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Endogenous Opioids Regulate the Expression of Inducible Nitric Oxide Synthase by Splenocytes[1]

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ABSTRACT

In the present study, we tested the hypothesis that lipopolysaccharide (LPS)-induced expression of nitric oxide synthase (iNOS) by splenocytes is modulated through the activation of endogenous opioids in the central nervous system. The initial studies determined the parameters of LPS-induced expression of iNOS by splenocytes. Rats were injected with LPS at doses of 0, 1, 10, 100, and 1000 μg/kg, and measures of both iNOS mRNA and protein showed a dose-dependent increase in expression. In a time course study, rats received 100 μg/kg LPS and were killed at 0, 2, 4, 8, and 16 h postinjection. Both iNOS mRNA and protein expression was detectable at the 2-h time point, with peak expression occurring at 8 h. To evaluate the involvement of endogenous opioids, the opioid receptor antagonist naltrexone was administered at 0, 0.1, 1, or 10 mg/kg s.c. in combination with LPS (100 μg/kg), with a second injection of naltrexone at the same dose 4 h after the injection of LPS. Naltrexone induced a pronounced dose-dependent reduction in iNOS mRNA and protein expression by splenocytes. The modulation of iNOS expression occurs via central opioid receptors as intracerebroventricular administration but not peripheral administration of *N*-methylnaltrexone, the quaternary form of naltrexone that does not readily cross the blood-brain barrier, reduced the expression of iNOS. For all of the manipulations, nitrite/nitrate levels in the plasma showed effects similar to those for iNOS mRNA and protein. Collectively, these findings indicate that central opioid receptors are involved in the in vivo regulation of splenic nitric oxide production.

In recent years, the involvement of nitric oxide in immune processes has become apparent. For example, the role of nitric oxide in infectious disease is demonstrated by studies showing that it is involved in the cytostatic activity of macrophages on parasitic growth (Vincendeau et al., 1992). The development of autoimmune disease also has been linked to alterations in the expression of nitric oxide, as spontaneous autoimmune disease in MRL-lpr/lpr mice has been shown to be dependent on enhanced expression of nitric oxide (Weinberg et al., 1994). In addition to the impact of nitric oxide directly on infectious organisms and immunoreactive tissues, nitric oxide has important regulatory functions in the immune system. Numerous studies have shown that nitric oxide limits the proliferative activity of lymphocytes (Albina and Henry, 1991; Pascual et al., 1993).

Despite extensive knowledge of the biochemistry of nitric oxide and the undeniable importance of nitric oxide for defense against disease, little is known about the diversity of in vivo factors involved in the regulation of nitric oxide production by cells of the immune system. Much attention has been focused on the role of cytokines in the regulation of nitric oxide production using in vitro culture systems. For example, it has been established that interferon-γ and tumor necrosis factor increase the production of nitric oxide by macrophages (Albina and Henry, 1991; Geller et al., 1993) and that interleukin-4 and interleukin-10 inhibit nitric oxide production by macrophages (Al-Ramadi et al., 1992; Gazzinelli et al., 1992; Oswald et al., 1992). However, there is evidence that explanations based on cytokine regulation alone do not adequately account for the in vivo expression of inducible nitric oxide. For example, Liu et al. (1993, 1997) found that lipopolysaccharide (LPS) treatment in vivo induces a very rapid expression of mRNA for inducible nitric oxide synthase (iNOS), the enzyme responsible for nitric oxide formation. This contrasts sharply with in vitro studies showing that LPS and cytokines are often relatively slow and weak stimulators of iNOS mRNA expression in a number of culture systems (Geller et al., 1993). Thus, the in vivo milieu provides an abundance of unexplored factors that serve as potent regulators of nitric oxide production in the immune system.

In some of our work, we have made the unique observation that the administration of morphine alters the production of nitric oxide by splenic macrophages (Fecho et al., 1994). In subsequent investigations, we showed that microinjection of

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ABBREVIATIONS: iNOS, inducible nitric oxide synthase; i.c.v., intracerebroventricular; RT, reverse transcription; PCR, polymerase chain reaction; LPS, lipopolysaccharide.

the *mu*-opioid receptor agonist [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin into the central nervous system produced an elevation of nitric oxide production by splenic macrophages, whereas the microinjection of *kappa*- and *delta*-selective opioid agonists did not significantly alter nitric oxide production (Schneider and Lysle, in press). The effect of [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin was blocked by the opioid receptor antagonist naltrexone, indicating that the effect was opioid receptor mediated (Schneider and Lysle, in press). These observations indicate that opioids modulate the production of nitric oxide by macrophages and that the effect is mediated by *mu*-opioid receptors in the central nervous system. These observations suggest a further hypothesis that the in vivo induction of iNOS expression involves endogenous opioid activity within the central nervous system.

The present study tests the hypothesis that iNOS expression involves endogenous opioid activity within the central nervous system by evaluating the effect of administration of the opioid receptor antagonist naltrexone on LPS-induced expression of iNOS by splenocytes. To evaluate the contribution of opioid receptors located in the central nervous system, the effect of intracerebroventricular (i.c.v.) and peripheral administration of *N*-methylnaltrexone on LPS-induced expression of iNOS by splenocytes was determined. *N*-methylnaltrexone is the quaternary form of naltrexone that does not readily cross the blood-brain barrier (Brown and Goldberg, 1985). The prediction, based largely on our prior work, was that blockage of opioid receptors, particularly those in the central nervous system, would inhibit the expression of nitric oxide synthase by splenocytes. Because the literature provided only limited information about the appropriate dose of LPS and the time course of LPS-induced expression of iNOS, the initial assessments determined the dose-effect relationship and time course of LPS-induced iNOS expression. To measure nitric oxide production in vivo, RT-PCR (reverse transcription-polymerase chain reaction) and Western blotting techniques was used to measure iNOS mRNA and protein expression in spleen tissue, and Griess reagent was used to measure nitrite/nitrate in plasma.

Materials and Methods

Animals. Male Lewis rats 65 days old and 225 to 250 g in weight were purchased from Charles Rivers Laboratories (Raleigh, NC). On arrival, animals were individually caged in a colony room where a reversed light-dark (12-h) cycle was maintained through artificial illumination. Food and water were provided ad libitum during both a 2-week acclimation period and the experimental procedure.

Drug Administration. To determine the doses and time course of LPS-induced expression of nitric oxide, rats received an s.c. injection saline or LPS serotype 055:B5 (Difco Laboratories, Detroit, MI) at doses of 1, 10, 100, or 1000 μg/kg ($n = 4$). The animals were killed 4 h after the injection. In a second study, rats received an s.c. injection of 100 μg/kg LPS, and the animals were killed immediately or 2, 4, 8, or 16 h after the injection ($n = 4$). For both studies, the spleen was obtained for the extraction of RNA and protein, and a sample of peripheral blood from the abdominal aorta was used for the determination of nitrite/nitrate levels.

To evaluate the effect of naltrexone, rats received an s.c. injection of saline or naltrexone at doses of 0.1, 1, or 10 mg/kg ($n = 4$) in combination with an s.c. injection LPS (100 μg/kg), with a second injection of naltrexone at the same dose 4 h after the injection of LPS. Eight hours after the injection of LPS, the rats were killed, and the spleen and a sample of peripheral blood were obtained for analysis.

To determine the involvement of central opioid receptors, *N*-methylnaltrexone was administer i.c.v. in combination with an s.c. injection of LPS (100 μg/kg). Rats were stereotaxically implanted with a single 22-gauge cannula (Plastics One) into the left lateral ventricle [anteroposterior +0.9, mediolateral +1.5, dorsoventral −3.2] under anesthesia induced with a 1 ml/kg injection of a 1:1 (v/v) mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml). Coordinates are expressed as millimeters from bregma (Paxinos and Watson, 1986). After a 1-week recovery period, rats were given an injection of saline or *N*-methylnaltrexone (0.01, 0.1, 1, or 10 μg) in a 5 μl volume via a 28-gauge (Plastics One) injector that protruded 1 mm beyond the tip of the cannula ($n = 4$). The injector was connected by a length of tubing to a Hamilton syringe. *N*-Methylnaltrexone was administered i.c.v. at the time of an s.c. injection LPS (100 μg/kg) with a second i.c.v. injection of *N*-methylnaltrexone 4 h later. Eight hours after the injection of LPS, rats were killed, and the spleen and a sample of blood were obtained for plasma. The selection of doses of *N*-methylnaltrexone were based on studies showing that an i.c.v. dose of 1.0 μg of *N*-methylnaltrexone is sufficient to block the immunomodulatory effect of morphine administration (Fecho et al., 1996a).

In a subsequent study, *N*-methylnaltrexone (10 mg/kg) or saline was administered s.c. at the time of an s.c. injection LPS (100 μg/kg) with a second injection of the same dose of *N*-methylnaltrexone 4 h later ($n = 5$). Eight hours after the injection of LPS, rats were killed, and the spleen and a sample of blood were obtained for plasma.

RT-PCR. Total RNA was extracted from a section of each spleen using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH), a modification of the original method described by Chomczynski and Sacchi (1987). The RNA was quantified spectrophotometrically (GeneQuant II; Pharmacia-Biotech, Piscataway, NJ). For the RT-PCR, RT was performed using Oligo(dT)$_{18}$ primer and Moloney murine leukemia virus-reverse transcriptase after the protocol of the 1st Strand cDNA Synthesis Kit from Clontech (Palo Alto, CA). The resulting cDNA was amplified for 20 cycles using a PCR primer set for iNOS, 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ and 5′-GGGTGTCAGAGTCTTGTGCCTTTGG-3′ that was synthesized by the Nucleic Acids Core Facility (Lineberger Cancer Center, University of North Carolina, at Chapel Hill). *Taq* DNA polymerase (Boehringer-Mannheim, Indianapolis, IN) was used in the reaction. The cycle temperatures were 94°C, 60°C, and 72°C for the denaturing, annealing, and extending temperatures, respectively. The cycle times were 30 s, 30 s, and 1.5 min for the denaturing, annealing, and extending times, respectively. Moreover, given the time required to start the relatively large number of samples, a hot-start method using Ampliwax PCR gems (Perkin-Elmer, Branchburg, NJ) was used. Positive and negative controls also were included. The PCR products were run on a 2.0% agarose gel in 0.5× Tris-Borate-EDTA buffer, stained with ethidium bromide (0.5 μg/ml), and visualized under ultraviolet transillumination. Product levels were quantified by densitometry using UltraLum (KS 3000) gel documentation system and Sigmagel software (Jandel Scientific). The data are expressed as a ratio of β-actin expression, which served as a housekeeping control. A PCR primer set from Clontech was used to amplify β-actin mRNA. The sequence of the β-actin primers was 5′-TTGTAACCAACTGGGACGATATGG-3′ and 5′-GATCTTGATCTTCATGGTGCTAGG-3′. The DNA molecular weight marker (DNA marker VI; Boehringer-Mannheim) was used to estimate the size of the products.

To verify the identity of the products, the PCR products were cloned using the Original Procaryotic TA Cloning Kit from InVitrogen (San Diego, CA). Plasmid DNA minipreps were prepared using Plasmid Mini Kits from Qiagen (Chatsworth, CA). DNA was sequenced at the University of North Carolina, Chapel Hill, Automated DNA Sequencing Facility on a model 373A DNA Sequencer (Applied Biosystems) using the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Systems).

Western Blot. For the iNOS protein determinations, protein was extracted from a section of spleen tissue using freeze/thaw lysis in

Tris buffer containing antiproteinases. Total protein was determined using the BioRad Protein Assay (BioRad, Hercules, CA). Then, 20 μg of protein was loaded on a sodium dodecyl sulfate-polyacrylamide gel and electrophoretically separated and blotted to nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was blocked with 10% dry milk and reacted with a polyclonal rabbit anti-iNOS antibody (Transduction Laboratories, Cincinnati, OH). The membrane then was incubated with donkey anti-rabbit antibody conjugated to horseradish peroxidase (Amersham, Cleveland, OH). Membranes were exposed to Hyperfilm ECL, and peroxidase activity was detected using the enhanced chemiluminescence analysis system (Amersham). A positive control containing protein extracted from the RAW 264.7 (ATCC TIB71; American Type Culture Collection, Rockville, MD) macrophage cell line that had been stimulated for 12 h with interferon-γ (10 ng/ml; Gibco Life Technologies, Gaithersburg, MD) and LPS (1.0 μg/ml serotype 055:B5; Difco Laboratories, Detroit, MI), along with Kaleidoscope prestained standards (BioRad), was used to identify the 130-kDA iNOS protein. Product levels were quantified by scanning the film and performing densitometry analysis (Sigmagel; Jandel Scientific).

Nitrite Production Assay. To provide an additional assessment of nitric oxide production, the level of nitrate/nitrite in plasma was determined using the Griess reagent assay (Green et al., 1982). Nitrate and nitrite are formed nonenzymatically when nitric oxide is exposed to oxygen; thus, plasma levels of these products indicate the level of nitric oxide production. Total nitrite/nitrate levels were determined by the conversion of nitrate to nitrite using nitrate reductase in the presence of reduced nicotinamide adenine dinucleotide phosphate and flavinadenine dinucleotide and then an assessment using Griess reagent (Tracey et al., 1995). More specifically, 6 μl of plasma was mixed with 44 μl of dH_2O, 20 μl of 0.31 M phosphate buffer (pH 7.5), and 10 μl of 0.86 mM reduced nicotinamide adenine dinucleotide phosphate (Boehringer-Mannheim), 10 μl of 0.11 mM flavinadenine dinucleotide (Sigma), and 10 μl of 1.0 U/ml nitrate reductase (Boehringer-Mannheim) in individual wells of a 96-well plate. Samples were incubated for 1.5 h at room temperature. Then, 200 μl of Griess reagent consisting of a 1:1 (v/v) solution of 1% sulfanilamide in 5.0% phosphoric acid and 0.1% *N*-(1-napthyl)ethylenedamine dihydrochloride in distilled water was added to the samples. Absorbance was measured, after 10 min of incubation at room temperature, at 550 nm using a Biotech plate reader (model EL312), and the micromolar (μM) concentration of nitrite was determined for each sample based on a standard curve. Recovery of nitrate was greater than 95% in this assay.

Statistical Treatment of Data. Analysis of variance was performed on all data sets. Standard polynomial contrasts were performed on those analyses that showed a significant main effect of dose of naltrexone or *N*-methylnaltrexone. The level of significance for all statistical tests was set at a probability of .05.

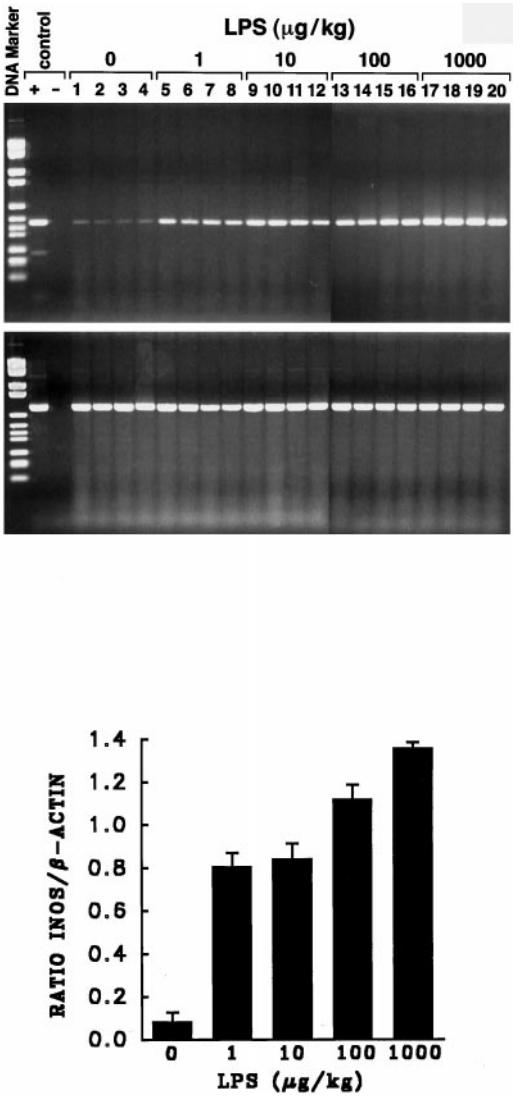

Fig. 1. Dose-dependent effect of LPS (0, 1.0, 10, 100, 1000 μg/kg) on the expression of iNOS mRNA as determined by RT-PCR. Rats were tested 8 h after the injection of LPS. Electrophoresis of the RT-PCR products is shown via an image of the ethidium bromide-stained agarose gel visualized under ultraviolet transillumination. The top image shows the results using the iNOS primers, and the bottom image shows the results for β-actin. The first lane of each gel is the DNA molecular weight marker (DNA marker VI; Boehringer-Mannheim). The amplified products using the iNOS primer set are shown in the subsequent lanes of the top image starting with a positive iNOS and negative control followed by the samples from the rats treated with increasing doses of LPS. There is a distinct product of the expected size (496 bp). The bottom image shows a distinct product of the expected size (764 bp) for β-actin.

Results

Figure 1 shows the dose-dependent effects of LPS on the expression of iNOS mRNA as determined by RT-PCR. The electrophoresis of the RT-PCR products is shown via an image of the ethidium bromide-stained agarose gel visualized under ultraviolet transillumination. The top image shows the results using the iNOS primers, and the bottom image shows the results for β-actin. The first lane of each gel is the DNA molecular weight marker (DNA marker VI; Boehringer-Mannheim). The amplified products using the iNOS primer set are shown in the subsequent lanes of the top image starting with a positive iNOS and negative control, followed by the samples from the rats treated with increasing doses of LPS. There is a distinct product of the expected size (496 bp). The bottom image shows a distinct product of the expected size (764 bp) for β-actin. The densitometric analysis of the bands showed no significant effect of LPS on the expression of β-actin; however, the analysis of the ratio of iNOS/β-actin expression showed a significant effect of LPS on iNOS expression: $F(4,15) = 55.65$ ($p < .0001$). Moreover, there was a significant linear component to the main effect of LPS [$F(1,15) = 180.53$, $p < .0001$] indicating a linear relationship between the dose of LPS and iNOS expression. The ratio data are shown in graphic form at the bottom of Fig. 1. The cloned iNOS PCR product was found to have 99% identity with the corresponding region of the reported sequence for rat iNOS (Genbank Accession No. D14051; Nunokawa et al., 1993).

Figure 2 shows the image and densitometric analysis of the

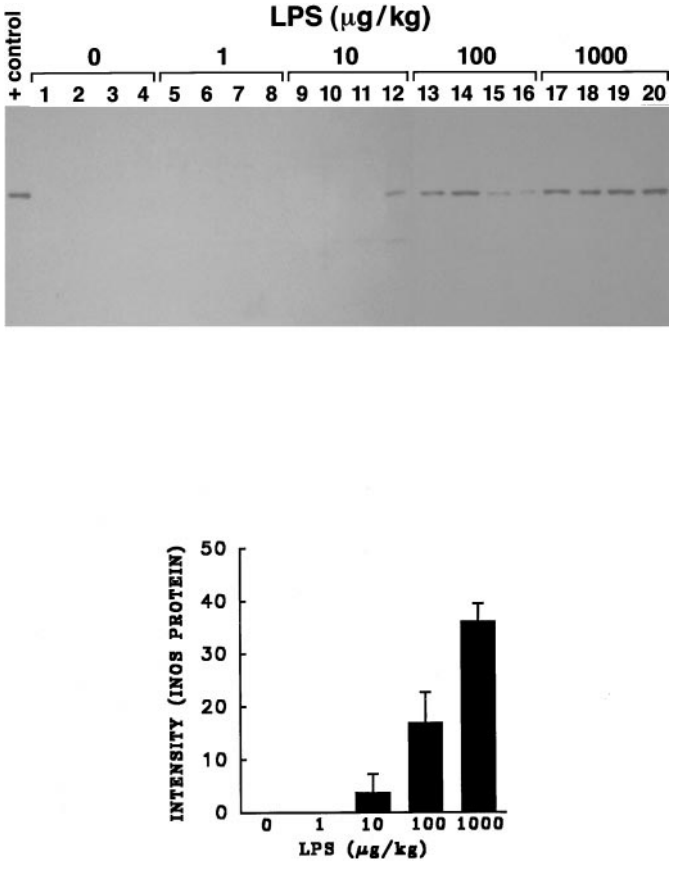

Fig. 2. Dose-dependent effect of LPS (0, 1.0, 10, 100, 1000 μg/kg) on the expression of iNOS protein as determined by Western blot. Rats were tested 8 h after the injection of LPS. The panel shows the image of the Hyperfilm from the chemiluminescence analysis. The positive control showed the expected 130-kDa band for iNOS protein. The graph shows the results of the densitometry analysis.

Western blot for iNOS protein. The positive control showed the expected 130-kDa band for iNOS protein. The densitometric analysis of the bands showed a significant effect of LPS on the expression of iNOS [$F(4,15) = 16.96$, $p < .0001$]. Moreover, there was a significant linear component to the effect of LPS [$F(1,15) = 56.34$, $p < .0001$] indicating a linear relationship between the dose of LPS and iNOS expression.

Figure 3 shows the effect of LPS on nitrite/nitrate levels in the plasma. The analysis showed a significant effect of LPS on the level of nitrite/nitrate in the plasma [$F(4,15) = 16.54$, $p < .0001$]. There was a significant linear component to the effect of LPS [$F(1,15) = 33.98$, $p < .0001$] indicating a linear relationship between the dose of LPS and nitrite/nitrate iNOS expression. There also was a significant quadratic component [$F(1,15) = 24.78$, $p < .001$] suggesting that the intermediate doses of LPS produced a slight decrease in the level of nitrite/nitrate, with the higher doses producing a pronounced increase in the levels.

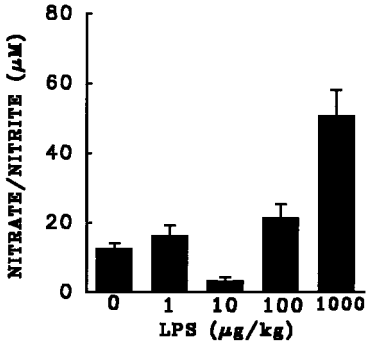

Fig. 3. Dose-dependent effect of LPS (0, 1.0, 10, 100, 1000 μg/kg) on nitrite/nitrate levels in the plasma expressed in μM. Rats were tested 8 h after the injection of LPS.

Figure 4 shows the time-dependent effect of LPS on the expression of nitric oxide. Figure 4A shows the ratio of iNOS/β-actin expression at the different time points. Figure 4B shows the results of densitometric analysis of the Western blot for iNOS protein. Figure 4C shows the time-dependent effect of LPS on nitrite/nitrate levels in the plasma. The analysis showed a significant effect of time on each measure [$Fs(4,15) < 3.46$, $p < .05$]. The results indicate that the peak expression of iNOS mRNA and protein and plasma nitrite/nitrate levels occurs at about 8 h after the injection of LPS.

Figure 5 shows the effect of naltrexone on LPS-induced expression of nitric oxide. Figure 5A shows the effect of LPS on the expression of iNOS mRNA. The analysis of the ratio of iNOS/β-actin showed a significant effect of naltrexone

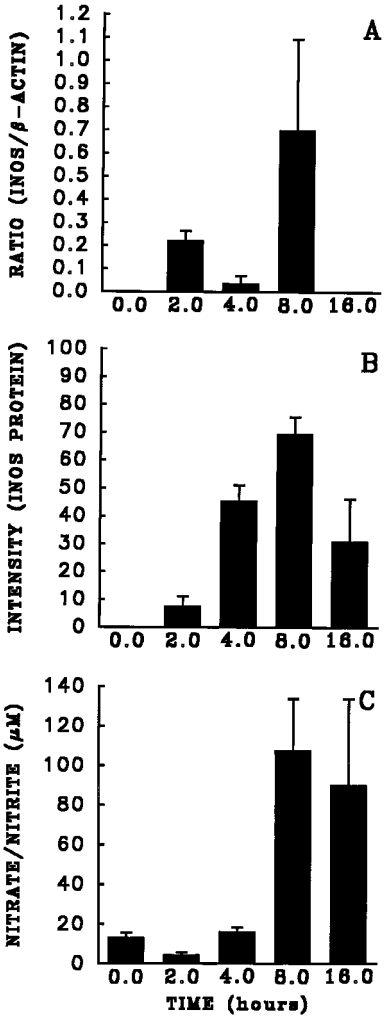

Fig. 4. Time-dependent effect of LPS (100 μg/kg) on the expression of nitric oxide. A, ratio of iNOS/β-actin expression at the different time points. B, results of densitometric analysis of the Western blot for iNOS protein. C, time-dependent effect of LPS on nitrite/nitrate levels in the plasma expressed in μM.

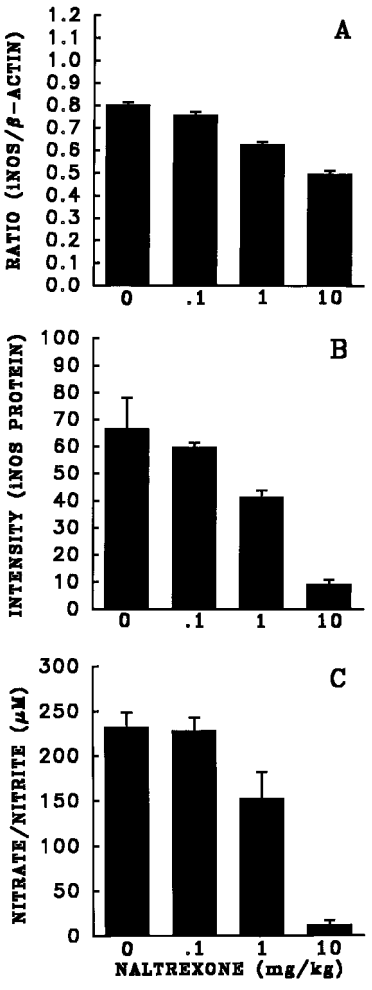

Fig. 5. Effect of naltrexone (0, 0.1, 1.0, 10 mg/kg) on the expression of nitric oxide induced by the injection of LPS (100 μg/kg). Naltrexone was injected at the same time as LPS, and the same dose of naltrexone was administer again 4 h later. Rats were killed 8 h after the injection of LPS. A, effects of LPS on the ratio of iNOS/β-actin mRNA expression as determine by RT-PCR. B, results of the densitometric analysis of the bands from the Western blot for iNOS protein. C, effect of naltrexone on serum nitrite/nitrate levels in the plasma expressed in μM.

[F(3,16) = 22.37, $p < .0001$]. There was a significant linear component to the main effect of naltrexone [F(1,16) = 65.08, $p < .0001$] indicating that increasing doses of naltrexone induced a decrease in iNOS mRNA expression. Contrasts showed that the dose of 0.1 mg/kg naltexone was not significantly different from the saline treatment, but the 1.0 mg/kg dose of naltrexone was significantly different from the saline treatment [F (1,16) = 21.07, $p < .001$]. Moreover, the naltrexone dose of 10 mg/kg was significantly different from the 1.0 mg/kg doses [F(1,16) = 7.73, $p < .03$]. The densitometric analysis of the bands showed no significant effect of naltrexone on the expression of β-actin, indicating that the change in the ratio was the result of a change in iNOS expression.

Figure 5B shows the results of the densitometric analysis of the bands from the Western blot for iNOS protein. The analysis showed that naltrexone induced a significant decrease in the expression of iNOS protein [F(3,16) = 22.27, $p < .0001$]. There was a significant linear component to the effect of naltrexone [F(1,16) = 58.97, $p < .0001$] indicating that the expression of iNOS protein was linearly related to the dose of naltrexone. The linear effect was confirmed by contrasts that showed the dose of 0.1 mg/kg naltexone was not significantly different from the saline treatment, but the 1.0 mg/kg dose of naltrexone was significantly different from the saline treatment [F(1,16) = 7.24, $p < .02$]. Moreover, the naltrexone dose of 10 mg/kg was significantly different from the 1.0 mg/kg doses [F(1,16) = 24.01, $p < .001$].

Figure 5C shows the effect of naltrexone on serum nitrite/nitrate levels in the plasma. The analysis showed that naltrexone induced a significant reduction in the level of nitrite/nitrate in the plasma [F(3,16) = 26.00, $p < .0001$]. There was a significant linear component to the effect of naltrexone [F(1,16) = 66.53, $p < .0001$] indicating that the plasma nitrite/nitrate was linearly related to the dose of naltrexone. Contrasts showed that the dose of 0.1 mg/kg naltexone was not significantly different from the saline treatment, but the 1.0 mg/kg dose of naltrexone was significantly different from the saline treatment [F(1,16) = 7.784, $p < .01$]. The naltrexone dose of 10 mg/kg was significantly different from the 1.0 mg/kg doses [F(1,16) = 24.30, $p < .001$].

Figure 6 shows the effect of i.c.v. administration of *N*-methylnaltrexone on LPS-induced expression of nitric oxide. Figure 6A shows that the effects of LPS on the expression of iNOS mRNA. The analysis of the ratio of iNOS/β-actin showed that *N*-methylnaltrexone induced a decrease in the ratio [F(4,15) = 8.00, $p < .0001$]. Moreover, there is a significant linear component to the main effect of *N*-methylnaltrexone [F(1,15) = 18.02, $p < .001$] indicating that increasing doses of *N*-methylnaltrexone induced a decrease in iNOS mRNA expression. Although there was a significant linear trend, the dose-effect function was more complicated, as contrasts showed that the dose of 0.01 μg of *N*-methylnaltexone was significantly different from the saline treatment [F(1,15) = 5.47, $p < .05$], but the 0.1-μg dose of *N*-methylnaltrexone was not significantly different from the saline treatment. Moreover, the *N*-methylnaltrexone dose of 1.0 μg was significantly different from the saline treatment [F(1,15) = 5.06, $p < .05$], and the 10-μg dose showed an even greater decrease in the expression compared with the 1.0-μg dose [F(1,15) = 6.50, $p < .03$]. The densitometric analysis of the bands showed no significant effect of *N*-methylnaltrexone on the expression of β-actin indicating that the change in the ratio was the result of a change in iNOS expression.

Figure 6B shows the results of the densitometric analysis of the bands from the Western blot for iNOS protein. The analysis showed a significant effect of *N*-methylnaltrexone on the expression of iNOS protein [F(3,15) = 5.74, $p < .01$]. Moreover, there was a significant linear component to the effect of *N*-methylnaltrexone [F(1,15) = 13.94, $p < .001$] indicating that the reduction in the expression of iNOS protein was linearly related to the dose of *N*-methylnaltrexone. Contrasts showed that the dose of 0.01 μg of *N*-methylnaltrexone was significantly different from the saline treatment [F(1,15) = 6.76, $p < .03$], but the 0.1-μg dose was not significantly different from the saline treatment. The *N*-methylnaltrexone dose of 1.0 μg was significantly different from the saline treatment [F(1,15) = 6.15, $p < .03$], and the 10-μg dose showed an even greater decrease in the expression compared with the 1.0-μg dose [F(1,15) = 8.78, $p < .02$].

Figure 6C shows the effect of *N*-methylnaltrexone on serum nitrite/nitrate levels in the plasma. The analysis showed that *N*-methylnaltrexone produced a significant reduction in

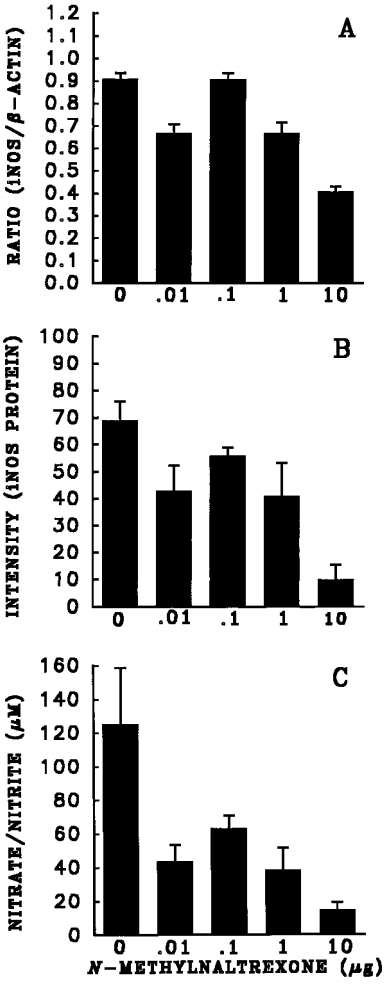

Fig. 6. Effect of i.c.v. administration of *N*-methylnaltrexone (0, 0.01, 0.1, 1.0, 10) on LPS-induced expression of nitric oxide. *N*-Methylnaltrexone was injected at the same time as LPS, and the same dose of *N*-methylnaltrexone was administered again 4 h later. Rats were killed 8 h after the injection of LPS. A, effect of *N*-methylnaltrexone on the ratio of iNOS/β-actin mRNA expression as determined by RT-PCR. B, results of the densitometric analysis of the bands from the Western blot for iNOS protein. C, effect of *N*-methylnaltrexone on serum nitrite/nitrate levels in the plasma expressed in μM.

the level of nitrite/nitrate in the plasma [$F(4,15) = 4.56$, $p < .05$]. There was a significant linear component to the effect of *N*-methylnaltrexone [$F(1,15) = 13.33$, $p < .01$] indicating that the reduction in the level of plasma nitrite/nitrate was linearly related to the dose of *N*-methylnaltrexone. Contrast showed that each dose of *N*-methylnaltrexone was significantly different from the saline treatment [$Fs(1,15) > 5.01$, $ps < 0.05$].

In contrast to the findings with i.c.v. administration of *N*-methylnaltrexone, the peripheral administration of *N*-methylnaltrexone (10 mg/kg) did not significantly alter the production of nitric oxide, as measured by iNOS mRNA expression using RT-PCR, Western blot for iNOS protein, and nitrite/nitrate production [$Fs < 1$], suggesting that peripheral opioid receptors are not involved in the regulation of nitric oxide production by splenocytes.

Discussion

The present study tested the hypothesis that LPS-induced expression of iNOS by splenocytes is modulated through the activation of endogenous opioids in the central nervous system. The initial studies showed that the injection of LPS produced dose- and time-dependent effects on the expression of both iNOS mRNA and protein in the spleen, as well as the accumulation of nitrite/nitrate in plasma. Interestingly, the opioid antagonist naltrexone was found to induce a pronounced dose-dependent reduction in iNOS mRNA and protein expression and the level of nitrite/nitrate in the plasma. Furthermore, the data indicate that the modulation of iNOS expression occurs via central opioid receptors; *N*-methylnaltrexone administered i.c.v. showed effects similar to those found with peripheral administration of naltrexone. Moreover, the peripheral administration of *N*-methylnaltrexone did not significantly alter the expression of iNOS mRNA, iNOS protein, or the level of plasma nitrate/nitrite. Taken together, these findings demonstrate that central opioid receptors are involved in the in vivo regulation of splenic nitric oxide production.

These findings raise several important issues, such as how peripheral injection of LPS stimulates central opioid receptors and how the activation of central opioid receptors induces alterations in the production of iNOS by splenocytes. Although the pathways by which LPS signals the brain have not been conclusively identified, there is growing evidence for an afferent pathway via the peripheral nervous system. For example, subdiaphragmatic vagotomy has been shown to decrease LPS- or interleukin-1-induced fever (Watkins et al., 1994; Sehic and Blatteis, 1996) and reduced food intake (Bret-Dibat et al., 1995). Furthermore, subdiaphragmatic vagotomy has been shown to decrease LPS- or cytokine-induced elevation of ACTH release, plasma corticosteroid levels, and hypothalamic norepinephrine depletion (Fleshner et al., 1995; Gaykema et al., 1995) and c-*fos* expression in the brain (Wan et al., 1994; Gaykema et al., 1995). Thus, it is possible that LPS induces activation of opioid receptors in the brain via the vagus nerve.

The involvement of central opioid receptors in the modulation of splenic nitric oxide also requires a neural/endocrine pathway by which central opioid activity induces alterations of immune status. The sympathetic nervous system and the hypothalamic-pituitary-adrenal axis are two possible pathways that may mediate central opioid-induced immune alterations. Activation of central opioid receptors has been shown to increase the activity of both the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis (George and Way, 1959; Appel et al., 1986). Moreover, lymphoid organs are innervated by sympathetic nerve fibers (Felten et al., 1985), and immunocytes express functional *alpha* and *beta* adrenergic receptors with which the sympathetic neurotransmitters epinephrine and norepinephrine interact (Hadden et al., 1970; McPherson and Summers, 1982; Abrass et al., 1985). The evidence from our laboratory indicates that *beta* adrenergic receptor antagonists block opioid-induced decreases in splenic lymphocyte proliferation, suggesting that some of the central effects of opioids are mediated by the sympathetic nervous system (Fecho et al., 1993). In addition, both chlorisondamine, a ganglionic blocker, and adrenalectomy antagonize the suppressive effects of morphine on the proliferative response of splenic lymphocytes (Fecho et al., 1996b). There also are a number of in vitro studies showing that exposure to catecholamines induce changes in macrophage activity. For example, it has

been shown that epinephrine and norepinephrine stimulate murine macrophages to suppress the growth of *Mycobacterium avium* (Miles et al., 1996). Other studies have shown that norepinephrine and epinephrine increase tumor necrosis factor and hydrogen peroxide production in rat macrophages (Hu et al., 1991; Costa Rosa et al., 1992). These results suggest that sympathoadrenal activity may be involved in the alterations of nitric oxide production after the administration of LPS. This suggestion is consistent with findings showing that the systemic injection of LPS at doses between 10 and 100 μg to rats induces an increase in splenic nerve activity up to about 175% of the control level (MacNeil et al., 1996).

Nitric oxide plays an important role in disease processes. For example, the role of nitric oxide in infectious disease is demonstrated by studies showing that it is involved in the cytostatic activity of macrophages on parasitic growth (Vincendeau et al., 1992). Moreover, mice lacking inducible nitric oxide synthase have markedly reduced resistance to parasitic and bacterial infection (MacMicking et al., 1995; Wei et al., 1995). In addition to the direct impact of nitric oxide on infectious organisms, nitric oxide has important regulatory functions in the immune system. There is evidence strongly supporting a role for nitric oxide in the suppression of antibody formation to tetanus toxoid and sheep red blood cells after immunization with *Salmonella typhimurium* (Al-Ramadi et al., 1992; Eisenstein et al., 1994). In addition, studies have shown that nitric oxide limits the proliferative activity of lymphocytes (Albina and Henry, 1991; Pascual et al., 1993). The present work suggests that opioid antagonists can be used to regulate nitric oxide production in vivo. This suggestion is consistent with other studies showing that ventriculocisternal administration of naloxone protects against severe hypotension during endotoxin shock (Janssen and Lutherer, 1980).

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