Inhibition of Lipopolysaccharide-Induced Nitric Oxide and Cytokine Production by Ultralow Concentrations of Dynorphins in Mixed Glia Cultures

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ABSTRACT
Dynorphins (dyn) are a major class of endogenous opioid peptides that modulate the functions of immune cells. However, the effects of dyn on the immune functions of glial cells in the central nervous system (CNS) have not been well characterized. Because nitric oxide (NO) and the proinflammatory cytokine tumor necrosis factor-α (TNF-α) produced by glial cells are involved in various pathophysiological conditions in the CNS, this study examined the effects of dyn on the production of NO and TNF-α from mouse glial cells treated with lipopolysaccharide (LPS). LPS induced a concentration-dependent increase in the production of NO or TNF-α from the mouse primary mixed glia cultures. Ultralow concentrations (10^{-16}-10^{-12} M) of dynorphin (dyn) A-(1–8) significantly inhibited the LPS-induced production of NO or TNF-α. The inhibitory effects of dyn A-(1–8) were not blocked by nor-binaltorphimine, a selective κ opioid receptor antagonist. U50-488H, a selective κ opioid receptor agonist, did not affect the LPS-induced production of NO or TNF-α. Ultralow concentrations (10^{-16}-10^{-12} M) of des-[Tyr^{1}]-dyn A-(2–17), a nonopioid analog that does not bind to κ opioid receptors, exhibited the same inhibitory effects as dyn A-(1–17) and dyn A-(1–8). These results suggest that dyn modulate the immune functions of microglia and/or astrocytes in the brain and these modulatory effects of dyn are not mediated by classical κ opioid receptors.

Numerous results suggest a bidirectional communication between the neuroendocrine and immune systems (Blalock, 1989; Stefano, 1989; Ader et al., 1990; Carr and Blalock, 1991). This evidence includes the observations that neuropeptides and neuroendocrine hormones can influence the functions of the monocytes and lymphocytes of the immune system, and cytokines produced by the activated immune cells can modulate the synthesis of neuropeptides. Specifically, the proinflammatory cytokine IL-1α increases the endogenous concentrations of the opioid peptide β-endorphin in the rat (Sacerdote et al., 1994). Endorphins enhance the response of T cells or bone marrow macrophages to mitogenic stimulation (Apte et al., 1990; Heijnen et al., 1991) and [D-Ala²]-methionine enkephalinamide, a metabolically stable analog of [Met²]-enkephalin, inhibits the production of reactive oxygen species in mouse peritoneal macrophages and human neutrophils (Zaitsev et al., 1991; Efannov et al., 1994). Work from our laboratory has shown that [Met²]-enkephalin inhibits LPS-stimulated IL-1β production by murine microglia (Das et al., 1995). Dynorphin (dyn) A-(1–8) significantly inhibited the LPS-induced production of NO or TNF-α.

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ABBREVIATIONS: LPS, lipopolysaccharide; NO, nitric oxide; TNF-α, tumor necrosis factor-α; IL, interleukin; dyn, dynorphin; nor-BNI, nor-binaltorphimine; CNS, central nervous system; HIV, human immunodeficiency virus; iNOS, inducible NO synthase.
Gehrmann et al., 1995). NO and the proinflammatory cytokine TNF-α produced by glial cells are involved in various pathophysiological conditions in the CNS. NO has pleiotropic effects in the CNS, including vasodilation, neurotransmission, cytotoxicity, and antimicrobial activity (Moncada et al., 1991). Excessive production of NO in the CNS could be toxic to many different cell types, including neurons (Dawson et al., 1993; Hewett et al., 1994; Bronstein et al., 1995; McMillian et al., 1995). TNF-α is a multifunctional cytokine, influencing both physiological and pathological conditions in the CNS. During diseases and trauma, TNF-α has been associated with lesioned white matter and microglial node formation (Selma and Raine, 1988). There are various agents that stimulate proinflammatory cytokine production in cells of the CNS. One of these stimuli is LPS, the bacterial endotoxin that is widely used to study experimentally induced infection, inflammation or tissue damage. Because NO and TNF-α produced by glial cells play important roles in the CNS, in our study, we examined the effects and mechanisms of action of dyn A-(1–8) on the LPS-induced production of NO and TNF-α in mouse primary mixed glia cultures.

Materials and Methods

Reagents. LPS (Escherichia coli O111:B4) was purchased from LIST Biological Laboratory, Inc. (Campbell, CA). Dyn A-(1–8), dyn A-(1–17), and des-[Tyr1]-dyn A-(2–17) were ordered from Peninsula Laboratories, Inc. (Belmont, CA). Naloxone and nor-BNI were purchased from Biochemicals, Inc. (Natick, MA), respectively. U50-488H was obtained from Mannheim Biochemicals, Indianapolis, IN). Naxolone and nor-BNI were purchased from Sigma Chemical Company (St. Louis, MO) and Research Biochemicals, Inc. (Natick, MA), respectively. U50-488H was obtained from Sigma Chemical Company. Fetal bovine serum and Dulbecco's modified Eagle's medium with Ham's nutrient mixture F12 were obtained from GIBCO-BRL (Gaithersburg, MD).

Cell cultures. Primary mixed glia cultures were prepared from the brains of newborn CD-1 mice (McMillian et al., 1992). Briefly, the whole brain was removed aseptically and the blood vessels and membranes were carefully removed. Brains were dissociated by treatment in ice-cold Ca2+- and Mg2+-free W3 buffer (145 mM NaCl, 5.4 mM KCl, 1 mM NaH2PO4, 15 mM HEPES, pH 7.4 and 11 mM glucose), and brain cells were pelleted by centrifugation. The cells were adjusted to a concentration of 106 cells/ml in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 media containing 10% heat-inactivated fetal bovine serum. One-half ml of the cell suspension (5 x 105 cells) was added into each well of 24-well Costar tissue culture plates. The medium was replenished 1 and 4 days after plating, and was changed every 3 days thereafter. Cells were used 13 to 15 days after plating. When antibodies against Mac-1 (Boehringer Mannheim Biochemicals, Indianapolis, IN), a marker for microglia, were used, about 30% of the cells in the cultures were Mac-1+ cells. Antibodies to glial fibrillary acidic protein (DAKO Corporation, Carpinteria, CA), a specific marker for astrocytes, stained 65% of the cells in the culture positively.

Stimulation of mixed glia cultures. The mixed glia cultures were stimulated as follows: 1) LPS (10 to 1000 mg/ml); 2) LPS (10 mg/ml) with dyn A-(1–8) (10–6 to 10–6 M); 3) LPS (10 mg/ml) with naloxone (10–10 to 10–8 M); 4) LPS (10 mg/ml) with nor-BNI (10–10 to 10–6 M); 5) LPS with dyn A-(1–8) (10–14 M), LPS with dyn A-(1–8) and nor-BNI (10–6 M); 6) LPS (10 mg/ml) with U50-488 (10–16 to 10–6 M); 7) LPS (10 mg/ml) with dyn A-(1–17) (10–16 to 10–6 M) or with dyn A-(2–17) (10–16 to 10–6 M).

Nitrïte assay. The production of NO was assessed as the accumulation of nitrite in the culture supernatants, using a colorimetric reaction with the Griess reagent (Stuehr and Nathan, 1989). The culture supernatants were collected after 48 hr of LPS-stimulation (Dawson et al., 1994) and mixed with equal volumes of the Griess reagent (0.1% N-[1-naphthyl]ethylene diamine dihydrochloride, 1% sulfanilamide and 2.5% H3PO4). The absorbance at 540 nm was measured with an UV MAX kinetic microplate reader (Molecular Devices, Menlo Park, CA). The nitrite concentration was determined from a sodium nitrite standard curve. The sensitivity of this assay was approximately 0.5 μM.

TNF-α assay. The culture supernatants were collected after 6 hr of LPS-stimulation for the measurement of TNF-α, which was based on previous studies (Chao et al., 1992a) and pilot studies in our laboratory. The quantity of TNF-α was measured with a mouse TNF-α enzyme-linked immunosorbent assay kit from Genzyme (Cambridge, MA). The detection limit for the TNF-α enzyme-linked immunosorbent assay kit was 15 pg/ml; the antibodies in the kit did not have any detectable cross-reactivity with other antigens.

Statistics. Data were expressed as the mean ± S.E. Analysis of variance followed by Dunnet's multiple comparison test was used for statistical comparisons. In some instances where it was appropriate, e.g., when there were only two groups in the comparison, the two-tailed Student's t test was used. P < .05 was considered significant.

Results

Our laboratory has established a mixed glia culture system, a model that more closely mimics the physiological conditions in the brain than do microglia-enriched or astrocyte-enriched cultures (McMillian et al., 1992; Das et al., 1995; Kong et al., 1996a; Kong et al., 1996b). To study the effects of LPS on the production of NO or TNF-α by glial cells, primary mixed glia cultures were treated with various concentrations of LPS (10–1000 ng/ml). The base-line releases (medium alone) of NO and TNF-α were below the detection limit of the assays, less than 0.5 μM and 15 pg/ml, respectively. LPS (10–1000 ng/ml) increased the levels of NO or TNF-α in a concentration-dependent manner (data not shown), indicating that glial cells activated by LPS produce NO and TNF-α.

The effects of dyn A-(1–8), an opioid receptor agonist, on the LPS-induced production of NO or TNF-α from glial cells. To investigate whether dyn modulate the immune functions of glial cells, this study examined the effects of dyn A-(1–8) (10–16 to 10–6 M) on the LPS (10 ng/ml)-induced production of NO and TNF-α in mouse primary mixed glial cell cultures. Dyn A-(1–8) inhibited the LPS-induced production of NO or TNF-α with a U-shaped concentration-response effect (fig. 1). The inhibitory effects of dyn A-(1–8) were at ultralow concentrations, 10–10 to 10–12 M (fig. 1). The maximal inhibition of NO and TNF-α secretion, 29.4 and 39.5% of the LPS-stimulated control levels, respectively, was observed at 10–14 M dyn A-(1–8). The maximal inhibition of IL-1α and IL-6 secretion, 32.3 and 25.4% of the LPS-stimulated control levels, respectively, was also observed at 10–14 M dyn A-(1–8) (data not shown).

The effects of naltrexone, a general opioid receptor antagonist, on the LPS-induced production of NO or TNF-α from glial cells. To study whether dyn A-(1–8) affected the production of NO or TNF-α from LPS-stimulated glial cells via opioid receptors, we intended to examine the effects of naltrexone, a general opioid receptor antagonist (μ, δ and κ types), on the dyn A-(1–8)-inhibited production of NO or TNF-α. The effects of naltrexone alone on the LPS-induced NO or TNF-α production were first examined. Naltrexone (10–10 to 10–5 M) concentration-dependently suppressed the LPS-induced production of NO or TNF-α (fig. 2). Specifically,
Naloxone reduced the LPS-induced production of NO and TNF-α by 55 and 21%, respectively (fig. 3). Compared with the medium control, there were no significant differences in the levels of lactate dehydrogenase, an indicator for cell injury or death, in naloxone-treated glial cells (data not shown), indicating that the selected doses of naloxone are not toxic to the cells. Because naloxone itself blocked the LPS-induced NO or TNF-α production, it cannot be used as an antagonist of opioid receptors to determine whether the inhibitory effects of dyn A-(1–8) are due to binding with classical κ receptors, the preferential opioid receptor for dyn A-(1–8). Therefore, this study examined the effects of nor-BNI, a selective κ opioid receptor antagonist, on the dyn A-(1–8)-inhibited production of NO or TNF-α from LPS-stimulated glial cells.

**Lack of effect of nor-BNI, a selective κ opioid receptor antagonist, on the dyn A-(1–8)-inhibited production of NO or TNF-α from LPS-stimulated glial cells.**

The effects of nor-BNI alone on the LPS-induced production of NO or TNF-α in primary mixed glia cultures were first examined. None of the three concentrations (10^{-10}–10^{-6} M) of nor-BNI affected the LPS-induced NO or TNF-α production (data not shown). The highest concentration of nor-BNI (10^{-6} M) was used to examine the effects of nor-BNI on the dyn A-(1–8)-inhibited production of NO or TNF-α from LPS-stimulated glial cells. The inhibitory effects of dyn A-(1–8) on the LPS-induced production of NO or TNF-α were not blocked by nor-BNI (fig. 3).

**Lack of effect of U50-488H, a selective κ opioid receptor agonist, on the LPS-induced production of NO or TNF-α from glial cells.**

This study also examined the effect of U50-488H, a selective κ opioid receptor agonist, on the LPS-induced NO or TNF-α production in mixed glial cultures. U50-488H (10^{-10}–10^{-6} M) did not affect the LPS-induced NO or TNF-α production (fig. 4).
The effects of dyn A-(1–17) and dyn A-(2–17) on the LPS-induced production of NO or TNF-α from glial cells. To further understand whether the κ receptor is involved in the inhibitory effects of dyn A-(1–8), this study examined the effects of dyn A-(1–17), an extended and fully active opioid analog of dyn A-(1–8), and dyn A-(2–17), an analog of dyn that does not bind to κ opioid receptors, on the LPS-induced NO or TNF-α production in mixed glia cultures. Both dyn A-(1–17) and dyn A-(2–17) suppressed the LPS-induced production of NO or TNF-α with a U-shaped dose-response effect (fig. 5). The inhibitory effects of both dyn A-(1–17) and dyn A-(2–17) were at ultralow concentrations (10^{-2} to 10^{-12} M) (fig. 5).

Discussion

In addition to their role in nociceptive/analgesic systems, dyn also modulate cell functions in the immune system (Prete et al., 1986; Foster and Moore, 1987; Tosk et al., 1993; Ichinose et al., 1995). However, few data have been provided about the effects of dyn on the immune functions of glial cells in the CNS (Chao et al., 1995a). Our study investigated the effects of dyn on the immune secretory functions of glial cells in the brain. Ultralow concentrations (10^{-16} to 10^{-12} M) of dyn inhibited the LPS-induced production of NO and TNF-α in murine primary mixed glia cultures. The inhibitory effects of dyn were not blocked by nor-BNI, a selective κ opioid receptor antagonist. U50-488H, a selective κ opioid receptor agonist, did not inhibit the LPS-induced production of NO and TNF-α. In addition, dyn A-(2–17), a nonopioid dyn analog, exhibited the same inhibitory effects as the classical opioid agonist dyn A-(1–17). These results suggest that dyn modulate immune functions of microglia and/or astrocytes in the brain and the modulatory effects of dyn are not mediated by classical κ opioid receptors.

The intriguing finding in our study was the inhibitory effects of ultralow concentrations (10^{-16} to 10^{-12} M) of dyn on the LPS-induced production of NO and TNF-α in the mixed glia cultures (figs. 1 and 5). Opioid peptide actions on immune cells at ultralow concentrations have recently been observed in several laboratories (Williamson et al., 1988; Zaitsev et al., 1991; Efano et al., 1994; Chao et al., 1995b). For example, β-endorphin at ultralow concentrations (10^{-18} to 10^{-14} M) enhanced the production of specific anti-herpes virus antibodies from human lymphocytes (Williamson et al., 1988). [D-Ala²]-Methionine enkephalinamide inhibited the production of reactive oxygen species in mouse peritoneal macrophages and human neutrophils at the low concentrations of 10^{-16} to 10^{-13} M (Zaitsev et al., 1991; Efano et al., 1994). Low concentrations (10^{-12} to 10^{-11} M) of [Met²]-enkephalin or dyn A-(1–13) inhibited the IL-6-induced up-regulation of HIV-1 expression in the chronically infected promonocyte clone U1 (Chao et al., 1995b). In our study, the inhibitory effects of dyn disappeared with increasing concentrations of dyn (above 10^{-12} M; figs. 1 and 5), indicating that dyn at ultralow concentrations and higher concentrations may interact with different types of receptors.

The receptors that bound ultralow concentrations of dyn are not characterized. All three of the major classes of opioid receptors, δ, κ and μ, as well as atypical opioid receptors have...
been characterized in the brain and cells of the immune system (Simon, 1986; Sibinga and Goldstein, 1988; Carr, 1991). Dyn are preferential agonists for the κ opioid receptor. However, our data that the inhibitory effects of the ultralow concentrations of dyn were not blocked by nor-BNI, a selective κ opioid receptor antagonist, and U50-488H, a selective κ opioid receptor agonist, did not inhibit the LPS-induced production of NO and TNF-α, indicate that the κ opioid receptors are not involved in these inhibitory effects of dyn (figs. 3 and 4). In addition, the observation that ultralow concentrations of dyn A-(2–17), a nonopioid dyn analog that is not able to bind to κ opioid receptors, exhibited the same inhibitory effects as the corresponding opioid agonist dyn A-(1–17) also indicates the lack of involvement of κ opioid receptors in these inhibitory effects of dyn (fig. 5). Furthermore, the ultralow concentrations (10^{-16}–10^{-12} M) of dyn-producing effective inhibition are well below the $K_i$ for most of the known opioid receptors ($K_i$ approximately 10^{-9} M). Taken together, our data suggest that a novel ultrahigh-affinity receptor mediates these inhibitory effects of dyn.

It is also interesting that naloxone, a opioid receptor antagonist (δ, κ and μ types), had inhibitory effects on the LPS-induced production of NO and TNF-α as had the dyn, which are opioid receptor agonists (fig. 2). Naloxone has been reported to inhibit antibody, IL-1β and T-lymphocyte chemotactic factor production as well as the IL-6-induced up-regulation of HIV-1 expression (Johnson et al., 1982; Brown and Van Epps, 1985; Das et al., 1995; Chao et al., 1995b). The inhibitory effects of naloxone on the LPS-induced production of NO and TNF-α from glial cells in this study suggest that endogenous opioid peptides are involved in the LPS-induced NO or cytokine production. Alternatively, naloxone may activate a novel receptor that then mediates the inhibition.

Reports describing the relative contributions of microglia vs. astrocytes to the production of NO and TNF-α differ. The induction of iNOS expression in astrocytes has been reported (Feinstein et al., 1994). However, the level of NO in interferon-γ-LPS-stimulated purified astrocyte cultures has been reported to be unmeasurable (Chao et al., 1992b). To verify the cellular sources of NO, our laboratory has done the immunocytochemical staining for iNOS followed by staining with the microglia marker Mac-1 or the astrocyte marker glial fibrillary acidic protein in microglia-enriched or astrocyte-enriched cultures, respectively. The expression of iNOS and the production of NO in the LPS-stimulated microglia-enriched cultures were significantly higher than those in the identically stimulated astrocyte-enriched cultures, indicating that microglia are more responsive than astrocytes to LPS that induces NO (Kong et al., 1996a). Lieberman et al. (1989) and Chung and Benveniste (1990) reported that astrocytes in brain cell cultures produce TNF-α. Hetier et al. (1990) and Giulian et al. (1994) reported that microglia, but not astrocytes, synthesize TNF-α in brain cell cultures. Our laboratory has used in situ hybridization for TNF-α mRNA in LPS-stimulated microglia-enriched or astrocyte-enriched cultures followed by immunocytochemistry for either Mac-1 or glial fibrillary acidic protein to verify the cellular sources of this cytokine. Our results demonstrated that the mRNA for TNF-α was induced by LPS mainly in Mac-1+ cells, indicating that microglia are the major sources of TNF-α (L.-Y. Kong, unpublished observations). Dyn may modulate immune functions of microglia and/or astrocytes in the brain. The types of glial cells on which dyn act need further study.

The modulation by dyn and naloxone on the LPS-induced production of NO and TNF-α from glia cells in our study indicates an additional interaction between the immune and neuroendocrine systems. Because the ultralow concentrations (10^{-16}–10^{-12} M) of dyn are well within the range of physiological concentrations of dyn in the brain, the data in this study suggest that dyn are endogenous immune modulators in the brain. The inhibitory effects of dyn may have physiological relevance to inflammation and brain injury in the CNS because NO and TNF-α are involved in various pathophysiological changes in the CNS. The mechanisms of the action of ultralow concentrations of dyn and the receptors involved require further study.

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References


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