Morphine Priming Rescues High-Dose Morphine-Induced Biological Perturbations

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In in vitro studies, macrophage morphine priming (MP; preincubation with low-dose morphine) attenuated the effects of high-dose morphine (HDM) on macrophage capabilities of killing and containment of phagocytosed bacteria. In in vivo studies, mice received either normal saline (control), HDM, or MP twice daily for 10 consecutive days. All HDM-treated mice showed a significant peritoneal bacterial leak; none of the control and only 2 of 12 mice receiving MP showed peritoneal bacterial leak. HDM-treated mice showed decreased macrophage migration into the peritoneal cavity; however, MP inhibited this effect. In in vitro studies, macrophages and bone-marrow cells harvested from HDM-treated mice showed not only enhanced apoptosis but also decreased migration across the filter of a Boyden chamber; nevertheless, MP inhibited these effects of HDM. MP also attenuated the proapoptotic effect of HDM; however, this effect was prevented by treatment with pyrrolidine derivative of dithiocarnamate, an inhibitor of nuclear factor-κB. These results suggest that MP provides protection against HDM-induced degradation of the host defense barrier through preservation of macrophage function.

Clinical reports have indicated that persons addicted to opiates are prone to infections [1, 2]. Opiates may enhance the occurrence of infections by both indirect and direct ways. Street opiates are often mixed with impurities and are at times administered with contaminated and unsterile needles [3, 4]. Because mononuclear phagocytes have been demonstrated to possess opiate receptors, the direct effect of opiates on these cells is increasingly recognized [5–8]. Moreover, morphine, an active metabolite of heroin, has been reported to modulate immune function by multiple ways. In in vitro as well as in vivo studies, morphine has been demonstrated to suppress the phagocytic capabilities of macrophages [9, 10] and chemotaxis of human monocytes [11]. We previously reported that morphine at higher concentrations promoted macrophage apoptosis [12, 13]. Other investigators have demonstrated that morphine induces sepsis in mice [14–16]. This effect of morphine may be partly linked to morphine-induced macrophage injury [17].

Morphine has been demonstrated to induce macrophage p38 MAP kinase (MAPK) phosphorylation in a dose-dependent manner [13]. Similarly, morphine has been reported to enhance macrophage-induced nitric oxide synthase (iNOS) expression and NO production. All of these effects of morphine occur at higher concentrations. Moreover, morphine has been shown to have a bimodal effect on cell growth [18, 19]. At lower concentrations, it stimulates cell survival, whereas, at higher concentrations, it induces apoptosis [18, 19]. Thus, theoretically, strategies that promote cell survival may not only promote the survival of macrophages but also attenuate the effects of morphine that are often associated with macrophage injury. We hypothesized that priming of macrophages with low-dose morphine (LDM)―to stimulate cell survival―might abrogate the injurious effect of high-dose morphine (HDM).

Recently, macrophage injury has been shown to be
linked to the degradation of the host defense barrier (peritoneal bacterial leak) in both mice and rats after the administration of HDM [17]. We propose that the priming of a mouse with LDM might rescue that mouse from HDM-induced peritoneal bacterial leak.

Morphine has been reported to decrease the number of murine peritoneal and alveolar macrophages [7]. This decrease in the number of macrophages may be related to a morphine-induced decrease in chemotaxis [11]. In addition, we recently reported that, at high concentrations, morphine causes apoptosis of peritoneal as well as bone-marrow macrophages [12, 13, 17]. Because apoptosed macrophages are functionally compromised, macrophages from other sources, such as blood and bone marrow, may not be able to migrate effectively to the site of the breach in the defense barrier (i.e., the peritoneal cavity). However, if priming of mice with LDM is able to preserve macrophage survival in an HDM milieu, it is likely that morphine priming (MP) might also maintain the normal rate of macrophage migration into the peritoneal cavity, even in the HDM milieu. To prove these hypotheses, we performed multiple studies pertaining to the migration and apoptosis of macrophages and bone-marrow cells and bacterial peritoneal leak in a mouse model of morphine-induced sepsis.

**MATERIAL AND METHODS**

**Mice.** Male FVB/N mice were housed in groups of 4 in a laminar-flow facility (Small Animal Facility, Long Island Jewish Medical Center, New Hyde Park, NY). Rodent diet 5001 (PMI Nutrition International) and fresh water were available ad libitum.

**Macrophage culture.** We used a murine macrophage cell line (J774.16 cells; American Type Culture Collection). Confluent macrophages were subcultured in Dulbecco’s modified Eagle medium (Life Technologies) that contained 10% fetal calf serum (FCS; Life Technologies), 50 U/mL penicillin, and 50 μg/mL streptomycin (Life Technologies).

**Colony and bacterial count.** Peritoneal fluid samples were diluted up to a 5-mL volume, and a sample was collected from each variable by a calibrated disposable inoculating loop (1 mL, 219052; Becton Dickinson Microbiology Systems) and inoculated onto blood agar plates. After 24 h, the number of colonies was counted in each plate. With the use of this method, each colony contained 1000 bacteria/mL.

**Experimental protocols.** Thirty-six FVB/N mice, weighing 25 g each, were used in 2 sets. Each set was composed of 18 mice in 3 groups: 6 mice received MP (a low dose [1 mg/kg body weight (BW)] 3 h before receiving HDM (40 mg/kg BW every 12 h)), 6 received HDM in the morning and in the evening, and 6 received normal saline in the morning and in the evening (control). To stimulate macrophage migration in the peritoneal cavity, on the eighth day, 3 mL of thioglycollate broth were administered intraperitoneally. On the tenth day, samples of peritoneal fluid were obtained under sterile conditions and inoculated by a 1-μL-calibrated inoculating loop onto agar plates. Macrophages were isolated from the peritoneal cavity of these mice as described elsewhere [20] and were evaluated for the occurrence of apoptosis by staining with H-33342 and propidium iodide [21]. In addition, bone-marrow cells were harvested as described elsewhere [17], and the occurrence of apoptosis was recorded.

**Evaluation of the effect of morphine on bone-marrow macrophage colony formation.** We and other investigators [22] have previously reported that morphine imparts a suppressive effect on the growth of bone-marrow macrophages. To evaluate the effect of MP, we studied the effect of mouse macrophage colony-stimulating factor (M-CSF) on the growth of bone-marrow macrophages harvested from control mice and those receiving MP and HDM. Eighteen mice, in groups of 6, received (subcutaneously) MP, HDM, or normal saline twice daily for 10 consecutive days. On the tenth day, mice were killed, femurs were removed under sterile conditions, and bone-marrow cells were harvested as described elsewhere [17].

**Evaluation of the effect of MP on bacterial killing and containment of macrophage phagocytosed bacteria.** To study the effect of morphine on the killing and containment of bacteria by macrophages, murine macrophages (10⁶ cells/petri dish) were incubated with opsonized clinical isolates of *Escherichia coli* (10⁸ microorganisms/mL) for 1 h at 37°C. Subsequently, cells were repeatedly washed, trypsinized, and washed again to remove any bacteria on the surface of macrophages. Aliquots of macrophages containing phagocytosed bacteria (MCPBs) were used to evaluate the effect of MP on HDM-induced reduced capability of bacterial killing.

Equal numbers of MCPBs were incubated in medium that contained either buffer or LDM (10⁻¹⁰ mol/L) for 2 h, followed by reincubation in medium that contained buffer, LDM, or HDM (10⁻⁶ mol/L) for 4 h. At the end of the incubation, to determine the level of escape of phagocytosed bacteria, samples of supernatants from control and morphine-treated cells were collected at 1, 2, 3, and 4 h and were then seeded onto agar plates with the use of a calibrated inoculating loop. To determine the survival level of phagocytosed bacteria, aliquots of trypsinized macrophages were collected at variable periods (1, 2, 3, and 4 h), homogenized, and inoculated onto agar plates. Agar plates were incubated at 37°C and were observed for growth of bacterial colonies after 24 h of incubation. Six series of experiments were performed.

**Apoptosis studies.** To determine the occurrence of apoptosis in macrophages, we used TUNEL staining as described elsewhere [21]. In vivo studies, apoptosed macrophages were immediately removed from the site of injury and incubated for 16 h in medium that contained 20% FCS. Subsequently, cells
Figure 1. A, Effect of morphine priming (MP) on a high-dose morphine (HDM)–induced tumor necrosis factor (TNF)–α production. Equal nos. of macrophages were incubated in medium that contained each variable for 8 h in the following sequences: buffer followed by buffer (B-F-B), buffer followed by low-dose morphine (LDM; 10^{-10} mol/L; BF-F-LD), LDM followed by LDM (LD-F-LD), buffer followed by HDM (10^{-6} mol/L; BF-F-HD), HDM followed by HDM (HD-F-HD), LDM followed by HDM (MP), pyrrolidine derivative of dithiocarnamate (PDTC; 100 μmol/L), PDTC followed by HDM (PDTC+HD), or PDTC followed by LDM (PDTC+LD). At the end of the incubation period, supernatants were collected and assayed for TNF-α. *P < .01, compared with control, BF-F-HD, HD-F-HD, MP, PDTC, PDTC+HD, and PDTC+LD. **P < .05, compared with control. ***P < .01, compared with control. B, Effect of MP on HDM-induced macrophage apoptosis. Equal nos. of macrophages were incubated in medium that contained each variable for 8 h in the following sequences: B-F-B (control), LD-F-LD, MP, or PDTC (100 μmol/L) plus LDM followed by HDM (PDTC+MP) or B-F-HD. Cells were then assayed for apoptosis. Results (means ± SEs) are from 4 sets of experiments, each performed in triplicate. *P < .001, compared with control, LDM, and MP.

were stained with H-33342 and propidium iodide, and the extent of macrophage injury was determined.

**Immunobulassay for tumor necrosis factor (TNF)–α.** To determine the effect of MP on HDM-induced TNF-α production, equal numbers of macrophages were incubated in medium that contained buffer, LDM, or LDM plus pyrrolidine derivative of dithiocarnamate (PDTC; 100 μmol/L; Sigma) for 6 h. Subsequently, cells were reincubated in medium that contained buffer or HDM for 8 h. At the end of the incubation period, supernatants were collected and assayed for TNF-α with the use of a TNF-α assay kit (R&D Systems). Cells were lysed, and the protein content was measured.

**In vitro evaluation of macrophage migration.** In these studies, equal numbers of peritoneal macrophages (5 x 10^5 cells/mL) under control or experimental conditions were incubated in 100 μL of medium in the upper compartment of a modified Boyden chamber. Two hundred microliters of medium that contained monocyte chemotactic protein–1 (10 ng/mL) was instilled in the lower compartment of the chamber. The chambers were kept in a 37°C, 5% CO_2 incubator for 120 min. At the end of the incubation period, cells were wiped off the upper surface of the filter with a cotton swab, and the undersurface was stained with Diff Quick (Baxter). The migrated macrophages were counted in 8 random fields, and the mean macrophage count was calculated.

**Protein extraction and Western blotting.** To determine the effect of morphine priming on P38 MAPK phosphorylation, equal numbers of macrophages (10^5 cells/well) were plated onto
6-well plates and grown to confluence, followed by treatment according to the experimental protocol. At the end of the incubation period, cells were washed, and protein extraction, blotting, and probing for P38 MAPK and phosphorylated P38 MAPK were performed as described elsewhere [13].

**Statistical analysis.** For comparison of mean values between groups, the unpaired *t* test was used. To compare values between multiple groups, analysis of variance was applied, and a Newman-Keuls multiple range test was used to calculate a *P* value. All values are means ± SEs, except where otherwise indicated. Statistical significance was defined as *P* < .05.

**RESULTS**

**Effect of MP on HDM-induced TNF-α production.** LDM stimulated macrophage TNF-α production (figure 1A). However, this effect of morphine was inhibited by PDTC, an inhibitor of NF-κB. Interestingly, priming of macrophages with LDM sustained macrophage TNF-α production, despite treatment with HDM (figure 1A). However, PDTC attenuated this effect of MP.

**Evaluation of the role of NF-κB activation on morphine-induced macrophage apoptosis.** Activation of NF-κB has been associated with better macrophage survival; therefore, we studied the role of NF-κB in morphine-induced macrophage apoptosis. As shown in figure 1B, HDM promoted macrophage apoptosis. However, MP modulated the proapoptotic effect of HDM. Interestingly, PDTC inhibited this effect of MP. These findings suggest that MP preserves macrophage survival in an HDM milieu. This effect of morphine priming seems to be mediated through the activation of NF-κB.

**Effect of MP on macrophage leak of phagocytosed bacteria.** To determine the morphine priming effect, equal numbers of MCPBs were incubated in medium that contained different variables (as described in figure 2A) for 6 h. MCPBs were assessed for bacterial escape at 1, 2, 3, and 4 h (figure 2A). HDM-treated MCPBs showed a greater leak of phagocytosed bacteria than did control, LDM-treated, and MP-treated MCPBs. These results suggest that MP prevents HDM-induced macrophage leak of bacteria.

**Effect of MP on macrophage killing of bacteria.** To determine the morphine priming effect, equal numbers of MCPBs were incubated in medium that contained different variables (as described in figure 2B) for 6 h. MCPBs were assessed for bacterial killing capability of macrophages at 1, 2, 3, and 4 h (figure 2B). HDM-treated MCPBs showed a higher number of surviving bacteria (decreased killing of bacteria) than did control, LDM-treated, and MP-treated MCPBs. These results suggest that MP prevents the HDM-induced inhibition of bacterial killing.

**Effect of MP on HDM-induced degradation of the host defense barrier.** All HDM-treated mice showed bacterial growth...
in their peritoneal fluid samples. None of the control mice showed any growth of bacteria in their peritoneal fluid samples. The mean ± SE colony counts are shown in figure 3A. Interestingly, only 2 of 12 MP-treated mice showed bacterial growth in their peritoneal fluid samples (figure 3A).

**Effect of MP on HDM-induced modulated migration.** HDM-treated mice showed decreased \((P < .001)\) migration of macrophages in the peritoneal cavity than control mice (figure 3B). However, MP-treated mice showed greater \((P < .001)\) migration into the peritoneal cavity than did HDM-treated mice (figure 3B).

Macrophages harvested from the HDM-treated mice showed decreased \((P < .001)\) migration across a filter of the modified Boyden chamber than macrophages isolated from control mice (figure 3C). However, macrophages harvested from mice treated with MP showed macrophage migration comparable to that of control macrophages (figure 3C).

To determine whether morphine modulates the chemotraction of macrophages into the peritoneal cavity, we studied the effect of peritoneal fluid samples from HDM-treated and control mice on the migration of murine macrophages across the filter of a Boyden chamber. Peritoneal fluid samples from HDM-treated mice showed greater macrophage migration \((37.5 ± 2.1 \text{ migrated macrophages/field})\) than those from control mice \((18.2 ± 1.2 \text{ migrated macrophages/field}; \ P < .001)\). These findings suggest that a decrease in the peritoneal fluid macrophage count in HDM-treated mice was not because of a lack of chemotraction to the peritoneal cavity. 

![Figure 3](https://academic.oup.com/jid/article-abstract/195/12/1860/811076)
**Effect of MP on HDM-induced peritoneal macrophage apoptosis.** Because morphine-induced degradation of the host defense barrier has been shown to correlate with macrophage injury, we evaluated macrophage survival in HDM- and MP-treated mice. HDM-treated mice showed an increased (P < .001) occurrence of peritoneal macrophage apoptosis, compared with control and MP-treated mice (figure 3D).

**Effect of MP on bone-marrow cell apoptosis.** Because bone-marrow cells act as a reservoir to provide additional macrophages at the site of inflammation, we studied the effect of MP on nucleated bone-marrow cell apoptosis. As shown in figure 4A, HDM-treated mice showed an increased occurrence of bone-marrow cell apoptosis, compared with control and MP-treated mice (P < .001).

**Effect of MP on colony formation by bone-marrow macrophages.** Bone-marrow macrophages isolated from control, HDM-treated, and MP-treated mice were treated with M-CSF for 4 days, and their growth was recorded. As shown in figure 4B, macrophages harvested from HDM-treated mice showed attenuated growth, compared with bone macrophages harvested from control or MP-treated mice.

**Evaluation of the role of P38 MAPK phosphorylation in MP-mediated macrophage survival.** Macrophages were treated under control and experimental conditions in the presence or absence of SB202190 (Calbiochem), a selective inhibitor of P38 MAPK phosphorylation, followed by morphologic evaluation for apoptosis. MP and SB202190 both attenuated the proapoptotic effect of HDM (figure 5A). Interestingly, SB202190 in combination with MP provided complete protection against HDM-induced macrophage apoptosis.

**Evaluation of the effect of MP on HDM-induced P38 MAPK phosphorylation.** Macrophages were treated under control, LDM, HDM, and MP conditions, followed by protein extraction, Western-blot preparation, and probing for P38 MAPK phosphorylation. As shown in figure 5B, HDM enhanced macrophage P38 MAPK phosphorylation. However, this effect of HDM was inhibited by MP.

**Evaluation of the effect of NOS inhibitors on MP-modulated macrophage apoptosis.** Macrophage NO production has been demonstrated to play a role in morphine-induced macrophage apoptosis [17]. We studied the role played by NO in MP-mediated macrophage survival. Macrophages were treated under control, LDM, HDM, and MP conditions with or without Nω-monomethyl-L-arginine (L-NMMA)/Nω-nitro-L-arginine methyl ester (L-NAME; both from Sigma) followed by assay for apoptosis. Both L-NMMA (figure 6A) and L-NAME (figure 6B) provided partial protection against HDM-induced macrophage apoptosis. However, MP in combination with L-NMMA/L-NAME completely inhibited the proapoptotic effect of HDM.

**DISCUSSION**

The present study demonstrates that HDM not only promotes macrophage apoptosis but also compromises macrophage capabilities of the containment and killing of phagocytosed bacteria. MP inhibited all of these effects of HDM. MP also maintained the macrophage capability of killing bacteria. All HDM-treated mice and 2 of 12 MP-treated mice showed a breach in the host defense barrier, whereas none of the control mice showed any degradation of the host defense barrier. HDM-treated mice showed decreased macrophage migration.
into the peritoneal cavity, as well as an increased occurrence of macrophage apoptosis, compared with control and MP mice. In addition, peritoneal macrophages/bone-marrow cells harvested from HDM-treated mice showed decreased migration across a filter, compared with respective cells harvested from control and MP-treated mice. These findings suggest that MP may be contributing to the maintenance of host defense barrier by promoting macrophage survival and preserving their function.

The mononuclear phagocyte system is an important component of the host’s defense. Monocytes migrate to the site of inflammation to phagocytose the invading bacteria. However, morphine at higher doses compromises the phagocytic capability of macrophages [9, 10]. In addition, macrophages may not be reaching in optimal numbers to phagocytose bacteria at the site of invasion. Migration of macrophages is a complex issue and involves multiple factors. First, morphine has been shown to decrease chemotaxis [11]. Second, morphine at higher doses may be reducing the adhesion of monocytes to endothelial cells by suppressing monocyte TNF-α production [23]. Third, the proapoptotic effect of morphine may also be com-

Figure 5. A, Role of P38 MAP kinase (MAPK) phosphorylation in macrophage apoptosis modulated by morphine priming (MP). Equal nos. of macrophages were incubated in medium that contained either buffer, low-dose morphine (LDM; 10−10 mol/L), or SB202190 (SB20; 5 μmol/L) for 4 h, followed by reincubation in medium that contained buffer, LDM, or high-dose morphine (HDM) for 12 h. At the end of the incubation period, cells were assayed for apoptosis. Results (means ± SEs) are from 4 series of experiments. *P < .001, compared with control, LDM, and SB20+MP. **P < .001, compared with HDM. ***P < .01, compared with HDM. ****P < .05, compared with MP. B, Effect of MP on HDM-induced P38 MAPK phosphorylation. Equal nos. of macrophages were incubated in medium that contained either buffer or LDM for 0.5 h, followed by reincubation in medium that contained buffer, LDM, or HDM for 0.5 h. At the end of incubation, cells were washed, protein was extracted, Western blots were prepared, and cells were probed for total and phosphorylated P38 MAPK. Top, macrophage expression of phosphorylated and total P38 MAPK under control and experimental conditions. Bottom, cumulative data from 3 sets of experiments. *P < .05, compared with control, LDM-treated, and MP-treated mice.
promising the functional status of macrophages as well as their precursors (nucleated bone-marrow cells). These factors(s), either alone or in combination, may have contributed to decrease macrophage migration into the peritoneal cavity in HDM-treated mice. However, we also considered a possibility of a lack of chemoattractants in the peritoneal fluid of HDM-treated mice. Contrary to our presumption, peritoneal fluid of HDM-treated mice showed greater chemoattractant properties. These observations are consistent with the findings of Hillburger et al. [24].

To maintain host defense, macrophages also serve an important function—killing of phagocytosed bacteria. Peterson et al. [25] demonstrated that morphine decreases macrophage respiratory burst activity and that it may compromise the capability of macrophage to kill phagocytosed bacteria. In the present study, we confirmed that HDM not only compromises macrophage killing of bacteria but also facilitates their leak. However, MP prevented these deleterious effects of HDM. We presume that MP may also be keeping the macrophage respiratory burst system intact. However, we have not tested this aspect of MP. It may be important to evaluate this effect of morphine on macrophage biology in the future studies.

We and other investigators have previously demonstrated that HDM-treated mice developed a leak of bacteria in their peritoneal cavity [14, 17]. Macrophages normally reside in the peritoneal cavity; thus, they are likely to phagocytose many of the leaked bacteria. Therefore, one may suggest that the lack of macrophage survival may have compromised the host defense barrier. Reynolds et al. [26] suggested that failure of macrophage activation plays a role in the degradation of the host defense barrier in an experimental animal model of obstructive jaundice. Conversely, preservation of macrophage function...
through decreased NO and increased TNF-α production is likely to maintain the host defense intact.

Morphine has been reported to modulate immune function by multiple ways, including modulation of phagocytosis, chemotaxis, and antibody and cytokine production by macrophages [9–13, 25, 27]. In addition, morphine may modulate macrophage function by altering the function of T cells [28, 29]. These effects of morphine in isolation, as well as in combination, may compromise the role of macrophages in the host defense system. Because injured macrophages are functionally compromised, many of the reported effects of morphine may also be indirectly contributed to by morphine-induced macrophage apoptosis.

Roy et al. [30] previously demonstrated a bimodal effect of morphine on macrophage NF-κB activation. Interestingly, morphine in nanomolar concentrations promoted TNF-α production, which was inhibitable by opiate receptor antagonists such as naloxone [30], whereas HDM (micromolar)–induced suppression of NF-κB was not inhibited by opiate receptor antagonists. Therefore, these investigators suggested that only the effect of LDM is mediated through opiate receptors. Recently, Roy and Barke [31], in a μ-opioid receptor knockout mouse model, showed that only some morphine-induced macrophage functions—TNF-α production and reduction of phagocytic capability—were deranged, whereas morphine-induced reduction of splenic and thymic cell numbers and mitogen-induced proliferation were not deranged. These results further confirm that morphine mediates its effect through both classical μ-opioid receptors and nonopioid-binding sites. Because the effect of LDM and HDM seems to proceed by different pathways, it seems logical that the effect of LDM may be sustainable despite an HDM-induced milieu.

Morphine has been reported to promote macrophage apoptosis through P38 MAPK phosphorylation [17]. In the present study, SB202190, a selective inhibitor of P38 MAPK phosphorylation, attenuated HDM-mediated macrophage apoptosis. As expected, HDM enhanced macrophage P38 MAPK phosphorylation, whereas MP attenuated this effect of HDM. Because LDM alone did not modulate macrophage P38 MAPK phosphorylation, it appears that there may be cross-talking between downstream signaling mediated by LDM and HDM. It may be interesting to explore this aspect in the future studies.

Morphine has also been reported to enhance macrophage iNOS expression as well as NO production in a dose-dependent manner [17]. Moreover, NOS inhibitors have been reported to inhibit HDM-induced macrophage apoptosis [12, 13]. In the present study, both L-NAME and L-NMMA also partially attenuated HDM-induced macrophage apoptosis. On the other hand, both L-NAME and L-NMMA in combination with MP completely inhibited the proapoptotic effect of HDM. It appears that MP and NOS inhibitors may be inhibiting the pro-apoptotic effect of HDM by acting at common and at different sites.

References

27. Stangl V, Gunther C, Jarrin A, et al. Homocysteine inhibits TNF-alpha-


