Oxyntomodulin attenuates TNF-α induced neuropathic pain by inhibiting the activation of the NF-κB pathway

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Received March 27, 2019; Accepted August 8, 2019

DOI: 10.3892/mmr.2019.10770

Abstract. Neuropathic pain is rarely diagnosed. Oxyntomodulin is peripherally and centrally distributed; however, the potential mechanisms underlying the effects of oxyntomodulin in attenuating nociception remain unclear; thus, we aimed to explore them in the present study. A neuropathic pain model in male C57BL/6 mice was induced by intrathecal injection of tumor necrosis factor-α (TNF-α), and the duration of nociceptive behavioral responses was measured with a stop-watch timer within 30 min. Western blotting was used to explore the protein levels of ionized calcium binding adaptor molecule-1 (IBA1), nuclear factor-κB (NF-κB) phosphorylated-p65, interleukin (IL)-6 and IL-1β. We performed reverse transcription-quantitative polymerase chain reaction and ELISA were performed to determine the mRNA and protein expression levels of IL-6 and IL-1β, respectively. An MTT assay was conducted to detect BV2 cell viability. Oxyntomodulin was observed to attenuate TNF-α-induced pain hypersensitivity in mice, as well as the expression of IBA1, NF-κB p-p65, IL-6 and IL-1β in the spinal cord. Oxyntomodulin exhibited no cytotoxicity on BV2 cells, and attenuated TNF-α-induced IL-6 and IL-1β production and release in BV2 cells and culture medium, respectively. Collectively, we proposed oxyntomodulin to attenuate TNF-α induced neuropathic pain associated with the release of glial cytokines IL-6 and IL-1β via inhibiting the activation of the NF-κB pathway.

Introduction

Neuropathic pain is defined as 'pain caused by a lesion or disease of the somatosensory system' by the International Association for the Study of Pain (1). Nociceptive pain involves peripheral noxious stimulation, for instance, by inflammatory factors (2). In 174 clinical trials, the efficacies of multiple drugs with analgesic effects, such as, local anesthetic drugs, opioids, antidepressants, NMDA receptor antagonists, botulinum toxin and topical capsaicin have been compared with placebo agents in patients with neuropathic pain (3). Despite the high prevalence of neuropathic pain, it is rarely diagnosed, and adequately and effectively treated. Thus, is important to develop novel potential therapies for treating patients with neuropathic pain.

Tumor necrosis factor (TNF)-α, which is a major proinflammatory cytokine and is produced during the transmission of pain (4), serves a role in the pathogenesis of neuropathic pain (5). Spinal nerve injury in rats induces mechanical allodynia and thermal hyperalgesia, which are accompanied with concomitant increases of interleukin (IL)-1β and TNF-α in the brain (6).

Increasing evidence has suggested that oxyntomodulin is peripherally and centrally distributed (7,8). Oxyntomodulin exhibits numerous functions. For instance, in mice administered a high-fat diet, intraperitoneal and intravenous treatment of oxyntomodulin ameliorates glucose intolerance (9), and increases insulin/glucose density in the plasma (10); additionally, oxyntomodulin regulates the intrinsic heart rate of mice (11). Furthermore, oxyntomodulin exhibits antinociceptive effects in mice (12). However, the potential molecules responsible for the attenuation of nociception by oxyntomodulin require further investigation of which we aimed to determine in the present study.

Materials and methods

Ethics statement. Each experimental procedure in the present study was approved by the Ethical Committee for the Use of Laboratory Animals of Ningbo No. 6 Hospital, and was conducted strictly according to the 'Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (13) and the ethical guidelines of the International Association for the Study of Pain (14)'.

Reagents. For *in vivo* experiments, 5 μl oxyntomodulin (0.2 μg/μl; Phoenix Pharmaceuticals) was dissolved in saline and administered to mice via an intrathecal (i.t.) injection according to a previous study (12). A total of 5 μl TNF-α (20 pg/μl; Sigma-Aldrich; Merck KGaA) was i.t. injected into mice (12).

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Key words: oxyntomodulin, pain, nuclear factor-κB signaling pathway

For *in vitro* experiments, BV2 cells were purchased from American Type Culture Collection and were treated with 0.01, 0.1 and 1 μM of oxyntomodulin (15). Then, 1 pg/μl TNF-α was added into the culture medium (16).

Animals. A total of 24 adult (8-week old, weight 20 g) male C57BL/6 mice were obtained from and housed in Ningbo No.6 Hospital. Mice were housed under controlled conditions with a temperature of 23±1˚C, humidity of 70±10% and a light-dark cycle of 12 h, with *ad libitum* access to water and food. All of the experiments in the current study were performed in the light phase of the light/dark cycle.

Mice were injected intrathecally with oxyntomodulin to determine the potential antinociceptive effects of oxyntomodulin. Thereafter, 24 mice were randomly divided into three different groups as described in a previously reported study (12): Control group, i.t. injection of 5 μl saline and 5 μl saline (n=8); TNF-α group, i.t. injection with 5 μl saline and 5 μl TNF-α (20 pg/μl) (n=8); oxyntomodulin + TNF-α group, i.t. injection with 5 μl oxyntomodulin (0.2 μg/μl) prior to 5 μl TNF-α (20 pg/μl) (n=8). Each mouse was euthanized by thoracic dislocation directly after the behavior tests (described below).

Nociceptive behavioral testing. Mice were acclimatized to the new condition for nociceptive behavioral testing for ~1 h before the experiments. After i.t. injection with TNF-α, mice were placed in a chamber (20 cm high, 20 cm diameter). Within half an hour, the cumulative response time of nociceptive behavioral responses of each mouse, including licking, scratching and biting episodes to the lumbar and caudal spinal cord were recorded with a stop-watch timer (17).

Western blotting. Lumbar 4-6 spinal cords were obtained mixed with radioimmunoprecipitation assay lysis (Roche Diagnostics) buffer containing phosphatase and proteinase inhibitors (Roche Diagnostics). The concentration of each protein sample was determined with a BCA kit (Beyotime Institute of Biotechnology). Protein samples (15 μg) were separated by 8% SDS-PAGE followed by transferring to a polyvinylidene difluoride (PVDF) membrane (EMD Millipore). The membranes were incubated with corresponding primary antibodies against IL-6 (cat. no. 12912; dilution 1:1,000), IL-1β (cat. no. 31202; dilution 1:1,000), phosphorylated-NF-κB (Ser536, cat. no. 3033; dilution 1:1,000), p65 (cat. no. 59674; dilution 1:1,000) and GAPDH (cat. no. 5174; dilution 1:1,000), which acted as a loading control. All the above primary antibodies were obtained from Cell Signaling Technology, Inc. and applied at 4˚C overnight. The antibody for IBA1 (cat. no. ab178846; dilution 1:1,000) was obtained from Abcam and applied at 4˚C overnight. Prior, the PVDF membrane was blocked with 5% skim milk at room temperature for ~1 h. The next day, the PVDF membrane was incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (cat. no. 7074; dilution 1:200, Cell Signaling Technology, Inc.) at room temperature for 1 h. Then, the protein bands were visualized via enhanced chemiluminescence (Thermo Fisher Scientific, Inc.). The quantity of each target protein band was analyzed by Image Quant LAS 500 (GE Healthcare Life Sciences).

Reverse transcription-quantitative-polymerase chain reaction (RT-qPCR). Total RNA was extracted from the lumbar 4-6 spinal cords and BV2 cells using TRIzol® (Thermo Fisher Scientific, Inc.). cDNA was synthesized from RNA with a PrimerScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocols. RT-qPCR was performed by SYBR Premix Ex Taq (Takara Bio, Inc.) and conducted by a CFX96™ real-time PCR system (Bio-Rad Laboratories, Inc.). The thermocycling conditions were: Denaturation (95˚C, 5 min), followed by 36 cycles of amplification (95˚C, 25 sec) and quantification (62˚C, 40 sec), the melting curve was them obtained (95˚C, 25 sec and 60˚C, 1 min). The $2^{-\Delta\Delta Cq}$ method was used to quantify the expression level of each gene (18). GAPDH acted as a control. The primer sequences were as listed: IL-6, forward, 5'-GGCTACTGCTTTCCCTACCC-3' and reverse, 5'-TTTTCTGCCAGTGCCTCTT-3'; IL-1β, forward, 5'-TCC AGGATGAGGACATGAGCAC-3' and reverse, 5'-GAACGT CACCCAGCAGGTTA-3'; GAPDH, forward, 5'-GTGATG CTGGTGCTGAGTATC-3' and reverse, 5'-GTGATGGCA TGGACKGTGG-3'.

Cell culture. BV2 microglial cells were seeded into 6-well plates, cultured with Dulbecco's Modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were maintained at 37˚C in an incubator with 5% CO_2 for 24 h. Cells ($3x10^5$) were divided into three groups and maintained at 37˚C in an incubator with 5% CO_2 with the treatments as followed: Control group, cultured with only culture medium; TNF-α group, treated with 1 pg/μl TNF-α at 37˚C for 24 h (16); oxyntomodulin + TNF-α group, treated with 0.01, 0.1 and 1 μM of oxyntomodulin at 37˚C at 24 h (15) prior to the treatment of 1 pg/μl TNF-α.

MTT assay. The effects of oxyntomodulin on BV2 cell viability were analyzed via an MTT assay (Sigma-Aldrich; Merck KGaA). Cells ($4x10^6$ cells/well) were first incubated for 8 h at 37˚C in an incubator with 5% CO_2. Thereafter, 10 μl MTT was added into each well and cells were incubated for further 4 h at 37˚C. The optical density (OD) at 570 nm was measured with a microplate reader (Bio-Rad 550; Bio-Rad Laboratories, Inc.).

ELISA. Supernatants of the culture medium were cleared of cells by spinning the 96-well plates at 400 x g at room temperature for 5 min. The supernatants were transferred to the round bottom, non-treated 96-well plates followed by spinning at 400 x g at room temperature for 5 min. Thereafter, the supernatants were transferred to a new 96-well plate for the subsequent experiments. The protein expression levels of IL-1β (SMLB00C, mouse IL-1 β/IL-1F2 Quantikine ELISA Kit) and IL-6 (SM6000B, mouse IL-6 Quantikine ELISA Kit) in the supernatants of the culture medium were tested by ELISA (R&D Systems, Inc.) according to the manufacturer's protocols. The OD at 490 nm was measured with a microplate reader (Spectra MAX 340PC Molecular Devices LLC).

Statistical analysis. Each experiment was repeated three times. Statistical analyses were conducted using GraphPad Prism 6

software (GraphPad Software, Inc.). Data were presented as the mean ± standard error of the mean. One-way ANOVA followed by a Student-Newman-Keuls test were applied for the analyses of differences among 3 or more groups. P<0.05 indicated a statistically significant difference.

Results

Oxyntomodulin attenuates TNF-α-induced pain hypersensitivity. The effects of oxyntomodulin on TNF-α-induced nociceptive behaviors in mice were assessed by nociceptive behavioral testing with a stop-watch timer. Compared with mice in the control group, mice in the TNF-α group presented acute licking, scratching, and biting the lumbar or caudal region which lasted for about half an hour; this was significantly longer than the control. On the contrary, a significant decrease in the duration of nociceptive behavior was reported following oxyntomodulin administration compared with TNF-α treatment alone (Fig. 1).

Oxyntomodulin decreases TNF-α-induced expression of IBA-1 and NF-κB p-p65 in the spinal cord. Over the past 15 years, neuropathic conditions have been proved to be related with the activation of glia in the central nervous system (CNS), which are important contributors to central sensitization (19). Molecular changes caused by glial activation leads to pain hypersensitivity, including upregulation of chemokine receptors and the release of glial cytokines (20).

Nuclear factor-κB is activated by IκB kinases, which then migrates to the nucleus (21). Spinal nerve injury in rats increases mechanical allodynia and thermal hyperalgesia which are accompanied with increases in NF-κB expression in the brain (6).

Therefore, we explored the expression levels of ionized calcium binding adaptor molecule-1 (IBA1, microglia marker) and NF-κB in the spinal cord of mice by western blotting. The results showed that, compared with the control group, TNF-α significantly induced the protein expression of IBA1 and NF-κB p-p65, but was attenuated by pretreatment with oxyntomodulin (Fig. 2A and B); no significant changes in NF-κB p65 expression were observed among the three groups.

Oxyntomodulin attenuates TNF-α-induced IL-6 and IL-1β production in the spinal cord. NF-κB activates the transcription of proinflammatory genes (18), while, NF-κB responds to ~150 different stimuli (22). Spinal nerve injury in rats induces mechanical allodynia and thermal hyperalgesia accompanied with concomitant increases of IL-1β and TNF-α in the brain (6). Several studies indicate that various cytokines can directly stimulate nociceptors (23,24). Cytokines (such as IL-1β and TNF-α) which are released after stress induce the activation of NF-κB (25).

Correspondingly, we detected the expression levels of IL-1β and TNF-α in the spinal cord of mice by RT-qPCR and western blotting. RT-qPCR demonstrated that compared with the control group, TNF-α significantly induced IL-6 and IL-1β mRNA expression in spinal cord, but was attenuated by oxyntomodulin administration (Fig. 3A and B). Western blotting demonstrated similar results to the findings of RT-qPCR (Fig. 3C and D).

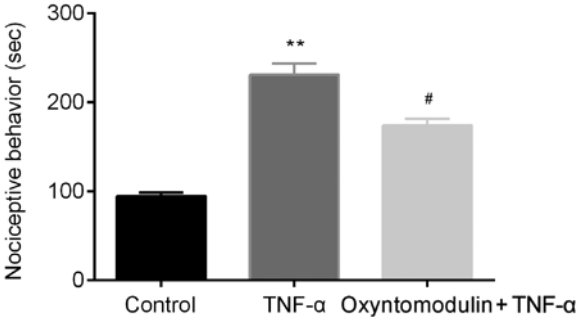

Figure 1. Oxyntomodulin attenuates TNF-α-induced pain hypersensitivity. Compared with the control group, TNF-α induced acute behavioral responses, which were attenuated by oxyntomodulin. **P<0.01 vs. Control; #P<0.05 vs. TNF-α. TNF-α, tumor necrosis factor-α.

Oxyntomodulin protects BV2 cells from TNF-α-induced toxicity. BV2 cell viability in different groups was investigated by an MTT assay. At 12 h after treatment, oxyntomodulin (0.01, 0.1 and 1 μM) did not affect BV2 cell viability as presented in Fig. 4A, indicating that oxyntomodulin exerted no toxicity on BV2 cells.

Compared with the control group, TNF-α significantly inhibited the cell viability of BV2 cells, which was rescued by oxyntomodulin treatment (0.01, 0.1 and 1 μM) (Fig. 4B).

Oxyntomodulin attenuates TNF-α-induced IL-6 and IL-1β production and release in BV2 cells and culture medium. Additionally, we detected the effects of oxyntomodulin on the release of glia cytokine. RT-qPCR was performed to determine the mRNA levels of IL-6 and IL-1β in BV2 cells. Compared with the control group, TNF-α significantly increased the mRNA levels of IL-6 and IL-1β in BV2 cells, which were significantly attenuated by oxyntomodulin treatment (0.1 and 1 μM) (Fig. 5A and B).

Furthermore, ELISA was used for the analyze the protein levels of IL-6 and IL-1β in the supernatants of the culture medium. The results demonstrated that compared with control group, TNF-α significantly increased protein levels of IL-6 and IL-1β, which were attenuated by oxyntomodulin treatment (0.1 and 1 μM) in the supernatants of culture medium (Fig. 5C and D).

Discussion

The effects of oxyntomodulin in the CNS have not been well elucidated (26). One previous study indicated that D-Ser2-oxytomodulin (a protease-resistant oxyntomodulin analogue) functions in the CNS. For instance, it improves locomotor activities and protects dopaminergic neurons in a mouse model of Parkinson's Disease (PD) (27). Although oxyntomodulin is expressed in the brain and the spinal cord (7,8), the function and potential molecules responsible for the function of oxyntomodulin in nociception remain to be further explored.

TNF-α which is a major proinflammatory cytokine that is produced during the transmission of pain (4), serves a role in the pathogenesis of neuropathic pain (5). In the present study, we reported that oxyntomodulin attenuated TNF-α-induced neuropathic pain in mice in nociceptive behavior tests; however,

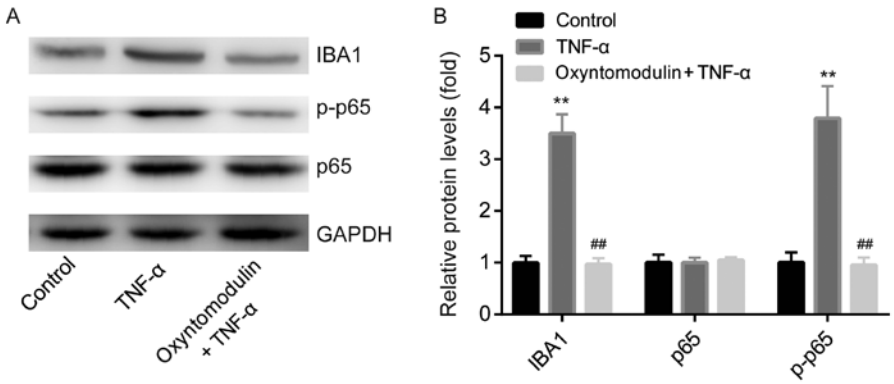

Figure 2. Oxyntomodulin decreases TNF-α-induced expression of IBA-1 and NF-κB p-p65 in the spinal cord. (A and B) Compared with the control group, TNF-α significantly induced the protein expression of IBA1 and nuclear factor-κB p-p65, which are attenuated by pretreatment with oxyntomodulin. **P<0.01 vs. Control; $^{\#\#}$P<0.01 vs. IBA1, ionized calcium binding adaptor molecule-1; p, phosphorylated; TNF-α, tumor necrosis factor-α.

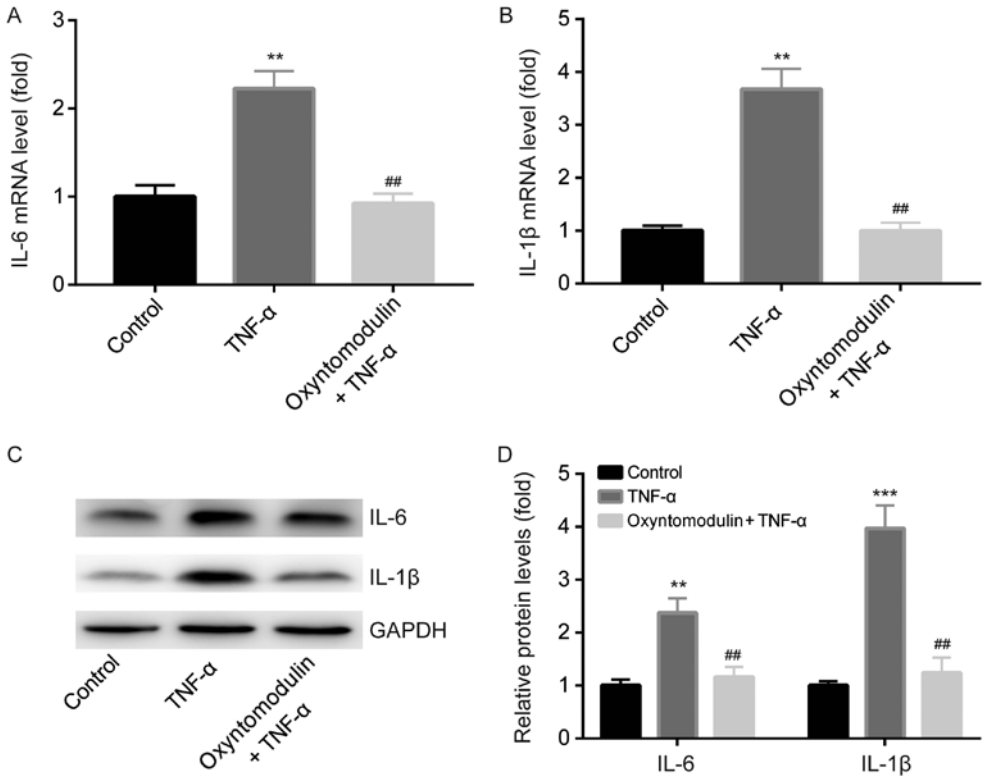

Figure 3. Oxyntomodulin attenuates TNF-α-induced IL-6 and IL-1β production in the spinal cord. (A and B) Compared with the control group, TNF-α induced IL-6 and IL-1β expression in the spinal cord, which was attenuated by oxyntomodulin administration. (C and D) The results of western blotting revealed similar trends in the protein expression of IL-6 and IL-1β to reverse transcription-quantitative polymerase chain reaction analysis of mRNA expression. **P<0.01, ***P<0.001 vs. Control; $^{\#\#}$P<0.01 vs. TNF-α. IL, interleukin; TNF-α, tumor necrosis factor-α.

the molecules that are responsible for the aforementioned changes remain to be further investigated.

As an important upstream molecule of NF-κB, TNF-α activates NF-κB, which further promotes the expression of IL-6 (28); TNF-α also leads to an increase of IL-1β expression (29). NF-κB plays a crucial role in the transcriptional regulation of various pro-inflammatory genes, including IL-1β and IL-6 (30). Our results showed that, compared with the control group, TNF-α induced the expression of NF-κB, as well as IL-6 and IL-1β, which were in agreement with the aforementioned findings, indicating that TNF-α induced the expression of IL-6 and IL-1β via activation of NF-κB (28-30).

Furthermore, the effects of TNF-α on nociceptive behaviors, and NF-κB, IL-6 and IL-1β levels were found to be attenuated by pretreatment of oxyntomodulin in the present study. These findings were in agreement with a recent study (12) and a report that investigated the potential treatment of oxyntomodulin on PD (27).

In conclusion, we revealed for the first time, to the best of our knowledge, that oxyntomodulin attenuates TNF-α-induced neuropathic pain (release of glial cytokines IL-6 and IL-1β) via inhibiting the activation of NF-κB pathway, suggesting a potential role of oxyntomodulin the clinical management of pain in the future.

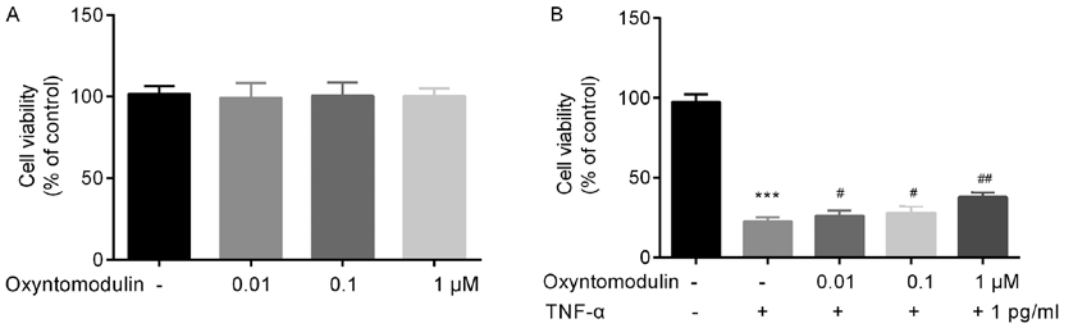

Figure 4. Oxyntomodulin protects BV2 cells from TNF-α-induced toxicity. Oxyntomodulin exerts no toxicity on BV2 cells. (A and B) Compared with the control group, TNF-α significantly decreased BV2 cell viability, which was rescued by oxyntomodulin treatment. ***P<0.001, vs. control; #P<0.05, ##P<0.01 vs. TNF-α. TNF-α, tumor necrosis factor-α.

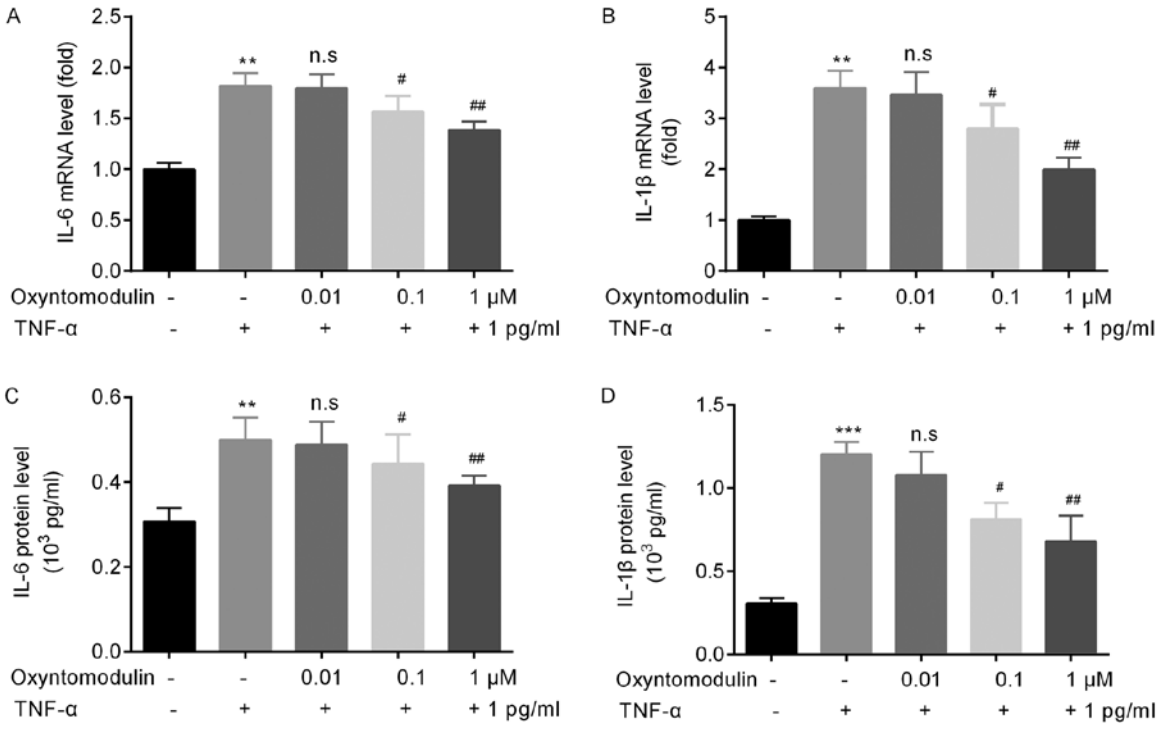

Figure 5. Oxyntomodulin attenuates TNF-α-induced IL-6 and IL-1β production in BV2 cells. (A and B) Compared with the control group, TNF-α significantly increases the mRNA expression of IL-6 and IL-1β, which was attenuated by oxyntomodulin in BV2 cells. (C and D) The results of ELISA were similar changes with those of reverse transcription-quantitative polymerase chain reaction. **P<0.01, ***P<0.001 vs. Control; #P<0.05, ##P<0.01 vs. TNF-α. IL, interleukin; n.s, no significance; TNF-α, tumor necrosis factor-α.

Unfortunately, there are several limitations in the present study: i) The time-dependent effects of oxyntomodulin on BV2 were not investigated, which could provide insight into the effects of intracellular cytokines; and ii) little evidence regarding the effects of oxyntomodulin on TNF-α-induced neuropathic pain has been reported; thus; further analysis should be conducted in the future.

Acknowledgements

Not applicable.

Funding

The present study was funded by Natural Science Foundation of Ningbo (grant no. 2009A610140).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ, LY, YC, CL and GY performed the experiments and analyzed the data. LY prepared the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee for the Use of Laboratory Animals of Ningbo No. 6 Hospital, and was conducted strictly according to the 'Guide for Care and Use of Laboratory Animals' published by the National

Institutes of Health (13) and the ethical guidelines of the International Association for the Study of Pain (14).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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