Femtomolar Concentrations of Dynorphins Protect Rat Mesencephalic Dopaminergic Neurons against Inflammatory Damage

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ABSTRACT

The hallmark of Parkinson’s disease is the death of nigral dopaminergic neurons, and inflammation in the brain has been increasingly associated with the pathogenesis of this neurological disorder. Dynorphins are among the major opioid peptides in the striato-nigral pathway and are important in regulating dopaminergic neuronal activities. However, it is not clear whether dynorphins play a role in the survival of nigral dopaminergic neurons. We have recently demonstrated that lipopolysaccharide (LPS) activates the brain immune cells microglia, in vitro and in vivo, to release neurotoxic factors to degenerate dopaminergic neurons. The purpose of this study was to explore the neuroprotective effect of dynorphins in the inflammation-mediated degeneration of dopaminergic neurons in rat midbrain neuron-glia cultures. LPS-induced neurotoxicity was significantly reduced by treatment with ultra low concentrations (10^{-13}–10^{-15} M) of the κ-opioid receptor agonist dynorphin A (1–17) or the receptor binding ineffective [des-Tyr^1]dynorphin A (2–17), but not by U50488, a synthetic κ-receptor agonist. The glia-mediated neuroprotective effect of dynorphins was further supported by the finding that femtomolar concentrations of dynorphins did not prevent the killing of dopaminergic neurons by 6-hydroxydopamine. However, ultra low concentrations of dynorphins inhibited LPS-induced production of superoxide. These results suggest a glia-mediated and conventional opioid receptor-unrelated mechanism of action for the neuroprotective effect of ultra low concentrations of dynorphins. Understanding the underlying mechanisms of action should further define the roles of dynorphins in the regulation of dopaminergic neurons and help devise novel strategies to combat neurodegenerative diseases.

The family of endogenous opioid peptides includes dynorphins, enkephalins, and β-endorphins that are widely distributed throughout the central nervous system (CNS), as well as peripheral tissues such as cardiac myocytes and heart tissues (Smith and Lee, 1988; Herz, 1993; Barron, 2000). In the CNS, opioid peptides are known to possess diverse biological activities, including effects on respiration, immune responses, and ion channel activity, with the best studied effects on the nociceptive/analgesic systems (Roy and Loh, 1996). Molecular cloning and ligand binding studies have identified three classes of receptors for opioids: δ, κ, and μ (Minami and Satoh, 1995; Jordan et al., 2000). They are all G-protein-coupled transmembrane receptors with distinctive affinities to various opioid peptides.

Biactive forms of opioid peptides are derived from precise proteolysis of inactive precursor polypeptides known as pro-
dynorphin, preproenkephalin, and prepro-opioiimelanocortin (Kieffer, 1995). In the case of dynorphins, prodynorphin is cleaved into smaller fragments with dynorphin A (1–17) being the most abundant form that possesses full biological and receptor binding activity. Dynorphin A (1–17) has a slight preference for binding κ over μ- and δ-opioid receptors with dissociation constants in the range of 10^{-9} M (Knapp et al., 1995; Minami and Satoh, 1995). Studies have shown that the N-terminal tyrosine in dynorphin A (1–17) is required for binding to opioid receptors (Naqvi et al., 1998). Diverse activities have been reported for dynorphins in various regions of the brain. In the hippocampus, dynorphins modulate granule cell-mediated excitatory transmission and the activity of multiple ion channels, which are important in mediating temporal lobe epilepsy (Simnato and Romualdi, 1996; Jeub et al., 1999; Terman et al., 2000). On the other hand, the biosynthesis of dynorphins is regulated in an activity-dependent manner (Hong, 1992). In the midbrain region, especially the nigrostriatal pathways, dynorphins are one of the major

ABBREVIATIONS: CNS, central nervous system; LPS, lipopolysaccharide; TH, tyrosine hydroxylase; 6-OHDA, 6-hydroxydopamine; SOD, superoxide dismutase; MEM, minimum essential medium; FBS, fetal bovine serum; TNFα, tumor necrosis factor-α; NO, nitric oxide; IR, immunoreactive.

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neurotransmitters, such as substance P and neuropeptides and are thought to behave like neurotransmitters (Gerfen and Young, 1988; Anderson and Reiner, 1990; Reiner et al., 1999). Studies from several groups have shown that dynorphins, along with other striatonigral neurotransmitters, such as substance P and γ-aminobutyric acid, regulate the activity of nigrostriatal dopamine projection through modulation of dopamine release (Reid et al., 1990; Steiner and Gerfen, 1998; You et al., 1999). Conversely, the nigrostriatal dopaminergic system also serves to regulate the levels of dynorphin through modulation of its biosynthesis (Li et al., 1990; Jiang et al., 1990; Engber et al., 1992).

The hallmark of Parkinson's disease is the highly selective and progressive degeneration of dopaminergic neurons in the substantia nigra. Despite decades of research, the etiology and pathogenesis of Parkinson's diseases remain poorly understood. In recent years, inflammation in the brain has been closely related to the development of this degenerative disease (McGeer et al., 1988; Jenner and Olanow, 1996). Brain inflammation mainly involves the activity of astroglia and microglia, the resident immune cells of the CNS. Under normal conditions, microglia play the role of immune surveillance and astroglia serve to maintain the survival of neurons by secreting nerve growth factors and buffering the action of neurotransmitters (Kreutzberg, 1996; Aloisi, 1999). However, glial cells become readily activated in response to injury and immunological challenges (Aschner, 1998). Activated microglia, especially microglia, produce a variety of proinflammatory and/or cytotoxic factors, including oxygen- and nitrogen-centered free radicals, cytokines, and eicosanoids (Chao et al., 1992; McGeer and McGeer, 1995; Mignetti and Levi, 1998; Liu et al., 2000a). Accumulation and/or overproduction of some if not all of these factors impact on neurons to induce their degeneration (Jeohn et al., 1998; Kim et al., 2000; Liu et al., 2000a). Astrocytes, on the other hand, are capable of producing various trophic factors that are beneficial to the survival of neurons (Ridet et al., 1997; Yoshida and Toya, 1997).

Using mixed neuron-glia cultures derived from rat mesencephalon, which encompasses the substantia nigra region of the brain, we have recently demonstrated that activation of glia induced by the inflammasome lipopolysaccharide (LPS) resulted in the degeneration of dopaminergic neurons (Kim et al., 2000; Liu et al., 2000a,b). Since dynorphins are one of the major modulators of the activity of striatonigral projections in the midbrain region, we set out to investigate their effect on the inflammation-mediated degeneration of dopaminergic neurons. In this report, we show that fentomolar concentrations of both an opioid receptor agonist dynorphin A (1–17) and the receptor binding ineffective dynorphin A (2–17) protect dopaminergic neurons from LPS-induced damage. The potential mechanisms of action for the neuroprotective effects of ultra low concentration of dynorphin peptides are discussed.

Materials and Methods

Reagents. Dynorphin A (1–17) and dynorphin A (2–17) were obtained from Phoenix Pharmaceuticals Co. (Belmont, CA). Cell culture ingredients were obtained from Life Technologies (Grand Island, NY). [7,8-3H]Dopamine (40 Ci/mmole) was purchased from PerkinElmer Life Science (Boston, MA). The polyclonal antibody against tyrosine hydroxylase (TH) was a kind gift from Dr. John Reinhard of Glaxo-Wellcome Co. (Research Triangle Park, NC). VECTASTAIN ABC kit and biotinylated secondary antibodies were obtained from Vector Laboratories ( Burlingame, CA). LPS (Escherichia coli 0111:B4) was obtained from Calbiochem (La Jolla, CA). Poly-d-lysine was purchased from Sigma (St. Louis, MO). 6-Hydroxydopamine (6-OHDA) was purchased from Aldrich (Milwaukee, WI). Polypropylene round-bottomed culture tubes and microcentrifuge tubes were purchased from Becton Dickinson Labware (Lincoln Park, NJ). Superoxide dismutase (SOD), partially acetylated ferricytochrome c, and U50488 were obtained from Sigma.

Primary Cultures. Rat mesencephalic mixed neuron-glia cultures were prepared from E14/15 Fischer 344 rat brains following our previously published protocol (Liu et al., 2000a). The mesencephalon was obtained aseptically, and the blood vessels and meninges were removed. Pooled mesencephalic tissues were dissociated by mild mechanical trituration in ice-cold calcium- and magnesium-free W3 buffer (145 mM NaCl, 5.4 mM KCl, 1 mM NaH2PO4, 15 mM HEPES, and 11 mM glucose; pH 7.4; 7.5 × 105 cells/well were seeded in poly-d-lysine-coated (20 μg/ml) 24-well culture cell plates (Costar, Cambridge, MA). Cells were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air in 0.5 ml (per well) of minimum essential medium (MEM) containing 10% heat-inactivated fetal bovine serum, 10% heat-inactivated horse serum, 1 g/l glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 50 U/ml penicillin, and 50 μg/ml streptomycin. Three days later, cultures were replenished with 0.5 ml (per well) of fresh medium. Six days later, cultures were used for treatment. Immunocytochemical analysis with cell-type specific antibodies (Liu et al., 2000a) indicated that at the time of treatment, the cultures were made up of approximately 15% microglia, 45% astrocytes, and 40% neurons, of which 1 to 2% were TH-positive neurons.

Treatment. Dynorphins were reconstituted in sterile-filtered (0.2 μm) phosphate-buffered saline (pH 7.4) containing 0.1% essentially fatty acid-free bovine serum albumin (Sigma). Stock solutions (500 μM) were immediately divided into small aliquots and stored at −70°C in sterile 1.5-ml polypropylene microcentrifuge tubes. For treatment of cultures, stock solutions were serially diluted (10×) in sterile round-bottomed polypropylene culture tubes with fresh culture medium containing 2% each of fetal bovine and horse serum. Cultures were pretreated with dynorphins for 30 min prior to treatment with LPS. Control cultures were treated with culture medium containing appropriate amounts of vehicle diluted in the same manner as dynorphins.

[3H]Dopamine Uptake Assay. High-affinity dopamine uptake was performed as described (Liu et al., 2000a). Briefly, cells were washed (two times) with warm Krebs-Ringer buffer (16 mM NaH2PO4, 16 mM Na2HPO4, 119 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl2, 1.2 mM MgSO4, 1.3 mM EDTA, and 5.6 mM glucose; pH 7.4). Cells were then incubated for 20 min at 37°C with 25 nM [3H]dopamine in Krebs-Ringer buffer. Afterward, cells were washed (three times) with ice-cold Krebs-Ringer buffer, lysed with 1 N NaOH, and mixed with CytoScint scintillation fluid (ICN, Costa Mesa, CA) to determine radioactivity. Nonspecific dopamine uptake was determined in the presence of the neuronal high-affinity dopamine uptake inhibitor maxindol (10 μM).

Measurement of Superoxide Production. The amount of superoxide was determined by measuring the SOD-inhibitable reduction of cytochrome c as previously described (Liu et al., 2000a). Briefly, neuron-glia cultures grown in 96-well plates (5 × 104 cells/well) were pretreated for 30 min with dynorphin in phenol red-free MEM containing 2% heat-inactivated FBS. Afterwards, the medium was removed, and 100 μl of phenol red-free MEM containing 2% heat-inactivated FBS, 50 μl of LPS (10 ng/ml), and 50 μl of ferricytochrome c (100 μM) in MEM containing 2% heat-inactivated FBS were added to each well. After incubation for 90 min at 37°C, the absorbance at 550 nm was read with a Spectra Max Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). All measurements were performed in quadruplicate. The amount of SOD-
inhibitable superoxide produced was calculated by subtracting the absorbance observed in the presence of SOD (300 U/ml) in quadruplicate sister wells from that observed in the absence of SOD.

**Nitrite and TNFα Assays.** The amount of TNFα released into the culture supernatant was determined as previously described (Liu et al., 2000a) using an enzyme-linked immunosorbent assay kit from Genzyme Diagnostics (Cambridge, MA). The production of nitric oxide (NO) was determined by measuring the level of accumulated nitrite, a metabolite of NO in the culture supernatant using the Griess reagent (Green et al., 1982) as described (Liu et al., 2000a).

**Immunocytochemical Analysis.** Cultures were immunostained for dopaminergic neurons with an anti-TH antibody as described (Liu et al., 2000a). Briefly, paraformaldehyde (3.7%)-fixed cells were treated for 10 min with hydrogen peroxide (1%), blocked for 20 min with phosphate-buffered saline containing 1% bovine serum albumin, 0.4% Triton X-100, and 4% normal goat serum, and then incubated overnight at 4°C with anti-TH antibody diluted (1:20,000) in blocking solution. The bound primary antibody was detected by incubation with biotinylated goat anti-rabbit secondary antibody (1:250) and then ABC reagents followed by development with 3,3′-diaminobenzidine and urea-hydrogen peroxide (Sigma). The images were analyzed with a Nikon Diaphot inverted microscope and recorded with a fitted CCD camera (DAGE-MTI, Michigan City, IN) operated through the MetaMorph Image System software (Universal Image and Co., West Chester, PA). For visual counting of the TH-positive neurons, nine representative areas per well were counted under the microscope at 100× magnification. For the measurement of the dendrite length, images of individual TH-positive neurons were recorded. The length of each fiber originated from each neuronal cell body and subsequent branches were measured, and a sum of total dendrite length for each neuron was calculated. For each well, 50 to 100 neurons were analyzed for total dendrite length.

**Statistical Analysis.** Statistical significance was assessed with an analysis of variance, followed by Bonferroni’s t test using the StatView program (Abacus Concepts, Inc., Berkeley, CA). A value of p < 0.05 was considered statistically significant.

### Results

**Effect of LPS on the Production of NO and TNFα and [3H]Dopamine Uptake of Mesencephalic Cultures.** Inflammation-mediated neurodegeneration required the activation of glia, especially microglial cells to produce cytotoxic factors that result in the death of neurons. Among the wide spectrum of proinflammatory and cytotoxic factors produced, release of TNFα and production of NO are reliable indicators of microglial activity. In this study, mesencephalic mixed neuron-glia cultures were treated with 1 to 100 ng/ml LPS. The release of TNFα and production of NO were determined 6 or 24 h post-LPS treatment, which were optimal time points, respectively, for maximum production (Liu et al., 2000a, 2001). As shown in Fig. 1A, significant amounts of both TNFα and nitrite were detected in LPS-treated cultures. Maximal levels of TNFα (8.8 ± 0.8 ng/ml) and nitrite (26.0 ± 0.5 μM) were detected in cultures treated with 10 ng/ml LPS, consistent with that observed in our previous studies (Kim et al., 2000; Liu et al., 2000a, 2001). LPS-induced activation of microglia was accompanied by a profound reduction in the capacity of cultures to take up [3H]dopamine, indicative of degeneration of dopaminergic neurons in the mixed neuron-glia cultures. At both 24 and 48 h, LPS induced a dose-dependent reduction in [3H]dopamine uptake (Fig. 1B). Significant decreases in [3H]dopamine uptake capacity were observed with cultures treated with 1 ng/ml LPS, and cultures treated with 100 ng/ml LPS for 48 h lost their uptake capacity by nearly 80% (Fig. 1B).

**Effect of Dynorphins on LPS-Induced Degeneration of Dopaminergic Neurons.** The effect of dynorphins on LPS-induced degeneration of dopaminergic neurons was first examined by preincubating cultures for 30 min with 10−18 to 10−16 M dynorphin A (1–17) prior to treatment with 10 ng/ml LPS. The capacity of the cultures for dopamine uptake was assayed 24 h later. As shown in Fig. 2, cultures treated with 10 ng/ml LPS for 24 h exhibited a 51.1% decrease in dopamine uptake compared with that of control cultures. However, treatment of cultures with 10−15 to 10−13 M dynorphin...
Fig. 2. Effect of dynorphin A (1–17) and dynorphin A (2–17) on LPS-induced reduction in [3H]dopamine uptake. Cultures were pretreated for 30 min with vehicle control or indicated concentrations of either dynorphin prior to treatment for 24 h with 10 ng/ml LPS, and dopamine uptake was then assessed. Results are mean ± S.E.M. of four separate experiments performed in triplicate. Dyn A, dynorphin A. *p < 0.001, compared with control; #p < 0.01 or †p < 0.001, compared with LPS-treated cultures.

A (1–17) prior to LPS markedly reduced the LPS-induced decrease in dopamine uptake capacity (Fig. 2). Dopamine uptake for cultures pretreated with 10–15 or 10–13 M dynorphin A (1–17) was 69.5 and 76.9% of control, respectively. Maximal effect was observed with cultures pretreated with 10–14 M dynorphin A (1–17), where dopamine uptake was 84.4% of control compared with 48.9% for LPS-treated cultures (Fig. 2). At the same range of concentrations (10–15–10–13 M), the effect of dynorphin A (1–17) on LPS-induced reduction in dopamine uptake was reproduced by dynorphin A (2–17), which is unable to bind conventional opioid receptors. No significant effect on LPS-induced decrease in dopamine uptake was observed with other concentrations of either dynorphin A (1–17) or dynorphin A (2–17). Therefore, subsequent studies were focused on the “ultra low” concentrations of these neuropeptides.

The neuroprotective effect of dynorphins was further examined by morphological analysis and counting of dopaminergic neurons following immunocytochemical staining. Cultures were treated for 30 min with 10–16 to 10–12 M dynorphin A (1–17) or dynorphin A (2–17) before treatment with 100 ng/ml LPS for 48 h. Although treatment with LPS (100 ng/ml; 48 h) significantly reduced dopamine uptake of the cultures, marked improvement was observed in cultures pretreated with dynorphin A (1–17) (10–12 to 10–15 M) prior to LPS treatment (Fig. 3A). At equal concentrations, dynorphin A (2–17) was as effective as dynorphin A (1–17).

No significant difference in dopamine uptake was observed between control cultures and cultures treated for 48 h with either 10–14 M dynorphin A (1–17) or dynorphin A (2–17). Immunocytochemical analysis of dopaminergic neurons with an antibody against tyrosine hydroxylase revealed that TH-immunoreactive (IR) neurons had elaborate dendrites (Fig. 3B). LPS treatment provoked the TH-IR neurons to lose most of the intricate dendrite network and possess only a few short stretches of neurites (Fig. 3B). Consistent with the improvement in dopamine uptake capacity, both dynorphin A (1–17) and dynorphin A (2–17) at 10–14 M significantly reduced the LPS-induced degeneration of TH-IR neurons. When the overall dendrite length of individual TH-IR neurons from different treatment conditions were compared, LPS treatment caused a 68% decrease in the total dendrite length per TH-IR neuron. However, as shown in Fig. 3C, a 19% and 18% decrease were observed in cultures pretreated with 10–14 M dynorphin A (1–17) and dynorphin A (2–17), respectively. In addition to the reduction of dopamine uptake and shortening of dendrites, LPS-induced degeneration of dopaminergic neurons also resulted in the loss of TH-IR neurons. As shown in Fig. 3C, compared to control cultures, LPS treatment (100 ng/ml; 48 h) resulted in a 47.3% loss of TH-IR neurons. The loss of TH-IR neurons was significantly less for cultures pretreated with either 10–14 M dynorphin A (1–17) or dynorphin A (2–17) (Fig. 3C).

Lack of Effect of a Synthetic κ-Opioid Receptor Agonist on LPS-Induced Degeneration of Dopaminergic Neurons. Dynorphin A (1–17) at pharmacological concentrations (10–10–10–6 M) exerts its biological activity through binding to distinctive G-protein-linked opioid receptors, a process that can be mimicked by synthetic and nonpolypeptide agonists such as (−)-U50488 (Taub et al., 1991). To determine whether the unique neuroprotective activity of femtomolar concentrations of dynorphins A (1–17) and dynorphin A (2–17) is a shared feature of opioid receptor agonists in general, the effect of (−)-U50488 on LPS-induced degeneration of dopaminergic neurons was examined. As shown in Fig. 4, treatment of cultures with 10–14 to 10–6 M (−)-U50488 did not have a significant effect on LPS (10 ng/ml; 24 h)-induced reduction in dopamine uptake. (−)-U50488 at 10–6 M was itself slightly toxic to dopamine neurons. In addition, no protective effect was observed for (+)-U50488, which is an ineffective stereoisomer of (−)-U50488 (Fig. 4).

Effect of Dynorphins on the Production of Free Radicals and Cytokines by LPS-Activated Glial Cells. Inflammation-mediated neurodegeneration involves the participation of proinflammatory and cytotoxic factors produced by activated glia, especially microglia. LPS activates glia, especially microglia, to produce cytokines such as TNFα and free radicals, such as superoxide and NO; these factors act on neurons to induce neurodegeneration (Chao et al., 1992; Jeohn et al., 1998; Liu et al., 2000a). As shown in Fig. 5, treatment of neuron-glia cultures with 10 ng/ml LPS markedly elevated the production of superoxide free radical. A significant inhibition of the LPS-stimulated superoxide production was observed in cultures pretreated (30 min) with either 10–15 to 10–13 M dynorphin A (1–17) or dynorphin A (2–17). Similar inhibitory profiles for LPS-stimulated production of superoxide were observed for dynorphin A (1–17) and dynorphin A (2–17) in microglia-enriched cultures (data not shown). In addition, the effect of femtomolar concentrations of dynorphins on the LPS-induced generation of TNFα and NO was examined. Both dynorphin A (1–17) and dynorphin A (2–17) exhibited a modest and statistically significant inhibition of LPS-induced release of TNFα, consistent with that observed in mouse cortical neuron-glial cultures (Kong
et al., 2000). At 6 h poststimulation with 10 ng/ml LPS, the levels of TNFα in cultures pretreated (30 min) with $10^{-14}$ M dynorphin A (1–17) and dynorphin A (2–17) were 88.5 ± 4.9% and 88.9 ± 5.8% of control, respectively, and were significantly lower than that of control cultures (100 ± 4.5%, n = 9, p < 0.05%). However, no significant reduction of LPS-stimulated nitrite production was observed with either $10^{-14}$ M dynorphin A (1–17) or dynorphin A (2–17) (data not shown).

Lack of Effect of Dynorphins on 6-Hydroxydopamine-Induced Degeneration of Dopaminergic Neurons. 6-OHDA is directly taken up by dopaminergic neurons and induces the degeneration of dopaminergic neurons through the generation of reactive oxygen species (Cadet and Brannock, 1998). Therefore, information on whether dynorphins were able to interfere with 6-OHDA-induced degeneration of dopaminergic neurons would help to determine if the presence of glial cells was required for the neuroprotective activity of femtomolar concentrations of dynorphins. Treatment of neuron-glial cultures with 10 to 50 μM 6-OHDA for 24 h resulted in a dose-dependent reduction in dopamine uptake (Fig. 6A). Pretreatment of cultures for 30 min with $10^{-16}$ to $10^{-12}$ M dynorphin A (1–17) or dynorphin A (2–17)
prior to treatment for 24 h with 30 μM 6-OHDA did not significantly affect the 6-OHDA-induced decrease in dopamine uptake (Fig. 6A). Similarly, the number of TH-IR neurons in cultures treated for 48 h with 10 to 50 μM 6-OHDA decreased in a dose-dependent manner (Fig. 6B). However, pretreatment with 10⁻¹⁴ M dynorphin A (1–17) or dynorphin A (2–17) did not significantly prevent 6-OHDA-induced degeneration of dopaminergic neurons (Fig. 6B).

Discussion

Dynorphins possess a wide spectrum of activity in both central and peripheral systems that include effects on nociceptive/analgesic systems, respiration, immune functions, neurotransmitter release, and ionic channel activity. In the midbrain region, dynorphins are one of the major neuromodulators present in the neuronal projections from the striatum to the substantia nigra that are important in the regulation of movement. Since a progressive and selective degeneration of nigral dopaminergic neurons is the pathological hallmark of Parkinson’s disease, it is of particular interest to investigate whether dynorphins play a role. In this study, we demonstrated that ultra low concentrations of dynorphin A (1–17) (10⁻¹⁵ to 10⁻¹³ M) protected dopaminergic neurons from degeneration induced by the inflammmagen LPS in rat mesencephalic neuron-glia cultures. Most importantly, the neuroprotective effect of ultra low concentrations of dynorphins may not be mediated through binding to the conventional opioid receptors (κ, μ, and δ), but rather through novel mechanisms of action. This conclusion is supported by several lines of evidence. First, dynorphin A (2–17), which is unable to bind opioid receptors, was equally effective as dynorphin A (1–17). Second, the effective concentrations of both dynorphins were in the range of 10⁻¹⁵ to 10⁻¹³ M, which are several orders of magnitude lower than that required for binding the G-protein-coupled opioid receptors (Kᵣ = 10⁻¹⁰–10⁻⁸ M). Third, the neuroprotective effect of dynorphins was not mimicked by synthetic κ-receptor agonists, such as U50488.

Inflammation in the brain has been frequently observed during the pathogenesis of several neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease, and human immunodeficiency virus-acquired immune defi-
be the most deleterious to neurons. Inhibition of the production of these factors, especially that of the free radicals, has been shown to protect neurons against degenerative insults (Jeohn et al., 2000; Liu et al., 2000a). In this study, both dynorphin A (1–17) and (2–17) were able to significantly inhibit the LPS-stimulated production of superoxide in the mesencephalic neuron-glia cultures, suggesting that inhibition of free radical generation may be more closely related to the mechanism of action for the neuroprotective effect of femtomolar dynorphins. Interestingly, this inhibitory effect on free radical generation by ultra low concentrations of dynorphins appears to be shared by another opioid peptide, enkephalin. Zaitsev and associates reported that femtomolar concentrations of [d-Ala²]metionine enkephalinamide, a stable analog of enkephalin, inhibited the respiratory burst in human neutrophils and mouse macrophages (Zaitsev et al., 1991; Efano et al., 1994). These inhibitory effects on free radical generation by femtomolar concentrations of dynorphins and enkephalins seemed to be distinct from the opioid receptor-dependent effects on free radical production observed for nanomolar concentrations of certain opioid receptor agonists (Hu et al., 1998).

Dynorphins are one of the major neurotransmitters present in the midbrain region that encompasses the striatonigral pathways. Degeneration of dopaminergic neurons in the substantia nigral area and dopamine terminals in the striatum is a pathological hallmark of Parkinson’s disease. Therefore, it is of particular interest to study whether dynorphins have an influence on the progressive degeneration of dopaminergic neurons. Between their well defined role of neurotransmitter and a potential role for neuroprotection, particular attention needs to be paid to the ranges of active concentrations. As a neurotransmitter, it is conceivable that nanomolar to even micromolar concentrations of dynorphins can be found at the nerve terminals at the moment of firing. On the other hand, it is also conceivable that dynorphins released from neurons may diffuse to the surrounding areas, and very low concentrations of “residual” dynorphins may be present around glial cells residing in the vicinity. These seemingly ultra low concentrations of dynorphins may serve to regulate some of the critical activity of glial cells.

The observation of biological activity at ultra low concentrations is not limited to dynorphins. In fact, modulatory activity at extraordinarily low concentrations has been reported for other peptides, including enkephalins (Zaitsev et al., 1991; Efanov et al., 1994; Das et al., 1995, 1997), β-endorphin (Williamson et al., 1988), substance P (Rameshwar et al., 1993), and pituitary adenylate cyclase-activating polypeptide (Yada et al., 1994; Kong et al., 1999). For example, a 27-amino acid fragment of the pituitary adenylate cyclase-activating polypeptide at $10^{-14}$ to $10^{-13}$ M significantly stimulated the release of insulin from rat islets (Yada et al., 1994). Incidentally, a significant number of these reports describe an immune modulatory effect of these peptides at ultra low concentrations. Rameshwar and coworkers (1993) reported that substance P at $10^{-13}$ M stimulated the production of interleukin-2 from murine lymphocytes. Williamson et al. (1998) discovered that β-endorphin enhanced antibody production in lymphocytes. Das and colleagues (1995, 1997) showed that enkephalins modulate antibody production in B cells and cytokine production in microglia. In understanding the potential mechanism of action of ultra low
concentrations of dynorphin A (1–17) and dynorphin A (2–17) protect dopaminergic neurons from inflammation-mediated damage in an opioid receptor-independent manner. Mechanistically, dynorphins may inhibit the activation of microglia to reduce their production of proinflammatory and cytotoxic factors such as superoxide free radical and TNFα. In addition, ultra low concentrations of dynorphins may stimulate astrocytes to secrete yet unidentified neurotrophic factors (Fig. 7). Since dynorphins are one the major mediators of the striato-nigral projections, which are important in the regulation of body movements, understanding the mechanism of action for ultra low concentrations of dynorphins will certainly pave a new path for devising novel therapeutic interventions for Parkinson’s disease.

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References

Knapp RJ, Malatsynska E, Collins N, Pang L, Wang JY, Hruby VJ, Roeseke WR and

Fig. 7. Proposed mechanism of action for the neuroprotective effect of ultra low concentrations of dynorphins.

concentrations of dynorphins, as well as other bioactive peptides, the existence of extremely high-affinity and distinctive cell surface binding sites/receptors has long been speculated. However, because of the potentially ultra high affinity of these binding sites/receptors, current technology has significantly hampered the quest to reveal their identities. In any case, further insights into their mechanisms of action may open a new chapter for the regulation of biological processes in the CNS and peripheral systems.

Besides microglia, activation of astrocytes is also closely related to the process of neurodegeneration. Astrocytes are known to produce trophic factors to promote the survival of neurons. One of the best studied trophic factors derived from astrocytes is the activity-dependent neurotrophic factor. Studies have shown that activity-dependent neurotrophic factor is effective at concentrations as low as 10−14 to 10−17 M (Gozes and Brenneman, 2000), which overlaps with the factor is effective at concentrations as low as 10−15 M (Chao et al., 1998; this study). The failure of dynorphins to prevent 6-OHDA from damaging dopaminergic neurons further suggests the involvement of non-neuronal cell types (i.e., glia) in the neuroprotective effect observed for ultra low concentrations of dynorphins.

In summary, this study demonstrates that ultra low concentrations of dynorphins may stimulate the production of highly effective neurotrophic factors to enhance neuronal survival.


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