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Attenuation of Morphine Tolerance, Withdrawal-Induced Hyperalgesia, and Associated Spinal Inflammatory Immune Responses by Propentofylline in Rats

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The activation of glial cells and enhanced proinflammatory cytokine expression at the spinal cord has been implicated in the development of morphine tolerance, and morphine withdrawal-induced hyperalgesia. The present study investigated the effect of propentofylline, a glial modulator, on the expression of analgesic tolerance and withdrawal-induced hyperalgesia in chronic morphine-treated rats. Chronic morphine administration through repeated subcutaneous injection induced glial activation and enhanced proinflammatory cytokine levels at the lumbar spinal cord. Moreover, glial activation and enhanced proinflammatory cytokine levels exhibited a temporal correlation with the expression of morphine tolerance and hyperalgesia. Consistently, propentofylline attenuated the development of hyperalgesia and the expression of spinal analgesic tolerance to morphine. The administration of propentofylline during the induction of morphine tolerance also attenuated glial activation and proinflammatory cytokines at the L5 lumbar spinal cord. These results further support the hypothesis that spinal glia and proinflammatory cytokines contribute to the mechanisms of morphine tolerance and associated abnormal pain sensitivity.

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INTRODUCTION

Neuronal plasticity associated with hyperalgesia and morphine tolerance has similar cellular and molecular mechanisms, suggesting predictable interactions between hyperalgesia and morphine tolerance (Mao *et al*, 1995; Mayer *et al*, 1999). The induction of pain facilitation by sustained opioid exposure contribute to the development of opioid antinociceptive tolerance, and manipulations that block enhanced pain also block antinociceptive tolerance (Ossipov *et al*, 2003). The role of glia and their secretory products, particularly proinflammatory cytokines, in the development of morphine tolerance, and morphine withdrawal-induced hyperalgesia, have been recently studied (Song and Zhao, 2001; Raghavendra *et al*, 2002). Both glial (microglia and astrocyte) activation and enhanced proinflammatory cytokine levels were observed following chronic morphine treatment at the lumbar spinal cord of the rat (Raghavendra *et al*, 2002). Inhibition of astrocytic activation or antagonizing the activity of proinflammatory cytokines (interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α) attenuated the development of morphine tolerance, and withdrawal-induced hyperalgesia in rats (Song and Zhao, 2001; Raghavendra *et al*, 2002; Johnston *et al*, 2003).

Propentofylline, a novel xanthine derivative with pharmacological effects different from those of the classical methylxanthines, theophylline, and caffeine, is known to modulate glial activity under pathological conditions. Propentofylline depresses the activation of microglia and astrocytes, which are associated with neuronal damage during ischemic injury (DeLeo *et al*, 1988; Schubert *et al*, 1997). Our previous studies demonstrated that propentofylline attenuated nerve injury-induced hyperalgesia, and spared the acute analgesic actions of morphine in rats following peripheral nerve injury (Sweitzer *et al*, 2001; Raghavendra *et al*, 2003b). The attenuation of nerve injury-induced hyperalgesia by propentofylline was also associated with its ability to suppress inflammatory immune responses at the lumbar spinal cord (Raghavendra *et al*, 2003b). Glial activation and increased proinflammatory cytokines were also observed during morphine tolerance and withdrawal-induced hyperalgesia. Hence, the present experiment was

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designed to evaluate the possible beneficial effects of propentofylline in the expression of morphine tolerance and morphine withdrawal-induced hyperalgesia in rats.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) weighing 175–200 g were used for these studies. The animals were allowed to habituate to the housing facilities for at least 1 week before the experiments began. Behavioral studies were carried out in a quiet room between 0900 and 1100. The Institutional Animal Care and Use Committee at Dartmouth College approved the procedures in this study. Efforts were made to limit distress and to use the minimum number of animals necessary to achieve statistical significance as set forth by the International Society for the Study of Pain guidelines (Covino *et al*, 1980).

Behavioral Tests

Mechanical sensitivity to non-noxious stimuli was measured by applying 2- and 12-g von Frey filaments (Stoelting, Wood Dale, IL, USA) on the plantar surface of the ipsilateral hind paw (Colburn *et al*, 1999). The number of paw withdrawals in three sets of 10 stimulations/each set to this normally non-noxious stimulus determined mechanical allodynia. Mechanical nociceptive thresholds were evaluated using an Analgesy-Meter (Ugo Basile, Comerio, Italy) as explained by Stein *et al* (1990). Rats were gently held and incremental pressure (maximum 250 g) was applied onto the dorsal surface of the ipsilateral hind paw. The pressure required to elicit paw withdrawal, the paw pressure threshold (PPT), was determined. Thermal nociceptive thresholds were evaluated by the hot water tail-flick test (Bian *et al*, 1999), which consisted of immersing the tail in water maintained at 49°C and recording the latency to a rapid flick. A 15-s cut-off time was used. In the noxious test paradigms, the mean of three consecutive measurements made, separated by 10 s, was determined and expressed as % maximal possible effect (%MPE) (refer to Statistical analyses). The entire behavioral testing was blinded with respect to the groups.

Experimental Design

Evaluation of expression of morphine tolerance, and withdrawal-induced hyperalgesia and allodynia in rats. Two groups ($n = 8$/group) of rats received subcutaneous (s.c.) injections of either saline or morphine (10 mg/kg; Sigma, St Louis, MO, USA) twice daily at 0800–0900 and 1600–1700 for 5 days to induce opioid tolerance. Chronic morphine withdrawal-induced hyperalgesia and allodynia in these animals were assessed 16 h after the last injection of morphine. Following the recording of morphine withdrawal-induced hyperalgesia and allodynia, animals were treated with morphine (5 μg) acutely via lumbar intrathecal (i.t.) injection to study the expression of morphine tolerance. Acute analgesic activity of i.t. morphine in morphine-tolerant and chronic saline-treated rats were evaluated by mechanical (Anlgesy-Meter) and thermal (tail-flick) test paradigms. Behavior recorded prior to the acute administration of i.t. morphine served as the basal latency.

Effect of propentofylline on the development of morphine tolerance and opioid withdrawal-induced hyperalgesia. A separate group of rats received either saline or morphine (10 mg/kg, s.c., twice daily for 5 days) and were treated once daily (at 1100–1200) with propentofylline (1 and 10 μg) or saline via direct lumbar puncture during induction of morphine tolerance. Chronic morphine withdrawal-induced hyperalgesia and allodynia in these animals were assessed 16 h after the last injection of s.c. morphine. Following the recording of morphine withdrawal-induced hyperalgesia and allodynia, animals were treated with morphine (5 μg) acutely via lumbar puncture to study the expression of morphine tolerance. Acute analgesic activity of i.t. administered morphine in morphine-tolerant and chronic saline-treated rats were evaluated by mechanical (Analgesy-Meter) and thermal (tail-flick) test paradigms. Behavior recorded prior to the acute administration of i.t. morphine served as the basal latency ($n = 8$ rats/group).

Evaluation of propentofylline treatment on chronic morphine-induced glial activation and proinflammatory cytokine levels at L5 lumbar spinal cord.

Tissue collection for real-time RT-PCR, RNase protection assay (RPA), and ELISA: To quantify mRNA for glial fibrillary acidic protein (GFAP), macrophage antigen complex-1 (Mac-1), and cytokine mRNA and cytokine protein levels, a separate group of rats was used ($n = 4$). Testing was blinded with respect to the groups. After behavioral testing, rats were killed by CO_2 asphyxiation followed by immediate decapitation. An 18-gauge needle was inserted into the caudal end of the vertebral column and the spinal cord was expelled with ice-cold phosphate-buffered saline. The spinal cord was frozen immediately on dry ice and stored at −80°C until homogenization. The L5 lumbar spinal cord was isolated from the intact frozen cord at the time of mRNA and protein quantification. Total RNA was isolated from the L5 lumbar spinal cord by the TRIzol extraction method (Invitrogen Corp., Carlsbad, CA, USA).

Real-time RT-PCR: Total RNA was treated with DNase I (Ambion, Austin, TX, USA) to remove DNA contamination before cDNA synthesis. The reverse transcription (RT) was carried out in a 100 μl total reaction volume containing RT buffer, dNTPs, multiscribe reverse transcriptase, RNase-free water and 10 μg of DNase-treated total RNA. The RT reaction was carried out at 25°C for 10 min, 37°C for 120 min, and 95°C for 5 min in the Mastercycler Gradient Eppendorf (Brinkmann Instrument Inc., NY, USA). Real-time PCR analysis was performed on an iCycler IQ™ Multicolor Real-Time PCR detection system (Bio-Rad, CA, USA). Gene-specific primers and probes were designed and were blasted against the GeneBank to confirm their species and gene specificity (Table 1). The primers and probes selected for this experiment met the G-C content requirement and had a melting temperature (T_m) of 60 and 70°C, respectively. The real-time PCR reactions were carried out using Platinum Taq DNA polymerase, 20 mM Tris HCl (PH

Table 1 Summary of Real-Time PCR Primers and Taqman Probes*

Gene	Primer/probe	Sequence
GFAP	Forward primer	5′-TGGCCACCAGTAACATGCAA-3′
	Reverse primer	5′-CAGTTGGCGGCGATAGTCAT-3′
	Probe	5′-CAGACGTTGCTTCCCGCAACGC-3′
Mac-1	Forward primer	5′-CTGCCTCAGGGATCCGTAAAG-3′
	Reverse primer	5′-CCTCTGCCTCAGGAATGACATC-3′
	Probe	5′-CCCGGGACAATGCCGCGAA-3′
GAPDH	Forward primer	5′-CCCCCAATGTATCCGTTGTG-3′
	Reverse primer	5′-TAGCCCAGGATGCCCTTTAGT-3′
	Probe	5′-TGCCGCCTGGAGAAACCTGCC-3′

The Taqman probe has a reporter fluorescent dye, FAM (6-carboxyfluorescein) at the 5′ end and a fluorescence dye quencher, TAMRA (6-carboxytetramethylrhodamine) at the 3′ end.

8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 μM dGTP, dCTP, and dATP, 400 μM of dUTP and 1 U of Uracyl DNA glycosylase (UDG), 900 nM of forward and reverse primers, 300 nM of TaqMan probe, and 10-fold dilution of cDNA (50 ng) from the RT step. Primers and probes for glyceraldehydes-3-phosphate (GAPDH), GFAP, and Mac-1 were obtained from Applied Biosystems. The relative standard curves generated by plotting the threshold value (C_T) *vs* the log of the amount of total cDNA added to the reaction (1–10 000 Pg), according to the protocol described in Bulletin #2 (Applied Biosystems, CA, USA), were used to compare the relative amount of target genes from control and chronic morphine-treated animals. The calculation of C_T, standard curve preparation, and quantification of mRNA in the samples were performed by the software provided with the iCycler system.

RNase protection assay: Assessment of the temporal cytokine mRNA expression in the L5 lumbar spinal cord was performed using a Ribonuclease Protection Assay technique. A MultiProbe RPAse kit was used (PharMingen, San Diego, CA, USA). Total RNA (15 μg) was hybridized to ^{32}P-labeled antisense RNA probes transcribed using the rat cytokine-1 (rCK-1) multiprobe template set (including IL-1α/β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, TNF-α/β, and IFN-γ, L32, GAPDH) resulting in double-stranded target RNA. After RNAse digestion, protected RNA and probe were resolved on a denaturing polyacrylamide gel and visualized by overnight autoradiography. Quantitative image analysis was employed to compare mRNA levels based on band intensities for each cytokine. The intensity of each band was measured using NIH Image software and assigned an arbitrary unit based on the measured intensity levels. The value for the normalized quantity for each band was obtained by dividing by the L32 housekeeping gene control. Individual mRNA concentration was calculated as the ratio of the expression compared with saline-treated animals in which normal values were weighted as 1.

Cytokine protein estimation by ELISA: Quantitative determination of IL-1β, TNF-α, and IL-6 protein was performed on the L5 spinal cord of the rat. Tissue homogenization was prepared as previously described (Raghavendra *et al*, 2002). In brief, weighed sections of L5 spinal cord were homogenized in a homogenization buffer consisting of a protease inhibitor (Boehringer Mannheim, Germany) using the Power Gen 125 tissue tearer (Fisher Scientific, Suwanee, GA, USA). Samples were spun at 20 000 *g* for 30 min at 4°C. Supernatant was aliquoted and stored at −80°C for future protein quantification. IL-1β and TNF-α (R&D systems, Minneapolis, MN, USA) and IL-6 (Biosource, Camarillo, CA, USA) protein concentrations were determined utilizing the quantitative sandwich enzyme immunoassay according to the manufacturer's protocol. IL-1β and IL-6 protein quantification was determined by comparing samples to the standard curve generated from the respective kits.

STATISTICAL ANALYSIS

Values are expressed as means ± SEM. Comparisons between groups were performed using analysis of variance (ANOVA) for repeated measurements followed by the Tukey–Kramer multiple comparisons test using InStat (GraphPad software, Inc., CA, USA). $p<0.05$ was considered significant. Acute i.t. morphine-induced analgesia in mechanical and thermal tests were expressed as the %MPE using the formula: $\%MPE = (WT-CT)/(CO-CT) \times 100$, where WT = withdrawal latency (in 's') or threshold (in 'g') after morphine/saline treatment, CT = latency before morphine/saline treatment and CO = the cut-off value (ie 250 g for mechanical test and 15 s for the tail-flick test). The use of MPE takes into account the differences in basal latencies between different groups of animals so that these differences do not bias the quantification of the antinociceptive effect of the morphine.

RESULTS

Propentofylline Attenuated the Development of Morphine Withdrawal-Induced Hyperalgesia and Expression of Spinal Antinociceptive Tolerance to Morphine

As in our previous study (Raghavendra *et al*, 2002), chronic administration of morphine (10 mg/kg, s.c., twice daily for 5 days) led to withdrawal-induced thermal and mechanical hyperalgesia and mechanical allodynia when recorded 16 h after the last injection. Chronic propentofylline treatment (10 μg, i.t. for 5 days) had no effect on nociceptive threshold in the saline-treated rats. However, propentofylline administration (1 and 10 μg, i.t. for 5 days) during induction of morphine tolerance significantly attenuated morphine withdrawal-induced noxious thermal ($F_{(4,35)} = 34.87$; $p<0.001$) and mechanical hyperalgesia ($F_{(4,35)} = 30.67$; $p<0.001$), and mechanical allodynia (for 2 g, $F_{(4,35)} = 47.45$; $p<0.001$: and for 12 g, $F_{(4,35)} = 93.22$; $p<0.001$) (Table 2).

Chronic administration of s.c. morphine (10 mg/kg, twice daily for 5 days) demonstrated antinociceptive tolerance to acute i.t. morphine (5 μg) in both noxious thermal and mechanical test paradigms (Figure 1). Propentofylline

treatment (10 μg, i.t. for 5 days) to chronic saline (s.c., twice daily for 5 days) administered rats had no effect on the antinociceptive action of acute i.t. morphine (5 μg). However, propentofylline (1 and 10 μg, i.t. for 5 days) treatment during the induction period of morphine tolerance (10 mg/kg, s.c., twice daily for 5 days) significantly attenuated the expression of spinal antinociceptive tolerance to i.t. morphine (5 μg) in both noxious thermal ($F_{(4,35)} = 106.2$; $p < 0.001$) and mechanical ($F_{(4,35)} = 106.2$; $p < 0.001$) test paradigms (Figure 1).

Propentofylline Treatment Attenuated Chronic Morphine-Induced Glial Activation at the L5 Lumbar Spinal Cord

Chronic morphine treatment significantly elevated the immunoreactive OX-42 and GFAP levels at the dorsal horn of the L5 lumbar spinal cord (Song and Zhao, 2001; Raghavendra *et al*, 2002). In the present study, by using the real-time RT-PCR technique, we observed significant increases in the Mac-1 and GFAP mRNA levels at the L5 lumbar spinal cord of chronic morphine-treated (10 mg/kg, twice daily for 5 days) rats compared to the saline-treated control group (Figure 2). Propentofylline (10 μg, i.t. for 5 days) had no effect on the expression of Mac-1 and GFAP in saline-treated rats. However, propentofylline administration during the induction phase of morphine tolerance significantly attenuated chronic morphine-induced upregulation of Mac-1 ($F_{(3,12)} = 7.267$; $p < 0.05$) and GFAP ($F_{(3,12)} = 15.3$; $p < 0.05$) (Figure 2).

Propentofylline Treatment Attenuated Chronic Morphine-Induced IL-1β and IL-6 Expression at the L5 Lumbar Spinal Cord

Chronic administration of morphine (10 mg/kg, twice daily for 5 days) significantly increased the expression of mRNA and protein levels of IL-1β ($p < 0.01$) and IL-6 ($p < 0.01$) at the L5 lumbar spinal cord compared to saline-treated rats (Figure 3, Tables 3 and 4). Although morphine treatment significantly ($p < 0.05$) enhanced the expression of TNF-α mRNA, TNF-α protein levels showed no significant difference as compared with chronic saline-treated animals. Propentofylline treatment (10 μg, i.t. for 5 days) had no significant effect on the expression of IL-1β, IL-6, and TNF-α in saline-treated rats. However, propentofylline treatment during the induction of morphine tolerance significantly attenuated morphine-induced mRNA upregulation for IL-1β ($p < 0.01$), IL-6 ($p < 0.05$) and TNF-α ($p < 0.05$) (Table 3),

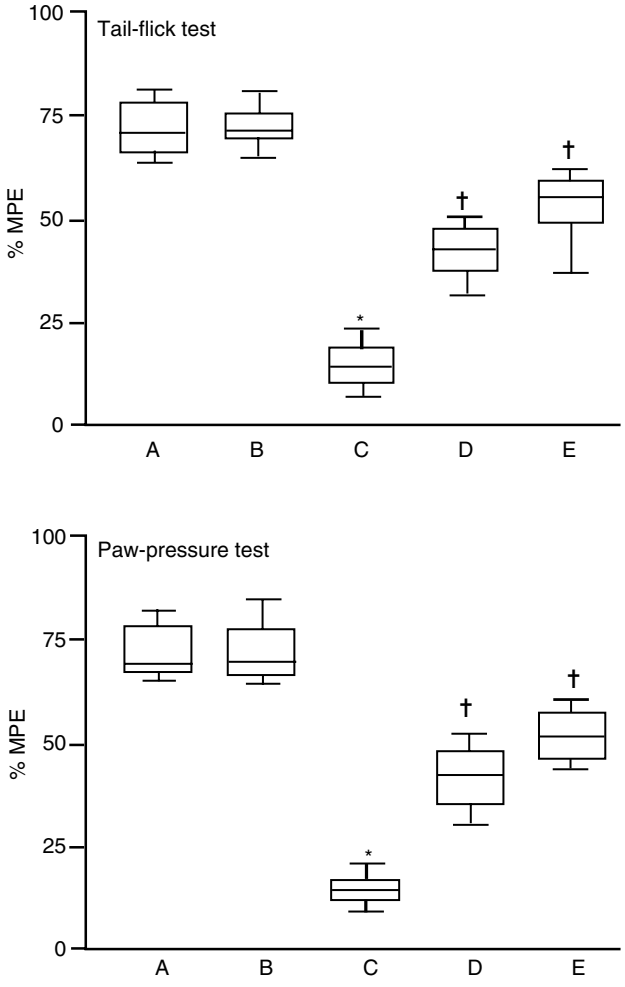

Figure 1 Propentofylline attenuates the expression of morphine tolerance. Rats received chronic saline (a), chronic propentofylline (10 μg) (b), chronic morphine (c), chronic morphine plus propentofylline (1 μg) (d), and chronic morphine plus propentofylline (10 μg) for 5 consecutive days. Acute antinociceptive action of i.t. morphine (5 μg) in these rats was evaluated against noxious thermal (tail-flick test) and mechanical (paw pressure test) stimuli on day 6 (for details, see Materials and methods). Values are mean (%MPE) ± SEM ($n = 8$). *$p < 0.001$ *vs* chronic saline (a), and $^{\dagger}p < 0.01$ *vs* chronic morphine-treated group (c).

Table 2 Effect of Propentofylline on Morphine Withdrawal-Induced Hyperalgesia and Allodynia

			Mechanical allodynia (g)	
Treatment	**Tail-flick latency (s)**	**Paw pressure latency (g)**	**2**	**12**
Saline	5.3 ± 0.2	113.5 ± 4.2	0	0.1 ± 0.2
Saline+propentofylline (10)	5.1 ± 0.3	119.4 ± 5.4	0	0
Morphine	3.7 ± 0.2*	69.8 ± 4.8*	3.9 ± 0.5*	6.9 ± 0.7*
Morphine+propentofylline (1)	4.6 ± 0.2^{+}	85.4 ± 5.9^{+}	1.6 ± 0.2^{+}	2.4 ± 0.4^{+}
Morphine+propentofylline (10)	4.9 ± 0.2^{+}	96.3 ± 4.7^{+}	1.2 ± 0.3^{+}	1.8 ± 0.6^{+}

Treatment consisted of two daily injections of morphine (10 mg/kg, s.c.) administered for 5 consecutive days. Propentofylline (1 and 10 μg) was administered intrathecally once daily during the induction of morphine tolerance. Withdrawal-induced hyperalgesia and allodynia were recorded 16 hr after the last injection of morphine or saline. Data are expressed as mean ± SEM ($n = 8$). *$p < 0.05$ *vs* saline, and $^{+}p < 0.05$ *vs* morphine-treated group.

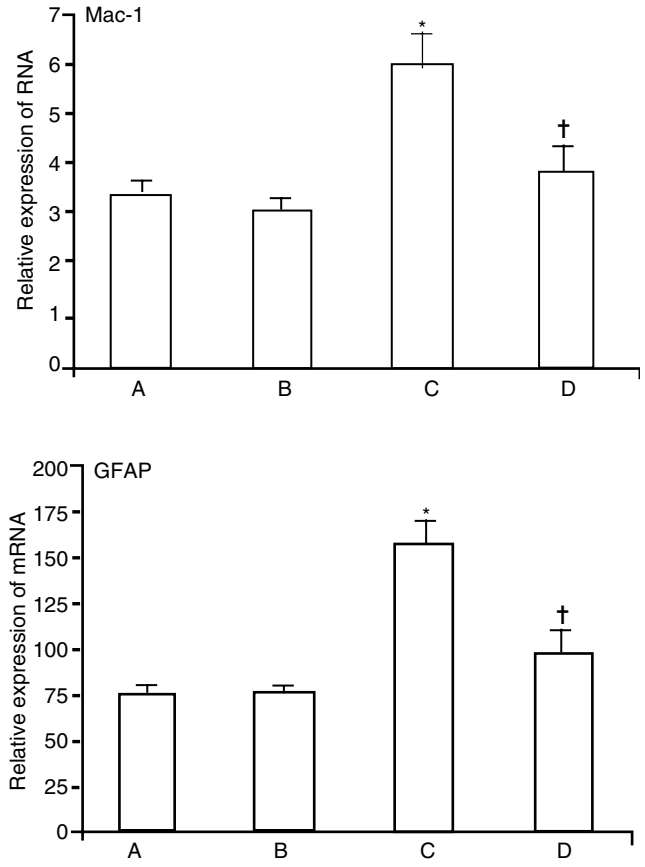

Figure 2 Propentofylline attenuates morphine-induced expression of mRNA for Mac-1 and GFAP at the L5 lumbar spinal cord. Rats received chronic saline (a), chronic propentofylline (10 μg) (b), chronic morphine (c), and chronic morphine plus propentofylline (10 μg) (d) for 5 consecutive days. On day 6, following behavioral observation, L5 spinal cord was harvested and processed for RNA quantification (for details, see Materials and methods). Values are mean (relative expression of mRNA) ± SEM ($n=4$). *$p<0.05$ *vs* chronic saline (a), and $^{\dagger}p<0.05$ *vs* chronic morphine-treated group (c) ($n=4$/group).

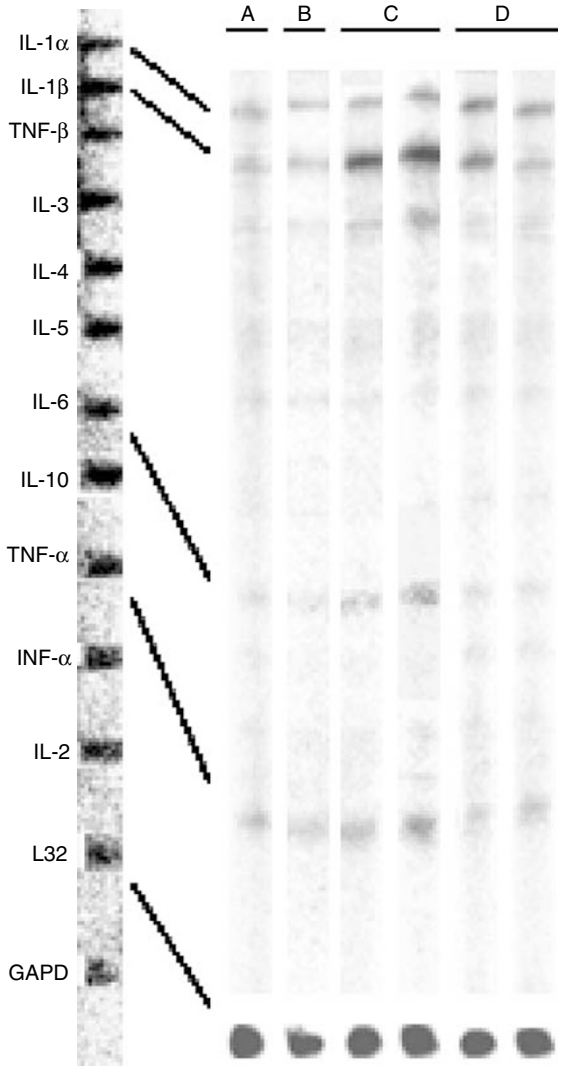

Figure 3 Representative RPA demonstrating cytokine mRNA expression in the L5 lumbar spinal cord of chronic saline (a), chronic propentofylline (10 μg) (b), chronic morphine (c), and chronic morphine plus propentofylline (10 μg)-treated rats (d).

and protein levels of IL-1β ($p<0.01$) and IL-6 ($p<0.01$) (Table 4).

DISCUSSION

Opioids are the most effective analgesics used to treat many forms of acute and chronic pain. The clinical use of opioid analgesics is often hampered by the development of analgesic tolerance that necessitates dose escalation regardless of the disease progression. Recently, we and other investigators showed that glial activation and subsequent proinflammatory immune responses at the lumbar spinal cord contribute to the development of morphine tolerance and morphine withdrawal-induced hyperalgesia (Song and Zhao, 2001; Raghavendra *et al*, 2002; Johnston *et al*, 2003). The present study demonstrates that chronic administration of propentofylline, a glial-modulating agent, administered during the induction of chronic morphine tolerance attenuated both: (1) the expression of spinal analgesic tolerance to morphine and associated inflammatory immune responses at the lumbar spinal cord, and (2) the development of morphine withdrawal-induced hyperalgesia and allodynia.

Glial Cell Modulation of Opioids

The activation of spinal glia, a characteristic response observed during central neuroimmune activation and neuroinflammation, may mediate and/or modulate the pathogenesis of persistent pain states (DeLeo and Yezierski, 2001; Watkins *et al*, 2001; Watkins and Maier, 2002; Raghavendra and DeLeo, 2003). Recent evidence suggests that glial cells may modulate opioid action. Studies showed that morphine primes microglia for enhanced production of TNF-α and nitric oxide (NO), inhibits microglial chemotaxis, and induces apoptosis of microglial cells (Chao *et al*, 1994, 1997; Magazine *et al*, 1996; Hu *et al*, 2002). In addition, chronic morphine activates spinal and cortical glial activity (Beitner-Johnson *et al*, 1993; Song and Zhao, 2001). I.t. administration of the HIV coat glycoprotein, gp120, a potent activator of microglia, reduced the analgesic

Table 3 Effect of Propentofylline (10 μg) on Chronic Morphine-Induced Upregulation of mRNA for IL-1β, IL-6, and TNF-α at the L5 Lumbar Spinal Cord

Treatment	IL-1β	IL-6	TNF-α
Propentofylline (B)	0.8 ± 0.12	1.1 ± 0.15	0.9 ± 0.18
Morphine (C)	1.92 ± 0.14**	1.85 ± 0.16**	1.48 ± 0.2*
Morphine+propentofylline (D)	1.3 ± 0.24^{+}	1.2 ± 0.22^{+}	1.1 ± 0.1^{+}

A multiprobe ribonuclease assay was performed using total mRNA from the L5 lumbar spinal cord of chronic saline (A), chronic propentofylline (B), chronic morphine (C), and chronic morphine plus propentofylline (D)-treated rats. Values for the normalized quantity for each band were obtained by dividing by the L32 housekeeping gene. Individual mRNA concentration in terms of fold activation was calculated as the ratio of their expression compared with the chronic saline (A)-treated animals, where normal values are weighed as 1. Values are mean ± SEM ($n=4$). *$p<0.05$ and **$p<0.01$ *vs* chronic saline (A), and $^{+}p<0.05$ *vs* chronic morphine (C)-treated group.

Table 4 Effect of Propentofylline (10 μg) on Chronic Morphine-Induced Upregulation of IL-1β, IL-6, and TNF-α Protein Levels at the L5 Lumbar Spinal Cord

Treatment	IL-1β	IL-6	TNF-α
Saline	43.5 ± 5.4	197.3 ± 17	25.8 ± 2.8
Propentofylline	50.1 ± 7.8	178.6 ± 21	27.2 ± 3.1
Morphine	73.5 ± 9.2*	359 ± 62.4*	36.6 ± 5.6
Morphine+propentofylline	56.4 ± 3.3^{+}	231 ± 46.7^{+}	29.4 ± 7.1

Values are pg/mg total protein ± SEM ($n=4$). *$p<0.01$ *vs* chronic saline and $^{+}p<0.05$ *vs* chronic morphine-treated group.

effectiveness of morphine (Johnston *et al*, 2003). In our previous study, we showed that morphine tolerance and its withdrawal-induced behavioral hyperalgesia/allodynia was associated with spinal microglial and astroglial activation (Raghavendra *et al*, 2002). The inhibition of spinal glial activation by fluorocitrate, a nonselective metabolic inhibitor of astrocytes partially reversed the development of morphine tolerance in rats (Song and Zhao, 2001). In accordance with the above observations, the present study demonstrated that propentofylline administered during chronic morphine treatment attenuated the expression of spinal analgesic tolerance to morphine and withdrawal-induced hyperalgesia. This was associated with its ability to suppress morphine-induced spinal glial activation.

Mechanisms for Morphine-Induced Glial Activation

The possible mechanisms for chronic morphine-induced glial activation are not known. Although the presence of μ-opioid receptors on microglia and astrocytes is reported (Peterson *et al*, 1998), studies showed that the acute administration of morphine does not induce spinal glial activation (Song and Zhao, 2001; Raghavendra *et al*, 2002). These findings suggest that chronic morphine-induced spinal glial activation could be a compensatory mechanism observed following its long-term administration. Similar to glial activation, a gradual increase in the downregulation of glial glutamate transporters, such as GLAST and GLT-1, was observed only after chronic morphine treatment (Mao *et al*, 2002). The significance of glial activation in the turnover of glial glutamate transporters is not known. However, the development of both neuropathy and morphine tolerance, which share similar molecular and cellular mechanisms, involves glial activation and decreased glial glutamate transporters at the lumbar spinal cord (Song and Zhao, 2001; Mao *et al*, 2002; Raghavendra *et al*, 2002; Sung *et al*, 2003). Also, like propentofylline, neuropathy-induced chronic pain and development of morphine tolerance are attenuated by glial activation inhibitors and glutamate transporter activators (Nakagawa *et al*, 2001; Song and Zhao, 2001; Mao *et al*, 2002; Sung *et al*, 2003; Raghavendra *et al*, 2003a, b).

Glia, upon activation, release various products, such as excitatory amino acids, NO, prostaglandins, proinflammatory cytokines, etc (Kreutzberg, 1996; Aloisi, 2001; Dong and Benveniste, 2001; Raghavendra and DeLeo, 2003). The role of these mediators in the development of morphine tolerance and opioid withdrawal-induced hyperalgesia is well documented (Mao *et al*, 1995; Powell *et al*, 1999; Raghavendra *et al*, 2002). In the present study, we observed that the propentofylline attenuation of morphine tolerance and its withdrawal-induced hyperalgesia was also associated with decreased spinal proinflammatory immune responses. Earlier studies demonstrated neuropathy-induced allodynia/hyperalgesia and decreased morphine analgesia were due to enhanced proinflammatory immune responses at the lumbar spinal cord (Raghavendra *et al*, 2002). Propentofylline attenuated allodynia/hyperalgesia and spared acute morphine analgesia in rats following nerve injury by modulating glial activity and suppressing proinflammatory cytokines (Raghavendra *et al*, 2003b). This suggests that propentofylline's attenuation of morphine tolerance and withdrawal-induced hyperalgesia could be secondary to the suppression of chronic morphine-induced spinal neuroimmune activation.

Opioid and Cytokine Interactions

An interaction between opioids and cytokines clearly exists. Morphine augments the production of proinflammatory cytokines by macrophages and microglia (Chao *et al*, 1994; Peng *et al*, 2000). Central or peripheral administration of IL-1β, IL-6, and TNF-α are known to induce hyperalgesia and allodynia in rodents (Oka and Hori, 1999). Recent studies show that augmentation of pronociceptive mechanisms by cytokines could modulate morphine analgesia. Raffa *et al* (1993) postulated that cytokines might interact with the opioid receptor and modulate its actions. Gul *et al* (2000) and Rady and Fujimoto (2001) observed a reduction in the analgesic effect of morphine after exogenous administration of IL-1β. Dynorphin, a mediator proposed to be responsible for the development of morphine tolerance (Vanderah *et al*, 2000), attenuates the analgesic effect of morphine through endogenous IL-1β (Laughlin *et al*, 2000; Rady and Fujimoto, 2001).

The selective inhibition of IL-1β or collective inhibition of proinflammatory cytokines during chronic morphine treatment attenuated the expression of morphine tolerance and its withdrawal-induced hyperalgesia (Raghavendra *et al*,

2002; Johnston *et al*, 2003). Alternatively, the role of IL-6 in modulating morphine actions is unclear. In IL-6 knockout mice, reduced morphine analgesia and early development of morphine tolerance were observed (Bianchi *et al*, 1999). IL-6 knockout mice used in that study showed increases in β-endorphin levels and decreases in μ-opioid receptor densities in certain brain regions compared to that of wild-type mice (Bianchi *et al*, 1999). This suggests that an altered opioid system in these animals could be responsible for differences in morphine actions compared to that of wild-type animals. Similar to our findings, elevation of IL-6 was observed following central or peripheral administration of morphine (Houghtling and Bayer, 2002; Zubelewicz *et al*, 2000), and IL-6 is known to induce hyperalgesia (Arruda *et al*, 1998; Oka and Hori, 1999).

Mechanisms of Action of Propentofylline

The mechanism of glial modulation and suppression of proinflammatory cytokines synthesis by propentofylline in chronic morphine-treated rats could be due to its inhibitory action on phosphodiesterase enzymes and subsequent augmentation of cAMP signaling. This is supported by the work in which administration of propentofylline mimicked the dibutyryl-cAMP action in inhibiting LPS-induced release of IL-1β, TNF-α, and oxygen radicals from cultured microglial cells (Si *et al*, 1996, 1998). As an atypical methylxanthine, propentofylline also functions as an adenosine reuptake inhibitor (Parkinson *et al*, 1993). Adenosine, by acting via A_1 and A_{2a} receptors, has been found to inhibit microglial proliferation (Si *et al*, 1996).

SUMMARY

The positive safety profile of long-term propentofylline treatment (Mielke *et al*, 1998) and its ability to spare morphine analgesia may have widespread clinical implications. Propentofylline may increase the options for opioid use to treat chronic pain; (a) by reducing opioid dose escalation, which is often associated with unwanted side effects (Raghavendra *et al*, 2003b), and (b) by attenuating the development of analgesic tolerance and hyperalgesia following chronic use of morphine. In summary, these data further support the role of immunocompetent glia and spinal neuroimmunologic processes in the development of morphine tolerance and morphine withdrawal-induced hyperalgesia.

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