-PBA or GSK2606414 Decreased the Expression of TNF-α, Interleukin 1β, and Interleukin 6 and Inhibited the Activation of Astrocytes in the Spinal Cord in Bone Cancer Pain Model

Studies have revealed the modulation of inflammatory mediators by endoplasmic reticulum stress. To determine whether endoplasmic reticulum stress could modulate neuroinflammation in mouse bone cancer pain model, we examined the expression of TNF-α, interleukin 1β, and interleukin 6 after injection. Treatment with the endoplasmic reticulum stress inhibitor 4-PBA decreased the expression of TNF-α, interleukin 1β, and interleukin 6 in the spinal cord (fig. 7, A and B) in comparison with bone cancer pain plus vehicle mice (TNF-α and interleukin 1β: $P < 0.001$ for 40-μg and 80-μg treatments; interleukin 6: $P = 0.036$ for 40-μg treatment, $P = 0.012$ for 80-μg treatment, $P < 0.001$ for 120-μg treatment; n = 6). Similar results were found in the GSK2606414-treated mice. The expression levels of TNF-α, interleukin 1β, and interleukin 6 decreased in the mice that received 100-μg and 200-μg

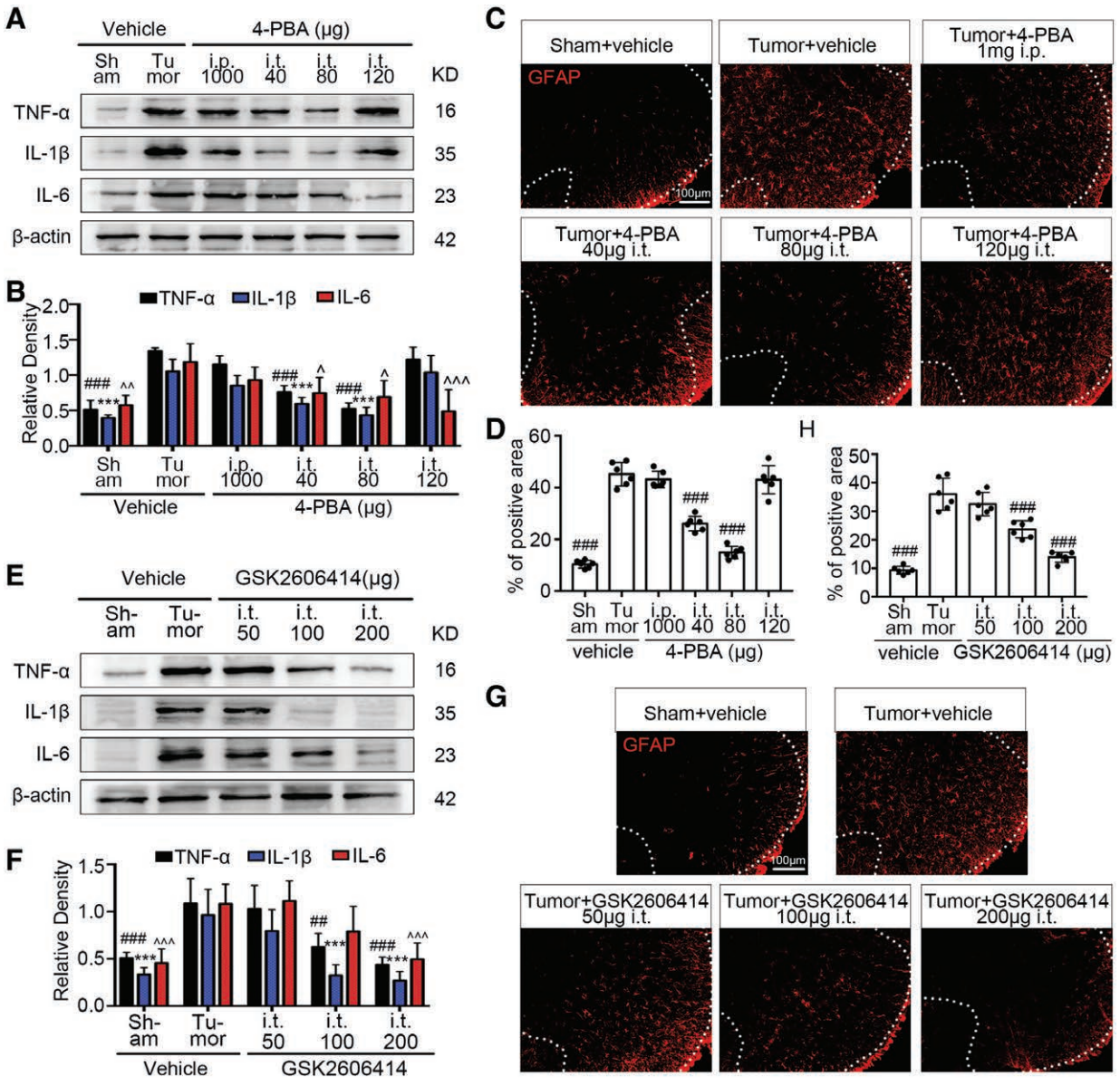

Fig. 7. Inhibition of endoplasmic reticulum stress by treatment with 4-PBA and GSK2606414 downregulated the expression of tumor necrosis factor-α (TNF-α), interleukin (IL) 1β, and IL-6, and inhibited the activation of astrocytes in the spinal cord in tumor group mice. (*A* and *E*) Representative blots of TNF-α, IL-1β, and IL-6 in the spinal cord 3 h after injection in tumor and sham group mice. (*B* and *F*) Quantification of TNF-α, IL-1β, and IL-6 in different groups. One-way ANOVA with Bonferroni *post hoc* test, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the expression of TNF-α in tumor plus vehicle mice; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the expression of IL-1β in tumor plus vehicle mice; $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$ compared with the expression of IL-6 in tumor plus vehicle mice; n = 6 per group. (*C* and *G*) Fluorescent photomicrographs of the astrocyte marker GFAP in the spinal cord 3 h after 4-PBA and GSK2606414 injection, respectively in tumor group mice; scale bar, 100 μm. (*D* and *H*) Quantification of the GFAP-positive area. One-way ANOVA with Bonferroni *post hoc* test, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with tumor plus vehicle mice; n = 6 per group. Data are expressed as mean ± SD.

treatment (TNF-α: $P = 0.002$ for 100-μg treatment, $P < 0.001$ for 200-μg treatment; interleukin 1β: $P < 0.001$ for 100-μg and 200-μg treatments; interleukin 6: $P < 0.001$ for 200-μg treatment; n = 6; fig. 7, E and F).

We also examined the activation of astrocytes and microglia in the spinal cord after treatment. The results showed that both 4-PBA and GSK2606414 could inhibit the activation of astrocytes ($P < 0.001$, n = 6, respectively; fig. 7, C, D, G, and H). There were no differences in the activation of microglia after treatment in comparison with that in the bone cancer pain plus vehicle mice ($P > 0.05$, n = 6, respectively; Supplemental Digital Content, fig. S5; http://

links.lww.com/ALN/C138). These results indicate that the analgesic effect of the inhibition of endoplasmic reticulum stress was modulated by reduced neuroinflammation in the spinal cord.

Discussion

In this study, we found activation of the RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α pathway of endoplasmic reticulum stress in spinal neurons, along with increased levels of inflammatory mediators TNF-α, interleukin 1β, and interleukin 6 in mouse bone cancer pain model. Stimulation of primary neurons with TNF-α (100 nM) and lipopolysaccharide (100 nM) resulted in the upregulation of the RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α pathway. Intrathecal administration of TNF-α (5 ng) and lipopolysaccharide (100 ng), respectively induced hyperalgesia and upregulation of endoplasmic reticulum stress markers in control mice. Inhibition of endoplasmic reticulum stress and the RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α pathway improved the nociceptive behaviors in a time- and dose-dependent manner, along with decreased expression of TNF-α, interleukin 1β, and interleukin 6 and suppressed activation of astrocytes in the spinal cord.

Endoplasmic reticulum stress is caused by various physiologic or pathologic conditions such as disturbance of Ca^{2+} homeostasis or exposure to oxidative stress and during protection of cells against various pathologic stresses. There are three endoplasmic reticulum stress sensors: RNA-dependent protein kinase-like endoplasmic reticulum kinase, inositol-requiring enzyme 1, and activating transcription factor 6, which are bound to BIP to maintain inactivated in normal conditions. When endoplasmic reticulum stress is triggered, these sensors are dissociated and three signaling pathways are activated.[29] Activation of endoplasmic reticulum stress markers and pathways have been reported in various diseases. Autophagy mediated by inositol-requiring enzyme 1–TRAF2-JNK pathway and RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α-C/EBP homologous protein (CHOP) pathway was suggested in the development of cancer.[30,31] Activation of inositol-requiring enzyme 1α–XBP1-JNK pathway and RNA-dependent protein kinase-like endoplasmic reticulum kinase–ATF4-TRB3 pathway was found in metabolic diseases.[32] Additionally, increased expression of BIP, p-RNA-dependent protein kinase–like endoplasmic reticulum kinase, and p-eukaryotic initiation factor 2α was found in various neurodegenerative diseases.[33]

Studies targeting endoplasmic reticulum stress in nociception development have been conducted. In a neuropathic pain model, endoplasmic reticulum stress was promoted in the spinal dorsal horn and mediated spinal sensitization.[34] Moreover, endoplasmic reticulum stress in the dorsal root ganglion contributed to the development of pain hypersensitivity after nerve injury.[35] In inflammatory pain, increased expression of BIP and p-eukaryotic initiation factor 2α was found in trigeminal ganglion.[10] Meanwhile, upregulation of BIP and activating transcription factor 6 was found in the superficial spinal dorsal horn in a formalin-induced rat pain model.[36] In this study, we observed activation of the RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α pathway and inositol-requiring enzyme 1 pathway in spinal neurons during the development of mouse bone cancer pain, but we observed no difference in activating transcription factor 6 expression. The activation of the RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α pathway persisted from days 14 to days 28, whereas the inositol-requiring enzyme 1 pathway was activated only on day 28. Therefore, we assumed that the RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α pathway was responsible for the progressive bone pain, whereas the inositol-requiring enzyme 1 pathway may be responsible for the terminal stage of mouse bone cancer pain.

Accumulating evidence indicates that inflammation plays a major role in nociception development. Activation of glial cells in the central nervous system leads to the release of proinflammatory mediators, which induce central sensitization and contribute to the development of chronic pain.[15] Additionally, excessive inflammation in the peripheral and central nervous system contribute to the initiation and maintenance of neuropathic pain.[37]

The mechanisms underlying endoplasmic reticulum stress and inflammation are interlinked.[38] Inflammation is the inducer of endoplasmic reticulum stress,[39] whereas persistent endoplasmic reticulum stress can activate and aggravate the inflammatory response. The relationship between endoplasmic reticulum stress and inflammation has been revealed in numerous diseases. Inflammation-induced endoplasmic reticulum stress was found in inflammatory bowel disease.[39] The modulation of the NLRP3 inflammasome by inositol-requiring enzyme 1α contributed to the development of Alzheimer disease. In the multiple sclerosis mouse model, endoplasmic reticulum stress-induced activation of the JAK1/STAT3 axis led to the expression of interleukin 6.[40] Moreover, endoplasmic reticulum stress-mediated inflammation in macrophages followed intracerebral hemorrhage.[41] However, few studies have explored the interaction between endoplasmic reticulum stress and inflammation in nociception development.

In this study, primary neuronal cells were stimulated with TNF-α and lipopolysaccharide, respectively to investigate whether endoplasmic reticulum stress could be triggered by inflammatory mediators in neurons. Increased expression of p-RNA-dependent protein kinase–like endoplasmic reticulum kinase and p-eukaryotic initiation factor 2α was found in primary spinal neurons after stimulation of TNF-α and lipopolysaccharide. Studies have revealed that cortical neuron was different from spinal neurons. For example, TNF-α caused

apoptosis in hippocampal neurons but not spinal cord neurons.[42] Meanwhile, TNF-α induced long-term potentiation in spinal cord neurons but long-term depression in hippocampal neurons.[43] In consideration of different mechanism of these neurons TNF-α acted on, we further investigated whether endoplasmic reticulum stress was induced in primary cortical neurons after TNF-α– and lipopolysaccharide- stimulation. Similar results were found as in primary spinal neurons.

Intrathecal injections of TNF-α and lipopolysaccharide were performed to further explore the effects of inflammatory mediators on endoplasmic reticulum stress. The dosages of TNF-α and lipopolysaccharide were used as described by Shen *et al*.[44] and Reeve et al.[45] Intrathecal injection of TNF-α 5 ng or lipopolysaccharide 100 ng induced hyperalgesia in control mice, along with increased expression of endoplasmic reticulum stress markers. These results indicate that endoplasmic reticulum stress was triggered by inflammatory mediators in nociception development. Moreover, decreased expression of TNF-α, interleukin 1β, and interleukin 6 and suppressed activation of astrocytes were noted after inhibition of endoplasmic reticulum stress by 4-PBA and GSK2606414, which indicated the modulation of inflammation by endoplasmic reticulum stress in bone cancer pain model. In summary, these results provide evidence for the close relationship between inflammation and endoplasmic reticulum stress in the development of mouse bone cancer pain.

4-PBA is a small molecule which can facilitate the correct folding of nascent proteins and suppresses endoplasmic reticulum stress. Studies have indicated that 4-PBA can alleviate hypertension,[46] attenuate endothelial inflammation and permeability in acute lung injury,[47] and attenuate neuropathic pain[11] and inflammatory pain[36] by inhibition of endoplasmic reticulum stress. GSK2606414 is the selective inhibitor of RNA-dependent protein kinase-like endoplasmic reticulum kinase–eukaryotic initiation factor 2α. Studies have revealed that GSK2606414 can reduce the levels of p-RNA-dependent protein kinase–like endoplasmic reticulum kinase and p-eukaryotic initiation factor 2α and restore protein synthesis rates.[29]

Studies have indicated the antinociceptive effects of intraperitoneal administration of 4-PBA in neuropathic pain at a dosage of 100 mg/kg,[11] but few studies have focused on the direct analgesic effect on the central nervous system. In this study, we injected 4-PBA intrathecally at three dosages (40 μg/5 μl, 80 μg/5 μl, 120 μg/5 μl) to investigate the dose effect on central nervous system. We also performed intraperitoneal administration to explore the analgesic effect *via* different modes of administration. As shown in the results, in comparison with intraperitoneal administration of 4-PBA, intrathecal administration appeared to show a better analgesic effect and longer analgesic time. Meanwhile, intrathecal administration of 4-PBA showed a time- and dose-dependent manner effect. Nociceptive behaviors were significantly improved at the dosage of 40 μg and 80 μg. However, there was no change in nociceptive behaviors at the dosage of 120 μg, which may have been caused by the complete inhibition of endoplasmic reticulum stress. As an adaptive mechanism, endoplasmic reticulum stress is a double-edged sword.[48] On the one hand, endoplasmic reticulum stress triggered by accumulation of unfolded proteins in the endoplasmic reticulum could mediate autophagy[49] and endoplasmic reticulum associated protein degradation,[50] and then degrade cytoplasmic constituents, damaged organelles, and intracellular pathogens and maintain the intracellular homeostasis. On the other hand, chronic or persistent endoplasmic reticulum stress could induce apoptosis by activating CHOP[51] and the caspase-12[52] pathway and ultimately lead to cell apoptosis. Thus, inhibition of endoplasmic reticulum stress appropriately to eliminate its harmful effects while maintaining the protective effects may be the key to endoplasmic reticulum stress as a therapeutic target.

Studies have indicated that estrogen and estrogen receptor played an important role in bone cancer pain model.[53] The relationship between endoplasmic reticulum stress and estrogen signaling has also been proposed. Endoplasmic reticulum stress could regulate estrogen signaling,[54] whereas estrogen could reduce endoplasmic reticulum stress.[55] To rule out the effect of estrogen, we used male mice in our study.

In conclusion, our results suggest that endoplasmic reticulum stress was activated in the spinal neurons by upregulation of inflammatory mediators in the spinal cord in mouse bone cancer pain model. Downregulation of neuroinflammation *via* the inhibition of endoplasmic reticulum stress attenuated nociception in bone cancer pain model. Our findings might partially explain the mechanism underlying the role of endoplasmic reticulum stress in pain development and indicate a potential analgesic approach targeting endoplasmic reticulum stress.

Research Support

Supported by the National Natural Science Foundation of China (Beijing, China) grant Nos. 81671087, 81471129, 81771142,81870875; National Key Research and Development Program of China (Beijing, China; SQ2018YFC200044), and a grant from Jiangsu Commission of Health (Jiangsu Provincial Key Medical Discipline, Jiangsu, China).

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Ma: Department of Anaesthesiology, Affiliated Drum Tower Hospital of Medical School of Nanjing University, 321 Zhong Shan Road, Nanjing 210008, Jiangsu Province, China. mazhengliang 1964@nju.edu.cn. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's

References

1. Sturge J, Caley MP, Waxman J: Bone metastasis in prostate cancer: Emerging therapeutic strategies. Nat Rev Clin Oncol 2011; 8:357–68
2. Rubens RD: Bone metastases: The clinical problem. Eur J Cancer 1998; 34:210–3
3. Mantyh P: Bone cancer pain: Causes, consequences, and therapeutic opportunities. Pain 2013; 154 Suppl 1:S54–62
4. Portenoy RK: Treatment of cancer pain. Lancet 2011; 377:2236–47
5. Kaneko M, Imaizumi K, Saito A, Kanemoto S, Asada R, Matsuhisa K, Ohtake Y: ER stress and disease: Toward prevention and treatment. Biol Pharm Bull 2017; 40:1337–43
6. Peng T, Liu X, Wang J, Liu Y, Fu Z, Ma X, Li J, Sun G, Ji Y, Lu J, Wan W, Lu H: Fluoxetine-mediated inhibition of endoplasmic reticulum stress is involved in the neuroprotective effects of Parkinson's disease. Aging (Albany NY) 2018; 10:4188–96
7. Hashimoto S, Ishii A, Kamano N, Watamura N, Saito T, Ohshima T, Yokosuka M, Saido TC: Endoplasmic reticulum stress responses in mouse models of Alzheimer's disease: Overexpression paradigm *versus* knockin paradigm. J Biol Chem 2018; 293:3118–25
8. Medinas DB, Rozas P, Martínez Traub F, Woehlbier U, Brown RH, Bosco DA, Hetz C: Endoplasmic reticulum stress leads to accumulation of wild-type SOD1 aggregates associated with sporadic amyotrophic lateral sclerosis. Proc Natl Acad Sci U S A 2018; 115:8209–14
9. Kroeger H, Chiang WC, Felden J, Nguyen A, Lin JH: ER stress and unfolded protein response in ocular health and disease. FEBS J 2019; 286:399–412
10. Yang ES, Bae JY, Kim TH, Kim YS, Suk K, Bae YC: Involvement of endoplasmic reticulum stress response in orofacial inflammatory pain. Exp Neurobiol 2014; 23:372–80
11. Inceoglu B, Bettaieb A, Trindade da Silva CA, Lee KS, Haj FG, Hammock BD: Endoplasmic reticulum stress in the peripheral nervous system is a significant driver of neuropathic pain. Proc Natl Acad Sci U S A 2015; 112:9082–7
12. Zhang E, Yi MH, Shin N, Baek H, Kim S, Kim E, Kwon K, Lee S, Kim HW, Chul Bae Y, Kim Y, Kwon OY, Lee WH, Kim DW: Endoplasmic reticulum stress impairment in the spinal dorsal horn of a neuropathic pain model. Sci Rep 2015; 5:11555
13. Bobinski F, Ferreira TAA, Córdova MM, Dombrowski PA, da Cunha C, Santo CCDE, Poli A, Pires RGW, Martins-Silva C, Sluka KA, Santos ARS: Role of brainstem serotonin in analgesia produced by low-intensity exercise on neuropathic pain after sciatic nerve injury in mice. Pain 2015; 156:2595–606
14. Biglari B, Swing T, Child C, Büchler A, Westhauser F, Bruckner T, Ferbert T, Jürgen Gerner H, Moghaddam A: A pilot study on temporal changes in IL-1β and TNF-α serum levels after spinal cord injury: The serum level of TNF-α in acute SCI patients as a possible marker for neurological remission. Spinal Cord 2015; 53:510–4
15. Ji RR, Nackley A, Huh Y, Terrando N, Maixner W: Neuroinflammation and central sensitization in chronic and widespread pain. Anesthesiology 2018; 129:343–66
16. Lu C, Liu Y, Sun B, Sun Y, Hou B, Zhang Y, Ma Z, Gu X: Intrathecal injection of JWH-015 attenuates bone cancer pain via time-dependent modification of pro-inflammatory cytokines expression and astrocytes activity in spinal cord. Inflammation 2015; 38:1880–90
17. Connor AM, Mahomed N, Gandhi R, Keystone EC, Berger SA: TNFα modulates protein degradation pathways in rheumatoid arthritis synovial fibroblasts. Arthritis Res Ther 2012; 14:R62
18. Park YJ, Yoo SA, Kim WU: Role of endoplasmic reticulum stress in rheumatoid arthritis pathogenesis. J Korean Med Sci 2014; 29:2–11
19. Kim C, Kim B: Anti-cancer natural products and their bioactive compounds inducing ER stress-mediated apoptosis: A review. Nutrients 2018; 10
20. Hotamisligil GS: Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell 2010; 140:900–17
21. Guthrie LN, Abiraman K, Plyler ES, Sprenkle NT, Gibson SA, McFarland BC, Rajbhandari R, Rowse AL, Benveniste EN, Meares GP: Attenuation of PKR-like ER kinase (PERK) signaling selectively controls endoplasmic reticulum stress-induced inflammation without compromising immunological responses. J Biol Chem 2016; 291:15830–40
22. Liu YP, Zeng L, Tian A, Bomkamp A, Rivera D, Gutman D, Barber GN, Olson JK, Smith JA: Endoplasmic reticulum stress regulates the innate immunity critical transcription factor IRF3. J Immunol 2012; 189:4630–9
23. Schwei MJ, Honore P, Rogers SD, Salak-Johnson JL, Finke MP, Ramnaraine ML, Clohisy DR, Mantyh PW: Neurochemical and cellular reorganization of the spinal cord in a murine model of bone cancer pain. J Neurosci 1999; 19:10886–97
24. Hylden JL, Wilcox GL: Intrathecal morphine in mice: A new technique. Eur J Pharmacol 1980; 67:313–6
25. Gu X, Mei F, Liu Y, Zhang R, Zhang J, Ma Z: Intrathecal administration of the cannabinoid 2 receptor agonist JWH015 can attenuate cancer pain and decrease mRNA expression of the 2B subunit

of N-methyl-D-aspartic acid. Anesth Analg 2011; 113:405–11
26. Huo W, Liu Y, Lei Y, Zhang Y, Huang Y, Mao Y, Wang C, Sun Y, Zhang W, Ma Z, Gu X: Imbalanced spinal infiltration of Th17/Treg cells contributes to bone cancer pain via promoting microglial activation. Brain Behav Immun 2019
27. Hu Y, Li G, Zhang Y, Liu N, Zhang P, Pan C, Nie H, Li Q, Tang Z: Upregulated TSG-6 expression in ADSCs inhibits the BV2 microglia-mediated inflammatory response. Biomed Res Int 2018; 2018:7239181
28. Ye J, Guan M, Lu Y, Zhang D, Li C, Zhou C: Arbutin attenuates LPS-induced lung injury via Sirt1/ Nrf2/ NF-κBp65 pathway. Pulm Pharmacol Ther 2019; 54:53–9
29. Almanza A, Carlesso A, Chintha C, Creedican S, Doultsinos D, Leuzzi B, Luís A, McCarthy N, Montibeller L, More S, Papaioannou A, Püschel F, Sassano ML, Skoko J, Agostinis P, de Belleroche J, Eriksson LA, Fulda S, Gorman AM, Healy S, Kozlov A, Muñoz-Pinedo C, Rehm M, Chevet E, Samali A: Endoplasmic reticulum stress signalling: From basic mechanisms to clinical applications. FEBS J 2019; 286:241–78
30. Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, Murakami T, Taniguchi M, Tanii I, Yoshinaga K, Shiosaka S, Hammarback JA, Urano F, Imaizumi K: Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol Cell Biol 2006; 26:9220–31
31. Kouroku Y, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H, Ogawa S, Kaufman RJ, Kominami E, Momoi T: ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell Death Differ 2007; 14:230–9
32. Ozcan L, Cristina de Souza J, Harari AA, Backs J, Olson EN, Tabas I: Activation of calcium/calmodulin-dependent protein kinase II in obesity mediates suppression of hepatic insulin signaling. Cell Metab 2013; 18:803–15
33. Cabral-Miranda F, Hetz C: ER stress and neurodegenerative disease: A cause or effect relationship? Curr Top Microbiol Immunol 2018; 414:131–57
34. Ge Y, Jiao Y, Li P, Xiang Z, Li Z, Wang L, Li W, Gao H, Shao J, Wen D, Yu W: Coregulation of endoplasmic reticulum stress and oxidative stress in neuropathic pain and disinhibition of the spinal nociceptive circuitry. Pain 2018; 159:894–906
35. Yamaguchi Y, Oh-Hashi K, Matsuoka Y, Takemura H, Yamakita S, Matsuda M, Sawa T, Amaya F: Endoplasmic reticulum stress in the dorsal root ganglion contributes to the development of pain hypersensitivity after nerve injury. Neuroscience 2018; 394:288–99
36. Zhou F, Zhang W, Zhou J, Li M, Zhong F, Zhang Y, Liu Y, Wang Y: Involvement of endoplasmic reticulum stress in formalin-induced pain is attenuated by 4-phenylbutyric acid. J Pain Res 2017; 10:653–62
37. Ellis A, Bennett DL: Neuroinflammation and the generation of neuropathic pain. Br J Anaesth 2013; 111:26–37
38. Rahmati M, Moosavi MA, McDermott MF: ER stress: A therapeutic target in rheumatoid arthritis? Trends Pharmacol Sci 2018; 39:610–23
39. Chotikatum S, Naim HY, El-Najjar N: Inflammation induced ER stress affects absorptive intestinal epithelial cells function and integrity. Int Immunopharmacol 2018; 55:336–44
40. Meares GP, Liu Y, Rajbhandari R, Qin H, Nozell SE, Mobley JA, Corbett JA, Benveniste EN: PERK-dependent activation of JAK1 and STAT3 contributes to endoplasmic reticulum stress-induced inflammation. Mol Cell Biol 2014; 34:3911–25
41. Yang Z, Liu Q, Shi H, Jiang X, Wang S, Lu Y, Zhang J, Huang X, Yu A: Interleukin 17A exacerbates ER-stress-mediated inflammation of macrophages following ICH. Mol Immunol 2018; 101:38–45
42. Liu Y, Zhou LJ, Wang J, Li D, Ren WJ, Peng J, Wei X, Xu T, Xin WJ, Pang RP, Li YY, Qin ZH, Murugan M, Mattson MP, Wu LJ, Liu XG: TNF-α differentially regulates synaptic plasticity in the hippocampus and spinal cord by microglia-dependent mechanisms after peripheral nerve injury. J Neurosci 2017; 37:871–81
43. Park CK, Lü N, Xu ZZ, Liu T, Serhan CN, Ji RR: Resolving TRPV1- and TNF-α-mediated spinal cord synaptic plasticity and inflammatory pain with neuroprotectin D1. J Neurosci 2011; 31:15072–85
44. Shen A, Zhou D, Shen Q, Liu HO, Sun L, Liu Y, Chen J, Yang J, Ji Y, Cheng C: The expression of tumor necrosis factor-alpha (TNF-alpha) by the intrathecal injection of lipopolysaccharide in the rat spinal cord. Neurochem Res 2009; 34:333–41
45. Reeve AJ, Patel S, Fox A, Walker K, Urban L: Intrathecally administered endotoxin or cytokines produce allodynia, hyperalgesia and changes in spinal cord neuronal responses to nociceptive stimuli in the rat. Eur J Pain 2000; 4:247–57
46. Naiel S, Carlisle RE, Lu C, Tat V, Dickhout JG: Endoplasmic reticulum stress inhibition blunts the development of essential hypertension in the spontaneously hypertensive rat. Am J Physiol Heart Circ Physiol 2019; 316:H1214–23
47. Leonard A, Grose V, Paton AW, Paton JC, Yule DI, Rahman A, Fazal F: Selective inactivation of intracellular BiP/GRP78 attenuates endothelial inflammation and permeability in acute lung injury. Sci Rep 2019; 9:2096
48. Jheng JR, Chen YS, Ao UI, Chan DC, Huang JW, Hung KY, Tarng DC, Chiang CK: The double-edged sword of endoplasmic reticulum stress in uremic sarcopenia through myogenesis perturbation. J Cachexia Sarcopenia Muscle 2018; 9:570–84

49. Bao Y, Pu Y, Yu X, Gregory BD, Srivastava R, Howell SH, Bassham DC: IRE1B degrades RNAs encoding proteins that interfere with the induction of autophagy by ER stress in Arabidopsis thaliana. Autophagy 2018; 14:1562–73
50. Van Hoewyk D: Defects in endoplasmic reticulum-associated degradation (ERAD) increase selenate sensitivity in Arabidopsis. Plant Signal Behav 2018; 13:e1171451
51. Tang J, Ge Y, Yang L, Xu X, Sui T, Ge D, Que J, Cao X: ER stress via CHOP pathway is involved in FK506-induced apoptosis in rat fibroblasts. Cell Physiol Biochem 2016; 39:1965–76
52. Datta D, Khatri P, Singh A, Saha DR, Verma G, Raman R, Mazumder S: Mycobacterium fortuitum-induced ER-Mitochondrial calcium dynamics promotes calpain/caspase-12/caspase-9 mediated apoptosis in fish macrophages. Cell Death Discov 2018; 4:30
53. Ono H, Nakamura A, Kanemasa T, Sakaguchi G, Shinohara S: Effect of estrogen on morphine- and oxycodone-induced antinociception in a female femur bone cancer pain model. Eur J Pharmacol 2016; 773:1–12
54. Raina K, Noblin DJ, Serebrenik YV, Adams A, Zhao C, Crews CM: Targeted protein destabilization reveals an estrogen-mediated ER stress response. Nat Chem Biol 2014; 10:957–62
55. Kooptiwut S, Mahawong P, Hanchang W, Semprasert N, Kaewin S, Limjindaporn T, Yenchitsomanus PT: Estrogen reduces endoplasmic reticulum stress to protect against glucotoxicity induced-pancreatic β-cell death. J Steroid Biochem Mol Biol 2014; 139:25–32