Amitriptyline Suppresses Neuroinflammation-dependent Interleukin-10-p38 Mitogen-activated Protein Kinase-Heme Oxygenase-1 Signaling Pathway in Chronic Morphine-infused Rats

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Background: This study explores the underlying mechanism of the antiinflammatory effect of amitriptyline in chronic mor-

Methods: Male Wistar rats were implanted with two intrathe-
cal catheters. One catheter was for the continuous infusion of saline, amitriptyline (15 μg/h), morphine (15 μg/h), p38 mitogen-activated protein kinase inhibitor SB203580 (0.5 μg/h), morphine plus amitriptyline, or morphine plus amitriptyline plus SB203580 for 5 days. The other catheter was used for daily intrathecal injection of anti-interleukin-10 (IL-10) antibody or heme oxygenase-1 inhibitor zinc protoporphyrin for 5 days.

Results: Amitriptyline/morphine coinfusion upregulated IL-10 protein expression in microglia; this was not observed in morphine-infused rats. Anti-IL-10 antibody effectively neutralized the amitriptyline-induced effect of chronic morphine-infused rats. In addition, coinfusion of amitriptyline restored the antinociceptive effect of morphine (a 4.8-fold right-shift of the morphine dose-response curve compared to a 7.8-fold right-shift in its absence), and the injection of anti-IL-10 antibody or coinfusion of SB203580 partially reversed the effect of amitriptyline on the antinociceptive effect of morphine in morphine-infused rats (a 17.9-fold and 15.1-fold right-shift in morphine dose-response curves). Anti-IL-10 antibody and SB203580 significantly inhibited the amitriptyline-induced p38 mitogen-activated protein kinase and heme oxygenase-1 expression and the associated antiinflammatory effect of amitriptyline. Daily injection of zinc protoporphyrin also demonstrated that it reverses the effect of amitriptyline in morphine’s antinociception and antiinflammation in chronic morphine-infused rats.

Conclusions: These results suggest that the antiinflammatory effect of amitriptyline on morphine tolerance, probably acting by increasing IL-10 expression, is mediated by p38 mitogen-activated protein kinase heme oxygenase-1 signal transduction cascade.

MORPHINE is one of the most potent analgesic drugs for clinical pain management. However, its use for chronic pain conditions is limited by the development of tolerance.1 Morphine tolerance is a complex physiologic response; besides opioid receptor uncoupling and down-regulation,2 glutamatergic receptor activation and neuroinflammation have also been demonstrated by us and others.3–6 Opioid-induced hyperalgesia is observed in tolerance; it is similar to the symptom observed in neuropathic pain, where opioids also have with limited analgesic effect. Poinflammatory cytokines and chemokines have been shown to modulate morphine tolerance and associated mechanical allodynia and thermal hyperalgesia.7 Administration of glial metabolic inhibitors, cytokine antagonists, antiinflammatory cytokine interleukin (IL)-10, or IL-1 receptor antagonist IL-1ra has been shown to attenuate the development of opioid tolerance and to prevent hyperalgesia and allodynia.3,8

Tricyclic antidepressants (TCAs) are commonly used for treating major depressive disorders and are also widely used in chronic pain states, such as neuropathic and inflammatory pains.9 TCAs produce analgesia by various mechanisms involving N-methyl-D-aspartate receptors, biogenic amines, opioids, inflammatory mediators, and substance P.10 Studies have shown that intrathecal amitriptyline administration effectively attenuates pain and thermal hyperalgesia in inflammatory and neuropathic pain in rats,11 particularly when combined with opiates and clonidine.12,13 Moreover, in our recent study, we found that morphine tolerance is associated with downregulation of spinal glutamate transporters (GLAST, GLT-1, and EAAC1) and increasing of excitatory amino acids aspartate and glutamate concentration in spinal cerebrospinal fluid dialysates.6 Coadministration of amitriptyline reversed the GLAST and GLT-1 expression and excitatory amino acids elevation, restoring the antinociceptive effect of morphine in chronic morphine-infused rats; this spinal microglia activation and increasing of cytokine tumor necrosis factor-α (TNFα), IL-1β, and IL-6 expression were also suppressed by the amitriptyline coinfusion.8 Furthermore, our recent study demonstrated spinal neuroinflammation, microglia activation, and induction of proinflammatory cytokine expression in percutaneous toxin-treated rats.14

IL-10 is a key molecule in controlling inflammation with a wide spectrum of biologic effects on lymphoid and myeloid cells.15 Previous study has shown that anti-inflammatory cytokine IL-10 is stimulated by antidepres-
An inhibitory effect of IL-10 on lippolysaccharide-induced thermal and mechanical hyperalgesia in mice was attributed to the inhibition of the production of proinflammatory cytokines, including TNF-α, IL-1β, and nerve growth factor. IL-10 was also demonstrated to attenuate the thermal hyperalgesia induced by chronic nerve constriction injury. In murine macrophages, IL-10 induces expression of heme oxygenase-1 (HO-1), a stress-induced protein with a potent antiinflammatory effect. Studies have shown that carbon monoxide and p38 mitogen-activated protein kinase (MAPK)-dependent mechanisms can positively feedback-modulate IL-10 and HO-1 activity, which amplifies the antiinflammatory capacity of IL-10. In the current study, therefore, we examined the effect of amitriptyline on inhibition of spinal cord neuroinflammation for its possible mechanisms. We particularly examined the role of IL-10 and its downstream p38MAPK-HO-1 signal transduction pathway in inhibiting proinflammatory cytokine expression in chronic morphine-infused rats. Hopefully, our results will provide the rationale for using of TCAs in clinical pain management, particularly in patients who suffer from neuropathic pain and need long-term opioid treatment.

Materials and Methods

Animal Preparation and Drug Administration

All experiments conformed to the Guiding Principles in the Care and Use of Animals of the American Physiology Society and were approved by the National Defense Medical Center Animal Care and Use Committee (National Defense Medical Center, Taipei, Taiwan). Animal preparation and intrathecal catheter implantation were as described in our previous study. Briefly, male Wistar rats (300–350 g) were anesthetized with phenobarbital (50 mg/kg, intra-peritoneal; Sigma, Saint Louis, MO), and two intrathecal catheters were implanted via the atlantooccipital membrane into the intrathecal space at the level of the lumbar enlargement of the spinal cord (L1–L2). Intrathecal catheters were constructed using an 8-cm polyethylene tube (0.008 inch ID, 0.014 inch OD) and a 3.5-cm silastic tube. The silastic tube was inserted into the polyethylene tube and sealed with epoxy resin and silicon rubber. One was connected to a miniosmotic pump for continuous infusion of saline (1 μl/h), amitriptyline (15 μg/h; Sigma), p38 MAPK inhibitor SB203580 (0.5 μg/h; Sigma), morphine (15 μg/h; Sigma), amitriptyline/morphine, or amitriptyline/morphine/SB203580 for 5 days. The other catheter was used for a single daily injection of the anti–IL-10 antibody (10 μg; 1-10 μg as indicated; Pierce-Endogen, Thermo Fisher Scientific Inc, Waltham, MA) or HO-1 antagonist zinc protoporphyrin (24 μg; 6-24 μg as indicated; Tocris Cookson Ltd., Bristol, United Kingdom) for 5 days. Rats received a mouse isotype-matched irrelevant mAb (Pierce-Endogen) as negative control. On day 5, after a discontinuation of drug infusion for 3 h, when tail-flick latency had returned to the baseline level (around 2 s), morphine’s dose-response curves were constructed. All rats were housed individually and maintained on a 12-h light/dark cycle with food and water freely available. Rats with any neurologic deficits were excluded from the study (< 10%).

Behavioral Tests

Tail-flick latency was measured using the hot water immersion test (52 ± 0.5°C) before drug infusion and daily after the start of infusion for 5 days. All rats were placed in plastic restrainers for the antinociceptive test. The average baseline tail-flick test latency was 2 ± 0.5 s in naïve rats, and the cutoff time was 10 s. The percentage of the maximal possible antinociceptive effect was calculated as (maximum latency − baseline latency)/ (cutoff latency − baseline latency) × 100. Antinociceptive dose-response curves were constructed for each study group.

Spinal Cord Preparation and Western Blot Analysis

After drug infusion, all rats were sacrificed, and the dorsal portion of the lumbar spinal cord enlargement was immediately removed and homogenized in ice-cold lysis buffer, consisting of 50 mM Tris, pH 7.5, 150 mM NaCl, 2% Triton X-100, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and phosphatase inhibitors. The lysate was centrifuged at 12,000g for 30 min at 4°C, and the supernatant was then used for Western blotting. The protein concentration of the samples was determined by the BCA method (Pierce, Thermo Fisher Scientific Inc, Waltham, MA) using bovine serum albumin as the standard. Equal amounts of total protein (20 μg) were adjusted to a similar volume with loading buffer (10% SDS, 20% glycerin, 125 mM Tris, 1 mM EDTA, 0.002% bromophenol blue, 10% β-mercaptoethanol), denatured by heating at 95°C for 5 min, separated on 10% sodium dodecyl sulfate–polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat milk and incubated overnight at 4°C with polyclonal rabbit anti-p44/p42 MAPK (1:1000), anti-phospho-p44/p42 MAPK (1:500), or anti-p38 MAPK (1:500) antibodies. The other antibodies included anti-SAPK/JNK (1:1000), anti-phospho-SAPK/JNK (1:500), anti-p38 MAPK (1:500), or anti-phosphor-p38 MAPK (1:500) antibodies (all from Cell Signaling, Danvers, MA) or polyclonal rabbit anti-HO-2 (1:2000) or monoclonal mouse anti-HO-1 (1:2000) antibodies (both from Stressgen, Victoria, British Columbia, Canada), and then incubated for 1 h at room temperature with appropriate horse radish peroxidase-conjugated secondary antibodies (1:2500; donkey antirabbit or anti-mouse immunoglobulin; Chemicon, Temecula, CA). Membrane-bound secondary antibodies were detected using the Chemiluminescence reagent (PerkinElmer LAS, Boston, MA) and visualized using a
chemiluminescence imaging system (Syngene, Cambridge, United Kingdom). Finally, the blots were incubated for 18 min at 56°C in stripping buffer (62.6 mM Tris-HCl, pH 6.7, 2% SDS, 100 mM mercaptoethanol) and reprobed with monoclonal mouse anti-β-actin antibody (1:2500; Sigma) as a loading control. The Western blot analysis was repeated three times. The density of each specific band was measured using a computer-assisted imaging analysis system (Gene Tools Match software, Syngene).

**Cytokine Proteins Measurement**

Spinal cord lysates were analyzed for four cytokines in a simultaneous multiplexed format using a microbead-based and flow-based protein detection system (Bio-Plex Suspension Array System, Bio-Rad Laboratories Inc., Hercules, CA) based on the Luminex xMAP technology (Luminex Molecular Diagnostics Inc., Toronto, Ontario, Canada). In this quantitative assay system, the surfaces of fluorescence-coded microbeads are conjugated to specific antibodies directed against four cytokines target. Each sample was first incubated with a mixture of all microbead types for 120 min at room temperature. After wash, samples were incubated with a mixture of secondary biotinylated detection antibodies also directed against each target for 30 min at room temperature, then washed again and incubated with a streptavidin-coupled phycoerythrin reporter system for 10 min at room temperature. After a final wash, the samples were resuspended in assay buffer and subjected to flow cytometric analysis. The Bio-Plex system instrumentation incorporates fluidics, laser excitation, fluorescence detection, and digital signal processing in a manner that allows for individual scanning and identification of microbeads. Each bead taken from the samples was identified on the basis of its internal fluorescence signature, and the phycoerythrin reporter signal associated with that bead was quantitated. A minimum 100 microbeads per each of the four targets were analyzed in each spinal cord sample. All spinal cord lysates were assayed in triplicate. Observed concentrations of each target cytokine were determined on the basis of an appropriate set of recombinant rat cytokine internal standard curves.

**Fluorescent Immunocytochemistry and Image Analysis**

After drug infusion, the rats were sacrificed by exsanguination under isoflurane anesthesia, and the lumbar spinal cord was immediately removed, embedded in optimal cutting temperature compound (Sakura Finetec Inc., Torrance, CA), and frozen at −20°C. Sections (5 μm) were prepared, air-dried on microscope slides for 30 min at room temperature, and fixed by immersion for 10 min in ice-cold acetone/methanol (1:1). After washing three times in ice-cold phosphate-buffered saline, the sections were incubated with blocking solution (0.01% Triton, 4% fetal bovine sera; 1 h). They were then double-labeled by incubation overnight at 4°C with (1) fluorescein isothiocyanate-labeled (green fluorescence) mouse monoclonal anti-rat CD11b/c antibodies (OX42 1:10, for macroglia; Chemicon, Temecula, CA) and unlabeled polyclonal goat anti-IL-10 antibodies (1:50; R&D System, Minneapolis, MN) and then for 1 h at room temperature with rhodamine-labeled guinea pig anti-goat immunoglobulin antibodies (1:200; R&D System), (2) FITC-labeled neuron-specific nuclear protein antibodies (NeuN 1:100; Chemicon) and unlabeled monoclonal mouse anti-HO-1 antibodies (1:500; Stressgen, Ann Arbor, MI) and then for 1 h at room temperature with rhodamine-labeled goat anti-mouse immunoglobulin antibodies (1:200; R&D System), or (3) unlabeled monoclonal mouse anti-HO-1 antibodies and unlabeled polyclonal rabbit anti-phopho-p38 MAPK antibodies (source) and then for 1 h at room temperature with FITC-labeled goat anti-mouse immunoglobulin antibodies and rhodamine-labeled anti-rabbit antibodies. After three phosphate-buffered saline rinses, the sections were incubated in 1% methanol in phosphate-buffered saline containing 20 μg/ml of 4′,6-diamidino-2-phenylindole (Sigma) (blue fluorescence). Images were captured using an Olympus BX 50 fluorescence microscope (Olympus Optical, Tokyo, Japan) and a Delta Vision deconvolution microscopic system operated by SPOT software (Diagnostic Instruments Inc. Sterling Heights, MI).

**Data and Statistical Analysis**

All values are presented as the mean ± SEM and analyzed using SigmaStat 3.0 (SYSTAT Software Inc., San Jose, CA). Tail-flick latency data were analyzed by using two-way analysis of variance (ANOVA) with post hoc Bonferroni test. Values for the analgesic dose of 50% of the maximal possible antinociceptive effect (AD50) were analyzed using a computer-assisted linear regression program SigmaPlot 10.0 (SYSTAT Software Inc.). The 95% confidence interval (CI) was calculated using pharmacologic calculations system PHARM/PCS version 4.2 (PharmSoft.Net, Wynnewood, PA). 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 and cytokine data were analyzed by one-way analysis of variance (ANOVA), followed by multiple comparisons with the Student-Newman-Keuls post hoc test. A significant difference was defined as a P value less than 0.05.

**Results**

**Neutralization of Microglia IL-10 Protein Expression by Anti-IL-10 Antibody in Amitriptyline/morphine Coinfused Rats**

As shown in figure 1A, analysis of lumbar spinal cord homogenates demonstrated that intrathecal daily injec-
Fig. 1. Neutralization of interleukin (IL)-10 protein expression by anti-IL-10 antibody in amitriptyline/morphine coinfused rats. (A) IL-10 protein level was measured in the different groups (n = 4 of each group). Coadministration of amitriptyline significantly decreases IL-10 expression in morphine-infused rats; anti-IL-10 antibody neutralizing the IL-10 expression induced by this combination. One-way analysis of variance (ANOVA) revealed significant differences among the six groups (F(5,18) = 24.35, P < 0.001). *** P < 0.001 as compared with saline-infused group; ### P < 0.001 as compared with morphine-infused group; +++ P < 0.001 as compared to the amitriptyline/morphine coinfused group. (B) Spinal cords were fixed and double-labeled with FITC-labeled anti-CD11/ OX42 (microglia, green fluorescence) and rhodamine-labeled goat anti-IL-10 antibody (red fluorescence). Coincidence of the two labels is seen in yellow (white arrows) (n = 4 of each group). The scale bar represents 20 μm. CONT = saline infusion; CONT + AMI = saline plus amitriptyline coinfusion; CONT + Anti-IL-10Ab = saline infusion plus anti-IL-10 antibody injection for 5 days; FITC = fluorescein isothiocyanate; MO = morphine infusion; MO + AMI = morphine plus amitriptyline coinfusion; MO + AMI + Anti-IL-10Ab = morphine plus amitriptyline coinfusion plus anti-IL-10 antibody injection for 5 days.

Fig. 2. The anti-interleukin (IL)-10 antibody and p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 partially reverses the potentiating effect of amitriptyline on the antinociceptive effect of morphine in chronic morphine-infused rats. (A) Time-course of tail-flick latencies of rats received continuous intrathecal infusion of saline (CONT), amitriptyline (CONT + AMI), SB203580 (CONT + SB), morphine alone (MO), morphine plus amitriptyline (MO + AMI), morphine plus amitriptyline plus SB203580 (MO + AMI + SB), or daily intrathecal injection of neutralization anti-IL-10 antibody (CONT/Anti-IL-10 Ab), or morphine plus amitriptyline plus Anti-IL-10 antibody (MO + AMI/Anti-IL-10 Ab) for 5 days (n = 6 of each groups). *** P < 0.001 as compared with saline-infused group; ### P < 0.001 as compared with morphine-infused group; +++ P < 0.001 as compared to the amitriptyline/morphine coinfused group. (B) On day 5, dose-response curves for morphine were constructed in rats from different drug infusion groups described above. The dose-response effect was presented as the percentage of the maximal possible antinociceptive effect (% of MPE). All data points are presented as the mean ± SEM (n = 12) of each group.

tion of anti-IL-10 antibody (10 μg/5 μl) alone did not affect IL-10 expression compared to saline-infused rats. However, coinfusion amitriptyline/morphine significantly increases the antiinflammatory cytokine IL-10 protein levels compared with saline-, amitriptyline-, and morphine-infused rats. Daily injection of anti-IL-10 antibody significantly neutralized the effect of amitriptyline in morphine-infused rats (F(5,18) = 24.35, P < 0.001). Additional experiments in which the rats received an isotype-matched irrelevant Ig show that the IL-10 protein level (956.7 ± 68.35 ng/ml) compared with amitriptyline/morphine-coinfused rats (906.67 ± 52.14 ng/ml) (P = 0.582, NS). As shown in figure 1B, weak immunocytochemical staining for microglia (OX42-positive cells; green) was distributed throughout the rat spinal cord, and the stained cells had the classic ramified shape of nonactivated microglia in saline-infused, amitriptyline-infused, and anti-IL-10 antibody injection-only rats. In contrast, strong OX42 staining with the amoeboid shape of activated microglia was observed throughout the section, but no IL-10 protein expression was apparent in chronic morphine-infused rats. Overlay of the sections stained for IL-10 and microglia showed colocalization of IL-10 with OX42 immunoreactivity (white arrows) in the spinal cord of amitriptyline plus morphine-infused rats, and additional treatment with anti-IL-10 antibody re-
Neutralization of IL-10 Expression or Blockade of p38 MAPK Pathway Partially Reverses the Effect of Amitriptyline on Morphine Tolerance

As shown in figure 2A, measured by the hot water immersion test, daily intrathecal injection of anti-IL-10 antibody, or continuous infusion of amitriptyline (15 μg/h) or SB203580 (0.5 μg/h) alone had no antinociceptive effect throughout the 5 days. As in our previous study, the maximal antinociceptive effect of morphine was seen on day 1, and maximal tolerance was observed from day 3 in morphine-infused rats (F(5,235) = 23.447, P < 0.001). Amitriptyline (15 μg/h) prevented the reduction of the antinociceptive effect of morphine seen in chronic morphine-infused rats. Daily injection of anti-IL-10 antibody or coinfusion of SB203580 with amitriptyline plus morphine blocked the maintenance of morphine’s antinociceptive effect by amitriptyline (F(47,235) = 6.231, P < 0.001). As shown in figure 2B and table 1, amitriptyline, anti-IL-10 antibody, and SB203580 alone did not affect the dose-response curve of morphine, with an AD50 of 1.13 μg in saline-infused rats, 1.3 μg in amitriptyline-infused rats, 1.24 μg in anti-IL-10 antibody injection rats, and 1.23 μg in SB203580-infused rats. In morphine-infused rats, the dose-response curve was shifted to the right by 77.8-fold compared to saline-infused rats (AD50, 87.96 μg), amitriptyline/morphine coinfusion shifted the dose-response curve to the left compared to morphine infusion alone (AD50, 5.47 μg), whereas injection of anti-IL-10 antibody or coinfusion of SB203580 produced a rightward shift compared to amitriptyline/morphine-infused rats (AD50, 20.23 μg and 17.03 μg, respectively).

Effect of Neutralizing Anti-IL-10 Antibody and SB203580 on MPAK and HO-1 Expression in Amitriptyline/morphine Coinfused Rats

Figure 3 shows that expression of external signal-regulated kinase (ERK), phospho-ERK (p-ERK), c-Jun N-terminal kinase (JNK), and phospho-JNK (p-JNK) protein levels were unaffected by the various treatments. (A and C) Typical blots show expression of ERK and p-ERK (A) and JNK and p-JNK (C) protein in the spinal cord dorsal horn of the five groups (n = 4 of each group). (B and D) Pooled densitometric results for ERK and p-ERK (B) and JNK and p-JNK (D), with the control band intensity assigned the value of 1. No significant difference was found among the groups. β-actin was used as the loading control. CONT = saline infusion; MO = morphine infusion; MO + AMI = morphine plus amitriptyline coinfusion; MO + AMI/anti-IL-10 Ab = morphine plus amitriptyline coinfusion plus anti-IL-10 antibody injection for 5 days; MO + AMI + SB = morphine plus amitriptyline plus SB203580 coinfusion.

Table 1. Morphine’s AD50 Values with the 95% CI after Various Drug Treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>AD50 (μg)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>1.13</td>
<td>1.0–1.24</td>
</tr>
<tr>
<td>CONT + AMI</td>
<td>1.3</td>
<td>1.1–1.6</td>
</tr>
<tr>
<td>CONT/anti-IL-10 Ab</td>
<td>1.24</td>
<td>1.1–1.4</td>
</tr>
<tr>
<td>CONT + SB</td>
<td>1.23</td>
<td>1.0–1.5</td>
</tr>
<tr>
<td>MO</td>
<td>87.96</td>
<td>82.3–93.6</td>
</tr>
<tr>
<td>MO + AMI</td>
<td>5.47</td>
<td>4.8–6.1</td>
</tr>
<tr>
<td>MO + AMI/anti-IL-10 Ab</td>
<td>20.23</td>
<td>18.9–21.7</td>
</tr>
<tr>
<td>MO + AMI + SB</td>
<td>17.03</td>
<td>13.6–20.4</td>
</tr>
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AD50 values were calculated from the antinociceptive dose-response curves shown in Figure 2B.

AD50 = morphine antinociceptive dose with a 50% maximum possible antinociceptive effect; CI = confidence interval; CONT = saline infusion; CONT + AMI = saline plus amitriptyline coinfusion; CONT + SB = saline plus p38 MAPK inhibitor SB 203580 coadministration; CONT/anti-IL-10 Ab = saline infusion plus anti-IL-10 antibody injection for 5 days; MO = morphine infusion; MO + AMI = morphine plus amitriptyline coinfusion; MO + AMI + SB = morphine plus amitriptyline plus SB203580 coinfusion; MO + AMI/anti-IL-10 Ab = morphine plus amitriptyline coinfusion plus anti-IL-10 antibody injection for 5 days.

Fig. 3. External signal-regulated kinase (ERK), phospho-ERK (p-ERK), c-Jun N-terminal kinase (JNK), and phospho-JNK (p-JNK) protein levels were unaffected by the various treatments. (A and C) Typical blots show expression of ERK and p-ERK (A) and JNK and p-JNK (C) protein in the spinal cord dorsal horn of the five groups (n = 4 of each group). (B and D) Pooled densitometric results for ERK and p-ERK (B) and JNK and p-JNK (D), with the control band intensity assigned the value of 1. No significant difference was found among the groups. β-actin was used as the loading control. CONT = saline infusion; MO = morphine infusion; MO + AMI = morphine plus amitriptyline coinfusion; MO + AMI/anti-IL-10 Ab = morphine plus amitriptyline coinfusion plus anti-IL-10 antibody injection for 5 days; MO + AMI + SB = morphine plus amitriptyline plus SB203580 coinfusion.
caused a slight, but significant increase in the level of phosphorylated p38 MAPK (p-p38 MAP) (fig. 4, A and C), but it had no effect on total p38 MAPK, HO-1, or HO-2 levels (fig. 4, B and D). In contrast, amitriptyline/morphine coinfusion significantly increased not only p-p38 MAPK expression (F(4,35) = 820, P < 0.001), but also total p38 MAPK expression (F(4,35) = 25.759, P < 0.001) (fig. 4, A and C) and HO-1 expression (F(4,35) = 84.821, P < 0.001) (fig. 4, B and D), and this injection of anti-IL-10 antibody or infusion of amitriptyline or SB203580 alone had no effect on protein expression (data not shown). As expected, HO-2 expression was not affected by any of the treatments (P = 0.824, NS) (fig. 4, B and D). The effects of amitriptyline/morphine coinfusion on p38 MAPK phosphorylation and HO-1 expression were completely blocked by both daily injection of anti-IL-10 antibody or coinfusion of the p38 MAPK inhibitor SB203580 (fig. 4, A–D). In the current study, we also found that daily intrathecal injection of various doses of anti-IL-10 antibody for 5 days dose-dependently reversed the amitriptyline-induced upregulation of p38 MAPK phosphorylation (F(4,35) = 723.242, P < 0.001) and HO-1 expression (F(4,35) = 39.368, P < 0.001) in morphine-infused rats (fig. 5, A and B). These results suggest that IL-10–induced p38 MAPK phosphorylation is involved in the increase in HO-1 expression in amitriptyline/morphine-infused rats.

**Zinc Protoporphyrin Reverses the Effect of Amitriptyline in HO-1 Expression and Morphone’s Antinociception in Chronic Morphine-infused Rats**

Daily intrathecal injection of various doses of zinc protoporphyrin for 5 days dose-dependently reversed the upregulation of HO-1 expression (F(3,35) = 41.056, P < 0.001; fig. 6A), and it blocked the maintenance of morphine’s antinociceptive effect by amitriptyline.
(F(29,145) = 6.682, P < 0.001) in morphine-infused rats (fig. 6B). The HO-2 expression (P = 0.388, NS) was not affected by zinc protoporphyrin injection. In agreement with the above results, the maximal antinociceptive effect of morphine was seen on day 1, and maximal tolerance was observed from day 3 in morphine-infused rats (F(5,145) = 19.902, P < 0.001). Coadministration of HO-1 antagonist zinc protoporphyrin (24 μg/5 μl) with amitriptyline plus morphine blocked the maintenance of morphine’s antinociceptive effect by amitriptyline and produced a rightward shift in dose-response curves (AD_{50}, 22.78 μg; table 2).

**Inhibition of IL-10 Expression, p38 MAPK Pathway, and HO-1 Expression Reverses the Suppressive Effect of Amitriptyline on Cytokine Production in Morphine-infused Rats**

As shown in figure 7, protein levels for the proinflammatory cytokines, TNF-α (F(5,18) = 58.817, P < 0.001), IL-1β (F(5,18) = 20.317, P < 0.001), and IL-6 (F(5,18) = 102.068, P < 0.001), were significantly increased in the morphine-infused rats, and this effect was significantly attenuated by amitriptyline coinfusion. Furthermore, anti-IL-10 antibody injection, SB203580 coinfusion, and HO-1 antagonist zinc protoporphyrin injection significantly abolished the suppressive effect of amitriptyline on proinflammatory cytokine production in morphine-infused rats.

**Amitriptyline/morphine Coinfusion Induces p38 MAPK and HO-1 Expression in Spinal Cord Neurons**

Fluorescence images of spinal cords from amitriptyline/morphine-coinfused rats, single-staining for neurons (green), HO-1 (red), or nuclei (4',6-diamidino-2-phenylindole, blue) are shown in the upper row in figure 8. As seen in the merged images, HO-1 expression colocalized with neurons (yellow on the merged image in panel D) and was around the nuclear membrane, with some translocated into the nucleus (purple in the merged image). The lower row...
Fig. 7. Anti-interleukin (IL)-10 antibody, SB203580, and heme oxygenase-1 (HO-1) antagonist zinc protoporphyrin reverse the suppressive effect of amitriptyline on proinflammatory cytokine production. Protein levels for tumor necrosis factor-α (TNFα), IL-1β, and IL-6 were measured in the different groups (n = 4 of each group). CONT = saline infusion; MO = morphine infusion; MO+AMI = morphine plus amitriptyline coinfusion; MO+AMI/Anti-IL-10 Ab = morphine plus amitriptyline coinfusion plus anti-IL-10 antibody injection for 5 days; MO+AM+SB = morphine plus amitriptyline plus SB203580 coinfusion; MO+AMI/ZnP = morphine plus amitriptyline coinfusion plus zinc protoporphyrin injection for 5 days. *** P < 0.001 compared with saline-infused group; ### P < 0.001 compared with morphine-infused group; $ P < 0.01$ compared with saline, morphine, and amitriptyline/morphine-infused group.

Fig. 8. Colocalization of heme oxygenase (HO-1) and p-p38 MAPK activators in neurons. Spinal cords from amitriptyline/morphine-coinfused rats were fixed and single-labeled with (4) fluorescein isothiocyanate (FITC)-labeled anti-neuronal nuclei (NeuN) antibody (neurons, green), (B and E) mouse anti-HO-1 antibody (red or green), (F) rabbit anti-phospho-p38 MAPK antibody (red), (C and G) or 4',6-diamidino-2-phenylindole (DAPI) (nuclei, blue), and images were obtained by immunofluorescent laser scanning fluorescence microscopy. D and H are the merged images for A–C and E–G, respectively. These images are representative of those seen in multiple fields for each treatment in four independent experiments. (Upper row) The majority of NeuN-positive cells show HO-1 labeling (yellow in the merged images), which is localized to the nucleus (blue in the merged images). (Lower row) HO-1 expression and p38 MAPK activation are seen in the same cells. The scale bar represents 20 μm. pp38 MAPK = phospho-p38 mitogen-activated protein kinase.

Discussion

In the current study, long-term morphine infusion significantly increased protein levels of proinflammatory cytokines, TNF-α, IL-1β, and IL-6, and caused slight but significant phosphorylation of p38 MAPK. Amitriptyline/morphine coinfusion increased the expression of IL-10 in nonactivated microglia, and increasing of p38 MAPK phosphorylation subsequently induced HO-1 expression in neurons, thus further inhibiting the expression of proinflammatory cytokines; however, the protein expression on ERK, p-ERK, JNK, and p-JNK proteins in the rat spinal cord were not affected. Neutralization of IL-10 expression and blockade of p38 MAPK pathway reverses the amitriptyline-induced increase in HO-1 expression and inhibition of proinflammatory cytokines expression, suggesting that the p38 MAPK pathway, but not the ERK or JNK pathways, is involved in the amitriptyline-mediated induction of HO-1 expression. Moreover, inhibition of IL-10 expression, p38 MAPK pathway, or HO-1 expression partially blocked the inhibitory effect of amitriptyline on the morphine’s antinoceptive tolerance, and it shifted the dose-response curve of morphine to the right from an AD50 of 5.47 μg (morphine plus amitriptyline) to 20.23 μg (morphine plus amitriptyline plus anti-IL-10Ab injection), 17.05 μg (morphine plus amitriptyline plus SB203580), and 22.8 μg (morphine plus amitriptyline plus zinc protoporphyrin injection). These results suggest that the suppressive effect of amitriptyline coinfusion on proinflammatory cytokine production in morphine-infused rats may act through increasing IL-10 protein expression in microglia, which activates the neuronal p38 MAPK pathway and increases HO-1 expression.

Studies have shown that TCAs inhibit the release of inflammatory mediators. Imipramine, clomipramine, and citalopram at low micromolar concentrations partially blocked IL-6, IL-1β, and TNF-α release from human blood monocytes and IL-2 and interferon-γ from T cells.20 Similarly, fluoxetine and amitriptyline were shown to inhibit release of nitric oxide and prostaglandin E2 from human synovial cells.21 A previously performed study also
showed that production of the antiinflammatory cytokine IL-10 was shown to be stimulated by antidepres-
sants.16 These changes resulted in a decrease of hyper-
sensitization of the nociceptive neurons in the spinal 
cord, and amitriptyline, when applied peripherally, also 
prevented c-fos expression in spinal cord in the formalin 
pain model.22 In the current study, we also demon-
strated that coinfusion of amitriptyline increased the 
expression of antiinflammatory cytokines IL-10 in mor-
phine-infused rats.

A previous study demonstrated that IL-10 inhibited 
proinflammatory cytokine production.25 Local injection 
of IL-10 abolishes the mechanical hyperalgesia induced 
by carrageenan administration in the hind paw of rats.24 
IL-10–encoding adenoviral vector not only attenuates 
the development of tolerance, hyperalgesia, and allo-
dynia; it also potentiates the analgesic effect of mor-
phine.7 Treatment with morphine has been shown to 
not alter IL-10 messenger RNA level and even reduces 
lipopolysaccharide-induced IL-10 secretion.25,26 In the 
current study, an increase in IL-10 protein expression 
was observed in some microglia in the spinal cord of 
amitriptyline/morphine coinfused rats, these being non-
activated microglia, as opposed to the activated forms 
seen in morphine-infused rats. Anti-IL-10 antibody neu-
tralized the amitriptyline induced IL-10 in activated mi-
croglia. It implies that the suppressive effect of amitrip-
tyline in morphine-infused rats acts by increasing IL-10 
protein expression.

A previous study showed that IL-10 exerts its antiin-
flammatory effect by upregulating HO-1 expression.19 
Antiinflammatory effect of HO-1 has been demonstrated 
in several inflammatory models.27,28 HO-1 overexpres-
sion, via carbon monoxide-cyclic guanosine monophos-
phate (CO-cGMP) pathway, produces a significant 
aninociceptive effect against formalin pain in mice.29–31 
These results show that HO-1 plays a crucial role in the 
host defense mechanism against inflammation; HO-1 ca-
tabolizes heme and produces antioxidants biliverdin and 
bilirubin, which also play a role in protection against 
tissue injury-induced inflammation. Carbon monoxide is a 
key molecule for the protective effect of HO-1.52,53 
Scavenging of carbon monoxide by hemoglobin signifi-
cantly reduces the effect of IL-10 on inhibiting the lip-
polysaccharide-induced TNF-α and nitric oxide produc-
tion and increasing MMP-9 expression in macrophages.19 
In addition, a recent study indicated that nuclear local-
ization of HO-1 protein causes upregulation of AP-1 gene 
and promotes cytoprotection against oxidative stress.34 
In our current study, we found that amitriptyline/mor-
phine coinfusion significantly increased IL-10 and HO-1 
expression, and it decreased the protein level of proin-
flammatory cytokines TNF-α, IL-1β, and IL-6. In contrast, 
the inhibition of IL-10 or HO-1 expression significantly 
downregulated HO-1 expression and increased TNF-α, 
IL-1β, and IL-6 protein level. These results suggest a role 
of HO-1 in the antiinflammatory effect of IL-10 in ami-
triptyline/morphine-coinfused rats. Taken together, car-
bon monoxide and HO-1 appear to be responsible for 
the protective effect of IL-10, and the detailed mecha-
nisms need to be determined.

An earlier study revealed that the JAK1/STAT3-dependent 
pathway is not sufficient for the antiinflammatory 
effect of IL-10 receptor signaling,35 and the IL-10-acti-
ved phosphatidylinositol-3-kinase and p70 S6 kinase 
pathways are not essential for the antiinflammatory ef-
effect of IL-10.36 Moreover, studies have suggested that 
IL-10 and HO-1 produce a positive feedback circuit to 
amply the antiinflammatory effect by upregulation of 
carbon monoxide-dependent and p38 MAPK-dependent 
mechanisms.19,37 The transcriptional activation of HO-1 is mediated by the ERK, JNK, and p38 MAPK path-
ways,38,39 and p38 MAPK is a key mediator of cellular 
stresses, such as inflammation and apoptosis. Both in 
vitro and in vivo studies have shown that p38 MAPK 
regulates the production of proinflammatory cytokines, 
nitric oxide, and prostaglandin E2 by increasing cytokine 
release or messenger RNA transcription.40–42 As known, 
increasing of proinflammatory cytokine expression in 
the spinal cord is associated with neuropathic pain and 
morphine tolerance.7,43 p38 MAPK is activated in neu-opathic pain by peripheral inflammation and nerve in-
jury.44–46 Morphine tolerance-associated microglia acti-
vation is also activated via p38 MAPK signaling pathway.47 
Inhibition of p38 MAPK was considered to be a potential therapeutic target for antiinflammatory 
therapy.48 However, contradictory results were re-
ported. Treatment with a p38 MAPK inhibitor resulted in 
an enhancement of TNF-α production in mast cells.49 
Moreover, inhibition of p38 MAPK resulted in a reduc-
tion of cytokine production by mast cells, but increase 
cytokine release from lipopolysaccharide-activated macrophages.50 These results suggest that p38 MAPK 
possesses both proinflammatory and antiinflammatory 
characteristics, and plays different roles in different 
inflammatory conditions, depending on the cell type 
and applied stimulus.

Communication between neurons and glia involves 
ion flux, neurotransmitters, cell adhesion molecules, and 
specialized signaling molecules released from synaptic 
and nonsynaptic regions of neurons.51 Morphine-
induced glia activation and proinflammatory cytokine re-
lease are also caused by the induction of neuronal frac-
talkine release.7 Fractalkine, expressed in spinal 
neurons, is a diffusible signal that activates nearby mi-
croglia and then releases proinflammatory cytokines.52 
Theses results suggest that morphine indirectly affects 
glia via chemokines conducting neuron-glia commu-
nication. Activated neuronal protein kinase Cγ (PKCγ) 
acts as an important factor to modulate the neuron-glia 
communication to increase astrocyte reactivity after re-
peated morphine treatment, and this neuron-glia com-

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munication may be responsible for the development of morphine tolerance. In the current study, we suggest that amitriptyline modulates neuroimmune responses via a two-way communication between glia and neurons. Amitriptyline stimulates microglia release of anti-inflammatory cytokine IL-10 via a paracrine-manner and increases neuron pho,p38 MAPK expression, which induces upregulation of HO-1 via carbon monoxide, exerts negative feedback control of microglia activity, and inhibits proinflammatory cytokine expression.

Activation of the neuroimmune system has been demonstrated to modulate morphine tolerance and consequently develops mechanical allodynia and thermal hyperalgesia. Administration of glial metabolic inhibitors, cytokine antagonist IL-10, or IL-1 receptor antagonist IL-1ra attenuates opioid tolerance and associated hyperalgesia and allodynia. Similarly, our previous study also demonstrated that amitriptyline inhibits microglia activation and proinflammatory cytokines expression, thus attenuating morphine tolerance. In our current study, we found that injection of anti-IL-10 antibody, or p38MAPK inhibitor SB203580, or HO-1 inhibitor zinc protoporphyrin increased proinflammatory cytokine expression in amitriptyline/morphine-coinfused rats and partially reversed the effect of amitriptyline on the antiinflammatory effect of morphine seen in chronic morphine-infused rats.

Currently available medications for the management of chronic pain, particularly those for neuropathic pain, are either inadequate or cause unbearable side effects, and TCAs are known to be effective adjuvant for the treatment of chronic neuropathic pain in clinical practice. The current study shows that amitriptyline inhibits proinflammatory cytokine expression by increasing IL-10 expression; it leads to phosphorylation of p38 MAPK that increases HO-1 expression and thus inhibits the expression of proinflammatory cytokines TNF-α, IL-1β, and IL-6 in morphine-tolerant rats. From our recent studies and the results of current study, we suggest that amitriptyline may be used as an adjuvant in combination with opioids for the treatment of chronic neuropathic pain conditions and patients who need long-term opioid administration for pain management in clinical practice. It explains why TCAs play an important role in the treatment of chronic neuropathic pain via systemic administration. The systemic effect and mechanisms of action of amitriptyline in morphine tolerance development and neuropathic pain should require further investigation.

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