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Role of P38 Mitogen-Activated Protein Kinase Phosphorylation and Fas-Fas Ligand Interaction in Morphine-Induced Macrophage Apoptosis

Pravin C. Singhal,* Madhu Bhaskaran,* Jaimita Patel,* Kalpesh Patel,* Balakuntalam S. Kasinath,* Senthil Duraisamy,† Nicholas Franki,* Krishna Reddy,* and Aditi A. Kapasi*

In this study, we evaluated the molecular mechanisms involved in morphine-induced macrophage apoptosis. Both morphine and TGF-β promoted P38 mitogen-activated protein kinase (MAPK) phosphorylation, and this phosphorylation was inhibited by SB 202190 as well as by SB 203580. Anti-TGF-β Ab as well as naltrexone (an opiate receptor antagonist) inhibited morphine-induced macrophage P38 MAPK phosphorylation. Anti-TGF-β Ab also attenuated morphine-induced p53 as well as inducible NO synthase expression; in contrast, Nω-nitro-l-arginine methyl ester, an inhibitor of NO synthase, inhibited morphine-induced P38 MAPK phosphorylation and Bax expression. Morphine also enhanced the expression of both Fas and Fas ligand (FasL), whereas anti-FasL Ab prevented morphine-induced macrophage apoptosis. Moreover, naltrexone inhibited morphine-induced FasL expression. In addition, macrophages either deficient in FasL or lacking p53 showed resistance to the effect of morphine. Inhibitors of both caspase-8 and caspase-9 partially prevented the apoptotic effect of morphine on macrophages. In addition, caspase-3 inhibitor prevented morphine-induced macrophage apoptosis. These findings suggest that morphine-induced macrophage apoptosis proceeds through opiate receptors via P38 MAPK phosphorylation. Both TGF-β and inducible NO synthase play an important role in morphine-induced downstream signaling, which seems to activate proteins involved in both extrinsic (Fas and FasL) and intrinsic (p53 and Bax) cell death pathways. The Journal of Immunology, 2002, 168: 4025–4033.

Heroin is widely abused on the streets as well as in recreational facilities. Recently, morphine, an active metabolite of heroin, has gained a prime status in the pain management of cancer patients. As the aged population is increasing so is the occurrence of cancer, and it appears that the use of morphine is likely to be increased. Thus, it is important to evaluate the biological effects of opiates on cells such as macrophages.

Opiate addicts are prone to bacterial infections (1–4). Because the mononuclear phagocyte system plays a critical role in the host’s defense against bacteria, modulation of monocyte function by morphine may be important. Opiates have been reported to modulate monocyte function in the form of decreased migration, phagocytosis, bacterial killing, inhibition of Ab formation, and altered production of cytokines (5–11). Previously, Nair et al. (12) and Yin et al. (13) demonstrated that morphine promotes the apoptosis of lymphocytes. Recently, we reported that morphine triggered macrophage/monocyte apoptosis (14, 15). In those studies we suggested that morphine-induced macrophage apoptosis is mediated through the activation of inducible NO synthase (iNOS),3 and expression of p53 and Bax (14). However, it appears that morphine uses multiple pathways to promote macrophage apoptosis.

We and other investigators previously reported that morphine stimulated the production of TGF-β by macrophages (15–18). Because TGF-β has been reported to act as an immune modulator, many of the effects of morphine may be mediated through the generation of TGF-β by monocytes (19, 20). TGF-β has been reported to trigger apoptosis in a variety of cells (21–23). TGF-β promotes the induction of apoptosis through downstream signaling by phosphorylation of mitogen-activated protein kinases (MAPKs) (24) and Smad proteins (25). Recently, we demonstrated that TGF-β also plays a role in morphine-induced macrophage apoptosis (15). In this study, we examined the role of P38 MAPK phosphorylation in morphine-induced macrophage apoptosis. To establish a causal relationship, we also studied the effect of SB 202190 and SB 203580, inhibitors of P38 MAPK phosphorylation, on morphine-induced macrophage apoptosis. In addition, we studied the effect of morphine on the expression of other proapoptotic proteins such as Fas and Fas ligand (Fasl). We have also made an attempt to delineate other pathways by which morphine may be tilting the balance toward apoptosis.

Materials and Methods

Macrophage culture

We used a murine macrophage cell line (J774 cells; American Type Culture Collection, Manassas, VA). Confluent macrophages were subcultured

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3 Abbreviations used in this paper: iNOS, inducible NO synthase; MAP, mitogen-activated protein; MAPK, MAP kinase; ERK, extracellular signal-related kinase; FasL, Fas ligand; l-NAME, Nω-nitro-l-arginine methyl ester; PI, propidium iodide; KO, knockout; C-Mφ, control macrophage; p53KO-Mφ, p53KO macrophage.
in DMEM (Life Technologies, Grand Island, NY) containing 10% FCS (Life Technologies), 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies).

To determine the role of FasL and p53, we evaluated the effect of morphine on peritoneal macrophages harvested from control (C57BL/6j, stock no. 000664; The Jackson Laboratory, Bar Harbor, ME), FasL-deficient (B6Smn.C3H-FasL^−/−, stock no. 001021; the background of this strain is statistically 96.9% C57BL/6j-like; The Jackson Laboratory), and p53-knockout (KO) (B6-129-TgP53^tm1^Tyj; The Jackson Laboratory) mice.

Experimental reagents

TGF-β1 was obtained from Collaborative Biomedical Products (Bedford, MA) and was used in concentrations of 1–10 ng/ml Anti-P38 MAPK and Abs to phospho-P38 MAPK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-TGF-β1, mouse monoclonal anti-p53, mouse monoclonal anti-Bax, rabbit polyclonal anti-Fasl, and goat polyclonal anti-Fas Abs, were also obtained from Santa Cruz Biotechnology. Inhibitors of caspase-8, -9, and -3, specific inhibitors of P38 MAPK (SB 203580 and SB 203580), and an inhibitor of mitogen-activated protein (MAP)extracellular signal-related kinase (ERK) kinase (PD 098059) were obtained from Calbiochem (La Jolla, CA). Mouse monoclonal anti-iNOS Ab was obtained from BD Transduction Laboratories (Lexington, KY). Morphine was dissolved in normal saline, stocked at a concentration of 10−8 M, and used in concentrations of 10−8–10−6 M (National Institute on Drug Abuse, Bethesda, MD). Curcumin (inhibitor of c-Jun-AP-1 complex) was used in a concentration of 5 μM (Sigma-Aldrich, St. Louis, MO).

Apoptosis studies

Morphologic evaluation of macrophage apoptosis was performed by staining cells with H-33342 (Molecular Probes, Portland, OR) and propidium iodide (PI; Sigma-Aldrich). H-33342 stains the nuclei of live cells and identifies apoptotic cells by increased fluorescence. Double staining by these two agents provides the percentage of live, apoptotic, and necrotic cells (14, 26). Cells were prepared under control and experimental conditions. At the end of the incubation period, cells were washed twice with ice-cold PBS and lysed in a modified radioimmunoprecipitation assay (RIPA) lysis buffer (containing 1 mM Tris (pH 8.0), 150 mM sodium chloride, 5 mM EDTA, 0.1 mM sodium orthovanadate, 1 mM orthovanadate, 1 mM PMSF, 0.1% aprotinin, and 1% Nonidet P-40) for 20 min on ice, protected from light, and then examined under UV light. The percentage of live, apoptotic, and necrotic cells was recorded in eight random fields by two observers unaware of the experimental condition.

To confirm the effect of morphine on macrophage apoptosis, control and morphine-treated macrophages (2 × 10^6 cells) were prepared for DNA isolation as described previously (27). DNA concentration was determined by reading the absorbance at 260 nm. DNA was electrophoresed on a 1.8% Tris boric acid, EDTA buffer containing 10 mM sodium EDTA, and used in concentrations of 10−8–10−6 M (National Institute on Drug Abuse, Bethesda, MD). Curcumin (inhibitor of c-Jun-AP-1 complex) was used in a concentration of 5 μM (Sigma-Aldrich, St. Louis, MO).

Protein extraction and Western blotting for total and phosphorylated P38 MAPK

Cells were incubated under control and experimental conditions (for all conditions, cells were pretreated with 10 ng/ml LPS for 12 h). Subsequently, cells were washed twice with ice-cold PBS and lysed in a modified radioimmunoprecipitation assay lysis buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, aprotinin 4 μg/ml, 1 mM orthovanadate, and a protease inhibitor mixture (Calbiochem). The cell lysates were centrifuged at 15,000 × g for 20 min and the supernatants of cell lysates containing 20 μg protein each were subjected to 10% SDS-PAGE and transferred onto Immobilon P membranes (Millipore, Marlboro, MA). The membranes were probed with anti-P38 MAPK (1:1,000; Santa Cruz Biotechnology), anti-phospho-P38 MAPK (1:1,000, New England Biolabs, Beverly, MA), anti-FasL (1:1,000; BD PharMingen, San Diego, CA), anti-Fas (1:1,000; Santa Cruz Biotechnology), anti-p53 (1:1,000; Santa Cruz Biotechnology), anti-iNOS (1:1,000; BD Transduction Laboratories), or anti-Bax (1:1,000; Santa Cruz Biotechnology) Abs. A HRP-conjugated appropriate secondary Ab (1:2,000) was used to detect immunoreactive bands using an ECL system from Pierce (Rockford, IL).

P38 MAPK assay

Equal amounts (150 μg) of cell lysates from control and treated cells were immunoprecipitated by incubating with anti-P38 MAPK Ab (Cell Signaling Technology, Beverly, MA) at 4°C for 3 h. Protein A-Sepharose beads were added and incubated at 4°C for 1 h. The immunobeads were washed three times in radioimmunoprecipitation assay buffer (consisting of 20 mM Tris (pH 7.5), 150 mM sodium chloride, 5 mM EDTA, 0.1 mM sodium orthovanadate, 1 mM PMSF, 0.1% aprotinin, and 1% Nonidet P-40) and twice in PBS. Beads were incubated with 2.5 mg/ml substrate, myelin basic protein (MBP), 25 μM cold ATP, and 1 μCi of [γ-32P]ATP in MAPK buffer (10 mM HEPES (pH 7.4), 10 mM magnesium chloride, 0.5 mM DTT, and 0.5 mM sodium orthovanadate) for 30 min at 30°C. Reaction was stopped by the addition of sample buffer. The reaction mixture was boiled for 5 min and subjected to SDS-PAGE using a 15% gel. The gel was dried and exposed to Kodak Biomax MR x-ray film (Kodak, Rochester, NY) with an image-intensifying screen. Western blotting with anti-P38 MAPK Ab was performed on an aliquot of assay lysates to assay loading.

Statistical analysis

For comparison of mean values between groups, the unpaired t test was used. To compare values among multiple groups, ANOVA was applied and a Newman-Keuls multiple range test was used to calculate a p value. All values are means ± SEM except where otherwise indicated. Statistical significance was defined as p < 0.05.

Results

Apoptotic studies

To determine the role of P38 MAPK phosphorylation in morphine-induced macrophage apoptosis, equal numbers of macrophages were incubated in medium containing vehicle (control), morphine (10−8 and 10−6 M), SB 202190 (5 μM), or morphine (10−8 and 10−6 M) plus SB 202190 for 24 h. At the end of the incubation period, cells were stained with H-33342 and PI. As shown in Fig. 1A, morphine promoted macrophage apoptosis. SB 202190, an inhibitor of P38 MAPK phosphorylation, inhibited (p < 0.001) the effect of morphine on macrophage apoptosis. These results suggest that morphine-induced macrophage apoptosis is mediated through P38 MAPK phosphorylation.

We also studied the effect of SB 203580 (another inhibitor of P38 MAPK phosphorylation) and PD 098059, the MAPK-ERK kinase inhibitor, on morphine-induced macrophage apoptosis. Equal numbers of macrophages were incubated in medium containing buffer (control), morphine (10−6 M), SB 203580 (1 μM), or SB 203580 plus morphine for 24 h. In parallel experiments, equal numbers of macrophages were incubated in medium containing buffer (control), morphine (10−6 M), PD 098059 (1 μM), or morphine plus PD 098059 for 24 h. Subsequently, cells were assayed for apoptosis. SB 203580 inhibited the effect of morphine on macrophage apoptosis (Fig. 1B), whereas PD 098059 did not modulate the effect of morphine (Fig. 1C).

To confirm the effect of morphine on macrophage apoptosis, equal numbers of macrophages were incubated in medium containing buffer, morphine (10−6 M), SB 202190 (1 μM), or SB 202190 plus morphine for 24 h. Subsequently cells were harvested and DNA was extracted and electrophoresed. As shown in Fig. 1D, morphine-treated cells showed DNA fragmentation into multiple integers of 180 bp (ladder pattern); however, control cells as well as cells treated with SB 202190 did not show fragmentation of DNA.

To determine the role of the c-Jun-AP-1 complex in morphine-induced macrophage apoptosis, we studied the effect of curcumin, an inhibitor of the c-Jun-AP-1 complex (28). Equal numbers of macrophages were incubated in medium containing buffer (control), morphine (10−8 and 10−6 M), curcumin (5 μM), or curcumin plus morphine for 24 h. At the end of the incubation period, cells were assayed for apoptosis. Curcumin partially inhibited the morphine-induced macrophage apoptosis (Fig. 2A).

P38 MAPK phosphorylation studies

To determine the effect of morphine on macrophage P38 MAPK phosphorylation, equal numbers of macrophages in 100-mm petri dishes were incubated in medium (DMEM plus 10% FCS) containing either vehicle (control) or variable concentrations of morphine (10−8–10−6 M) for 60 min. At the end of the incubation,
Macrophages were incubated in medium containing vehicle (control), morphine ($10^{-5}$–$10^{-6}$ M), or SB 202190 (SB20; 5 μM) with or without morphine for 24 h ($n = 3$). Subsequently, cells were stained with H-33342 and PI. *, $p < 0.001$ compared with control, morphine ($10^{-6}$ M), SB20, and morphine ($10^{-5}$ M) plus SB20; **, $p < 0.001$ compared with control, morphine ($10^{-5}$ M), and SB20 plus morphine ($10^{-5}$ M). B, Effect of SB 203580 on morphine-induced macrophage apoptosis. Macrophages were incubated in medium containing vehicle (control), morphine ($10^{-5}$ M), or SB 203580 (1 μM) with or without morphine for 24 h. Subsequently, cells were assayed for apoptosis. *, $p < 0.001$ compared with control, SB 203580, and morphine plus SB 203580. C, Effect of PD 098059 on morphine-induced macrophage apoptosis. Macrophages were incubated in medium containing vehicle (control), morphine ($10^{-5}$ M), or PD 098059 (PD098; 1 μM) with or without morphine for 24 h ($n = 3$). Subsequently, cells were assayed for apoptosis. *, $p < 0.001$ compared with control and PD098. D, Effect of SB 202190 on morphine-induced macrophage DNA fragmentation. Macrophages were incubated in medium containing vehicle (control), morphine ($10^{-5}$ M), or SB 202190 (5 μM) with or without morphine for 24 h. At the end of the incubation period DNA was extracted and electrophoresed. Lane 1, A molecular marker. Lanes 2 and 3, Control and morphine-treated cells. Lanes 4 and 5, Cells treated with SB 202190 and SB 202190 plus morphine, respectively.

cells were prepared for Western blotting and probed with anti-P38 MAPK and phospho-P38 MAP Abs. As shown in Fig. 3A, morphine promoted macrophage P38 MAPK phosphorylation. To evaluate whether this effect of morphine is mediated through the generation of TGF-β by macrophages, equal numbers of macrophages in 100-mm petri dishes were incubated in medium (DMEM plus 10% FCS) containing vehicle (control), variable concentrations of morphine ($10^{-5}$–$10^{-6}$ M), anti-TGF-β Ab, or morphine plus anti-TGF-β Ab for 60 min. At the end of the incubation, cells were prepared for Western blotting and probed with P38 MAPK and phospho-P38 MAP Abs. As shown in Fig. 3B, morphine enhanced the phosphorylation of P38 MAPK; however, anti-TGF-β Ab inhibited the phosphorylation of P38 MAPK under a morphine-stimulated state.

To determine the direct effect of TGF-β on the macrophage P38 MAPK pathway, equal numbers of J774 cells were incubated in medium (DMEM plus 10% FCS) containing either vehicle (control) or variable concentrations of TGF-β (1, 5, and 10 ng/ml) for 60 min. At the end of the incubation period, cells were prepared for Western blotting and probed with anti-P38 MAPK or phospho-P38 MAP Abs. As shown in Fig. 4A, TGF-β enhanced the phosphorylation of P38 MAPK.

To evaluate the specificity of morphine-induced P38 MAPK phosphorylation, equal numbers of macrophages were incubated in medium containing either vehicle (control) or morphine ($10^{-8}$–$10^{-6}$ M) with or without SB 202190 for 60 min. In parallel experiments, equal numbers of macrophages were incubated in medium containing either vehicle (control) or TGF-β (5 ng/ml) with or without SB 202190 for 60 min. At the end of the incubation period cells were prepared for Western blotting and probed with anti-P38 MAPK and phospho-P38 MAPK Abs. As shown in Fig. 4B, SB 202190 attenuated P38 MAPK phosphorylation under a morphine-stimulated state.

To determine the role of TGF-β in the constitutive activation of P38 MAPK, we determined the effect of anti-TGF-β Ab on the activation of P38 MAPK under a basal and a morphine-stimulated state. In addition, we determined the direct effect of TGF-β on P38 MAPK activation. Equal numbers of macrophages were incubated in medium containing buffer (control), morphine ($10^{-8}$–$10^{-6}$ M), anti-TGF-β Ab (1 μg/ml), anti-TGF-β1 Ab plus morphine ($10^{-8}$ M), anti-TGF-β1 Ab plus morphine ($10^{-6}$ M), or TGF-β1 (1 ng/ml) for 60 min. Subsequently, cells were lysed and a P38 MAPK assay was performed. As shown in Fig. 5, morphine enhanced P38 MAPK activity; however, anti-TGF-β1 Ab attenuated this effect of morphine. Interestingly, anti-TGF-β1 Ab-treated cells showed a lower P38 MAPK activity when compared with control cells, thus suggesting that TGF-β plays a role in the constitutive activation of TGF-β.
we previously demonstrated that NOS inhibitors \( \text{L-NAME} \) and \( \text{G-nitro-L-arginine} \) partially inhibited the morphine-induced P38 MAPK phosphorylation. As shown in Fig. 6, \( \text{L-NAME} \) partially inhibited the morphine-induced P38 MAPK phosphorylation.

**Role of Fas and FasL in morphine-induced macrophage apoptosis**

To determine the role of FasL in morphine-induced macrophage apoptosis, equal numbers of macrophages were incubated in medium containing either buffer (control) or \( \text{L-NAME} \) (1 mM) with or without morphine \( (10^{-8} - 10^{-6} \text{ M}) \) for 1 h. At the end of the incubation period, cells were harvested, proteins were extracted, and Western blots were generated and probed for P38 MAPK and phospho-P38 MAPKs. As shown in Fig. 6A, \( \text{L-NAME} \) partially attenuated the morphine-induced P38 MAPK phosphorylation.

**Role of Fas and FasL in morphine-induced macrophage apoptosis**

To confirm the role of FasL in morphine-induced macrophage apoptosis, we evaluated the effect of morphine on peritoneal macrophages isolated from control and FasL-deficient mice. Equal numbers of macrophages harvested from control and FasL-deficient mice were incubated in medium containing variable concentrations of morphine \( (0 - 10^{-6} \text{ M}) \) for 16 h. Subsequently, cells were stained for apoptosis and necrosis. As shown in Fig. 6B, morphine promoted macrophage expression of FasL in control macrophages treated with morphine \( (10^{-6} \text{ M}) \); \( * \), \( p < 0.001 \) compared with control macrophages treated with morphine \( (10^{-4} \text{ M}) \).

**Role of p53 in morphine-induced macrophage apoptosis**

We previously reported that morphine promotes the expression of Fas and FasL, equal numbers of macrophages were incubated in medium containing either buffer (control) or variable concentrations of morphine \( (10^{-8} - 10^{-6} \text{ M}) \) for 60 min. Subsequently, cells were prepared for Western blots and probed for Fas and FasL. As shown in Fig. 6C and D, morphine promoted macrophage expression of both FasL and Fas.

**Role of p53 in morphine-induced macrophage apoptosis**

We previously reported that morphine enhances the expression of p53 in macrophages (14). To confirm the role of p53 in morphine-induced macrophage apoptosis, we evaluated the effect of morphine on peritoneal macrophages isolated from control (C57BL/6; The Jackson Laboratory) and p53-KO (B6-129-Trp53\(^{tm1Tyj} \)) mice. Equal numbers of macrophages harvested from control macrophage (C-Mb) and p53-KO-macrophage (p53KOMb) mice were incubated in medium containing either buffer (control) or variable concentrations of morphine...
mum containing buffer (control), morphine (10^{-8}-10^{-6} M) for 60 min (n = 4). Subsequently, cells were prepared for Western blotting and probed with anti-P38 MAPK and phospho-P38 MAPK Abs. Representative blots are shown depicting the effect of morphine on macrophage total and phospho-P38 MAPKs. *, p < 0.01 compared with control; **, p < 0.001 compared with control. B, Effect of anti-TGF-β Ab on morphine-induced macrophage P38 MAPK phosphorylation under basal and morphine (endogenous TGF-β)-stimulated states. Macrophages were incubated in medium containing vehicle (control), variable concentrations of morphine (10^{-8}-10^{-4} M), or anti-TGF-β Ab with or without morphine for 60 min (n = 3). At the end of the incubation period, cells were stained for apoptosis. As shown in Fig. 7A, morphine induced greater apoptosis in C-Mφ when compared with p53KO-Mφ. These results confirm that p53 also plays a role in morphine-induced macrophage apoptosis.

Role of Bax in morphine-induced macrophage apoptosis

To determine whether morphine-induced macrophage apoptosis is mediated through extrinsic (caspase-8) or intrinsic (caspase-9) pathways, we studied the effect of inhibitors of caspase-8 and caspase-9. Equal numbers of macrophages were incubated in medium containing buffer (control), morphine (10^{-8} M), caspase-8 inhibitor (1 μM), caspase-9 inhibitor (1 μM), morphine plus caspase-8 inhibitor, or morphine plus caspase-9 inhibitor for 24 h. Subsequently, cells were stained for apoptosis. As shown in Fig. 7B, inhibitors of caspase-8 and caspase-9 partially inhibited the morphine-induced macrophage apoptosis.

To determine the role of caspase-3 in morphine-induced macrophage apoptosis, equal numbers of macrophages were incubated in medium containing either buffer (control) or morphine (10^{-8}-10^{-6} M) with or without caspase-3 inhibitor (5 μM) for 16 h. At the end of the incubation period, cells were stained for apoptosis. Caspase-3 inhibitor attenuated the morphine-induced macrophage apoptosis (Fig. 7C).

Role of TGF-β in morphine-induced iNOS and p53 expression

To determine the relationship between morphine-induced p53/iNOS expression and TGF-β, we studied the effect of anti-TGF-β Ab on morphine-induced macrophage p53 and iNOS expression. Equal numbers of macrophages were incubated in medium containing buffer (control), morphine (10^{-8} and 10^{-6} M), or anti-TGF-β (1 μg/ml) with or without morphine (10^{-8} and 10^{-6} M) for 1 h. Subsequently, proteins were extracted and Western blots were prepared and probed for p53 and iNOS. As shown in Fig. 8, morphine promoted the expression of p53 and iNOS; however, these effects of morphine were inhibited by anti-TGF-β Ab.

Role of opiate receptors in morphine-induced P38 MAPK phosphorylation and FasL expression

To determine the role of opiate receptors in morphine-induced macrophage P38 MAPK phosphorylation, equal numbers of macrophages were incubated in medium containing buffer (control), morphine (10^{-8} M), naltrexone (10^{-6} M), or morphine plus naltrexone for 60 min. Subsequently, proteins were extracted and Western blots were prepared and probed for total and phospho-P38 MAPKs. As shown in Fig. 9A, morphine promoted P38 MAPK phosphorylation under basal and morphine (endogenous TGF-β)-stimulated states. Macrophages were incubated in medium containing vehicle (control), variable concentrations of morphine (10^{-8}-10^{-6} M), or anti-TGF-β Ab with or without morphine for 60 min (n = 3). At the end of the incubation period, cells were stained for apoptosis. Caspase-3 inhibitor attenuated the morphine-induced macrophage apoptosis (Fig. 7C).

Role of caspases in morphine-induced macrophage apoptosis

FIGURE 3. A, Effect of morphine on macrophage P38 MAPK phosphorylation. Macrophages were incubated in medium containing either vehicle (control) or variable concentrations of morphine (10^{-8}-10^{-6} M) for 60 min (n = 4). Subsequently, cells were prepared for Western blotting and probed with anti-P38 MAPK and phospho-P38 MAPK Abs. Representative blots are shown depicting the effect of morphine on macrophage total and phospho-P38 MAPKs. *, p < 0.01 compared with control; **, p < 0.001 compared with control. B, Effect of anti-TGF-β Ab on morphine-induced macrophage P38 MAPK phosphorylation under basal and morphine (endogenous TGF-β)-stimulated states. Macrophages were incubated in medium containing vehicle (control), variable concentrations of morphine (10^{-8}-10^{-4} M), or anti-TGF-β Ab with or without morphine for 60 min (n = 3). At the end of the incubation period, cells were stained for apoptosis. Caspase-3 inhibitor attenuated the morphine-induced macrophage apoptosis (Fig. 7C).
phosphorylation. However, this effect of morphine was partially inhibited by naltrexone. These results indicate that morphine-induced P38 MAPK phosphorylation may be mediated through opiate receptors.

To determine the role of opiate receptors in morphine-induced FasL expression, equal numbers of macrophages were incubated in medium containing either buffer (control) or morphine (10^{-6} - 10^{-8} M) with or without naltrexone (10^{-6} M) for 60 min. Subsequently, proteins were extracted and Western blots were generated and probed for FasL. Morphine promoted FasL expression, whereas naltrexone inhibited morphine-induced macrophage FasL expression (Fig. 9B). These results suggest that morphine-induced FasL expression may be mediated through opiate receptors.

**Discussion**

This study demonstrates that morphine promotes P38 MAPK phosphorylation via opiate receptors through TGF-β- and iNOS-mediated downstream signaling, which appears to have an ongoing "cross-talk." Because morphine enhanced the expression of Fas, FasL, p53, and Bax, it appears that morphine-induced macrophage apoptosis is associated with the activation of proteins involved in both extrinsic and intrinsic cell death pathways.

**FIGURE 5.** Evaluation of the role of TGF-β in constitutive activation of P38 MAPK. Macrophages were incubated in medium containing buffer (control), morphine (10^{-6} - 10^{-8} M), anti-TGF-β Ab (A-TGF Fab, 1 μg/ml), anti-TGF-β Ab plus morphine (10^{-8} M), anti-TGF-β Ab plus morphine (10^{-6} M), or TGF-β1 (TGF-β1; 1 ng/ml) for 60 min. Subsequently, cells were lysed and P38 MAPK activity assay was performed. Western blotting with anti-P38 MAPK Ab was performed on an aliquot of assay lysates to assess loading. Upper panel, The effect of different variables on macrophage P38 MAPK activity (kinase assay). Lower panel, A Western blot indicating loading of P38 MAPK in the corresponding lysate sample.

**FIGURE 4.** A, Direct effect of TGF-β on macrophage P38 MAPK phosphorylation. Macrophages were incubated in medium containing either vehicle (control) or variable concentrations of TGF-β (1, 5, and 10 ng/ml) for 60 min (n = 3). At the end of the incubation period cells were prepared for Western blotting and probed with anti-P38 MAPK or anti-phospho-P38 MAPK Abs. Representative blots of phospho-P38 MAPK and total P38 MAPK are shown depicting the effect of TGF-β (*, p < 0.05 compared with TGF-β (1–5 ng/ml)). B, Effect of SB 202190 on morphine-induced macrophage P38 MAPK phosphorylation. Macrophages were incubated in medium containing either buffer (control) or morphine (10^{-6} - 10^{-8} M) with or without SB 202190 for 60 min (n = 3). At the end of the incubation period cells were prepared for Western blotting and probed with anti-P38 MAPK or anti-phospho-P38 MAPK Abs. Representative blots of phospho-P38 MAPK and total P38 MAPK are shown depicting the effect of SB 202190 (*, p < 0.05 compared with respective morphine concentrations).

**FIGURE 6.** A, Effect of L-NAME on morphine-induced macrophage P38 MAPK phosphorylation. Macrophages were incubated in medium containing either buffer (control) or L-NAME (LN; 1 mM) with or without morphine (M; 10^{-8} - 10^{-6} M) for 1 h. At the end of the incubation period cells were harvested, protein were extracted, and Western blots were generated and probed for P38 MAPK and phospho-P38 MAPKs. B, Effect of L-NAME on morphine-induced Bax expression. Macrophages were incubated in medium containing either buffer or L-NAME (LN; 1 mM) with or without morphine (10^{-8} and 10^{-6} M) for 16 h. At the end of the incubation period, Western blots were generated and probed for Bax. C, The effect of morphine on macrophage FasL expression. Macrophages were incubated in medium containing either buffer or morphine (M; 10^{-8}) for 1 h. At the end of the incubation period cells were harvested, protein were extracted, and Western blots were generated and probed for FasL. D, The effect of morphine on macrophage Fas. Macrophages were incubated in medium containing either buffer (control) or variable concentrations of morphine (10^{-10} - 10^{-6} M) for 60 min. Subsequently, cells were prepared for Western blots and probed for Fas.
Welters et al. (29) recently reported that morphine inhibits LPS-induced NF-κB activation in murine macrophages. NF-κB has been considered to be a transcription factor for genes associated with survival. Roy et al. (30) also previously reported that morphine at higher concentrations not only inhibits activation of NF-κB but also suppresses the production of TNF-α. TNF-α has been reported to inhibit macrophage apoptosis (31). It appears that morphine acts as a double-edged sword, stimulating the production of TGF-β, a proapoptotic cytokine, while attenuating the production of TNF-α, an antiapoptotic cytokine.

Signals from various stimuli are conducted to the nucleus to regulate gene expression through a distinct set of MAPK signal transduction cascades. These include the ERKs, the c-Jun NH2-terminal kinases, and P38 kinase (32, 33). With respect to cell growth, the important targets of MAPKs are transcription factors such as c-fos and c-jun. c-fos and c-jun families of transcription factors form functional heterodimeric transcription complexes known as AP-1 (34) and determine whether the outcome of the signal will promote proliferation or apoptosis. Inflammatory cytokines such as TGF-β or other stress signals activate serine/threonine kinase, which is related to the kinases of the ERK pathway (33). The MAPKs are highly specific for their transcription factor substrates; for example, ERK phosphorylates Elk-1 but not activated transcription factor 2, whereas c-Jun NH2-terminal kinase phosphorylates c-Jun but not c-fos. Stress signals can also stimulate another protein kinase, P38 (35). Previously, morphine has been shown to activate c-fos expression in the rat brain (36); however, in these studies upstream and downstream signaling was not investigated and thus it is difficult to comment on the morphine-induced upstream MAPK pathways (36).

It is the balance between phosphorylated and dephosphorylated forces that determines whether the transcription of apoptosis-associated genes will be sustained or switched off. In the present study, morphine promoted P38 MAPK phosphorylation. Interestingly, morphine-induced P38 MAPK phosphorylation was inhibited by anti-TGF-β Abs. Moreover, TGF-β directly enhanced the phosphorylation of P38 MAPK. Because these kinases have been demonstrated to propagate proapoptotic signals and promote the transcription of cell death-associated genes, we suggest that TGF-β mediates morphine-induced macrophage apoptosis through the phosphorylation of P38 MAPK kinases. The specificity of this

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![Figure 7](image-url)  
FIGURE 7. A. Effect of morphine on macrophages harvested from control and p53KO mice. Macrophages harvested from C-M6 and p53KO-M6 mice were incubated in medium containing either buffer (control) or variable concentrations of morphine (10^-8 to 10^-6 M) for 16 h. At the end of the incubation period cells were stained for apoptosis. *, p < 0.05 compared with respective control; **, p < 0.05 compared with respective control; ***, p < 0.001 compared with respective control; Aa, p < 0.001 compared with control; Ab, p < 0.05 compared with control plus morphine (10^-8 M); Ac, p < 0.001 compared with control plus morphine (10^-8 to 10^-6 M). B. Effect of caspase-8 and -9 inhibitors on morphine-induced macrophage apoptosis. Macrophages were incubated in medium containing buffer (control), morphine (10^-8 M), caspase-8 inhibitor (C-8inh; 1 μM), caspase-9 inhibitor (C-9inh; 1 μM), morphine plus caspase-8 inhibitor, or morphine plus caspase-9 inhibitor for 24 h. Subsequently, cells were stained for apoptosis. Inhibitors of caspase-8 and caspase-9 partially inhibited the morphine-induced macrophage apoptosis. *, p < 0.001 compared with control; **, p < 0.001 compared with morphine alone. C. Effect of caspase-3 inhibitor on morphine-induced macrophage apoptosis. Macrophages were incubated in medium containing either buffer (control) or morphine (10^-8 to 10^-6 M) with or without caspase-3 inhibitor (C3inh; 5 μM) for 16 h (n = 3). At the end of the incubation period cells were stained for apoptosis. *, p < 0.001 compared with control; **, p < 0.001 compared with morphine (10^-8 M); ***, p < 0.001 compared with morphine (10^-8 M); ****, p < 0.001 compared with morphine (10^-6 M).

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![Figure 8](image-url)  
FIGURE 8. Evaluation of the effect of anti-TGF-β Ab on morphine-induced p53 and iNOS expression. Macrophages were incubated in medium containing buffer (control), morphine (10^-8 and 10^-6 M), or anti-TGF-β (AT-ab; 1 μg/ml) with or without morphine (10^-8 and 10^-6 M) for 1 h. Subsequently, proteins were extracted and Western blots were prepared and probed for p53 and iNOS. A, Morphine increased the expression of p53; however, anti-TGF-β attenuated this effect of morphine. B, Morphine enhanced the expression of iNOS, but this effect of morphine was attenuated by anti-TGF-β Ab.

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![Figure 9](image-url)  
FIGURE 9. A. Evaluation of the role of opiate receptors in morphine-induced macrophage P38 MAPK phosphorylation. Macrophages were incubated in medium containing buffer (control), morphine (10^-8 M), naloxone (Nal; 10^-6 M), or morphine plus naloxone for 60 min. Subsequently, proteins were extracted and Western blots were prepared and probed for total and phospho-P38 MAPKs. Upper panel, The effect of morphine and naloxone on P38 MAPK phosphorylation. Lower panel, Total P38 MAPKs under similar conditions. B, Evaluation of the role of opiate receptors in morphine-induced FasL expression. Macrophages were incubated in medium containing either buffer (control) or morphine (10^-8 and 10^-6 M) with or without naloxone (10^-6 M) for 60 min. Subsequently, proteins were extracted and Western blots were generated and probed for FasL.
effect of TGF-β was confirmed by the inhibitory effect of SB 202190 on P38 MAPK phosphorylation under both the TGF-β and morphine-stimulated states. Alternatively, TGF-β may have promoted the phosphorylation of Smad proteins. Phosphorylated Smad proteins may interact with c-Jun, thus activating the transcription of proapoptotic proteins (24, 25).

Previously, various investigators reported that morphine enhances DNA damage in lymphocytic cells (37–39). These investigators suggest that opiates might be either decreasing the capacity to repair the damaged DNA or enhancing sensitization for DNA damage (37–39). Interestingly, TGF-β inhibits the expression of FasL in lymphocytes, and thus it appears that morphine-induced lymphocyte apoptosis is unlikely to be mediated through TGF-β (40). However, in vivo, theoretically, morphine-induced macrophage FasL expression may participate in the induction of apoptosis of bystander lymphocytes (41).

It appears that morphine promotes macrophage apoptosis through multiple pathways. We previously reported not only that morphine promoted macrophage iNOS expression but that morphine-induced macrophage apoptosis was also inhibited by NOS inhibitors l-NAME and N⁴-monomethyl-L-arginine monoacetate (14). Other investigators have also reported the role of NO in the induction of macrophage apoptosis (42). We also demonstrated that morphine-induced macrophage apoptosis was associated with increased expression of p53 and Bax (14). Expression of wild-type p53 appears to be linked to apoptosis promoted by DNA-damaging agents (43). P53 has been demonstrated to be a direct transcriptional activator of the Bax gene, a cell death promoter gene. Thus it is plausible that morphine-induced expression of p53 and Bax are linked. Because Bax is a protein belonging to the Bel-2 family, an important player of the mitochondrial (intrinsic) pathway, it appears that an intrinsic pathway also plays a role in morphine-induced macrophage apoptosis. This notion is further supported by morphine-induced enhanced expression of iNOS and p53 (proteins linked to the mitochondrial pathway).

In the present study, P38 MAPK activation appears to play an important role in morphine-induced macrophage apoptosis. Both morphine and TGF-β promoted P38 MAPK activity, whereas anti-TGF-β Ab inhibited P38 MAPK activity in both the stimulated and basal states; therefore, it appears that TGF-β induces the constitutive activation of P38 MAPK in macrophages. Because anti-TGF-β Ab inhibited morphine-induced P38 MAPK activation, it appears that morphine-induced P38 MAPK activation is mediated through TGF-β generation.

We previously reported that morphine stimulated the production of TGF-β by macrophages and that morphine-induced apoptosis was mediated through TGF-β (15). In those studies, anti-TGF-β Ab also inhibited morphine-induced Bax expression, thus suggesting the role of TGF-β-mediated downstream signaling in the activation of the mitochondrial pathway. In the present study we found that morphine-induced expression of Bax was inhibited by l-NAME. Thus, it appears that morphine-induced Bax production is also mediated through iNOS. Because anti-TGF-β Ab as well as l-NAME inhibited the morphine-induced P38 MAPK phosphorylation it appears that both TGF-β and iNOS contribute to the phosphorylation of P38 MAPK.

The binding of FasL to Fas results in the recruitment of an adapter protein, Fas-associated death domain, which associates with a proenzyme form of caspase-8 and leads to the activation of caspase-8, leading to the activation of downstream caspasers and subsequent degradation of nuclear proteins (44). In this study, morphine enhanced macrophage expression of both Fas and FasL, whereas anti-FasL Ab inhibited the morphine-induced macrophage apoptosis. In addition, peritoneal macrophages harvested from FasL-deficient mice showed resistance to the apoptotic effect of morphine. Thus, it appears that an extrinsic pathway (Fas and FasL interaction) may also be contributing to morphine-induced macrophage apoptosis.

Interestingly, morphine-induced FasL expression was inhibited by l-NAME, thus suggesting a link to the iNOS-mediated downstream signaling in the activation of the FasL-mediated (extrinsic) pathway. In contrast, l-NAME also inhibited morphine-induced FasL expression. These findings suggest that iNOS-mediated downstream signaling contributes to the activation of both the intrinsic (Bax, a protein belonging to intrinsic pathway) and extrinsic (FasL, a protein participating in the extrinsic pathway) pathways. Fig. 10 displays a hypothetical scheme suggested for morphine-induced macrophage apoptosis. These studies need to be further extended to delineate the details of these pathways.

Previous reports suggest that morphine-induced macrophage apoptosis is mediated through opiate receptors (14). This study delineates the involved mechanisms. Naltrexone, an opiate receptor antagonist, inhibited the effect of morphine on P38 MAPK phosphorylation and FasL expression. Because both FasL expression and P38 MAPK phosphorylation in response to morphine are key events in the induction of macrophage apoptosis it appears that opiate receptors play a role in morphine-induced macrophage apoptosis.

We conclude that morphine promotes P38 MAPK phosphorylation via opiate receptors through TGF-β- and iNOS-mediated downstream signaling. Because morphine activates proteins involved in extrinsic as well as intrinsic cell death pathways it appears that both pathways contribute to morphine-induced macrophage apoptosis.

References