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Lipopolysaccharide Induced Septicemia in Chronic Morphine Treated Rats

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*Lipopolysaccharide Induced Septicemia in Chronic Morphine
Treated Rats*

by

Submitted in partial fulfillment of the requirements for the degree of Master of
Science in Biology from the Department of Biology of Seton Hall University

Frank Ocasio

August, 2003

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Approval Page

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*Indicates figures provided by Dr. Sulie L. Chang;

Abstract

Opiate-addicts have been known to show increased susceptibility to bacterial infection. Lipopolysaccharide (LPS), which is a cell wall component in gram-negative bacterial, is a potent stimulator of inflammation. We investigated how treatment with morphine alters LPS-induced inflammatory responses in the rat. Chronic morphine exposure alone elevated serum endotoxin levels. Animals treated with morphine and LPS (250 µg/kg) developed hypothermia, decreased mean arterial pressure (MAP), increased plasma thrombin anti-thrombin III (TAT) complex, and approximately 67% exhibited progressive intramicrovascular coagulation. Morphine also enhanced LPS-induced leukocyte endothelial adhesion (LEA), suppressed leukocyte flux and corticosterone production, and elevated interleukin-1 β , tumor necrotic factor- α , and interleukin-6 serum levels. This study presents both the molecular and cellular mechanisms underlying the potentiated LPS-induced inflammation and accelerated progression to septic shock seen with chronic morphine exposure.

Introduction

Morphine is an opiate commonly used for analgesic purposes. Physicians frequently prescribe morphine and other opiate analogs for post-operative and long-term pain management. Morphine's addictive properties make it subject to abuse as its use can bring about a profound sense of euphoria. Morphine tolerance, in which a higher dose of drug is required for the same effect, begins with the initial treatment and becomes apparent after 2 – 3 weeks with regular therapeutic doses (Katzung, 1992). With the onset of tolerance comes a certain drug dependence whereby frequent drug administration is required in order to avoid the negative consequences of withdrawal such as chills, sweats, nausea, and vomiting. It has been known for decades that this chronic opiate-tolerant state carries with it a series of negative immunologic consequences. Studies have shown that morphine suppresses lymphocyte trafficking (Flores et al., 1995), the lymphocyte proliferative response to mitogens (Bryant et al., 1991; Fecho et al., 1995), natural killer T-cell activity (Gomez-Flores and Weber, 1999; Yokota et al., 2000), the production of antibodies (Bussierre et al., 1993), the total number of circulating leukocytes (LeVier et al., 1994; Fecho and Lysle, 2002), and atrophy of the spleen and thymus (Patel et al., 1996).

Morphine-induced immunosuppression increases patient susceptibility to bacterial infection (Hilberger et al., 1997). Bacterial infections usually remain local but can become systemic if the pathogens gain access to the circulatory system. This condition is called bacteremia (Wheeler et al., 1999). Lipopolysaccharide (LPS) is a component of gram-negative bacteria that is shed from the outer cell wall and is a potent endotoxin (Lukasiewicz and Lugowski, 2003). While LPS has no direct toxicity, it leads to the

activation of the complement system and the coagulation cascade, and induces immune cells, such as macrophages and neutrophils, to release proteins termed endogenous mediators of sepsis (Parrillo, 1990). The term, sepsis, refers to bacteremia that produces a systemic immunological response. Septic patients suffer from either high or low body temperature, and an elevated heart rate, respiratory rate, and white blood cell count. At the molecular level, tumor necrosis factor-alpha (TNF- α), which is released from macrophages, is the primary mediator of sepsis (Wheeler et al., 1999). TNF- α activates the inflammatory response by causing vasodilation through the release of bradykinin and nitric oxide. TNF- α also increases vascular permeability, enhances leukocyte-endothelial interaction, and activates platelets (Parillo, 1993). In addition, TNF- α stimulates macrophages and endothelial cells to release interleukin-1 (IL-1), an endogenous pyrogen which acts on the hypothalamus causing the fever usually associated with bacterial infections. IL-1, in turn, triggers the release of other pro-inflammatory cytokines, such as interleukin-6 (IL-6), and prostaglandins (Ato et al., 2002; Li et al, 2002; Itoh et al., 2003). Initially this cascade of events is protective, acting to contain and eliminate the infection. However, as the bacteria continue to proliferate and exude increasing amounts of endotoxin, sepsis can progress to septic shock (Wang et al., 2003; Asakura et al., 2003).

Septic shock is a deadly response to bacterial infection. The mortality rate is high, and it is one of the most common causes of death in the intensive care unit (Wheeler et al., 1999). As many as 40% of patients in septic shock will die even with extensive treatment and antibiotic therapy. Septic shock causes a dramatic decrease in blood pressure and the onset of disseminated intravascular coagulation (DIC). The hypotension is caused by an excessive increase in vascular permeability, vasodilation,

and decreased peripheral resistance all primarily mediated through the actions cytokines such as IL-1 and TNF- α . (Wheeler et al., 1999). DIC ensues due to activation of the coagulation cascade via the Hageman factor (factor XII), leading to thrombosis, tissue ischemia, and eventual multi-organ failure (Parillo 1993; Yamaguchi et al., 2000). Another complication of DIC is excessive bleeding and hemorrhage through the depletion of platelets and anti-coagulant factors, such as anti-thrombin III. Anti-thrombin III binds to circulating thrombin forming a complex of thrombin and anti-thrombin III (TAT) thus preventing thrombin from further potentiating the coagulation cascade. In the pathogenesis of septic shock, while the concentration of endotoxin or LPS is important, it is the inflammatory response to the endotoxin that is the key factor in determining the progression of sepsis.

Inflammation is a critical component of innate immunity, being one of the initial responses to invading pathogens. Innate immunity refers to the non-specific host defenses which include barriers such as the skin and mucous membranes, immune cells such as natural killer cells and macrophages, blood proteins like those involved in the complement cascade and interferons, and inflammation. The innate immune response is capable of killing a broad range of invading microbes and serves to ready the adaptive immune response through the production and activation of cytokines such as IL-1, IL-6, IL-8, and TNF- α . Inducing functional leukocyte-endothelial interaction (LEI) is essential for the generation of an effective inflammatory response (Eppihimer and Granger, 1997). Under normal conditions, LEI is minimal with the majority of erythrocytes and leukocytes being restricted to the lumen of the blood vessel (House and Lipowsky, 1987; Nazziola and House, 1992; Rosenkranz and Mayada, 1999). As a result of vascular

injury or infectious challenge with endotoxin, vasoactive peptides, chemoattractant factors, or pro-inflammatory cytokines the incidence of LEI increases. The process of LEI includes: (1) leukocyte marginalization where leukocytes leave the main flow (2) leukocyte rolling on the vascular endothelium through the expression of selectins on leukocytes and endothelial cells; (3) leukocyte adhesion via the expression of integrin, ICAM, and VCAM; and (4) leukocyte extravasation, which refers to the PECAM-1-mediated migration of leukocytes out of the vascular lumen and along a chemotactic gradient leading toward the infecting pathogen (Muller et al., 1993; Salas et al., 2002; Smith et al., 2002). A functional LEI response to infection would consist of an increase in leukocyte-endothelial adhesion (LEA) accompanied by a decrease in leukocyte flux (FLUX, representing the number of leukocytes traveling past a particular point in the venule per unit of time). This model has been used as an indicator of an intact and functional immune response. However, few studies have successfully examined the LEI response during the development of DIC due to the exacerbated LEA, decreased leukocyte flow, and systemic coagulation.

While the inflammatory response is necessary for the containment and clearance of infections and the healing of injuries, regulatory mechanisms must be in place to ensure that the inflammatory response is not excessive. A major pathway through which the central nervous system regulates the immune system is the hypothalamic-pituitary-adrenal (HPA) axis (Bateman et al., 1989). The HPA axis controls the hormonal stress response, the mechanism by which the CNS exerts its modulatory actions on the immune response (Sternberg et al., 1992). The pathway originates within an area of the hypothalamus called the paraventricular nucleus (PVN). The PVN secretes corticotropin-

releasing hormone (CRH) into the hypophyseal blood supply where it stimulates the anterior pituitary to release adrenocorticotrophic hormone (ACTH) [Katsuura et al., 1988; McCoy et al., 1994; Lacosta et al., 1998]. Then ACTH enters the blood stream and induces the adrenal glands to synthesize and release glucocorticoids, i.e., cortisol in the human and corticosterone in the rat (Hansen et al., 2002). Glucocorticoids such as corticosterone exert powerful anti-inflammatory effects. Previous studies have shown that chronic morphine treatment desensitizes the HPA axis, leading to desensitization of IL-1 β -induced activation of FOS immunoreactivity in the PVN (Chang et al., 1996) and potentiation of IL-1 β - induced LEA in the rat mesentery (House et al., 2001). Chronic morphine treatment has also been shown to induce sepsis in mice (Hilberger et al., 1997).

The current study demonstrates that rats chronically treated with morphine and given a subsequent intraperitoneal (i.p.) injection of LPS show increased LEI, suppressed production of corticosterone, and elevated levels of TNF- α , IL-1 β , and IL-6. In addition, LPS-challenged rats treated with chronic morphine developed a decrease in mean arterial blood pressure (MAP), reduced body temperature, and plasma TAT concentrations significantly greater than placebo-treated rats. Chronic treatment with morphine alone resulted in an increase in serum endotoxin levels. Taken together, these data provide insight into the physiologic phenomena associated with the progression of sepsis in morphine-tolerant individuals at both molecular and cellular levels.

Materials and Methods

Treatment of animals. Harlan Sprague-Dawley (Indianapolis, IN) normal adult male rats, adrenalectomized (ADX, adrenalectomized at Harlan Sprague-Dawley) and SHAM (rats that underwent the same surgical procedure but retained their intact adrenal glands), 250-300 g, were housed in a temperature controlled environment with a 12:12 hour light-dark cycle, and fed ad libitum a standard rat diet and water. All animals were acclimated for 5 to 7 days prior to any experimental procedures. ADX rats received 0.9% filtered saline instead of water in order to maintain normal physiological levels of sodium. Animal studies were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) at Seton Hall University.

Chronic morphine treatment followed by LPS. Animals were randomly assigned to receive morphine sulfate or placebo pellets (75 mg morphine sulfate per pellet) [National Institute of Drug Abuse, Rockville, MD] via subcutaneous implantation. Treatment was administered in a 2 + 4 regimen where animals were given 2 pellets on day 1 and 4 pellets on day 2 of either morphine or placebo. This regimen of chronic morphine treatment has been shown to produce a high degree of tolerance and dependence (Zadina et al., 1995). In some of our studies, an additional group of animals that received no treatment with morphine or placebo was included as a control and is referred to as naive animals. On Day 5, each group of rats was randomly assigned to receive either LPS (250 µg/kg i.p.) [from *Salmonella typhimurium*; lot 39H4110; Sigma, St. Louis, MO] or an equal volume of saline. Two hours after the injection, the animals were prepared for

intravital microscopy or sacrificed and truncal blood collected for serum and plasma extraction.

Intravital microscopy. Rats were anesthetized with of sodium pentobarbital (45 mg/kg) [Veterinary Laboratories, Inc., Lenexa, KS] administered via intra-peritoneal injection. After 10 min, a tail pinch test was performed to see if the animal was adequately anesthetized. Ventral fur was removed using electric hair clippers, and the animals were placed on a warm heating pad. A tracheotomy was performed to facilitate respiration. The carotid artery was cannulated for determination of blood pressure. The mesentery was exteriorized through a mid-sagittal abdominal incision and draped over a transparent Plexiglas shelf. Exteriorized tissues were continuously topically suffused with a 1% Ringer's gelatin drip solution (in mM, 3.43 KCl, 22.85 NaHCO₃, 4.19 HEPES, 0.28 CaCl₂, Sigma, St. Louis, MO) maintained at 37° C. The intestine and the areas outside the region of interest were covered with gauze (Johnson & Johnson, New Brunswick, NJ), moistened, and kept warm with drip solution. The rat mesentery was observed with a Nikon UM3 metallurgic microscope adapted for intravital microscopy. A Nikon 40X water immersion lens was used to view the field on a Panasonic TR-930A high-resolution video monitor. Vessel length and width were measured using the video image shearing technique (IPM Model 908) on and off line. Video image shearing refers to when a cross-section of the video image of the venule being examined is displaced or "sheared" either horizontally or vertically so as to have the opposing walls of the venule overlap. The image displacement is quantified in microns. Post-capillary venules ranging from 20 – 40 µm were selected. All microcirculatory events were recorded using a Panasonic

VWCD-52 video camera and a JVC HR-S3500U videocassette recorder. Leukocyte FLUX was determined off-line by videotape playback by counting the number of leukocytes rolling past a line drawn perpendicular to the vessel of interest during a 30-sec interval. Leukocytes sticking to the endothelium for more than 5 sec during the duration of the hemodynamic measurements were counted as adhering WBCs, and expressed as the number of LEA per 100 μm vessel length. Intravital microscopy was used to visualize the microvascular trees of the mesentery. Systemic mean arterial pressure (MAP) was measured using a strain-gauge transducer (Century Technology, CP-01) connected to the rat's carotid artery. MAP was calculated as the sum of 1/3 systolic pressure and 2/3 diastolic pressure.

An obvious retardation of blood flow accompanied with intermittent coagulation was used as a gross indicator of DIC.

Induction of acute inflammation. During intravital microscopy, inflammation in the mesenteric vessels was achieved by administration of 10^{-7} M n-formyl-methionyl-leucyl-phenylalanine (FMLP). FMLP is a chemotactic agent that was suffused for a 2 min interval to induce the immune response and establish that the normal inflammatory response was intact. FMLP was prepared the day of the experiment by dissolving the powder in DMSO in a ratio of 1mg FMLP to 100 microlites of DMSO. At the concentration used, DMSO does not effect leukocyte endothelial interaction (LEI).

Enzyme-linked immunosorbant assay for cytokines (ELISA). ELISA kits for rat TNF- α , IL-1 β , and IL-6 were obtained from R & D Systems (Minneapolis, MN). At the end

of each experimental protocol treatment, truncal blood was collected into 15ml vials. The blood samples were centrifuged at 4° C (1000 x g) for 10 min. Serum was stored in aliquots at -80 ° C until use. ELISA assays were performed according to the kit manufacturer's procedures.

Radioimmunoassay (RIA) for the measurement of serum corticosterone. RIA of corticosterone levels was performed using a commercial kit and protocol from ICN Pharmaceuticals, Inc. (Orangeburg, NY). Briefly, rat serum was diluted, [¹²⁵I]- tracer was added, and anti-corticosterone was then added in that order. The sample was thoroughly vortexed, centrifuged at 1000 x g for 15 min, and the supernatant decanted. The precipitate was counted in a Wallace Wizard I470 Automatic gamma counter.

TAT immunoassay. The Enzygnost TAT microenzyme immunoassay kit (Dade Behring, Inc.) was used to determine plasma levels of human thrombin-antithrombin III complex (TAT) in the naïve, placebo, and morphine treated rats. Ravanat et al. (1996) found that TAT was present in a variety of species including the rat. Plasma samples, standards, and controls were added to a microtiter plate coated with rabbit anti-human thrombin antibodies; the plate was washed, and peroxidase conjugated rabbit anti-human ATIII antibody solution was added. Next, the plate was washed, and the chromogen POD (o-phenylenediamine dihydrochloride) solution was added; the chromogen reaction was stopped with 0.5N sulfuric acid, and the absorbance was measured at 492 nm. Plasma TAT concentrations were obtained from the standard curve via their respective absorbance values.

Endotoxin Assay. Using the Pyrochrome© kit with diazo-coupling (Associates of Cape Cod, Inc.), we assessed serum levels of the endotoxin, LPS, in naïve, placebo, and chronic morphine treated rats. Serum samples were first divided into two categories, filtered (0.2 µm pore size) and unfiltered. Two fold serial dilutions were made for the standards (1, 0.5, 0.25, 0.125, and 0.063 EU/ml). Then, serum samples, standards, and controls were added to a 96-well microplate, and incubated with an equal volume of Pyrochrome© reagent. The incubation was stopped with nitrite in HCl, and then N- (1-Naphthyl)-ethylenediamine (NEDA) was added, forming a magenta diazotized derivative. The absorbance was read at 540 nm. Serum endotoxin concentrations were obtained from the standard curve by comparing the respective absorbance values.

Statistical analysis. Data are presented as the mean \pm SE. Comparisons of data were made by one-way analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc test, or by a paired or unpaired Student's t-test (Mann-Whitney). Significance was assessed at either the 95% ($p < 0.05$) or 99% ($p < 0.01$) confidence level. Significance was defined as a probability of 0.05 or less.

Results

The effect of chronic morphine exposure in combination with LPS on the incidence of

DIC. To determine the extent of coagulation in chronic morphine-treated rats during a challenge with LPS, we used intravital microscopy to observe the mesenteric microvasculature of placebo and morphine-treated rats after LPS injection (Fig 1).

Following a single i.p. injection of LPS (250 µg/kg), 67% of the morphine-treated animals demonstrated disseminated intravascular coagulation (DIC) in their mesentery. By comparison, no placebo-treated animals showed DIC following an LPS injection.

The effect of LPS administration on rat plasma concentrations of TAT in both placebo

and chronic morphine treated rats. To further evaluate the extent of coagulation in chronic morphine-treated rats during systemic infection, we measured plasma TAT levels in placebo- and chronic morphine-treated rats following either normal saline or LPS injection (250 µg/kg; i.p.) [Fig. 2]. There was no significant difference in TAT concentrations between placebo and chronic morphine-treated animals after an injection with normal saline. Treatment with LPS elevated plasma TAT levels in both placebo and chronic morphine treated rats; however, the increase from baseline was only significant in the chronic morphine-treated rats.

The effect of chronic morphine treatment on rat serum endotoxin levels. To examine the possibility of a bacterial component being responsible for the enhanced responses to treatment with a non-pyrogenic dosage of LPS, we measured the serum endotoxin concentration in serum samples (with and without filtering) collected from naïve,

placebo, and chronic morphine-treated rats. There was relatively little difference in endotoxin concentrations in the unfiltered sera of naïve and placebo rats (Fig. 3A). The animals given chronic morphine treatment had significantly higher serum endotoxin levels (Fig. 3A). However, the morphine-induced increase was removed upon filtering of the sera, resulting in similar endotoxin concentrations among all 3 treatment groups (Fig. 3B).

Effects of LPS on LEA and FLUX in morphine- and placebo-treated rats. Both indicators of functional immune responses, LEA and FLUX, were used to investigate LPS-stimulated alterations in the inflammatory processes of naïve, placebo, and chronic morphine-treated animals. Figure 6 shows representative photomicrographs taken of the mesentery of animals given placebo followed by either saline or LPS (Figs. 4A and 4C, respectively), or animals given chronic morphine followed by saline or LPS (Figs. 4B and 4D, respectively). Topical suffusion of FMLP, a chemoattractant, caused a significant increase in LEA and a decrease in FLUX in all three groups (data not shown). These results demonstrated the mesenteric vasculature's integrity was maintained during the course of the intravital microscopic observation. Intraperitoneal injection of LPS (250 µg/kg) significantly enhanced LEA in both placebo- and morphine-treated rats (Fig. 5). Chronic morphine treatment resulted in a significant potentiation of LPS-induced LEA compared to the animals given placebo (Fig. 5). Rats given chronic morphine or placebo showed similar leukocyte FLUX compared to the naïve rats (Fig. 6). Injection of LPS caused a significant attenuation of FLUX in both animals given chronic morphine and

those given placebo (Fig. 6). However, animals given chronic morphine alone did not show a significant alteration in either LEA (Fig. 5) or FLUX (Fig. 6)

Adrenalectomy alters LPS-induced LEA/FLUX responses. To observe the effects of the HPA-axis on the regulation of the inflammation, we measured LPS-induced LEA and FLUX responses in adrenalectomized (ADX) and SHAM rats. An injection with LPS produced a transient increase in LEA (Fig. 7) as well as a gradual decrease in FLUX (Fig. 8) in the SHAM rats. However, ADX rats demonstrated a significant decrease in LEA at 2 hours (Fig. 7), and a greater reduction in FLUX at 1 and 2 hours following injection with LPS (Fig. 8).

Chronic morphine- and LPS-induced alterations in rat serum corticosterone levels.

The serum concentration of corticosterone in naïve rats and in animals given either morphine or placebo pellets followed by an i.p. injection of LPS (250 µg/kg) was measured (Fig. 9). Rats given morphine or placebo pellets produced serum corticosterone levels only slightly higher than those in the naïve animals. Following an i.p. injection of LPS (250 µg/kg), rats given either morphine or placebo pellets had significantly higher serum concentrations of corticosterone (472.9 ± 31.2 and 573.9 ± 14.5 ng/mL, respectively) than the animals given normal saline (46.4 ± 25.0 and 59.0 ± 11.8 ng/mL, respectively). However, the increase seen in morphine-treated rats was significantly less than that in the animals given placebo pellets.

The effect of chronic morphine treatment on rat serum levels of the pro-inflammatory cytokines, IL-1 β , TNF- α , and IL-6. To determine the effects of chronic morphine on inflammatory processes at the molecular level, we examined the effects of morphine treatment and/or LPS on the serum levels of three pro-inflammatory cytokines, IL-1 β , TNF- α , and IL-6, in naive rats and in animals treated with morphine or placebo pellets. The effect of chronic morphine alone on serum cytokine levels was measured over 5 days (Fig. 10A, B, and C). Levels of IL-1 β , TNF- α , and IL-6 increased only slightly with a subsequent reduction to baseline after 3 – 5 days. The serum concentrations of all three cytokines was negligible in the naïve rats and in the animals given morphine or placebo pellets followed by an injection of normal saline (Fig. 11A, B and C). Chronic exposure to morphine did not alter the basal level of serum cytokines. Following an i.p. injection of LPS (250 μ g/kg), both morphine- and placebo-treated animals had a significantly higher serum concentration in these cytokines compared to the animals given normal saline. In addition, rats given morphine pellets had significantly elevated serum concentrations of IL-1 β , TNF- α , and IL-6 (515.1 \pm 68.1, 253.5 \pm 39.7, 9673.7 \pm 1774.9 pg/ml, respectively) in response to the LPS injection compared to animals given placebo pellets (287.6 \pm 38.2, 93.7 \pm 20.8, 2438.6 \pm 735.8 pg/ml, respectively).



Figure 1



Figure 1.

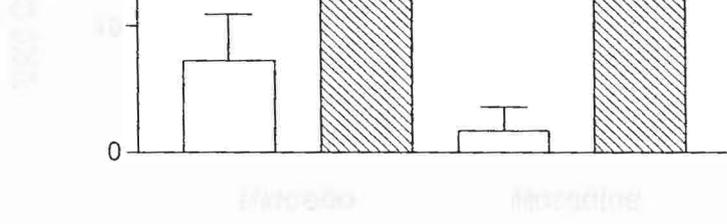


Figure 1.

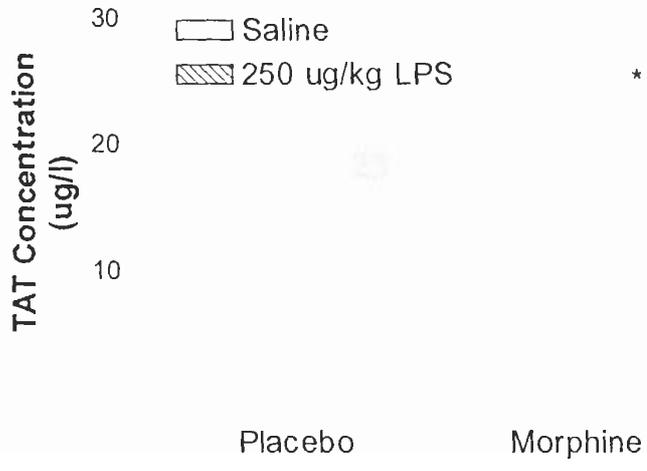


Figure 2.

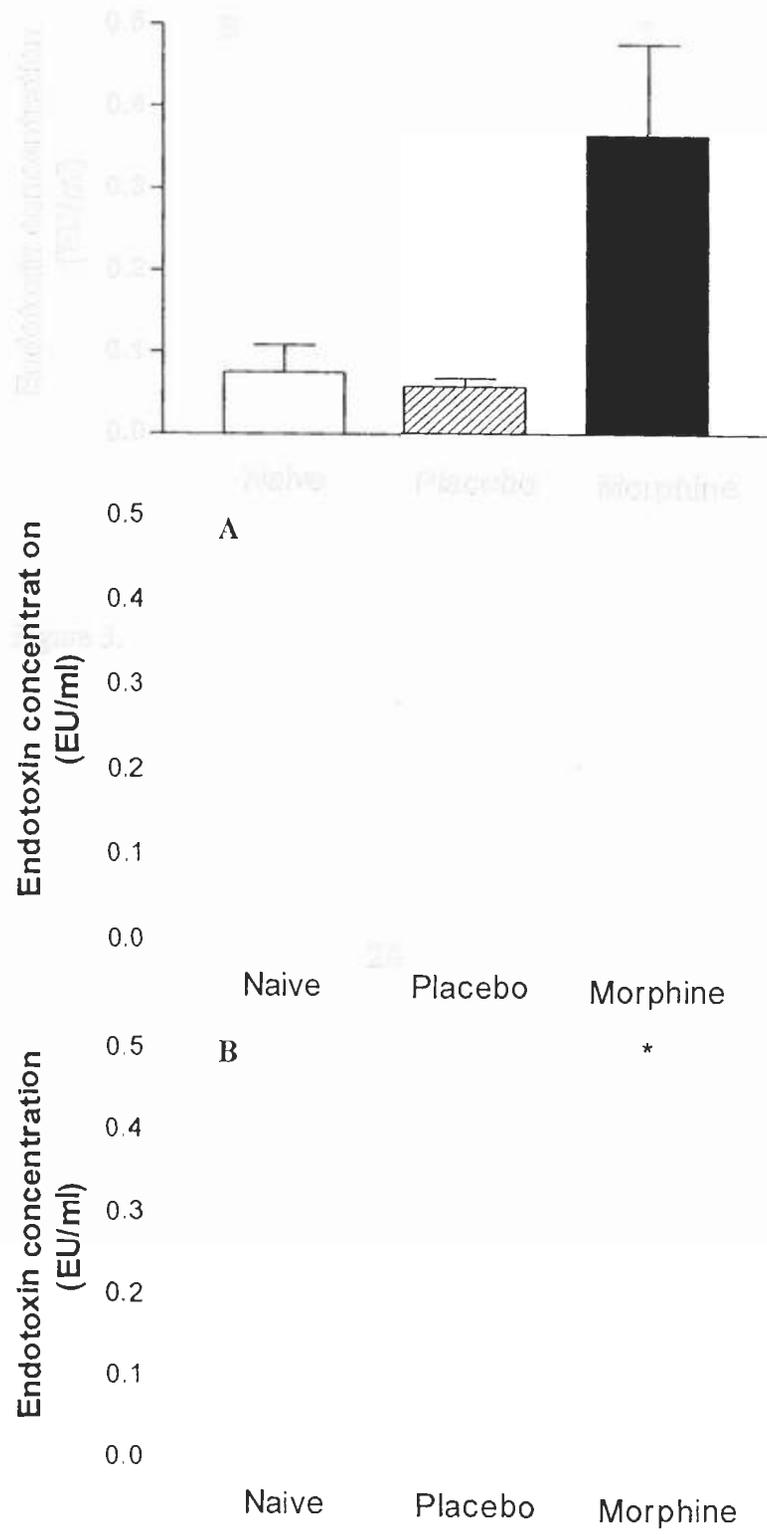


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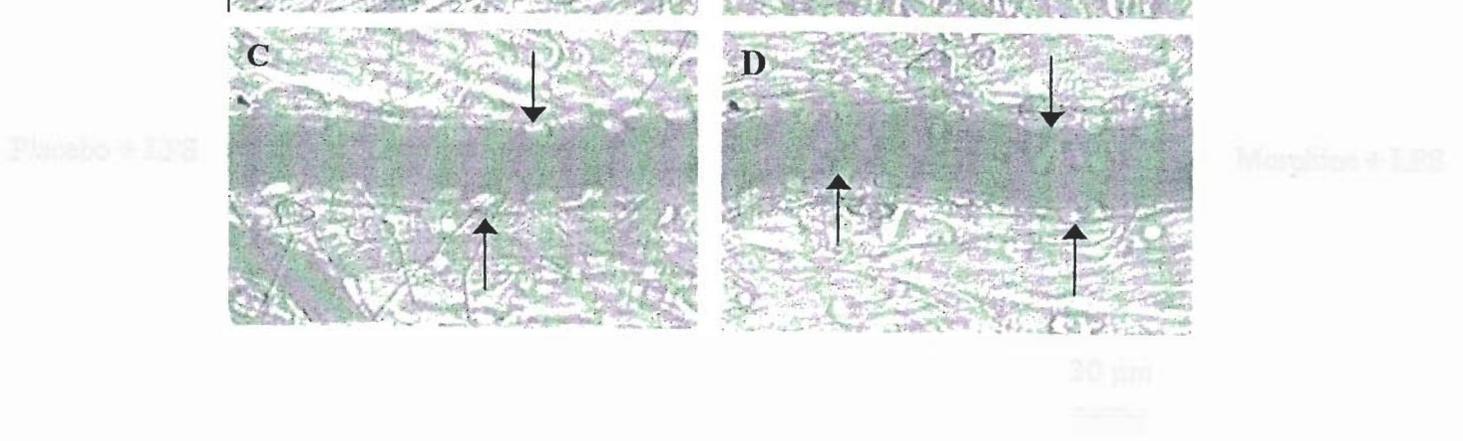


Figure 4.

Placebo+ NS

Morphine + NS

25

Placebo + LPS

Morphine + LPS

30 μm



Figure 4.



Figure 5.

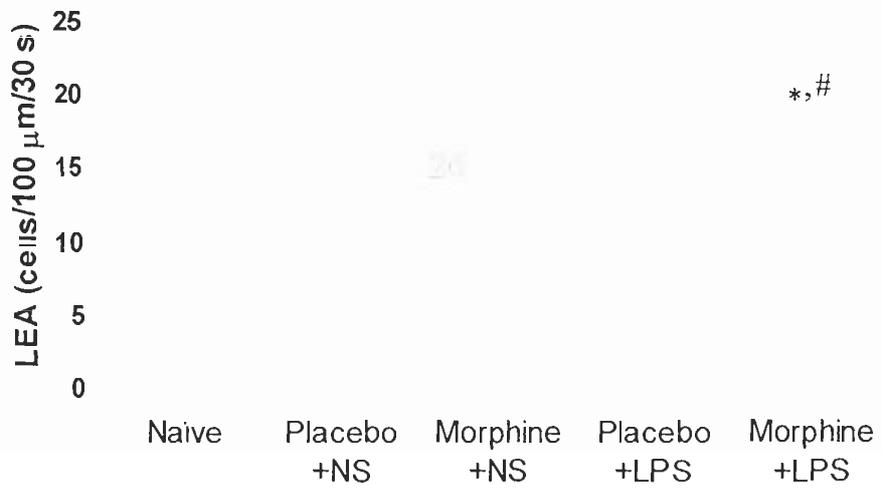


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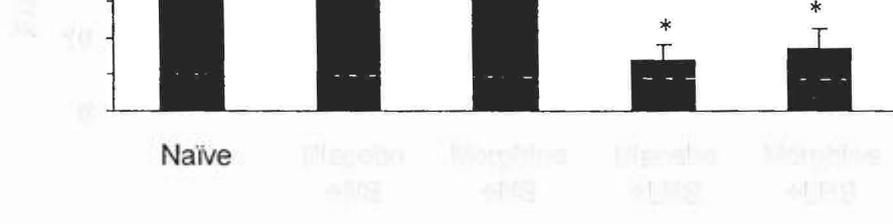


Figure 6

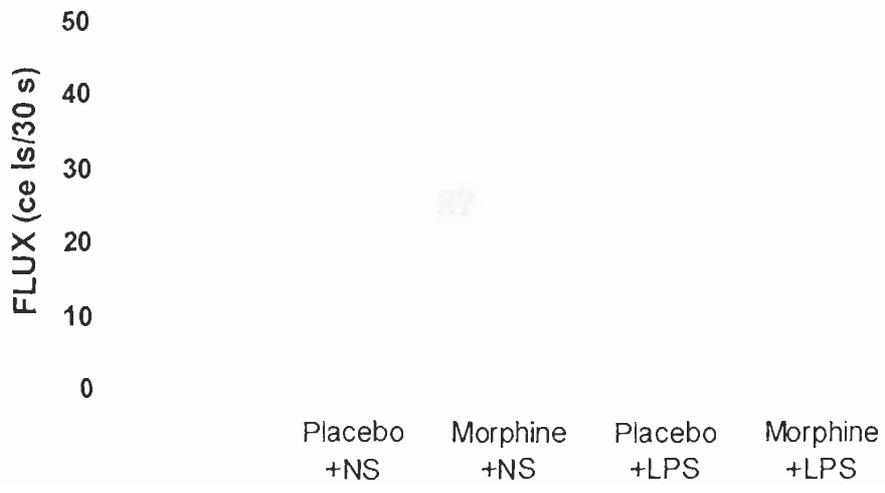


Figure 6.

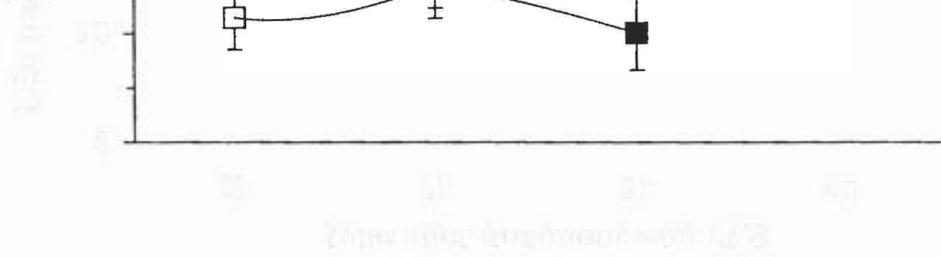


Figure 7.

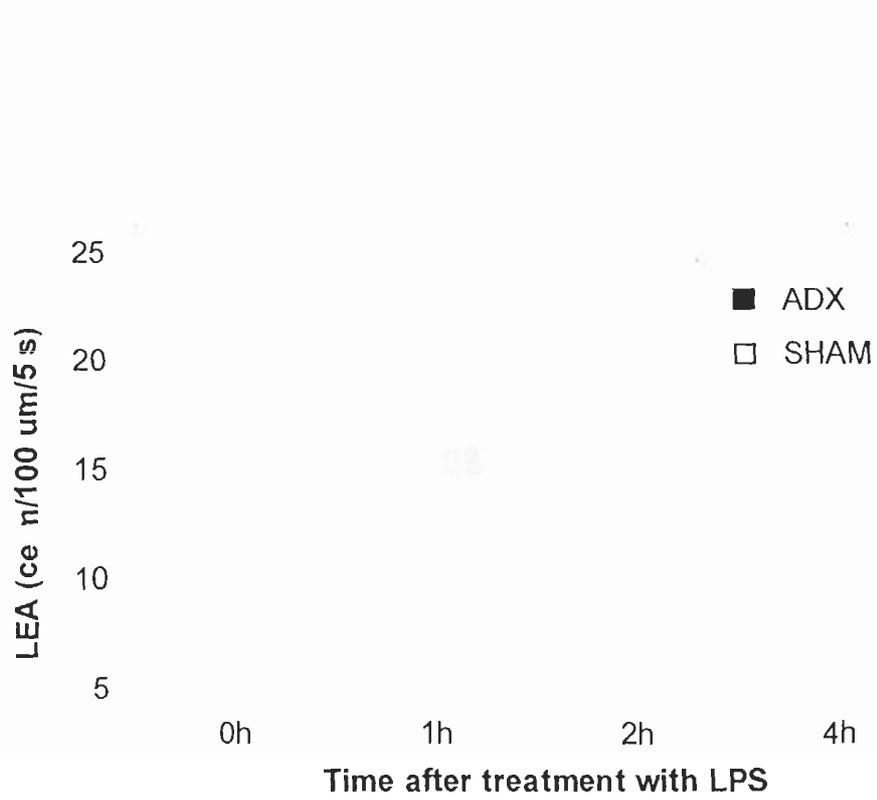


Figure 7.

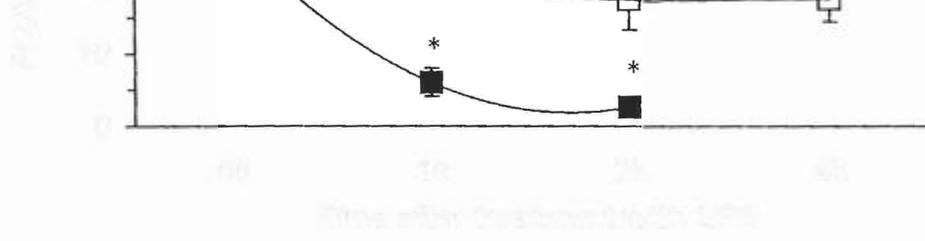


Figure 8.

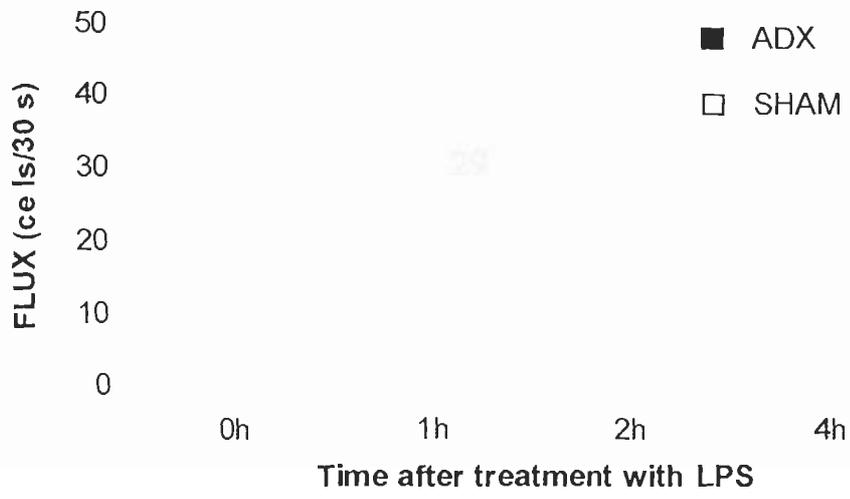


Figure 8.

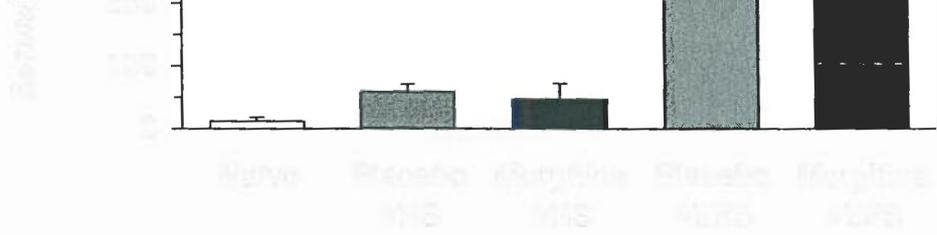


Figure 9.

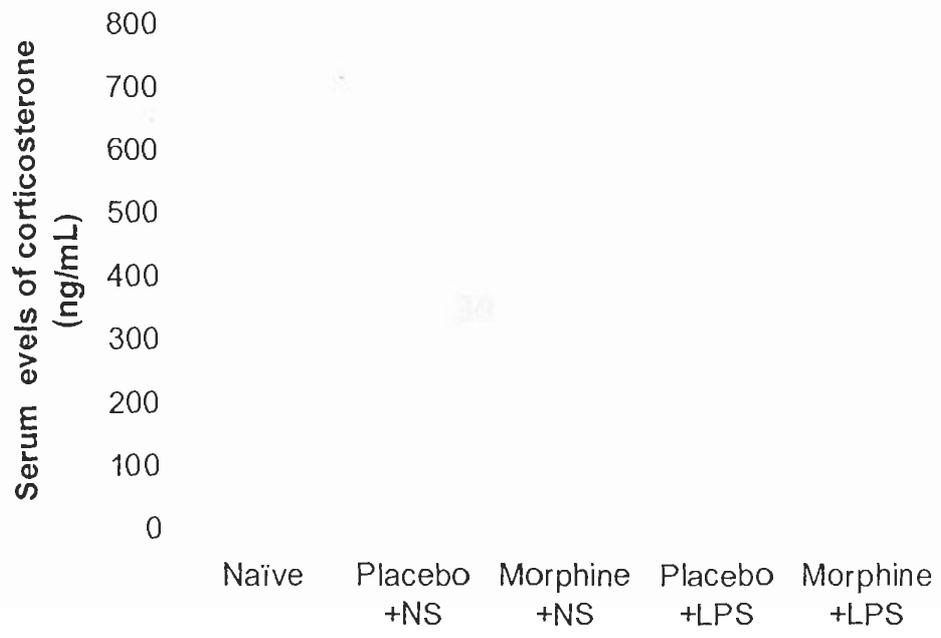


Figure 9.

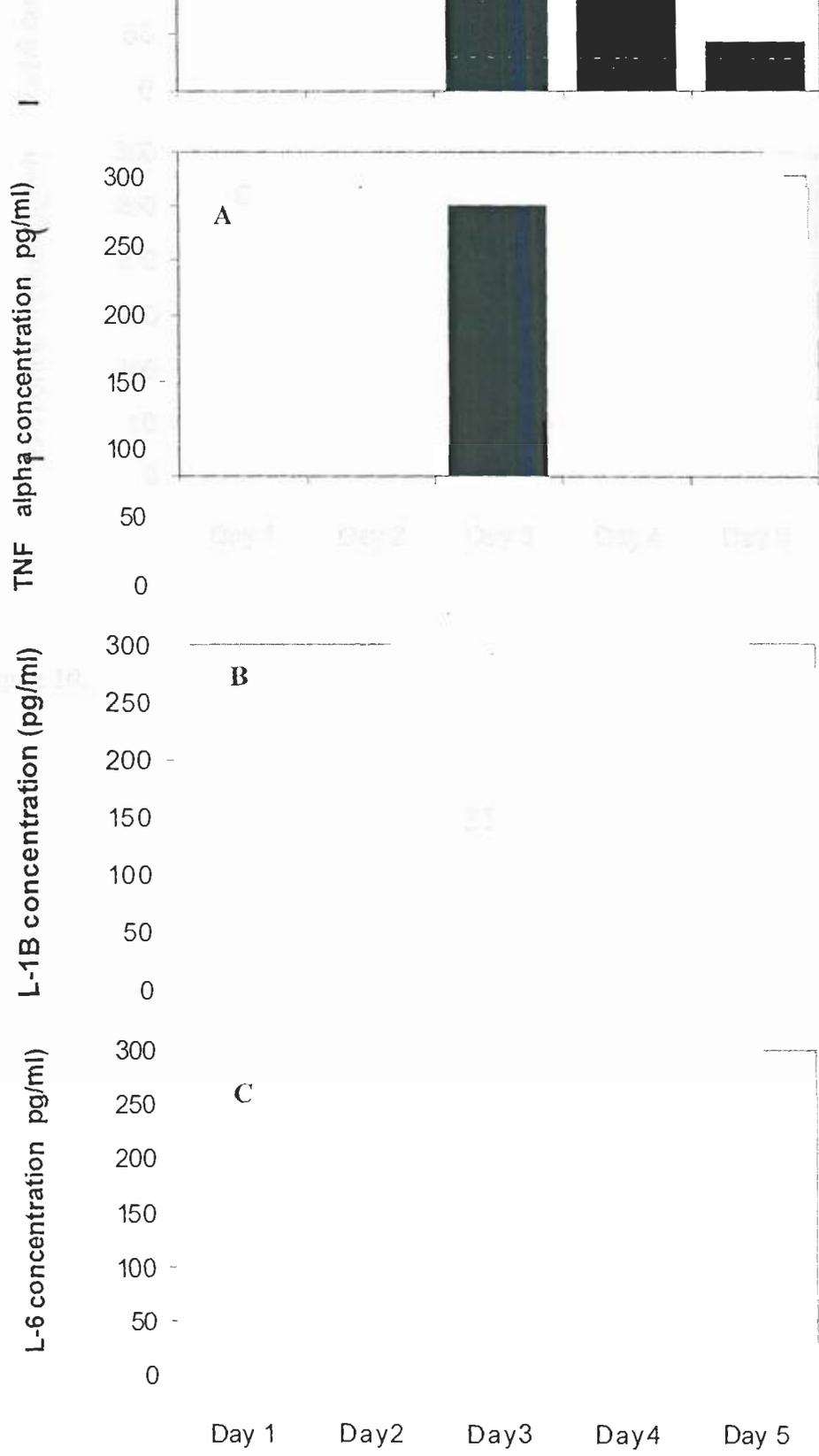


Figure 10.

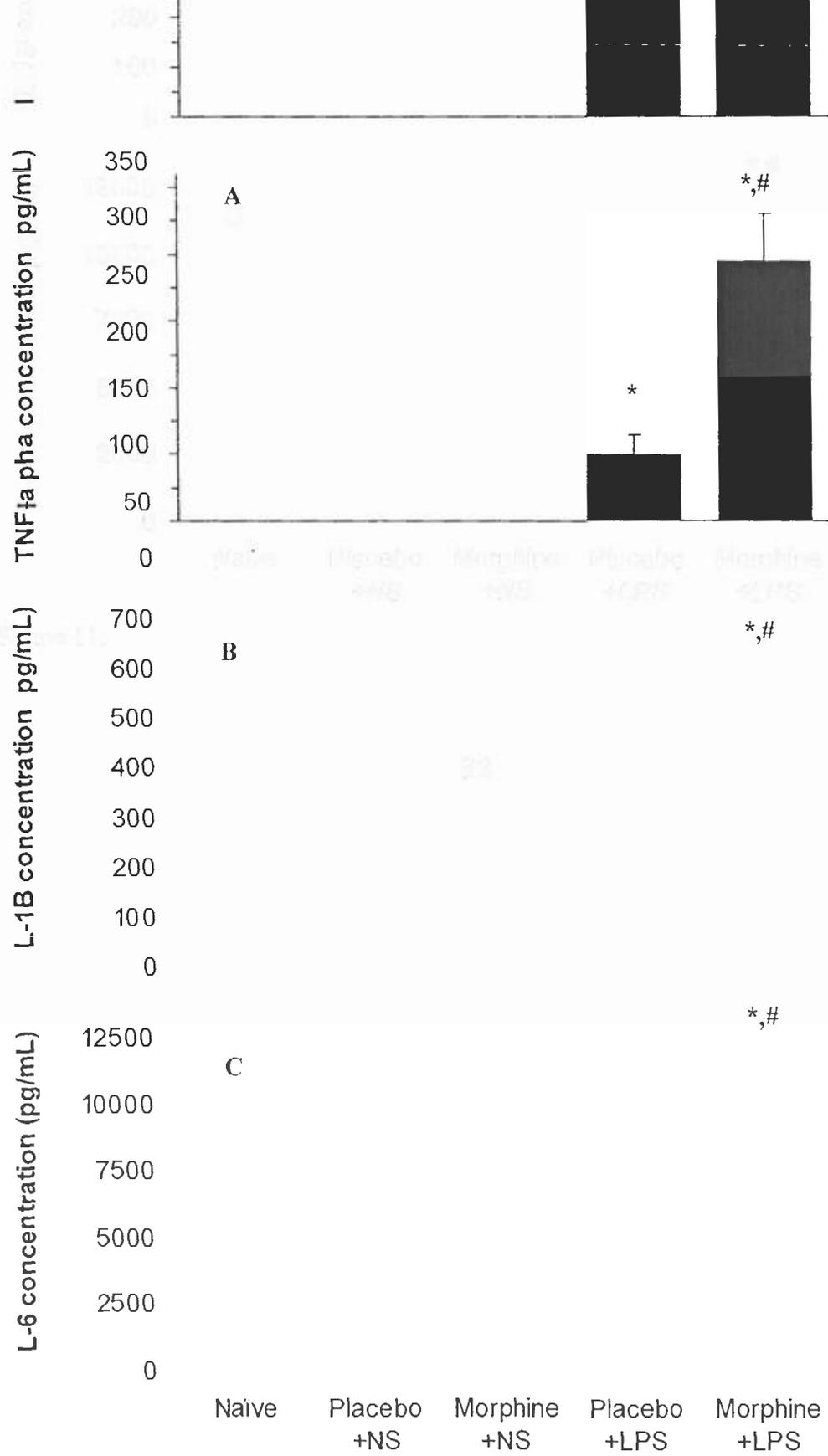


Figure 11.

Discussion

The inflammatory response of the innate immune system is necessary for the walling-off and elimination of bacterial infections. The bacterial endotoxin, LPS, initiates a cascade of systemic, cellular, and molecular events that ultimately manifest in the typical physiological signs and symptoms of infection, i.e., fever. LPS, an endotoxin shed from gram-negative bacteria, exerts major toxic effects via its activation of the immune response. A compromised immune response, as seen following chronic morphine exposure (Hilberger et al., 1997), can potentially accelerate the progression to septic shock during acute infection. In this study, we demonstrated the effects of chronic morphine treatment on the development of sepsis and septic shock in response to treatment with LPS at a non-pyrogenic dosage.

We first examined the effects of an i.p. injection of LPS (250 μ g/kg) on body temperature and MAP. Treatment with LPS did not significantly alter the body temperature of placebo treated rats. However, 30 min after the LPS injection, chronic morphine treated rats showed a significant depression in body temperature that continued for 240 min. We examined the likelihood of an attenuated flow or perfusion throughout the circulatory system by measuring the MAP. Chronic morphine treatment resulted in a significant decrease in MAP following both normal saline and LPS injections, with the greatest reduction in MAP being detected subsequent to LPS treatment. A reduction in MAP, and therefore, flow, increased the occurrence of blood coagulation. Hence, under intravital microscopy, we discovered an increase in the number of mesenteric post-capillary venules showing extensive coagulation in chronic morphine treated rats given a challenge with LPS. Further investigation demonstrated that following an LPS injection,

chronic morphine treated rats showed a 67% increase in DIC compared to placebo treated rats. We then utilized TAT as a measure of coagulability in order to assess the effects of chronic morphine on the progression of DIC during an LPS immune challenge. There was not a significant difference between placebo and chronic morphine treated rats given normal saline. However, the LPS-induced potentiation in plasma TAT levels was only significant in chronic morphine treated animals. It appears that during infection with LPS, chronic morphine treatment results in increased plasma TAT levels, and consequently, DIC. Even with a non-pyrogenic dose of LPS, chronic morphine treatment significantly enhanced the systemic effects of infection.

The serum endotoxin concentrations of naïve, placebo, and chronic morphine treated rats were then measured in order to assess whether the hypothermia, hypotension, and increase in DIC might also be due to an underlying infection attributable to morphine treatment alone. Both naïve and placebo animals had negligible serum endotoxin levels. Conversely, treatment with chronic morphine resulted in a significant increase in the concentration of serum endotoxin. Filtering the serum removed the rise in endotoxin concentration associated with morphine treatment. This confirmed that the elevated endotoxin concentration seen in the assay was of bacterial origin. This is in accordance with a previous report by Hilberger et al. (1997) showing that morphine treatment resulted in the colonization of enteric bacteria in other organs including the liver, spleen, and peritoneal cavity, as well as an enhanced sensitivity to the lethal effects of LPS. Morphine has been shown to act on μ -opioid receptors in myenteric plexus, submucosal plexus, and circular muscle layer in the gut (Ruoff et al., 1991), causing a slowed peristalsis and facilitating bacterial translocation (Yukioka et al., 1987; Runkel et al., 1993). Therefore,

a subsequent bacterial infection will result in a more severe response due to the chronic morphine-induced bacteremia.

As previously mentioned, LEI is a functional indicator of an effective inflammatory response. We measured LEA and FLUX in naïve, placebo, and chronic morphine treated rats to ascertain the effects of chronic morphine and LPS at the cellular level. Local suffusion of the bacterial peptide, FMLP, led to an enhancement of LEA and a reduction in FLUX in all treatment groups (data not shown). This demonstrates that all groups of animals possessed a functional immune system. Injection of a 250µg/kg dose of LPS produced a significant increase in LEA and reduction in FLUX in both placebo and morphine treated animals. Though morphine treatment alone did not significantly increase LEA or reduce FLUX, chronic morphine with a subsequent injection of LPS produced the most dramatic potentiation of LEA compared to the placebo and LPS treated rats. These results are consistent with our previous reports showing that chronic morphine treatment desensitizes the HPA axis-mediated inhibition of the inflammatory response (House et al., 2001). Without an additional exogenous challenge, the impairment of the HPA axis by chronic exposure to morphine apparently does not elicit a different basal LEI, which may be due to physiological “adaptation” in an opiate tolerant state. Systemic adaptation of many physiological events following chronic treatment with morphine has been well documented (Zadina et al., 1995). Bryant et al. (1991) showed that the elevation of circulating corticosterone following morphine pelleting is sustained for 72 h, and then declines to the serum level observed in placebo-pelleted mice.

Disruption of the HPA axis can have a substantial effect on LEI. To investigate chronic morphine-mediated desensitization of the HPA axis, we measured LEA and FLUX in ADX and SHAM rats given an i.p. dose of LPS. SHAM rats, whose HPA axis was intact, demonstrated an increase in LEA and decrease in FLUX that continued for up to 4 h after LPS injection. Furthermore, ADX rats showed an even greater reduction in FLUX at 1 and 2 h after LPS injection, and demonstrated a significant attenuation in LEA at 2 h post-injection. It seems that removal of the adrenal glands produced an inappropriate LEA response due to the lack of an intact HPA axis. These data are consistent with the observations that the corticosteroid, hydrocortisone, significantly attenuates the elevated LEA in ADX animals (Suzuki et al., 1995), and supports our argument that disruption of the HPA axis can potentiate the LEI response to an immune challenge.

Since adrenalectomy and morphine treatment both alter LEI, we examined the effect of chronic morphine on serum levels of the end product of HPA axis activation, corticosterone. Naïve, placebo, and chronic morphine treated animals given normal saline showed no variation in serum corticosterone levels. An injection with LPS resulted in a significant potentiation in the concentration of corticosterone in both the placebo and morphine treated groups. In response to LPS, the elevation of serum corticosterone levels was significantly less in the animals given morphine than in the rats given placebo.

LPS stimulates immune cells, i.e., macrophages, to release a variety of cytokines. In response to endotoxin, macrophages release cytokines, such as, TNF- α , IL- β , and IL-6, which play a key role in the recruitment of the body's defenses against infection (Ato

et al., 2002; Li et al., 2002; Itoh et al., 2003). However, these cytokines are also central to the development of septic shock (Katsuyama et al., 1999; Wang et al., 2003). We examined the effect of chronic morphine treatment alone and in combination with LPS on serum cytokine levels. Chronic morphine treatment over a 5-day period produced a transient yet negligible increase in all 3 cytokines. Naïve, placebo, and chronic morphine treated rats given normal saline did not show a significant difference in cytokine concentrations. LPS treatment produced a significant increase in serum cytokine concentrations in both placebo and chronic morphine treated animals. The greatest potentiation in the levels of TNF- α , IL-1 β , and IL-6 were observed with chronic morphine treated rats injected with LPS. The increase in cytokines following LPS injection in morphine treated animals may be a result of the reduced corticosterone. Glucocorticoids have been shown to inhibit the secretion and actions of various cytokines. Suppression of LPS-induced corticosterone production in morphine-treated animals could prevent the inhibition of cytokine production, which would normally operate in control placebo animals. We have previously shown that injection of IL-1 β ablates the negative feedback of glucocorticoids on immune responses via the desensitization of the HPA axis, but also potentiates the positive effects exerted on immune responses such as LEA (Chang et al., 1998; House et al., 2001).

Summary

In summary, chronic exposure to morphine desensitized the HPA response to an exogenous challenge, and consequently suppressed the production of glucocorticoids, leading to an enhanced secretion of pro-inflammatory cytokines in response to an immune challenge. These cytokines exert positive effects on immune responses, including LEI. Morphine-treated animals, in response to an LPS challenge, demonstrated an enhanced LEI response prior to the development of DIC. In the absence of an external infection, chronic morphine administration alone resulted in bacteremia that was still manageable by the functional yet compromised immune response. This initial increase in serum endotoxin created a “pre-septic” state during which a subsequently immune challenge led to a dramatically enhanced inflammatory response. These animals also developed hypothermia and hypotension, which, collectively, can lead to septic shock. With morphine treatment, the LPS stimulated an increase in LEI, elevation in the cytokines, TNF- α , IL-1 β , and IL-6, and a reduction in serum corticosterone; all of these responses act synergistically to further enhance the development of sepsis and septic shock. These studies substantiate our hypothesis that the immune response is potentiated when the HPA axis is compromised, and provide valuable insight into some of the molecular and cellular mechanisms underlying how attenuation of the HPA axis in the opiate addict may accelerate the progression of septic shock.

References

- Asakura, H, Suga Y, Yoshida T, Ontachi Y, Mizutani T, Kato M, Ito T, Morishita E, Yamazaki M, Miyamoto K, Nakao S. 2003. Pathophysiology of disseminated intravascular coagulation (DIC) progresses at a different rate in tissue factor-induced and lipopolysaccharide-induced DIC models in rats. *Blood Coagul. Fibrinolysis*. 14:221-228.
- Ato, M., Iwabuchi, K., Shimada, S., Mukaida, N., Onoe, K. 2002. Augmented expression of tumour necrosis factor-alpha induced by lipopolysaccharide in spleen of human monocyte chemoattractant protein-1 transgenic mouse enhances the lipopolysaccharide sensitivity of the marginal zone macrophages. *Immunology*. 106:554-563.
- Bateman A, Singh A, Kral T, Solomon S. 1989. The immune-hypothalamic-pituitary-adrenal axis. *Endocr. Rev.* 1:92-112.
- Bussierre, J.L., Adler, M.W., Rogers, T.J., Eisenstein, T.K. 1993. Cytokine reversal of morphine-induced suppression of the antibody response. *J. Pharmacol. Exp. Ther.* 264:591-597.
- Bryant, H.U., Bernton, E.W., Kenner, J.R., Holaday, J.W. 1991. Role of adrenal cortical activation in the immunosuppressive effects of chronic morphine treatment. *Endocrinol.* 128:3253-3258.
- Chang, S.L., Patel, N.A., Romero, A.A., Thompson, J., Zadina, J.E. 1996. FOS expression induced by interleukin-1 or acute morphine treatment in the rat hypothalamus is attenuated by chronic exposure to morphine. *Brain Res.* 736:227-236.

- Chang, S.L., Wu, G.D., Patel, N.A., Vidal, E.L., Fiala, M. 1998. The effects of interaction between morphine and interleukin-1 on the immune response. In *Drugs of Abuse, Immunomodulation, and AIDS*. W.H. Friedman, ed. Plenum Press, New York, pp. 67-72.
- Eppihimer, M.J., Granger, D.N. 1997. Ischemia/reperfusion-induced leukocyte-endothelial interactions in postcapillary venules. *Shock*. 1:16-25.
- Fecho, K., Lysle, D.T. 2002. Morphine-induced enhancement in the granulocyte response to thioglycollate administration in the rat. *Inflammation*. 6:259-271.
- Fecho, K., Maslonek, K.A., Dykstra, L.A., Lysie, D.T. 1995. Mechanisms whereby macrophage-derived nitric oxide is involved in morphine-induced suppression of splenic lymphocyte proliferation. *J. Pharmacol. Exp. Ther.* 2:477-483.
- Flores, L.R., Wahl, S.M., Bayer, B.M. 1995. Mechanisms of morphine-induced immunosuppression: effect of acute morphine administration on lymphocyte trafficking. *J. Pharmacol. Exp. Ther.* 3:1246-1251.
- Gomez-Flores, R., Weber, R.J. 1999. Inhibition of interleukin-2 production and downregulation of IL-2 and transferrin receptors on rat splenic lymphocytes following PAG morphine administration: a role in natural killer and T cell suppression. *J. Interferon. Cytokine. Res.* 6:625-630.
- Hansen, M.K., Nguyen, K.T., Fleshner, M., Goehler, L.E., Gaykema, R.P., Maier, S.F., Watkins, L.R. 2000. Effects of vagotomy on serum endotoxin, cytokines, and corticosterone after intraperitoneal lipopolysaccharide. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2:R331-336.

- Hilberger, M.E., Adler, M.W., Truant, A.L., Meissler, Jr., J.J., Sathishchandran, V., Rogers, T.J., Eisenstein, T.K. 1997. Morphine induces sepsis in mice. *J. of Infectious Diseases*. 176:183-188.
- House, S.D., Lipowsky, H.H. 1987. Leukocyte-endothelium adhesion: microhemodynamics in the mesentery of the cat. *Microvasc. Res.* 34:363-379.
- House, S.D., Mao, X., Wu, G., Espinelli, D., Li, W.X., Chang, S.L. 2001. Chronic morphine potentiates the inflammatory response by disrupting interleukin-1beta modulation of the hypothalamic-pituitary-adrenal axis. *J. Neuroimmunol.* 118:277-285.
- Itoh, K., Udagawa, N., Kobayashi, K., Suda, K., Li, X., Takami, M., Okahashi, N., Nishihara, T., Takahashi, N. 2003. Lipopolysaccharide promotes the survival of osteoclasts via Toll-like receptor 4, but cytokine production of osteoclasts in response to lipopolysaccharide is different from that of macrophages. *J. Immunol.* 170:3688-3695.
- Katsuura, G., Gottscha, P.E., Dahl, R., Arimura, A., 1988. Adrenocorticotropin release induced by intracerebroventricular injection of recombinant human interleukin-1 in rats: possible involvement of prostaglandin. *Endocrinology* 122:1773-1779.
- Katzung, W. 1992. Legal and illegal drugs--status, selected problems, trends. *Z. Arztl. Fortbild. (Jena)*. 86:689-699.
- Lacosta, S., Merali, Z., Anisman, H., 1998. Influence of interleukin-1 beta on exploratory behaviors, plasma ACTH, corticosterone, and central biogenic amines in mice. *Psychopharmacology* 137:351-361.

- Nazziola, E., House, S.D., 1992. Effects of hydrodynamics and leukocyte-endothelium specificity on leukocyte-endothelium interactions. *Microvasc. Res.* 44:127-142.
- LeVier, D.G., McCay, J.A., Stern, M.L., Harris, L.S., Page, D., Brown, R.D., Musgrove, D.L., Butterworth, L.F., White, K.L., Munson, A.E. 1994. Immunotoxicological profile of morphine sulfate in B6C3F1 female mice. *Fund. App. Toxicol.* 22:525-542.
- Li, W.D., Ran, G.X., Teng, H.L., Lin, Z.B. 2002. Dynamic effects of leflunomide on IL-1, IL-6, and TNF-alpha activity produced from peritoneal macrophages in adjuvant arthritis rats. *Acta. Pharmacol. Sin.* 23:752-756.
- McCoy, J.G., Matta, S.G., Sharp, B.M., 1994. Prostaglandins mediate the ACTH response to interleukin-1-beta instilled into the hypothalamic median eminence. *Neuroendocrinology* 60:426-435.
- Muller, W.A., Weigl, S.A., Deng, X., Phillips, D.M. 1993. PECAM-1 is required for transendothelial migration of leukocytes. *J. Exp. Med.* 178:449-460.
- Nazziola, E., House, S.D., 1992. Effects of hydrodynamics and leukocyte-endothelium specificity on leukocyte-endothelium interactions. *Microvasc. Res.* 44:127-142.
- Parrillo, J.E. 1990. Septic shock in humans: advances in the understanding of pathogenesis, cardiovascular dysfunction, and therapy. *Ann. Intern. Med.* 113:227-42.
- Parrillo, J.E. 1993. Pathogenic mechanisms of septic shock. *N. Engl. J. Med.* 328:1471-1477.
- Patel, N.A., Romero, A.A., Zadina, J.E., Chang, S.L. 1996. Chronic exposure to morphine attenuates expression of interleukin-1 beta in the rat hippocampus. *Brain Res.* 712:340-344.

- Ravanat, C., Freund, M., Schuhler, S., Grunert, P., Meyer, L. and Cazenave, J.P. 1996. Species specific immunoassays to measure blood platelet and coagulation activation in the rat. *Thromb. Haemost.* 76:1090-1095.
- Rosenkranz, A.R., Mayada, T.N., 1999. Leukocyte–endothelial cell interactions: lessons from knockout mice. *Exp. Nephrol.* 7:125–136.
- Runkel, N.S.F., Moody, F.G., Smith, G.S., Rodriguez, L.F., Chen, Y., Larocco, M.T. 1993. Alterations in rat intestinal transit by morphine promote bacterial translocation. *Dig. Dis. Sci.* 38:1530–1536.
- Ruoff, H.J., Fladung, B., Demol, P., Weihrauch, T.R. 1991. Gastrointestinal receptors and drugs in motility disorders. *Digestion.* 48:1–17.
- Salas, A., Shimaoka, M., Chen, S., Carman, C.V., Springer, T.A. 2002. Transition from rolling to firm adhesion is regulated by the conformation of the I domain of the integrin lymphocyte function-associated antigen-1. *J. Biol. Chem.* 52:50255-50262.
- Smith, M.L., Smith, M.J., Lawrence, M.B., Ley, K. 2002. Viscosity-independent velocity of neutrophils rolling on p-selectin in vitro or in vivo. *Microcirculation.* 6:523-536.
- Sternberg, E.M., Chrousos, G.P., Wilder, R.L., Gold, P.W. 1992. The stress response and the regulation of inflammatory disease. *Ann. Intern. Med.* 117:854–866.
- Suzuki, H., Zweifach, B.W., Forrest, M.J., Schmid-Schonbein, G.W. 1995. Modification of leukocyte adhesion in spontaneously hypertensive rats by adrenal corticosteroids. *J. Leukocyte Biol.* 57:20-26.

- Yokota, T., Uehara, K. and Nomota, Y. 2000. Intrathecal morphine suppresses NK cell activity following abdominal surgery. *Can. J. Anaesth.* 47:303-308.
- Wang, J.H., Doyle, M., Manning, B.J., Blankson, S., Wu, Q.D., Power, C., Cahill, R., Redmond, H.P. 2003. Cutting edge: bacterial lipoprotein induces endotoxin-independent tolerance to septic shock. *J. Immunol.* 170:14-18.
- Wang, H., Yu, M., Ochani, M., Amelia, C.A., Tanovic, M., Susarla, S., Li, J.H., Wang, H., Yang, H., Ulloa, L., Al-Abed, Y., Czura, C.J., Tracey, K.J. 2003. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. *Nature.* 421 :384-388.
- Wheeler, A.P., Bernard, G.R. 1999. Treating patients with septic shock. *N. Engl. J. Med.* 340:207-214.
- Yamaguchi, K., Majima, M., Katori, M., Kakita, A., Sugimoto, K. 2000. Preferential consumption of coagulation factors I, V, and VIII in rat endotoxemia. *Shock.* 14:535-543.
- Yokota, T., Uehara, K. and Nomota, Y. 2000. Intrathecal morphine suppresses NK cell activity following abdominal surgery. *Can. J. Anaesth.* 47:303-308.
- Yukioka, H., Rosen, M., Evans, K.T., Leach, K.G., Hayward, M.W.J., Saggi, G.S. 1987. Gastric emptying and small bowel transit times in volunteers after intravenous morphine and nalbuphine. *Anaesthesia.* 42:704-710.
- Zadina, J.E., Kastin, A.J., Harrison, L.M., Ge, L.J., Chang, S.L. 1995. Opiate receptor changes after chronic exposure to agonists and antagonists. *Ann. NY Acad. Sci.* 757:353-361.