Leukemia inhibitory factor (LIF) potentiates antinociception activity and inhibits tolerance induction of opioids

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Abstract

Background: The efficacy of opioids typically decreases after long-term use owing to the development of tolerance. Glial activation and the upregulation of proinflammatory cytokines are related to the induction of tolerance. We investigated the effect of leukemia inhibitory factor (LIF) on morphine analgesia and tolerance.

Methods: LIF concentrations in rat spinal cords were measured by polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) after morphine administration. LIF distribution was examined using confocal microscopy. To evaluate the effects of LIF on morphine analgesia and tolerance, LIF was intrathecally administered 30 min before morphine injection. The analgesic effect of morphine was evaluated by measuring tail-flick latency. Human LIF concentrations from the cerebrospinal fluid (CSF) of opioid tolerant patients were also determined by specific ELISA.

Results: Chronic morphine administration upregulated LIF concentrations in rat spinal cords. Intrathecal injection of LIF potentiated the analgesic action of morphine. Patch clamp recording of spinal cord slices showed that LIF enhanced DAMGO ([D-Ala², N-MePhe⁴, Gly-ol]-enkephalin)-induced outward potassium current. The development of tolerance was markedly suppressed by exogenous LIF, whereas neutralizing the endogenously released LIF with anti-LIF antibodies accelerated the tolerance induction. Moreover, LIF concentrations in the CSF of opioid-tolerant patients were higher than those in the opioid-naive controls.

Conclusions: Intrathecal administration of LIF potentiated morphine antinociceptive activity and attenuated the development of morphine tolerance. Upregulation of endogenously released LIF by long-term use of opioids might counterbalance the tolerance induction effects of other proinflammatory cytokines. LIF might be a novel drug candidate for inhibiting opioid tolerance induction.

Key words: glia; leukemia inhibitory factor; opioid
Opioid analgesics, such as morphine, provide effective pain relief and are considered the therapy of choice for managing moderate-to-severe pain, particularly cancer pain. However, clinical use of opioid analgesics is usually restricted by their side-effects, especially the development of tolerance, which arises because of chronic use. As tolerance develops, a higher opioid dose is required to achieve the same analgesic level, which might lead to serious side-effects and physical dependence. Several hypotheses have been proposed for explaining the tolerance mechanism: receptor desensitization,3 upregulation of NMDA receptors,4 and downregulation of glutamate receptors.5 Gial cells have also been reported to mediate morphine tolerance.6–8

Chronic morphine treatment stimulates glial cell activation, leading to the release of proinflammatory cytokines.9–10 These findings suggest that cytokines may play a pivotal role in tolerance development.

Leukemia inhibitory factor (LIF), a gp130 member of IL-6 family, is involved in inflammation,11 pain,12 and neurogenesis13 and its receptors are widely expressed in various regions of the central nervous system.14 Recent studies also indicate that LIF is a key mediator in sensory neurones.15 Here we examined the potential effect of LIF on morphine-induced analgesic activity and tolerance.

Methods

Animals

Adult male Sprague–Dawley rats (250–275 g) were purchased from BioLASCO Taiwan Co., Ltd. All procedures were performed in accordance with the International Association for the Study of Pain (IASP) and the NIH guidelines on laboratory animal welfare. Rats were anaesthetized with isoflurane. The occipital and the spinal cord was rapidly removed from the vertebral column, and the spinal cord was quickly dissected and immersed in a chilled cutting solution for the rats to be used in the study. The spinal cord was then cut into the L4–L6 spinal cord segments using a TRIzol® kit (MDBio Inc., Taipei, Taiwan). Single-stranded cDNA was synthesized using a two-step MMLV reverse transcriptase system (Promega, Madison, WI, USA). Fifty nanograms of cDNA was mixed with 200 nM specific primers and SYBR® Green PCR Master Mix (Roche Molecular system, NJ, USA). Quantitative PCR assays were performed in triplicate on a StepOnePlus sequence detection system (Applied Biosystems, CA, USA). The change in gene expression relative to the control was calculated using the 2−ΔΔCt method.

The specific PCR primers used were as follows: rat GAPDH: sense, GGCAAGTTCAATGGCAACTG; antisense, TGGTGAAGACG CCACTGACCT. rat LIF: sense, AGTTGTGCCCTGCCTGTTGG; antisense, GTCACTTGGGCGCCACATAG.

To measure endogenous LIF expression, the L4–L6 spinal cord segments were isolated, and an extraction buffer (#9806, Cell Signaling Technology, MA, USA) was added to the tissue. Samples were homogenized and centrifuged at 4°C. The supernatant was transferred to a fresh tube. Rat LIF concentrations were determined using a specific ELISA kit (SEA085Ra, USCN Life Science Inc., Wuhan, Hubei, PRC).

Drug administration

Morphine hydrochloride (Factory for Controlled Drugs, Taiwan Food and Drug Administration) was dissolved in sterile endotoxin-free water. Recombinant rat LIF (rLIF) was purchased from EMD Millipore (LIF3010). To evaluate the effect of LIF on acute morphine analgesic action, exogenous LIF (10 µg in 5 µl) was slowly injected through the intrathecal catheter, followed by a flush of 10 µl sterile saline. The morphine (2 mg kg−1) was injected subcutaneously 30 min later. Subsequently, tail-flick latency was assessed at 15 min intervals (Supplementary data, Fig. 1A).

For the tolerance studies, exogenous LIF was injected as mentioned above, and morphine (10 mg kg−1 day−1, s.c.) was injected 30 min later. The antinociceptive activity of morphine was measured using a tail-flick test 30 min after morphine injection (Supplementary data, Fig. 1B).

To neutralize endogenously released LIF, a specific anti-LIF antibody (AB-449-NA, R&D) was used. Normal goat IgG (AB-108-C, R&D Systems) was used as a control. Both control goat IgG and anti-LIF (5 µg in 5 µl) were infused through an intrathecal catheter 30 min before morphine injection (10 mg kg−1 day−1, s.c.). Tail-flick latency was measured 30 min after morphine administration.

Antinociception test

Thermal nociception was evaluated using a tail-flick apparatus (Columbus Instruments, Columbus, OH, USA). Rats were gently restrained with their tail positioned directly under a heat source (heat intensity setting at 15, approximately 60°C). The time it took for the rats to flick their tails were recorded, with a 15-s cut-off time to minimize the risk of tissue damage. The analgesic response of morphine was calculated as a percentage of the maximum possible effect (MPE) using the following equation:

\[
\text{MPE} = \left( \frac{\text{test latency} - \text{baseline latency}}{\text{cut - off time} - \text{baseline latency}} \right) \times 100.
\]

Electrophysiology in spinal cord slices

Adult male SD rats (150–200 g) were anaesthetized with isoflurane, and the spinal cord was rapidly removed from the vertebral canal through hydraulic extrusion, as reported in a previous study.17 The fresh spinal cord containing the L4–L6 vertebrae, was quickly dissected and immersed in a chilled cutting solution as previously described.18 Coronal spinal cord slices (300–350-µm-thick) were cut using a microslicer (DTK-1000, Dosaka, Kyoto, Japan) and subsequently transferred to a holding chamber
with artificial CSF (aCSF). These slices were incubated in oxygenated aCSF (bubbled with 95% O₂ and 5% CO₂) at room temperature for at least one hour before measurements were obtained.

The spinal cord slices were then transferred to the recording chamber. Individual neurones were visualized under the 40X water immersion objective of the infrared differential interference contrast camera of an upright microscope (BX51WI, Olympus, Tokyo, Japan). Whole-cell recordings of the dorsal horn neurones were obtained with a microelectrode amplifier (Axopatch 200B, Molecular Devices, CA, USA). Recording electrodes (5–8 MΩ) were pulled from borosilicate glass capillaries using a horizontal puller (P97, Sutter Instrument, CA, USA) and filled with an internal solution. Signals were filtered at 2 Hz and digitized at 5 Hz. To record the potassium currents, tetrodotoxin (1 µM) was added to a bath solution to block the voltage-gated sodium channels. Neurones were clamped at −60 mV, and an outward potassium current was generated by applying 200 ms depolarizing steps from −50 mV to +50 mV at 10 mV increments. The recordings were analysed offline using the pClamp software (Molecular Devices). Detailed information on the composition of the solutions and the procedure is attached in the Supplementary data.

**Immunohistochemistry**

After morphine treatment (10 mg kg⁻¹ day⁻¹, s.c.) for five consecutive days, the animals became tolerant to morphine, as
determined by the tail-flick test. The rats were then anaesthetized and perfused with 4% paraformaldehyde, and the L4-L6 spinal cord segments were removed for immunohistochemistry. The sections were incubated overnight with Iba-1 antibody (019-19741, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 4°C. They were then incubated with biotinylated horse anti-goat IgG for one h at room temperature. Immunohistochemical staining was performed using an avidin–biotin solution for one h (ABC kit, Vector Laboratories, Burlingame, CA, USA).

To investigate the localization of LIF, spinal cord sections were stained with primary goat-anti-LIF (SC-1336, Santa Cruz, CA, USA) and mouse-anti-CD11b (MCA275R, AbD Serotec, Oxford, UK) antibodies, and then incubated with secondary antibodies conjugated with Alexa Fluor-488 or 546, respectively. The fluorescent images were obtained using confocal microscopy (TCS SPS; Leica, Heidelberg, Germany).

Human study
After obtaining approval from the relevant institutional research ethics committee (NTUH 201004056R), the prospective case-control study was conducted at the National Taiwan University Hospital, Taipei, Taiwan. Participant recruitment and sample collections were carried out from Jan 2011 to Oct 2014. Fully informed consent was obtained before collection of the CSF samples. Opioid tolerance was defined as a daily opioid requirement greater than 100 mg i.v. morphine or other equipotent opioids, such as transdermal fentanyl, as previously described.10 The patient profiles are listed in Supplementary data, Table 1.

The LIF concentrations in human CSF were measured using a specific human ELISA kit (DLF00, R&D Systems).

Statistical analyses
All animal study data were presented as mean (SEM). Data were tested using the ANOVA or repeated measure ANOVA when appropriate. Post hoc tests for different dosages and time points were performed using the Holm-Sidak’s or Bonferroni correction. Human LIF concentrations in the CSF from opioid-tolerant patients and opioid-naive control subjects were compared, using an independent samples Student’s t-test. P<0.05 was considered statistically significant.

Results
Long-term morphine exposure has been reported to increase the release of many cytokines and chemokines.7 We examined the concentrations of endogenously released LIF after morphine treatment. As shown in Fig. 1, LIF concentrations were increased when a higher dose of morphine was administered (10 mg kg⁻¹ day⁻¹, s.c.). The expression of LIF mRNA in the treated rats showed a 4.1-fold increase on Day four, compared with controls (Fig. 1A). In addition, the expression of LIF increased from 1260 pg mg⁻¹ tissue on day 0 to 3700 pg mg⁻¹ tissue on day eight (Fig. 1B). The lower dose of morphine (2 mg kg⁻¹ day⁻¹, s.c.) exerted a smaller effect on LIF expression. These results showed that the expression of endogenous LIF in rat spinal cords is dose- and time-dependently increased after morphine treatment.

We also used confocal microscopy to localize the expression of LIF. After chronic morphine treatment, we found that endogenous LIF is expressed mainly from the microglia of the spinal dorsal horn (Fig. 1C).

To examine the effect of LIF on morphine analgesia, a lower morphine dose (2 mg kg⁻¹, s.c.) was used, and the analgesic action only reached 60% of the MPE obtained by using a 10 mg kg⁻¹ dose. However, adding exogenous LIF enhanced the analgesic effect of morphine, restoring it to 100% MPE at 15 min (Fig. 2A). When an anti-LIF antibody was used to neutralize endogenously released LIF before the morphine injection, the acute analgesic efficacy of morphine was not affected (Fig. 2B). These results suggest that LIF is expressed at very low concentrations in the nervous system under normal physiological conditions.15 To preclude the possibility of exogenous LIF alone

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![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Fig 2** Exogenous LIF enhances morphine analgesic efficacy in rats. Exogenous LIF was intrathecallly administered 30 min before morphine treatment (2 mg kg⁻¹, s.c.). The antinociceptive effect of morphine was measured at different time intervals after morphine treatment. (a) Exogenous LIF enhanced the analgesic action and reached 100% MPE at 15 min (P-value=0.63, P<0.001). (b) Administration of anti-LIF antibody did not affect the morphine analgesic effect (P-value=0.73, P=0.63). (c) Intrathecal administration of exogenous LIF alone did not affect the basal tail-flick latency. Data are presented as mean [SEM (n=5–8)]. *, P<0.05 compared with control at corresponding time points.
affecting basal pain thresholds, LIF was intrathecally injected to the lumbar spinal cord without morphine treatment. There was no change to the tail-flick latency in response to LIF administration alone (Fig. 2c).

After binding to the µ-opioid receptors (MOR), opioids can induce a voltage-dependent outward potassium current. Drugs such as diazoxide and minoxidil may act as potassium channel openers and thus amplify the antinociceptive potency of morphine. Therefore, we examined whether the effect of exogenous LIF on the morphine analgesic action is a result of changes to the potassium channels. We found that the antinociceptive effect of morphine was mainly mediated by the MOR (Supplementary data, Fig. 2). Therefore, we used DAMGO, a µ-opioid receptor selective agonist which potentiates the outward potassium current. It was found that exogenous LIF enhanced the effect of DAMGO on the outward potassium current (Fig. 3).

As endogenous LIF concentrations increased after chronic morphine treatment, we further examined the effect of LIF on tolerance induction by daily morphine administration. We found that the maximal analgesic action of morphine lasted three days, the tail-flick latency then decreased to 60% on day four, and gradually returned to baseline by day seven in control rats (Fig. 4). However, exogenous LIF markedly delayed the development of tolerance. In rats receiving exogenous LIF, no sign of tolerance was detected until Day nine, and MPE had not returned to the baseline by the end of the study period (Day 16) (Fig. 4a). LIF administration alone did not affect the tail-flick latency (Fig. 4b). As shown in Fig. 1, morphine administration increased endogenous LIF expression in the spinal cord; we thus used a specific antibody to neutralize the endogenously released LIF. Intrathecal administration of an anti-LIF antibody accelerated the induction of morphine tolerance. Under these conditions, the maximal analgesic action of morphine lasted for only two days, and gradually declined to 25% MPE on Day four, whereas the control IgG-infused rats still retained 60% MPE on Day four. Anti-LIF antibody alone also had no effect on the tail-flick latency (Fig. 4b).

Long-term morphine treatment increased glial activation in the spinal cord, which may be related to tolerance induction. We found that after five consecutive days of chronic morphine treatment to induce tolerance, microglial activation marker Iba-1 expression in the spinal cord was higher in the morphine-treated rats than in the opioid-naive controls. The microglial activation caused by the chronic morphine treatment was suppressed by intrathecal LIF administration (Fig. 5). Calcitonin gene related-peptide (CGRP), which is known to be involved in pain transmission, is upregulated after chronic morphine treatment.

To validate the findings in the animal study, a human case-control study was conducted to clarify the role of LIF in chronic opioid treatment. Twenty patients with different opioid tolerance

![Fig 3](https://example.com/fig3.png)

**Fig 3** Exogenous LIF potentiates the effect of DAMGO on outward potassium current in the dorsal horn neurones. (A) µ-opioid receptor selective agonist DAMGO at 1 µM enhanced outward potassium current compared with the control. LIF alone did not affect the potassium current. Co-perfusion of LIF (10 ng ml⁻¹) with DAMGO (1 µM) further enhanced the effect of DAMGO on the outward potassium current. (B) Voltage-current curves. Quantitative results are shown in (C). Normalized peak outward potassium currents were recorded at +50 mV (F (3,27)=17.06, P<0.001). Data are presented as mean [SEM (n=7–8)]. *, P<0.05 compared with the control group. #, P<0.05 compared with DAMGO alone.
levels were included in the opioid-tolerant group. An additional 10 age-compatible healthy participants, with no history of opioid use in the preceding three months, were defined as the opioid-naive control group. Compared with the opioid-naive group, LIF concentrations in the CSF were significantly higher in opioid-tolerant patients (78.8 (17.3) pg ml\(^{-1}\) vs. 11.3 (1.3) pg ml\(^{-1}\), \(P=0.01\), Fig. 6A). Furthermore, there was a positive correlation between the daily equivalent opioid dose and LIF expression (Fig. 6B).

**Discussion**

While the underlying mechanism of opioid tolerance is unclear, the association between opioid tolerance and glial activation-induced cytokine upregulation has been extensively investigated.\(^6\) In the current study, we found that repetitive morphine injections led to a time-dependent increase of endogenous LIF expression in rat spinal cords. Although LIF is generally considered to be a proinflammatory cytokine,\(^28\) to our surprise, exogenous LIF administration significantly potentiated the acute morphine analgesic action and delayed the development of tolerance to chronic morphine treatment. In addition to the findings in the animal study, we also found that endogenous LIF concentrations in the CSF were upregulated amongst the opioid-tolerant patients and were positively correlated to the daily opioid dose in the human translational study.

Peripheral nerves are excited in response to pain stimuli. The pain signal is transmitted to the presynaptic terminal in the dorsal horn, causing the release of neuroexcitatory substances, which in turn cause glial activation.\(^29\) Glial cells are quiescent under normal condition, but release various substances after activation, such as cytokines, NO, reactive oxygen species (ROS), and excitatory amino acids (EAA\(s\)).\(^9\) Moreover, glial cells can indirectly influence glial cells via neuronal-glial signalling. Fractalkine, a neuron-released chemokine, is upregulated by chronic morphine treatment.\(^16\) Once fractalkine is released, it binds to its receptor CX3CR1 in microglia\(^31\) and increases cytokine production. Drugs such as fluorocitrate or minocycline can suppress morphine tolerance by inhibiting glial cell activation.\(^7\) Proinflammatory cytokines play a pivotal role in pain modulation\(^13\) and regulate morphine analgesia. Acute morphine treatment on lumbar dorsal spinal cord sections enhances the expression of cytokines and chemokines, such as IL-1β, IL-6, TNF-α, CXCL1, and IL-10.\(^9\) Furthermore, cytokine and chemokine expression was upregulated in rats receiving chronic morphine treatment. We have recently reported that CXCL1 concentrations are markedly higher in patients with opioid tolerance.\(^10\) The administration of IL-1 receptor antagonist, anti-IL-6 neutralizing antibody, and TNF-α soluble receptors can suppress morphine-induced tolerance.\(^34\) These results indicate a complex
immune response between neurone and glial cell after morphine treatment, in which cytokines have an important role. We have showed that chronic morphine treatment enhanced the expression of endogenous LIF in rats and humans. Administering anti-LIF antibodies accelerated morphine tolerance in the rat model. We have demonstrated that endogenously released LIF can counterbalance other tolerance-inducing cytokines, such as IL-1 and TNF-α. According to previous studies, most of the cytokines stimulated by morphine treatment have been indicated as the cause of tolerance induction. However, IL-10 has been found to act as an anti-inflammatory cytokine which attenuates morphine tolerance. LIF may have a similar mode of action to IL-10, which inhibits over-activation of glia cells after morphine stimulation.

Our study had one major limitation: although LIF has been implicated in both pain and cancer progression, our human study groups consisted of opioid-tolerant cancer patients and opioid-naïve surgical patients. Based on our study protocol, it is difficult to interpret whether the upregulation of LIF is related to cancer or to opioid tolerance. However, it is unethical to recruit opioid naïve cancer patients and to collect their CSF, and it is difficult to find and recruit chronic non-cancer pain patients with strong opioid use. That being said, chronic pain would also be a confounding factor because our opioid naïve participants were in pain free and the CSF samples were collected during scheduled spinal anaesthesia for the surgical removal of implants from healed bone fractures. In the present study, we tried to minimize the confounding effect of cancer by recruiting patients with similar disease severity. Additionally, we chose patients with no CNS involvement of cancer, to exclude the possibility that CSF LIF concentrations were affected by CNS metastasis. We found a strong positive correlation between the CSF LIF concentrations and opioid dosage, indicating that upregulation of LIF expression might be related to chronic high doses of opioids. In parallel, our animal translational studies showed that LIF concentrations in the CSF were significantly upregulated in tumour free rats receiving chronic morphine treatment. These results correlated well with the data from the human study.

In summary, our data provide strong evidence that both endogenously released and exogenously administered LIF may have regulatory roles in morphine tolerance. Compared with other proinflammatory cytokines, LIF not only enhances the acute effect of morphine but also delays the development of tolerance. These findings may provide novel strategies for alleviating the problem of opioid tolerance.

Authors’ contributions
Study design/planning: H.J.T., C.P.L., H.H.L., W.M.F.
Data analysis: H.J.T.
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