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ORIGINAL RESEARCH

Involvement of endoplasmic reticulum stress in formalin-induced pain is attenuated by 4-phenylbutyric acid

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Background: Endoplasmic reticulum (ER) stress is involved in many neurological and inflammatory responses. Peripheral inflammatory responses can induce central sensitization and trigger inflammatory pain. However, there is little research on the relationship between ER stress and inflammatory pain. In this study, we examined whether the ER stress response is involved in peripheral inflammatory pain using a formalin-induced rat pain model.

Methods: Rats were divided into the following five groups: control, formalin, formalin + vehicle, formalin + 4-phenylbutyric acid (4-PBA) (40 mg/kg) and formalin + 4-PBA (100 mg/kg). Formalin-induced pain was assessed behaviorally by recording licking activity. The expression levels of immunoglobulin-binding protein (BIP), activating transcription factor-6 (ATF6), phosphorylated inositol-requiring enzyme-1 (p-IRE1), phosphorylated protein kinase RNA-like ER kinase (p-PERK) and c-fos were quantitatively assessed by Western blot, and the distribution of BIP, ATF6 and c-fos in the lumbar enlargement of spinal cord were identified by immunohistochemistry in spinal dorsal horn slices. In addition, the concentrations of nitric oxide (NO) and prostaglandin E2 (PGE2) in the spinal cord were tested by biochemical measurement and enzyme-linked immunosorbent assay (ELISA), respectively.

Results: Intraperitoneal injection of 4-PBA at the dose of 100 mg/kg before formalin injection significantly decreased nociceptive behavior in the second phase compared with control, formalin, formalin + vehicle and formalin + 4-PBA (40 mg/kg) ($P<0.05$). Western blot showed that formalin injection significantly upregulated the expression of BIP, ATF6, p-PERK and c-fos in the spinal cord. This upregulation was reduced by peritoneal injection of 4-PBA ($P<0.05$), while expression of p-IRE1 was not altered by formalin treatment. Immunohistochemistry revealed markedly increased staining density for BIP, ATF6 and c-fos in the superficial spinal dorsal horn after formalin injection. This was significantly decreased by administration of 4-PBA ($P<0.05$). Compared with the formalin + vehicle group, 4-PBA inhibited the release of NO and PGE2 in the spinal cord ($P<0.05$).

Conclusion: These results suggest that ER stress is involved in formalin-induced inflammatory pain and that inhibition of ER stress may attenuate central sensitization induced by peripheral inflammatory stimulation.

Keywords: endoplasmic reticulum stress, formalin-induced pain, 4-phenylbutyric acid, central sensitization

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Introduction

Endoplasmic reticulum (ER) stress is triggered by the accumulation of unfolded or misfolded proteins, alterations in calcium homeostasis, exposure to free radicals or glucose deprivation, all of which can disturb the correct functions of the ER. Such ER stress initiates an evolutionarily conserved signaling cascade called the unfolded

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protein response (UPR), which is a self-protective signaling pathway.[1,2] This pathway includes three main proteins that act as signal transducers, activating transcription factor-6 (ATF6), protein kinase RNA-like ER kinase (PERK), and inositol-requiring protein-1 (IRE1), and an important ER chaperone known as immunoglobulin-binding protein/glucose regulated protein 78 (BIP/GRP78).[3]

The chemical chaperone 4-phenylbutyric acid (4-PBA) is approved by the US Food and Drug Administration (FDA) for use in humans.[4] Chemical chaperones are small molecules that have the ability to stabilize mutant proteins and facilitate their folding.[5] Both in vitro and in vivo, administration of 4-PBA alleviates ER stress, blocks neuropathic pain,[6–8] protects against cerebral ischemic injury[9] and prevents cardiac fibrosis.[10] Moreover, 4-PBA acts against ER stress-induced autophagy in gingival fibroblasts.[11] Such findings show that 4-PBA can attenuate ER stress and consequently alleviate ER stress-related pathophysiology and disease.

Injection of formalin into the hind paw in rodents is a widely used model of inflammatory pain that shows a biphasic pain response.[12] The first phase is mainly caused by peripheral noxious stimulation, and the second phase depends primarily on central sensitization in the spinal dorsal horn.[13] Previous studies have shown that formalin-induced tissue injury in the spinal cord increases calcium influx following *N*-methyl-D-aspartate (NMDA) receptor activation, contributing to the central sensitization that is characteristic of the second phase.[14] Ca^{2+}-influx also initiates an enzymatic cascade that finally triggers the release of nitric oxide (NO), prostaglandins (PGs) and the induction of c-fos expression.[15,16] NO and PGs have been proven to further increase the sensitivity of dorsal horn neurons and finally central sensitization,[17] and the early gene *c-fos* has been demonstrated to be a suitable indicator for the presence of nociceptive neurons activated by formalin injection.[18] ER stress has been shown to be involved in a range of diseases, such as neurodegenerative disorders, including Parkinson's disease,[19] Alzheimer's disease[2] and neuropathic and inflammatory pain.[7,20,21] However, there is little evidence to show whether and how ER stress is involved in formalin-induced pain and central sensitization. In addition, in vivo and in vitro studies have demonstrated that NMDA receptor activation can induce ER stress by activating transducing proteins.[22] Indeed, NMDA receptor antagonist, such as *S*-methyl-*N*,*N*-diethyldithiocarbamate sulfoxide (DETC-MeSO), exerts potent neuroprotective effects by attenuating ischemia-induced UPR signaling.[23] In light of these findings, we hypothesized that the ER stress response might be involved in formalin-induced pain and play a critical role in central sensitization caused by the activation of NMDA receptors in the spinal dorsal horn. We further predicted that 4-PBA would be effective at inhibiting ER stress and consequently attenuating formalin-induced pain.

Materials and methods

Ethics statement

This study and the experiments were approved by the Animal Care and Use Committee of Central South University, and animal handling procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Reagents and animals

4-PBA and polyethylene glycol 400 (PEG 400) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies (Ab) against BIP (78 kDa), ATF6 (90 kDa), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary Ab were purchased from Proteintech, Wuhan, China. Phosphorylated PERK (p-PERK; Thr 981, 125 kDa) Ab was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Phosphorylated IRE-1 (p-IRE1; phosphor S724, 110 kDa) Ab was purchased from Abcam, Cambridge, UK. c-fos (62 kDa) Ab was purchased from Bioworld Technology, Inc, Minneapolis, MN, USA. Dimethylsulfoxide (DMSO) was purchased from Amresco, Solon, OH, USA.

A total of 80 male Sprague Dawley rats (weighing 200–250 g) were purchased from Central South University Animal Service (Changsha, China). All rats were housed in a quiet, specialized animal room, with a constant temperature (25°C) and humidity on a 12:12 hour light/dark cycle, with free access to food and water.

Formalin test

Formalin test experiments were performed 1 week after acclimation in the animal room. Rats were placed in a Plexiglas box for ~30 minutes for acclimatization. After 30 minutes, rats received 50 μL of 5% formalin, injected subcutaneously (sc) into the plantar surface of the right hind paw.

Rats were administered 4-PBA at doses of 100 mg/kg and 40 mg/kg (dissolved in a 5% DMSO, 20% PEG 400 solution at a concentration of 10 mg/mL) by intraperitoneal injection, 30 minutes before formalin injection. Control group animals were injected in the hind paw with 50 μL of 0.9% saline, while the formalin + vehicle group received an intraperitoneal injection of 10 mL/kg vehicle (5% DMSO, 20% PEG 400) only. Rats were sacrificed an hour after formalin injection for subsequent histological and biochemical assessments.

Behavioral assessment

Formalin-induced pain is typically characterized by two phases of nociceptive behaviors: 1) an initial acute phase (the first phase, the first 5 minutes after the formalin injection) was followed by a relative short quiescent period, which lasts ~5 minutes. The first phase is mainly caused by peripheral noxious stimulation; 2) the second phase, which begins ~10 minutes after formalin injection and lasts for ~50 minutes. The main mechanism of the second phase is enhanced neuronal responsiveness, which is known as central sensitization.

Behaviors were recorded every 5 minutes following formalin injection for a total duration of ~1 hour and scored according to the pain rating scale of Abbott et al.[13] The durations of licking and biting behaviors for the injected hind paw were recorded every 5 minutes after injection, up to ~1 hour post-injection. The first phase and the second (tonic) phase were recorded between 0–10 minutes and 10–60 minutes post-injection, respectively.

Western blotting analysis

After anesthetization with 10% chloral hydrate (3 mL/kg), the L4–L6 section of the spinal cord was dissected from the rats. Tissue from rats in each group was homogenized in radioimmunoprecipitation assay (RIPA) buffer (Beyotime Technology, Shanghai, China) with 1% protease inhibitor cocktail (ethylene diamine tetraacetic acid-free; Roche, Basel, Switzerland) and phosphatase inhibitor cocktail (PhosSTOP; Roche). Homogenates were left to stand for 30 minutes and then centrifuged at 12,000 rpm for 15 minutes at 4°C. Afterward, the supernatants were collected and the protein concentration was measured with the bicinchoninic acid (BCA) protein assay kit (Beyotime Technology). A total of 20–40 μg protein from each sample was loaded and separated on either a 12% or 8% Bis-Tris sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (Beyotime Technology). The separated proteins were then transferred to a 0.45 μm polyvinylidene fluoride (PVDF) membrane (Millipore, Boston, MA, USA). Each membrane was blocked with 5% defatted milk for 2 hours at room temperature and subsequently incubated in the presence of primary Ab overnight at 4°C (dilution factors: 1:2000 for GAPDH and BIP; 1:200 for p-PERK; 1:300 for ATF6; 1:1000 for p-IRE1 and 1:500 for c-fos). After washing in Tris-buffered saline with 0.1% Tween (TBST), the membrane was incubated for 120 minutes at room temperature with HRP-conjugated goat anti-mouse or goat anti-rabbit secondary Ab (diluted 1:5000). After washing again with TBST, membranes were rinsed and Ab-reactive bands were visualized using a chemiluminescent HRP substrate (ECL; Millipore). Western blotting bands were analyzed according to their mean gray value using the BandScan 4.0 system (Glyko, Toronto, Canada), with GAPDH used as the control protein.

Immunohistochemistry

Rats in different groups were anesthetized using a high dose of 10% chloral hydrate (3 mL/kg) ~1 hour after formalin injection. Immediately after this, they were transcardially perfused with heparinized 0.9% saline, followed by perfusion with 4% paraformaldehyde. The lumbosacral enlargement (L4–L6) of the spinal cord was removed immediately and then post-fixed with 4% paraformaldehyde overnight at 4°C, before being embedded in paraffin.

Paraffin-embedded lumbar enlargements of the spinal cord were cut into slices that were 4 μm thick. Slices were put in a 60°C oven for 30 minutes and washed three times in dimethylbenzene for 10 minutes each, before being rehydrated using the following alcohol gradient wash sequence: 3 × 100% ethanol, 95% ethanol, 75% ethanol, 50% ethanol, for 5 minutes each. After rinsing with 0.01 M phosphate–buffered saline (PBS) (pH 7.4) three times (5 minutes each), tissue antigens were retrieved using a citrate buffer (CW Biotech, Beijing, China) in a water bath heated to 98°C for 18 minutes. For immunohistochemical analysis, endogenous peroxidases were removed from the slices by immersing them in 3% H_2O_2 at room temperature for 30 minutes and then incubated with primary Ab for BIP (1:100), ATF6 (1:20) and c-fos (1:100) at 4°C overnight. Next, slices were incubated with goat anti-rabbit secondary antibody immunoglobulin (ZSGB-BIO, Beijing, China). Diaminobenzidine tetrahydrochloride (DAB; ZSGB-BIO) was used for visualization. Finally, all slices were cover slipped for visualization by optical microscopy.

Enzyme-linked immunosorbent assay (ELISA) and NO assay

Inflammatory cytokine (prostaglandin E2 [PGE2]) expression in the spinal cord was tested using ELISA kits (Elabscience, Wuhan, China). The protocol was carried out following the manufacturer's instructions. Optical density (OD) was detected at 450 nm (A450), and the standard curve was calibrated according to Protein A standard. The concentration of PGE2 in the samples was then determined by comparing the OD of the samples to the standard curve. The concentration of NO was detected using the NO assay kit (Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions. The absorbance was measured at 550 nm.

Statistical analysis

Data from the behavioral tests, immunohistochemistry and Western blotting are shown as mean ± standard error. GraphPad Prism 6 (GraphPad Software, Inc, San Diego, CA, USA) and IPP 6.0 (Media Cybernetics, Rockville, MD, USA) were used for statistical analyses. A two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post-test was used to analyze the behavioral data. A one-way ANOVA followed by Tukey's multiple comparison post-test was used for comparisons of mean values. Differences were considered statistically significant at $P<0.05$.

Results

Pain behavior in the second, but not first, phase of formalin-induced pain is attenuated by 4-PBA

Formalin intra-plantar injection usually induces two distinct periods of high licking activity: the first phase, which begins immediately after the injection of formalin and lasts for 10 minutes, and the second phase, which begins after 10 minutes and lasts for 50 minutes after injection.[12] To determine the level of acute inflammatory pain following unilateral formalin intra-plantar injection, we measured the duration of licking time every 5 minutes for 1 hour and the total licking time in each of the two phases, between 0–10 minutes and 10–60 minutes.

We administered 4-PBA 30 minutes before injection of 50 μL of 5% formalin. Pretreatment with 4-PBA (100 mg/kg) before formalin injection significantly decreased nociceptive behavior in the second phase (Figure 1A and C), compared with other treatment groups: control, formalin, formalin + vehicle and formalin + 4-PBA (40 mg/kg). It is worth noting that 4-PBA, both at the dose of 100 mg/kg and 40 mg/kg, had no effect on pain behavior during the first phase of the formalin test (Figure 1A and B).

ER stress is involved in the central sensitization response of the formalin test and is reduced by 4-PBA administration

It is widely accepted that the mechanism underlying the second phase of the formalin test is central sensitization of the spinal cord. To better understand this process, we performed Western blot analyses to investigate whether ER stress-mediated pathways are activated in the ipsilateral side of the lumbar enlargement of the spinal cord. The levels of the ER stress chaperone protein BIP and signaling pathway proteins ATF6 and p-PERK were increased in the formalin group compared to the saline control group (Figure 2A, B, C and E). Treatment with 4-PBA alongside formalin significantly decreased the expression of BIP, ATF6 and p-PERK, relative to the formalin or formalin + vehicle groups; however, the expression of p-IRE1 showed no difference in the four groups (Figure 2A, B, C, E and F). In combination with the behavioral results showing that 4-PBA attenuates the second phase of nociceptive behavior (Figure 1A and C), these results suggest that ER stress is involved in the central sensitization response of the formalin test.

Activation of the NMDA receptor plays a key role in central sensitization, leading to calcium influx followed by a series of biochemical reactions including transcription of the *c-fos* gene and the release of NO and PGE2.[14,24] To investigate this, we measured the expression of c-fos protein in the ipsilateral lumbar enlargement of the spinal cord using Western blotting. The results showed that the level of c-fos protein was significantly increased after formalin injection compared with the saline control group. In contrast, 4-PBA administration before formalin injection reduced the level of c-fos compared with the formalin and formalin + vehicle groups (Figure 2A and D). These results suggest that the modulation of c-fos expression by the ER stress response is one mechanism influencing central sensitization of the spinal cord.

Expression of BIP, ATF6 and c-fos in the spinal dorsal horn is increased in the formalin test

Central sensitization is triggered by inputs from nociceptive afferents and characterized by a reduced threshold of dorsal horn neurons to noxious stimulation.[14,25] Therefore, we examined the expression of BIP, ATF6 and c-fos in the spinal dorsal horn, 1 hour after formalin injection, using immunohistochemical analysis. Formalin injection induced a marked upregulation of BIP, ATF6 and c-fos in the ipsilateral superficial lumbar dorsal horn, whereas only low staining density for BIP, ATF6 and c-fos could be detected in the saline control group (Figure 3A1–2, B1–2, C1–2). Administration of 4-PBA before formalin injection decreased the expression of BIP, ATF6 and c-fos in the superficial lumbar dorsal horn (Figure 3A3–4, B3–4, C3–4 and D–F). These results suggest that ER stress markers are elevated in the ipsilateral spinal dorsal horn during formalin-induced pain and further support the conclusion that the ER stress response is involved in central sensitization.

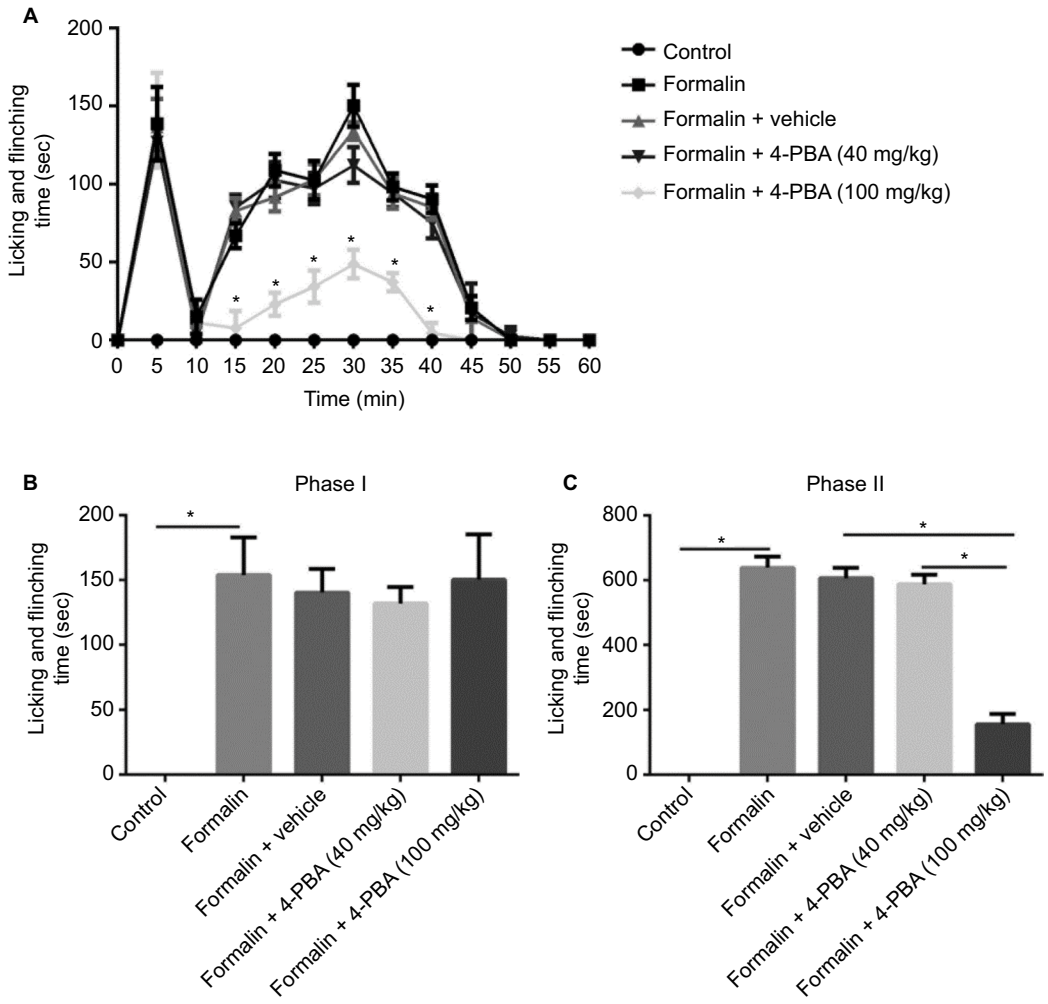

Figure 1 Pretreatment of 4-PBA (100 mg/kg) attenuates formalin-induced pain.

Notes: Intraperitoneal pretreatment with the ER stress inhibitor, 4-PBA (100 mg/kg), attenuates the second phase of nociceptive responses induced by 5% formalin intraplantar injection, but 4-PBA (40 mg/kg) shows no effect on nociceptive behaviors. (**A**) Time course of biphasic nociceptive responses (*P<0.05 versus control, formalin, formalin + vehicle and formalin + 4-PBA (40 mg/kg); two-way ANOVA with Dunnett's multiple comparisons test; n = 5 per group). (**B**) Both doses of 4-PBA (40 mg/kg and 100 mg/kg) have no remission effect on the first phase of formalin-induced nociceptive behaviors (*P<0.05 versus formalin, one-way ANOVA with Tukey's multiple comparison test; n = 5 per group). (**C**) Intraperitoneal pretreatment with 4-PBA at 100 mg/kg but not at 40 mg/kg significantly reduces tonic pain in the formalin test (*P<0.05, one-way ANOVA with Tukey's multiple comparison test; n = 5 per group).

Abbreviations: 4-PBA, 4-phenylbutyric acid; ER, endoplasmic reticulum; ANOVA, analysis of variance; sec, seconds; min, minutes.

4-PBA attenuates the release of NO and PGE2 in the ipsilateral spinal cord after formalin injection

NO and PGs are thought to be key mediators in the induction and maintenance of nociceptive transmission and central sensitization in the formalin test.[14] To examine this, we measured the concentration of NO and PGE2 in the ipsilateral spinal cord using biochemical measurement and ELISA, respectively. We found that the release of NO and PGE2 both increased after formalin injection, while 4-PBA (100 mg/kg) treatment before formalin injection decreased the level of both NO and PGE2 compared to the formalin and formalin + vehicle groups (Figure 4). Meanwhile, the formalin and formalin + vehicle groups did not differ from one another. These results suggest that 4-PBA reduces the release of NO and PGE2, corroborating the notion that inhibition of the ER stress response attenuates central sensitization.

Discussion

To explore the role of ER stress in the development of formalin-induced pain, we inhibited the ER stress response by intraperitoneal injection of 4-PBA before formalin injection. We found that formalin injection significantly elevated the expression level of the ER stress markers BIP, ATF6 and p-PERK in the ipsilateral lumbar enlargement of the spinal cord, while the expression level of p-IRE1 showed

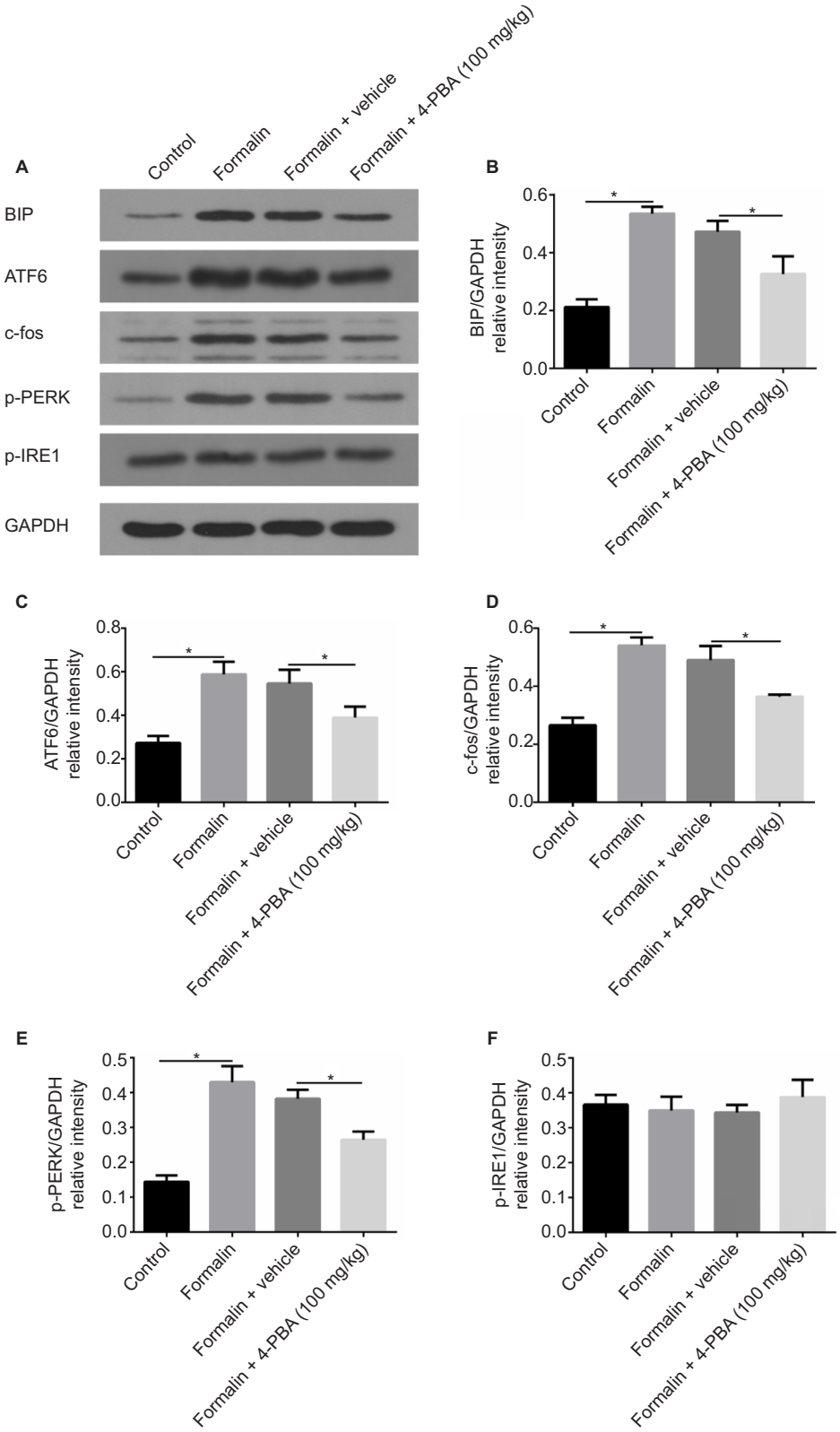

Figure 2 After formalin injection, 4-PBA blocks prominent signs of elevated ER stress in the spinal cord and pain-related behaviors and suppresses markers of ER stress and c-fos levels.

Notes: Markers of ER stress (BIP, ATF6 and p-PERK) and central sensitization-related protein c-fos from the lumbar enlargement of the spinal cord were measured by Western blotting. (**A**–**E**) The levels of BIP, ATF6, p-PERK and c-fos increase in the ipsilateral lumbar enlargement of the spinal cord in the formalin group compared to the control group, 1 hour after injection of 50 µL of 5% formalin. Intraperitoneal injection of 4-PBA at 30 minutes before formalin injection inhibits the expression of BIP, ATF6, p-PERK and c-fos in the formalin + 4-PBA (100 mg/kg) group compared with the formalin + vehicle group. (**A** and **F**) The expression level of p-IRE1 showed no change between the four groups. GAPDH was used as a loading control. Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparisons test; n = 5 per group; *$P<0.05$.

Abbreviations: 4-PBA, 4-phenylbutyric acid; ER, endoplasmic reticulum; BIP, immunoglobulin-binding protein; ATF6, activating transcription factor-6; p-PERK, phosphorylated protein kinase RNA-like ER kinase; p-IRE1, phosphorylated inositol-requiring enzyme-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance.

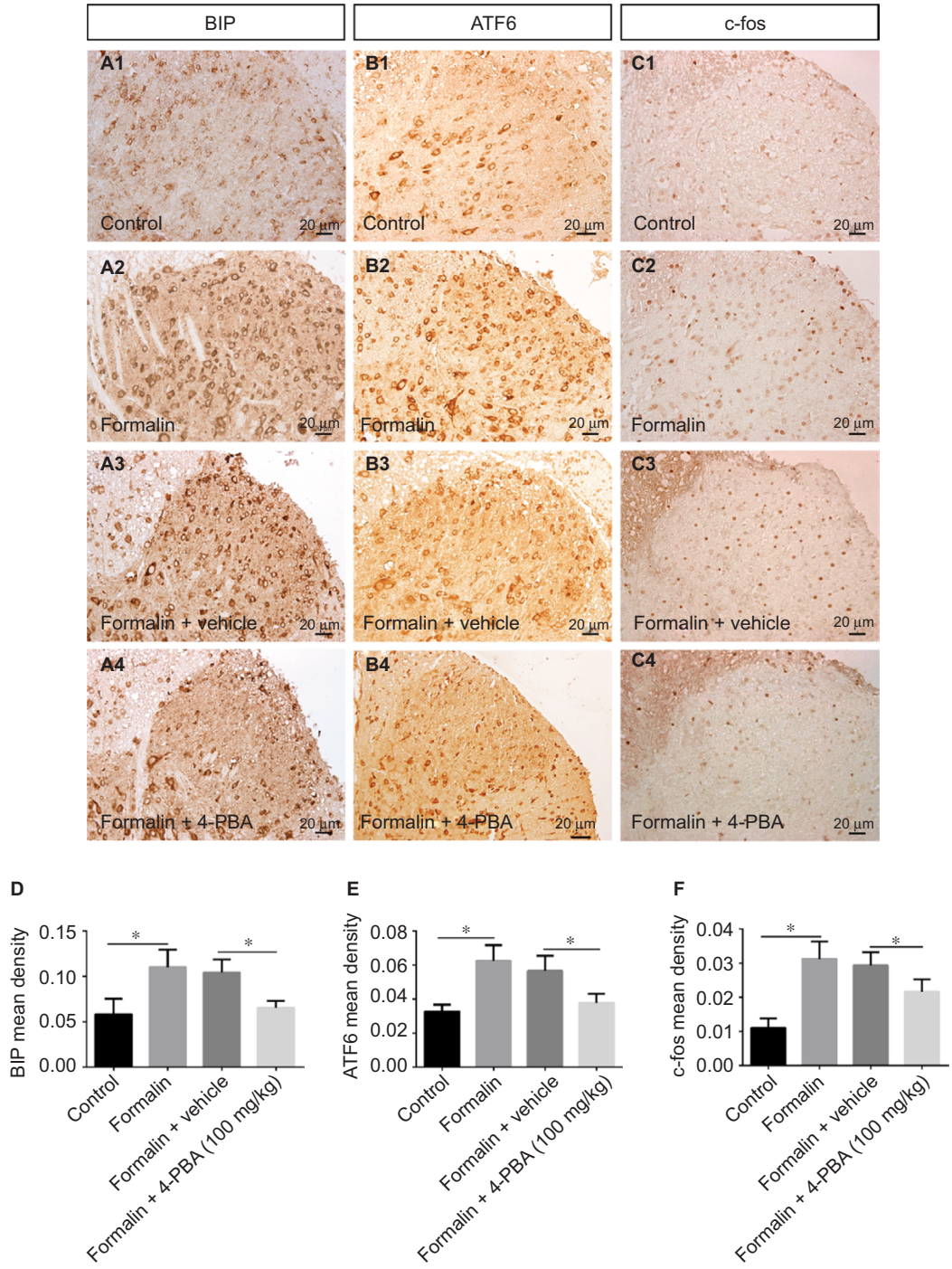

Figure 3 4-PBA attenuates the expression of BIP, ATF6 and c-fos in the rat spinal dorsal horn 1 hour after formalin injection.
Notes: (**A**–**C**) Weak staining for (**A**1) BIP, (**B**1) ATF6 and (**C**1) c-fos can be observed in the dorsal horn of the spinal cord in the control group, especially in the superficial dorsal horn. (**A**–**F**) A 50 μL of 5% formalin hind paw injection induces (**A**2) BIP, (**B**2) ATF6 and (**C**2) c-fos upregulation in the ipsilateral lumbar spinal dorsal horn, compared to the control group. In addition, injection of 4-PBA (100 mg/kg) reduces the expression of (**A**4) BIP, (**B**4) ATF6 and (**C**4) c-fos in the spinal dorsal horn compared with formalin + vehicle group (**A**3, **B**3 and **C**3). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparisons test; n = 5 per group; *$P<0.05$.
Abbreviations: 4-PBA, 4-phenylbutyric acid; BIP, immunoglobulin-binding protein; ATF6, activating transcription factor-6; ANOVA, analysis of variance.

no difference, suggesting that the signaling pathway for IRE1 is not activated in formalin-induced pain. This could be explained by the role of IRE1 signaling in cell apoptosis and death[3] but not in formalin-induced pain. Furthermore, the ER stress inhibitor 4-PBA greatly decreased the levels of BIP, ATF6 and p-PERK (Figure 2). However, 4-PBA had no effect on pain behaviors during the first phase of the formalin test (Figure 1). A possible reason may be that the first phase occurs as a result of direct injury to the nerve endings by formalin.[26] Additionally, accurate behavioral observations were difficult during this phase, since it lasts only 5–10 minutes. Meanwhile, 4-PBA treatment before

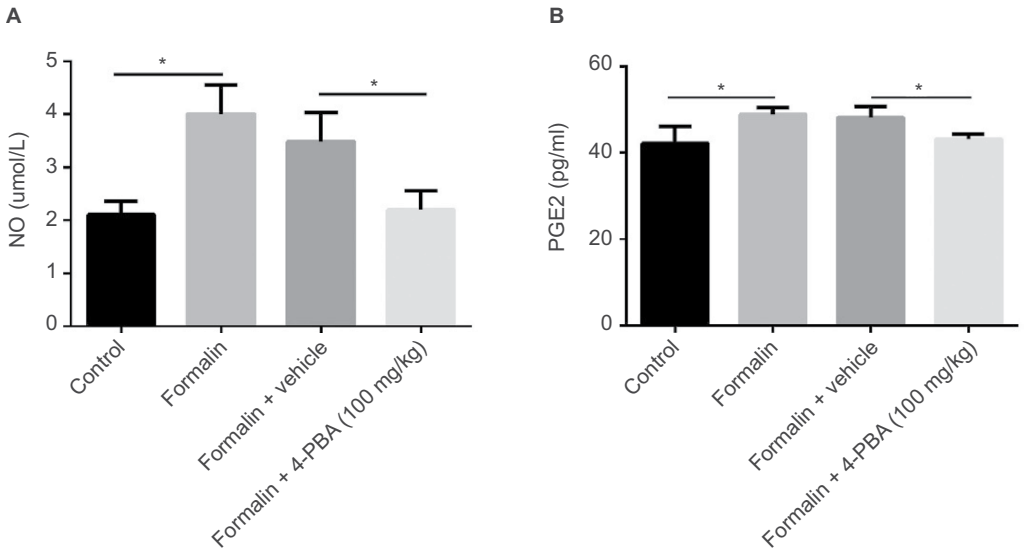

Figure 4 Administration of 4-PBA reduces the release of NO and PGE2 in the ipsilateral spinal cord.
Notes: Administration of 4-PBA (100 mg/kg) attenuates the release of (**A**) NO and (**B**) PGE2 in the formalin + 4-PBA (100 mg/kg) group compared with the formalin + vehicle group. Concentrations of NO and PGE2 were measured by biochemical measurement and ELISA assays. Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparisons test; n = 5 per group; *$P<0.05$.
Abbreviations: 4-PBA, 4-phenylbutyric acid; NO, nitric oxide; PGE2, prostaglandin E2; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance.

formalin injection significantly reduced nociceptive pain behaviors in the second (tonic) phase of the pain response (Figure 1) and decreased the expression of BIP and ATF6 in the lumbosacral enlargement of the spinal dorsal horn (Figure 3). These results support the idea that ER stress is involved in central sensitization caused by activity of the NMDA receptor, which is widely accepted to play an important role in the second phase.

Repetitive activation of the NMDA receptor leads to calcium influx into the cell,[14] which then activates Ca^{2+}/calmodulin-dependent protein kinases, resulting in the transcription of the immediate early gene *c-fos*.[24] In neurons, increasing the intracellular free calcium can lead to fast and profound changes in the transcription of genes such as *c-fos*. The precise signaling pathway is as follows: 1) elevated intracellular calcium activates calmodulin; 2) activated calmodulin regulates a calmodulin-dependent protein kinase (CaMK), which phosphorylates cyclic AMP response element-binding protein (CREB) at Ser133; 3) leading to the transcriptional activation of *c-fos*.[16,27] Alterations in the ER Ca^{2+} store induces ER stress, and if severe ER stress occurs, the 1,4,5-trisphosphate (IP3) receptor 1 (IP3R1), which is the most ubiquitously expressed Ca^{2+}-release channel, can become hypersensitized, finally resulting in excessive calcium flow out of the ER and aggravating the calcium overload.[28] Liu et al[29] found that in periprosthetic osteolysis, ER stress is linked to the Ca^{2+}–c-fos pathway and 4-PBA reduces c-fos and Ca^{2+} levels. The in vitro findings of He et al[30] suggest that ER stress can be induced by thapsigargin (TG), accompanied by a rapid increase in the levels of *c-fos* messenger RNA (mRNA). These reports support the notion that ER stress influences central sensitization by altering calcium homeostasis, thus inducing the transcription of *c-fos* mRNA; 4-PBA may, through inhibiting the ER stress response, consequently attenuate calcium overload in the cytoplasm, thereby reducing the level of c-fos. Our results are consistent with previous findings in showing that the level of c-fos in the spinal dorsal horn is increased by the formalin test and that 4-PBA treatment before formalin injection inhibits the ER stress response, reducing c-fos expression and attenuating pain in the second phase of the formalin test (Figures 2 and 3).

Furthermore, NO and PGE2 have been shown to be key mediators involved in the induction and maintenance of spinal nociceptive transmission and central sensitization induced by formalin injection. These effects are evoked by an enzymatic cascade initiated by Ca^{2+}-influx, which is followed by NMDA receptor activation after peripheral tissue injury.[31] Kuda et al[32] found that PGE2 concentration was significantly elevated in the medium of Chinese hamster ovary (CHO) cells after TG treatment, which simultaneously induced ER stress. ER stress can stimulate the expression of cyclooxygenase-2, which is the key enzyme for the production of PGE2.[33,34] Those studies support the hypothesis that ER stress could upregulate release of PGE2. Previous studies showed that excessive NO could disturb calcium homeostasis in ER, thus activating ER stress pathway.[35] Our results show that formalin injection increases

the level of both NO and PGE2 in the lumbar enlargement of the spinal cord, which could be attenuated by 4-PBA treatment (Figure 4). These results strongly support a role for ER stress in central sensitization that results from an alteration to calcium hemostasis. They furthermore suggest that the ER stress inhibitor 4-PBA can stabilize calcium hemostasis and thus attenuate central sensitization. However, our study does not supply full evidence of the necessary association between NO and the ER stress response; further research is required to elucidate their exact relationship.

Conclusion

Inflammation and central sensitization play important roles in the development of inflammatory pain, and the mechanisms underlying these processes are an important topic for current and future investigation. In this study, we showed that ER stress is strongly involved in formalin-induced pain and that 4-PBA can attenuate pain behaviors and central sensitization. This suggests that 4-PBA might be a useful therapeutic agent for counteracting central sensitization. However, further studies are required to completely understand how the ER stress response relates to the formalin test and central sensitization and exactly how the ER stress inhibitor 4-PBA attenuates formalin-induced nociceptive behaviors.

Acknowledgment

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Disclosure

The authors report no conflicts of interest in this work.

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