Angiotensin II receptor type 1 blocker candesartan improves morphine tolerance by reducing morphine-induced inflammatory response and cellular activation of BV2 cells via the PPARγ/AMPK signaling pathway

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Abstract. Morphine is the most common drug of choice in clinical pain management; however, morphine tolerance presents a significant clinical challenge. The pathogenesis of morphine tolerance is known to be closely associated with angiotensin II receptor type 1 (AT1R) in microglia. As an AT1R antagonist, candesartan may serve an important role in regulating morphine tolerance. Therefore, the present study aimed to investigate the role of candesartan in morphine tolerance, and to explore the underlying mechanism. To meet this aim, BV2 microglial cells were treated with morphine or candesartan alone, or as a combination, and the expression levels of AT1R in BV2 cells were detected by reverse transcription-quantitative PCR (RT-qPCR) and western blotting. The levels of inflammatory cytokines tumor necrosis factor-α, interleukin (IL)-1β and IL-6 were subsequently detected by ELISA and western blotting. In addition, immunofluorescence analysis, western blotting and RT-qPCR were used to detect the expression levels of the BV2 cell activation marker, ionized calcium-binding adaptor molecule 1 (IBA-1). Western blotting was also used to detect the expression levels of peroxisome proliferator-activated receptor-γ/AMP-activated protein kinase (PPARγ/AMPK) signaling pathway-associated proteins. Finally, the cells were treated with the PPARγ antagonist GW9662 and the AMPK inhibitor compound C to further explore the mechanism underlying the effects of candesartan on improving morphine tolerance. The expression levels of AT1R were revealed to be significantly increased following morphine induction; however, candesartan treatment inhibited the expression levels of AT1R, the levels of inflammatory cytokines and the protein expression levels of IBA-1 in morphine-induced BV2 cells in a dose-dependent manner. These processes may be associated with activation of the PPARγ/AMPK signaling pathway. Taken together, the present study revealed that treatment with candesartan reduced morphine-induced inflammatory response and cellular activation of BV2 cells via PPARγ/AMPK signaling.

Introduction

Opioids, such as morphine, are important drugs for the treatment of clinical pain. The main limitation associated with the long-term use of morphine is the occurrence of physiological tolerance and dependence (1). Morphine tolerance may develop through the use of multiple administration routes, the use of different doses and various durations (2). Morphine tolerance leads to poor analgesic effects, constipation, drowsiness, skin itching, and even to morphine addiction and other adverse reactions (3). Therefore, how to effectively reduce the occurrence of morphine tolerance provides the main focus of the present study.

The main mechanisms underlying morphine tolerance are opioid receptor desensitization and endocytosis (4), alteration of the glutamate receptor (5) and glial activation (6). In addition, previous studies have shown that the release of inflammatory factors has an important role in morphine-induced analgesic tolerance (7,8). Activated microglial cells are able to produce numerous pro-inflammatory cytokines, including interleukin (IL)-6, tumor necrosis factor-α (TNF)-α and IL-1β, which contribute towards the development of morphine tolerance (9). Intrathecal injection of microglial inhibitors has previously been shown to significantly reduce tolerance to chronic opioids (10). Therefore, effectively inhibiting the activity of microglia is of great importance in terms of alleviating morphine tolerance.
A previous study demonstrated that angiotensin (Ang) II receptor type 1 (AT1R) is expressed in microglial cells (11). The angiotensin-converting enzyme (ACE)/Ang II/AT1R axis can lead to activation of the renin angiotensin system (RAS) thus activating vasoconstriction, inflammation, fibrosis, cell growth and migration (12). Notably, renin cleaves Ang I from angiotensinogen, which is further cleaved to Ang II by Ang-converting enzyme, and Ang II ultimately produces two receptors: AT1R and AT2R (13). Previous studies have shown that the brain RAS mediates microglial polarization. For example, Ang II can activate NADPH oxidase complexes of microglial cells through the AT1R, which leads to an enhancement of neuroinflammatory responses (14,15). In concordance with this finding, AT1R activation has been shown to intensify microglial inflammatory responses, oxidative stress and dopaminergic degeneration in the mitochondrial permeability transition pore model of Parkinson's disease, and AT1R blockers were shown to inhibit these responses (16-18). Therefore, it was hypothesized that regulation of the AT1R may exert an influence on the development of morphine tolerance.

Candesartan is an AT1R antagonist, the pharmacological action of which is to antagonize Ang II-induced vasoconstriction by binding to vascular smooth muscle AT1R, thereby reducing peripheral vascular resistance (19). A recent study reported that candesartan is able to regulate neuroinflammatory responses through inhibiting the release of pro-inflammatory cytokines and stimulating anti-inflammatory cytokines in lipopolysaccharide (LPS)- and interferon-γ-stimulated BV2 cells (20). Candesartan has also been shown to reduce microglial activation in young and aged animals (21).

Therefore, it was hypothesized that candesartan may reduce the inflammatory responses and microglial activation that are induced by morphine tolerance. The present study sought to address these hypotheses.

Materials and methods

Cell culture. BV2 microglia cells were obtained from the BeNa Culture Collection; Beijing Beina Chunglian Institute of Biotechnology and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.) at 37°C in an atmosphere containing 5% CO₂/95% air. BV2 cells were treated with morphine (Northeast Pharmaceutical Group Co., Ltd.) at concentrations of 50, 100 and 200 µM for 24 h at 37°C, and 200 µM was ultimately selected as the optimal concentration of morphine (22). For further experiments, 1x10⁵ cells were plated in a 6-well plate overnight and the next morning BV2 cells were treated with candesartan (1 or 5 µmol/l) or morphine (200 µM) alone for 24 h, or were co-treated with candesartan and morphine, at 37°C for 24 h. The cells were divided into the control, 1 µmol/l candesartan, 5 µmol/l candesartan, 200 µM morphine, 200 µM morphine + 1 µmol/l candesartan and 200 µM morphine + 5 µmol/l candesartan treatment groups. Untreated cells were regarded as the control group. In an alternative set of experiments, BV2 cells were treated with morphine (200 µM) the following morning after plating with or without candesartan (1 and 5 µmol/l; MedChemExpress) for 24 h at 37°C (23). The cells were divided into the control, 200 µM morphine, 200 µM morphine + 1 µmol/l candesartan and 200 µM morphine + 5 µmol/l candesartan treatment groups. Untreated cells were regarded as the control group. For the mechanistic studies, the BV2 cells were pre-treated with the peroxisome proliferator-activated receptor-γ (PPARγ) antagonist, GW9662 (10 mM; MedChemExpress) or with the AMPK inhibitor, compound C (1 mM; MedChemExpress) for 30 min at 37°C. The BV2 cells were divided into the control, 200 µM morphine, 200 µM morphine + 5 µmol/l candesartan, 200 µM morphine + 5 µmol/l candesartan + GW9662, and 200 µM morphine + 5 µmol/l candesartan + compound C treatment groups. Untreated cells were regarded as the control group.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted from BV2 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) combined with treatment with DNase (Promega Corporation). cDNA was synthesized from 1 µg total RNA in a 20-µl reaction volume using the ImProm-II™ Reverse Transcription System (Promega Corporation). All procedures were performed according to the manufacturer's instructions. qPCR was performed using a LightCycler 480 SYBR-Green 1 Master mix (cat. no. 4070516001; Roche Diagnostics). The qPCR thermocycling reaction conditions were as follows: 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 64°C for 1 min and elongation at 72°C for 1 min, and a final extension step at 72°C for 7 min, before maintaining the reaction mixture at 4°C. RNA expression was quantitatively analyzed using the 2⁻ΔΔCq method (24). The primer sequences were as follows: AT1R, forward 5'-GCC GTGCGTCAAGTTATCTT-3' and reverse 5'-CAGGAACTT TGCCCCCTTGC-3'; ionized calcium-binding adaptor molecule 1 (IBA-1), forward 5'-TGAGGAGATTTCAACAGAAGC TGA-3' and reverse 5'-CCCTCAGACGCTGTGGTCCTT-3'; GAPDH, forward 5'-CCCTTAAGAGGATGCTGC-3' and reverse 5'-ACTGTGCGCGTTAAT-3'. Western blot analysis. The BV2 cells were lysed with RIPA buffer (Thermo Fisher Scientific, Inc.), and the protein concentration was detected using a BCA kit (Beyotime Institute of Biotechnology). Proteins (20 µg) were separated by SDS-PAGE on 10% gels and were then transferred to PVDF membranes (Merck KGaA). Subsequently, the membranes were blocked with 5% non-fat milk for 1.5 h at 37°C. Primary antibodies were then incubated with the membranes at 4°C overnight. The next day, after washing, the PVDF membranes were incubated with Goat Anti-Mouse IgG H&L (HRP)-conjugated secondary antibody (1:5,000 dilution; cat. no. ab7063; Abcam) or Goat Anti-rabbit IgG H&L (HRP)-conjugated secondary antibody (1:2,000 dilution; cat. no. ab6721; Abcam). The proteins were visualized using an ECL detection solution (Merck KGaA) and were analyzed with ImageJ software (version 1.46; National Institutes of Health). The primary antibodies used were as follows: Anti-AT1R (cat. no. ab124505; 1:2,000), anti-TNF-α (cat. no. ab255275; 1:2,000), anti-IL-1β (cat. no. ab254360; 1:2,000), anti-IL-6 (cat. no. ab259341; 1:2,000), anti-PPARγ (cat. no. ab178860; 1:2,000), anti-phosphorylated (p)-AMP-activated protein kinase (AMPK; cat. no. ab133448;
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1:2,000), anti-AMPK (cat no. ab32047; 1:2,000), anti-IBA-1 (cat no. ab178846; 1:2,000) and anti-GAPDH (cat no. ab8245; 1:5,000) (all from Abcam). The intensities of the protein bands were normalized against those of GAPDH.

ELISA. Briefly, BV2 cells were seeded into 96-well plates (5x10^{3} cells/well) and were stimulated with the relevant treatment the next day. After centrifuging at 2,000 x g for 5 min at 4˚C, the cell supernatants were collected. The levels of TNF-α (cat no. H052-1), IL1-β (cat no. H001) and IL-6 (cat no. H007-1-1) in the supernatant were assessed using ELISA kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer’s instructions.

Immunofluorescence (IF) staining. The cells were fixed with 4% formaldehyde for 30 min at 4˚C and then permeabilized with 0.1% Triton X-100 in PBS for 15 min at 4˚C. After blocking with 10% FBS for 30 min at 37˚C, the cells were incubated with anti-IBA (cat no. ab178846; 1:200) and anti-AT1R primary antibodies (cat no. ab124505; 1:200) (both from Abcam) at 4˚C overnight. Subsequently, the cells were incubated with goat anti-rabbit IgG H&L Alexa Fluor® 488 antibody (cat no. ab150077; 1:1,000; Abcam) for 30 min at 37˚C. The nuclei were stained with DAPI after IF staining for 15 min at room temperature. Images were visualized under a fluorescence microscope (Eclipse 80i; Nikon Corporation).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software, Inc.). Data are presented as the mean ± standard deviation and all experiments were repeated three times. One-way analysis of variance analysis followed by Tukey’s post-hoc test of variance was used to compare the differences among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Candesartan inhibits the expression of AT1R in morphine-induced BV2 cells. After BV2 cells were treated with morphine at different doses (50, 100 and 200 µM), the expression levels of AT1R were detected by RT-qPCR, western blotting and IF staining. The results revealed that the AT1R expression levels were increased in the morphine treatment groups compared with those in the control group (Fig. 1A-C). The expression of AT1R was most significantly increased in the 200 µM morphine treatment group; therefore, this concentration of morphine was chosen for subsequent experiments. Subsequently, the effects of different
concentrations of candesartan on the expression levels of AT1R in morphine-induced cells were investigated. These experiments revealed that the expression levels of AT1R in BV2 cells were significantly decreased following treatment with 1 and 5 µmol/l candesartan compared with those in the control group. Candesartan also caused a significant decrease in AT1R expression levels in co-treated BV2 cells compared with those in the 200 µM morphine treatment group (Fig. 1D and E).

Candesartan dose-dependently inhibits the inflammatory response in morphine-induced BV2 cells. ELISA and western blot analysis were then used to detect the intracellular levels of the inflammatory factors. The results obtained revealed that the levels of inflammatory cytokines were significantly increased in the 200 µM morphine group compared with those in the control group. However, further administration of candesartan reversed the morphine-induced increases in the levels of inflammatory factors (Fig. 2A and B).

Candesartan dose-dependently inhibits cell activation in morphine-induced BV2 cells. Subsequently, IF analysis was used to detect the expression levels of IBA-1, a marker of microglial activation. IBA-1 expression in the 200 µM morphine treatment group was markedly increased compared with that in the control. After further administration of candesartan, the expression levels of IBA-1 were markedly decreased (Fig. 3A). RT-qPCR and western blot analysis were subsequently used to determine the expression levels of IBA-1 at the mRNA and protein levels. The results showed that candesartan dose-dependently inhibited the expression of IBA-1 in morphine-induced BV2 cells.
to verify the expression levels of IBA-1. These experiments revealed that the expression trend of IBA-1 was consistent with that revealed in the IF staining experiments (Fig. 3B and C).

Candesartan activates the expression of PPARγ and AMPK in morphine-induced BV2 cells. Mechanistic studies were subsequently performed to investigate whether candesartan could exert effects on the expression levels of PPARγ and AMPK in morphine-induced BV2 cells. The expression levels of PPARγ and p-AMPK in the PPARγ/AMPK signaling pathway were significantly decreased following treatment with morphine compared with those in the control group. Following candesartan treatment (1 or 5 µmol/l) in morphine-treated cells, PPARγ and p-AMPK expression levels were dose-dependently elevated. Conversely, after further treatment with the PPARγ antagonist GW9662, the trends in the altered expression levels of PPARγ and p-AMPK were reversed compared with that in the 200 µM morphine + 5 µmol/l candesartan group (Fig. 4). Therefore, it was possible to conclude that candesartan could activate expression of the PPARγ and AMPK proteins in the PPARγ/AMPK signaling pathway in morphine-induced BV2 cells.

Candesartan attenuates inflammatory factors in morphine-induced BV2 cells via the PPARγ/AMPK signaling pathway. To further explore the mechanism, inhibitors of the PPARγ/AMPK signaling pathway were added to the cells; namely, the PPARγ antagonist GW9662 and the AMPK inhibitor compound C. According to the dose-dependent effect of candesartan on morphine-induced BV2 cells, candesartan at a concentration of 5 µmol/l was selected for cell treatment in the following experiments. The levels of TNF-α, IL-1β and IL-6 in the 200 µM morphine + 5 µmol/l candesartan + GW9662, and 200 µM morphine + 5 µmol/l candesartan + compound C groups were significantly increased compared with those in the 200 µM morphine + 5 µmol/l candesartan treatment group (Fig. 5A and B). These findings indicated that inhibition of PPARγ or AMPK reversed the suppressive effects of candesartan on the inflammatory response in morphine-induced BV2 cells.

Candesartan attenuates microglial activation in morphine-induced BV2 cells via PPARγ/AMPK signaling. IBA-1 expression in the 200 µM morphine + 5 µmol/l candesartan + GW9662, and 200 µM morphine + 5 µmol/l candesartan + compound C groups were markedly increased compared with that in the 200 µM morphine + 5 µmol/l candesartan group (Fig. 6A). Subsequently, RT-qPCR and western blot analysis were performed, and the results obtained revealed the same trends as identified in the IF staining analysis (Fig. 6B and C). These findings suggested that inhibition of PPARγ or AMPK reversed the suppressive effects of candesartan on microglial activation in morphine-induced BV2 cells.

Discussion

Opioids are the first choice of drug for the clinical treatment of severe cancer pain and perioperative pain (25). Morphine, as one of the most widely used opioid drugs for reducing pain, has a short half-life and a variety of dosage forms (26). However, the major limitation of long-term use of morphine is its gradual loss of efficacy, which is known as morphine drug tolerance (27). To make further medical advances, resolving the issue of morphine tolerance is essential.

Activation of microglia has been reported to have an important role in morphine tolerance. Morphine is able to activate microglia by acting on morphine receptors or Toll-like receptor-4 (TLR-4) on the surface of microglia (28). Activated microglia release a large number of cytokines and...
inflammatory factors, including IL-1β, IL-6 and TNF-α, which act on their corresponding receptors, resulting in increased excitability of pain-associated neurons and pain. Subsequently, the analgesic effect of morphine is weakened and the process of tolerance is accelerated (7). Therefore, morphine tolerance may be effectively circumvented through inhibiting microglial activation and the inflammatory response. In the present study, it was shown that the expression of inflammatory factors in BV2 microglia cells was increased and microglia were activated following morphine induction.

Microglia express µ-opioid receptors (29), as well as TLR-4 (30) and P2X receptors (31). It has been shown that blocking TLR-4 can lead to a reduction in morphine tolerance (32,33). Furthermore, activation of the NLRP3 inflammasome, which is dependent on TLR-4/P2X7 receptors in the spinal cord, can lead to promotion of the development of morphine tolerance (34). These findings suggested that the regulation of microglial receptors may have an influence on the development of morphine tolerance. A previous study reported that AT1R is expressed in microglia (35). Inhibition of AT1R has been shown to alleviate neuroinflammation in various neurological diseases, and AT1R antagonists may inhibit microglia-mediated neuroinflammatory responses and microglial activation in neurological diseases (36,37). In addition, it has been reported that morphine can regulate the activation of AT1R and vitamin D receptor to induce T-cell apoptosis (38). Therefore, it was reasonable to hypothesize that regulation of AT1R may also affect morphine tolerance. In the present study, it was revealed that the expression levels of AT1R in morphine-induced BV2 cells were significantly increased with an increase in the dose of morphine used for induction. However, following treatment with the AT1R antagonist candesartan, the expression levels of AT1R were decreased. Moreover, candesartan was also shown to dose-dependently inhibit morphine-induced BV2 cell inflammation and activation. A previous study demonstrated that candesartan is able to reduce the release of inflammation cytokines in the brain in Alzheimer's disease (23). Candesartan has also been shown to improve stroke-induced neuronal injury by transferring microglia to the M2 phenotype (20). In addition, candesartan has been reported to inhibit LPS-induced neuroinflammation in rats, and to also inhibit the LPS-induced BV2 cell inflammatory response (39). Notably, candesartan is a common drug used in the clinical treatment of elderly hypertension, which is known to have a good safety profile. The use of the AT1R blocker candesartan could reduce the cost of drug development, which is beneficial for the clinical treatment of morphine tolerance.

Candesartan is an AT1R blocker, and it has been shown that interference with AT1R can reduce atherosclerotic damage in diabetic mice via activating PPARγ (40). Activation of PPARγ has also been reported to improve morphine analgesia and morphine tolerance (41,42). Moreover, PPARγ can activate AMPK signaling (43,44). Notably, metformin, an AMPK activator, has been reported to reverse the increase in AT1R detected in response to a high-fructose diet (45). AT1R antagonists can regulate the proliferation and migration of vascular smooth muscle cells through AMPK/mTOR (46), and blocking AT1R can effectively inhibit activation of the AMPK/p38 MAPK/MCP1/ER pathway in macrophages (47). Activation of AMPK signaling, in itself, may also reduce morphine tolerance (48). Therefore, effective activation of AMPK may have an important role in reducing morphine tolerance. Consequently, the mechanism through which candesartan could regulate morphine-induced microglial activation and the inflammatory response was further explored in the present study by treating cells with the PPARγ antagonist GW9662 and the AMPK inhibitor compound C. These experiments revealed that GW9662 and compound C were able to significantly reverse the inhibitory effects of candesartan on morphine-induced microglial inflammation and activation, suggesting that candesartan may activate PPARγ/AMPK.
signaling via inhibiting AT1R, thereby reducing morphine tolerance.

The present study has some limitations. Firstly, some indicators were not detected. Notably, OX-42 and IBA-1 are both important markers of microglia; however, the present study only detected the expression of IBA-1; therefore, the role of OX-42 will be further verified in future experiments. Furthermore, the levels of inflammatory cytokines TNF-α, IL-1β and IL-6 were detected; however, the levels of NF-κB, IFN or IL-1 were not detected, which we aim to further verify in future experiments. In addition, morphine significantly decreased AT1R at the transcriptional level, which indicates that morphine may inactivate the transcription factor for AT1R; the candidates...
for transcription factors of ATR1 will be further discussed in future experiments.

In conclusion, to the best of our knowledge, the present study was the first to explore the effects of the AT1R blocker candesartan on the inflammation and activation of microglia induced by morphine, in order to determine the association between AT1R, microglial activation and morphine tolerance, and the underlying mechanism was explored. The present study demonstrated that candesartan reduced the morphine-induced inflammatory response and cellular activation of BV2 cells via the PPARγ/AMPK signaling pathway, suggesting that candesartan may improve morphine tolerance. Consequently, the present study provided a theoretical basis for the use of candesartan in the treatment of morphine tolerance.

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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

WZ and ZZ contributed to the conception and design of the present study, analyzed and interpreted the data, and critically revised the manuscript for important intellectual content. FS, JY and SS contributed to designing the study, analyzed the data, and drafted and revised the manuscript. WZ and ZZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.
Ethics approval and consent to participate
Not applicable.

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Competing interests
The authors declare that they have no competing interests.

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