Hippocampal Cholinergic Neurostimulating Peptide Suppresses Acetylcholine Synthesis in T Lymphocytes

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Lymphocytic cholinergic system has important roles in T cell functions, including immune responses and proliferation and differentiation of immune cells. T lymphocytes exclusively produces acetylcholine (ACh) via choline acetyltransferase (ChAT), activating their muscarinic and nicotinic ACh receptors (mAChRs and nAChRs, respectively) on the T cells themselves, and on other immune cells including B lymphocytes, dendritic cells and macrophages. In fact, our recent study shows that autocholine released from T lymphocytes evoked spontaneous increases in intracellular Ca\(^{2+}\) via nAChRs, which participates in interleukin (IL)-2 production and cell proliferation.

Hippocampal cholinergic neurostimulating peptide (HCNP) is an undecapeptide cleaved from N-terminal of phosphatidylethanolamine-binding protein 1 (PEBP1). HCNP enhances ACh synthesis through upregulation of ChAT expression in septo-hippocampal cholinergic neurons and participates in neuronal development and differentiation. Although PEBP1 and HCNP appears to be distributed ubiquitously in tissues and cells including spleen, its functions in immune cells have not been understood. In the present study, we observed that PEBP1 is also expressed in human and murine T cells. Long-term exposure to HCNP suppressed ChAT expression in MOLT3 human leukemic T cells, resulting in decreased release of ACh. HCNP also decreased the expression of extracellular signal-regulated kinase (ERK). Thus, HCNP appears to suppress lymphocytic cholinergic signaling, which might act as an immune modulator.

Key words hippocampal cholinergic neurostimulating peptide; acetylcholine; T lymphocyte; choline acetyltransferase

MATERIALS AND METHODS

Materials Synthetic HCNP was purchased from Bachem (H-8555).

Cell Culture C57BL/6J mice were obtained from Japan SLC, Inc. (Japan). The protocols used in this study were approved by the Ethical Committees of Doshisha Women's College of Liberal Arts (No. Y15012, Y16002). Murine sple-
nocyes were obtained from male C57BL/6J mice (3 months old) as described previously.\(^3\) MOLT3 human leukemic T cell and murine splenocytes were then cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 µg/mL streptomycin at 37°C under a humidified atmosphere with 5% CO\(_2\). In addition, when culturing murine splenocytes, 2-mercaptoethanol (100 µM) was added to the medium.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting** Murine splenocytes and MOLT3 cells were lysed in 20 mM Tris–HCl (pH 7.4) with 2% SDS. The lysates were subjected to 4–12% Bis-Tris SDS-PAGE (Thermo Fisher Scientific, 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, Japan) for 1 h at room temperature and 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 (FUJIFILM Las-3000, FUJIFILM, Japan).

**Immunocytochemistry** Murine splenocytes and MOLT3 cells plated on poly-d-lysine-coated glass-bottom dishes were fixed with 4% paraformaldehyde for 20 min at 4°C, permeabilized, and blocked and then incubated first with rabbit polyclonal anti-PEBP1 antibody (Abgent, U.S.A.) overnight at 4°C and then with Alexa-488-conjugated goat anti-rabbit immunoglobulin G (IgG) (Thermo Fisher Scientific), and allophycocyanin (APC)-conjugated anti-CD8 antibody (53-6.7, Thermo Fisher Scientific), phycoerythrin and then with Alexa-488-conjugated goat anti-PEBP1 antibody (Abgent, U.S.A.) overnight at 4°C.

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**Radioimmunoassay for ACh** MOLT3 cells (1×10^8 cells) were incubated with HCNP (300 pg/mL) for 6 d in the presence of 1 µM diisopropylfluorophosphate, an ACh esterase inhibitor. The culture supernatant (1 mL) was mixed with 500 µL of 1.2 n perchloric acid, after which the solution was kept on ice for 15 min and then centrifuged at 15000×g for 30 min at 4°C. The ACh content of the extracts was determined with a RIA using [3H]ACh (specific activity: 2.81 TBq/mmol, GE Healthcare, U.S.A.) and antiserum against ACh raised in a rabbit immobilized with choline hemiglutarate-bovine serum albumin conjugates.\(^9\)

**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., U.S.A.). Differences between two groups were evaluated using Student’s t-test, and between 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**

**PEBP1, HCNP Precursor Protein, Is Expressed in MOLT3 Human Leukemic T Cells and Murine Splenocytes** HCNP is generated from PEBP1 by the action of chymotrypsin-like enzyme intracellularly or after being secreted extracellularly.\(^5,8\) To assess expression of PEBP1 in T cells and splenocytes, we performed Western blotting using anti-PEBP1 antibodies. PEBP1 appears to be expressed in both MOLT3 human leukemic T cells and murine splenocytes (Fig. 1A). PEBP1 was expressed in several types of cells, including CD4\(^+\) and CD8\(^+\) T cells, in murine spleen (Fig. 1B) and was broadly distributed in the cytoplasm of MOLT3 cells (Fig. 1C). HCNP was previously described as a cytoplasmic protein that also associates with the plasma and endoplasmic reticulum membranes,\(^8\) which is consistent with our observed cytoplasmic localization of PEBP1 in MOLT3 cells and splenocytes.

**Long-Term Exposure to HCNP Decreases ACh Content in MOLT3 Cells by Suppressing ChAT Expression** In hippocampal cholinergic neurons, HCNP produced through cleavage of the N-terminal domain of PEBP1 fosters development of the cholinergic phenotype by increasing ChAT expression.\(^5,6\) To assess the effect of HCNP in T cells, MOLT3 cells were exposed to HCNP (1–300 pg/mL) for 1, 3 or 6 d. Exposure to HCNP for 6 d, but not 1 and 3 d, suppressed levels of ChAT mRNA in MOLT3 cells at concentrations ranging from 3 to 300 pg/mL (approximately 2.56 to 256 pm), which is consistent with the concentrations that increased ChAT expression in cultured hippocampal neurons\(^9\) (Fig. 2A). This suppressive effect of HCNP was also observed in splenocytes (Fig. 2B). Consistent with its ability to suppress ChAT mRNA expression, exposure to 300 pg/mL HCNP for 6 d reduced ChAT proteins by 40% and the ACh content as compared to control in MOLT3 cells (Figs. 2C, D). Long-term exposure to HCNP did not affect cell viability in MOLT3, indicating that the suppressive effect of HCNP on ChAT expression results from its cytotoxic effect (data not shown).

**Long-Term Exposure to HCNP Decreases Extracellular Signal-Regulated Kinase (ERK) Expression in MOLT3 Cells** We next investigated the HCNP-involved signaling pathway modulating ChAT expression. HCNP activates M, mACHRs in the heart, exerting a negative inotropic effect under basal conditions and counteracting adrenergic-induced...
positive inotropism.\textsuperscript{8,9} We tested whether scopolamine, a mAChR antagonist, suppressed HCNP-induced ChAT gene suppression. However, scopolamine did not affect the HCNP effect (Fig. 3A). ChAT gene expression is regulated by mitogen-activated protein kinase (MAPK) cascade.\textsuperscript{11} In fact, U0126, a MEK inhibitor, decreased ChAT mRNA levels in MOLT3 cells (Fig. 3B). Western blotting showed that exposure to HCNP for 6 d decreased ERK expression, leading to decreases in the level of phosphorylated (activated) ERK (Fig. 3C). This suggests HCNP acts by suppressing ERK transcription, which in turn leads to decrease in T cell expression of ChAT.

In summary, PEBP1, a HCNP precursor protein, is expressed in several immune cell types present in the murine spleen. Although it is unknown whether HCNP is released from its precursor protein expressed in these cells, long-term exposure to HCNP has a negative effect on ACh synthesis by suppressing ChAT expression in T lymphocytes. In hip-
pocampal neurons, HCNP increases ChAT expression, resulting in the increased ACh production. This discrepancy is probably due to multiple ChAT mRNA transcripts (R-, N1-, N2-, and M-types) initiated by three distinct promoter regions and produced by alternative splicing of 5'-noncoding exons. M-type is the most abundant transcript, followed by R-type and N-types, in the brain and spinal cord, while only N2-type is expressed in MOLT-3. The different promoter for ChAT and N-types, in the brain and spinal cord, while only N2-type is the most abundant transcript, followed by R-type.

Our previous study using mAChR and nAChR-deficient mice revealed that lymphocytic cholinergic system influences diverse immune responses. Given the fact that HCNP suppresses ChAT expression and then ACh contents in T lymphocytes, HCNP acts as an immune modulator by inhibiting cholinergic signaling in T cells.

Fig. 3. Long-Term Exposure to HCNP Decreases ERK Expression in MOLT3 Cells

A. ChAT mRNA levels in MOLT3 cells exposed to 300 pg/mL HCNP in the absence or the presence of 10 μM scopolamine for 6 d. ChAT mRNA levels were assessed using real-time PCR. Shown are means±S.E.M. (n=3). ***p<0.001 vs. Control. B. ChAT mRNA levels in MOLT3 cells exposed to 300 pg/mL HCNP or 10 μM U0126 for 6 d. ChAT mRNA levels were assessed using real-time PCR. Shown are means±S.E.M. (n=3). ***p<0.001 vs. Control. C. Left: Western blots showing ChAT and ERK expression in MOLT3 cells exposed to 300 pg/mL HCNP for 6 d. GAPDH was used as a loading control. Right: Graph showing relative levels of ChAT in MOLT3 cells with or without exposure to 300 pg/mL HCNP for 6 d. ChAT protein was quantified using densitometry and normalized to GAPDH. Shown are means±S.E.M. (n=3). *p<0.05, ***p<0.001 vs. Control.