

Effect of ethanol extracts of *Hericium erinaceus* mycelium on morphine-induced microglial migration

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Received November 13, 2018; Accepted August 6, 2019

DOI: 10.3892/mmr.2019.10745

Abstract. Microglia serve important roles in chronic pain signal transduction pathways. Glia cells, especially microglia, seem to share mechanisms that lead to chronic pain and morphine-induced tolerance. Evidence has suggested that downregulating cytoskeleton activity in microglia provides pain relief in chronic pain and morphine tolerance. The purpose of the present study was to evaluate the effect of ethanol extracts of *Hericium erinaceus* (EHE) mycelium on morphine-induced BV2 microglial cell activation. BV2 cells were starved for 4 h in DMEM before being incubated with 100 ng/ml EHE for 30 min, followed by 1 μ M morphine for 2 h. Subsequently, the cells were harvested and used for migration experiments and western blotting. The results showed that 1 μ M morphine enhanced BV2 cell activation and chemotactic reaction, and it increased histone deacetylase 6 (HDAC6) expression and heat shock protein 90 (HSP90) deacetylation as well as HSP90 cleavage. Pretreatment with 100 ng/ml EHE significantly inhibited the morphine-stimulated effects on BV2 cells. The present study demonstrated that EHE

inhibited morphine-induced BV2 activations by regulating the HDAC6/HSP90 deacetylation signal transduction pathway.

Introduction

Opioids are useful in medicine for alleviating acute and chronic pains. However, they are limited by their associated side effects, particularly allodynia and hyperalgesia. The underlying cellular and molecular mechanisms of opioid tolerance and chronic neuropathic pain remain to be understood. Evidence suggests that opioid tolerance and chronic neuropathic pain share a general cellular mechanism (1) that increases sensitivity to pain via spinal cord microglia activation (2). Therefore, it is necessary to understand the detailed mechanisms of opioid tolerance and chronic neuropathic pain in order to develop new pain therapies.

Previous studies reported that glia-modulating agents, such as ionized calcium binding adaptor molecule 1, cluster of differentiation 11b (CD11b) (3) and pro-inflammatory cytokines (4) can decrease morphine tolerance by inhibiting rat spinal microglia activity. In impaired central nervous systems, injury induces a gradual transformation of resting microglia to the active state. Activating microglia not only changes their morphology, but also enhances their adhesion and chemotaxis mobility (5). Those processes depend on the velocity of cytoskeleton assembly and disassembly. It was demonstrated that the histone deacetylase 6 (HDAC6)/heat shock protein 90 (HSP90) signaling pathway was involved in the microglia migration process (6). It has been suggested that activated microglia play a role in the development of morphine tolerance and/or hyperalgesia. Our previous study and other previous studies demonstrated that co-administration of an anti-inflammatory drug with morphine is a strategy for enhancing the antinociception of morphine, which also attenuates tolerance (7,8) and neuropathic pain (9) by suppressing pro-inflammatory cytokine expression and microglia activation.

Hericium erinaceus (*H. erinaceus*) is an edible mushroom with notable medicinal properties. It has been consumed as

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Key words: neuroinflammation, morphine, neuropathic, ruffling, cytoskeleton

food and used in traditional Chinese herbal medicine in China without toxic side effects (10). Evidence shows that *H. erinaceus* has several beneficial biological effects, such as neuroprotection (11), anti-oxidation (12), immune modulation and anticancer activity (13). *H. erinaceus* suppresses synthesis of inflammatory mediators by inhibiting oxidative stress (14) and the inducible nitric oxide synthase/p38 MAPK pathway (15), which suggested that *H. erinaceus* may have analgesic properties. Additionally, in Alzheimer's APP^{swe}/PS1^{dE9} transgenic mice, *H. erinaceus* significantly diminished the number of amyloid- β plaque-activated microglia (16). The ethanol extracts of *H. erinaceus* (EHE) mycelium, especially Erinacine A, have demonstrated epinephrine-stronger nerve growth factor-inducing activities *in vitro* and *in vivo* (17). This result suggested that EHE has a potential effect on pain management in patients who develop morphine tolerance. This is the first study, to the best of our knowledge, to evaluate the effect of EHE on morphine-induced BV2 microglial cell activation and migration. It was observed that EHE suppresses BV2 microglial cell activation and migration by regulating the HDAC6 and HSP90 deacetylation signaling pathway.

Materials and methods

Heridium erinaceus. *H. erinaceus*, which was purchased from The Bioresources Collection and Research Center (BCRC no. 35669) in The Food Industry Research and Development Institute, was maintained on potato dextrose agar at 26°C for 15 days (18). After incubation, a mycelial agar block (1 cm³) was removed, transferred to a 2-L Erlenmeyer flask containing 1.3 L synthetic medium (composed of 0.25% yeast extract, 4.5% glucose, 0.5% soybean powder, 0.25% peptone and 0.05% MgSO₄, adjusted to pH 4.5) and incubated for 5 days at 26°C on a rotary shaker (1.12 x g; Firstek; cat. no. S203). The fermentation process was then scaled up from a 2-L shake flask to 500-L and 20-ton fermenters for 5 and 12 days, respectively. At the end of the fermentation process, the mycelia were then harvested, lyophilized, ground into a powder and stored in a desiccator at room temperature (18). The dry powder was extracted four times with 90% ethanol under reflux. The *H. erinaceus* powder was then extracted four times with 95% ethanol at 40 KHz and 26°C with 1 h of sonication each time. All ethanol solutions were concentrated under a vacuum in order to obtain brown extracts.

Cell culture and reagents. The mouse BV2 microglial cell line was provided by Professor Chih-Shung Wong (Fu-Jen Catholic University). The cell line was verified in our laboratory; it was positive for MAC1 and CD11b expression, but negative for the expression of the astrocyte marker glial fibrillary acidic protein and oligodendrocyte marker galactocerebrosidase. BV2 mouse microglia cells were grown in DMEM (Gibco; Thermo Fisher Scientific, Inc.) and supplemented with 100 μ g/ml penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a 5% CO₂ incubator. The medium was changed every 3 days.

Drug delivery for culture cells. Cultured cells were washed 3 times with serum-free DMEM (blank medium) and starved for 4 h. Then, the cells were incubated with different

concentrations (ranging between 1 ng and 1 μ g) of EHE for 30 min, followed by a 2-h incubation with morphine at different concentrations (10–100 μ M) for 2 h. (Sigma-Aldrich; Merck KGaA). Subsequently, the cells were gently harvested in PBS/1 mM EDTA and used for a migration assay and western blot analysis.

Chemotaxis assay. The cells were incubated for 30 min in serum-free DMEM with or without 100 ng/ml EHE, and then incubated for 2 h with 1 μ M morphine. After incubation, cell survival was further determined by trypan blue staining (0.4% trypan blue and 3 min at room temperature) followed by cell counting. The treated cells (1x10³) in serum-free DMEM were seeded in the top wells of a 48-well microchemotaxis chamber (Neuro Probe, Inc.), and DMEM containing 10% FBS was added to the bottom wells of the chamber. The cells in both chambers were incubated for 1 h at 37°C in 5% CO₂. The number of BV2 microglial cells that migrated to the underside of the filter (25x80 mm polycarbonate membrane, 3.0 μ m pore size; Osmonics; cat. no. K80SH58050) was counted. The membranes were rinsed with PBS, and the migrated cells were then fixed with 100% methanol at room temperature for 10 min and stained with crystal violet for 20 min at room temperature (Sigma-Aldrich; Merck KGaA). In total, 6 random fields were counted for each condition using an Olympus BX 50 fluorescence microscope (Olympus Corporation; magnification, x20).

Image assay. Cell images were captured under a light microscope at x10 and x40 magnification and at least three wells from each experimental group were selected. (Olympus Corporation).

Cell lysis and western blotting. The cells were lysed in 200 μ l 1X Laemmli sample buffer (Bio-Rad Laboratories, Inc.) comprised of 2-mercaptoethanol. The protein concentration of the samples was measured using the bicinchoninic acid assay (Pierce; Thermo Fisher Scientific Inc.; cat. no. 23225). In total, 15–25 μ g protein was boiled at 95°C for 5 min, separated by SDS-PAGE on 10% gels, and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked with 5% non-fat milk in buffer (50 mM Tris-HCl, 154 mM NaCl and 0.05% Tween 20) at room temperature for 1 h. Then, the membranes were incubated at 4°C overnight with various primary antibodies; goat polyclonal antibodies against mouse HDAC6 (Santa Cruz Biotechnology, Inc.; cat. no. sc-5258; 1:200 in blocking buffer) and rabbit polyclonal antibodies against HSP90, CD11b (Abcam; cat. no. ab13495 and ab128797; 1:200 in blocking buffer) and acetylated-HSP90 (LifeSpan BioSciences, Inc.; cat. no. LS-C380587; 1:100 in blocking buffer). The membranes were then incubated for 1 h at room temperature with the corresponding horseradish peroxidase-conjugated donkey anti-goat or goat anti-rabbit immunoglobulin G antibodies, as appropriate (all from Abcam; cat. nos. ab205723 and ab205718; 1:2,000 in 5% non-fat milk in TBS with 0.05% Tween-20). Membrane-bound secondary antibodies were detected using Chemiluminescence Plus reagent (PerkinElmer, Inc.) and visualized using a chemiluminescence imaging system (Syngene Europe). Finally, the blots were incubated for 18 min at 56°C in stripping buffer (62.6 mM Tris-HCl, pH 6.7, 2% SDS, 100 mM mercaptoethanol) and

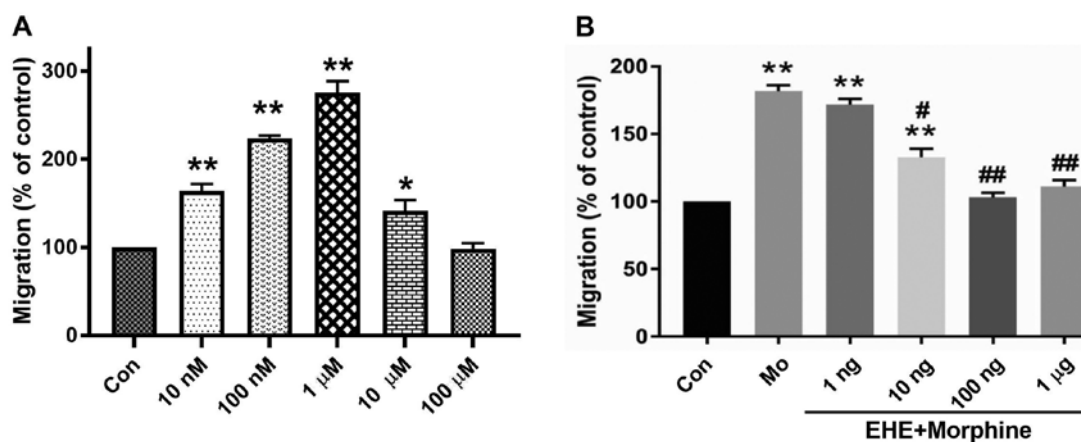


Figure 1. Effect of EHE on morphine-induced BV2 cell activation. (A) Concentration-response effect of morphine on BV2 cell migration. The cells were incubated for 2 h with serum-free medium alone (Con) or with medium containing 10 and 100 nM, and 1, 10 or 100 μ M Mo. * P <0.05, ** P <0.001 vs. Con group. (B) Concentration-response effect of EHE on morphine-induced BV2 cell migration. The cells were incubated for 2 h with blank medium alone (Con), incubated with 1 μ M Mo, or pretreated for 30 min with a particular concentration of EHE and then incubated with 1 mM Mo (EHE+Mo). A cell migration assay was performed, and the migrated cells were counted. Data are presented as the mean \pm SEM. n =18. ** P <0.001 vs. Con group; # P <0.05, ## P <0.001 vs. Mo group. EHE, ethanol extracts of *H. erinaceus*; Mo, morphine; Con, control.

re-probed with mouse monoclonal anti- β -actin antibody (Sigma-Aldrich; Merck KGaA; cat. no. A1978; 1:10,000 in blocking buffer) at room temperature for 1 h and horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Abcam; cat. no. ab205719; 1:3,000 in 5% non-fat milk in TBS with 0.05% Tween-20) as the loading control. The relative densities of the protein bands were analyzed using a computer-assisted imaging analysis system (GeneTools Match software, 4.3.7, Syngene).

Statistical analysis. All experiments were conducted at least three times, and data are presented as the mean \pm SEM. For immunoreactivity data, the intensity of each test band was expressed as the optical density relative to that of the average optical density for the corresponding control band. For statistical analysis, immunoreactivity was analyzed using one-way ANOVA, followed by multiple comparisons with the Student-Newman-Keuls post hoc test. The statistical analysis was performed using SigmaStat 3.0 software (Systat Software, Inc.). P <0.05 was considered to indicate a statistically significant difference.

Results

EHE mycelium inhibits morphine-induced microglia chemotaxis. BV2 microglial Morphine stimulated cell migration in a concentration-dependent manner up to 1 μ M, and then reduced cell migration at higher doses (Fig. 1A). Pretreatment with 100 ng/ml EHE significantly inhibited morphine-induced microglia migration from 181.7 \pm 7.63 to 103.0 \pm 6.08% (P =0.0002; Figs. 1B and 2). All subsequent experiments were performed using 1 μ M morphine and 100 ng/ml EHE.

Morphine-induced microglia activation is inhibited by EHE. In the cell morphology study, BV2 cells exhibited shapes that were bipolar rod-like or globular after seeding overnight. At 24 h after seeding, the cells were starved for 4 h prior to treatment. They were pre-incubated with blank medium or

with 100 ng/ml EHE for 30 min; then they were incubated for 2 h after morphine was added. As shown in Fig. 3, the cell morphology was altered from ramified cells (Fig. 3A and B) to an amoeboid shape with a larger cell body and retracted processes (Fig. 3C). Moreover, morphine treatment not only induced BV2 cell activation with membrane ruffling (black arrows), it activated the microglia as well. In addition, EHE pretreatment markedly inhibited microglia activation and cell membrane ruffling (Fig. 3D).

As shown in Fig. 4, the immunoblot analysis of BV2 protein extracts showed that CD11b expression was significantly increased (2.70 \pm 0.23; P <0.001) in the morphine-treated cells, compared with the control group. This effect was significantly decreased by EHE pre-treatment (1.46 \pm 0.12; P <0.05 compared with the control group; P <0.001 compared with the morphine-treated group).

Morphine induces HDAC6 expression in BV2 cells. HDAC6 has been documented to affect cell migration through regulation of HSP90 (19). As shown in Fig. 5, morphine treatment increased HDAC6 expression compared with the control group from 1 to 1.6 \pm 0.13 (P =0.0003) and it was inhibited by EHE pretreatment (1.02 \pm 0.05; P <0.001 compared with the morphine group). The present results demonstrated that morphine affects HDAC6-mediated processes.

EHE reverses morphine-stimulated HSP90 cleavage and deacetylation. Morphine treatment not only increased HDAC6 expression, it additionally decreased HSP90 expression (0.68 \pm 0.012; P =0.00001) compared with the control group and further led to HSP90 fragmentation (Fig. 6). Compared with the morphine group, EHE pretreatment significantly inhibited the morphine-induced cleavage of HSP90 and lower HSP90 expression (0.98 \pm 0.06; P <0.05). As Fig. 7 shows, the morphine treatment stimulated deacetylation of HSP90 (0.42 \pm 0.06; P =0.0006, compared with the untreated control), which was significantly inhibited by EHE pretreatment (0.95 \pm 0.076; P <0.005, compared with the morphine control).

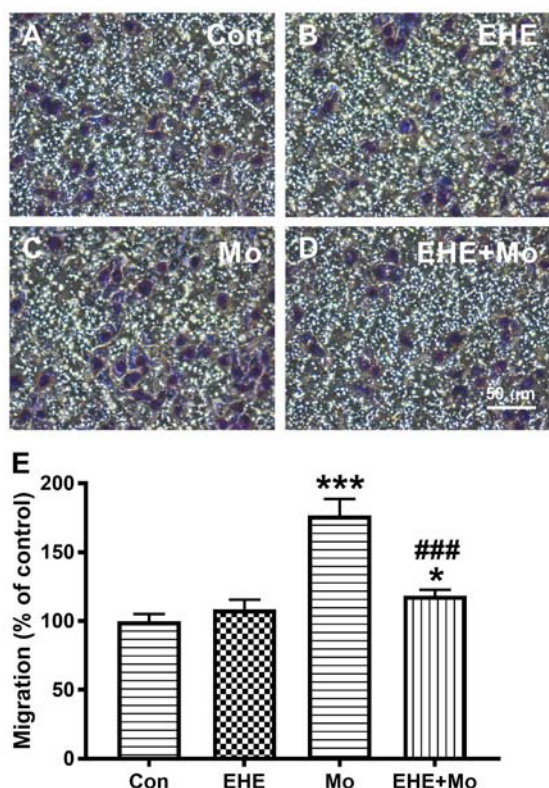


Figure 2. EHE inhibits Mo-induced BV2 cell migration. Images of trypan blue-stained migrated BV2 cells at the bottom of the polycarbonate membrane in the (A) Con, (B) EHE, (C) Mo and (D) EHE+Mo groups. (E) Quantification of the data. * $P < 0.05$, *** $P < 0.005$ vs. Con group; ### $P < 0.005$ vs. Mo group. Data are presented as the mean \pm SEM. $n = 12$. EHE, ethanol extracts of *H. erinaceus*; Mo, morphine; Con, control.

Discussion

Microglia are now recognized for playing key roles in the formation and maintenance of morphine tolerance, and are associated with hyperalgesia (20). Activated microglia are a source of production of pro-inflammatory cytokines, such as tumor necrosis factor- α , interleukin (IL)1- β and IL-6 (7), which attenuate antinociception induced by morphine, and thus contribute to the development of morphine tolerance and associated pain sensitization. Therefore, suppressing microglia activation has been considered an effective therapeutic approach in mitigating morphine tolerance and neuropathic pain. In the present study, *H. erinaceus* mycelium demonstrated potent inhibitory effects on morphine-stimulated BV2 cells in multiple aspects; it markedly attenuated morphine-induced membrane ruffling and migration of BV2 microglial cells. The present data suggested that EHE has potential as an analgesic adjuvant in pain management.

Reactive microgliosis is associated with changes of cell morphology, enhanced adhesion and migration processes (6). A previous study demonstrated that microtubule stabilization plays an active role in axon formation in primary hippocampal neurons of rats (21). These findings suggested that the stabilization of α -tubulin and microtubules plays a crucial role in cellular processes, as well as morphogenesis, compartmentalization and organelle movement. It has been reported that chronic morphine treatment results in

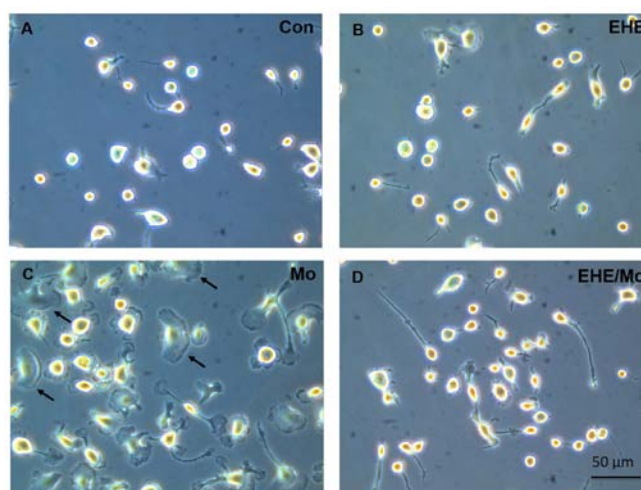


Figure 3. EHE inhibits Mo-induced BV2 cell membrane ruffling. BV2 cells were washed with blank medium 3 times and starved for 4 h in the same medium, then incubated (A) for 2.5 h with blank medium, (B) for 2.5 h with medium containing EHE, (C) for 30 min with blank medium and then for 2 h with medium containing Mo, or (D) for 30 min with EHE and then for 2 h with Mo. Black arrows indicate the ruffling membranes of BV2 cells. EHE, ethanol extracts of *H. erinaceus*; Mo, morphine; Con, control.

a significant decrease in both mRNA and protein levels of α -tubulin, and the microtubule-associated protein τ in rat striatum (22). Long-term morphine administration markedly decreases neurofilament light polypeptide expression, a major component of neuronal cytoskeleton neurofilaments, and results in neuronal damage (23). In our previous study, morphine treatment decreased acetylated α -tubulin levels in EOC13.31 microglia cells, and these effects were correlated with an increase in activation and migration of microglia (6). In addition, it was observed that α -tubulin stabilization directly correlated with cell function. According to these results, it was hypothesized that chronic morphine administration directly affects microglia cell cytoskeleton stabilization and consequent activation.

Cell chemotaxis plays an essential role in immune surveillance, tissue localization and tumor metastasis (19). Most migrating cells are polarized and show a leading edge or frontal flat lamella, which is the site of protrusive activity. A previous study used microglia cells to examine the effect of morphine on microglia chemotaxis and to explore the role of immunocompetent cells in the actions of opioids (24). The results of that previous study showed that morphine stimulated microglia migration by modulating P2X purinoceptor 4 receptor signaling. Our previous study demonstrated that morphine changes the morphology of EOC13.31 microglia, and causes membrane ruffling and chemotaxis (6). Membrane ruffling exists at the lead edge of a migrating cell as a driving force of chemotaxis (25). Similar results were found in the present study. The results demonstrated that morphine increased BV2 microglial cell migration and membrane ruffling. Pretreatment with EHE inhibited BV2 cell migration and activation. This suggested that EHE may regulate cytoskeleton dynamics.

HDAC6 is a member of the class IIb HDAC family. HDAC6 removes acetyl groups from substrates other than histones; it is a unique isoenzyme with specific physiological roles.

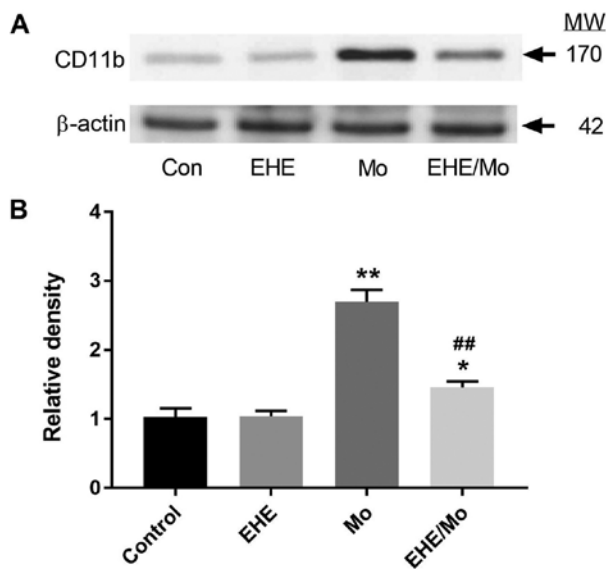


Figure 4. EHE blocks the Mo-induced CD11b expression in BV2 cells. (A) Typical western blot of CD11b levels in a whole cell lysate (25 μ g protein loaded per lane). β -actin was used as the protein loading control. (B) The protein levels of CD11b were quantitatively expressed after being standardized to β -actin (n=3 at each concentration point). The data are expressed as the mean \pm SEM of three independent experiments. *P<0.05, **P<0.001 vs. Con group; ##P<0.001 vs. Mo group. EHE, ethanol extracts of *H. erinaceus*; Mo, morphine; Con, control.

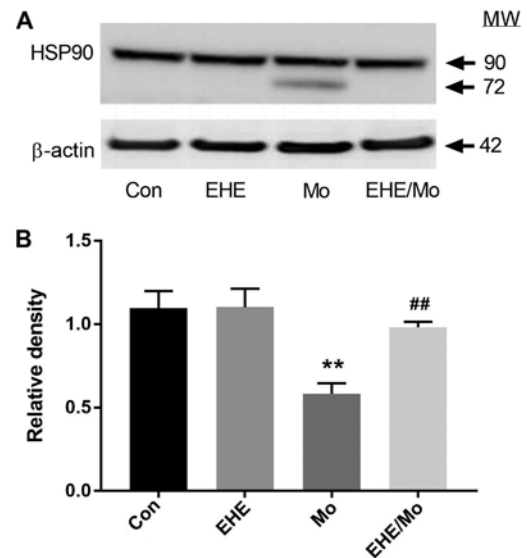


Figure 6. EHE inhibits the Mo-induced decrease in HSP90 expression in BV2 cells. (A) Typical western blot of HSP90 levels in a whole cell lysate (15 μ g protein loaded per lane). β -actin was used as the protein loading control. (B) The protein levels of HSP90 were quantitatively expressed after being standardized to β -actin (n=3 at each concentration point). All data are expressed as the mean \pm SEM of three independent experiments. **P<0.001 vs. Con; ##P<0.005 vs. Mo group. EHE, ethanol extracts of *H. erinaceus*; HSP90, heat shock protein 90; Mo, morphine; Con, control; MW, molecular weight.

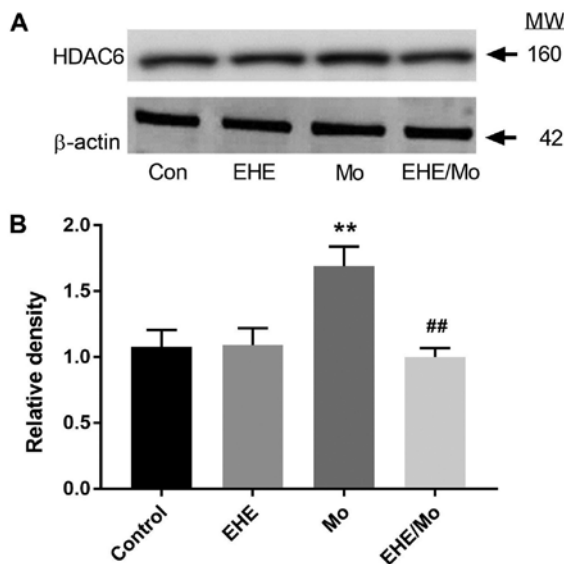


Figure 5. EHE blocks the Mo-induced HDAC6 expression in BV2 cells. (A) Typical western blot of HDAC6 levels in a whole cell lysate (25 μ g protein loaded per lane). β -actin was used as the protein loading control. (B) The protein levels of HDAC6 were quantitatively expressed after being standardized to β -actin (n=3 at each concentration point). The data are expressed as the mean \pm SEM of three independent experiments. **P<0.001 vs. Con group; ##P<0.001 vs. Mo. group. EHE, ethanol extracts of *H. erinaceus*; HDAC6, histone deacetylase 6; Mo, morphine; Con, control; MW, molecular weight.

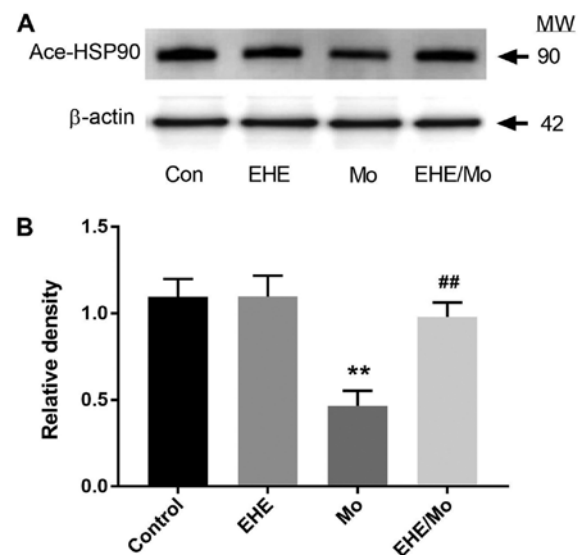


Figure 7. EHE blocks the Mo-induced decrease in Ace-HSP90 levels. (A) Typical western blot of Ace-HSP90 levels in a whole cell lysate (15 μ g protein loaded per lane). β -actin was used as the protein loading control. (B) The protein levels of Ace-HSP90 were quantitatively expressed after being standardized to β -actin (n=3 at each concentration point). Data are expressed as the mean \pm SEM for three independent experiments. **P<0.001 vs. Con; ##P<0.005 vs. Mo group. EHE, ethanol extracts of *H. erinaceus*; Ace, acetylated; HSP90, heat shock protein 90; Mo, morphine; Con, control; MW, molecular weight.

HDAC6 deacetylates various non-histone substrates, such as α -tubulin and HSP90, and is involved in protein trafficking and degradation, and cell shaping and migration (19,26). A previous study indicated that decreased tubulin and HSP90 acetylation was correlated with cell migration, cell growth and survival (26). Similarly, our recent study showed that

HDAC6 overexpression in morphine-treated microglial cells was accompanied by inhibited acetylation of α -tubulin and enhanced microglia activity (6). In the present study, it was also demonstrated that chronic morphine treatment of BV2 microglial cells induced upregulation of HDAC6, promoted

HSP90 deacetylation and enhanced microglia reactivity. It was hypothesized that HDAC6 deacetylase serves an important role in microtubule configuration and cell migration. A previous study found that HDAC6 is localized in the protrusive structures of polarized cells as the leading edge and membrane ruffles (27). In agreement, the present study observed that EHE suppressed morphine-induced HDAC6 expression. According to these results, it was hypothesized that the inhibitory effect of EHE on morphine-stimulated microglia activation and migration is by inhibiting the HDAC6/HSP90 deacetylation signaling transduction pathway.

HSP90 is an ATP-dependent molecular chaperone, an essential cytosolic protein under normal conditions, and it markedly increases during stress (28). HSP90 works together with a group of co-chaperones. Co-chaperones modulate HSP90 activity, which facilitates client protein maturation. Post-translational modifications of HSP90 contribute to protein folding and serve various cellular processes, such as signal transduction and protein degradation (29). Acetylation is a reversible modification regulated through the opposing action of acetyltransferases and deacetylases (30). In general, HSP90 serves to regulate both the inactivation and activation of client proteins. Therefore, acetylation weakens HSP90 interaction with client proteins, which results in the inactivation of the client proteins (31). In the present study, it was determined that morphine induced microglial activation and migration, upregulated HDAC6 expression, and induced HSP90 fragmentation and deacetylation; this may explain the action of HSP90 related to cytoskeleton stabilization. A previous study showed that HSP90 protected tubulin against thermal denaturation and keeps it in a state compatible with microtubule polymerization (32) in nephrotoxin-induced injuries of renal cell models. This result suggests that HSP90 may contribute to the regulation of microtubule cytoskeleton polymerization and depolymerization. HSP90 is a protein associated with a variety of stress responses and defense mechanisms of cells (33). Based on the results of the present study, morphine treatment downregulated HSP90 expression and induced HSP90 fragmentation in BV2 microglia cells, which were accompanied with cell activation and migration. Therefore, it was suggested that morphine treatment may cause a stress response in BV2 cells. Pretreatment with EHE significantly inhibited this stress response. In conclusion, the present data revealed a novel mechanism in which morphine mediates BV2 migration via the HDAC6/HSP90 deacetylation signaling pathway.

The limitation of the present study is that there is no *in vitro* method available yet that includes all hallmarks of homeostatic microglia. A large collection of microglia cell lines has been generated over the years, including BV2, N9, EOC-13.31 and IMG (34,35). Most microglia cell lines obtained from neonatal or embryonic central nervous system sources are unlikely to reflect the phenotype of adult or elderly microglia. Despite these limitations, the BV2 cells have been used for many years to study neuroinflammation and neurodegenerative disorders (34,35). The neuroscience field is increasingly acknowledging that modulation of neuroinflammation is a promising strategy to beneficially alter the disease course of neuroinflammation and neurodegenerative disease. Although there are advantages and limitations of existing models across

different species, microglia remain key components of the neuroinflammatory response, leading to an intensive research interest in this particular cell type.

In conclusion, in the present study, it was demonstrated that EHE inhibits morphine-induced BV2 activations by regulating the HDAC6/HSP90 deacetylation signaling transduction pathway. The present findings suggested that EHE, as an analgesic adjuvant, may be a potential therapeutic agent for pain management in morphine-treated patients.

Acknowledgements

The authors are grateful for the use of the laboratories provided to us by the Proteomics Laboratory, Cathay Medical Research Institute, Cathay General Hospital, Xizhi, New Taipei City, Taiwan.

Funding

The present study was supported by grants provided by The Grape King Bio Ltd.-Da Yeh University Collaboration Project (grant nos. DON-0411 and DON-0604) and by Da Yeh University (grant no. ORD-105032).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CHY and RYT participated in the design of the present study and performed the statistical analysis. LWS, CML, CMC and CCC conducted the study and analyses, and collected important background information. TPY, JNL, SLT and WPC analyzed and interpreted the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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