Hippocampal Cholinergic Neurostimulating Peptide Suppresses LPS-Induced Expression of Inflammatory Enzymes in Human Macrophages

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Hippocampal cholinergic neurostimulating peptide (HCNP) is a secreted undecapeptide produced through proteolytic cleavage of its precursor protein, HCNPpp. Within hippocampal neurons, HCNP increases gene expression of choline acetyltransferase (ChAT), which catalyzes acetylcholine (ACh) synthesis, thereby modulating neural activity. HCNPpp also appears to be expressed in various immune cells. In the present study, we observed that HCNPpp is expressed in U937 human macrophage-like cells and that HCNP exposure suppresses lipopolysaccharide (LPS)-induced gene expression of ChAT. The opposite action is also seen in T lymphocytes, which suggest that HCNP appear to suppress cholinergic system in immune cells. In addition, HCNP suppresses LPS-induced gene expression of inflammatory enzymes including cyclooxygenase 2 (COX2) and inducible nitric oxide (NO) synthase (iNOS). The suppressive effect of HCNP may reflect suppression of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling activated by LPS. Thus, HCNP may have therapeutic potential as an anti-inflammatory drug.

Key words hippocampal cholinergic neurostimulating peptide; cyclooxygenase2; macrophage

INTRODUCTION

Hippocampal cholinergic neurostimulating peptide (HCNP) is an undecapeptide generated by cleavage of the N-terminal of HCNP precursor protein (HCNPpp).1,2) HCNP was originally identified in hippocampal neurons, where it induces expression of choline acetyltransferase (ChAT) to increase the production of acetylcholine (ACh), leading to maturation of the neurons.1,2) Notably, decreased expression of HCNP and its precursor has been seen in the brains of patients with Alzheimer’s disease,3) suggesting that HCNP is regulated physiologically and pathologically, and that it affects neural activity through ACh synthesis. HCNPpp is also reportedly expressed in a variety of other cells and tissues.4,5) For example, Goumon et al. reported that HCNP is released together with adrenaline from adrenal chromaffin cells and that it acts on M2 muscarinic ACh receptors (mACHRs) in the heart, mediating a negative inotropic effect under basal conditions and counteracting adrenergically induced positive inotropic.

Immune cells express both ChAT and AChRs, which constitute a non-neuronal cholinergic system that modulates immune and inflammatory function.6,7) We previously reported that T cells and other immune cells express HCNPpp, and that long-term exposure to HCNP reduces the ACh content of T cells by reducing ChAT gene expression.8) Here, we assessed expression of HCNPpp in U937 human macrophage-like cells and investigated the effects of HCNP on resting cells and on cells activated using lipopolysaccharide (LPS).

MATERIALS AND METHODS

Cell Culture U937 human macrophage-like cells were cultured in RPMI 1640 containing 7% fetal bovine serum (FBS), 100 units/mL penicillin and 100 µg/mL streptomycin at 37°C under a humidified atmosphere with 5% CO2.

Materials and Antibodies Synthetic HCNP was purchased from Bachem (Bubendorf, Basel-Landschaft, Switzerland). LPS was from Sigma-Aldrich (St. Louis, MO, U.S.A.). Anti-extracellular signal-regulated kinase (ERK), anti-phosphorylated ERK, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and anti-cyclooxygenase (COX)2 antibodies were all from Cell Signaling Technology (Danvers, MA, U.S.A.).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting U937 cells were lysed in 20 mM Tris–HCl (pH 7.4) with 2% SDS. Phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) was added to the lysis buffer to prevent dephosphorylation of ERK. The lysates were then subjected to 4–12% Bis-Tris SDS-PAGE (Thermo Fisher Scientific, Waltham, MA, U.S.A.), after which the separated proteins were transferred to nitrocellulose membranes (Thermo Fisher Scientific). The membranes were blocked using Blocking One (Nacalai Tesque) for 1 h at room temperature and then incubated first with primary antibodies at 4°C overnight and then with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h. The blots were developed using SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific), after which the chemiluminescence was detected using an ECL system (Amersham Imager 600, Cytiva, Marlborough, MA, U.S.A.).

Real-Time PCR Total RNAs were extracted from U937 cells using Sepasol RNA II Super (Nacalai Tesque), and cDNAs were prepared by reverse transcription using a Prime Script RT reagent Kit (TaKaRa Bio, Kusatsu, Shiga, Japan) with a S1000 Thermal Cycler (Bio-rad, Hercules, CA, U.S.A.). Real-time PCR analysis was conducted using CYBR premix EX taq, FAM-labeled probes, and predesigned primers (TaKaRa Bio) with a Thermal Cycler Dice Real Time System (TaKaRa).
Bio). The primer pairs were as follows: for ChAT (HA221706) 5'-AGC CCT GCC GTG ATC TTT G-3' and 5'-GCA CAG TCA GTG GGA ATG GAG T-3'; for HCNPpp (HA243336) 5'-CCA GCA AGG ATC CCA AAT-3' and 5'-CTG CGT GTA AAC CAG CCA GAC A-3'; for COX2 (HA293877) 5'-AGC CGT GTA GGA AGG ATC CCA AAT-3' and 5'-AAA CCC ACA GTG CTT GAC ACA GA-3'; inducible nitric oxide (NO) synthase (iNOS) (HA282449) 5'-CAG GCA ACG CCA GAA GAT CTC-3' and 5'-TG CTCAG CAC GAC ACA CA-3'; GAPDH (HA067812) 5'-GCA CCG TCA AGG CTG AGA AC-3' and 5'-TGA TGA AGA CGC CAG TGG A-3'.

**Statistical Analysis**  Data are presented as means ± standard error of the mean (S.E.M.). All representative experiments were repeated three times. Statistical analysis was performed using SigmaPlot (Systat Software Inc., San Jose, CA, U.S.A.). Differences between two groups were evaluated using Student’s t-test, and among three or more groups using one- and two-way ANOVA with post hoc Dunnett’s or Tukey’s test, respectively. Values of p < 0.05 were considered significant.

**RESULTS AND DISCUSSION**

Real-time PCR analysis revealed that HCNPpp in expressed in U937 macrophage-like cells to the same degree as in MOLT3 human T lymphoid cells (Fig. 1). This suggests that HCNP may be released from U937 cells after proteolytic cleavage of HCNPpp.

In MOLT3 cells, long-term exposure to HCNP results in a decreased levels of ChAT expression. In U937 cells, no ChAT expression was seen under resting conditions, but it was dramatically increased in response to 1 µg/mL LPS (Fig. 2A). HCNP did not affect ChAT mRNA levels in U937 cells under resting conditions, but it significantly suppressed LPS-induced ChAT gene expression (Fig. 2A). This suggests that, as in T cells, HCNP negatively regulates cholinergic signaling in U937 macrophages by suppressing ChAT gene expression. Because macrophages activated by LPS via Toll-like receptor 4 (TLR4) release several pro-inflammatory cytokines and mediators, including prostanoids and NO, we next investigated whether HCNP exposure affects expression of two inducible enzymes, COX-2 and iNOS, in U937 cells. We found that HCNP suppressed LPS-induced COX2 and iNOS gene expression, though HCNP had no effect on their expression in unstimulated cells (Fig. 2B). Correspondingly, HCNP also decreased LPS-induced expression of COX2 protein (Fig. 2C). HCNP activates myocardial M2 mAChRs, thereby exerting a negative inotropic effect under basal conditions and counteracting adrenergically induced positive inotropism. However, scopolamine, a mAChR antagonist, did not inhibit HCNP-induced suppression of ChAT and COX2 gene expression (Fig. 3A). This suggests HCNP does not act via mAChRs in macrophages.

Because LPS-induced TLR4 activation leads in turn to activation (phosphorylation) of ERK, we tested whether HCNP...
exposure to LPS. 14,15) Thereby, decreased ChAT gene expression increases ChAT expression in hippocampal neurons, resulting in cholinergic signaling in immune cells. On the contrary, HCNP Gq/11-coupled mAChR activation appears to enhance the release of adrenergic agonists on cardiac function, 6,10) we found that M2 mAChRs are not involved in HCNP-mediated suppression of COX2 and ChAT gene expression. Instead, the effect may reflect suppression of signaling triggered by TLR4 activation. HCNP is generated by cleaving the N-terminus of HCNPpp, which is alternatively referred to as Raf kinase inhibitory protein (PEBP) and reportedly inhibits the mitogen-activated protein kinase (MAPK)/ERK by interacting with Raf-1 and mitogen-activated protein extracellular kinase (MEK), as well as the nuclear factor-kappaB (NFκB) pathway. 2,12,13) Our findings suggest HCNP may retain some of the functionality of HCNPpp. Alternatively, HCNP may act as an antagonist preventing LPS binding to TLR4. In addition, we revealed that HCNP also suppresses ChAT gene expression in macrophages. Similar results were obtained from T cells, 9) suggesting that HCNP negatively regulates cholinergic signaling in immune cells. On the contrary, HCNP increases ChAT expression in hippocampal neurons, resulting in the increased Ach production. 25) Several species of ChAT mRNAs transcribed from three distinct promoters and produced by alternative splicing of noncoding exons are reportedly expressed in human. 10) The transcriptional regulation of ChAT gene with different promoters might account for the opposite response to HCNP between immune cells and neurons. Macrophages release several cytokines and inflammatory mediators in response to LPS. Our results suggest that HCNP suppresses induction of related inflammatory mediators, including iNOS and COX2. Although HCNP reportedly acts as a M2 mAChR agonist that suppresses the inotropic effect of adrenergic agonists on cardiac function, 6,10) we found that M2 mAChRs are not involved in HCNP-mediated suppression of COX2 and ChAT gene expression. Instead, the effect may reflect suppression of signaling triggered by TLR4 activation. HCNP is generated by cleaving the N-terminus of HCNPpp, which is alternatively referred to as Raf kinase inhibitory protein (PEBP) and reportedly inhibits the mitogen-activated protein kinase (MAPK)/ERK by interacting with Raf-1 and mitogen-activated protein extracellular kinase (MEK), as well as the nuclear factor-kappaB (NFκB) pathway. 2,12,13) Our findings suggest HCNP may retain some of the functionality of HCNPpp. Alternatively, HCNP may act as an antagonist preventing LPS binding to TLR4. In addition, we revealed that HCNP also suppresses ChAT gene expression in macrophages. Similar results were obtained from T cells, 9) suggesting that HCNP negatively regulates cholinergic signaling in immune cells. Macrophages express muscarinic and nicotinic ACh receptors (mACHRs and nAChRs, respectively). 2,10) Non-neuronal cholinergic system is involved in the regulation of immune and inflammatory responses. Especially, in macrophages, G4-coupled mAChR activation appears to enhance the release of cytokines such as tumor necrosis factor-α (TNF-α) after exposure to LPS. 14,15) Thereby, decreased ChAT gene expression by HCNP might reduce Ach production and release in immune cells, which partially and indirectly suppress LPS-induced gene expression of inflammatory enzymes. Determining where in the pathway which HCNP acts will be the aim of a future study.

In summary, our findings suggest that HCNP acts as an immunosuppressant in macrophages, providing possible evidence of the therapeutic potential of HCNP.

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Conflict of Interest The authors declare no conflict of interest.

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