

p38 β Mitogen-Activated Protein Kinase Signaling Mediates Exenatide-Stimulated Microglial β -Endorphin Expression

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ABSTRACT

Recent discoveries established that activation of glucagon-like peptide-1 receptors (GLP-1Rs) mediates neuroprotection and antinociception through microglial β -endorphin expression. This study aimed to explore the underlying signaling mechanisms of microglial β -endorphin. GLP-1Rs and β -endorphin were coexpressed in primary cultures of microglia. Treatment with the GLP-1R agonist exenatide concentration-dependently stimulated microglial expression of the β -endorphin precursor gene proopiomelanocortin (POMC) and peptides, with EC₅₀ values of 4.1 and 7.5 nM, respectively. Exenatide also significantly increased intracellular cAMP levels and expression of *p*-protein kinase A (PKA), *p*-p38, and *p*-cAMP response element binding protein (CREB) in cultured primary microglia. Furthermore, exenatide-induced microglial expression of POMC was completely blocked by reagents that specifically inhibit adenylyl cyclase and activation of PKA, p38, and CREB. In addition, knockdown of p38 β (but not p38 α) using short interfering RNA (siRNA) eliminated exenatide-induced microglial

p38 phosphorylation and POMC expression. In contrast, lipopolysaccharide increased microglial activation of p38, and knockdown of p38 α (but not p38 β) partially suppressed expression of proinflammatory factors (including tumor necrosis factor- α , interleukin-1 β , and interleukin-6). Exenatide-induced phosphorylation of p38 and CREB was also totally blocked by the PKA inhibitor and siRNA/p38 β , but not by siRNA/p38 α . Seven-day intrathecal injections of siRNA/p38 β (but not siRNA/p38 α) completely blocked exenatide-induced spinal p38 activation, β -endorphin expression, and mechanical allodynia in rats with established neuropathy, although siRNA/p38 β and siRNA/p38 α were not antiallodynic. To our knowledge, our results are the first to show a causal relationship between the PKA-dependent p38 β mitogen-activated protein kinase/CREB signal cascade and GLP-1R agonism-mediated microglial β -endorphin expression. The differential role of p38 α and p38 β activation in inflammation and nociception was also highlighted.

Introduction

Glucagon-like peptide-1 receptors (GLP-1Rs) in pancreatic islet β cells have been implicated in the treatment of type 2 diabetes mellitus (Koole et al., 2013; de Graaf et al., 2016). Activation of GLP-1Rs in the central nervous system also mediates neuroprotection in preclinical animal models of neurodegenerative disorders, including Parkinson disease, Alzheimer disease, amyotrophic lateral sclerosis, multiple sclerosis, peripheral neuropathy, and ischemia and stroke (Kim et al., 2009; Harkavyi and Whitton, 2010; Hölscher, 2012; Hansen et al., 2015; Jia et al., 2015). Our laboratory also recently revealed that agonism of spinal GLP-1Rs by peptidic, nonpeptidic, and herbal iridoid agonists produced

antinociception in a variety of rodent pain models of neuropathy, inflammation, bone cancers, and diabetes (Gong et al., 2014b; Zhu et al., 2014; Fan et al., 2015; Xu et al., 2017). The GLP-1R in pancreatic islet β cells evokes insulin synthesis during episodes of hyperglycemia (Baggio and Drucker, 2007; Lee and Jun, 2014). In contrast, activation of GLP-1Rs in the hippocampus and spinal dorsal horn leads to microglial expression of β -endorphin (Gong et al., 2014b; Jia et al., 2015). However, the signaling mechanisms underlying GLP-1R-mediated microglial β -endorphin expression remain to be determined.

The GLP-1R belongs to class B of the G protein-coupled receptor family, with signaling via multiple G proteins, including G_{as}, G_{ai}, G_{ao}, and G_{aq/11} (Hällbrink et al., 2001). Multiple signal transduction pathways have been characterized for GLP-1R-induced insulin synthesis (Baggio and Drucker, 2007); among these, cAMP/protein kinase A (PKA) signaling through G_{as} was identified as a classic pathway for

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ABBREVIATIONS: ANOVA, analysis of variance; CREB, cAMP response element binding protein; Ct, cycle threshold; DMEM, Dulbecco's modified Eagle's medium; DOTAP, 1,2-dioleoyl-3-trimethylammonium propane; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLP-1R, glucagon-like peptide-1 receptor; H-89, *N*-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride; IBA-1, ionized calcium-binding adapter molecule 1; IL, interleukin; JNK, c-Jun N-terminal kinase; KG501, [3-[[4-(4-chlorophenyl)carbamoyl]naphthalen-2-yl] dihydrogen phosphate; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; PKA, protein kinase A; POMC, proopiomelanocortin; SB203580, 4-[5-(4-fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1*H*-imidazol-4-yl]pyridine; siRNA, short interfering RNA; SP600125, anthra[1-9-*cd*]pyrazol-6(2*H*)-one; TNF, tumor necrosis factor; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[[2-aminophenylthio]butadiene.

insulin secretion (Drucker et al., 1987; Koole et al., 2013). Moreover, cAMP response element binding protein (CREB) signaling was shown to be a crucial transcription factor for expression of insulin and the β -endorphin precursor proopiomelanocortin (POMC) in pancreatic and pituitary cells (Kraus and Höllt, 1995; Dalle et al., 2011). These findings prompted us to illustrate the causal association between the cAMP/PKA/CREB signaling pathway and exenatide-induced β -endorphin expression in microglia.

Mitogen-activated protein kinases (MAPKs), including the p38, c-Jun N-terminal kinase (JNK) 1/2, and extracellular signal-regulated kinase (ERK) 1/2 isoforms (Johnson and Lapadat, 2002), play critical roles in microglial activation (Milligan and Watkins, 2009). However, GLP-1R-induced p38 activation promoted insulin secretion in insulinoma cells (Kemp and Habener, 2001), whereas it inhibited rapamycin-induced p38 activation in pancreatic β cells (Kawasaki et al., 2010). ERK1/2 was markedly activated in β cells and adipose tissue macrophages (Montrose-Rafizadeh et al., 1999; MacDonald et al., 2002) or was inactivated (Lee et al., 2012). By inhibiting JNK1/2 activation, GLP-1R activation in pancreatic β cells and macrophages was anti-inflammatory and antiapoptotic (Kawasaki et al., 2010; Lee et al., 2012). Previous data on p38 involvement in the antinociceptive effect of the GLP-1R iridoid agonist shanzhiside methylester (Fan et al., 2016) led us to explore the roles of MAPK activation in exenatide-mediated β -endorphin expression in microglia.

The p38 MAPK family consists of four members: p38 α , p38 β , p38 δ , and p38 γ . Among these four isoforms, only p38 α and p38 β are mainly expressed in microglia in the central nervous system (Dong et al., 2014). Although p38 α and p38 β share approximately 80% homology of their protein sequences, they exhibit differential biologic functions (Li et al., 2008). Knockout/mutation of p38 α (but not p38 β) attenuated microglial expression of proinflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, and IL-1 β (Bachstetter et al., 2011; Xing et al., 2011), and activated microglia-induced neuron degeneration (Xing et al., 2013). In contrast, p38 β was recently identified to play a critical role in the survival of endothelial cells, myocytes, and fibroblasts (Wang et al., 1998; Si and Liu, 2009). Accordingly, identification of the roles of p38 α and p38 β in exenatide-induced β -endorphin expression is an area worthy of exploration.

This study mainly aimed to characterize the signal transduction mechanisms in cultured primary microglia by which exenatide stimulates microglial expression of β -endorphin, with a focus on the involvement of MAPK subtypes and p38 MAPK isoforms as well as their upstream and downstream signals. In parallel to β -endorphin expression, we assessed the effect of exenatide on intracellular cAMP levels and phosphorylation of PKA, MAPKs, and CREB. To reveal the causal association, selective inhibitors of each signaling molecule were employed to intervene in β -endorphin expression. Since selective inhibitors of p38 β and p38 α activation are not available (O'Keefe et al., 2007; Coulthard et al., 2009), RNA interference technology was employed to measure p38 α and p38 β phosphorylation of and intervention in exenatide-induced β -endorphin expression and antinociception in neuropathy. To our knowledge, our results are the first to demonstrate that the cAMP/PKA/p38 β /CREB signal transduction pathway entirely mediates exenatide-induced β -endorphin expression and we highlight differential roles of p38 α and p38 β in inflammation and nociception.

Materials and Methods

Drugs. Exenatide was obtained from Kaijie Bio-Pharmaceuticals (Chendu, China). The specific adenylate cyclase inhibitor 2',5'-dideoxyadenosine, PKA inhibitor H-89 (*N*-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride), JNK1/2 inhibitor SP600125 (anthra[1-9-*cd*]pyrazol-6(2*H*)-one), ERK1/2 inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene), CREB inhibitor KG501 ([3-[(4-chlorophenyl)carbamoyl]-naphthalen-2-yl] dihydrogen phosphate), and p38 inhibitor SB203580 (4-[5-(4-fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1*H*-imidazol-4-yl]-pyridine) were purchased from Selleck Chemicals (Houston, TX). We followed references and chose the above specific inhibitors at certain concentrations/doses (see below), although their specificity on microglial cells may not be validated.

Laboratory Rodents. Male 1-day-old neonatal and adult (8- to 10-week-old) Wistar rats were obtained from the Experimental Animal Institute (Shanghai, China). The adult rats were placed in a humidity- and temperature-controlled environment on a 12-hour light/dark cycle, with water and food ad libitum. The adult rats were accustomed to the environment for 3–5 days before surgery and were randomly assigned to research groups. The animal procedures were approved by the Shanghai Jiao Tong University Experimental Animal Care and Welfare Committee and followed National Institutes of Health regulatory animal care guidelines.

Primary Cultures of Microglia. As the cortex harvested more cells, its microglial cells were collected for this study. Briefly, the cortex was harvested from neonatal rats and its meninges were removed. The isolated cortex tissues were then minced and incubated in 0.05% trypsin. Dissociated cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), penicillin (100 U/ml), and streptomycin (100 μ g/ml). Suspended cells were plated in 75-cm² tissue culture flasks (1 \times 10⁷ cells/flask) precoated with poly(L-lysine) (0.1 mg/ml) and were incubated in a humidified atmosphere with 5% CO₂ at 37°C, with replenishment each 3 days. After 8 days of culture, confluent mixed glial cultures were collected as floating suspensions by shaking (260 rpm) at 37°C for 2 hours. The unattached cells were removed by serum-free DMEM after the aliquots were placed on plates (Gong et al., 2014b). Harvested microglial cells showed a characteristic morphology of a small cell body endowed with thin processes, with a purity of more than 95% measured by ionized calcium-binding adapter molecule 1 (IBA-1) immunostaining.

Isolation and Reverse Transcription of RNA and Real-Time Quantitative Polymerase Chain Reaction Measurements. Total RNAs of primary microglia and spinal homogenates were isolated and purified on ice using TRIzol reagent (Invitrogen). Reverse transcription was performed using a quantitative reverse transcription polymerase chain reaction (PCR) kit (Toyobo, Osaka, Japan), and real-time quantitative PCR measurements were performed using RealMasterMix (SYBR Green I; Toyobo). The following primer sequences were used: p38 β (forward: 5'-CTCCCTGGCACCCATGAAAT-3'; reverse: 5'-GACACATCCGTGCATTCGTG-3'; NM001109532.2), p38 α (forward: 5'-AGTGCCTCGAACCCTG-3'; reverse: 5'-GGGT CACCAGGTACACATCG-3'; NM031020.2), *POMC* exons 2–3 (forward: 5'-CCCTCCTGCTTC AGACCTCCA-3'; reverse: 5'-TCTC-TTCTCCGCACGC CTCT-3'; Sitte et al., 2007), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) (forward: 5'-CCAAGGTCATCATG ACGAC-3'; reverse: 5'-TCCACAGTCTTCTGAGTGGC-3'; Gong et al., 2014b), TNF- α (forward: 5'-CCCCGACTATGTGCTCCTCAC-3'; reverse: 5'-AGGGCTCTTGATGGCGGA-3'), IL-1 β (forward: 5'-GGAAGG CAGTGTCACTCATTGTG-3'; reverse: 5'-GGTCTCATCCTGGAA GCTCC-3'), and IL-6 (forward: 5'-GGGACTGATGTTGTGACAGCC-3'; reverse: 5'-CATATGTAATTAAGCCTCCGACTGTG-3'; Raghavendra et al., 2004). All primers were validated to be specific by melting curves. Relative expression was calculated with the 2^{- $\Delta\Delta$} cycle threshold (Ct) method after normalizing targeting Ct values with *gapdh* Ct values (Gong et al., 2014b).

Western Blotting. Homogenized spinal lumbar enlargements (L4–L6) and cultured microglia were lysed in immunoprecipitation analysis buffer containing the protease inhibitor phenylmethylsulfonyl fluoride and phosphatase inhibitor cocktail A/B. Protein supernatants were obtained and a bicinchoninic acid assay was used to measure their concentrations (Beyotime Institute of Biotechnology, Jiangsu, China) (Holz et al., 1995, 1999). Proteins were separated by SDS-PAGE (10%) and further transferred to polyvinylidene fluoride membranes using an electrophoretic method. The membrane was blocked by skim milk (5%) in Tris-buffered saline containing Tween 20 (0.1%) and was further incubated with the primary antibody against p-PKA (1:500; Santa Cruz Biotechnology, Dallas, TX), p-p38 (1:1000; Cell Signaling Technology, Danvers, MA), p-JNK1/2 (1:1000; Cell Signaling Technology), p-ERK1/2 (1:1000; Cell Signaling Technology), p-CREB (1:1000; Cell Signaling Technology), p38 β (1:500; Proteintech Group, Chicago, IL), p38 α (1:1000; Cell Signaling Technology), and GAPDH (1:5000; Protein Tech Group) overnight at 4°C by slight shaking. Protein bands were visualized under an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) after 1-hour incubation at 37°C with DyLight 680–conjugated anti-mouse IgG (1:10,000; Rockland Immunochemicals, Gilbertsville, PA) and DyLight 800–conjugated anti-rabbit IgG (1:10,000; Rockland Immunochemicals). Protein band intensity was measured using ImageJ software (National Institutes of Health, Bethesda, MD), and the relative protein expression level was calculated after normalization to the GAPDH protein. Protein samples from three to four batches of cultured cells were used for Western blotting (Gong et al., 2014b; Fan et al., 2016).

Immunofluorescence Staining. Cultured primary microglia were seeded on poly(L-lysine)-coated coverslips placed at the bottom of 12-well plates (5×10^4 cells/well). After overnight culture, cells were fixed in 4% paraformaldehyde for 1 hour and were further incubated in phosphate-buffered saline containing 10% goat serum and 0.5% X-100 for 1 hour. The cell flasks were then incubated with rat GLP-1R antibody (1:200; Abcam, Cambridge, UK), β -endorphin antibody (1:200; Abcam), and IBA-1 antibody (1:200; Merck Millipore, Temecula, CA) at 4°C overnight, followed by incubation with Alexa 555–conjugated goat anti-rabbit secondary antibody (1:200; Life Technologies, Carlsbad, CA) and Alexa 488–conjugated goat anti-mouse secondary antibody (1:200; Life Technologies) for 1 hour at 37°C. Expression of GLP-1R, β -endorphin, and IBA-1 was visualized under a laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany). Nucleic staining reagent 4',6-diamidino-2-phenylindole (1 μ g/ml; Sigma-Aldrich, St. Louis, MO) was used to stain cell nuclei. Colocalization was identified using ImageJ software equipped with a colocalization finder, under which colocalized pixels appeared white (Gong et al., 2014b).

cAMP Accumulation Assay. After 30-minute incubation, exenatide-stimulated intracellular cAMP levels in cell lysates containing 3-isobutyl-1-methylxanthine (a specific inhibitor of the cyclic nucleotide phosphodiesterase), were measured using a commercial enzyme immunoassay kit (R&D Systems, Minneapolis, MN) (Koole et al., 2010). Total intracellular protein concentrations were determined using the standard bicinchoninic acid assay (Beyotime Institute of Biotechnology).

β -Endorphin Assay. Exenatide-induced β -endorphin expression in cultured primary microglia was determined using an enzyme-linked fluorescence immunoassay kit (Phoenix Pharmaceuticals, Burlingame, CA). Based on the manufacturer's instructions, β -endorphin had no cross-reactivity with Leu-enkephalin (0%) or Met-enkephalin (0%) but showed 60% and 100% cross-reactivity with γ -endorphin and α -endorphin, respectively. Cultured primary microglia were placed in 24-well plates (1×10^5 cells/well) and washed with warm DMEM (1 ml) in the presence of *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (15 mM) and bovine serum albumin (2 mg/ml). β -endorphin titers in the supernatants were determined using a fluorescence assay after microglial cells were stimulated with exenatide for 2 hours (Chen et al., 2012b; Gong et al., 2014b).

RNA Interference. GenePharma (Shanghai, China) designed and synthesized the short interfering RNA (siRNA) targeting p38 α /

MAPK14 and p38 β /MAPK11, as well as the nonspecific oligonucleotides (oligos), in the following sequences: p38 α /MAPK14 (5'-GCACGAGAAUGUGAUUGGUTT-3'/5'-ACCAAUCACAUUCUC-GUGCTT-3'), p38 β /MAPK11 (5'-GCACGAGAACGUCAUAGGATT-3'/5'-UCCUAUGACGUUC UCGUGCTT-3'), and the nonspecific oligos 5'-UUCUCCGAACGUGUCACGUT T-3'/5'-ACGUGACACGUUCG-GAGAATT-3'. To formulate siRNA, the lipofectin 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (Avanti Polar Lipids, Alabaster, AL) was added according to the manufacturer's instructions. For microglial transfection, the cells were seeded into 24-well/6-well plates and the siRNA-DOTAP complex was added with a supplement of basic DMEM medium (final siRNA concentration, 5 μ g/ml) and incubated for 5 hours. Cells were further cultured for 24 hours routinely after transfection. For the spinal transfection, the siRNA-DOTAP complex was injected intrathecally for 7 days successively.

Rat Intrathecal Catheterization and Injection. A PE-10 catheter (Clay Adams, Parsippany, NJ) was inserted intrathecally into the rat's lumbar spine under isoflurane anesthesia run by a gas anesthesia system (Ugo Basile, Comerio, Italy). Correct placement of the catheter was confirmed by intrathecal administration of lidocaine 2 days after recovery from anesthesia. Rats that had no motor impairments after catheterization and developed immediate paralysis of bilateral hindlimbs after lidocaine were selected for this study. For intrathecal administration, 10 μ l drug solution was administered, followed by a 15- μ l normal saline flush (Wei et al., 2016).

Rat Model of Neuropathy and Mechanical Threshold Assessment. Rats were anesthetized under inhaled isoflurane anesthesia. The left spinal nerves (L5 and L6) were isolated and tightly ligated with 6-0 silk sutures. The lumbar fascia and skin were closed with 4-0 resorbable polyglactin sutures after ligation and the rats were allowed to recover (Kim and Chung, 1992). Since intrathecal delivery was needed in neuropathic rats in this study, intrathecal catheterization was performed at the same time just before spinal nerve injury. After surgery, rats that had no major motor impairments and had significant mechanical allodynia in the ipsilateral hindpaws with withdrawal thresholds < 8 g were chosen for the investigation. Drug testing started 1 week after spinal nerve ligation.

To assess mechanical allodynia, an examiner who was blinded to the treatment groups tested mechanical thresholds in both hindpaws using a 2290 CE electrical von Frey hair (IITC Life Science, Woodland Hills, CA), which could generate a force ranging from 0.1 to 90 g. The evoked withdrawal thresholds were determined by stimulation of the hindpaw when the rat stood on a metal grid. Increasing force was applied to stimulate the footpad until a sudden withdrawal response and the lowest force was the threshold, which was averaged from triplicate measurements within approximately 30 seconds (Zhang et al., 2013).

Statistical Analysis. To determine the half-effective concentration (EC₅₀) of the concentration-response curve, values of the response (*y*) were fitted by nonlinear least-squares curves to the following: $y = a + bx$, where $x = [C]^n / (EC_{50}^n + [C]^n)$, yielding a minimum residual sum of squares of deviations from the theoretical curve. The EC₅₀ and *b* (maximum effect) values were projected (Wang and Pang, 1993).

Data are shown as means \pm S.D. Unpaired and two-tailed *t* tests and one-way analysis of variance (ANOVA) were used to evaluate statistical significance. The post hoc Student–Newman–Keuls test was performed when statistical significance was achieved for the drug (concentration or dose) in the ANOVA analysis. GraphPad Prism software (version 7.00; GraphPad Software Inc., La Jolla, CA) was used for the concentration-response analysis and statistical evaluation. *P* < 0.05 was considered statistically significant.

Results

Exenatide Stimulated β -Endorphin Overexpression in Primary Microglia. Peptidic, nonpeptidic, and small molecule agonists of GLP-1Rs stimulated β -endorphin expression and release in microglia originating from the cortex (Fan

et al., 2015, 2016), hippocampus (Jia et al., 2015), and spinal cord (Gong et al., 2014b). To confirm coexpression of GLP-1R and β -endorphin, we performed double immunofluorescence staining of the respective GLP-1R and the microglial marker IBA-1, β -endorphin and IBA-1, and β -endorphin and GLP-1R in cultured primary microglia originating from the cortex. GLP-1Rs (Fig. 1, A–D) and β -endorphin (Fig. 1, E–H) were colocalized in microglial cells (labeled by IBA-1), and β -endorphin was also colocalized with GLP-1Rs in microglia (Fig. 1, I–L).

We further constructed a concentration-response curve of the GLP-1R peptidic agonist exenatide (0.3, 1, 3, 10, 30, and 100 nM) on β -endorphin expression and release in cultured primary microglia. POMC gene expression in microglia and β -endorphin levels in the cell culture medium were measured 2 hours later using real-time quantitative PCR and a fluorescence immunoassay assay kit, respectively. The time point selected was based on the higher measurement sensitivity after POMC and β -endorphin accumulation. Incubation with exenatide concentration-dependently increased POMC expression, with an EC_{50} of 4.1 nM (Fig. 1M). In agreement with POMC expression, the β -endorphin level in the cell culture medium was concentration-dependently elevated, with an EC_{50} of 7.4 nM (Fig. 1N). We thus selected the submaximal concentration of 10 nM for the later studies of signaling mechanisms. Because exenatide stimulated POMC expression and β -endorphin release in parallel, POMC expression was only measured for the following studies.

cAMP/PKA Signaling Mediated Exenatide-Stimulated POMC Overexpression. Incubation of exenatide (10 and 100 nM) with cultured primary microglia for 30 minutes

maximally elevated the intracellular cAMP level measured using a commercial fluorescence immunoassay kit ($P < 0.05$, one-way ANOVA followed by post hoc Student–Newman–Keuls tests; Fig. 2A). Exenatide-stimulated PKA activation was later determined using Western blotting. Thirty-minute treatment with exenatide (10 nM) significantly increased PKA phosphorylation by 80% ($P < 0.05$, unpaired and two-tailed t test; Fig. 2B). To further determine whether activation of cAMP/PKA signaling was causally associated with exenatide-stimulated POMC expression, pharmacological inhibition of adenylate cyclase and PKA phosphorylation was tested. Although treatment with the specific adenylate cyclase inhibitor 2',5'-dideoxyadenosine (100 μ M) and PKA inhibitor H-89 (10 μ M) (Mitsuya et al., 1987; Chijiwa et al., 1990; Engh et al., 1996; Liu et al., 2012, 2014) did not affect basal POMC expression, their pre-treatment (1 hour earlier) completely attenuated exenatide-increased expression of POMC ($P < 0.05$, one-way ANOVA; Fig. 2, C and D).

p38 Phosphorylation Mediated Exenatide-Stimulated POMC Overexpression. To explore whether MAPK signaling played a causal role in exenatide-induced β -endorphin overexpression measured by using Western blotting, phosphorylation of p38, JNK1/2, and ERK1/2 was analyzed. Treatment with exenatide (10 nM) for 15, 30, or 60 minutes could time-dependently stimulate p38 phosphorylation, with a peak effect at 30 minutes ($P < 0.05$, one-way ANOVA; Fig. 3A). However, it did not significantly alter phosphorylation of either ERK1/2 or JNK1/2 during the observation period up to 60 minutes (Fig. 3, B and C). A time point of 30 minutes was then selected for the later phosphorylation measurements.

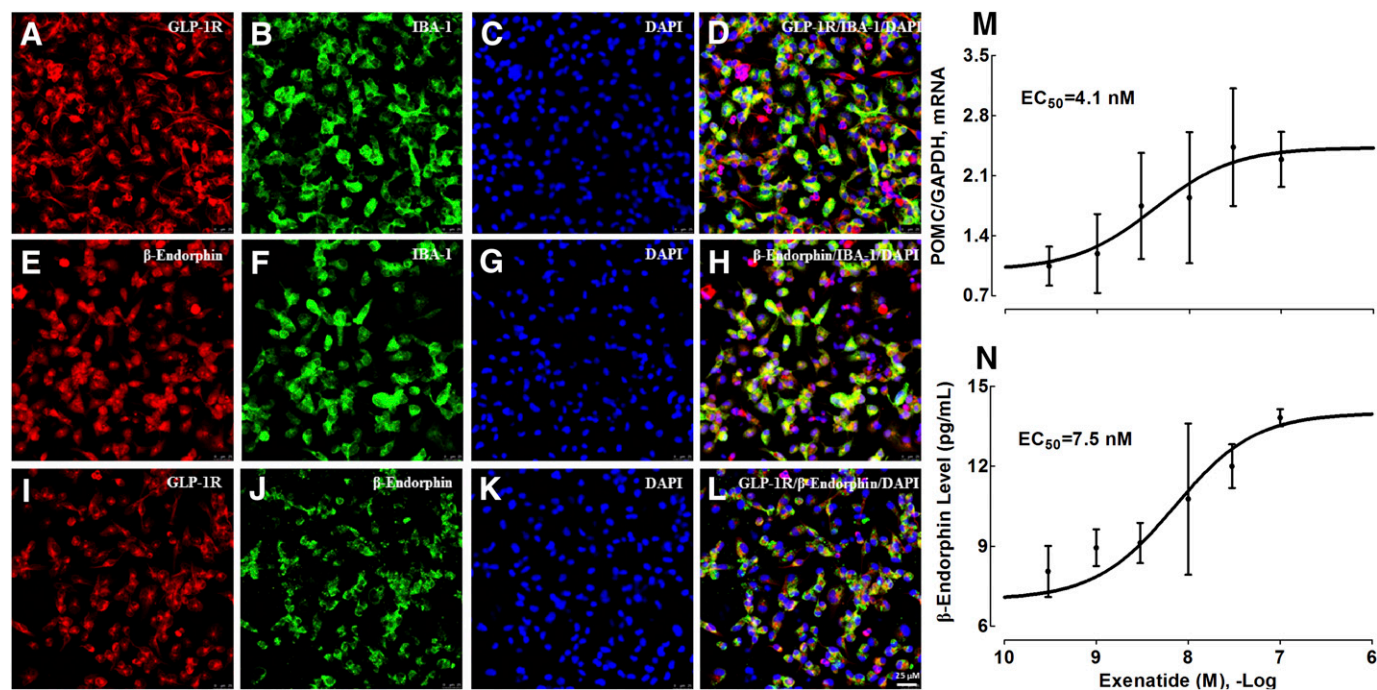


Fig. 1. Representative photomicrographs of expression of the GLP-1R and β -endorphin in primary microglia (A–L), and stimulatory effects of exenatide on β -endorphin precursor gene (POMC) expression (M) and β -endorphin release (N) in primary cultures of microglia. Primary microglial cells were collected from the cortex of 1-day-old neonatal rats. For the immunostaining study, double immunofluorescence staining of GLP-1R and IBA-1 (A–D), β -endorphin and IBA-1 (E–H), and β -endorphin and GLP-1R (I–L) was performed, and DAPI staining was used to identify cell nuclei. Scale bar, 25 μ M. To assess the stimulatory effect of exenatide, cultured primary microglial cells were incubated with gradient concentrations of exenatide (0.3, 1, 3, 10, 30, and 100 nM) for 2 hours. POMC expression in microglia and β -endorphin levels in the microglial culture medium were determined using real-time quantitative PCR and a commercial fluorescence immunoassay kit, respectively. Data are presented as means \pm S.D. ($n = 3$ in each treatment). DAPI, 4',6-diamidino-2-phenylindole.

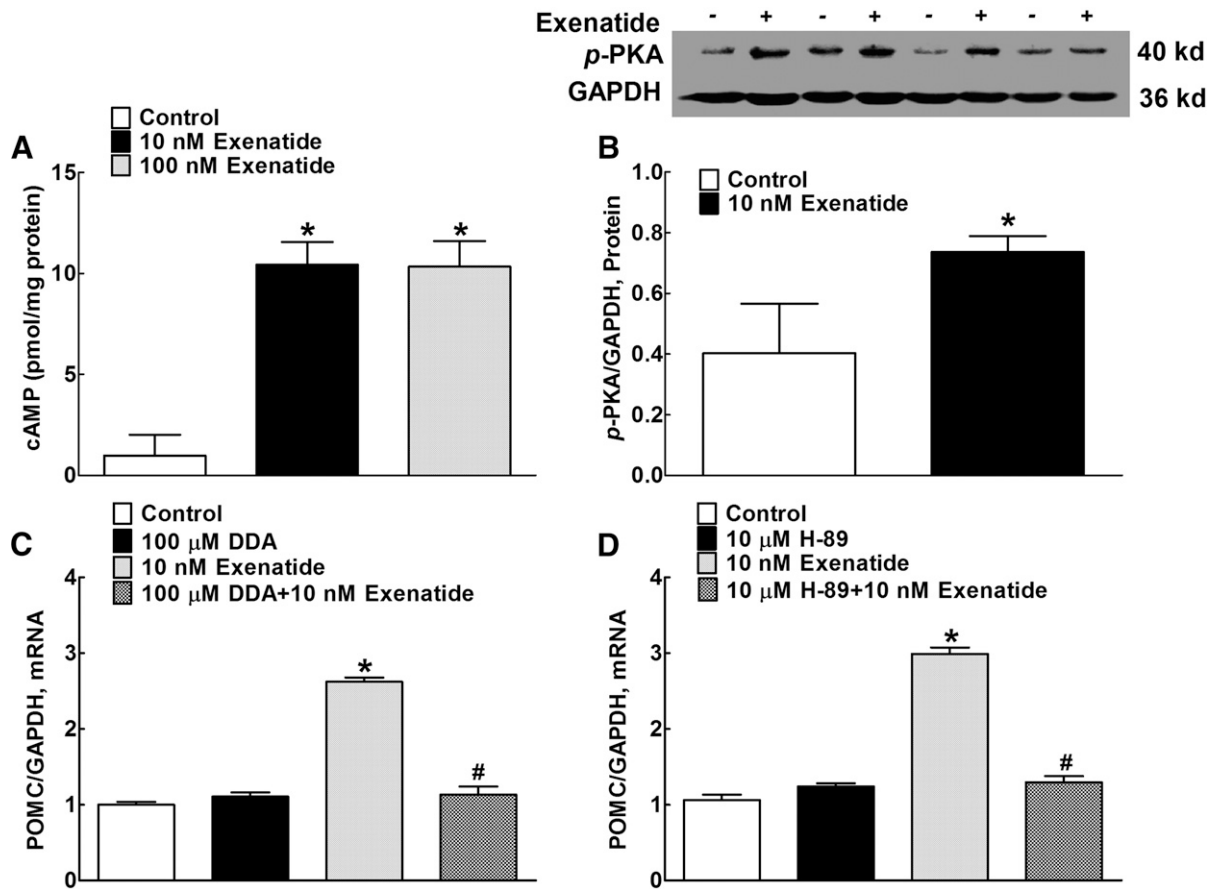


Fig. 2. Stimulatory effects of exenatide on intracellular cAMP levels (A) and PKA phosphorylation (B), and blockade effects of the adenylate cyclase inhibitor DDA (C) and PKA inhibitor H-89 (D) on exenatide-stimulated β -endorphin overexpression in primary cultures of microglia. Primary microglial cells were collected from the cortex of 1-day-old neonatal rats. For cAMP and *p*-PKA measurements, cultured primary microglia were incubated with exenatide for 30 minutes. Intracellular cAMP levels and *p*-PKA expression were measured using a fluorescence immunoassay kit and Western blotting, respectively. The representative gels are shown at the top of the figure. To test the blockade effects, DDA (100 μ M) and H-89 (10 μ M) were incubated with microglia for 1 hour before exenatide (10 nM) treatment. Two hours later, POMC gene expression was determined by real-time quantitative PCR. Data are means \pm S.D. ($n = 3$ to 4 in each treatment). * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the exenatide treatment group (unpaired and two-tailed *t* test and one-way ANOVA followed by post hoc Student–Newman–Keuls tests, respectively). DDA, 2',5'-dideoxyadenosine.

To further explore whether p38 activation was causally associated with exenatide-mediated POMC overexpression, pharmacological inhibition on activation of p38, JNK1/2, and ERK1/2 was tested. Incubation with the selective p38 inhibitor SB203580 (50 μ M) (Pyo et al., 1999; Lali et al., 2000; Yang et al., 2007), ERK1/2 inhibitor U0126 (50 μ M) (DeSilva et al., 1998), and JNK1/2 inhibitor SP600125 (50 μ M) (Bennett et al., 2001) did not alter basal POMC expression (Fig. 3, D–F). However, pretreatment (1 hour earlier) with SB203580 completely blocked exenatide-stimulated overexpression of POMC ($P < 0.05$, one-way ANOVA; Fig. 3D). In contrast, neither U0126 nor SP600125 significantly suppressed exenatide-increased POMC expression (Fig. 3, E and F).

To confirm the PKA dependence of exenatide-stimulated p38 activation in cultured primary microglia, incubation with exenatide (10 nM) for 30 minutes significantly stimulated p38 phosphorylation, which was completely blocked by pretreatment (1 hour before) with 10 μ M H-89 ($P < 0.05$, one-way ANOVA; Fig. 3G).

p38 β Phosphorylation Mediated Exenatide-Stimulated POMC Overexpression. As shown in Fig. 4A, transfection with siRNA/p38 α for 5 hours did not significantly alter p38 β mRNA expression compared with the nonspecific oligo control,

but it markedly reduced expression of p38 α mRNA by 57% ($P < 0.05$, one-way ANOVA). Similarly, siRNA/p38 α reduced the levels of p38 α (but not p38 β) protein by 58% ($P < 0.05$, one-way ANOVA; Fig. 4B). Moreover, compared with the nonspecific oligo control, transfection with siRNA/p38 β significantly reduced expression of p38 β (but not p38 α) mRNA and protein by 57% and 53%, respectively ($P < 0.05$, one-way ANOVA; Fig. 4, C and D).

We performed an additional study aimed to reveal the specific effects of p38 α and p38 β silencing on exenatide-stimulated p38 activation and POMC expression. Transfection with siRNA/p38 β completely blocked exenatide-stimulated total p38 phosphorylation, compared with the nonspecific oligo control ($P < 0.05$, one-way ANOVA; Fig. 4E). In contrast, siRNA/p38 α failed to alter exenatide-induced total p38 phosphorylation. In addition, transfection with siRNA/p38 β (but not siRNA/p38 α) completely attenuated exenatide-stimulated overexpression of POMC ($P < 0.05$, one-way ANOVA; Fig. 4F).

p38 α Phosphorylation Mediated Lipopolysaccharide-Stimulated Overexpression of Proinflammatory Cytokines. We also tested the possible attenuation of siRNA/p38 α and siRNA/p38 β on microglial expression of proinflammatory

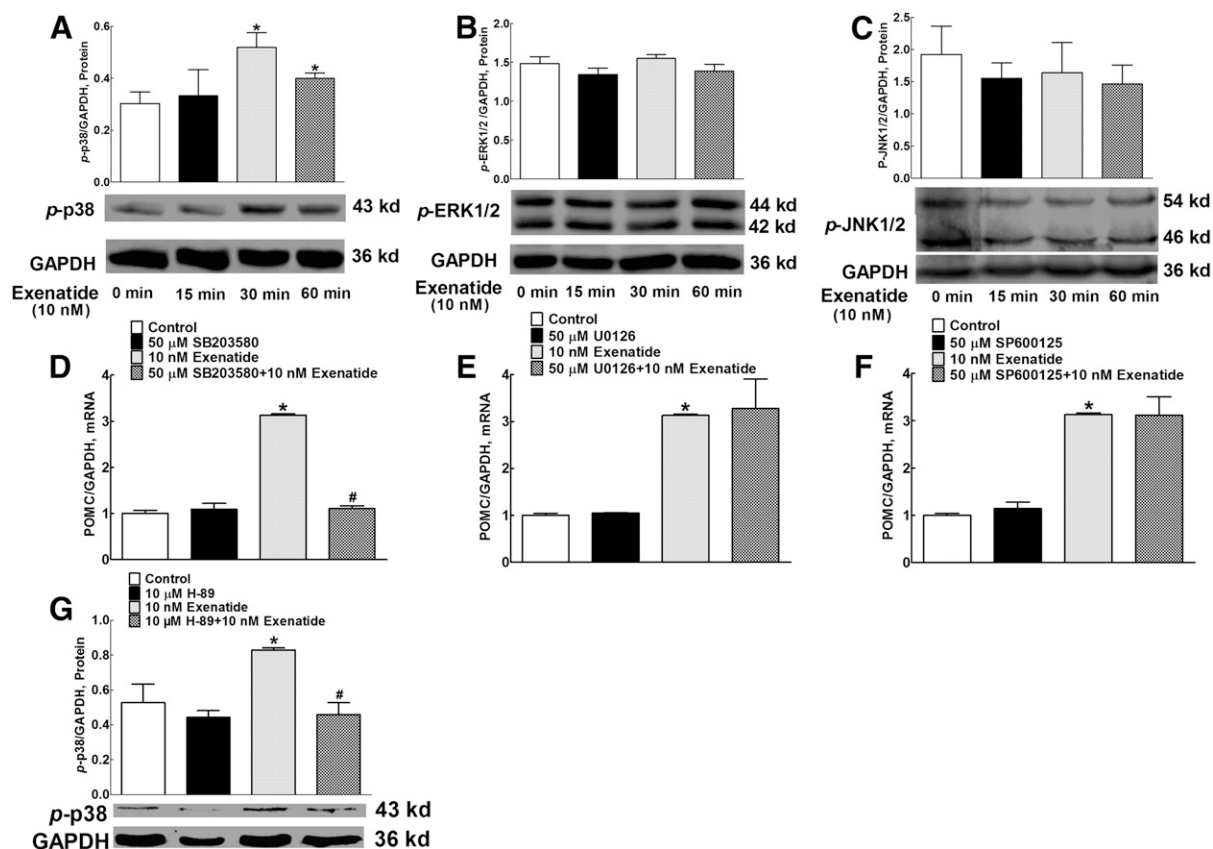


Fig. 3. Stimulatory effects of exenatide on phosphorylation of p38 (A), ERK1/2 (B), and JNK1/2 MAPK (C), and blockade effects of the p38 inhibitor SB203580 (D), ERK1/2 inhibitor U0126 (E), JNK1/2 inhibitor SP600125 (F), and PKA inhibitor H-89 (G) on exenatide-increased p38 phosphorylation and POMC mRNA expression in primary cultures of microglia. Primary microglial cells were collected from the cortex of 1-day-old neonatal rats. To measure MAPK phosphorylation, exenatide (10 nM) was incubated with microglia for 15, 30, and 60 minutes and phosphorylation of p38, ERK1/2, and JNK1/2 was determined using Western blotting. To test the blockade effects, SB203580 (50 μM), U0126 (50 μM), SP600125 (50 μM), and H-89 (10 μM) were incubated with microglia 1 hour before exenatide (10 nM) treatment. p38 phosphorylation or POMC mRNA expression was determined 0.5 or 2 hours later using Western blotting or real-time quantitative PCR. Data are means ± S.D. ($n = 3$ in each treatment), and the representative gels are shown in their respective panels. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the exenatide treatment group (one-way ANOVA followed by post hoc Student–Newman–Keuls tests).

cytokines. Treatment with lipopolysaccharide (LPS; *Escherichia coli* strain O26:B6; Sigma-Aldrich) for 1 hour significantly increased microglial total p38 phosphorylation, which was reduced 43% by pretransfection with siRNA/p38α ($P < 0.05$, one-way ANOVA). In contrast, knockdown of the p38β gene failed to affect LPS-stimulated p38 phosphorylation (Fig. 5A). Furthermore, treatment with LPS in microglia dramatically increased the expression of TNF-α, IL-1β, and IL-6 by 20-, 36-, and 880-fold, respectively. Pretransfection with siRNA/p38α partially attenuated LPS-induced overexpression of TNF-α, IL-1β, and IL-6 by 40%, 33%, and 24%, respectively ($P < 0.05$, one-way ANOVA). However, siRNA/p38β was not able to significantly reduce LPS-induced overexpression of proinflammatory cytokines (Fig. 5, B–D).

p38β Phosphorylation Mediated Exenatide-Induced Spinal POMC Overexpression and Mechanical Antiallodynia. To further confirm the causal role of p38β isoforms in exenatide-mediated spinal POMC overexpression and mechanical antiallodynia, specific siRNA/p38α and siRNA/p38β were employed in rats with neuropathy established 1 week earlier. Five groups of rats with neuropathy received consecutive 7-day intrathecal injections of vehicle, nonspecific oligos (5 μg/d), siRNA/p38α (5 μg/d), or

siRNA/p38β (5 μg/d). Mechanical withdrawal thresholds were measured once daily in both hindpaws prior to each siRNA injection. Multidaily intrathecal injections of either siRNA/p38α or siRNA/p38β were not able to alter basal withdrawal thresholds in both hindpaws (Fig. 6A). On the eighth day, the rats received a single bolus intrathecal injection of normal saline (10 μl) or exenatide (100 ng) and their hindpaws were subjected to mechanical stimuli 1 hour after injection. Intrathecal exenatide produced marked mechanical antiallodynia in the ipsilateral hindpaws, which was completely suppressed by knockdown of the p38β gene ($P < 0.05$, one-way ANOVA). In contrast, 7-day intrathecal injections of siRNA/p38α failed to significantly suppress exenatide-induced mechanical antiallodynia (Fig. 6B).

Upon completion of the behavioral assessment, spinal cord enlargements were isolated and divided into two parts for measurement of the expression of p38α and p38β (mRNA and protein) and POMC mRNA. The remaining portion was further divided into the contralateral and ipsilateral sides for measurement of p38 phosphorylation. Compared with the nonspecific oligo control, the consecutive 7-day intrathecal injections of siRNA/p38α effectively reduced expression of p38α mRNA and protein by 68% and 54%, respectively ($P < 0.05$, one-way ANOVA), without a significant reduction in

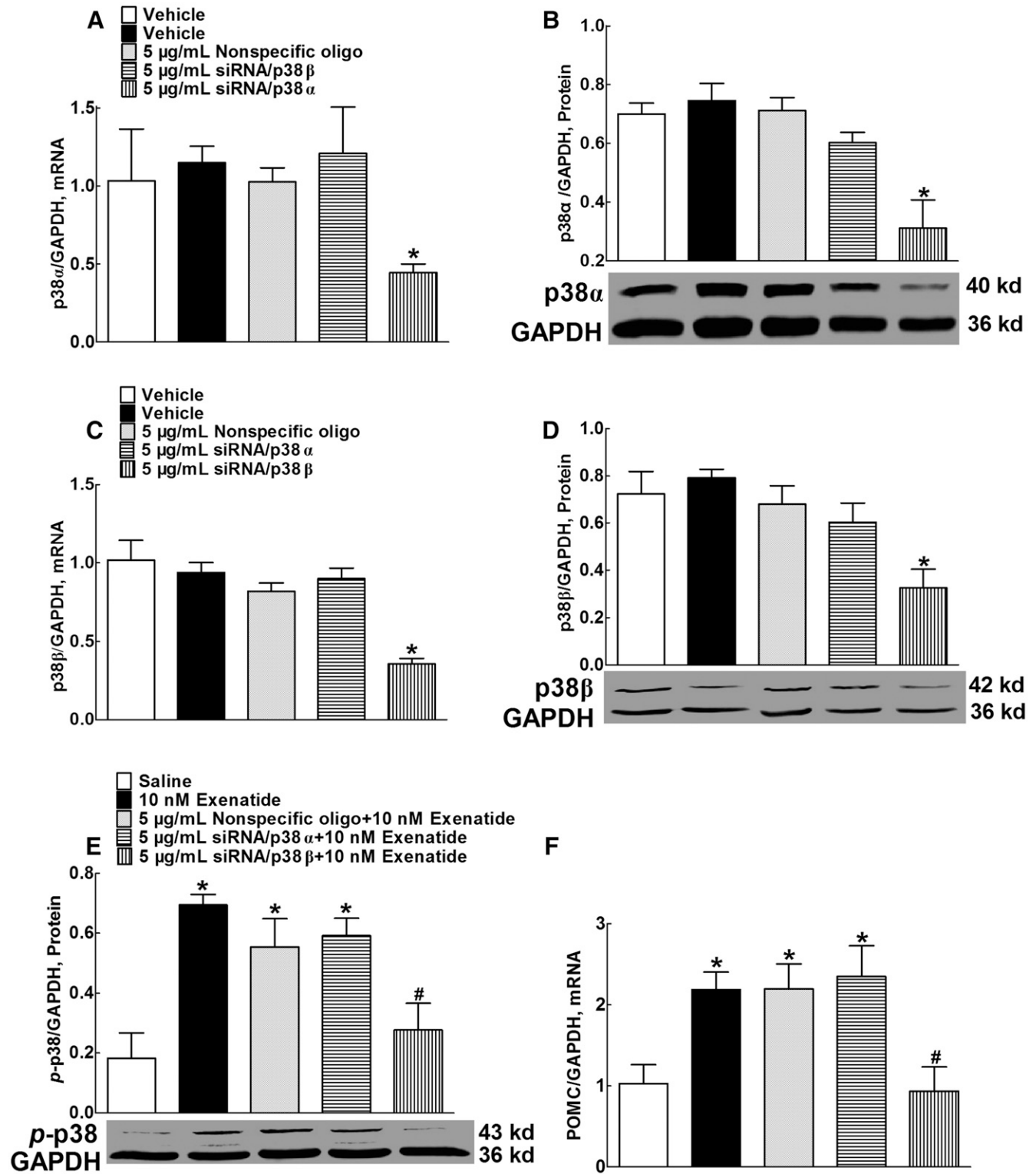


Fig. 4. Blockade effects of siRNA/p38 α and siRNA/p38 β on p38 α (A and B) and p38 β (C and D) gene and protein expression, exenatide-increased p38 phosphorylation (E), and β -endorphin gene expression (F). Primary microglial cells were collected from the cortex of 1-day-old neonatal rats. For the gene silencing study, equal concentrations (5 μ g/ml) of siRNA/p38 α , siRNA/p38 β , and nonspecific oligos were transfected with microglia by using DOTAP for 5 hours. p38 α and p38 β gene and protein expressions were determined by using real-time quantitative PCR and Western blotting, respectively, after further 24 hours interference. To test the blockade effects, siRNA/p38 α and siRNA/p38 β interference was performed in cultured microglia for 24 hours before exenatide (10 nM) challenge. p38 phosphorylation or expression of POMC was analyzed 0.5 or 2 hours after exenatide treatments by Western blotting or real-time quantitative PCR. Data are means \pm S.D. ($n = 3$ to 4 in each treatment), and the representative gels are shown in their respective panels. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the exenatide or LPS treatment group (one-way ANOVA followed by post hoc Student–Newman–Keuls tests).

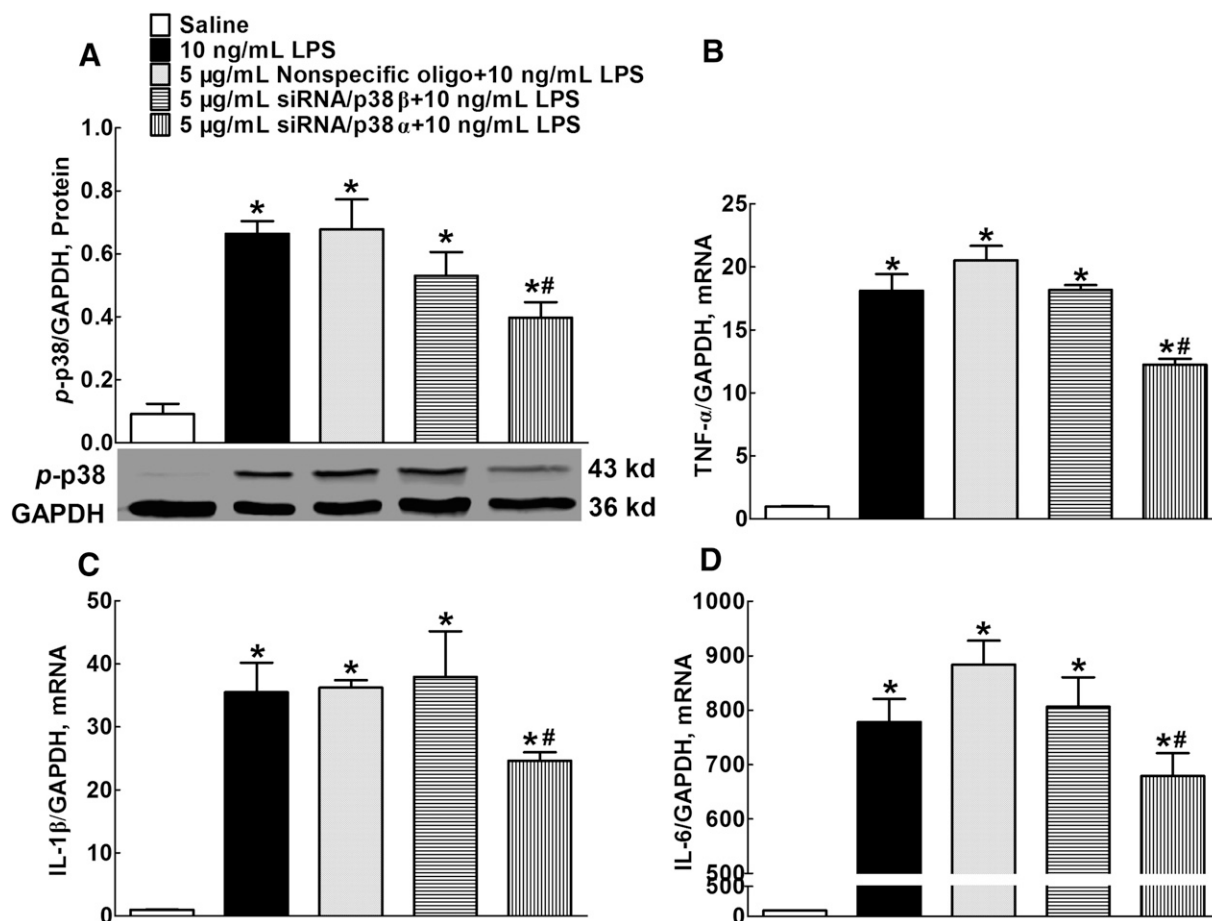


Fig. 5. Blockade effects of siRNA/p38 α and siRNA/p38 β on LPS-increased p38 phosphorylation (A) and expression of TNF- α (B), IL-1 β (C), and IL-6 (D). Primary microglial cells were collected from the cortex of 1-day-old neonatal rats. For the gene silencing study, equal concentrations (5 μ g/ml) of siRNA/p38 α , siRNA/p38 β , and the nonspecific oligos were transfected with microglia by using DOTAP for 5 hours. To test the blockade effects, siRNA/p38 α and siRNA/p38 β interferences were performed in cultured microglia for 24 hours before LPS (10 ng) challenge. p38 phosphorylation or expression of proinflammatory cytokines was analyzed 0.5 or 2 hours after LPS treatment by Western blotting or real-time quantitative PCR. Data are means \pm S.D. ($n = 3-5$ in each treatment), and the representative gels are shown in their respective panels. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the exenatide or LPS treatment group (one-way ANOVA followed by post hoc Student–Newman–Keuls tests).

p38 β mRNA and protein expression (Fig. 6, C and D). On the other hand, siRNA/p38 β reduced p38 β gene and protein expression by 40% and 47%, respectively, compared with the nonspecific oligo control ($P < 0.05$, one-way ANOVA), without reducing p38 α gene and protein expression (Fig. 6, E and F).

Further analyses were undertaken to test the possible blockade effects of p38 isoform gene silencing on exenatide-stimulated spinal p38 phosphorylation in the spinal cord of rats that were subjected to spinal nerve ligation 2 weeks earlier. As shown in the representative gels, the expression of p38 total phosphorylation in the contralateral spinal cord was not apparently different from that in the ipsilateral spinal cord. However, intrathecal exenatide significantly elevated total p38 phosphorylation in both the contralateral and ipsilateral spinal cord by the same degree. The stimulatory effect of exenatide was completely suppressed by intrathecal injection of siRNA/p38 β ($P < 0.05$, one-way ANOVA; Fig. 6G). In contrast, intrathecal siRNA/p38 α failed to significantly reduce exenatide-increased spinal total p38 activation. Figure 6H shows summarized group results from scanned gels.

Moreover, we aimed to uncover which p38 isoform was responsible for exenatide-stimulated spinal POMC overexpression. In agreement with cultured primary microglia,

intrathecal exenatide increased spinal expression of POMC mRNA, which was entirely inhibited by knockdown of the spinal p38 β gene, compared with the nonspecific oligo control ($P < 0.05$, one-way ANOVA). In contrast, intrathecal siRNA/p38 α was unable to significantly attenuate exenatide-stimulated spinal POMC overexpression (Fig. 6I).

CREB Phosphorylation Mediated Exenatide-Stimulated POMC Overexpression.

We further tested whether exenatide-induced microglial POMC overexpression was via CREB phosphorylation. Treatment with exenatide (10 nM) in cultured primary microglia for 30 minutes significantly elevated CREB phosphorylation by 113% ($P < 0.05$, unpaired and two-tailed t test; Fig. 7A). Exenatide also significantly increased expression of POMC mRNA by 2.4-fold, which was completely attenuated by pretreatment 1 hour earlier with the specific CREB inhibitor KG501 (25 μ M) (Best et al., 2004) ($P < 0.05$, one-way ANOVA; Fig. 7B).

We further confirmed whether CREB was a downstream acceptor of cAMP/PKA signaling. Incubation with exenatide (10 nM) stimulated microglial CREB phosphorylation by 110%, which was completely blocked by pretreatment (1 hour earlier) with H-89 (10 μ M) ($P < 0.05$, one-way ANOVA; Fig. 7C).

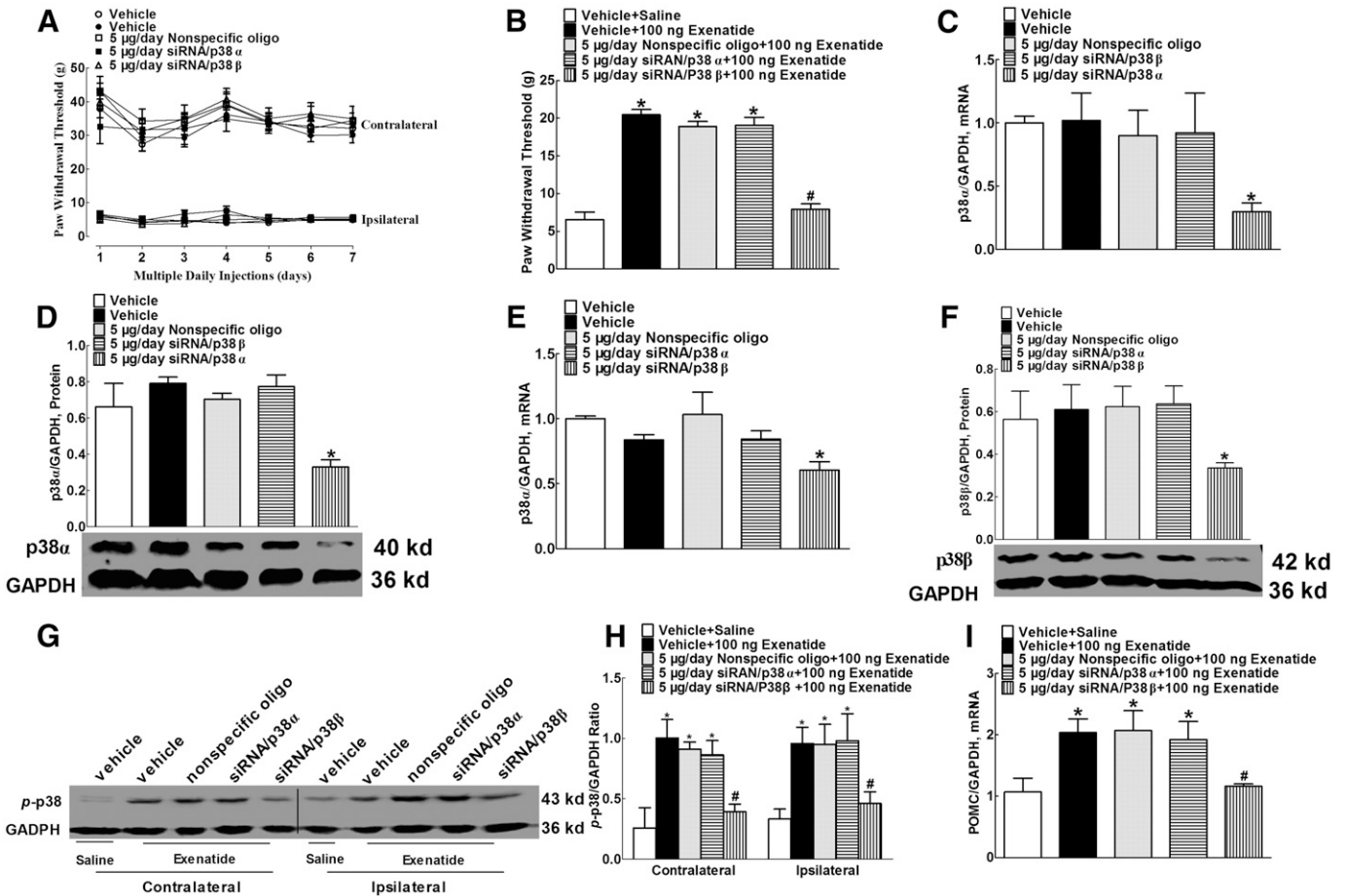


Fig. 6. Effects of multiple daily intrathecal injections of siRNA/p38 α and siRNA/p38 β MAPK on baseline paw withdrawal thresholds (A), exenatide-induced antinociception (B), spinal p38 α (C and D) and p38 β (E and F) gene and protein expression, and exenatide-induced spinal p38 phosphorylation (G and H) and POMC expression (I) in rats with established neuropathy. Rats received multiple daily intrathecal injections of vehicle (DOTAP, 40 μ g/d), nonspecific oligos (5 μ g/d), siRNA/p38 α (5 μ g/d), and siRNA/p38 β (5 μ g/d) for 7 days. Baseline paw withdrawal thresholds were measured once daily by using electronic von Frey filaments. On the eighth day, a single bolus of exenatide (100 ng) was injected intrathecally and mechanical nociceptive behaviors were quantified 1 hour after injection. Spinal lumbar enlargements were immediately obtained after completion of behavioral assessments. Expression of p38 α , p38 β , and POMC mRNA, levels of p38 α and p38 β protein, and phosphorylation p38 were determined by using real-time quantitative PCR and Western blotting, respectively. Data are means \pm S.D. ($n = 5$ to 6 in each group) and the representative gels are shown in their respective panels. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the exenatide treatment group (one-way ANOVA followed by post hoc Student–Newman–Keuls tests).

Finally, we determined whether CREB was activated by p38 β . As shown in Fig. 7D, incubation of exenatide (10 nM) in cultured microglial cells significantly stimulated CREB phosphorylation, which was completely blocked by pretransfection (5 hours earlier) with siRNA/p38 β , compared with the nonspecific oligo control ($P < 0.05$, one-way ANOVA). Distinctly, knockdown of p38 α did not significantly alter exenatide-increased CREB phosphorylation.

Discussion

GLP-1R agonism by peptidic agonists GLP-1-(7–36) and exenatide, nonpeptidic agonist WB4-24 (He et al., 2012), and iridoid agonists of herbal origin (including catalpol and shanzhiside methylester) produce antinociception and neuroprotection through β -endorphin overexpression from the spinal cord and hippocampus (Gong et al., 2014a, b; Fan et al., 2015, 2016; Jia et al., 2015). Our study further identified the cAMP/PKA/p38 β /CREB signaling that mediated

exenatide-induced microglial β -endorphin expression. Furthermore, we also revealed the antinociceptive role of p38 β .

Although β -endorphin is expressed in neurons (Fichna et al., 2007), microglia (Fan et al., 2015), and astrocytes (Hauser et al., 1990), GLP-1R-induced β -endorphin expression occurs only in microglial cells originating from the cortex (Fan et al., 2015), hippocampus (Jia et al., 2015), or spinal cord (Gong et al., 2014b). We further demonstrated coexpression of GLP-1Rs and β -endorphin in microglia. Moreover, treatment with exenatide concentration-dependently increased spinal POMC expression and β -endorphin release, with EC_{50} values of 4.1 and 7.5 nM, close to those for insulin expression in pancreatic islets (Baggio and Drucker, 2007). These results provide histologic and functional couplings for the microglial GLP-1R/ β -endorphin pathway.

cAMP/PKA signaling through G_{as} has been identified as a classic pathway associated with GLP-1R-stimulated insulin expression in pancreatic β cells (Koole et al., 2013). In this study, exenatide markedly increased intracellular cAMP levels and activated PKA, in parallel with POMC expression.

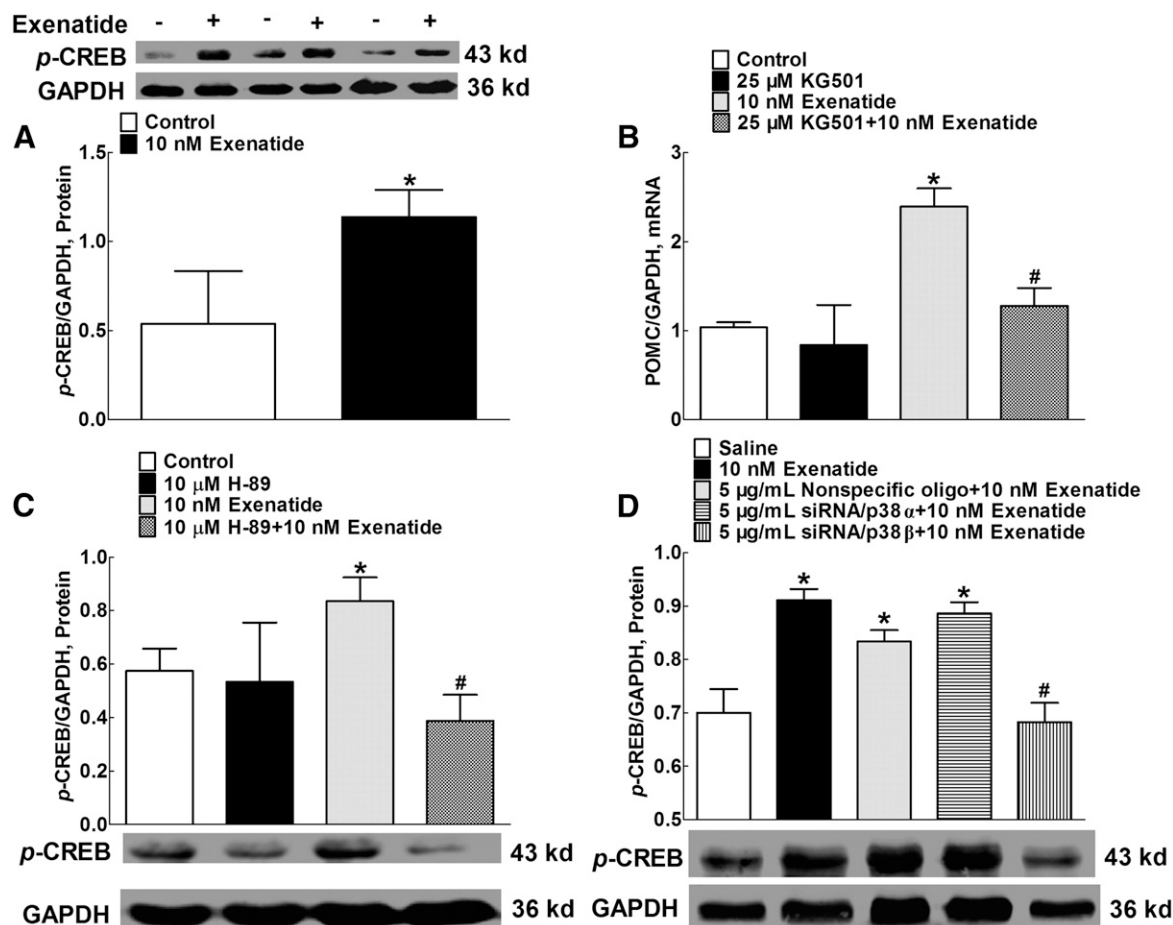


Fig. 7. Stimulatory effects of exenatide on CREB phosphorylation (A), and blockade effects of the CREB inhibitor KG501 on exenatide-induced β -endorphin gene (POMC) overexpression (B), and PKA inhibitor H-89 (C) and gene silencers siRNA/p38 α and siRNA/p38 β (D) on exenatide-stimulated CREB phosphorylation in primary cultures of microglia. Primary microglial cells were isolated from the cortex of 1-day-old neonatal rats. For CREB phosphorylation, Western blotting was used to determine incubation of exenatide (10 nM) with microglial cells for 30 minutes and CREB phosphorylation. To test the blockade effects, KG501 (25 μ M) and H-89 (10 μ M), or siRNA/p38 α (5 μ g/ml) and siRNA/p38 β (5 μ g/ml) in a formulation with the transfection reagent (DOTAP, 50 μ M), were incubated with microglial cells 1 or 24 hours before exenatide (10 nM) treatment. CREB phosphorylation or POMC expression was analyzed by using Western blotting or real-time quantitative PCR. Data are means \pm S.D. ($n = 3$ to 4 in each treatment) and the representative gels are shown in their respective panels. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the exenatide treatment group (unpaired and two-tailed t test and one-way ANOVA followed by post hoc Student–Newman–Keuls test).

Furthermore, pharmacological inhibition of cAMP production and PKA activation completely inhibited exenatide-induced POMC expression. Thus, cAMP/PKA signaling mediates GLP-1R-induced microglial POMC expression, along with GLP-1R-induced insulin expression in pancreatic β cells (Drucker et al., 1987).

Involvement of MAPKs in GLP-1R activation is complicated and controversial (MacDonald et al., 2002; Kawasaki et al., 2010; Lee et al., 2012). In this study, activation of p38 (but not JNK1/2 or ERK1/2) mediated exenatide-induced p38 phosphorylation and POMC expression, which were completely blocked by the selective p38 (but not JNK1/2 or ERK1/2) inhibitor. The results are consistent with our previous findings in neuropathic rats, in which intrathecal GLP-1R iridoid agonist shanzhiside methylester specifically stimulated spinal phosphorylation of p38 (but not JNK1/2 or ERK1/2) and expression of β -endorphin, which were entirely blocked by the p38 (but not ERK1/2 and JNK1/2) inhibitor (Fan et al., 2016). Collectively, p38 phosphorylation mediates exenatide-induced microglial POMC overexpression. Moreover, exenatide-induced p38 activation was nearly entirely reduced

by H-89, indicating an upstream induction role of cAMP/PKA signaling in p38 phosphorylation. These results are supported by previous findings in which p38 was activated after cAMP-PKA signaling by activation of family A of the G protein-coupled receptors, such as β -adrenergic receptors (Yamauchi et al., 1997; Yin et al., 2006; Hattori et al., 2016).

Our finding that p38 β plays a crucial role in exenatide-induced microglial expression of β -endorphin and mechanical allodynia is particularly compelling. Exenatide in primary microglia induced marked activation of total p38, shows no deviation from previous findings in pancreatic β cells and Chinese hamster ovary cells (Macfarlane et al., 1997; Montrose-Rafizadeh et al., 1999). However, knockdown of p38 β but not p38 α completely attenuated exenatide-stimulated total p38 phosphorylation. Furthermore, intrathecal injection of exenatide also caused similar activation of p38 in the contralateral and ipsilateral spinal cord. Consistent with the results in primary microglia, exenatide-induced total p38 phosphorylation in contralateral and ipsilateral spinal cord was completely blocked by 7-day intrathecal siRNA/p38 β , but not by siRNA/p38 α . These results indirectly indicate that

exenatide-stimulated phosphorylation of total p38 is entirely originated from the p38 β isoform. Moreover, knockdown of p38 β (but not p38 α) also completely blocked exenatide-increased POMC expression in primary microglia, as well as spinal POMC expression and mechanical antiallodynia in neuropathic rats. These results solidify our postulation that exenatide specifically induces spinal p38 β phosphorylation, which subsequently mediates β -endorphin overexpression and antinociception. The broad impact of spinal p38 β activation on endogenous opioid secretion and subsequent antinociception is supported by recent observations in which intrathecal siRNA/p38 β blocked cynamidone A- or bulleyacotine A-induced spinal microglial total p38 activation and β -endorphin or dynorphin A expression, and spinal antinociception (Huang et al., 2017; Li et al., 2017).

It is interesting to note that partial knockdown of p38 β (40%–70% in our *in vitro* and *in vivo* settings) was able to fully inhibit exenatide-induced total p38 activation and POMC expression in primary microglia, as well as spinal POMC expression and mechanical antiallodynia in neuropathic rats. Although it is possible that our endpoints may not be sufficiently sensitive to distinguish partial inhibition from full inhibition, it is more likely that the p38 β protein remaining after RNA interference does not functionally serve to trigger the downstream signaling, and siRNA could just affect p38 β levels by reducing the *de novo* and “active” form rather than the existing and “inactive” form. To illustrate the mechanisms, we need to perform spatial and temporal experiments using different concentrations of siRNA/p38 β and exenatide to determine the minimal p38 protein level that is required for exenatide to execute biofunctions. Nevertheless, the separation phenomenon of knockdown of spinal microglial p38 β has also been shown in formalin and substance P nociception (Svensson et al., 2005) and in bulleyacotine A (Li et al., unpublished data) and cynamidone A antinociception (Huang et al., unpublished data). Moreover, partial knockdown of spinal GLP-1Rs, α 3 glycine receptors, or D-amino acid oxidase also completely inhibited their mediation of antinociception or nociception (Chen et al., 2012a; Zhang et al., 2013; Gong et al., 2014b).

In agreement with previous findings (Bachstetter et al., 2011; Xing et al., 2011), treatment with LPS in cultured primary microglia markedly activated p38 and dramatically stimulated expression of TNF- α , IL-6, and IL-1 β , which were partially attenuated by knockdown of p38 α but not p38 β , suggesting that LPS stimulated phosphorylation of p38 partially through p38 α and the remaining activity may not be involved in p38 β . The partial involvement of p38 α on LPS-induced total p38 activation and overexpression of proinflammatory cytokines does not appear due to its partial knockdown, because nearly all of the MAPK members are involved in LPS-induced microglial activation (Johnson and Lapadat, 2002; Nikodemova et al., 2006). Indeed, knock-out/mutation of p38 α (but not p38 β) only partially reduced LPS-induced proinflammatory cytokine production (O’Keefe et al., 2007; Li et al., 2008; Xing et al., 2011). Hence, GLP-1R agonism by exenatide specifically activates p38 β , which fully mediates POMC overexpression; in contrast, in addition to other signaling molecules (but not p38 β), LPS activates p38 α , which partially induces overexpression of proinflammatory cytokines. The multitude of these results highlights differential roles of p38 α and p38 β in inflammation and nociception.

CREB belongs to the basic leucine-zipper family of transcription factors and is a key transcription element for insulin gene expression after GLP-1R agonism (Shaywitz and Greenberg, 1999; Dalle et al., 2011). POMC expression was also stimulated by corticotrophin-releasing factor through the cAMP/PKA/CREB signaling in the anterior pituitary (Kraus and Höllt, 1995). We thus further detailed the involvement of CREB in the regulation of exenatide-mediated POMC overexpression and its p38 β dependence. Exenatide stimulated CREB phosphorylation and mediated POMC overexpression, the latter of which was completely attenuated by the CREB inhibitor. Although PKA was originally identified to be a direct activator of CREB phosphorylation at serine residue 133, ramification of the cAMP/PKA signaling is also involved in the diversity of signaling molecules including p38 (Delghandi et al., 2005; Dalle et al., 2011). In our study, exenatide-stimulated POMC overexpression was entirely attenuated by the p38 inhibitor and knockdown of p38 β (but not p38 α) and, more specifically, exenatide-induced CREB phosphorylation was completely suppressed by silencing p38 β but not p38 α . Therefore, CREB phosphorylation is intermediated through p38 β , rather than direct PKA phosphorylation. Similar findings have been reported in which p38 β and p38 δ (but not p38 α) were essential for arsenite-stimulated CREB activation in mouse epidermal cells (Che et al., 2013). Moreover, the blockade effect of H-89 also supports PKA dependence for p38 β to activate CREB. In summary, the G $_s$ /cAMP/PKA/p38 β /CREB signaling pathway entirely mediates GLP-1R-induced microglial β -endorphin overexpression and subsequent neuroprotection and antinociception (Fig. 8).

Spinal p38 activation induces overexpression of proinflammatory cytokines, which is associated with neuropathic pain (Ji and Suter, 2007; Ji et al., 2009). However, debate continues regarding whether p38 and its isoforms could be targeted for

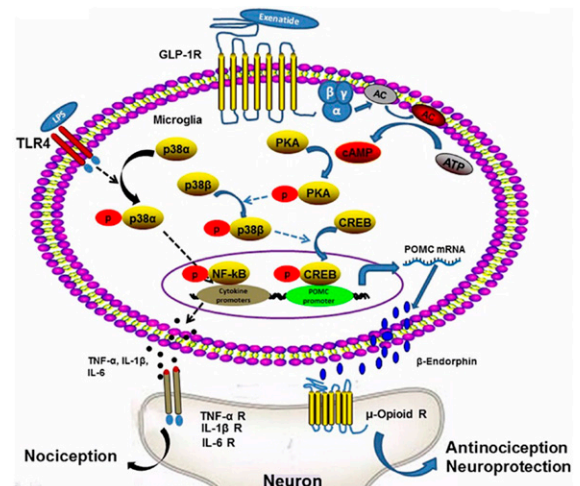


Fig. 8. Proposed signal transduction pathways for GLP-1R- and TLR4 receptor-induced expressions of β -endorphin and proinflammatory cytokines in microglia. After agonism of GLP-1Rs, the cAMP/PKA, p38 β (but not p38 α) MAPK, and CREB signals are successively activated, which mediate β -endorphin expression and subsequent neuroprotection and antinociception (blue lines). On the other hand, p38 α MAPK and NF- κ B signals are successively activated after agonism of TLR4 receptors by LPS, which partially mediate expression of proinflammatory factors including TNF- α , IL-1 β , and IL-6 and subsequent nociception (black line). TLR, Toll-like receptor.

the treatment of neuropathic pain (Schäfers et al., 2003; Galan-Arriero et al., 2014). p38 inhibitors minocycline and SB203580 are generally not antinociceptive when neuropathy is established, although they may be effective in preventing initiation of neuropathic pain (Schäfers et al., 2003; Mei et al., 2011; Fan et al., 2016). p38 phosphorylation in the ipsilateral spinal cord was not significantly elevated in neuropathic rats approximately 14 days after peripheral nerve injury, which is supported by the previous finding that spinal p38 was phosphorylated between 5 hours and 3 days after spinal nerve ligation and its activation returned to baseline in 5 days (Schäfers et al., 2003). Furthermore, 7-day intrathecal administration of either siRNA/p38 α or siRNA/p38 β did not alter withdrawal thresholds in ipsilateral paws. These findings support the notion that phosphorylation of p38 and its α or β isoform may not mediate nociception or antinociception in established neuropathy. However, silencing spinal p38 β (but not p38 α) by using the antisense oligonucleotides was shown to attenuate bone cancer pain, tissue injury, and inflammatory hyperalgesia induced by formalin, substance P, and carrageenan (Svensson et al., 2005; Fitzsimmons et al., 2010; Dong et al., 2014). In contrast, systemic LPS-induced production of IL-1 β and TNF- α was not altered by genetic knockout of p38 β (O'Keefe et al., 2007; Xing et al., 2013). The reasons for these controversial findings are not known but might be associated with different spinal p38 β phosphorylation levels in these pain models at different stages.

Authorship Contributions

Participated in research design: Wu, Wang.

Conducted experiments: Wu, Mao, Fan.

Performed data analysis: Wu, Wang.

Wrote or contributed to the writing of the manuscript: Wu, Wang.

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