Curcumin Attenuates Opioid Tolerance and Dependence by Inhibiting Ca^{2+} /Calmodulin-Dependent Protein Kinase II α Activity

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Received August 11, 2014; accepted November 25, 2014

ABSTRACT

Chronic use of opioid analgesics has been hindered by the development of opioid addiction and tolerance. We have reported that curcumin, a natural flavonoid from the rhizome of *Curcuma longa*, attenuated opioid tolerance, although the underlying mechanism remains unclear. In this study, we tested the hypothesis that curcumin may inhibit Ca^{2+} /calmodulin-dependent protein kinase II α (CaMKII α), a protein kinase that has been previously proposed to be critical for opioid tolerance and dependence. In this study, we used state-of-the-art polymeric formulation technology to produce poly(lactic-co-glycolic acid) (PLGA)-curcumin nanoparticles (nanocurcumin) to overcome the drug's poor solubility and bioavailability, which has made it extremely difficult for studying in vivo pharmacological actions of curcumin. We found that PLGA-

curcumin nanoparticles reduced the dose requirement by 11- to 33-fold. Pretreatment with PLGA-curcumin (by mouth) prevented the development of opioid tolerance and dependence in a dose-dependent manner, with ED₅₀ values of 3.9 and 3.2 mg/kg, respectively. PLGA-curcumin dose-dependently attenuated already-established opioid tolerance (ED₅₀ = 12.6 mg/kg p.o.) and dependence (ED₅₀ = 3.1 mg/kg p.o.). Curcumin or PLGA-curcumin did not produce antinociception by itself or affect morphine (1–10 mg/kg) antinociception. Moreover, we found that the behavioral effects of curcumin on opioid tolerance and dependence correlated with its inhibition of morphine-induced CaMKII α activation in the brain. These results suggest that curcumin may attenuate opioid tolerance and dependence by suppressing CaMKII α activity.

Introduction

Opioid analgesics such as morphine have been used widely for treating moderate-to-severe pain. Although opioids are highly efficacious for treating acute pain, their chronic use has been hindered by the development of opioid tolerance and dependence, the underlying mechanisms of which are not fully understood (Tang et al., 2006a; Wang et al., 2006).

Previous work by our laboratory and others demonstrated that Ca^{2+} /calmodulin-dependent protein kinase II α (CaMKII α) is critically important for the development and maintenance of opioid tolerance, opioid dependence, and opioid-induced hyperalgesia (Chen et al., 2010). CaMKII α is a multifunctional serine/threonine protein kinase that is abundantly expressed in the central nervous system. CaMKII α activity in the spinal cord and brain was found to be elevated after prolonged treatment with morphine (Wang et al., 2003; Liang et al., 2004; Tang et al.,

2006b). Spinal and supraspinal inhibition of CaMKII α were further demonstrated to be effective in preventing and reversing opioid tolerance and dependence in rodent models (Wang et al., 2003; Tang et al., 2006b).

Curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a natural flavonoid component found in the rhizome of *Curcuma longa* (Zingiberaceae or ginger family). A number of pharmacological effects have been reported for curcumin, including antioxidant, anti-inflammatory, chemotherapeutic, and possibly even antinociceptive effects (Asher and Spelman, 2013; Marchiani et al., 2014). Several recent publications suggest that long-term treatment with curcumin is effective in attenuating opioid tolerance and dependence, although the underlying mechanism is not clear (Matsushita and Ueda, 2009; Lin et al., 2011; Liang et al., 2013). Interestingly, curcumin has been recently found to inhibit the Ca^{2+} -dependent and -independent kinase activities of CaMKII based on cell-free assays (Mayadevi et al., 2012). We hypothesize that curcumin may attenuate opioid tolerance and dependence by inhibiting CaMKII α in the central nervous system.

Despite the various reported pharmacologic actions, curcumin is not widely used as a therapeutic agent, likely due to its relatively low solubility and bioavailability (Anand et al., 2007) and lack of understanding of its mechanism of action. With the requirement of high doses in pharmacologic studies and poor solubility, it is difficult to independently confirm

This work was supported in part by the National Institutes of Health National Center for Complementary and Alternative Medicine [Grant K07-AT003647]. Nanoparticle formation was supported by a University of Illinois at Chicago Proof of Concept award. Mechanistic CaMKII study received funds from the National Science Foundation of China (81328009). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of National Center for Complementary and Alternative Medicine or the National Institutes of Health (NIH). The final peer-reviewed manuscript is subject to the NIH Public Access Policy.
dx.doi.org/10.1124/jpet.114.219303.

ABBREVIATIONS: CaMKII, Ca^{2+} /calmodulin-dependent protein kinase II; LC/MS, liquid chromatography/mass spectrometry; MPE, maximal possible effect; NMDA, *N*-methyl-D-aspartate; pCaMKII, phosphorylated CaMKII; PLGA, poly(lactic-co-glycolic acid).

pharmacologic actions and ascertain the exact dose producing these effects. We have recently developed several polymeric nanoparticles encapsulating curcumin, including poly(lactic-co-glycolic acid) (PLGA)-curcumin (Shen et al., 2013). In this study, we have more thoroughly characterized PLGA-curcumin in two rodent models of opioid tolerance and dependence.

Materials and Methods

Morphine sulfate and naloxone HCL were purchased from Hospira (Lake Forest, IL). Curcumin, PLGA (acid terminated; poly(lactic acid): poly(glycolic acid) 50:50, w/w; Mol. Wt. 7000–17,000), tetrahydrofuran, trehalose, leucine, and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Animals. All experiments were performed after approval by the University of Illinois at Chicago Institutional Animal Care and Use Committee, and complied with policies and recommendations of the International Association for the Study of Pain and National Institutes of Health guidelines. Male ICR mice weighing 21–25 g were obtained from Harlan Laboratories (Indianapolis, IN), housed in standard conditions with a light/dark cycle (lights on at 5:00 AM and off at 7:00 PM), and provided with food and water ad libitum prior to experimental procedures.

Production and Characterization of PLGA-Curcumin Nanoparticles. PLGA-curcumin nanoparticles were generated by using a multi-inlet vortex mixer as previously described (Shen et al., 2013). One stream was 0.2% weight percent PLGA and 0.2% weight percent curcumin dissolved in tetrahydrofuran. The other three inlet streams were deionized water as an antisolvent to precipitate the drug compound and PLGA. The volumetric flow rate of streams 1 and 2 was 6 ml/min, and it was 54 ml/min for streams 3 and 4. Freeze drying of the nanoparticle suspensions was carried out in a freeze dryer (FreeZone 1 Liter Console Freeze Dry Systems; Labconco, Kansas City, MO) at a vacuum pressure and -47°C . Trehalose and leucine were used to prevent nanoparticle permanent aggregation during the freeze-drying process. PLGA-curcumin nanoparticles were homogeneously resuspended using bath sonication for 10 minutes before use.

Drug loading, encapsulation efficiency of curcumin in nanoparticles, and nanoparticle size and size distributions were measured as previously described (Shen et al., 2013).

Antinociception Tests. The tail-flick test was performed to assess basal nociception and morphine-induced antinociception as previously described (Tang et al., 2006b; Yang et al., 2011). In brief, the distal one-third of mouse tail was immersed into a 52°C water bath, and the latency to a quick tail-flick response was recorded. Morphine-induced antinociception was determined 30 minutes after an injection of morphine (10 mg/kg s.c.) and expressed as the percentage of maximal possible effect (MPE) according to the following formula:

$$\% \text{MPE} = 100^{\circ} (\text{postdrug latency} - \text{predrug latency}) / (\text{cutoff} - \text{predrug latency}).$$

A cutoff time of 12 seconds was applied to prevent tissue damage.

Acute Opioid Tolerance and Dependence. Mice were made acutely tolerant to and dependent on opioids by the treatment of a large dose of morphine sulfate (100 mg/kg s.c.). Morphine tolerance and dependence developed and peaked around 4–6 hours (Shukla et al., 2006; Tang et al., 2006b; Yang et al., 2011). An equal volume of saline was given to control mice. To assess tolerance to opioids, mice received a test dose of morphine sulfate (10 mg/kg s.c.) 4.5 hours later, and the antinociceptive effect was measured. Tolerance to morphine was demonstrated by a significant reduction of antinociceptive effect. Dependence to opioids was revealed by naloxone-precipitated withdrawal jumping. Mice were treated with naloxone (10 mg/kg i.p.) 5 hours after the injection of morphine sulfate (100 mg/kg s.c.) and were then placed in glass cylinders. Mice were observed for a 15-minute period with the number of vertical jumps recorded. To prevent the development of

morphine tolerance and dependence, PLGA-curcumin (2–20 mg/kg p.o.) was given 15 minutes before the induction dose of morphine sulfate (100 mg/kg s.c.). To reverse the established acute morphine tolerance and dependence, PLGA-curcumin (2–20 mg/kg p.o.) was given 15 minutes before the test dose of morphine sulfate (10 mg/kg s.c.) or naloxone.

Chronic Opioid Tolerance and Dependence. Mice were treated with morphine sulfate (10 mg/kg s.c.) twice a day for 5 consecutive days to induce opioid tolerance and dependence (Herz and Teschemacher, 1973; Yang et al., 2011). Control mice received saline. Morphine tolerance and naloxone-precipitated withdrawal were evaluated as described earlier. On the experiment day, PLGA-curcumin (2–20 mg/kg) was given by gastric gavage (p.o.) 15 minutes before the test dose of morphine or naloxone.

Liquid Chromatography/Mass Spectrometry Analysis of Curcumin in Brain Tissue. Mice were sacrificed 5 minutes after the administration of PLGA-curcumin (20 mg/kg p.o.) or saline, and brain tissues were collected immediately. Brain tissues were homogenized with saline and extracted using liquid-liquid extraction. In brief, ethyl acetate was added to the homogenized tissue sample and mixed vigorously for 10 minutes. The mixtures were then centrifuged at 35,000 rpm for 10 minutes, and supernatants were collected. Vacufuge (Eppendorf, Hauppauge, NY) was used to concentrate the samples. Naive brain tissue spiked with a known amount of curcumin was used as the standard. Curcumin in tissue samples was quantified by negative ion tandem mass spectroscopy, using a Triple Quad LC Mass Spectrometer (6410QQQ; Agilent Technologies, Santa Clara, CA). Identification of curcumin was performed by multiple reactions monitoring using suitable transitions ($367 > 216.9 \text{ m/z}$).

Immunoblotting Analysis. Prefrontal cortex sections were quickly dissected on ice and frozen on dry ice for western blotting analysis as described previously (Luo et al., 2008; Chen et al., 2010). Tissues were homogenized in ice-cold radioimmunoprecipitation assay buffer in the presence of protease inhibitors and phosphatase inhibitors and centrifuged. Protein content in the supernatant was determined by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Samples (20 μg of protein) were separated by 12% SDS-PAGE and electrotransferred onto a polyvinylidene difluoride membrane. The membrane was preblocked using 5% bovine serum albumin (Sigma-Aldrich) in 20 mM Tris-buffered saline (pH 7.6) containing 0.1% Tween 20. Antibodies including a rabbit anti-(T286)phosphorylated CaMKII α (pCaMKII α) antibody (1:1000; Santa Cruz Biotechnology, Dallas, TX), a mouse anti- β -actin antibody (1:10,000; Thermo Fisher Scientific, Waltham, MA), and horseradish peroxidase-conjugated donkey anti-rabbit (for pCaMKII α) or anti-mouse (for β -actin) secondary antibodies (1:10,000; Santa Cruz Biotechnology) were used. The specificities of the anti-(T286)pCaMKII α antibody were characterized in transgenic mice (CaMKII α T286A) lacking the phosphorylation site (Chen et al., 2010). An enhanced chemiluminescence detection system (Thermo Fisher Scientific) was applied for detection. Enhanced chemiluminescence signals were captured by a ChemiDoc system and analyzed with the Quantity One program (Bio-Rad, Hercules, CA). Ratios of the optical densities of pCaMKII α to those of β -actin were calculated for each sample.

Statistics. All data are presented as the mean $\pm$ S.E.M. Comparisons between treatment groups were analyzed using a one-way analysis of variance followed by Dunnett's post-hoc test. Statistical significance was established at the 95% confidence limit.

Results

Unformulated Curcumin Prevented Acute Opioid Tolerance and Dependence. First, an acute model of opioid tolerance and dependence was used to investigate whether curcumin could prevent the development of opioid tolerance and dependence. To induce acute opioid tolerance, mice received a large dose of morphine sulfate (100 mg/kg s.c.), and 4.5 hours later, showed significantly reduced antinociception to

morphine (10 mg/kg) ($19.1 \pm 4.9\%$ MPE, $P < 0.001$) compared with MPE in the control mice pretreated with saline ($91.5 \pm 4.4\%$ MPE) (Fig. 1A). Mice were treated with unformulated curcumin (20–400 mg/kg p.o.) 15 minutes before the induction dose of morphine. Mice treated with curcumin (20 mg/kg p.o.) developed morphine antinociceptive tolerance ($22.6 \pm 5.2\%$ MPE versus $91.5 \pm 4.4\%$ MPE in the saline group, $P < 0.001$) and displayed a significant number of naloxone-precipitated withdrawal jumps (82.7 ± 11.7 versus 13.0 ± 4.9 in the saline group, $P < 0.001$) (Fig. 1). In mice treated with curcumin (200 or 400 mg/kg p.o.), morphine (100 mg/kg) did not produce antinociceptive tolerance ($75.9 \pm 12.4\%$ and $81.1 \pm 7.0\%$ MPE, not significant from the saline-treated group, $P < 0.001$ versus morphine alone) (Fig. 1). In those mice, naloxone-precipitated withdrawal jumping was significantly reduced (46.3 ± 10.8 and 37.0 ± 12.8 versus 80.4 ± 7.4 in the morphine group, $P < 0.05$ and $P < 0.01$, respectively), suggesting that curcumin at high doses prevented the development of acute morphine tolerance and dependence (Fig. 1). The ED_{50} of curcumin is estimated to be 44.2 mg/kg (tolerance) and 109.0 mg/kg (dependence) (Fig. 3).

PLGA-Curcumin Nanoparticles Prevented Acute Opioid Tolerance. The major problem in working with curcumin was its poor solubility and bioavailability; therefore,

the drug at very high doses was required in pharmacologic experiments. We found that PLGA-curcumin nanoparticles significantly improved the solubility of the compound. In this study, we compared the relative potency of unformulated versus PLGA-curcumin nanoparticles in attenuating the development of opioid tolerance and dependence. Mice received PLGA-curcumin nanoparticles at three different doses (2, 6, and 20 mg/kg p.o.) 15 minutes before the induction dose of morphine. In mice receiving the highest dose of PLGA-curcumin nanoparticles (20 mg/kg p.o.) and morphine (100 mg/kg s.c.), opioid tolerance was almost absent with largely intact morphine-induced antinociception ($79.9 \pm 9.8\%$ MPE, $P < 0.001$ versus the morphine-alone group; not significant versus the saline group) (Fig. 2A). PLGA-curcumin nanoparticles at 6 mg/kg also significantly attenuated morphine antinociceptive tolerance ($64.5 \pm 14.6\%$ MPE, $P < 0.05$ versus the morphine-alone group; not significant from the saline group) (Fig. 2A). Pretreatment with PLGA-curcumin nanoparticles at a lower dose (2 mg/kg p.o.) was ineffective in modulating opioid tolerance ($32.3 \pm 11.1\%$ MPE, not significant versus the morphine-alone group) (Fig. 2A). These data suggest that curcumin encapsulated in PLGA nanoparticles prevented morphine tolerance, and the effect is dose-dependent

Fig. 1. Prevention of acute opioid tolerance (A) and dependence (B) by curcumin at high doses. Separated groups of six mice were pretreated with curcumin (20, 200, 400 mg/kg p.o.) or saline before the treatment with morphine sulfate (100 mg/kg s.c.) or saline to induce acute opioid tolerance and dependence. Curcumin (200, 400 mg/kg) significantly attenuated opioid antinociceptive tolerance (A) and physical dependence (B), whereas it was not effective at 20 mg/kg. Data are expressed as the mean $\pm$ S.E.M. *** $P < 0.001$ compared with the saline group; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ compared with the morphine (MS) group.

Fig. 2. Prevention of acute opioid tolerance (A) and dependence (B) by PLGA-curcumin nanoparticles. Separate groups of eight mice were pretreated with PLGA-curcumin nanoparticles (2–20 mg/kg p.o.) or saline for 15 minutes. Acute opioid tolerance and dependence were established by treatment with morphine sulfate (100 mg/kg s.c.) or saline for 4.5 hours. PLGA-curcumin nanoparticles (6, 20 mg/kg p.o.) significantly prevented the development of opioid tolerance (A) and dependence (B) in a dose-dependent manner. Data are expressed as the mean $\pm$ S.E.M. * $P < 0.05$; *** $P < 0.001$ compared with the saline group; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ compared with the morphine (MS) group.

with an estimated ED_{50} of 3.9 mg/kg (p.o.), which is 11 times lower than the ED_{50} of unformulated curcumin (Fig. 3A).

PLGA-Curcumin Nanoparticles Prevented Acute Opioid Dependence. In these experiments, mice pretreated with morphine (100 mg/kg) or saline were challenged with naloxone (10 mg/kg i.p.) 5 hours later. Morphine-pretreated mice exhibited a significantly higher number of naloxone-precipitated withdrawal jumps (83.6 ± 9.4) compared with mice that received saline (14.9 ± 4.6 , $P < 0.001$), which is indicative of the development of acute dependence on morphine (Fig. 2B). In mice copretreated with PLGA-curcumin nanoparticles (2, 6, 20 mg/kg p.o.) and morphine, the numbers of naloxone-precipitated withdrawal jumps were reduced to 53.5 ± 14.4 , 36.3 ± 7.9 , and 31.2 ± 8.5 , respectively (Fig. 2B). These data indicated that curcumin encapsulated in PLGA nanoparticles dose-dependently blocked the development of acute physical dependence, with an estimated ED_{50} value of 3.2 mg/kg, which is 33 times lower than the ED_{50} of unformulated curcumin (Fig. 3B). Therefore, nanoparticles exhibited significantly improved curcumin solubility and higher potency than the unformulated curcumin.

Effect of PLGA-Curcumin Nanoparticles on Basal Nociception, Morphine Antinociception, and Locomotor Activity. There are several potential confounding factors in interpreting the results presented in the previous sections, because free or nanoencapsulated curcumin may 1) produce

antinociception, 2) interfere with morphine antinociception, and 3) impair locomotor activity. Therefore, we directly determined whether free or nanoencapsulated curcumin produced antinociception or interfered with morphine antinociception. Separate groups of mice received either nanocurcumin (20 mg/kg p.o.) or free curcumin (400 mg/kg p.o.), followed by morphine (0, 1, 3, or 10 mg/kg s.c.). Mice treated with nanocurcumin (20 mg/kg p.o.) or free curcumin (400 mg/kg p.o.) showed no difference in basal tail-flick withdrawal latencies compared with the saline-treated mice ($P > 0.05$) (Fig. 4A). Neither free nor nanocurcumin altered antinociception produced by morphine (1, 3, or 10 mg/kg s.c.) ($P > 0.05$) (Fig. 4B).

We further tested the effect of curcumin and nanocurcumin on locomotor activity in a rotarod test (Chen et al., 2010). Mice were placed on an accelerating rotarod, and the latencies to fall off were recorded 0.5, 1, 2, and 4 hours after the administration of nanocurcumin (20 mg/kg p.o.) or free curcumin (400 mg/kg p.o.). The locomotor coordination of the mice treated with PLGA-curcumin nanoparticles or unformulated curcumin was indistinguishable from the saline-treated mice ($P > 0.05$, $n = 6$) (Fig. 4C).

Therefore, nanocurcumin (20 mg/kg p.o.) or curcumin (400 mg/kg p.o.) did not produce nociception, interfere with morphine antinociception, or impair locomotor activity.

PLGA-Curcumin Nanoparticles Reversed Acute Opioid Tolerance and Dependence. We further examined whether PLGA-curcumin nanoparticles can reverse the established opioid tolerance and dependence in the acute model. Mice were given nanocurcumin (2, 6, 20 mg/kg p.o.) 15 minutes before the test dose of morphine or naloxone. Nanocurcumin (20 mg/kg p.o.) completely restored the morphine antinociception ($86.3 \pm 9.5\%$ MPE, $P < 0.001$) in morphine-pretreated mice, compared with the tolerance control group ($21.1 \pm 4.3\%$ MPE) (Fig. 5A). Nanocurcumin at a lower dose (6 mg/kg p.o.) produced a partial effect ($45.9 \pm 13.0\%$ MPE). No effect was found in mice treated with the lowest dose (2 mg/kg) of nanocurcumin ($26.5 \pm 10.4\%$ MPE, $P > 0.05$) (Fig. 5A).

PLGA-curcumin particles (20 and 6 mg/kg p.o.) also significantly attenuated the established physical dependence on morphine, as revealed by naloxone-precipitated withdrawal jumping, resulting in 23.0 ± 8.0 ($P < 0.001$) and 29.7 ± 7.2 ($P < 0.01$) jumps (Fig. 5B). In comparison, naloxone produced 88.6 ± 7.6 jumps in the mice treated with morphine alone (Fig. 5B). Nanocurcumin at the lowest dose (2 mg/kg p.o.) did not have a significant effect (60.7 ± 21.7 jumps) (Fig. 5B). The ED_{50} of PLGA-curcumin nanoparticles is estimated as 12.6 and 3.1 mg/kg for reversing the established acute morphine tolerance and dependence, respectively.

Liquid Chromatography/Mass Spectrometry Analysis of Curcumin in Brain Tissue after PLGA-Curcumin Administration. To investigate whether PLGA-curcumin can be taken up in the brain, we directly determined the level of curcumin in brain tissues after PLGA-curcumin treatment using liquid chromatography/mass spectrometry (LC/MS). Mice were treated with PLGA-curcumin (20 mg/kg p.o.) or saline, and brain tissues were collected 5 minutes later. Brain samples from mice treated with PLGA-curcumin contained a significant amount of curcumin ($367 > 216.9$ m/z transition at ~ 9.8 -minute acquisition time) (Fig. 6C) compared with no curcumin peak in the naïve brain tissue (Fig. 6A). The naïve brain tissues spiked with a known amount of standard curcumin (8 ng/g) had an identical peak (Fig. 6B). This suggests

Fig. 3. Dose-response curve of unformulated curcumin and PLGA-curcumin nanoparticles. Dose-response curves for the effects of unformulated curcumin and PLGA-curcumin nanoparticles on the acute morphine tolerance (A) and dependence (B) were plotted on a log-dose scale. ED_{50} values were calculated based on the dose-response curve. PLGA-curcumin nanoparticles left shifted the dose-response curve and showed higher potency than unconjugated curcumin in preventing both acute morphine tolerance and dependence.

Fig. 4. Effect of PLGA-curcumin nanoparticles on basal nociception, morphine antinociception, and locomotor activity. (A) Separate groups of six mice received (p.o.) PLGA-curcumin nanoparticles (20 mg/kg), curcumin (400 mg/kg), or saline. The tail-flick test was performed 1, 2, and 4 hours after drug administration. Neither PLGA-curcumin nanoparticles (20 mg/kg) nor curcumin (400 mg/kg) changed the basal nociception (A) in the mice. (B) Separate groups of six mice received (p.o.) PLGA-curcumin nanoparticles (20 mg/kg), curcumin (400 mg/kg), or saline, followed by morphine sulfate (1–10 mg/kg s.c.) 15 minutes later. PLGA-curcumin nanoparticles or curcumin did not interfere with antinociceptive effects produced by morphine. (C) Effects of PLGA-curcumin nanoparticles and curcumin on locomotor activity were assessed by the rotarod test in separate groups of six mice. PLGA-curcumin nanoparticles (20 mg/kg p.o.) or curcumin (400 mg/kg p.o.) did not affect the locomotor activities. MS, morphine.

Fig. 5. Reversal of acute opioid tolerance (A) and dependence (B) by PLGA-curcumin nanoparticles. Groups of eight mice received morphine sulfate (100 mg/kg s.c.) to induce acute opioid tolerance and dependence. PLGA-curcumin nanoparticles (2–20 mg/kg p.o.) or saline was administered 15 minutes before a test dose of morphine (10 mg/kg s.c.) or naloxone (10 mg/kg i.p.). PLGA-curcumin nanoparticles dose-dependently reversed the acute opioid tolerance (A) and opioid dependence (B). Data are expressed as the mean $\pm$ S.E.M. * P < 0.05; ** P < 0.01; *** P < 0.001 compared with the saline group; ## P < 0.01; ### P < 0.001 compared with the morphine (MS) group.

that curcumin infiltrated the blood-brain barrier into the brain within 5 minutes of PLGA-curcumin administration.

PLGA-Curcumin Nanoparticles Reduced CaMKII α Activation in Acute Opioid Tolerance and Dependence.

To investigate the hypothesis that curcumin can inhibit CaMKII α , which may mediate the effect seen in opioid tolerance and dependence, we determined the CaMKII α activity in the prefrontal cortex using western blotting analysis. Activation of CaMKII α was determined by the amount of pCaMKII α . The pCaMKII α immunoreactivity was significantly elevated in the acute opioid tolerance and dependence state. Either pretreatment or acute treatment with nanocurcumin (20 mg/kg p.o.) significantly reduced pCaMKII α in mice that are dependent on/tolerant to morphine (Fig. 7). These data suggest that PLGA-curcumin nanoparticles may attenuate acute opioid tolerance and dependence by inhibiting CaMKII α activity.

PLGA-Curcumin Nanoparticles Attenuate Opioid Tolerance and Dependence in a Chronic Model.

To eliminate the possibility that the promising effect of PLGA-curcumin nanoparticles was limited to acute morphine tolerance and dependence, we next studied the effects of PLGA-curcumin nanoparticles in a chronic model of opioid tolerance and

Fig. 6. LC/MS analysis of curcumin in brain tissue after PLGA-curcumin administration. Mice were treated with PLGA-curcumin (20 mg/kg p.o.) or saline, and brain tissues were collected 5 minutes later. Blank brain tissue has no peak (A), whereas standard curcumin added in blank brain tissue peaked around 9.8 minutes of acquisition time (B). Brain samples from mice treated with PLGA-curcumin have a peak of curcumin at 9.8 minutes (C). MRM, multiple reaction monitoring.

dependence. Mice developed antinociceptive tolerance to and physical dependence on opioids within days after receiving twice-daily injections of morphine (10 mg/kg per injection s.c.) (Herz and Teschemacher, 1973; Yang et al., 2011). On day 6, morphine (10 mg/kg s.c.) antinociception was significantly reduced in mice chronically treated with morphine ($14.2 \pm 4.2\%$ MPE, $P < 0.001$) compared with saline-treated mice ($97.6 \pm 2.4\%$ MPE) (Fig. 8A). Administration of PLGA-curcumin nanoparticles (20 mg/kg p.o.) significantly attenuated the established chronic morphine tolerance ($77.9 \pm 8.8\%$ MPE, $P < 0.001$ versus the morphine group), whereas nanocurcumin at lower doses (6 and 2 mg/kg p.o.) showed marginal effects on morphine tolerance ($43.5 \pm 17.9\%$ and $17.6 \pm 16.0\%$ MPE, $P > 0.05$ from morphine alone) (Fig. 8A).

Nanocurcumin (20 and 6 mg/kg p.o.) significantly blocked naloxone-precipitated withdrawal jumping (28.2 ± 7.7 , $P < 0.001$ and 42.5 ± 10.4 , $P < 0.01$ compared with the morphine group), although at the lowest dose (2 mg/kg) it was not effective (72.2 ± 6.5 , $P > 0.05$) (Fig. 8B). Therefore, PLGA-curcumin nanoparticles dose-dependently attenuated chronic morphine tolerance and dependence, with estimated ED_{50} values of 7.2 and 4.0 mg/kg, respectively.

PLGA-Curcumin Nanoparticles Attenuated Morphine-Induced Activation of CaMKII α in the Chronic Model of Opioid Tolerance and Dependence. To correlate the effect of PLGA-curcumin nanoparticles in chronic opioid tolerance and dependence with the activity of CaMKII α , we tested the

pCaMKII α immunoreactivity in these mice. Chronic treatment with morphine significantly enhanced the activity of CaMKII α in the prefrontal cortex (Fig. 8C). PLGA-curcumin nanoparticles (20 mg/kg p.o.) significantly reduced morphine-induced pCaMKII α immunoreactivity in these mice (Fig. 8C), suggesting that the pharmacological effect of PLGA-curcumin nanoparticles in attenuating chronic opioid tolerance and dependence correlated with its ability to inhibit CaMKII α activity. Since CaMKII α has been implicated as a critical regulator of opioid tolerance and dependence, our data suggest that PLGA-curcumin nanoparticles attenuated opioid tolerance and dependence by inhibiting CaMKII α .

Discussion

In the current study, we investigated the effects of nanocurcumin at three different doses (2, 6, and 20 mg/kg p.o.) in two mouse models of morphine tolerance and dependence, comparing the effects with free curcumin at three much higher doses (20, 200, and 400 mg/kg p.o.). Nanoencapsulation of curcumin lowered the ED_{50} approximately 11 (tolerance) to 33 (dependence) times.

We demonstrated that PLGA-curcumin nanoparticles not only prevented but also reversed opioid antinociceptive tolerance and physical dependence in mice. Since curcumin (up to 400 mg/kg) or nanocurcumin (up to 20 mg/kg) alone did not produce antinociception, interfere with morphine antinociception, or impair

Fig. 7. Pretreatment (A) or acute treatment (B) with PLGA-curcumin nanoparticles inhibited supraspinal CaMKIIα activity. Prefrontal cortex samples were taken to determine CaMKIIα activity. (A) For preventing acute opioid tolerance and dependence, mice were pretreated with PLGA-curcumin nanoparticles (20 mg/kg p.o.) or saline 15 minutes before the large dose of morphine (100 mg/kg s.c.). Mice were sacrificed and prefrontal cortex samples were collected 4.5 hours later. Supraspinal CaMKIIα activity was significantly enhanced in morphine-treated mice. PLGA-curcumin nanoparticles (20 mg/kg p.o.) effectively prevented the elevation of CaMKIIα activity. (B) For reversing acute opioid tolerance and dependence, mice were treated with a large dose of morphine (100 mg/kg s.c.). After 4.5 hours, mice received PLGA-curcumin nanoparticles (20 mg/kg p.o.) or saline 15 minutes before the tissue collection. PLGA-curcumin nanoparticles (20 mg/kg p.o.) significantly reduced the activation of CaMKIIα in acute opioid-tolerant and -dependent mice. Densitometry ratio (arbitrary unit) over actin was first calculated, and was further normalized to that of control (fixed to 1). Final data are expressed as the mean ± S.E.M. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with the saline group; ## $P < 0.01$; ### $P < 0.001$ compared with the morphine (MS) group. CaMKIIα^{-/-}, negative control using brain tissues from CaMKIIαT286A mice (Chen et al., 2010).

locomotor activity, we concluded that the action of free or nanoencapsulated curcumin was due to its interactions with endogenous mechanisms that are important for promoting and maintaining opioid tolerance and dependence.

We further demonstrated that the behavioral effect of PLGA-curcumin nanoparticles correlated with its inhibition of CaMKIIα activity in the central nervous system. These data are consistent with the findings from our previous studies that have implicated a role for CaMKIIα in the development and

Fig. 8. Reversal of chronic opioid tolerance (A), dependence (B), and supraspinal CaMKIIα activation (C) by PLGA-curcumin nanoparticles. Separate groups of six mice were treated with morphine sulfate (10 mg/kg s.c.) twice a day for 5 consecutive days to induce chronic opioid tolerance and dependence. Mice received PLGA-curcumin nanoparticles (2–20 mg/kg p.o.) or saline 15 minutes before a test dose of morphine (10 mg/kg s.c.) or immediately before naloxone (10 mg/kg i.p.) on day 6. Established chronic opioid tolerance (A) and dependence (B) were significantly reversed by PLGA-curcumin in a dose-dependent manner. (C) Chronic morphine exposure significantly increased the phosphorylation of CaMKIIα, which was attenuated by acute treatment with PLGA-curcumin nanoparticles (20 mg/kg p.o.). Densitometry ratio (arbitrary unit) over actin was first calculated, and was further normalized to that of control (fixed to 1). Final data are expressed as the mean ± S.E.M. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with the saline group; ## $P < 0.01$; ### $P < 0.001$ compared with the morphine (MS) group.

maintenance of opioid tolerance and dependence (Wang et al., 2003; Tang et al., 2006b). CaMKIIα is colocalized with the μ-opioid receptor in the anatomic areas that are critical for pain processing, such as the superficial layer of the spinal dorsal horn and the dorsal root ganglia (Bruggemann et al., 2000). It has been reported that constitutively active CaMKIIα increased

agonist-induced desensitization of the μ -opioid receptor in cellular studies (Koch et al., 1997), and phosphorylation of the μ -opioid receptor by colocalized CaMKII α may contribute to the development of opioid tolerance and dependence (Bruggemann et al., 2000).

CaMKII α may interact with the *N*-methyl-D-aspartate (NMDA) receptors in the initiation process of opioid tolerance and dependence (Trujillo and Akil, 1991; Gutstein and Trujillo, 1993). Ca²⁺ influx through the activation of the NMDA receptor may lead to CaMKII α autophosphorylation at Thr286 and resultant full activation of the kinase (Strack et al., 2000). In turn, activated CaMKII α can phosphorylate and activate the NMDA receptor, forming a positive feed-forward loop between CaMKII α and the NMDA receptor (Kitamura et al., 1993; McGlade-McCulloh et al., 1993).

Although the beneficial actions of inhibiting the NMDA receptor (Trujillo and Akil, 1991; Gutstein and Trujillo, 1993) or CaMKII α (Wang et al., 2003; Tang et al., 2006b; Chen et al., 2009) in opioid tolerance, dependence, and in chronic pain have been unequivocally demonstrated and replicated by independent studies, little progress has been made in translating these findings to clinical use. In many attempts, drug toxicity was cited as a culprit for lack of success. We have taken an approach in drug repurposing whereby clinically used drugs were screened and studied for inhibiting CaMKII α . The goal is to identify clinically used drugs with CaMKII α inhibitory activity for alleviating problems associated with opioids, such as opioid tolerance, opioid dependence, and opioid-induced hyperalgesia (Chen et al., 2010). Here, we took another approach to identify the pharmacologic mechanisms and efficacy of a traditionally used, orally effective, relatively safe botanical ingredient that is not found in the U.S. pharmacopoeia. Moreover, we presented a method to improve compound druggability by formulating polymeric nanoparticles.

In this study, we have demonstrated that one-time administration of PLGA-curcumin (20 mg/kg p.o.) is effective in preventing acute morphine tolerance and dependence, and in reversing both established acute and chronic morphine tolerance and dependence. It has been reported in the literature that chronic treatment with curcumin at a dose of 100 mg/kg p.o. or 50 mg/kg i.p. effectively attenuated chronic morphine tolerance and dependence (Matsushita and Ueda, 2009; Liang et al., 2013). However, another group reported that chronic curcumin treatments at low doses (25 mg/kg i.p.) attenuated morphine tolerance, but high doses of curcumin (400 mg/kg i.p.) aggravated morphine tolerance (Lin et al., 2011). Since curcumin has a poor solubility and bioavailability, the actual doses of curcumin exhibiting the effect may be difficult to obtain precisely in those studies. We used curcumin encapsulated in PLGA, which greatly improves the solubility and bioavailability of curcumin, and we found that significantly lower doses of curcumin were needed to generate the comparable pharmacologic effects as evidenced by the dramatic left shift in dose-response curves.

This is our first study aiming to establish the mechanism of curcumin and efficacy of PLGA-curcumin. We correlated curcumin's effect on morphine tolerance and dependence with the significantly reduced supraspinal CaMKII α phosphorylation. Furthermore, using the LC/MS analysis, we demonstrated that curcumin, administered in PLGA-curcumin constructs, was able to infiltrate the blood-brain barrier and become available in the brain within 5 minutes after its administration.

In addition to the brain, curcumin may also inhibit CaMKII α in the spinal cord. Our preliminary data indicated that curcumin attenuated opioid-induced hyperalgesia by modulating spinal CaMKII α activation (data not shown). Therefore, the brain may not be the only site of action for curcumin in attenuating CaMKII α activity and opioid tolerance and dependence.

Although the current study was not designed to determine the pharmacokinetic profiles of PLGA-curcumin beyond the one-point LC/MS analysis, it has been reported that the $t_{1/2}$ of curcumin is around 1.45 hours in rodents (Anand et al., 2007). In our experiments, acute action was tested within an hour after PLGA-curcumin, and we found it to be highly efficacious, so $t_{1/2}$ does not appear to be a problem. In the pretreatment, PLGA-curcumin or curcumin was given immediately before morphine, and we recorded a powerful effect of these interventions in preventing the development of opioid tolerance/dependence; therefore, the presence of curcumin during the early time was sufficient to block tolerance to and dependence on morphine.

Curcumin is commonly used in traditional Asian cuisine, and its reported bioactivity profile includes antioxidant, anti-inflammatory, chemotherapeutic, and neuroprotective actions. It has been suggested that curcumin abolished morphine analgesic tolerance along with the morphine-induced up-regulation of brain-derived neurotrophic factor transcription (Matsushita and Ueda, 2009). Liang et al. (2013) reported that daily administration of curcumin reduced opioid-induced hyperalgesia, tolerance, and physical dependence, possibly by inhibiting histone acetyltransferase. Other studies found that curcumin inhibited histone deacetylase (Liu et al., 2005; Chen et al., 2007, 2013; Lee et al., 2011). It has also been suggested that curcumin blocked corticosterone-induced phosphorylation of CaMKII in cultured hippocampal neurons (Xu et al., 2009) and inhibited CaMKII autophosphorylation in vitro (Mayadevi et al., 2012), although such a mechanism has not been studied in vivo. Since we have previously found a critical role of CaMKII α in opioid tolerance and dependence, we tested the hypothesis that curcumin's inhibitory action on CaMKII α may be a mechanism attenuating the initiation or maintenance of opioid tolerance and dependence. Indeed, we found that curcumin was highly efficacious in inhibiting CaMKII α in mice that were made tolerant to and dependent on morphine.

In addition to opioid tolerance and dependence, curcumin has been reported to attenuate hyperalgesia in mice with chronic pain, including nerve injury-induced neuropathic pain (Zhao et al., 2012) and diabetic neuropathic pain (Sharma et al., 2006; Banafshe et al., 2014). Although neuropathic pain and opioid tolerance and dependence are distinct central nervous system processes, they could potentially share a common contributor, such as synaptic long-term potentiation, for which CaMKII α is required (Wang and Wang, 2003; Lisman et al., 2012). Indeed, CaMKII α has been found to be essential and required for chronic inflammatory pain (Luo et al., 2008), nerve injury-induced neuropathic pain (Chen et al., 2009), and opioid-induced hyperalgesia (Chen et al., 2010).

In summary, our study demonstrated that PLGA-curcumin nanoparticles, at relatively low doses, prevented and reversed opioid antinociceptive tolerance and physical dependence, correlating with its inhibitory actions on supraspinal CaMKII α . PLGA-curcumin is significantly more potent than unformulated curcumin. Moreover, its high solubility allows us to obtain the precise doses that are required to produce these pharmacologic

effects. These data provide a plausible molecular mechanism for the action of curcumin in *in vivo* preclinical models of opioid tolerance and dependence. The rational design, formulation, and characterization of stable PLGA-curcumin nanoparticles not only provide a pharmacologic reagent, but more importantly can be further developed for blocking opioid dependence and for improving chronic pain therapies by attenuating tolerance to opioid drugs.

Authorship Contributions

Participated in research design: Hu, Liu, Wang.

Conducted experiments: Hu, Huang, Szymusiak.

Performed data analysis: Hu, Wang.

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

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