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Urocortin Modulates Inflammatory Response and Neurotoxicity Induced by Microglial Activation

Mei-Jen Wang,2,3*, Shinn-Zong Lin,2* Jon-Son Kuo,2‡ Hsin-Yi Huang,*§ Shi-Hang Tseng,* Chia-Hsin Liao,*§ Der-Cherng Chen,† and Wu-Fu Chen¶

Microglia are the major inflammatory cells in the brain. Recent studies have highlighted the reciprocal roles of other brain cells in modulating the microglial inflammatory responses. Urocortin (UCN) is a member of the corticotropin-releasing hormone (CRH) family of neuropeptides that function to regulate stress responses. In the present study, we demonstrated that expression of UCN in rat substantia nigra was found to be localized principally to dopaminergic neurons. In cell culture models, the CRH receptors were expressed in microglia, and CRHR expression was up-regulated by treatment with LPS. Thus, it might be proposed that UCN regulates cellular communication between dopaminergic neurons and microglia. We show that femtomolar concentrations of UCN could inhibit LPS-induced TNF-α production in cultured microglia. Investigation of the underlying signaling pathway that mediated the anti-inflammatory effect of UCN the involved PI3K/Akt and glycogen synthase kinase-3β pathway, but not cAMP pathway. Furthermore, UCN protected dopaminergic neurons against LPS-induced neurotoxicity by inhibiting microglial activation in LPS-treated mesencephalic neuron-glia cultures. These results suggest that endogenous UCN and its receptors might be involved in a complex network of paracrine interaction between dopaminergic neurons and glia. The Journal of Immunology, 2007, 179: 6204–6214.

UCN is able to tether microglia to neurons, especially dopaminergic neurons in the SN. However, it is unknown whether UCN is localized almost exclusively in neurons and its receptors exist in both neurons and microglia, suggesting that UCN may provide an approach of limiting the inflammatory response (4–8).

Urocortin (UCN),4 a 40 aa peptide, is a corticotropin-releasing hormone (CRH)-related peptide that was first identified in rat midbrain (9). UCN acts as an endogenous ligand for the type 2 CRH receptor (CRHR2) and is implicated in various functions, such as appetite suppression, anxiety, and cardiovascular regulation (10). UCN has been shown to be distributed in a variety of rat brain regions (9, 11). The effects of UCN are mediated by two high-affinity receptors, CRHR1 and CRHR2, which are expressed in neurons and microglia in the brain (12–16). Both CRHRs are G protein-coupled receptors linked to a number of intracellular signaling pathways (13, 17). UCN has been shown to possess a direct anti-inflammatory effect in rat Kupffer cells (18). Recently, accumulating studies showed that UCN can protect cultured hippocampal and cortical neurons against neurotoxin-induced cell death (15, 19).

Activated microglia are thought to contribute to dopaminergic neuron loss in the substantia nigra (SN) (20). Microglia are present in large numbers within the brain, but they are not distributed with a uniform density in all major divisions of the brain. The higher density of resting microglia in the SN compared with other brain regions might be one of the reasons why dopaminergic neurons are more vulnerable to microglia-mediated neurotoxicity (21, 22). In the brain, UCN is localized almost exclusively in neurons and its receptors exist in both neurons and microglia, suggesting that UCN might regulate signaling between neurons and microglia. Our investigation demonstrated that UCN was principally expressed in dopaminergic neurons in the SN. However, it is unknown whether UCN can be able to tether microglia to neurons, especially dopaminergic neurons, as observed between UCN and CRHR-bearing cells in the periphery (18).

TNF-α is a proinflammatory cytokine that is up-regulated in the brain in response to various insults or injury. This cytokine is mainly expressed by activated microglia around the injured area. Within the brain, TNF-α might modulate the inflammatory processes by further activation of microglia and astrocytes (23, 24). Increased TNF-α production has been implicated in the pathology of neurodegenerative diseases such as Parkinson’s disease (25, 26). In the present study, we investigated whether UCN regulates microglial inflammatory responses through decreasing microglial...
TNF-α secretion. In addition, we also elucidated the molecular mechanisms of this modulation.

Materials and Methods

**Materials**

LPS from *Escherichia coli* serotype O111:B4, LY294002, Nf449 and 2',5'-dideoxyadenosine (ddATP) were from Calbiochem. Rat UCN, polyclonal mouse anti-tyrosose hydroxylase (TH), and polyclonal rabbit anti-UCN Abs were obtained from Sigma-Aldrich. Polyclonal mouse anti-neuron-specific nuclear protein (NeuN) and polyclonal rabbit anti-microtubule-associated protein-2 Abs were from Chemicon International. Monoclonal rabbit anti-glia fibrillary acidic protein Ab was from Abcam. Monoclonal mouse anti-ED1 Ab was purchased from Serotec. Polyclonal goat anti-CRHR1 Ab was obtained from Santa Cruz Biotechnology. Polyclonal rabbit anti-CRHR2 Ab was purchased from Novus Biologicals. Abs against ERK and phospho-ERK were from Promega. All other Abs were from Cell Signaling Technology. Cell culture ingredients were purchased from Invitrogen Life Technology. [3H]Dopamine (DA) (30 Ci/μmol) was bought from PerkinElmer Life Sciences. The biotinylated secondary Abs and Vectastain avidin-biotin-peroxidase kit were from Vector Laboratories. All other reagents were purchased from Sigma-Aldrich.

**Microglia cultures**

Microglia were prepared from ventral mesencephalon of 1-day-old Sprague–Dawley rat as previously described (27). Briefly, ventral mesencephalic tissues, devoid of meninges and blood vessels, were dissociated by a mild mechanical triturator. The isolated cells (5 × 10^5) were seeded in 150-cm² culture flasks in DMEM containing 10% FBS, 50 μM penicillin, and 50 μg/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The medium was changed 4 days later. Upon reaching confluence (12–14 days), microglia were separated from astrocytes by shaking the flasks for 2 h at 180 rpm. Detached cells later. Upon reaching confluence (12–14 days), microglia were separated from astrocytes by shaking the flasks for 2 h at 180 rpm. Detached cells were plated into 24-wells plate at a density of 2 × 10^5 cells/well and then incubated for 24 h before treatment. Murine BV-2 microglial cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator under 5% CO₂. Confluent cultures were trypsinized. Cells were plated into 24-well plate at a density of 2 × 10^5 cells/well and then incubated for 24 h before treatment.

**Mesencephalic neuron-glia cultures**

Primary rat ventral mesencephalic neuron-glia cultures were prepared following a previously described protocol with some modifications (27). Briefly, ventral mesencephalic tissues were dissected from embryonic day 14- or day 15-Sprague Dawley rats and dissociated enzymatically (0.1% trypsin) and mechanically. Cells were seeded to 24-well (3 × 10⁵/well) culture plates precoated with poly-t-lysine (20 μg/ml) and maintained in a 0.5 ml/ml of MEM supplemented with 10% heat-inactivated FBS and 10% heat-inactivated horse serum, 1 μl glucose, 2 mm T-glatamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cultures were replenished with 0.5 ml/well fresh medium 3 days later and were used for treatment 6 days later. For neuroprotective effects assay, mesencephalic cultures were maintained in 1 ml/well of MEM containing 2% FBS, 2% horse serum, 2 mm T-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were pretreated with vehicle or 1 μM UCN for 30 min, then cells were treated with 10 ng/ml LPS for 3 days. Degeneration of dopaminergic neurons were assessed by measuring the ability of cultures to take up [3H]Dopa, and counting the number of TH-positive cells following immunostaining in mesencephalic cultures.

**Real-time RT-PCR analysis**

The level of TNF-α gene expression was quantified using real-time RT-PCR analysis as described by Walker (28). Briefly, total RNA was extracted from microglia cultures with a cold RNA extraction solution (Ultraspec RNA; Biotecx Laboratories). Total RNA was reverse transcribed with M-MLV reverse transcriptase and oligo(dT) primers (Superscript First-Strand Synthesis System; Invitrogen Life Technologies). The primers sequences are as follows: mouse TNF-α, 5′-TTC TGT CTA CTC GTC GAT TTC GGC GTG ATC GTA-3′ and 5′-GCA GTG AGA TCA TCT TCT CGA-3′; and rat β-actin, 5′-TTG TAA CCA ACT GGG ATA TGG-3′ and 5′-GAT CTT GAT CAT GGT AGG-3′. The SYBR green DNA PCR kit (Applied Biosystems) was used for real-time PCR analysis. The relative differences in expression between groups were analyzed on the basis of threshold cycle time (Ct) values normalized with β-actin.

Otherwise, one-step RT-PCR analysis were performed to determine the expression of CRHRS mRNA (SuperScript One-Step RT-PCR System kit; Invitrogen Life Technologies). The primer sequences are as follows: rat CRHR1, 5′-CAA CAC GAC AAA CAA TGG-3′ and 5′-GCA AGA GGA CAA AGG-3′ (197 bp fragment); rat CRHR2, 5′-TCA TCA CCA CCT TCA TCC-3′ and 5′-CAG CCT TCT ACA AAC ATC-3′ (158 bp fragment); rat β-actin, 5′-TTG TAA CCA ACT GGG ATA TGG-3′ and 5′-GAT CTT GAT CAT GGT AGG-3′ (764 bp fragment); mouse CRHR1, 5′-ATC CTC ATG ACC AAA AAT CTC CG-3′ and 5′-TGA AGA CCC TGG AGA CC-3′ (157 bp fragment); mouse CRHR2, 5′-CTA CAC CTA CTG CAC CAC CAC GAC C-3′ and 5′-TTC GCA GTG TGA GTT GAC C-3′ (190 bp fragment); and mouse β-actin, 5′-GTC GCC GGC TCT AGG CAC CAA-3′ and 5′-CTC TCT GTT GAT GTC ACC CAC GAT TTC-3′ (540 bp fragment). After PCR amplification, the products were visualized by electrophoresis in 3% agarose gel and staining with 0.5 μg/ml ethidium bromide. Specific genes were verified by their predicted size.
Measurement of intracellular cAMP

Cells were preincubated for 90 min in serum-free DMEM. After this time, the medium was replaced with serum-free DMEM containing 1 mM 3-isobutyl-1-methyxanthine (Sigma-Aldrich) to inhibit cAMP phosphodiesterase activity. After 60 min, medium was replaced with fresh DMEM containing 2% FBS, 1 mM 3-isobutyl-1-methyxanthine, and various concentrations of UCN, and cells were incubated for 10 min at 37°C. Intracellular cAMP was measured using the Amersham Biosciences cAMP EIA system, according to the manufacturer’s instructions.

Preparation of cell extracts

Cells cultured in 10-cm petri dishes were washed twice with ice-cold PBS and lysed in 800 μl of lysis buffer (50 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 3 mM benzamidine, 1 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 100 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 5 μg/ml pepstatin A). After incubation on ice for 30 min, cell lysates were centrifuged and the supernatants were collected. Protein concentration of samples was determined by Bradford assay (Bio-Rad), and samples were equilibrated to 2 mg/ml with lysis buffer.

Western blotting

Western blot analysis was conducted using Abs against UCN, CRHRs, phosphorylation of the members of MAPK family, phosphorylated site of Akt (Ser473) and GSK-3β (Ser9). Abs against the members of MAPK family, total Akt or total GSK-3β were used as internal controls to determine loading efficiency. Protein samples containing 100 μg of protein were separated on 10–15% SDS-polyacrylamide gels and transferred to immobilon polyvinylidene difluoride membranes (Millipore). The membranes were incubated in TBST buffer (0.1 M Tris-HCl (pH 7.4), 0.9% NaCl, 0.1% Tween 20) supplemented with 5% dry skim milk for 1 h to block nonspecific binding. After rinsing with TBST buffer, they were incubated with primary Abs. The membranes were washed twice with TBST buffer followed by incubation with appropriate streptavidin-HRP-conjugated secondary Abs. The Ag-Ab complexes were detected by using a chemiluminescence detection system (ECL; Amersham Biosciences). The intensity of the band was quantified with a densitometric analysis, and calculated as the OD times the area of band.

Histology

During deep anesthesia, rats were perfused through the left ventricle with saline followed by 4% paraformaldehyde. Brains were removed and then postfixed in 4% paraformaldehyde at room temperature for 2 h, cryoprotected in 30% (w/v) sucrose (4°C), frozen and stored at −80°C. Serial (30 μm) coronal sections were cut on a freezing sliding microtome. Free-floating sections (30 μm) were processed for immunofluorescent labeling. Tissue sections were blocked with 3% donkey normal serum and 2% BSA in PBS and incubated overnight with primary Abs. After washing, secondary Abs conjugated to the fluorescent markers FITC and rhodamine (Jackson ImmunoResearch Laboratories) were applied to sections for 1 h. Sections were then washed, mounted on slides, cover slipped with Vectashield mounting medium (Vector Laboratories), and examined with confocal microscope (Zeiss).
**Immunocytochemistry**

Dopaminergic neurons were detected with anti-TH Ab. Briefly, cells were fixed with 3.7% paraformaldehyde followed by blocking with PBS containing 0.4% Triton X-100, 2% BSA, and 3% normal goat serum. After blocking, cells were incubated with primary Ab at 4°C for overnight. The bound primary Ab was visualized by incubation with an appropriate biotinylated secondary Ab followed by the Vectastain avidin-biotin-peroxidase reagents and color development with 3,3′-diaminobenzidine. The number of TH-positive cells was counted in the entire surface area of a culture well.

**Uptake assays for [3H]DA**

Cells were washed twice with warm Krebs-Ringer buffer (16 mM sodium phosphate, 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)), and then incubated with [3H]DA in 96-well tissue culture plates for 10 min at room temperature in 1 part 0.1% naphthylethylenediamine, 1 part 1% sulfanilamide in 5% of culture supernatant was reacted with an equal volume of Griess reagent

**Cytokines assay**

Primary microglia and BV-2 microglia were stimulated with LPS in the absence or presence of UCN, and supernatants were collected and kept frozen in aliquots at −80°C until use. Release of TNF-α, IL-1β, IL-6, and IL-10 were measured with a commercial ELISA kit from R&D Systems, according to the manufacturer’s instructions.

**NO assay**

NO production was assayed by measuring the concentrations of the stable NO metabolite, nitrite, in the conditioned medium. Briefly, 100 μl of culture supernatant was reacted with an equal volume of Griess reagent (1 part 0.1% naphthylethylenediamine, 1 part 1% sulfanilamide in 5% H3PO4) in 96-well tissue culture plates for 10 min at room temperature in the dark. The absorbance at 540 nm was determined using a microplate reader (spectraMAX 340; Molecular Devices).

**Statistical analysis**

All data are expressed as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Scheffe’s test. A value for p < 0.05 was considered statistically significant.

**Results**

**Cellular localization of UCN and CRHRs in the adult SN**

UCN was first cloned from rat midbrain (9). To test the cellular localization of UCN and its receptors in rat SN, we evaluated the expression of these proteins by double-immunohistochemistry. The results showed that UCN was expressed in neuronal cells, and almost all TH-immunoreactive cell bodies in the SN showed immunoreactivity for UCN (Fig. IA). The ratio of TH-immunoreactive neurons to UCN-immunoreactive cells in the SN was ~81%. The expression of UCN in the SN was also confirmed by Western blotting (Fig. 1B). Although the CRHRs were not detectable by immunohistochemistry (data not shown), Western blot analysis showed that these proteins were indeed expressed in the SN (Fig. 1B).

**Femtomolar concentrations of UCN inhibit LPS-induced TNF-α production in microglia**

To investigate whether neuronally derived UCN might play a role in mediating interactions between dopaminergic neurons and microglia. We first performed RT-PCR analysis to establish whether both CRHR1 and CRHR2 are expressed in the cultured primary microglia and BV-2 cells and to determine the relative abundance of CRHR1 and CRHR2 mRNAs within this sample. We observed CRHR2 mRNA was expressed in both unstimulated BV-2 microglia and primary microglia, whereas CRHR1 mRNA was not readily detected. Stimulation with LPS resulted in an increase in CRHR2 mRNA expression (Fig. 2A). Because TNF-α is a major molecule induced by various inflammatory stimuli and it plays a central role in various inflammatory diseases (25, 26), we tested the hypothesis that UCN might modulate microglial activation by examining the effect of UCN on LPS-induced TNF-α release in BV-2 microglia. BV-2 cells were pretreated for 30 min with 10−15–10−7 M UCN before treatment with 10 ng/ml LPS. As shown in Fig. 2B, cotreatment with UCN decreased the content of LPS-induced TNF-α release in a concentration-dependent manner ranging from 10−15 to 10−8 M. Significant inhibition of the TNF-α production occurred with UCN at concentrations as low as 1 fM, and maximal effect was observed with cultures pretreated with 10−8 M. To investigate whether the reduction in TNF-α protein in BV-2 cells following treatment with UCN is due to the suppression of TNF-α mRNA expression, real-time RT-PCR analysis was performed to assess the effect of UCN on the expression of TNF-α mRNA levels. The data showed that UCN had no inhibitory effect on LPS-induced TNF-α mRNA expression.
decrease in TNF-α of control cAMP levels (167 ± 0.17 ng/ml. 200 nM NF449, or 10 nM ddATP) /H9251 LPS-induced increase in TNF-α cAMP analog, with LPS. Fig. 4 shows dibutyryl cAMP inhibited LPS-induced increase in TNF-α in a dose-dependent manner.

Next, we performed experiments to determine whether UCN induces cAMP production in BV-2 microglia at concentrations in the range from 10⁻¹⁵ to 10⁻⁸ M. Treatment of cultured BV-2 microglia with UCN caused a significant increase in cellular cAMP levels at concentrations >10⁻¹⁰ M (Fig. 4B). The results raise the possibility that the inhibition of LPS-induced TNF-α release by UCN at lower concentrations (10⁻¹⁰⁻¹⁰⁻¹⁰ M) might be due to the inhibition of cellular cAMP. To further confirm this hypothesis, we examined the effects of compounds that inhibit the activation of G proteins and adenylate cyclase on the ability of UCN to attenuate TNF-α production in LPS-stimulated BV-2 microglia. Pretreatment of the cultures with NF449, a selective antagonist of Gs, or the adenylate cyclase inhibitor ddATP could not reverse UCN-induced TNF-α decrease (Fig. 4C). Similarly, this phenomenon was also observed in primary microglia cultures (Fig. 4D). These data taken together suggest that the inhibition of LPS-induced TNF-α is not mediated through production of cAMP.

UCN does not inhibit LPS-induced MAPKs activation

It has been shown that LPS-induced TNF-α production by microglia involves phosphorylation of p38 MAPK and ERK (30). To investigate whether these kinases are modulated by UCN,
BV-2 cells were pretreated with UCN (1 pM) for 30 min followed by stimulation with LPS. Activation of three MAPKs, including p38, ERK, and JNK, was analyzed by Western blotting. The results showed that UCN was not able to block LPS-induced phosphorylation of three MAPK (data not shown).

UCN blocks LPS-caused phosphorylation of Akt and glycogen synthase kinase (GSK)-3β

Because UCN exerts its anti-inflammatory action in LPS-stimulated microglia is not dependent on cAMP and MAPK signaling pathways. The intracellular mechanisms that is required for down-regulation of TNF-α production merits to elucidate. The PI3K is well known to control translation through regulation of eukaryotic initiation factor (eIF)-4E and 4E-binding protein 1 (4E-BP1) phosphorylation (31). Recently, LPS has been shown to activate PI3K/Akt signaling in microglia (32). To test whether the PI3K pathway is regulated by UCN, we examined the phosphorylation of Akt, a well-known target of PI3K. The results showed that LPS stimulation of BV-2 cells resulted in a time-dependent phosphorylation of Akt. Pretreatment with UCN markedly suppressed Akt phosphorylation caused by LPS (Fig. 5A).
FIGURE 7. Lithium blocks the inhibition of UCN on LPS-induced GSK-3β phosphorylation and TNF-α production. A, BV-2 cells were stimulated with 10 nM LiCl for the various times as indicated. B, Cells were treated with 10 ng/ml LPS for 60 min without or with 30 min pretreatment with UCN (1 pM) alone or UCN (1 pM) and LiCl (10 mM). Whole cell lysates were prepared and subjected to Western blotting using Abs specific for phosphorylated (Ser9) or total form of GSK-3β. C and D, Cells were treated with 10 ng/ml LPS for 6 (C) or 2 h (D) without or with 30 min pretreatment with UCN (1 pM) alone or UCN (1 pM) and various concentrations of lithium chloride. TNF-α release (C) was determined by ELISA. Data are represented as the mean ± SEM of three independent experiments. *p < 0.05; **p < 0.1; comparing UCN with UCN plus lithium chloride-treated cultures. The levels of TNF-α in LPS-treated alone cells were 22 ± 0.09 ng/ml. The expression of TNF-α mRNA (D) was quantified by real-time RT-PCR analysis as described in Fig. 2. Data are the mean ± SEM of three independent experiments and are expressed as a percentage of LPS.

GSK-3β is one of the major downstream elements of the PI3K/Akt pathway, and its activity can be inhibited by Akt-mediated phosphorylation of GSK-3α at Ser37 and GSK-3β at Ser9 (33, 34). GSK-3β phosphorylation plays an important role in the control of translation by regulation of eIF-2B (35–37). Recent studies indicated that LPS can inactivate GSK-3β by inducing phosphorylation at Ser9 in human alveolar macrophages and THP-1 monocytic cells (38, 39). Therefore, we sought to determine whether UCN could regulate LPS-induced GSK-3β phosphorylation. As shown in Fig. 5B, LPS induced a time-dependent phosphorylation of GSK-3β on Ser9 (inactivation), which, however, did not correlate with the kinetics of Akt activation by LPS. LPS significantly increased levels of phosphorylated GSK-3β within 20 min instead of 30 min, at which time Akt was activated. Preincubation of BV-2 cells with UCN abrogated LPS-induced inactivation of GSK-3β, thereby retaining the kinase in its active (dephosphorylated) state.

**LY294002, a PI3K inhibitor, mimics UCN action in LPS-treated BV-2 microglia**

To study whether PI3K is a critical signaling molecule in the inhibitory effect of UCN, BV-2 cells were pretreated with a PI3K inhibitor LY294002 and then stimulated with LPS. We found that LY294002 treatment completely abolished LPS-induced Akt activation, whereas the increase in GSK-3β phosphorylation was not significantly blocked (Fig. 6, A and B). This finding suggests that LPS-induced GSK-3β phosphorylation is largely independent of PI3K/Akt in BV-2 microglia. Furthermore, LPS-induced TNF-α production was inhibited by LY294002 in a dose-dependent manner, whereas the mRNA levels were unaltered. (Fig. 6, C and D).

**Lithium blocks UCN action in LPS-stimulated BV-2 microglia**

Lithium is a direct inhibitor of GSK-3β, and it also causes increased phosphorylation of GSK-3β on Ser9 (40–42), which inhibits GSK-3β activity (33, 35, 43). The investigation of GSK-3β phosphorylation evoked by lithium treatment of BV-2 microglia is shown in Fig. 7A. We found that lithium treatment caused increased GSK-3β phosphorylation in a time-dependent manner. Furthermore, the decrease in LPS-induced GSK-3β phosphorylation caused by UCN treatment was reversed by lithium (Fig. 7B). To determine whether the regulation of GSK-3β phosphorylation is involved in UCN-induced inhibition on TNF-α release, BV-2 cells were pretreated with lithium followed by stimulation with LPS and UCN. Fig. 7C shows UCN-induced decrease in TNF-α production after LPS exposure was significantly increased after cultures were pretreated with lithium. Finally, we further investigated whether the blocking effect caused by lithium is resulted from a transcriptional event, the expression of TNF-α mRNA was assessed by real-time RT-PCR analysis. The results showed that treatment of BV-2 cells with lithium alone or in the presence of LPS and UCN did not significantly alter the expression of TNF-α mRNA (Fig. 7D).

We further used primary microglia to confirm both PI3K/Akt and GSK-3β-mediated the anti-inflammatory effect of UCN. LPS induced Akt and GSK-3β phosphorylation, and treatment with
UCN abolished Akt and GSK-3β phosphorylation caused by LPS (Fig. 8A). In microglia stimulated with LPS, TNF-α release was significantly suppressed in cells treated with LY294002 (Fig. 8B). In addition, treatment with lithium blocked the inhibition of UCN on LPS-caused TNF-α production as observed in mouse BV-2 cells (Fig. 8B).

**UCN prevents dopaminergic neuron death caused by LPS-induced toxicity**

Microglia are the major source of inflammatory factors that mediate LPS-induced neurotoxicity in mesencephalic neuron-glia cultures (22). To determine whether the inhibitory effect of UCN on LPS-induced microglial activation could be also observed in mesencephalic neuron-glia cultures, cell cultures were pretreated with UCN followed by treatment with LPS. As shown in Fig. 9A, pretreatment of cultures with 1 pM UCN significantly alleviated the loss of TH-positive neurons. Moreover, UCN could suppress LPS-induced microglial inflammatory responses. These findings raise the possibility that UCN may protect mesencephalic dopaminergic neurons from neurotoxicity mediated by activated microglia.

**FIGURE 8.** UCN reduces LPS-induced TNF-α release via PI3K/Akt and GSK-3β pathways in primary microglia. A, Primary microglia were pretreated with 1 pM UCN for 30 min followed by exposure to 10 ng/ml LPS for another 30 min. Whole cell lysates were prepared and subjected to Western blotting using Abs specific for phosphorylated Akt and GSK-3β. B, Cells were pretreated with 10 μM LY294002 or 10 mM LiCl for 30 min before the exposure to LPS (10 ng/ml) in the presence or absence of 1 pM UCN for another 6 h. TNF-α release was determined by ELISA. Data are represented as the mean ± SEM of three independent experiments. ***, p < 0.01 or ##, p < 0.01 compared with LPS- or LPS plus UCN-treated cultures, respectively. The levels of TNF-α in LPS-treated alone cells were 2.39 ± 0.17 ng/ml.

**FIGURE 9.** UCN protects cultured primary dopaminergic neurons from LPS-induced neurotoxicity. Mesencephalic neuron-glia cultures were pretreated with various concentrations (4 and 8) or 1 pM (C) of UCN for 30 min followed by treatment with 10 ng/ml LPS for another 6 h (TNF-α) or 72 h (immunostain and DA uptake). TNF-α release was determined by ELISA (A). Degeneration of dopaminergic neurons was evaluated by counting the number of TH-positive neurons (B) and by measuring the [3H]DA uptake (C). D, LPS-induced neurodegeneration is mediated by TNF-α. Mesencephalic neuron-glia cultures were pretreated with various concentrations of anti-TNF-α Ab for 1 h before LPS (10 ng/ml) application. After 72 h of incubation, dopamine uptake was assessed. Data are represented as the mean ± SEM of three independent experiments. ***, p < 0.01 indicates significant difference compared with LPS-treated cultures. The levels of TNF-α in LPS-treated alone cells were 1.90 ± 0.11 ng/ml.
mesencephalic neuron-glia cultures were pretreated with anti-TNF-α Ab for 1 h before LPS application. We found that anti-TNF-α Ab significantly inhibited LPS-induced reduction of DA uptake (Fig. 9D). Our results clearly demonstrate that LPS-stimulated microglia caused dopaminergic cells death is mediated by TNF-α.

Discussion
In the present study, we have demonstrated that in LPS-treated microglia cultures UCN could inhibit TNF-α secretion at concentrations as low as 1 nM. The investigation of the intracellular mechanisms demonstrated that inhibition of both Akt and GSK-3β phosphorylation caused by LPS contributes to UCN-mediated decrease in LPS-induced TNF-α production. Furthermore, we also found that activation of the cAMP pathway is not an essential mechanism responsible for UCN-mediated anti-inflammatory actions. Control of microglial neurotoxicity by UCN could protect dopaminergic neurons against LPS-induced toxicity.

UCN has been shown to be distributed in a variety of rat brain regions including the supraoptic nucleus, the median eminence, Edinger-Westphal nucleus, and the sphenoid nucleus (9, 11). In this study, we demonstrated that dopaminergic neurons are the primary source of expression of UCN in the SN. Moreover, the present results demonstrated that the CRHR2 is the predominant receptor expressed in microglia cultures. Because UCN acts as an endogenous ligand for CRHR2 (10), herein examined the possibility of interactions between dopaminergic neurons and microglia by examining whether UCN could modulate microglial inflammatory responses. Indeed, we found that ultra low concentration of UCN (10⁻¹⁵ M) could significantly inhibit LPS-induced proinflammatory cytokines and NO production in microglia. These results suggest that dopaminergic neurons-derived UCN could act as a physiological regulator to prevent excessive inflammatory microglial responses.

All of the known effects of UCN involve receptor-coupled activation of adenylate cyclase and an increase in cellular levels of cAMP (9, 29). Exogeneous addition of cAMP analog could attenuate TNF-α release in LPS-treated primary microglia and BV-2 cells. These findings raise the possibility that UCN inhibitory effect on LPS-induced TNF-α release may be mediated through induction of cAMP production. UCN at 10⁻¹⁹ M significantly stimulated the accumulation of cAMP, and increased it in a dose-dependent manner. However, its inhibitory effect began at very low concentrations (10⁻¹⁵ M). Therefore, it was of interest to examine whether the effect of UCN is linked to the cAMP pathway or some other transduction system. Accordingly, we used specific inhibitors to block the cAMP production. Our data showed that neither NF449 nor dATP could abolish the inhibition of TNF-α production by extremely low concentrations of UCN in both microglial cells. These results suggest that some other mechanisms but not cAMP pathway are involved in the inhibitory effect of UCN. Whether UCN effects is mediated through cAMP production or also involve an additive inhibitory mechanism as dosage increasing needs further investigation.

Regulation of proinflammatory gene expression in a biological system is a balance between positive and negative signal transduction pathways. MAPKs are known to play a critical role in cytokine production (30, 44). Recent studies showed that PI3K/Akt is also involved in LPS-TLR4-mediated cytokine expression in macrophages and microglia (32, 45, 46). To understand the molecular mechanisms underlying the anti-inflammatory effect of UCN, we studied the effect of UCN on LPS-induced activation of MAPKs and PI3K/Akt pathways. We found that UCN did not inhibit LPS-induced phosphorylation of all three MAPKs, but it markedly attenuated phosphorylated levels of Akt, a downstream target of PI3K. Using pharmacological approaches, we further demonstrated that LY294002, a PI3K inhibitor, could mimic UCN action in LPS-treated microglia. In contrast to previous and our studies showing that the PI3K/Akt pathway positively regulates expression of proinflammatory gene in macrophages and microglia (32, 47, 48), a recent report demonstrated that inhibition of the PI3K/Akt pathway enhances the LPS induction of TNF-α expression in human monocytes (39). These differences may reflect the use of different cell types. By showing the inhibitory effect of UCN on LPS-induced PI3K/Akt activation, the present study reveals a part of the mechanism of suppressive action of UCN in brain inflammation.

UCN, a serine/threonine kinase originally identified as a regulator of glycogen metabolism, is also a member of the Wnt signaling pathway (49, 50). There are two highly homologous mammalian isoforms of GSK-3, GSK-3α and GSK-3β. GSK-3α and GSK-3β are unusual in that they can be inactivated through phosphorylation of Ser21 and Ser9. Protein kinases known to phosphorylate GSK-3α/GSK-3β include Akt, protein kinase A, p70⁵⁶ kinase, p90RSK, and protein kinase C (51, 52). Several growth factors, including insulin, fibroblast growth factor, epidermal growth factor, and platelet-derived growth factor, cause increased inhibitory phosphorylation of GSK-3β (50). Recently, LPS has been shown to induce GSK-3β phosphorylation in human alveolar macrophages (38). GSK-3β is known to play a pivotal role in numerous cellular functions ranging from glycogen metabolism and modulation of microtubule dynamics to the regulation of cell survival and neural polarity (51–54). Furthermore, GSK-3β is also responsible for the phosphorylation of eIF-2B, which plays an important role in translation regulation (35–37).

Our study showed that exposure of microglia to LPS resulted in GSK-3β phosphorylation at Ser21 and Ser9, and this phosphorylation was abrogated by UCN pretreatment. Phosphorylation of GSK-3β induced by lithium reversed UCN-mediated decrease in phosphorylated level of GSK-3β and the inhibition of TNF-α secretion, suggesting that GSK-3β also contributes to UCN-induced anti-inflammatory actions. Our data are consistent with a very recent report showing that overexpression of GSK-3β in endothelial cells significantly inhibits TNF-α and IL-1β-induced expression of IL-6, MCP-1, and VCAM1 (55). However, Guha and Mackman (39) have shown that GSK-3β positively regulates TNF-α expression in LPS-stimulated human monocytes. These findings indicate that the role of GSK-3β in inflammatory responses may depend on the cell type. Furthermore, inhibition of PI3K/Akt (with LY294002) did not block LPS-induced GSK-3β phosphorylation, indicating that increased GSK-3β phosphorylation by stimulation with LPS in BV-2 microglia is not mediated through activation of Akt. These results suggest that both PI3K/Akt and GSK-3β signaling pathways participate in the inhibition of UCN-mediated LPS-induced TNF-α production. In addition, the present data our knowledge are the first to show the negative regulatory role of GSK-3β in inflammatory reactions in microglia, suggesting that GSK-3β could be an important therapeutic target for treatment of neuroinflammatory diseases.

The posttranscriptional regulation involving translational efficiency appears to play a critical role in the regulation of TNF-α expression (56, 57). Phosphorylation of eIF-4E and its binding protein 4E-BP1 is an important step in controlling the rate of initiation of translation in mammalian cells (31). Phosphorylation of 4E-BP1 dissociates it from eIF-4E, relieving the translational inhibition. A role of 4E-BP1 in the translation of TNF-α mRNA could be inferred by the finding that LPS enhanced the phosphorylation of 4E-BP1 in macrophages (58). The PI3K/Akt pathway is
involved in the phosphorylation of 4E-BP1. Inhibition of this pathway would thus inhibit translation by preventing this translational derepression. Furthermore, GSK-3β phosphorylates eIF-2B, leading to a shutdown of cellular protein synthesis (35–37). In this report, we present data to show that UCN inhibited LPS-induced Akt and GSK-3β phosphorylation. Our findings in microglia, where UCN reduced the production of TNF-α protein but had no effect on mRNA levels, might not be inconceivable. Further studies are necessary to elucidate the significance of Akt and GSK-3β signaling inhibition by UCN in relation to LPS-stimulated TNF-α translation.

Accumulating evidences suggest that activated microglia is implicated in several neurodegenerative diseases (1, 2). There is some in vitro and in vivo studies support the proposal that blocking microglial activation may be neuroprotective (20, 59). Therefore, we were interested to determine whether the inhibitory effect of UCN on LPS-induced microglial activation could be translated into neuroprotective. Our data showed that UCN increased the survival of dopaminergic neurons in LPS-treated mesencephalic neuron-glia cultures, suggesting the possible functions of UCN as an intrinsic anti-inflammatory neuropeptide in the CNS.

An active role for microglia during selective dopaminergic neuron loss certainly correlates with the fact that the SN represents a brain region particularly concentrated with microglia (22). UCN is an active role for microglia during selective dopaminergic neuron loss certainly correlates with the fact that the SN represents a brain region particularly concentrated with microglia (22). UCN is an intrinsic anti-inflammatory neuropeptide in the CNS.

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Disclosures

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