

# Morphine enhances LPS-induced macrophage apoptosis through a PPAR $\gamma$ -dependent mechanism

MINGYING LIN\*, KEQIONG DENG\*, YA LI and JING WAN

Department of Cardiology, Zhongnan Hospital of Wuhan University, Wuhan, Hubei 430071, P.R. China

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**Abstract.** Morphine has been widely used for the treatment of pain and extensive studies have revealed a regulatory role for morphine in cell apoptosis. However, the molecular mechanisms underlying morphine-mediated apoptosis remain to be fully elucidated. The present study aimed to investigate the effects of morphine on lipopolysaccharide (LPS)-induced bone marrow-derived macrophage (BMDM) apoptosis and to determine the role of the peroxisome proliferator-activated receptor (PPAR) $\gamma$  signaling pathway in this process. BMDMs were isolated from BALB/c mice and stimulated with LPS. Hoechst 33342 staining and flow cytometric analysis were performed to evaluate the effects of morphine on LPS-induced apoptosis of BMDMs. Caspase activity assays were used to determine the involvement of the apoptosis pathway. The expression levels of caspase-3, caspase-8, caspase-9 and PPAR $\gamma$  were analyzed using western blotting. Finally, GW9662, a specific PPAR $\gamma$  antagonist, was used to determine whether the regulatory effects of morphine on LPS-induced BMDM apoptosis were PPAR $\gamma$ -dependent. The results of the present study revealed that morphine increased the apoptosis of LPS-stimulated BMDMs. Morphine upregulated the expression levels and activity of caspase-3 in LPS-stimulated BMDMs, but downregulated the expression levels and activity of caspase-8. Morphine treatment also upregulated LPS-induced PPAR $\gamma$  expression levels in BMDMs. Finally, the stimulatory effects of morphine on LPS-induced apoptosis and caspase-3/9 activation were

markedly reduced by GW9662. In conclusion, the findings of the present study indicated that morphine significantly promoted LPS-induced BMDM apoptosis and caspase-3/9 activation. These results suggested that the intrinsic pathway of apoptosis may be involved in the proapoptotic effects of morphine on LPS-stimulated BMDMs, which may be dependent, at least partially, on PPAR $\gamma$  activation.

## Introduction

Morphine is widely used as an analgesic and acts by activating opioid receptors in the central nervous system (1). In addition to providing pain relief, morphine has also been reported to modulate apoptosis in various types of cells, including immune, neuronal and cancer cells, suggested its potential effects on immunomodulation, nerve damage and tumor progression (2-4). Although opioid receptors are essential for opioid-induced effects, an increasing number of studies have demonstrated that other mechanisms beyond opioid receptors may be involved in morphine-mediated cell apoptosis (5-7). However, the underlying molecular mechanisms remain to be fully elucidated.

Apoptosis, a type of programmed cell death, is crucial for maintaining growth, development and homeostasis within the body (8). Macrophages, which are important innate immune cells, have been reported to exert regulatory effects on various pathological processes beyond immunomodulation, and the dysregulation of macrophage apoptosis was found to contribute to multiple diseases including atherosclerosis, diabetic kidney disease and hepatitis (9-11). Previous studies have demonstrated that lipopolysaccharide (LPS) is an activator of apoptosis in macrophages (12-14). Morphine was also reported to induce macrophage apoptosis; however, the specific mechanisms were not elucidated (15). To the best of our knowledge, the effects of morphine on LPS-induced macrophage apoptosis have not been investigated to date.

Peroxisome proliferator-activated receptor (PPAR) $\gamma$ , which belongs to the steroid-lipid nuclear receptor family, is predominantly found in adipose tissue and plays a crucial role in adipocyte differentiation, lipid metabolism and insulin resistance (16,17). PPARs regulate gene expression by heterodimerizing with retinoid X receptors and binding to specific PPAR response elements in the promoter regions of specific target genes (16,17). PPAR $\gamma$  was also discovered to be highly expressed in macrophages and the activation of PPAR $\gamma$

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*Correspondence to:* Dr Jing Wan, Department of Cardiology, Zhongnan Hospital of Wuhan University, 169 Donghu Road, Wuchang, Wuhan, Hubei 430071, P.R. China  
E-mail: wanjing\_zn@163.com

\*Contributed equally

*Abbreviations:* AMI, acute myocardial infarction; BMDM, bone marrow-derived macrophage; IACUC, Institutional Animal Care and Use Committee; LPS, lipopolysaccharide; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$

*Key words:* morphine, macrophage, caspases, peroxisome proliferator-activated receptor, apoptosis

triggered the apoptosis of macrophages through activation of the proliferator-activated receptors (18-20). In addition, a previous study indicated that the development of analgesic tolerance to morphine may be regulated by PPAR $\gamma$  (21). However, whether PPAR $\gamma$  is involved in morphine-induced apoptosis remains unclear.

The present study was undertaken to investigate the possible effects of morphine on LPS-induced bone marrow (BM)-derived macrophage (BMDM) apoptosis and to determine the role of the PPAR $\gamma$  signaling pathway in this process, hoping to uncover novel potential therapeutic and clinical applications for morphine.

## Materials and methods

**BMDM isolation.** All animals were maintained under specific pathogen-free (SPF) barrier conditions. All animal experiments were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Wuhan University. A total of 8 male BALB/c mice (age, 7-8 weeks; weight, 20.23 $\pm$ 1.01 g) were sacrificed by cervical dislocation, and the femurs and tibiae were removed. After flushing the medullar cavity, BM cells were collected and cultured in DMEM, high glucose (cat. no. 11965092; Gibco; Thermo Fisher Scientific, Inc.) supplemented with L-glutamine (Gibco; Thermo Fisher Scientific, Inc.), 10% heat-inactivated FBS (HyClone; Cytiva), 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and 20 ng/ml mouse macrophage colony-stimulating factor recombinant protein (eBioscience; Thermo Fisher Scientific, Inc.). The cells were incubated at 37°C in an atmosphere containing 5% CO $_2$  for 7 days to induce macrophage differentiation. BMDMs were subsequently harvested (22) and the purity was analyzed by flow cytometry for the expression of F4/80 (>90%). All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the Zhongnan Hospital of Wuhan University (Wuhan, China; approval no. AF165).

**Chemicals and BMDM treatment.** Morphine sulfate was obtained from the National Institutes for Food and Drug Control. LPS and GW9662 were purchased from Sigma-Aldrich; Merck KGaA. For the exposure to morphine, BMDMs were subjected to overnight incubation with 1  $\mu$ M morphine, followed by stimulation with 0.5  $\mu$ g/ml LPS for 24 h. For the treatment with the PPAR $\gamma$  inhibitor, BMDMs were pretreated with 1  $\mu$ M GW9662 for 4 h prior to morphine and/or LPS stimulation.

**Hoechst 33342 staining.** To evaluate the morphological changes of apoptotic BMDMs, Hoechst staining was performed as described previously (23). Briefly, BMDMs cells were seeded on chamber slides, treated with 1  $\mu$ M GW9662 for 24 h. BMDMs were then fixed with 4% formaldehyde for 15 min at room temperature and washed three times with PBS. Subsequently, the cells were incubated with Hoechst 33342 dye for 5 min in the dark. The cells were washed three times with PBS, and the slides were then visualized using a fluorescence microscope (Olympus Corporation; magnification, x40 and x100). For semi-quantification, apoptotic scores were counted from five randomly selected

fields by direct counting of 500 cells in each sample using a blinded method (23). The percentage of apoptotic cells was calculated as the number of apoptotic cells divided by the number of total cells.

**Flow cytometric analysis of apoptosis.** Apoptosis was measured using the FITC-Annexin V Apoptosis Detection kit (BD Biosciences) as previously described (24). BMDMs were harvested by centrifugation at 1,000 x g for 5 min at 4°C and washed twice with ice-cold PBS. The cells were resuspended in binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl $_2$ ] at a concentration of 1x10 $^6$  cells/ml, and then gently mixed and incubated with 5  $\mu$ l Annexin V-FITC (BD Biosciences) and 10  $\mu$ l propidium iodide (PI; BD Biosciences) in the dark at room temperature for 15 min. After washing the cells with 1X binding buffer to remove the excess FITC-Annexin V and PI, apoptotic cells were analyzed using flow cytometry (FACS Via Flow Cytometer; BD Biosciences) within 1 h to determine the levels of apoptosis. The fluorescence of FITC and PI were measured using the FL-1 and FL-2 channels, respectively. Apoptosis was quantified by the percentage of the population shifting to fluorescence positivity. The percentage of apoptotic cells was calculated as the percentage of early and late apoptotic cells. The data were analyzed using CytExpert software 2.0 (Beckman Coulter, Inc.).

**Caspase activity assay.** The activities of caspase-3, -8 and -9 in BMDMs subjected to the indicated treatments were detected using caspase-Glo 3/7, caspase-Glo 8 and caspase-Glo assay kits (cat. nos. G8090, G8200 and G8210, respectively; Promega Corporation) according to the manufacturer's protocols. BMDMs were seeded at a density of 1x10 $^3$  cells in 96-well plates and allowed to adhere overnight. Following incubation with the indicated drugs, caspase-Glo reagent was added to the cells at a 1:1 ratio. The contents were mixed and the cells were incubated for 1 h at room temperature. The luminescence signal of each sample was then detected using a Veritas plate-reading luminometer (Turner BioSystems) according to the manufacturer's instructions (25). The relative percentage of luminescence intensity was calculated by comparison to the vehicle control (26).

**Western blotting.** Total protein was extracted from cells using RIPA lysis buffer (Sigma-Aldrich; Merck KGaA) and stored at -80°C. Total protein concentration was determined using a BCA assay and proteins (20  $\mu$ g per lane) were separated via SDS-PAGE (12%). The separated proteins were subsequently electrotransferred onto nitrocellulose membranes (Amersham; Cytiva) and blocked in TBS solution containing 5% non-fat milk for 1 h at room temperature. The membranes were then incubated with the appropriate primary antibodies overnight at 4°C. Following incubation with the primary antibodies anti-PPAR $\gamma$  (1:1,000; cat. no. 95128; Cell Signaling Technology, Inc.), anti-caspase-3 (1:1,000; cat. no. 9661; Cell Signaling Technology, Inc.), anti-caspase-8 (1:1,000; cat. no. 8592; Cell Signaling Technology, Inc.), and anti- $\beta$ -actin (1:1,000; cat. no. 4970, Cell Signaling Technology, Inc.), the membranes were incubated with the relevant species-specific horseradish peroxidase-conjugated

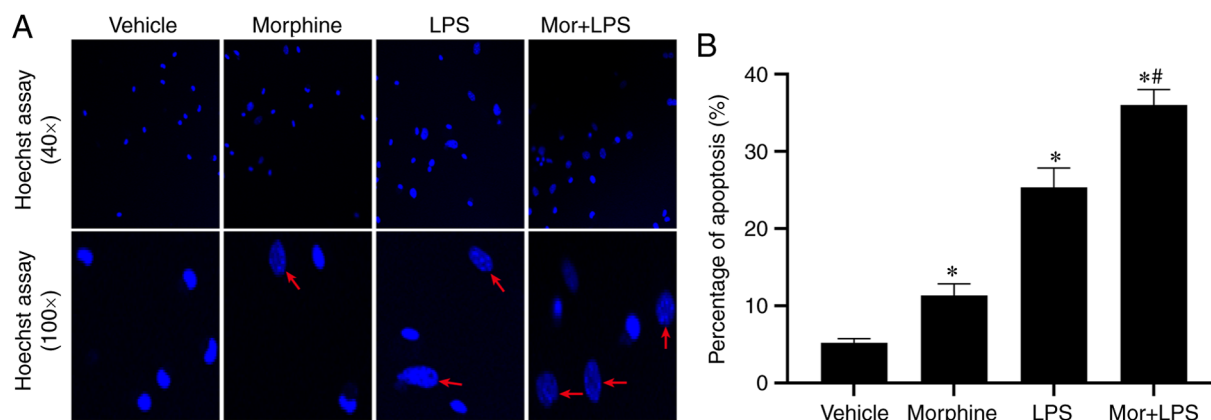


Figure 1. Morphine increases the apoptosis of LPS-induced BMDMs. (A) Representative images of Hoechst 33342 staining in BMDMs treated with vehicle, morphine, LPS, or morphine + LPS for 24 h. Upper panels, magnification x40; lower panels, magnification x100. Arrows, apoptotic cells. (B) Semi-quantification of BMDM apoptosis in the indicated groups. Data are presented as the mean  $\pm$  SD and were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. \* $P < 0.05$  vs. vehicle and # $P < 0.05$  vs. LPS. BMDMs, bone marrow-derived macrophages; LPS, lipopolysaccharide.

secondary antibodies (1:1,000; cat. nos. 7074 and 7076; Cell Signaling Technology, Inc.) for 1 h at room temperature. The membranes were subsequently washed and protein bands were visualized using a chemiluminescence detection system (Amersham; Cytiva). The expression levels of specific proteins were normalized to the expression levels of  $\beta$ -actin.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism software, version 8.0 (GraphPad Software, Inc.). Data are presented as the mean  $\pm$  SD of three independent experiments. Comparisons among multiple groups were assessed by one-way or two-way ANOVA followed by Bonferroni's post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Morphine promotes apoptosis in LPS-induced BMDMs.** To investigate the effects of morphine on LPS-induced BMDMs, the cells were incubated with morphine and/or LPS, and the levels of apoptosis in each group were analyzed by Hoechst 33342 staining. In the vehicle control group, few apoptotic cells were observed, and the majority of the nuclei displayed uniform morphology, intact membrane and even chromatin distribution (Fig. 1A). Following treatment with morphine or LPS alone, an increasing number of apoptotic cells with morphological changes and uneven staining were detected, particularly in the LPS group. Moreover, the characteristics of apoptosis were more prominent in BMDMs challenged with LPS and morphine in combination. In the combined treatment group, Hoechst 33342 staining revealed a larger number of blue fluorescent cells with fragmented nuclei, chromatin condensation and apoptotic body formation. Statistical analysis of the percentage of apoptotic cells revealed that, compared with the control group, LPS stimulation significantly increased the levels of apoptosis in BMDMs, which were further stimulated by morphine treatment (Fig. 1B and C). The apoptosis of BMDMs was further quantified by flow cytometric analysis following

Annexin V-FITC and PI double staining. As shown in Fig. 2, morphine treatment significantly increased the percentage of apoptotic cells. Taken together, these findings indicated the potential stimulatory effects of morphine on LPS-induced BMDM apoptosis.

**Morphine promotes the activation of caspase-3 in LPS-induced BMDMs.** The caspase-3 protease is the predominant effector caspase involved in the execution of apoptosis, and may be activated by both the extrinsic and intrinsic apoptotic pathways (27). To determine whether morphine treatment affected the activation of caspase-3 in LPS-induced BMDMs, caspase-3 activity was determined using the caspase-Glo 3/7 assay kit. The results revealed that morphine treatment further promoted the LPS-induced activation of caspase-3 in a time-dependent manner (Fig. 3A). To validate these findings, the expression levels of caspase-3 were analyzed using western blotting. Exposure to morphine or LPS alone did not markedly upregulated the expression levels of cleaved caspase-3, whereas the expression levels were significantly higher in the combined treatment group compared with either treatment alone (Fig. 3B and C). These results indicated the potentially important role of caspase-3 in the effects of morphine on LPS-induced BMDMs.

**Morphine treatment reduces caspase-8 activity in LPS-activated BMDMs.** Caspase-8 is a crucial upstream initiator that cleaves and activates the effector caspase-3 in the death receptor-triggered extrinsic pathway (27). Therefore, the activity and expression levels of caspase-8 were investigated in the present study. Notably, morphine or LPS treatment, alone or in combination, significantly reduced the activity of caspase-8 at 2, 6, 8 and 12 h (Fig. 4A). Western blotting demonstrated that the expression levels of active caspase-8 subunit p18 were downregulated in the LPS, morphine and combined treatment groups (Fig. 4B and C). These results suggested that the effects of morphine on BMDM apoptosis may be independent of caspase-8 activation, indicating the potential involvement of the intrinsic pathway in the apoptotic events.

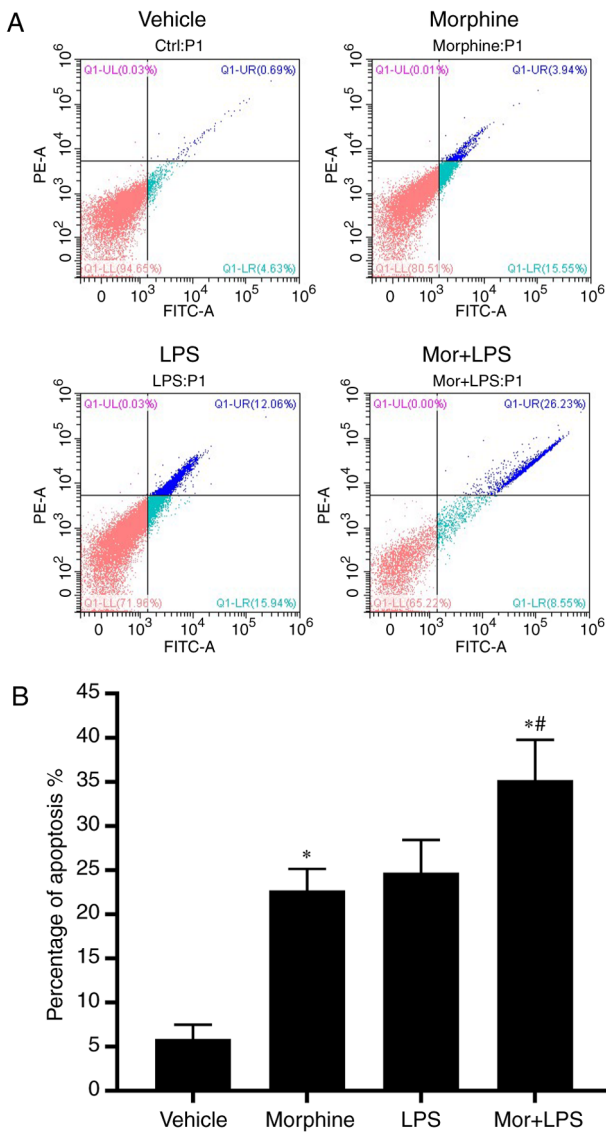


Figure 2. Flow cytometric analysis of apoptosis in BMDMs. (A) Flow cytometry following Annexin V-FITC and propidium iodide double staining was used to analyze apoptosis in BMDMs stimulated by morphine and LPS, alone or in combination, for 24 h. (B) Quantification of apoptosis in BMDMs (%) in the indicated groups. Data are presented as the mean  $\pm$  SD and were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. \* $P < 0.05$  vs. vehicle and \*\* $P < 0.05$  vs. LPS. BMDMs, bone marrow-derived macrophages; LPS, lipopolysaccharide.

*Morphine treatment upregulates LPS-induced PPAR $\gamma$  expression in BMDMs.* PPAR $\gamma$  was previously reported to be involved in the tolerance to morphine analgesia (21). Considering the crucial role of PPAR $\gamma$  in the regulation of macrophage apoptosis (18-20), the present study hypothesized that PPAR $\gamma$  may participate in the proapoptotic effect of morphine in LPS-stimulated BMDMs. Western blotting was performed to determine the protein expression levels of PPAR $\gamma$  in each group. Compared with the vehicle control group, exposure to morphine or LPS treatment alone upregulated the protein expression levels of PPAR $\gamma$ , although the observed difference was not deemed statistically significant. Moreover, the upregulation of PPAR $\gamma$  was significantly enhanced in the BMDMs challenged with morphine in combination with LPS, suggesting a potential

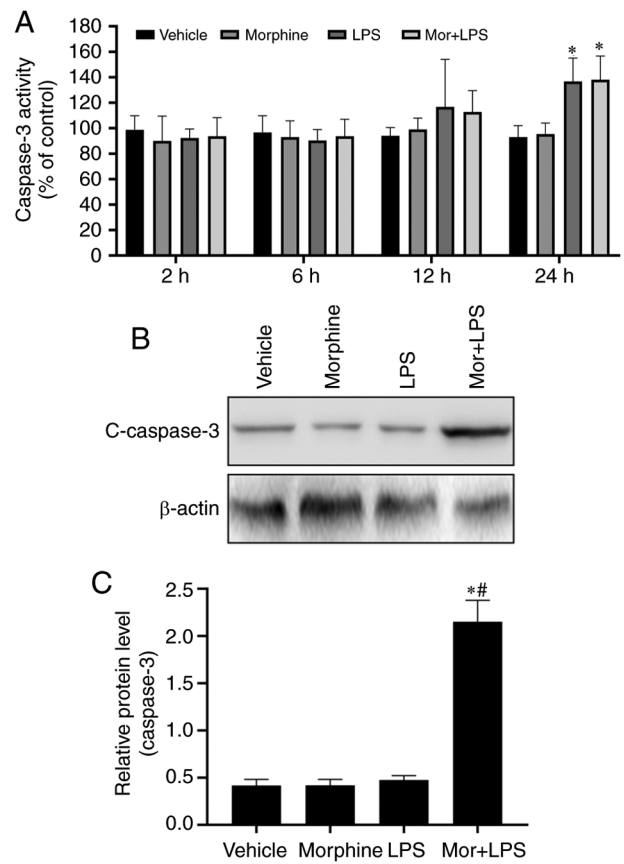


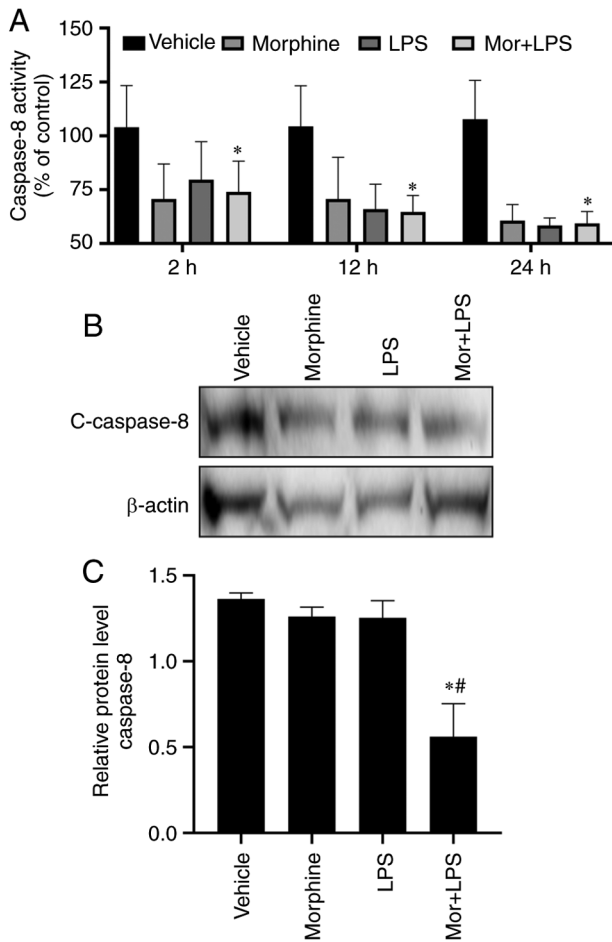
Figure 3. Morphine promotes the activation of caspase-3 in LPS-induced BMDMs. (A) Caspase-3 activity was measured in BMDMs treated with vehicle, morphine, LPS or morphine + LPS at 2, 6, 12 or 24 h. (B) Representative western blots of cleaved caspase-3 expression levels in BMDMs stimulated with the indicated treatments for 24 h. (C) Semi-quantification of the western blots of C-caspase-3 expression levels in BMDMs stimulated with the indicated treatments for 24 h. Data are presented as the mean  $\pm$  SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test. \* $P < 0.05$  vs. vehicle and \*\* $P < 0.05$  vs. LPS. BMDMs, bone marrow-derived macrophages; LPS, lipopolysaccharide; C-caspase, cleaved caspase.

role of PPAR $\gamma$  in the regulatory effect of morphine on LPS-stimulated BMDMs (Fig. 5A and B).

*Proapoptotic effect of morphine is dependent on PPAR $\gamma$  activation.* To validate the role of PPAR $\gamma$  in morphine-induced BMDM apoptosis, BMDMs were pre-incubated with GW9662, a specific PPAR $\gamma$  antagonist, for 4 h prior to the treatment with morphine and/or LPS. As shown in Fig. 6, the morphine-induced increased percentage of apoptotic cells was significantly reduced by GW9662 treatment, suggesting that morphine-induced apoptosis of LPS-activated BMDMs may be mediated via PPAR $\gamma$  activation.

*PPAR $\gamma$  antagonist reverses morphine-induced activation of caspase-3/9 in LPS-treated BMDMs.* The effects of PPAR $\gamma$  on morphine-induced caspase activation were further evaluated. As mentioned above, morphine treatment enhanced LPS-induced caspase-3 activation, which was subsequently reversed by GW9662 pretreatment (Fig. 7A and B). A similar result was observed regarding the activation of caspase-9, a major upstream initiator of caspase-3 in the intrinsic pathway



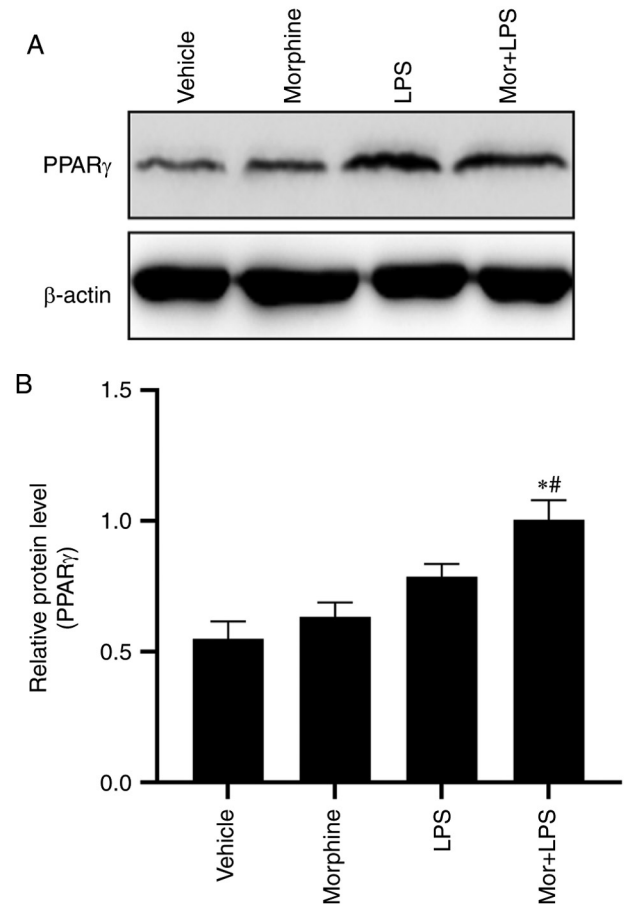


**Figure 4.** Morphine treatment reduces caspase-8 activity in LPS-induced BMDMs. (A) Caspase-8 activity was measured in BMDMs treated with vehicle, morphine, LPS or morphine + LPS at 2, 12 or 24 h. (B) Representative western blots of C-caspase-8 expression levels in BMDMs stimulated with the indicated treatments for 24 h. (C) Semi-quantification of the western blots of C-caspase-8 expression levels in BMDMs stimulated with the indicated treatments for 24 h. Data are presented as the mean  $\pm$  SD and were analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test. \* $P < 0.05$  vs. vehicle and # $P < 0.05$  vs. LPS. LPS, lipopolysaccharide; BMDMs, bone marrow-derived macrophages; C-caspase, cleaved caspase.

of apoptosis (28,29). In addition, LPS stimulation significantly increased the activity of caspase-9, which was further enhanced by LPS + morphine treatment (Fig. 7C and D). GW9662 significantly abrogated the stimulatory effect of morphine on LPS-induced caspase-9 activation (Fig. 7C and D). However, GW9662 treatment exerted no statistically significant effect on caspase-8 activity (Fig. 7E and F). Taken together, these results provided further evidence to suggest that the intrinsic pathway of apoptosis may be involved in the proapoptotic effects of morphine on LPS-induced BMDMs, which may be dependent, at least partially, on PPAR $\gamma$  activation.

## Discussion

Morphine has been widely used in the clinical setting as an analgesic, and numerous studies have supported a role for morphine in the regulation of apoptosis (30,31). Although opioid receptors are crucial for opioid-mediated effects, the molecular mechanisms underlying opioid-induced cell



**Figure 5.** Morphine treatment upregulates LPS-induced PPAR $\gamma$  expression levels in BMDMs. (A) Representative western blots and (B) semi-quantification of PPAR $\gamma$  expression levels in BMDMs treated with vehicle, morphine, LPS or morphine + LPS for 24 h. Data are presented as the mean  $\pm$  SD and were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons test. \* $P < 0.05$  vs. vehicle and # $P < 0.05$  vs. LPS. BMDMs, bone marrow-derived macrophages; LPS, lipopolysaccharide; PPAR $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ .

apoptosis remain to be fully elucidated. The results of the present study demonstrated that morphine enhanced LPS-induced apoptosis. Further investigations revealed that morphine treatment potentiated LPS-induced caspase-3 and caspase-9 activation, but inhibited the activity of caspase-8, suggesting the involvement of the intrinsic apoptosis pathway in the apoptotic events. In addition, morphine exposure resulted in the upregulation of PPAR $\gamma$  expression levels. More importantly, the stimulatory effects of morphine on LPS-induced apoptosis and caspase-3/9 activation were significantly reduced by GW9662, a PPAR $\gamma$  antagonist. Taken together, these results revealed a regulatory role for morphine in LPS-induced BMDM apoptosis and provided evidence supporting the involvement of PPAR $\gamma$  in the mechanisms underlying these observed effects.

To date, at least two major mechanisms have been discovered to be involved in the initiation of apoptosis: The extrinsic (death receptor-induced) and intrinsic (mitochondria-mediated) pathways (8,32). Both pathways are dependent on the activation of the caspase family of the cysteine proteases, which together act as a proteolytic cascade to dismantle and remove dying cells (8,30). In the extrinsic

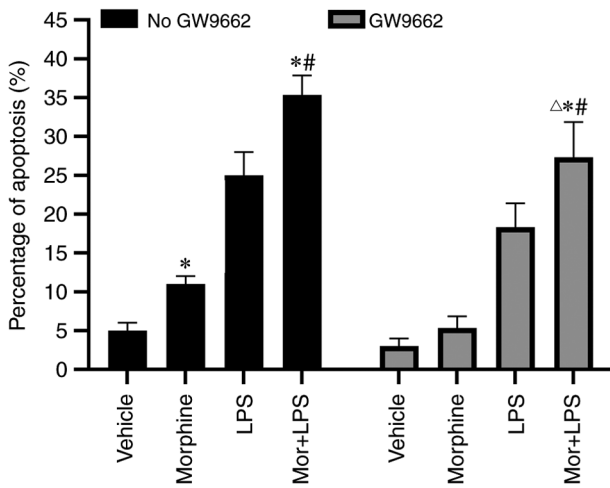


Figure 6. Proapoptotic effect of morphine is dependent on PPAR $\gamma$  activation. Quantification of apoptosis in BMDMs stimulated with vehicle, morphine, LPS or morphine + LPS with or without treatment with the PPAR $\gamma$  inhibitor, GW9662 (1  $\mu$ M). Data are presented as the mean  $\pm$  SD and analyzed by two-way ANOVA followed by Bonferroni's multiple comparisons test. \*P<0.05 vs. vehicle and GW + vehicle; #P<0.05 vs. LPS and GW + LPS;  $\Delta$ P vs. LPS + morphine without GW9662. BMDMs, bone marrow-derived macrophages; LPS, lipopolysaccharide; PPAR $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ .

pathway, death receptor stimulation leads to the recruitment and activation of caspase-8, which subsequently promotes the proteolysis of the downstream effector caspase-3 or other caspases, such as caspase-1 and -7. In addition, caspase-8 cleaves and activates the BH3-only protein, Bid, which can translocate to the mitochondria and promote cytochrome *c* release, resulting in caspase-9 and caspase-3 activation (8,32). LPS, a well-established ligand of Toll-like receptor 4, is a powerful stimulator of macrophages, which plays a key role in regulating macrophage activation and the resultant inflammatory response (33). It was also reported that LPS may play a proapoptotic role by enhancing caspase-3 activity and, thus, inducing the apoptosis of macrophages (12,14). The results of the present study revealed that morphine treatment acted synergistically with LPS and potentiated LPS-induced caspase-3/9 activation in BMDMs. Conversely, morphine or LPS treatment, alone or in combination, significantly reduced the activity and expression levels of caspase-8. Taken together, these results indicated the involvement of the intrinsic pathway in the proapoptotic effects of morphine in coordination with LPS. Considering the potent effects of LPS on BMDM-mediated inflammation, future studies should aim to further determine the potential function of morphine in LPS-induced inflammation in BMDMs.

PPAR $\gamma$ , a member of the nuclear hormone receptor superfamily, regulates cell proliferation, differentiation and death (16,17). Previous studies have demonstrated that the activation of PPAR $\gamma$  by agonists promoted macrophage apoptosis, and the proapoptotic effect may be dependent on negatively regulating NF- $\kappa$ B signaling, induction of cathepsin L and regulation of vitamin D3-upregulated protein-1 expression levels (18-20). Although the mechanisms underlying PPAR $\gamma$ -mediated apoptosis remain to be fully elucidated, recent evidence has demonstrated that

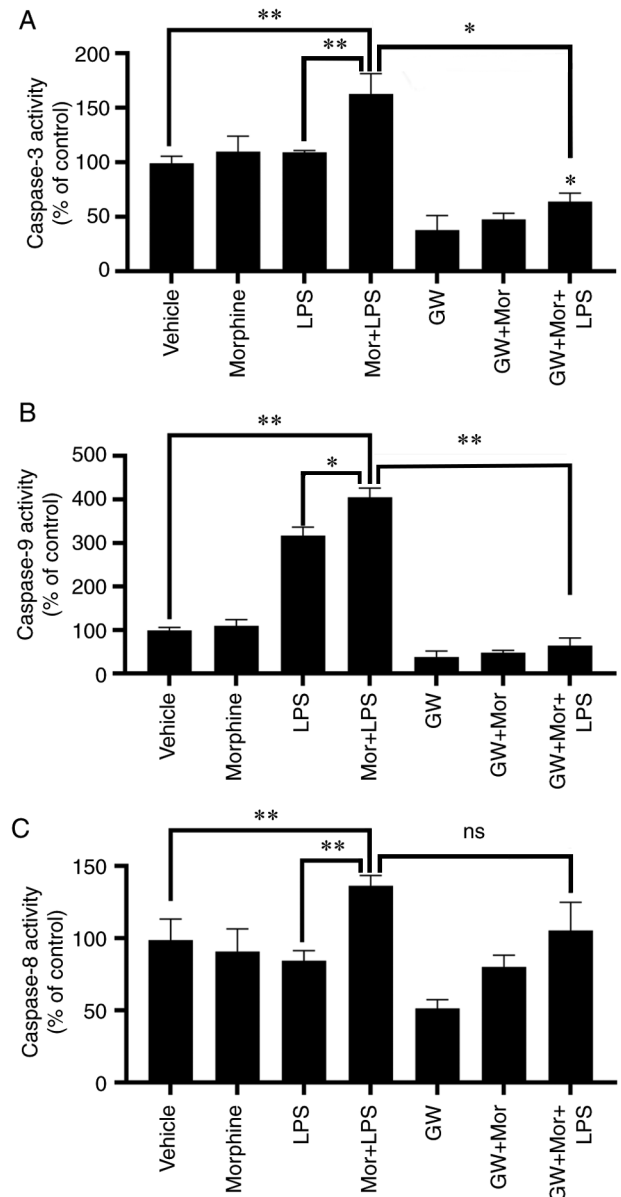


Figure 7. PPAR $\gamma$  antagonist reverses morphine-induced activation of caspase-3 and -9 in LPS-induced BMDMs. (A) Caspase-3, (B) caspase-9 and (C) caspase-8 activities were analyzed in BMDMs treated with vehicle, morphine, LPS or morphine + LPS, with or without treatment with the PPAR $\gamma$  inhibitor, GW9662 (1  $\mu$ M). Data are presented as the mean  $\pm$  SD. Data are presented as the mean  $\pm$  SD and were determined by one-way ANOVA and Bonferroni's post hoc test. \*P<0.05, \*\*P<0.01 and ns indicated no significance (P>0.05) vs. vehicle or the two groups connected by the umbrella lines in the figure. BMDMs, bone marrow-derived macrophages; LPS, lipopolysaccharide; PPAR $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ ; ns, not significant.

PPAR $\gamma$  activation promoted caspase-3 activity, while the transfection with PPAR $\gamma$  antisense oligonucleotides resulted in the downregulation of caspase-9 activity, indicating that the proapoptotic effects of PPAR $\gamma$  may be dependent, at least in part, on the caspase-mediated pathway (18-20). The findings of the present study demonstrated that the exposure to morphine upregulated PPAR $\gamma$  expression levels in LPS-induced BMDMs. Moreover, pretreatment with GW9662, a selective PPAR $\gamma$  antagonist, markedly abolished the stimulatory effects of morphine on LPS-induced apoptosis and caspase-3/9

activation. It has been reported that PPAR $\gamma$  plays a key role in the modulation of morphine tolerance (34). In the present study, PPAR $\gamma$  was found to be involved in the regulation of morphine-induced cell apoptosis, which may provide novel insight into the possible mechanisms underlying the biological function of morphine. However, to the best of our knowledge, the mechanism through which morphine upregulates PPAR $\gamma$  expression in BMDMs has not been reported to date. Thus, further research is required to elucidate the exact mechanism underlying the regulatory role of morphine in PPAR $\gamma$  expression.

Morphine is the most well-characterized and commonly used analgesic in the clinical setting (1). Following in-depth analysis of the pharmacological effects of morphine, a large number of studies have reported its numerous biological functions, including regulation of autophagy and neuro-immune modulation (35,36). Morphine is recommended for the alleviation of chest pain during acute coronary syndromes (37,38). In addition to pain relief, several previous studies have demonstrated a cardioprotective effect of morphine treatment (39-41). In animal studies, morphine was reported to protect against ischemia-reperfusion injury by significantly reducing infarct size and improving heart function (42,43). The results of the present study revealed a regulatory effect of morphine on PPAR $\gamma$  expression and the resultant apoptosis of BMDMs, which may expand the potential clinical applications of morphine to include acute myocardial infarction (AMI). Numerous previous studies have suggested an anti-atherogenic role of PPAR $\gamma$  in macrophages by reducing the inflammatory response (14,18,44). In addition, macrophage apoptosis, as the predominant pathway for macrophage removal from the plaque, was found to play a crucial role in atherosclerosis progression and plaque stability (45,46). During the early stages of AMI, the administration of morphine was found to not only exert an analgesic effect, but also improve myocardial injury, alleviate the local inflammatory response and promote plaque stability, which may be attributed to its regulatory function over PPAR $\gamma$  expression and macrophage apoptosis. Therefore, further studies are required to provide novel insight into the potential therapeutic and clinical applications of morphine beyond its analgesic properties.

In conclusion, the findings of the present study demonstrated that morphine treatment enhanced the LPS-induced apoptosis of BMDMs. Regarding the molecular mechanisms underlying morphine-mediated apoptosis of LPS-activated BMDMs, it was demonstrated that the co-administration of morphine facilitated the LPS-induced activation of caspase-3/9, but inhibited caspase-8 activity, indicating the involvement of the intrinsic pathway in the apoptotic events. In addition, morphine treatment upregulated LPS-induced PPAR $\gamma$  expression levels in BMDMs, while the PPAR $\gamma$  antagonist, GW9662, markedly abrogated the stimulatory effects of morphine on LPS-induced apoptosis and caspase activation. Taken together, these results provide what is, to the best of our knowledge, the first evidence to suggest that the intrinsic pathway of apoptosis may be involved in the proapoptotic effects of morphine on LPS-induced BMDMs, which may be dependent, at least partially, on PPAR $\gamma$  activation.

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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

JW designed the study; MYL and YL performed the experiments; KQD analyzed the data; MYL performed the biological analysis; MYL and KQD collected and analyzed the data; MYL, KQD and YL sacrificed the mice and performed the caspase assays; MYL performed the western blot experiments; KQD determined the quality of the BMDMs and performed the western blot experiments; MYL and JW wrote the manuscript. MYL and JW confirm the authenticity of all the raw data. All the authors have read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

## Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of the Zhongnan Hospital of Wuhan University (Wuhan, China; approval no. AF165).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References

1. Puig S and Gutstein HB: Opioids: Keeping the good, eliminating the bad. *Nat Med* 23: 272-273, 2017.
2. Tuerxun H and Cui J: The dual effect of morphine on tumor development. *Clin Transl Oncol* 21: 695-701, 2019.
3. Plein LM and Rittner HL: Opioids and the immune system-friend or foe. *Br J Pharmacol* 175: 2717-2725, 2018.
4. Bajic D, Commons KG and Soriano SG: Morphine-enhanced apoptosis in selective brain regions of neonatal rats. *Int J Dev Neurosci* 31: 258-266, 2013.
5. Li Y, Li H, Zhang Y, Sun X, Hanley GA, LeSage G, Zhang Y, Sun S, Peng Y and Yin D: Toll-like receptor 2 is required for opioids-induced neuronal apoptosis. *Biochem Biophys Res Commun* 391: 426-430, 2010.
6. Osmanoglu O, Yildirim MK, Akyuva Y, Yildizhan K and Naziroglu M: Morphine induces apoptosis, inflammation, and mitochondrial oxidative stress via activation of TRPM2 channel and nitric oxide signaling pathways in the hippocampus. *Mol Neurobiol* 57: 3376-3389, 2020.



7. Weng HL and Wang MJ: Effects of microRNA-338-3p on morphine-induced apoptosis and its underlying mechanisms. *Mol Med Rep* 14: 2085-2092, 2016.
8. Singh R, Letai A and Sarosiek K: Regulation of apoptosis in health and disease: The balancing act of BCL-2 family proteins. *Nat Rev Mol Cell Biol* 20: 175-193, 2019.
9. Gonzalez L and Trigatti BL: Macrophage apoptosis and necrotic core development in atherosclerosis: A rapidly advancing field with clinical relevance to imaging and therapy. *Can J Cardiol* 33: 303-312, 2017.
10. Davis TME, Peters KE and Lipscombe R: Apoptosis inhibitor of macrophage and diabetic kidney disease. *Cell Mol Immunol* 16: 521, 2019.
11. Li Z and Weinman SA: Regulation of hepatic inflammation via macrophage cell death. *Semin Liver Dis* 38: 340-350, 2018.
12. George L, Ramasamy T, Sirajudeen KNS and Manickam V: LPS-induced apoptosis is partially mediated by hydrogen sulphide in RAW 264.7 murine macrophages. *Immunol Invest* 48: 451-465, 2019.
13. Xaus J, Comalada M, Valledor AF, Lloberas J, López-Soriano F, Argilés JM, Bogdan C and Celada A: LPS induces apoptosis in macrophages mostly through the autocrine production of TNF-alpha. *Blood* 95: 3823-3831, 2000.
14. Du P, Li SJ, Ojcius DM, Li KX, Hu WL, Lin X, Sun AH and Yan J: A novel Fas-binding outer membrane protein and lipopolysaccharide of *Leptospira interrogans* induce macrophage apoptosis through the Fas/FasL-caspase-8/-3 pathway. *Emerg Microbes Infect* 7: 135, 2018.
15. Bhat RS, Bhaskaran M, Mongia A, Hitosugi N and Singhal PC: Morphine-induced macrophage apoptosis: Oxidative stress and strategies for modulation. *J Leukocyte Biol* 75: 1131-1138, 2004.
16. Ahmadian M, Suh JM, Hah N, Liddle C, Atkins AR, Downes M and Evans RM: PPAR $\gamma$  signaling and metabolism: The good, the bad and the future. *Nat Med* 19: 557-566, 2013.
17. Wang SB, Dougherty EJ and Danner RL: PPAR $\gamma$  signaling and emerging opportunities for improved therapeutics. *Pharmacol Res* 111: 76-85, 2016.
18. Mahmood DFD, Jguirim-Souissi I, Khadija EH, Blondeau N, Diderot V, Amrani S, Slimane MN, Syrovets T, Simmet T and Rouis M: Peroxisome proliferator-activated receptor gamma induces apoptosis and inhibits autophagy of human monocyte-derived macrophages via induction of cathepsin L: Potential role in atherosclerosis. *J Biol Chem* 286: 28858-28866, 2011.
19. Billiet L, Furman C, Larigauderie G, Copin C, Page S, Fruchart JC, Brand K and Rouis M: Enhanced VDUP-1 gene expression by PPAR $\gamma$  agonist induces apoptosis in human macrophage. *J Cell Physiol* 114: 183-191, 2008.
20. Chinetti G, Griglio S, Antonucci M, Torra IP, Delerive P, Majd Z, Fruchart JC, Chapman J, Najib J and Staels B: Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages. *J Biol Chem* 273: 25573-25580, 1998.
21. de Guglielmo G, Kallupi M, Scuppa G, Stopponi S, Demopoulos G, Gaitanaris G and Ciccocioppo R: Analgesic tolerance to morphine is regulated by PPAR $\gamma$ . *Br J Pharmacol* 171: 5407-5416, 2014.
22. Weischenfeldt J and Porse B: Bone marrow-derived macrophages (BMM): Isolation and applications. *CSH Protoc* 2008: pdb.prot5080, 2008.
23. Wan J, Xiao Z, Chao S, Xiong S, Gan X, Qiu X, Xu C, Ma Y and Tu X: Pioglitazone modulates the proliferation and apoptosis of vascular smooth muscle cells via peroxisome proliferator-activated receptor-gamma. *Diabetol Metab Syndr* 6: 101, 2014.
24. Pazar B, Ea HK, Narayan S, Kolly L, Bagnoud N, Chobaz V, Roger T, Lioté F, So A and Busso N: Basic calcium phosphate crystals induce monocyte/Macrophage IL-1 $\beta$  secretion through the NLRP3 inflammasome in vitro. *J Immunol* 186: 2495-2502, 2011.
25. Li CG, Yan L, Mai FY, Shi ZJ, Xu LH, Jing YY, Zha QB, Ouyang DY and He XH: Baicalin inhibits NOD-like receptor family, pyrin containing domain 3 inflammasome activation in murine macrophages by augmenting protein kinase a signaling. *Front Immunol* 8: 1409, 2017.
26. Guo S, Sun X, Cheng J, Xu H, Dan J, Shen J, Zhou Q, Zhang Y, Meng L, Cao W and Tian Y: Apoptosis of THP-1 macrophages induced by protoporphyrin IX-mediated sonodynamic therapy. *Int J Nanomedicine* 8: 2239-2246, 2013.
27. Fulda S and Debatin KM: Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene* 25: 4798-4811, 2006.
28. Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E and Boise LH: Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. *BMC Cell Biol* 14: 32, 2013.
29. Kurokawa M and Kornbluth S: Caspases and kinases in a death grip. *Cell* 138: 838-854, 2009.
30. Yenikomshian HA, Curtis EE, Carrougher GJ, Qiu Q, Gibran NS and Mandell SP: Outpatient opioid use of burn patients: A retrospective review. *Burns* 45: 1737-1742, 2019.
31. Maher DP, Walia D and Heller NM: Morphine decreases the function of primary human natural killer cells by both TLR4 and opioid receptor signaling. *Brain Behavior Immunity* 83: 298-302, 2020.
32. Kroemer G and Martin SJ: Caspase-independent cell death. *Nat Med* 11: 725-730, 2005.
33. Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaeili SA, Mardani F, Seifi B, Mohammadi A, Afshari JT and Sahebkar A: Macrophage plasticity, polarization, and function in health and disease. *J Cell Physiol* 233: 6425-6440, 2018.
34. Javadi S, Ejtemaieimehr S, Keyvanfar HR, Moghaddas P, Aminian A, Rajabzadeh A, Mani AR and Dehpour AR: Pioglitazone potentiates development of morphine-dependence in mice: Possible role of NO/cGMP pathway. *Brain Res* 1510: 22-37, 2013.
35. Zhao L, Zhu Y, Wang D, Chen M, Gao P, Xiao W, Rao G, Wang X, Jin H, Xu L, *et al*: Morphine induces Beclin 1-and ATG5-dependent autophagy in human neuroblastoma SH-SY5Y cells and in the rat hippocampus. *Autophagy* 6: 386-394, 2010.
36. Koob GF: Neurobiology of opioid addiction: Opponent process, hyperkatifeia, and negative reinforcement. *Biol Psychiat* 87: 44-53, 2020.
37. Task Force on the management of ST-segment elevation acute myocardial infarction of the European Society of Cardiology (ESC); Steg PG, James SK, Atar D, Badano LP, Blömmström-Lundqvist C, Borger MA, Di Mario C, Dickstein K, Ducrocq G, *et al*: ESC guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation. *Eur Heart J* 33: 2569-2619, 2012.
38. O'Gara PT: 2013 ACCF/AHA guideline for the management of ST-elevation myocardial infarction: A report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines (vol 127, pp e362, 2013). *Circulation* 128: e362-e425, 2013.
39. Murphy GS, Szokol JW, Marymont JH, Avram MJ and Vender JS: Opioids and cardioprotection: The impact of morphine and fentanyl on recovery of ventricular function after cardiopulmonary bypass. *J Cardiothor Vasc Anesth* 20: 493-502, 2006.
40. Tanaka K, Kersten JR and Riess ML: Opioid-induced cardioprotection. *Curr Pharm Design* 20: 5696-5705, 2014.
41. Rentoukas I, Giannopoulos G, Kaoukis A, Kossyvakis C, Raisakis K, Driva M, Panagopoulou V, Tsarouchas K, Vavetsi S, Pyrgakis V and Deftereos S: Cardioprotective role of remote ischemic preconditioning in primary percutaneous coronary intervention enhancement by opioid action. *JACC Cardiovasc Interv* 3: 49-55, 2010.
42. Wang Y, Wang L, Li JH, Zhao HW and Zhang FZ: Morphine alleviates myocardial ischemia/reperfusion injury in rats by inhibiting TLR4/NF- $\kappa$ B signaling pathway. *Eur Rev Med Pharmac* 23: 8616-8624, 2019.
43. Chen ZL, Li TZ and Zhang BX: Morphine postconditioning protects against reperfusion injury in the isolated rat hearts. *J Surg Res* 145: 287-294, 2008.
44. Oppi S, Nusser-Stein S, Blyszczuk P, Wang X, Jomard A, Marzolla V, Yang K, Velagapudi S, Ward LJ, Yuan XM, *et al*: Macrophage NCOR1 protects from atherosclerosis by repressing a pro-atherogenic PPAR $\gamma$  signature. *Eur Heart J* 41: 995-1005, 2020.
45. Subramanian M, Thorp E and Tabas I: Identification of a non-growth factor role for GM-CSF in advanced atherosclerosis promotion of macrophage apoptosis and plaque necrosis through IL-23 signaling. *Circ Res* 116: e13-e24, 2015.
46. Linton MF, Babaev VR, Huang JS, Linton EF, Tao H and Yancey PG: Macrophage apoptosis and efferocytosis in the pathogenesis of atherosclerosis. *Circ J* 80: 2259-2268, 2016.

