Intrathecal Etanercept Partially Restores Morphine’s Antinociception in Morphine-Tolerant Rats via Attenuation of the Glutamatergic Transmission

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BACKGROUND: Long-term exposure to morphine leads to analgesic tolerance. In addition to an opioid receptor conformational change, enhancing the glutamatergic signal transmission is also involved in morphine tolerance. Tumor necrosis factor-α (TNF-α) has been demonstrated to correlate with neuronal plasticity via activation of glutamatergic transmission. We examined the effect of etanercept, a tumor necrosis factor-α inhibitor on morphine tolerance in rats.

METHODS: Male Wistar rats were implanted with 2 intrathecal (IT) catheters, and 1 IT catheter was connected to a mini-osmotic pump, used for either morphine infusion (15 μg/h) or saline (1 μL/h) infusion for 5 days. On day 5, either etanercept (50 μg) or saline (10 μL) was injected after discontinued morphine infusion. Three hours later, acute morphine (15 μg/10 μL, IT) treatment was given and all rats received a nociceptive tail-flick test.

RESULTS: The results showed that acute etanercept (50 μg) treatment caused a significant antinociceptive effect of morphine in morphine-tolerant rats. Western blotting indicated that etanercept attenuated the downregulation of membrane glutamate transporters GLT-1 and GLAST in morphine-tolerant rats. Etanercept also inhibited the upregulation of surface AMPA-receptor and N-methyl-D-aspartate–receptor subunits, including GluR1/GluR2 and NR1/NR2A.

CONCLUSIONS: These results demonstrate that etanercept partially restores the antinociceptive effect of morphine in morphine tolerance after a morphine challenge. Etanercept has potential for use in the clinical management of pain, particularly in patients who require long-term opioid treatment, and the effectiveness of which can be hampered by tolerance. (Anesth Analg 2011; X:000–000)

Opioids are potent analgesics and widely used for pain management. Long-term use of opioids leads to tolerance, which limits their utility. Morphine tolerance is a pharmacological phenomenon characterized by a shift of the dose–response curve to the right with a larger 50% effective dose.1 Koob and Bloom2 described 2 possible mechanisms of drug tolerance: within-system and between-system adaptation. Investigations of the mechanisms of within-system opioid adaptation include examination of the role of intracellular adenyl cyclase and cyclic adenosine monophosphate–dependent protein kinase A,3–5 G protein uncoupling,6,7 β-arrestin binding and internalization of opioid receptors,8–10 and μ-opioid receptor oligomerization.11,12 Studies of between-system adaptation have focused on glutamatergic synaptic transmission via activation of N-methyl-D-aspartate (NMDA) receptor function,13–17 downregulation of glutamate transporters (GTs),17,18 glia activation with increasing proinflammatory cytokine expression,19,20 and apoptosis.18

Tumor necrosis factor (TNF)-α, a 17-kDa peptide, is constitutively expressed in neurons and glial cells of the central nervous system21 and is released from neurons and glia upon stimulation.22–24 In H4 astroglia cells, TNF-α and corepressor N-myc suppress EAAT2/GLT-1 gene expression.25 TNF-α may also enhance synaptic strength by increasing the expression of the AMPA receptor.26 Hence, TNF-α could regulate the expression of GTs and receptors and influence the function of the glutamatergic system. Previous reports suggest that TNF-α has an important role in the function of the neural circuit by modulating glutamatergic synaptic transmission.27,28

TNF inhibitors have been widely used for the treatment of rheumatoid arthritis (RA) and other inflammatory autoimmune diseases.29 Etanercept (Enbrel; Amgen, Thousand Oaks, CA, and Wyeth) is a recombinant soluble p75 receptor that binds to the released TNF-α and prevents its interaction with membrane receptors, thereby modulating TNF-α–mediated signal transduction.30 Etanercept has potential for treatment of inflammatory conditions characterized by an excess of TNF-α, such as ankylosing spondylitis,31 Crohn disease,32 and Alzheimer disease.33 Etanercept is approved for the treatment of RA, juvenile RA, psoriasis, ankylosing spondylitis, and psoriatic arthritis in the United States. In the present study, we examined the effect of a
single intrathecal (IT) etanercept injection on the antinociceptive effect of morphine in morphine-tolerant rats and its underlying mechanisms.

METHODS

Animal Preparation and IT Drug Delivery
Eight-week-old male Wistar rats (300–350 g, n = 112) were sedated with pentobarbital (50 mg/kg, intraperitoneally; Sigma, St. Louis, MO) and then under isoflurane anesthesia. All rats were implanted via the atlantooccipital membrane with 2 IT polyethylene (PE)-10 catheters (0.8 cm), which were advanced caudally to the lumbar enlargement of the spinal cord. The level of L1-2 spinal bony structure corresponds to the spinal cord segments L5, L6, and S1-3, which are responsible for the tail flick reflex. The ends of the catheters were externalized and fixed to the dorsal aspect of the head. One IT catheter was connected to a mini-osmotic pump, implanted in the intercapsular region, and used for either morphine (15 μg/h) or saline (1 μL/h) infusion for 5 days. After the operation, all rats were housed individually and maintained on a 12-hour light/dark cycle with food and water freely available. During the experiment, any rats that developed any abnormal behavior (such as poor appetite, decreased stool passage, decreased daily activity, frequent vocalizations, or flaccidity), motor deficit, or obstructed IT catheter were excluded from the experiments. The use of rats conformed to the Guiding Principles in the Care and Use of Animals of the American Physiological Society and was approved by the National Defense Medical Center Animal Care and Use Committee.

Construction of the IT Catheter
The IT catheter was constructed using an 8-cm PE tube (0.008-in. inner diameter, 0.014-in. outer diameter; Spectranetics, Colorado Springs, CO) and a 3.5-cm silastic tube (Dow Corning, Midland, MI). The silastic tube was inserted into the PE tube and the joint sealed with epoxy resin and silicon rubber. The dead space of the IT catheter was approximately 8 μL.

Nociceptive Test
The antinociceptive test was modified and adapted from our previous studies.19,35 Tail-flick latency, by the hot water immersion test (52°C ± 0.5°C), was measured before drug infusion and daily after the start of drug infusion for 5 days. The baseline latency was approximately 1.95 ± 0.04 seconds with a cutoff time of 10 seconds. Rats were placed in plastic restrainers for drug injection and antinociception assessment. All drug infusions were performed at the rate of 1 μL/h via a mini-osmotic pump (Alzet, Cupertino, CA). On day 5, the IT catheter for drug infusion was cut, and either etanercept (50 μg in 10 μL; Enbrel, Amgen and Wyeth) or saline (10 μL) was administered via the other IT catheter. Three hours later, a morphine challenge test (15 μg/10 μL saline) was performed and antinociceptive response was examined for each study group. The tail-flick latency was recorded at 30, 60, 90, and 120 minutes after morphine administration. The areas under the curve (AUCs) were from time 0 to 120 minutes for measurement of the antinociceptive effect of morphine.

Spinal Cord Preparation
Three hours after discontinuation of the IT drug infusion and simultaneous infusion of etanercept or saline treatment on day 5, rats were killed by exsanguinations under isoflurane anesthesia (ABBOTT; Abbott Laboratories, Ltd., Queenborough, Kent, UK). Laminectomy was then performed at the lower edge of the 12th thoracic vertebra; the L5-S1 segment of the spinal cord was removed immediately and separated into ventral and dorsal parts. The dorsal parts were stored at −80°C until used for Western blotting.

Preparation of Spinal Cord Plasma Membrane, Cytosolic, and Nuclear Fractions for Western Blotting Analysis
Each dorsal section of the spinal cords was fractionated into cytosolic, membrane, and nuclear fractions with the cytoplasmic, nuclear, and membrane compartment protein extraction kit as recommended by the manufacturer (BioChain Institute, Inc., Hayward, CA). The membrane fraction was checked by Western blotting with anti-EGFR (epidermal growth factor receptor) (MBL, Naka-ku, Nagoya, Japan) for specificity.

Western Blotting Analysis
The membrane fractions were denatured by heating at 95°C for 10 minutes in an equal volume of sodium dodecyl sulfate (SDS) sample buffer, then separated on SDS–10% polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane (ImmobilonTM; Millipore, Bedford, MA). The membrane was blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 and incubated overnight at 4°C with polyclonal guinea pig antirat GLT-1 (1:5000) or antirat GLAST antibodies (1:5000), polyclonal rabbit antirat EAAC1 antibodies (1:5000) or rabbit antirat GluR1 (1:5000), or GluR2 (1:5000), or NR1 (1:5000) (all from Chemicon, Temecula, CA), then with the corresponding horseradish peroxidase–conjugated secondary antibody (1:5000, donkey antiguinea pig or goat antirabbit immunoglobulin G or goat antimouse immunoglobulin G, all from Chemicon) for 1 hour at room temperature. After reaction with ECL solution (Amersham, Arlington Heights, IL), the bound antibody was visualized using a chemiluminescence imaging system (Syngene, Cambridge, UK). Finally, the blots were incubated at 56°C for 18 minutes in stripping buffer (0.0626 mol/L Tris-HCl, pH 6.7, 2% SDS, 0.1 mol/L mercaptoethanol) and reprobed with a monoclonal mouse anti-EGFR (Sigma) as the loading control. The optical density of each specific band was measured using a computer-assisted imaging analysis system (GeneTools Match software; Syngene).

Data and Statistical Analysis
All data are presented as the mean ± SEM where n is the number of rats used. Tail-flick latency data were analyzed using 2-way analysis of variance with post hoc Bonferroni test. A significant difference was defined as a P value <0.05. For immunoreactivity data, the intensity of each test band was expressed as the relative optical density...
calculated with respect to the average optical density for the corresponding control band. For statistical analysis, immunoreactivity was analyzed using 1-way analysis of variance, followed by multiple comparisons with the Bonferroni test. A significant difference was defined as a \( P < 0.05 \).

RESULTS

**Etanercept Restored the Antinociceptive Effect of Morphine in Chronic Morphine-Infused Rats**

Similar to our previous studies, \(^{17,19}\) morphine challenge (15 \( \mu \)g, IT) produced an antinociceptive effect in saline-infused rats (AUC = 934.5 ± 13.12 minutes \( \times \) seconds), but not in morphine-infused rats (AUC = 286.43 ± 9.40 minutes \( \times \) seconds) 3 hours after discontinuation of pump infusion on day 5. Etanercept (50 \( \mu \)g) did not alter the tail-flick latencies in saline-infused rats and morphine-infused rats before morphine challenge, as shown in Figure 1A. However, a significant antinociceptive effect of the morphine challenge was observed in rats that received etanercept (50 \( \mu \)g) and showed an AUC = 622.61 ± 22.52 minutes \( \times \) seconds, as shown in Figure 1B.

**Etanercept Treatment Inhibited the Downregulation of Membrane GTs After Morphine Challenge in Chronic Morphine-Infused Rat Spinal Cord**

We found a 50% to 60% reduction on the expression of membrane GTs GLAST, GLT-1, and EAAC1 in the dorsal horn of morphine-tolerant rat spinal cords (Fig. 2). This phenomenon was not observed in rats that received IT saline infusion followed by either saline or etanercept treatment. Etanercept (50 \( \mu \)g) not only restored the level of GLAST and GLT-1 expression in the dorsal horns of morphine-tolerant rat spinal cords, but exceeded the levels in the control group. However, the expression of EAAC1 was unaffected by etanercept (50 \( \mu \)g) in morphine-tolerant rats.

**Chronic Morphine Infusion Upregulates Surface AMPA- and NMDA-Receptor Subunit Expression in Rat Spinal Cords**

We also examined the expression of ionotropic glutamate receptors on the plasma membrane in the dorsal horn of...
morphine-tolerant rat spinal cords. The time-course changes of AMPA and NMDA receptors in chronic morphine-infused rats showed that there was an increase in the expression of GluR1, GluR2, and NR1 subunits on days 1, 3, and 5 (Fig. 3, A and B; \( P < 0.05 \)).

**Etanercept Attenuated AMPA and NMDA Receptor Expression on the Plasma Membrane of Chronic Morphine-Infused Rat Spinal Cord**

On day 5, the expression of GluR1 was increased by 3.83-fold, GluR2 increased by 2.27-fold, and NR1 increased by 2.17-fold after morphine infusion, compared with the control group (Fig. 4, A and B). The enhancement on the expression of AMPA- and NMDA-receptor subunits on the plasma membrane was completely abolished by etanercept (50 \( \mu \)g) treatment in morphine-tolerant rats (Fig. 4, A and B; \( P < 0.05 \)).

**DISCUSSION**

In this study, we demonstrated that the TNF-\( \alpha \) inhibitor etanercept partially restores the antinociceptive effect of morphine in morphine-tolerant rats. Etanercept prevented not only the downregulation of the glial membrane GTs GLT-1 and GLAST, but also attenuated the upregulation of surface AMPA and NMDA receptors. Etanercept has been used to treat RA since 1998\(^{32} \) and has been widely studied in the treatment of inflammatory disorders. A pilot study indicated that perispinal administration of etanercept dramatically improves cognitive function of Alzheimer patients.\(^{33} \) In our present study, IT etanercept (50 \( \mu \)g) pretreatment restored the antinociceptive effect of morphine in morphine-tolerant rats.

Emerging evidence suggests that opioid tolerance and abnormal pain sensitivity may share common cellular mechanisms and be mediated, at least in part, through the glutamatergic receptor system.\(^{13,17,37} \) Glutamate is a major excitatory neurotransmitter in the mammalian central nervous system. Excess glutamate in the synaptic cleft leads to NMDA receptor activation and \( \text{Ca}^{2+} \) influx, which causes neuronal plasticity and even excitotoxicity\(^{38} \). Glutamate homeostasis is crucial in maintaining efficient synaptic communication and prevention of neurotoxicity.\(^{39} \) Glutamate is rapidly cleared from the synaptic cleft by diffusion and reuptake in normal intact brain tissue without compromised energy supply.\(^{40} \) The reuptake is accomplished by GTs localized on the cytoplasmic membrane of astrocytes and neurons.\(^{40,41} \) Mao et al.\(^{18} \) demonstrated a correlation among GT downregulation, morphine tolerance, and thermal hyperalgesia in chronic...
morphine-treated but not saline-treated rats. Similarly, our previous studies also demonstrated that coinfusion of the NMDA antagonist MK801, dexamethasone, and amitriptyline prevented GT downregulation, and the subsequent EAA elevation in the rat spinal CSF dialysates after morphine challenge, thus retained the antinociceptive effect of morphine. In the present study, we examined the membrane GT expression in morphine-tolerant rats instead of total lysate because only GTs on the plasma membrane reuptake glutamate from the synaptic cleft. In addition, our previous study demonstrated a rapid GT trafficking from cytoplasm onto the plasma membrane by amitriptyline pretreatment before the morphine challenge in morphine-tolerant rats. We therefore examined the membranous GTs and found that chronic morphine infusion led to downregulation of membrane GLAST, GLT-1, and EAAC1 in the dorsal horn of rat spinal cords, whereas etanercept inhibited the downregulation of glial GLAST and GLT-1 but not neuronal EAAC1. These data are supported by the work of Sitcheran et al. who demonstrated that TNF-α induced IkB degradation, which triggered nuclear factor (NF)-κB nuclear translocation and suppressed GLT-1 expression in H4 astroglialoma cells. Furthermore, increasing the TNF-α concentration leads to a reduction of GLAST protein, in a dose-dependent manner, which could be prevented by preincubation with neutralizing TNF-α antibody. The authors assumed that downregulation of GLAST resulted in an excess of extracellular glutamate and triggered excitotoxicity through NMDA receptors in neuron and AMPA/kainate receptors in oligodendrocytes, and concluded that TNF-α had an important role in central nervous system inflammatory disorders via reduction of glutamate uptake. Similar to our previous study, we found that etanercept (50 μg) effectively suppressed microglia activation and proinflammatory cytokines TNF-α, interleukin (IL)-1β, and IL-6 mRNA expression. Taken together with our present results, we suggest that etanercept (50 μg) treatment, via attenuation of TNF-α expression, suppresses the downregulation of glial GLAST and GLT-1 in morphine-tolerant rats. As is known, NF-κB and activator protein-1 are the most important transcriptional factors related to the TNF-α signaling pathway. Central glucocorticoid receptors modulated downregulation of spinal EAAC1 after peripheral nerve injury through diminished NF-κB expression. Intriguingly, we found that amitriptyline could induce GT upregulation including GLAST, GLT-1, and EAAC1 through NF-κB-dependent activation. Moreover, chronic morphine exposure also induced E3 ligase activation via cyclic adenosine monophosphate/protein kinase A signaling and resulted in a ubiquitin-proteasome system mediating degradation of EAAC1. This means that there are more mechanisms, besides the NF-κB pathway, involving EAAC1 regulation after chronic morphine administration, which demand more studies to elucidate the underlying signaling pathway.

In our present study, there was a significant increase in the expression of AMPA- and NMDA-receptor subunits in the spinal cords of chronic morphine-infused rats beginning on day 1 and lasting for 5 days. In contrast, Beattie et al. found that TNF-α enhances synaptic strength by increasing the expression of the AMPA receptor, but not the NMDA receptor; they suggested that mobility of NMDA receptors was less than AMPA receptors because of differential attachments. Compared with the brief exposure (10 minutes) of TNF-α in the study by Beattie et al., our results indicated that chronic IT morphine infusion is long enough to enhance the surface expression of both AMPA and NMDA receptors, which contributes to the reduction of morphine’s antinociceptive effect. Our study confirmed that NMDA receptors are important for neuronal plasticity and spinal morphine tolerance development. Stellwagen et al. also demonstrated that TNF-α preferentially increased the synaptic expression of GluR2-lacking AMPA receptors, which are more permeable to Ca2+ and simultaneously downregulate surface GABAA receptors, thus resulting in a reduction of inhibitory synaptic transmission. These results are consistent with our results, which showed that the GluR1 surface expression is apparently augmented compared with GluR2 subunits. Induction of GluR1 expression of AMPA receptors by TNF-α increases the neurons’ vulnerability to excitotoxicity, which is mediated through acid sphingomyelinase and the NF-κB pathway. GluR2 expression affected calcium permeability through AMPA receptors, and the increase of GluR2-lacking AMPA receptors will alter the function of the synaptic AMPA receptors. Our results imply that TNF-α not only influences AMPA receptor expression but also modulates the amount of GluR2-abundant AMPA receptors, which control Ca2+ influx. NMDA receptors are highly permeable to Ca2+, and the NR1 subunit of the NMDA receptor is an essential functional unit of the NMDA receptor. As mentioned, NMDA receptors are involved in the mechanism of morphine tolerance and NMDA receptor antagonists have been demonstrated to inhibit morphine tolerance development. Lim et al. further observed an increase of NMDA receptor NR1 subunit expression in chronic morphine-infused rats’ spinal cord dorsal horn, which was mediated by glucocorticoid receptor activation in a time-dependent manner. Shimoyama et al. demonstrated that morphine tolerance was attenuated by deletion of the NMDA receptor NR1 subunit via IT administration of antisense oligonucleotide. Similarly, we also found that etanercept attenuated the upregulation of the NMDA receptor NR1 subunit expression in chronic morphine-infused rats. We suggested that this increase of glutamate availability by TNF-α was attributable to downregulation of membrane GTs, in accordance with upregulation of surface AMPA and NMDA receptors in chronic morphine-infused rats, which contributes to the excessive activation of glutamatergic receptors and then reduces the antinociceptive effect of morphine. Conceivably, activation of the glutamatergic receptor system will enhance the intracellular mechanisms of morphine tolerance, such as interaction with μ-opioid receptors through Ca2+ influx, which activate protein kinase C, nitric oxide synthase, and relevant gene regulation. The glutamate-mediated excitotoxicity with excessive influx of calcium ions was proposed to be vital in neuropathic pain. We proposed that the restoration of the antinociceptive effect of morphine by etanercept (50 μg) pretreatment in chronic morphine-infused rats occurs by inhibiting the TNF-α-mediated glutamatergic
Etanercept attenuates glutamatergic transmission

In summary, our results indicate that TNF-α has an important role in morphine tolerance, and etanercept pretreatment might diminish TNF-α signaling and partially restore the antinociceptive effect of morphine in chronic morphine-infused rats. Moreover, we found that enhancement of synaptic strength is the result of the increase of glutamate availability and expression of AMPA and NMDA receptors. Etanercept preserves the antinociceptive effect of morphine by inhibiting glutamatergic synaptic transmission. Based on the present study, we suggest that etanercept may be clinically useful in the management of patients who experience chronic pain and need long-term morphine therapy.

DISCLOSURES

Name: Ching-Hui Shen, MD.
Contribution: Participated in study design and conduct of the study, and wrote the manuscript.

Name: Ru-Yin Tsai, PhD.
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Contribution: Study design, data analysis, and manuscript revision.

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26. Beattie MS, Stellwagen D, Morishita W, Stocker R, Trentz O, Koszmann T. Pro-inflammation cytokines.57 Our previous study also demonstrated that etanercept attenuated the proinflammatory cytokine TNF-α, IL-1β, and IL-6 mRNAs expression.44 These results were consistent, but further analysis of the underlying molecular mechanisms of etanercept is needed to elucidate the interaction between TNF-α and morphine tolerance.

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