Resveratrol reduces morphine tolerance by inhibiting microglial activation via AMPK signalling


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Abstract

Background: Evidence has accumulated indicating that microglia within the spinal cord play a critical role in morphine tolerance. The present study investigated the effects and possible mechanisms of 5′ adenosine monophosphate-activated protein kinase (AMPK) activator resveratrol and AICAR to inhibit microglial activation and to limit the decrease in antinociceptive effects of morphine.

Methods: The microglial cell line BV-2 was used. Cytokine expression was measured using quantitative polymerase chain reaction. Cell signalling was assayed by Western blot and immunohistochemistry. The antinociception and morphine tolerance were assessed in CD-1 mice using the hot plate and tail-flick tests.

Results: (1) Morphine induces robust BV-2 cell activation, as evidenced by increased p38 mitogen-activated protein kinase phosphorylation, nuclear factor-κB translocation and mRNA expression of pro-inflammatory cytokines [including interleukin-1β (IL-1β), IL-6 and tumour necrosis factor-α], inducible nitric oxide synthase and Toll-like receptor-4, and these changes are inhibited by resveratrol. (2) Resveratrol activates AMPK to suppress morphine-induced BV-2 cell activation. AICAR, another AMPK activator, can mimic the effects of resveratrol, whereas compound C, an AMPK inhibitor, reverses the inhibitory effects of resveratrol treatment. (3) Systemic or spinal administration of resveratrol with morphine significantly blocks microglial activation in the spinal cord and then attenuates the development of acute and chronic morphine tolerance in both male and female mice.

Conclusion: Resveratrol directly suppresses morphine-induced microglial activation through activating AMPK, resulting in significant attenuation of morphine antinociceptive tolerance.

1. Introduction

Opioids such as morphine are powerful analgesic drugs commonly used for severe pain management. However, marked reduction in the analgesic properties of opiates resulting from repeated use significantly hinders the prolonged clinical use of these drugs. Although the exact mechanisms of opiate tolerance remain unknown, it has been suggested that the activation of microglia in response to morphine and the consequent production of pro-inflammatory cytokines plays a key pathogenic role.

In-depth characterization indicates that morphine exposure induces the robust activation of spinal...
microglia, which secrete large amounts of pro-inflammatory cytokines including interleukin-1β (IL-1β), IL-6, and tumour necrosis factor-α (TNF-α), ATP and nitric oxide (NO), accelerating the development of morphine tolerance (Berrios et al., 2008). It has been demonstrated that the intrathecal (i.t.) injection of minocycline (a microglial inhibitor) significantly reduces analgesic tolerance during chronic opioid administration (Raghavendra et al., 2002; Sun et al., 2012). However, prolonged use of minocycline causes severe side effects, including diphtheritic enteritis and autoimmune disorders. Therefore, novel, potent and safe drug is urgently needed for the prevention of morphine tolerance through microglial inhibition.

5′-Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric Ser/Thr protein kinase activated by alterations in cellular AMP:ATP ratio and serves as an energy sensor that regulates energy homeostasis and metabolic stress. Once activated, AMPK inhibits ATP-consuming anabolic processes such as protein translation. AMPK activation achieves these effects mainly through inhibition of mammalian target of rapamycin (mTOR) signalling. AMPK activation has also been indicated to the inhibition of mitogen-activated protein kinase (MAPK) signalling (Melemedjian et al., 2011). MAPK family, including extracellular signal-regulated protein kinase (ERK), p38 MAPK, c-Jun N-terminal kinase and extracellular signal-regulated protein kinase 5 (ERK5), have been shown to play a crucial role in morphine-induced neuroinflammation and tolerance (Chen and Sommer, 2009). AICAR, an AMPK activator, shows marked inhibition on lipopolysaccharide (LPS)-mediated production of pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS) and NO in rat glial cells, leading to the inhibition of nuclear translocation of nuclear factor-κB (NF-κB) (Giri et al., 2004; Chang et al., 2011; Zong et al., 2012). Interestingly, morphine could activate Toll-like receptor-4 (TLR-4) to increase release of pro-inflammatory cytokines in BV-2 cells in a manner similar to LPS (Wang et al., 2012). Moreover, there have been multiple studies exhibiting the role played by AMPK activators in alleviation of acute and neuropathic pain (Tillu et al., 2012; Melemedjian et al., 2011). It has been indicated that there are striking similarities between neuropathic pain and morphine tolerance (Watkins et al., 2005). Therefore, we hypothesized that activation of AMPK signalling pathway may represent a novel pharmacological strategy to suppress morphine tolerance through attenuation of morphine-induced microglial activation.

To test this hypothesis, we have utilized resveratrol, a natural polyphenol widely found in red grapes and wine that has previously been shown to increase AMPK activity (Tillu et al., 2012), to evaluate the effect of AMPK activation on morphine-induced microglial activation and tolerance.

Herein, we demonstrate that resveratrol suppresses morphine-induced microglial activation in a dose-dependent manner. Moreover, the impact of resveratrol on morphine-induced microglial activation was due to its effect on AMPK activation. We also show that resveratrol inhibits both acute and chronic morphine tolerance. These data suggest that AMPK activator, such as resveratrol, may have utility in the treatment of morphine-induced neuroinflammation and further implicate AMPK as a novel target for the treatment of morphine tolerance.

2. Materials and methods

2.1 Ethics statement

All procedures were strictly performed in accordance with the regulations of the ethics committee of the International Association for the Study of Pain and the Guide for the Care and Use of Laboratory Animals (The Ministry of Science and Technology of China, 2006). All animal experiments were approved by Nanjing University Animal Care and Use Committee and were designed to minimize suffering and the number of animals used.

2.2 Animals

CD-1 mice (weighing 20–25 g; the Experimental Animal Center at Xuzhou Medical College, Jiangsu Province, China)
were used in this study. Mice were housed 5–6 per cage under pathogen-free conditions with soft bedding under controlled temperature (22 ± 2 °C) and photoperiods (12:12-h light–dark cycle). Mice were allowed to acclimate to these conditions for at least 2 days before inclusion in the experiments. For each group of experiments, mice were matched by age and body weight.

2.3 Materials

Fetal bovine serum (FBS) and other cell culture media and supplements were purchased from Hyclone (Logan, UT, USA). Morphine was purchased from Shenyang First Pharmaceutical Factory, Northeast Pharmaceutical Group Company (Shenyang, China). AICAR (5-Aminomidazole-4-carboxamide 1-b-D-ribofuranoside Acadesine N′-(b-D-Ribofuranosyl)-5-aminomidazole-4-carboxamide), Compound C (6-[4-(2-Piperidin-1-yloxy)phenyl]-3-pyridin-4-ylpyrazol[1,5-a]pyrimidine) and resveratrol (3′,4′,5′-Trihydroxy-trans-stilbene, 5-[(1E)-2-(4-Hydroxyphenyl) ethenyl]-1,3-benzenediol) were purchased from Sigma (St. Louis, MO, USA).

2.4 Western blotting

To identify temporal expression of ionized calcium-binding adaptor molecule 1 (IBA-1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the phosphorylated protein levels of AMPK, p38, whole cell protein extract lysates were analysed as described before (Liu et al., 2010). In brief, samples (cells or spinal cord segments at L1–L6) were collected and washed with ice-cold phosphate buffered saline (PBS) before lysed in RIPA (radio immunoprecipitation assay) lysis buffer [Beyotime, Nantong, Jiangsu Province, China; 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mmol/L phenylmethylsulfonyl fluoride, 0.15 U/mL aprotinin and 1 mg/mL pepstatin]. Then, whole sample lysates were collected for protein expression assay with the primary antibodies [GAPDH, 1:1000 from Sigma; p-AMPK (Tyr172), 1:1000, Cell Signaling Technology (Danvers, MA, USA); p-p38 (Tyr182), 1:800, Epitomics (Burlingame, CA, USA); IBA-1, 1:1000, Wako Pure Chemical Industries (Mie-gun, Japan)]. GAPDH was performed as a loading control. The filters were developed using ECL reagents (PerkinElmer, Waltham, MA, USA) with secondary antibodies from Chemicon (Billerica, MA, USA). Data were analysed with the Molecular Imager (Gel DocTM XR, 170-8170) and the associated software Quantity One-4.6.5 (BioRad Laboratories, Berkeley, CA, USA).

2.5 Immunohistochemistry

Under deep anaesthesia, mice were transcardially perfused with PBS followed by 4% paraformaldehyde with 1.5% picric acid in 0.16 mol/L phosphate buffer (PB) (pH 7.2–7.4, 4 °C), and then the L4 and/or L5 lumbar segment was dissected out and post-fixed in the same fixative overnight. The embedded blocks were sectioned (30 μm thick) and processed for immunofluorescence. Sections from each group (5 mice in each group) were incubated with rabbit anti-IBA-1 polyclonal antibody (1:100; Wako Pure Chemical Industries). Rabbit IgG (1:200; Vector Laboratories, Cambridgeshire, UK) was used as an isotype control. Then, the free-floating sections were washed three times in 50 mmol/L Tris–HCl (pH = 7.4) PBS, and incubated in the secondary antibody for 2 h at room temperature or overnight at 4 °C. After washing three times in PBS, the samples were studied under a confocal microscope (Leica TCS SP2, Leica Biosystems, Solms, Germany) for morphological details of the immunofluorescence staining on the spinal cord. Images were randomly coded and transferred to a computer for further analysis.

2.6 Analysis of mRNA levels by quantitative real-time PCR

Samples (cells or spinal cord segments at L1–L6) were homogenized in Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and total RNA was treated by DNaseI (Invitrogen Life Technologies) and subjected to quantitative polymerase chain reaction (PCR), which was performed with ABI Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Green I dye (Biotium, Inc. Hayward, CA, USA), and threshold cycle numbers were obtained using ABI Prism 7300 SDS software, version 1.0. The specific primer sequences for IL-1β, IL-6, TNF-α, IL-4, IL-10, TGF-β, iNOS, Toll-like receptor 4 (TLR-4) and GAPDH are listed in Supporting Information Table S1. Conditions for amplification were one initial step of 95 °C for 10 s, 95 °C denature for 5 s, 60 °C anneal/extension for 30 s, and 40 cycles for all the primers. The samples were analysed in triplicate. GAPDH gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. The mean value of the replicates for each sample was calculated and expressed as cycle threshold (CT); cycle number at which each PCR reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference (ΔCT) between the CT value of the sample for the target gene and the mean CT value of that sample for the endogenous control (GAPDH) (Hu et al., 2010; Wang et al., 2012).

2.7 Cell cultures

BV-2 cells were incubated under humidified 5% CO₂ and 95% O₂ at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% FBS and 1% streptomycin and penicillin (Invitrogen). Twenty-four hours prior to experimentation, the culture media was replaced by 0.5% FBS high-glucose DMEM.

2.8 NF-κB activation assay

BV-2 cells were fixed with ice-cold methanol and were permeabilized with 0.25% Triton X-100/PBST. After blocking
with 1% bovine serum albumin (BSA) in PBST for 60 min, the coverslips were incubated for 2 h at room temperature with the p65/RelA antibody diluted in 1% BSA (1:50). The coverslips were then exposed to the FITC-conjugated anti-rabbit IgG (1:100, at room temperature for 60 min) after being washed three times with PBS. After that, the coverslips were stained with 1 μg/mL DAPI (4′,6-diamidino-2-phenylindole, a fluorescent DNA dye to mark nucleus) for 1 min and then fluorescent confocal images were acquired using a Olympus FV1000 confocal system (Hu et al., 2010).

2.9 Behavioural assessment of antinociceptive effects

To test for the possible effects of resveratrol on the pain threshold and the initial analgesic response to morphine, we used hot plate testing to evaluate thermal nociception in female mice as previously described (Liu et al., 2009, 2010). Briefly, mice were placed on a 55 °C hot plate apparatus, and the latency to lick a paw or jump was measured. A cut-off time of 30 s was set to avoid tissue damage. Mice were tested every 30 min for 2 or 3 h. Data were calculated as percentage of maximal possible effect (%MPE), which was calculated by the following formula: 100% × [(Drug response time – Basal response time)/(30 s – Basal response time)] = %MPE. Morphine was administered [10 mg/kg, subcutaneous (s.c.)] 30 min before testing. Resveratrol was administered intraperitoneally at 15 min before morphine was given.

The second analgesia test was tail-flick latency testing in male mice. The test was performed by gently holding the mouse in a terrycloth towel and immersing between 2 and 3 cm from the tip of the tail into warm water (52 °C). Response latencies were recorded at the removal of the whole tail from the water as previously described (Stone et al., 1997). A cut-off time of 10 s was set to avoid tissue damage. Data were calculated as percentage of maximal possible effect (% MPE), which was calculated by the following formula: 100% × [(Drug response time – Basal response time)/(10 s – Basal response time)] = %MPE. The experimenters were blinded to the treatment.

2.10 Acute and chronic morphine tolerance tests

For the test of chronic tolerance, repetitive morphine injection (10 mg/kg, s.c.) was given daily for 7 days and analgesic effect was measured (55 °C hot plate) 30 min after each injection in female mice. Resveratrol (40, 80 or 160 mg/kg) was administered intraperitoneally 15 min before morphine was given once a day from day 1 to day 7.

Chronic tolerance was also tested with tail-flick assay following repetitive treatment of morphine (10 μg/10 μL/mouse, i.t.) daily for 7 days, and the analgesic effect was measured 30 min after each injection in male mice. Resveratrol and AICAR were dissolved in DMSO and diluted in PBS (final concentration of DMSO for i.t. administration was 0.5%). Resveratrol, AICAR or the vehicle (0.5% DMSO-PBS) were injected intrathecally (each in 10 μL), respectively, by means of lumbar puncture at the intervertebral space of L4–5 or L5–6 for multiple injections using a stainless steel needle (30 gauge) attached to a 25-μL Hamilton syringe.

Acute morphine tolerance was tested immediately after morphine treatment at 10 mg/kg (s.c.) following a high-dose morphine challenge at 100 mg/kg (s.c.) 24 h before. Female mice were placed on a 55 °C hot plate apparatus, and the latency to lick a paw was measured at 30, 60, 90 and 120 min after morphine treatment (10 mg/kg, s.c.). Resveratrol (40, 80 or 160 mg/kg) was administered intraperitoneally once at 15 min before the morphine challenge at 100 mg/kg. From the %MPE scores, we calculated an area under the curve (AUC) for 0–120 min after morphine injection to indicate the degree of analgesia as described before (Ueda et al., 2000).

2.11 Statistics

SPSS Rel 15 was used to perform all the statistical analyses (SPSS Inc., Chicago, IL, USA). Alteration of expression of the proteins detected and the behavioural responses to morphine withdrawal were tested with one-way analysis of variance (ANOVA) and the differences in latency over time among groups were tested with two-way ANOVA, followed by Bonferroni’s post-hoc tests. The mean fluorescent pixels of IBA-1 were analysed by Image Pro Plus 6.0. Results are expressed as mean ± SEM of three independent experiments in triplicate. Results described as significant are based upon a criterion of p < 0.05.

3. Results

3.1 Resveratrol suppresses inflammatory reactions enhanced by morphine in BV-2 cells

To investigate the in vitro effects of resveratrol on morphine-induced microglial activation, we used the immortalized murine microglial cell line BV-2, which were derived from primary mouse microglial cells (Blasi et al., 1990; Wang et al., 2012). BV-2 cells have a similar reaction pattern with that of primary microglia after stimulation with LPS (Henn et al., 2009). It has also been used to investigate the efficiency of morphine on microglia (Bokhari et al., 2009). In the present study, morphine treatment (200 μmol/L, 6 h) induced robust activation in BV-2 cells, which was characterized by up-regulated mRNA expression of IL-1β, TNF-α, IL-6 and iNOS (Fig. 1A), increased phosphorylation of p38 MAPK (Fig. 1B) and translocation of p65 NF-κB from the cytoplasm to the nucleus (Fig. 1D). Moreover, resveratrol given before morphine administration significantly reduced these effects (Fig. 1A, B and D). Furthermore, the increase of TLR-4 mRNA expression induced by morphine was markedly suppressed by resveratrol (Fig. 1C).
We also examine the potential effects of resveratrol on anti-inflammatory mediators (IL-4, IL-10 and TGF-β). As shown in Supporting Information Fig. S1a, morphine with or without resveratrol treatment showed no notable effects on mRNA expression of IL-4, IL-10 and TGF-β in BV-2 cells. Resveratrol (20 μmol/L) alone also had no marked effects on the expression of these cytokines.

### 3.2 Resveratrol activates AMPK to attenuate the morphine-induced microglial activation

Since resveratrol has been reported to play a protective role in the central nerve system by affecting the activities of AMPK (Chang et al., 2011; Yuan et al., 2012), we hypothesized that AMPK activation by resveratrol might play a crucial role in suppressing microglial activation. We found that resveratrol could significantly increase phosphorylation of AMPK at Thr172 in BV-2 cells in a dose-dependent manner (Fig. 2A). Furthermore, our results demonstrated that AICAR (20 μmol/L), the specific AMPK activator, inhibited morphine-induced p38 phosphorylation (Figs. 2B and 3A) and attenuated NF-κB p65 translocation from cytosol to nucleus (Fig. 3B). AICAR also suppressed the expression of pro-inflammatory cytokines (Fig. 2C). These results suggest that AMPK activation is effective in inhibiting inflammatory signals in microglia.

Moreover, we assessed whether an AMPK inhibitor, compound C, could prevent or alter the suppressive effects of resveratrol on morphine-activated BV-2 cells. Our data demonstrated that compound C (20 μmol/L, co-addition with resveratrol to the culture) significantly reversed the inhibitory effects of resveratrol (Fig. 2B and C), whereas compound C alone showed no notable effects on naïve BV-2 cells. These results indicate that resveratrol may inhibit activated microglia exposed to morphine via activating AMPK signalling.

### 3.3 Resveratrol attenuates spinal microglial activation associated with morphine chronic treatment

Furthermore, we extended our experiments to evaluate the role of resveratrol on morphine-induced microglial activation in vivo. Our Western blot and...
immunofluorescence data showed that, compared to the naive control, mice with repeated morphine exposure (10 mg/kg, given subcutaneously each day for 7 days) showed significant microglial activation in the spinal cord. The activation was manifested as an increased IBA-1 (a microglial marker) fluorescence density (Fig. 4A) and an up-regulated IBA-1 protein level (Fig. 4B). In addition, resveratrol [160 mg/kg, intraperitoneal (i.p.), 15 min before each injection of morphine on each day] attenuated these pathological changes significantly (compared to morphine alone group; Fig. 4A and B) while not affecting naive mice (p > 0.05). These results indicate that pretreatment with resveratrol could suppress activation of spinal microglia induced by chronic morphine exposure in vivo.

We further examined the impact of resveratrol on the up-regulation of pro-inflammatory cytokines, iNOS and TLR-4 mRNA level induced by chronic morphine exposure. Consistently, our quantitative real-time PCR analysis data demonstrated that, compared to naive control, mice exposed to morphine (10 mg/kg each day for 7 days) had increased mRNA expression levels of the inflammatory molecules, whereas repetitive administration of resveratrol (i.p., 15 min before each injection of morphine each day) suppressed them effectively in a dose-dependent manner. Western blot analysis data showed that resveratrol could significantly reduce the increased phosphorylation of p38 MAPK enhanced by chronic morphine exposure (Fig. 4B). Furthermore, as shown in Supporting Information Fig. S1b, morphine or resveratrol showed no notable effects on the mRNA level of IL-4, IL-10 and TGF-β in the spinal cord.

Importantly, resveratrol treatment for 15 min could significantly increase phosphorylation of AMPK at Thr172 in spinal cord in a dose-dependent manner (Fig. 4D). This may be the reason for the immune suppressor activity of resveratrol.

3.4 Systemic resveratrol administration alleviates acute and chronic morphine tolerance

We continued to examine whether the analgesic effects of morphine could be enhanced by resveratrol.

Figure 2 Effects of resveratrol on morphine-induced microglial activation are mediated by 5′ adenosine monophosphate-activated protein kinase (AMPK) activation. (A) Resveratrol increased AMPK-Tyr172 phosphorylation level in BV2 cells in a dose-dependent manner. BV2 cells were collected and analysed 6 h after incubation with resveratrol (0.2, 2, 20 μmol/L). Representative Western blot bands and a data summary (n = 4) for p-AMPK are shown. (B) Effects of AICAR or compound C (20 μmol/L) on phosphorylated p38 in morphine-activated BV-2 cells with or without resveratrol. AICAR or compound C pretreatment occurred 30 min before morphine administration. Representative Western blot bands and a data summary (n = 4) for p-p38 are shown. (C) Effects of AICAR or compound C (20 μmol/L) on the mRNA expression of interleukin-1β (IL-1β), IL-6, tumour necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS) and Toll-like receptor-4 (TLR-4) in morphine-activated BV-2 cells with or without resveratrol. Significant difference was revealed following one-way analysis of variance (*p < 0.05; **p < 0.01; Bonferroni’s post-hoc tests).
Resveratrol (up to 160 mg/kg) did not alter the pain threshold or the initial morphine-induced analgesia (Fig. 5A). However, repetitive treatment with resveratrol significantly reduced the decreased analgesia resulted by morphine in a dose-dependent manner (Fig. 5B). Specifically, the MPE observed at 30 min in chronically morphine-treated mice on day 7 was 19.81%, whereas mice co-administrated with resveratrol (80 or 160 mg/kg) simultaneously had MPE of 32.16% and 37.54%, respectively (Fig. 5C). The analgesia was further represented by AUC units. Significant attenuation of chronic tolerance but no effects on initial analgesia by resveratrol was shown in Fig. 5D.

The effect of resveratrol on acute morphine tolerance was also investigated by challenging mice with high-dose morphine (100 mg/kg, s.c.) followed by a lower dose (10 mg/kg, s.c.) 24 h later (Mor100-10). The peak analgesic effect and the AUC for mice treated with Mor100-10 were markedly decreased compared to those of mice treated with 10 mg/kg morphine (Mor10) only (Fig. 5E and F). However, administration of resveratrol that had been given 15 min before the 100 mg/kg morphine injection exhibited a significant attenuation of morphine antinociception compared to the Mor100-10 control group (Fig. 4E and F).

3.5 Spinal administration of resveratrol and AICAR attenuates chronic tolerance

To provide additional evidence and confirmation for AMPK-mediated effect in suppression morphine-induced microglial activation, resveratrol and AICAR were administrated intrathecally to evaluate their effects on morphine tolerance and microglial activation induced by i.t. injection. Male CD1 mice developed tolerance to the analgesic effects of morphine during the 7-day period of chronic treatment (10 μg/10 μL/mouse, i.t.), whereas resveratrol or AICAR given 15 min (i.t.) before morphine challenge significantly rescued the analgesia in a dose-dependent manner (Fig. 6A and C). Moreover, repeated morphine exposure resulted in robust up-regulation of IBA-1 protein, which was significantly inhibited by resveratrol or AICAR (Fig. 6B and D). It should be noted that spinal administration of resveratrol and AICAR alone showed no notable effects on the pain threshold or IBA-1 expression in mice (Supporting Information Fig. S2a and b).

4. Discussion

In the present study, the principal findings are as follows: (1) AMPK activation with resveratrol or
AICAR has a significant inhibitory impact on morphine-evoked microglial activation and abolishes morphine-induced up-regulated p38 MAPK phosphorylation, NF-κB nuclear translocation and pro-inflammatory cytokine expression in microglia; (2) AMPK is required for resveratrol-mediated inhibition on activated microglia; and (3) the development of acute and chronic morphine tolerance is significantly attenuated by resveratrol in a dose-dependent manner. To the best of our knowledge, we provide the first experimental evidence that AMPK activation by resveratrol can directly attenuate morphine-induced microglial activation, as well as partially prevent morphine acute analgesic tolerance. This study may provide a new strategy for inhibiting microglial activation to increase clinical efficacy of opioids.

Mounting compelling evidence indicates that spinal microglia plays a crucial role in the pathogenesis of morphine tolerance (Cui et al., 2006; Horvath et al., 2010; Zhou et al., 2010). Microglia inhibitor minocycline and p38 inhibitor SB203580 significantly attenuate morphine-induced pro-inflammatory cytokines synthesis and inhibits morphine tolerance. We found that resveratrol remarkably down-regulated the increase in phosphorylated levels of p38 MAPK in morphine-treated BV2 cells. Consistently, morphine treatment increased IL-1β, TNF-α, IL-6 and iNOS mRNA expression in BV2 cells, which was abolished with resveratrol pretreatment. These findings are consistent with previous study, which demonstrates that resveratrol decreases the LPS-induced activation of p38 MAPK along with reduced expression iNOS and pro-inflammatory cytokines (Zong et al., 2012).

Interestingly, previous studies have shown that p38 could regulate transcriptional activity of NF-κB (Saha et al., 2007). Thus, on the basis of our results that resveratrol inhibited morphine-induced p38 MAPK phosphorylation in BV2 cells, we further investigated its effect on NF-κB activation. The mammalian NF-κB family members include NF-κB1 (p105 and p50), NF-κB2 (p100 and p52), RelA (p65), RelB and c-Rel, which become activated by upstream signals from
diverse immune receptors, such as ligand-triggered TLRs, IL-1 receptors, TNF receptors and antigen receptors (Liu et al., 2014; Yuan et al., 2013). Upon ligand engagement, signal transduction events are initiated, which lead to the release and translocation of the active NF-κB dimer into the nucleus to up-regulate target gene transcription (Dejardin et al., 1999; Chen et al., 2005). It has been reported that the activation of NF-κB may play a key role in pro-inflammatory cytokines production in response to morphine treatment in microglial cells (Tai et al., 2008). Indeed, our study revealed that, for NF-κB p65 level, morphine markedly led to an increase in nucleus, which was effectively reversed by resveratrol treatment (Fig. 1). These results indicate that resveratrol can attenuate morphine-mediated NF-κB activation, which has been implicated as the first signal priming the synthesis of inflammatory cytokines in microglia.

However, it is still unclear how resveratrol inhibits the morphine-induced microglial activation. Previous studies have shown that resveratrol is a highly potent and efficacious activator of AMPK (Zang et al., 2006; Dasgupta and Milbrandt, 2007) and its metabolic effects are dependent upon α-subunit AMPK expression (Um et al., 2010). Therefore, we had a hypothesis that resveratrol might inhibit p38/NF-κB signalling partly through its effect on AMPK. Our data showed that (1) resveratrol increased APMK phosphorylation in a dose-dependent manner (Fig. 2A); (2) AICAR, another AMPK activator, significantly inhibited morphine-induced p38/NF-κB activation and cytokines expression in BV-2 cells (Fig. 3); (3) the inhibitory effects of resveratrol on activated microglia were markedly reversed by the AMPK inhibitor compound C in BV-2 cells (Fig. 2B and C). Altogether, our data supported an inhibitory effect of resveratrol on activated microglia mainly through AMPK signalling.

We further investigated whether resveratrol could inhibit morphine-induced microglial activation in vivo. The existing literature has suggested that microglia, when activated, exhibit increased expression of the microglial markers CD11b and IBA-1, with a consequent increase in the levels of pro-inflammatory factors (Cui et al., 2006, 2008). Our data showed that resveratrol notably inhibited microglial activation, suppressing the up-regulated IBA-1 expression and increased pro-inflammatory cytokines in the dorsal horn of spines (Fig. 4). Moreover, we provide the first evidence that resveratrol also remarkably attenuated morphine-induced expression of TLR-4 mRNA level both in vitro and in vivo (Figs. 1 and 4). It is reported that there is potentially an alternative pain processing in female mice when TLR-4 is specifically challenged by LPS (Sorge et al., 2011). However, our data suggested that male mice also developed morphine tolerance and could be attenuated by AMPK activators like...
their female controls (Fig. 6). These results suggest that AMPK activation with resveratrol is efficient to the attenuation of morphine tolerance by resveratrol both in male and in female mice.

It is worth noting that morphine with or without resveratrol showed no notable effects on the expression of anti-inflammatory cytokines (IL-4, IL-10, TGF-β) both in vivo and in vitro in our experiment (Supporting Information Fig. S1). This is parallel to the previous report (Raghavendra et al., 2002; Rege et al., 2013) but inconsistent with other studies (Jin et al., 2012; Little et al., 2013). This inconsistency might be due, in part, to the differences in the animal species, dosage and route of administration, or due to the low levels of these negative regulators in spines. Collectively, our data suggest that resveratrol might suppress morphine-induced microglial activation mainly through inhibiting pro-inflammatory cytokines synthesis but not enhancing anti-inflammatory cytokines.

Morphine tolerance involves many processes. In a positive feedback loop, activation of microglia in response to morphine resulted in a robust production of pro-inflammatory cytokines, which leads to sensor neurons extra-excitation in the spinal cord (Kawasaki et al., 2008; Zhong et al., 2010; Zhang et al., 2011) and hinders the efficiency of morphine analgesia (Matsushita et al., 2013). In the present study, we demonstrated that intraperitoneal (i.p.) administration of resveratrol at doses of 80–160 mg/kg significantly reversed the decreased antinociception induced by repeated administration of morphine (Fig. 5B–F). Notably, this compound did not alter the pain threshold or initial morphine-induced analgesia in naive mice (Fig. 5A), by which it was indicated for clinical safety. We also evaluated the roles of spinal administration of resveratrol and AICAR on morphine chronic tolerance and microglial activation in male mice, and obtain similar results to those in female mice (Fig. 6).
Taken together, although a previous document has reported that resveratrol pretreatment provided a significant antinociceptive effect of morphine in morphine-tolerant rats (Tsai et al., 2012), here we further observed the efficiency of resveratrol on decreased morphine analgesia with both system and local administration, in both chronic and acute tolerance conditions, thus to present resveratrol as a potential drug candidate to enhance the clinical utility of opioid drugs.

There are several literature evidence demonstrating poor bioavailability of resveratrol following systemic injection, which is also sensitive to physiological factors (Azorin-Ortuno et al., 2011; Lin and Ho, 2011). This raises the question whether systemic resveratrol administration is directly responsible and efficient to activate AMPK in the spinal cord and to attenuate neuroinflammation evoked by morphine. We examined the impact of resveratrol on phosphorylated AMPK in the spinal cord after i.p. injection and observed a significant increase of AMPK phosphorylation at Thr172 in the spinal cord in a dose-dependent manner (Fig. 4D). Resveratrol and its metabolites have been detected in the central nervous system (Abd El-Mohsen et al., 2006; Frozza et al., 2010; Juan et al., 2010) and showed neuroprotective effects against Aβ administration, oxidative stress, etc., with a systemic administration (Frozza et al., 2013). So the effects of resveratrol on morphine tolerance may be due to, at least in part, its inhibition on the spinal-activated microglia.

The mechanisms of opiate tolerance are complex and involve many factors at the levels of receptors, ion channels, cell and neural networks. Although we cannot rule out other sites of action and other mechanisms of resveratrol that might directly or indirectly lead to the observed effects, e.g., the inhibition on Na+ channels or ERK/mTOR signalling in dorsal root ganglion neurons (Kim et al., 2005; Tillu et al., 2012), the efficacy of resveratrol in suppressing microglial activation may provides important clues, at least in part, in elucidating the effects of resveratrol on morphine tolerance.

5. Conclusions

In summary, our data demonstrate that activation with resveratrol, a naturally occurring compound, reduces tolerance to the analgesic effects of morphine through mechanisms that are associated with the inhibition of p38 MAPK/NF-κB signalling and the inhibition of pro-inflammatory cytokine expression in the microglia. Moreover, these effects are mediated through the regulation of AMPK activity. Therefore, resveratrol represents a potential drug candidate to reduce morphine tolerance and enhance the clinical utility of opioid drugs.

Author contributions

W.L., X.W. and X.S. conceived and designed the experiments. Y.H., C.J., J.T., C.W. and P.W. performed the experiments. Y.H. and X.W. analysed the data. G.Z., N.J., X.W. and W.L. contributed reagents/materials/analysis tools. Y.H., W.L., X.W. and S.X. wrote the paper. All authors read and approved the final manuscript.

References


Reveratrol attenuates morphine tolerance


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:
mRNA expression of the IL-4 IL-10 and TGF-β in BV-2 cells. Cells were pretreated with resveratrol for 15 min following challenge with morphine (200 μmol/L), BV-2 cells were collected and analysed 6 h after morphine treatment. The levels of IL-4, IL-10 and TGF-β mRNA in BV-2 cells were determined using real-time quantitative PCR. GAPDH was used as an invariant control. (b) Resveratrol showed no notable effects on the mRNA expression of the IL-4, IL-10 and TGF-β in mice following chronic morphine tolerance treatment. Morphine was injected subcutaneously (10 mg/kg) once a day for 7 days, resveratrol was administered intraperitoneally daily at 15 min before the morphine each injection at 40, 80 or 160 mg/kg. The lumbar spines (L1–L6) were collected and analysed 120 min after the last morphine administration. The levels of IL-4, IL-10 and TGF-β mRNA in BV-2 cells were determined using real-time quantitative PCR. GAPDH was used as an invariant control.

**Figure S2.** Spinal administration of resveratrol and AICAR alone showed no notable effects on the pain threshold and IBA-1 expression in mice. (a) Intrathecal injection of resveratrol and AICAR alone showed no notable effects on the pain threshold. Animals were administered once daily resveratrol (200 μmol/L, 10 μL/mice), AICAR (200 μmol/L, 10 μL/mice) or vehicle (0.5% DMSO diluted with saline) intrathecally for 7 days. Tail-flick latency in mice was measured 15 min after each i.t. injection once a day. Data were calculated as percentage of maximal possible effect (%MPE). (b) Intrathecal injection of resveratrol and AICAR alone showed no notable effects on spinal microglial activation in mice. Animals were administered once daily resveratrol (200 μmol/L, 10 μL/mice), AICAR (200 μmol/L, 10 μL/mice) or vehicle (0.5% DMSO diluted with saline) intrathecally for 7 days. The lumbar spines (L1–L6) were collected and analysed 120 min after the last i.t. injection. Representative Western blot bands and a data summary (n = 4) for IBA-1 are shown.

**Table S1.** Primers for real-time RT-PCR.