Role of Heme Oxygenase–1 in Morphine-Modulated Apoptosis and Migration of Macrophages

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We examined the role of heme oxygenase (HO)–1 in morphine-induced decrease in macrophage migration. Morphine promoted expression of HO-1 in murine macrophages. Morphine-receiving mice (MRCs) showed decreased (P < .001) macrophage migration and increased (P < .001) occurrence of macrophage apoptosis. In in vitro studies, peritoneal macrophages harvested from MRCs also showed decreased (P < .001) migration, compared with those from control mice. Bone marrow cells isolated from MRCs showed not only decreased (P < .001) migration but also increased apoptosis. Pretreatment of MRCs with hemin not only decreased migration of macrophages further but also enhanced the apoptosis of peritoneal macrophages. On the other hand, pretreatment of MRCs with zinc protoporphyrin attenuated the effect of morphine on both macrophage migration and the occurrence of apoptosis. In in vitro studies, pretreatment of macrophages with hemin exacerbated morphine-induced apoptosis, whereas pretreatment with zinc protoporphyrin attenuated morphine-induced macrophage apoptosis.

Opiate addicts are prone to infections, and at times these infections may be fatal [1, 2]. The increased incidence of infection was once attributed to the use of nonsterile and contaminated needles by drug addicts. Other investigators have emphasized the role of impurities in street heroin [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 in multiple ways. In in vitro and in vivo studies, morphine has been demonstrated to suppress the phagocytic capabilities of macrophages [9, 10] and chemotaxis of human monocytes [11]. Because morphine has been demonstrated to induce many of its biological effects through oxidative stress, it may be important to develop strategies to modulate morphine-induced oxidative stress.

Microsomal heme oxygenase (HO) is the rate-limiting enzyme for heme degradation in mammals [12]. HO cleaves heme into biliverdin and releases free iron and carbon monoxide. Two isoforms have been identified that show differences in regulation and localization [13–15]. HO-1 is an inducible form previously known as “heat-shock protein 32.” It is an integral part of the antioxidant response element of cells. It is upregulated by a variety of factors, including hypoxia, hyperoxia, heat shock, cytokines, heavy metals, hydrogen peroxide, UV irradiation, and its substrate, heme [16–22]. Because heme synthesis and degradation are essential for cytochromes and other heme-containing enzymes, such as catalase and nitric oxide synthase,
HO-1 is expressed in many cells. Moreover, heme is pro-oxidant and induces cytotoxicity [23]. Thus, expression of HO-1 after oxidative stress may be a protective response. Furthermore, the products of heme cleavage (bilirubin and biliverdin) may act as antioxidants [24, 25]. Because 11–12 mol of NADPH are consumed in an HO reaction to reduce heme iron to activate molecular oxygen, it appears that HO-1 may play a protective role under conditions of oxidative stress [26].

It is increasingly recognized that HO-1 down-regulates the inflammatory response [27–29]. In a rat model of pleurisy, up-regulation of HO-1 significantly attenuated acute cellular inflammation [30]. Similarly, up-regulation of HO-1 led to the amelioration of chronic vascular inflammation associated with vascular rejection in antibody-induced transplant arteriosclerosis [31]. On the other hand, in a model of cardiac xenotransplant rejection, the deficiency of HO-1 exacerbated the inflammatory response [32]. Similarly, in a rat model of tubulointerstitial inflammation, deficiency of HO-1 enhanced the chronic inflammation [33]. Because monocyte migration plays an important role in the development of the inflammatory response, it appears that HO-1 may be modulating the migration of monocytes. Some investigators have reported that morphine inhibits the migration of monocytes/macrophages [11]. This effect of morphine has been reported to be mediated by the inhibition of chemotaxis [11]. We suggest that morphine-induced macrophage apoptosis might also be contributing to morphine-induced inhibition of migration. In the present study, we have evaluated the effect of HO-1 expression on the morphine-induced modulation of macrophage apoptosis and migration.

**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) and fresh water were available ad libitum. The pathogen-free status of each mouse was determined by culture of peritoneal fluid and blood samples for any bacterial growth before the start of the experimental protocols.

**Experimental protocols.** Eight FVB/N mice (weighing 25 g each) were used in each set of experiments. Three sets of experiments were carried out. Each set involved 2 groups of mice: 4 mice received normal saline, and the other 4 mice received normal saline containing 20 mg of morphine (donated by the National Institute on Drug Abuse) daily for 10 days; doses were administered subcutaneously under sterile conditions. To stimulate macrophage migration into the peritoneal cavity, on the eighth day, 3 mL of thioglycollate was administered intraperitoneally. Forty-eight hours later, macrophages were harvested from the peritoneal cavity, as described elsewhere [34–36], and counted in a hemocytometer. Morphological (staining with Hoechst 33342 [H-33342; Molecular Probes] and propidium iodide [PI; Sigma Chemical]) and DNA fragmentation assays [34–37] were carried out to evaluate apoptosis. In addition, bone marrow cells were harvested as described elsewhere [35]. Morphological assay of bone marrow cells was carried out by staining with H-33342 and PI.

To determine the role of HO-1 in the migration of macrophages into the peritoneal cavity, 24 mice in groups of 4 received either normal saline, normal saline containing morphine (20 mg/kg), normal saline containing hemin (50 μM/kg; Sigma), normal saline containing zinc protoporphyrin (ZnP, 50 μM/kg; Sigma), normal saline containing morphine and hemin, or normal saline containing morphine and ZnP daily for 10 days. However, mice that received a combined therapy (hemin and morphine or ZnP and morphine) received either hemin or ZnP 1 day prior to the administration of morphine. On the eighth day, 3 mL of thioglycollate was administered intraperitoneally. Forty-eight hours later, peritoneal macrophages were harvested, counted, and assayed for the occurrence of apoptosis, and the results were recorded.

**Apoptosis studies.** Morphological evaluation of macrophage apoptosis was performed by staining cells with H-33342 and PI, as described elsewhere [34–36]. Cells were prepared under control and experimental conditions. At the end of the incubation period, cells were stained with H-33342 and PI, and the percentage of live, apoptotic, and necrosed cells was recorded in 8 random fields by 2 observers unaware of the experimental conditions.

To determine the effect of HO-1 modulation on morphine-induced macrophage apoptosis, equal numbers of murine macrophages (J774; American Type Culture Collection) were incubated in medium containing either buffer (control), morphine (10⁻⁸ M), hemin (5 μM), ZnP (50 μM), morphine and hemin, or ZnP and morphine (when morphine was used in combination, cells were primed for 16 h with either hemin or ZnP prior to morphine treatment) for 16 h, followed by the apoptosis assay. Four series of experiments were performed.

**DNA fragmentation assay: gel electrophoresis.** Gel electrophoresis is a simple method that is specific for isolation and confirmation of DNA fragments from apoptotic cells [37]. Because this method only picks up DNA fragments, loading of samples that do not contain any DNA fragments is not seen. DNA was isolated from equal numbers of macrophages harvested under control and experimental conditions. DNA was dissolved in gel loading buffer and separated by electrophoresis in 1.6% agarose gels.

**Protein extraction and Western blotting for expression of HO-1.** To determine the effect of morphine on macrophage HO-1 expression, equal numbers of macrophages (J774) were incubated in medium containing either vehicle or variable concentrations of morphine (10⁻¹⁴–10⁻⁶ M) for 16 h. At the end
of the incubation period, cells were washed, and protein extraction and blotting were performed as described elsewhere [34–36]. The blots were treated with 0.5% bovine serum albumin and 0.1% Tween 20 in 1× PBS for 60 min at room temperature and then incubated with the primary monoclonal antibody to HO-1 (Stressgen) for 2 h at 25°C. A horseradish peroxidase–conjugated secondary antibody was applied for 1 h at room temperature. The blots were then developed using a chemiluminescence detection kit (Amersham) and exposed to Kodak X-OMAT AR film. The blots were stripped and reprobed for β-actin. Quantitative densitometry was performed on the identified band using a computer-based measurement system. Ratios of HO-1 to actin were calculated.

Measurement of HO enzyme activity. HO activity was measured by the bilirubin generation method [24, 25]. In brief, equal numbers of macrophages were incubated in medium that contained either vehicle or variable concentrations of morphine (10⁻¹⁰–10⁻⁶ M) for 16 h. At the end of the incubation period, cells were washed, scraped, and centrifuged (1000 g for 10 min at 4°C). The cell pellet was suspended in MgCl₂ (2 mM) phosphate (100 mM) buffer (pH 7.4) and sonicated on ice prior to centrifugation at 18,800 g for 10 min at 4°C. The supernatant was added to a reaction mixture (400 μL) containing rat liver cytosol (2 mg), hemin (20 μM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 U), and NADPH (0.8 mM) for 1 h at 37°C in the dark. The formed bilirubin was extracted with chloroform, and the change in optical density at 464–530 nm was measured. HO activity is expressed as picomoles of bilirubin formed per microgram of cell protein per hour.

In vitro evaluation of macrophage/bone marrow cell migration. In our studies, equal numbers of peritoneal macrophages (1 × 10⁶ 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 containing equal numbers of macrophages were incubated in the upper compartment of the Boyden chamber. The lower compartment contained 200 μL of peritoneal fluid samples of either control or morphine-receiving mice. The chambers were kept in an incubator for 2 h, followed by assay of the migrated macrophages. Peritoneal fluid samples from 6 control and 6 morphine-receiving mice were examined.

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 P values. All values are mean ± SE, except where otherwise indicated. P < .05 was considered to be statistically significant.

RESULTS

Migration studies. Morphine-receiving mice showed a decrease (P < .001) in the migration of macrophages into the peritoneal cavity, compared with control mice (figure 1A). Peritoneal macrophages harvested from morphine-receiving mice showed decreased (P < .001) migration across the filter of a modified Boyden chamber, compared with macrophages isolated from control mice (figure 1D). Similarly, bone-marrow cells isolated from morphine-receiving mice showed attenuated (P < .001) migration across the filter, compared with bone-marrow cells harvested from control mice (figure 1C).

Apoptosis studies. Morphine-receiving mice showed an increased (P < .001) occurrence of apoptosis of peritoneal macrophages, compared with control mice (figure 2A). This effect of morphine was further confirmed by DNA fragmentation assay (figure 3A). Macrophages harvested from morphine-treated mice showed increased integer multiples of 180 bp in a ladder pattern (figure 3A). Morphine-receiving mice also showed an increased (P < .001) occurrence of bone-marrow cell apoptosis, compared with control mice (figure 2C).

Studies pertaining to HO-1. As shown in figure 4A, morphine modulated macrophage HO-1 expression in a bimodal way. Similarly, morphine modulated HO activity in a bimodal way (figure 4B). At concentrations of 10⁻¹²–10⁻¹⁰ M, morphine enhanced macrophage HO-1 expression as well as HO activity. On the other hand, at higher concentrations, morphine inhibited macrophage HO-1 expression as well as HO-activity.

Hemin-receiving mice showed decreased (P < .001) migration of macrophages into the peritoneal cavity (figure 1B). Pretreatment of morphine-receiving mice with hemin further de...
compared with morphine (figure 1). Pretreatment of morphine-receiving mice with ZnP attenuated (P < .001) the effect of morphine on peritoneal macrophages (figure 2B). DNA gel electrophoresis was used to confirm the effect of hemin and ZnP treatment on peritoneal macrophage apoptosis under control and morphine-treated states (figure 3B). Macrophages harvested from morphine-receiving mice showed increased integer multiples of 180 bp. Pretreatment of morphine-receiving mice with hemin enhanced the proapoptotic effect of morphine (figure 3B), whereas pretreatment of morphine-receiving mice with ZnP inhibited morphine-induced macrophage DNA fragmentation (figure 3B).

In in vitro studies, HO-1 induction enhanced (P < .001) morphine-induced macrophage apoptosis (figure 2D). On the other hand, HO-1 inhibition (pretreatment with ZnP) attenuated morphine-induced macrophage apoptosis (figure 2D).

**FIGURE 1.** A, Effect of morphine on macrophage migration in the peritoneal cavity. Peritoneal macrophages were harvested from control and morphine-treated mice (for 10 days; as described in Materials and Methods) and counted. B, Effect of heme oxygenase (HO)-1 modulation on macrophage migration in the peritoneal cavity. Mice received normal saline, hemin, zinc protoporphyrin (ZnP), morphine + hemin, or morphine + ZnP (as described in Materials and Methods) daily for 10 days. Peritoneal macrophages were harvested and counted. *P < .001, compared with control, ZnP, and morphine + hemin; **P < .01, compared with morphine + ZnP; ***P < .001, compared with control, ZnP, and morphine + hemin; ****P < .001, compared with morphine, hemin, and morphine + ZnP. C, Effect of morphine on the migration of bone-marrow cells across a filter. Mice received either normal saline or morphine daily for 10 days. On the tenth day, bone-marrow cells from control and morphine-treated mice were harvested, and migration studies were carried out in a modified Boyden chamber (as described in Materials and Methods). D, Effect of morphine and HO-1 induction on macrophage migration across a filter. Migration studies on macrophages harvested from control, morphine-receiving, or hemin-receiving mice were carried out (as described in Materials and Methods). *P < .001, compared with control. For each study, 3 sets of experiments were performed.

**DISCUSSION**

The present study demonstrates that morphine promotes the expression of HO-1 in macrophages. Morphine-receiving mice showed decreased macrophage migration into the peritoneal cavity, as well as increased occurrence of macrophage apoptosis. In addition, peritoneal macrophages, as well as bone marrow cells harvested from morphine-receiving mice, showed decreased migration across a filter, compared with control cells. Hemin-receiving mice also showed decreased migration of macrophages into the peritoneal cavity. Pretreatment of morphine-receiving mice with hemin not only contributed to a decrease in peritoneal accumulation of macrophages but also enhanced macrophage apoptosis. Conversely, pretreatment of morphine-receiving mice with ZnP, an inhibitor of HO-1 activity, attenuated the effect of morphine on both macrophage migration and the occurrence of apoptosis. Of interest, peritoneal fluid samples from morphine-receiving mice showed greater chemoattraction, which suggests that a decrease in the migration of macrophages in morphine-receiving mice may not be related to an altered chemoattraction. In in vitro studies, macrophage modulation of HO-1 also altered the effect of morphine on macrophage apoptosis.

The mononuclear phagocyte system is an important component of the host defense. Monocytes migrate to the site of inflammation to phagocytose and kill invading bacteria. The chemokine-induced macrophage migration is dependent on the concentration gradient between the milieu around monocytes and the site of inflammation. Perez-Castrillon et al. [11] demonstrated that morphine decreases the migration of macrophages by diminishing chemotaxis. In addition, we have demonstrated that morphine decreases the spreading and adhesion of mesangial cells, which behave like macrophages [38]. Because the adhesion of monocytes initiates diapedesis of monocytes through the endothelial fenestrae, morphine-induced modu-
Morphine-Induced Macrophage Migration

Figure 2. A, Effect of morphine on apoptosis of peritoneal macrophages. Peritoneal macrophages were harvested from control and morphine-treated mice (daily for 10 days) and evaluated for apoptosis (as described in Materials and Methods). Three sets of experiments were carried out. B, Effect of heme oxygenase (HO)-1 modulation on morphine-induced peritoneal macrophage apoptosis. Mice received normal saline, morphine, hemin, zinc protoporphyrin (ZnP), morphine + hemin, or morphine + ZnP daily for 10 days (as described in Materials and Methods). On the tenth day, peritoneal macrophages were harvested, counted, and assayed for apoptosis. *P < .001, compared with control and morphine + ZnP; **P < .001, compared with morphine. C, Effect of morphine on bone marrow cell apoptosis. Bone marrow cells were harvested from control and morphine-treated mice (daily for 10 days) and evaluated for apoptosis as described in Materials and Methods. Three series of experiments were carried out. D, Effect of HO-1 modulation on morphine-induced macrophage apoptosis. Equal nos. of murine macrophages (J774) were incubated in media containing either buffer (control), morphine (10^{-8} M), hemin (5 \mu M), ZnP (50 \mu M), morphine + hemin, or morphine + ZnP (cells were primed with hemin or ZnP for 16 h prior to the addition of morphine) for 16 h followed by apoptosis assay. Four sets of experiments were carried out. *P < .001, compared with control and ZnP + morphine. **P < .01, compared with morphine.

Ligation of adhesion is likely to affect macrophage migration. Moreover, morphine at higher concentrations also decreases macrophage production of tumor necrosis factor–α [39], a cytokine that enhances the adhesion of monocytes to endothelial cells. In addition, morphine enhances apoptosis of both monocytes and precursors of monocytes (bone marrow cells). Because apoptosed macrophages are functionally compromised, morphine-induced monocyte/bone marrow cell apoptosis also may have contributed to the attenuated migration of macrophages into the peritoneal cavity. Thus, morphine seems to affect the migration of macrophages in multiple ways.

Peterson et al. [40] demonstrated that morphine decreases respiratory burst in mononuclear phagocytes. Because free radicals released during a respiratory burst (associated with phagocytosis of bacteria) are responsible for the bacterial killing properties of macrophages, morphine is likely to compromise the bacterial killing properties of macrophages. Other investigators as well as ourselves have reported that morphine compromises these properties [35, 41]. In the present study, morphine promoted the expression of HO-1 by macrophages. Because induction of HO-1 is associated with the generation of antioxidants, morphine is likely to neutralize the effect of free radicals such as superoxide (generated as a part of the respiratory burst phase), which is required for bacterial killing. The present study further provides a mechanistic insight into the hypothesis proposed by other investigators.

MCP-1, transforming growth factor (TGF)-β, and metalloproteinase-9 have been reported to be important chemoattractants regulating monocyte and T cell chemotaxis [42–44]. Free radicals such as hydrogen peroxide and nitric oxide have been reported to act as second messengers for transcription of MCP-1 and TGF-β [45, 46]. Thus, it is possible that morphine-induced modulation of oxidative stress may also modulate the generation of these chemoattractants. Therefore, in the present study, we evaluated the chemoattractant potential of the peritoneal fluid of control and morphine-receiving mice. To our surprise, the peritoneal fluid of morphine-receiving mice showed greater chemoattractant properties compared with that
Figure 3. A, A representative gel showing the effects of morphine on peritoneal macrophage DNA fragmentation. Lanes 1 and 6, molecular markers; lane 2, macrophages harvested from control mice; and lanes 3–5, macrophages harvested from morphine-receiving mice show classic ladder pattern. B, A representative gel showing the effects of zinc protoporphyrin (ZnP) and hemin on macrophage DNA fragmentation in morphine-receiving mice. Lanes 1 and 8, molecular markers; lane 2, control; lane 3, hemin alone; lane 4, morphine alone; lane 5, pretreatment with hemin followed by morphine; lane 6, ZnP alone; and lane 7, pretreatment with ZnP followed by morphine. Hemin enhanced the effect of morphine, whereas ZnP inhibited morphine-induced macrophage DNA fragmentation.

Figure 4. A, Dose-response effect of morphine on macrophage heme oxygenase (HO)–1 expression. Equal nos. of murine macrophages were treated with either buffer (control) or morphine (10⁻¹⁴–10⁻⁶ M) for 16 h. Subsequently, Western blots were prepared and probed for HO-1 and actin. The upper panel shows the effect of morphine on macrophage HO-1 expression under control and morphine-stimulated (10⁻¹⁴–10⁻⁶ M) states. The middle panel shows the β-actin content of macrophages under similar conditions. The lower panel shows a bar diagram indicating ratios of HO-1 to actin under the above-mentioned conditions. B, Dose-response effect of morphine on macrophage HO activity. Equal nos. of macrophages were treated with either buffer (control) or morphine (10⁻¹⁴–10⁻⁶ M) for 16 h. Subsequently, HO activity was measured. Results (mean ± SE) are from 3 series of experiments. *P< .001, compared with control and morphine (10⁻⁶ M); **P< .001, compared with control and morphine (10⁻¹⁴, 10⁻¹²–10⁻⁶ M); ***P< .001, compared with control and morphine (10⁻⁶ M); ****P< .01, compared with control.

Hilburger et al. [47] also demonstrated that morphine-receiving mice had greater migration of splenic macrophages in the peritoneal cavity. Thus, it was not the lack of chemoattraction that was contributing to the decrease in the migration of macrophages into the peritoneal cavity of morphine-receiving mice.

Other investigators, as well as ourselves, have demonstrated that morphine-receiving mice developed a leak of bacteria in their peritoneal cavities [35, 48]. Because macrophages normally reside in the peritoneal cavity, they are likely to phagocytose many of the leaked bacteria. However, the phagocytosis of bacteria by macrophages has been shown to be associated with increased macrophage HO-1 expression [30]. It may well be a compensatory response to combat oxidative stress associated with a respiratory burst (a phenomenon associated with phagocytosis of bacteria by macrophages). Because morphine also induces the macrophage expression of HO-1, an additive effect of morphine-induced HO-1 expression may have further compromised the migration of macrophages. In the present study, both morphine and hemin also showed an additive effect in decreasing the migration of macrophages into the peritoneal cavity.

Willis et al. [30] demonstrated the effect of HO-1 activity on the migration of mononuclear cells (MNCs) in the pleural cavity of rats. These investigators showed that the induction of HO-1 by ferriprotoporphyrin was associated with a decrease in the accumulation of MNCs in a dose-dependent manner. On the other hand, inhibition of HO-1 activity by tin protoporphyrin was associated with an increase in the accumulation of MNCs in a dose-dependent manner. Those investigators suggested that the modulation of HO-1 induction directly affects the migration of MNCs. The present study further supports this notion.

We conclude that morphine promotes HO-1 expression by macrophages. Increased HO-1 activity is associated with decreased macrophage migration and increased macrophage apoptosis, whereas decreased HO-1 activity is associated with increased macrophage migration and decreased macrophage apoptosis.
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