Human antimicrobial peptide LL-37 induces glial-mediated neuroinflammation

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ABSTRACT

LL-37 is the sole cathelicidin-derived antimicrobial peptide found in humans. It becomes active upon C-terminal cleavage of its inactive precursor hCAP18. In addition to antimicrobial action, it also functions as an innate immune system stimulant in many tissues of the body. Here we report that hCAP18 and LL-37 are expressed in all organs of the human body that were studied with the highest basic levels being expressed in the GI tract and the brain. Its expression and functional role in the central nerve system (CNS) has not previously been reported. We found increased expression of LL-37 in IFNγ-stimulated human astrocytes and their surrogate U373 cells, as well as in LPS/IFNγ-stimulated human microglia and their surrogate monocyte-derived THP-1 cells. We found that treatment of microglia, astrocytes, THP-1 cells and U373 cells with LL-37 induced secretion of the inflammatory cytokines IL-1β and IL-6; the chemokines IL-8 and CCL-2, and other materials toxic to human neuroblastoma SH-SY5Y cells. The mechanism of LL-37 stimulation involves activation of intracellular proinflammatory pathways involving phospho-P38 MAP kinase and phospho-NFκB proteins. We blocked the inflammatory stimulant action of LL-37 by removing it with an anti-LL-37 antibody. The inflammatory effect was also prevented by treatment with inhibitors of PKC, PI3K and MEK-1/2 as well as with the intracellular Ca^{2+}-chelator, BAPTA-AM. This indicates involvement of these intracellular pathways. Our data suggest that LL-37, in addition to its established roles, may play a role in the chronic neuroinflammation which is observed in neurodegenerative diseases such as Alzheimer's and Parkinson's disease.

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1. Introduction

Cathelicidins are a special class of proteins that are part of the innate immune system of mammals. They consist of a signal peptide, a cathelin domain, and an active C-terminal antimicrobial peptide [1]. In humans there is only one representative of this class of proteins. It has been identified as human cationic antimicrobial protein (hCAP18) [1,2]. LL-37, the active C-terminal component of hCAP18, is released upon cleavage of the inactive precursor. LL-37 is so-named because the first two of the 37 amino acids in the peptide are leucines. The anti-pathogenic activity of the peptide has been demonstrated extensively against Gram-negative and

Gram-positive bacteria, numerous viruses, and fungi [reviewed by 3,4]. It is released from epithelial cells directly; and also into the phagosomes of immunocompetent cells where it can come into direct contact with foreign organisms that have been phagocytosed.

These anti-pathogenic functions of LL-37 are effective in a wide range of organs such as the pulmonary and digestive systems, the genitourinary system, esophageal tissue, salivary glands, skin and ocular surfaces [3,5,6]. It has been proposed that LL-37 produced by glia and other cells in the central nervous system may play an important role in the innate immune response against pathogens during bacterial infections [7]. Here we show that organs of the human body routinely express hCAP18 and its cleaved product LL-37. The highest base levels are observed in the GI tract and brain. Presumably this reflects a standby function which can immediately respond to pathogenic challenges.

There is increasing evidence that LL-37 plays a substantially greater role than that of an endogenous antibiotic. It appears to be an important signaling molecule for innate immune system responses. It induces activation of extracellular signal-regulated

kinase and p38 kinase signaling pathways in monocytes [8] and keratinocytes [9]. Its production in keratinocytes is increased after application of IL-1α or IL-6. Its production is also increased by exposure to LPS [3,10]. It reduces secretion of TNFα and reactive oxygen species in lipopolysaccharide (LPS)-treated macrophages, presumably due to its strong binding to LPS [1].

LL-37 is important as a host defense peptide because it has toxicity to bacterial cells, fungi, viruses and also cancerous host cells, given its known importance for the function of natural killer cells [11]. The cathelicidins are also known to be an element of snake venoms [12], which would indicate naturally toxic biophysical aspects. In its role as an innate immune system effector, LL-37 is known to spike during acute infections and/or injury.

Given this context, and its unique role in human physiology, it is important to determine whether LL-37 may play a role in neuroinflammation. Neuroinflammation is known to be induced by injuries to brain with subsequent release from activated microglia and astrocytes, pro-inflammatory cytokines, chemokines and other potentially toxic materials. Indeed, most neuropathological states resulting in neurodegeneration are associated with inflammatory responses mediated by over-activated glial cells. For example, such inflammation has been shown in AD, PD, and ALS [13–15].

To explore the possible role of LL-37 in neurological disorders, we first investigated whether LL-37 production was induced by the stimulation of microglia and astrocytes with lipopolysaccharide/interferon-gamma (LPS/IFNγ) or IFNγ, respectively. We found that such induction took place. We next found that treatment with LL-37 caused microglia and astrocytes to release proinflammatory cytokines, chemokines and other materials toxic to SH-SY5Y cells by activating intracellular inflammatory pathways involving P38 MAPK and NFκB proteins. We then conducted pharmacological studies indicating that an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and activation of phosphinositide 3-kinase (PI3K), protein kinase C (PKC), and MEK-1/2 are involved in this LL-37-mediated glial neuroinflammatory response.

2. Materials and methods

2.1. Materials

The source and study of all human tissues was done in accordance with policies and procedures for human studies approved by ethics committees of the University of British Columbia. The brain and other organs of three autopsy cases were studied for the expression of LL-37 and hCAP18. Case 1 was an apparently healthy male age 55 who died suddenly of a myocardial infarction. Case 2 was a male age 80 who died of pneumonia. Case 3 was a male age 62 with type 1 diabetes who died of advanced Alzheimer disease. For living human cells from surgical cases, microglia and astrocytes were obtained from aspirated temporal lobe tissue lying superficial to an epileptic focus undergoing removal.

All reagents were purchased from Sigma (St. Louis, MO) unless stated otherwise. The following substances were used in the assays: diaphorase (EC 1.8.1.4, from Clostridium kluyveri, 5.8 U/mg solid), p-iodonitrotetrazolium violet (INT), nicotinamide adenine dinucleotide (NAD⁺), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). LL-37 was purchased from Anaspec Inc. (Fremont, CA).

2.2. Cell culture and experimental protocols

The human THP-1, U373, NT-2 and SK-N-MC cell lines were obtained from the American Type Culture Collection (ATCC). These are standard surrogate cell lines for human microglia, astrocytes, and neurons respectively. The human neuroblastoma SH-SY5Y cell line was a gift from Dr R. Ross, Fordham University, NY. All cells except for the NT-2 cells were grown in DMEM/F12 medium containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) in a humidified 5% CO_2 and 95% air incubator. NT-2 cells were grown in DMEM medium containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen) under humidified 5% CO_2 and 95% air.

Human astroglia and microglia were isolated from surgically resected temporal lobe tissue. Protocols for culturing these cells have been described in detail previously [16]. Tissues were first incubated in a trypsin solution, then pelleted, resuspended and passed through a nylon filter. They were pelleted once more, resuspended in DMEM/F12 medium with 10% FBS containing gentamicin, and added to tissue culture plates. Microglial cells adhered first. The non-adherent cells along with myelin debris were transferred into new culture plates. Astrocytes adhered next and were allowed to grow by replacing the medium once a week. New passages of cells were generated by harvesting confluent astrocyte cultures using a trypsin–EDTA solution. The purity of astrocytes and microglia was examined by immunostaining with specific antibodies. For astrocytes, anti GFAP antibody (1/4000, DAKO, Mississauga, Ontario), and for microglia anti CR3/43 antibody (1/2000, DAKO, Mississauga, Ontario) were utilized. The purity of microglia and astrocytes were 97% and 99%, respectively. Human astrocytes from up to the fifth passage were used in the study.

Human astrocytes, THP-1 cells, and U373 cells (5×10^5 cells), as well as human microglia (5×10^4 cells) were seeded into 24-well plates in 800 μl of DMEM/F12 medium containing 5% FBS. The cells were treated with various concentrations of LL-37 (0.1–30 μg/ml). After incubation for 2 days, the supernatants (400 μl) were transferred to undifferentiated human neuroblastoma SH-SY5Y cells (2×10^5 cells per well). The cells were incubated for a further 72 h and MTT and LDH assays were performed as described below. For comparative experiments with differentiated SH-SY5Y cells, they were first treated with retinoic acid at 5 μM for 4 days [17].

For some experiments, microglia and astrocytes were treated with LL-37 plus an inhibitor of PI3K, wortmanin (1 μM, Calbiochem, La Jolla, CA) [18], a PKC inhibitor, bisindolylmaleimide (1 μM, Calbiochem, La Jolla, CA) [19] or the MEK-1/2 kinase inhibitor, U0126 (10 μM, Calbiochem, La Jolla, CA) [19]. For possible involvement of Ca^{2+} we used a Ca^{2+} chelator BAPTA-AM (300 μM) [20]. The mixtures were incubated for 6 h before the cell supernatants were transferred to SH-SY5Y cells. After 72 h incubation, SH-SY5Y cell viability assays were carried out. As controls, microglia and astrocytes were treated with the above inhibitors without LL-37 for 6 h before the cell supernatants were transferred to SH-SY5Y cells. After 72 h incubation, SH-SY5Y cell viability assays were carried out.

To investigate further the specific effects of LL-37 on glial mediated toxicity toward SH-SY5Y cells, experiments were carried out where LL-37 was added together with an anti LL-37 antibody (200 μg/ml, Hycult Biotech, Plymouth Meeting, PA). After 2 days incubation, cell free supernatants were collected and the released cytokine levels measured. SH-SY5Y cell viability was also examined using the MTT and LDH assays after these cells were exposed to the conditioned medium.

2.3. Cell viability assay

The viability of SH-SY5Y cells following incubation with glial cell supernatants was evaluated by the lactate dehydrogenase
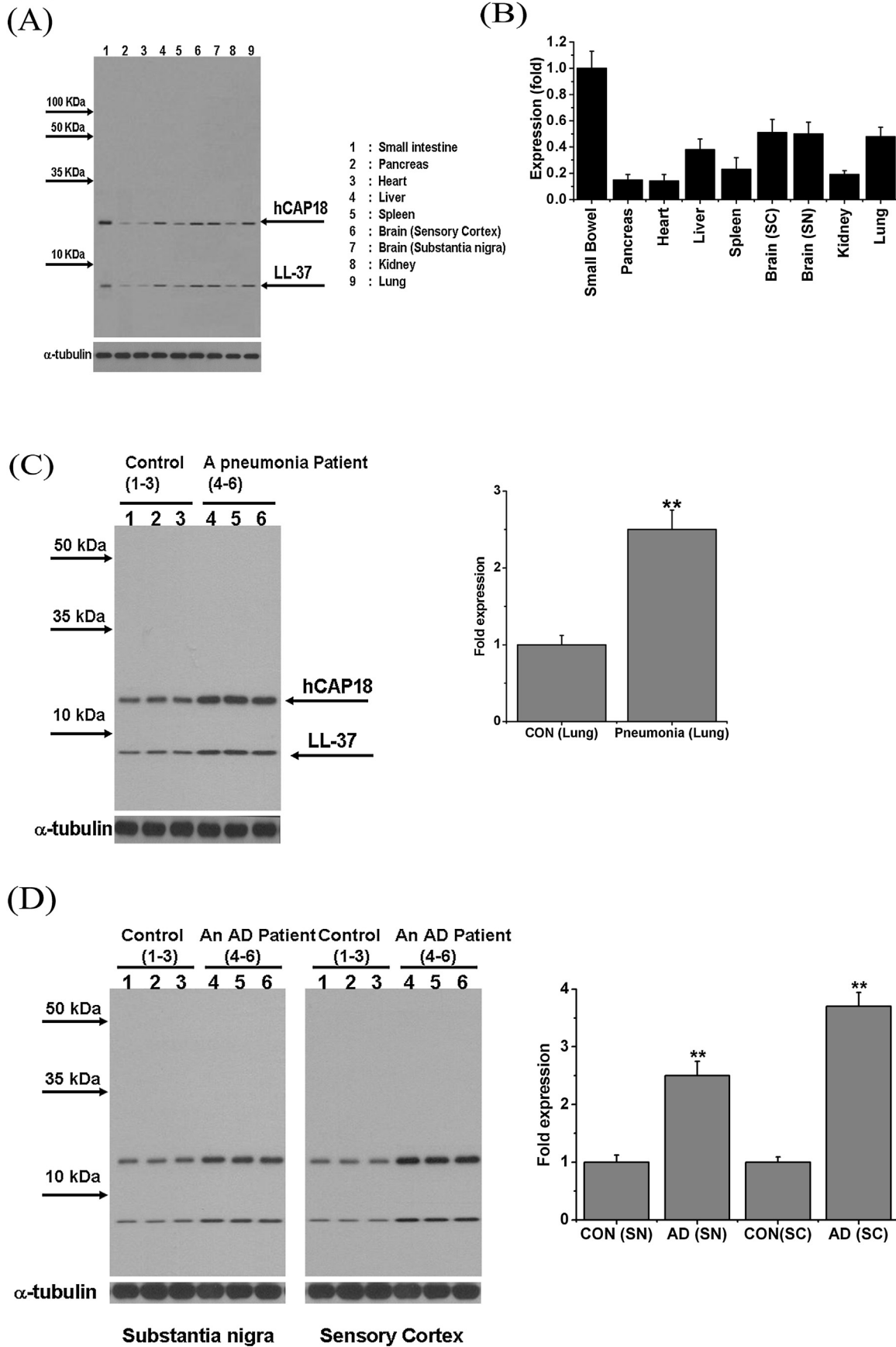
(A)

1	: Small intestine
2	: Pancreas
3	: Heart
4	: Liver
5	: Spleen
6	: Brain (Sensory Cortex)
7	: Brain (Substantia nigra)
8	: Kidney
9	: Lung

(B)

(C)

(D)

Fig. 1. Expression of hCAP18 and LL-37 in human organs. (A) Typical results representative of three independent experiments. (B) Quantitative results. The densities of bands were compared with those of the small bowel which was the tissue with the highest expression. Note that expression of the proteins was higher in small intestine, liver, lung and brain (substantia nigra and sensory cortex) than other organs examined. (C) Expression of hCAP18 and LL-37 in lung in normal and pneumonia patients. Right panel:

(LDH) release and MTT assays as previously described in detail [21]. The amount of LDH released was expressed as a percentage of the value obtained in comparative wells where cells were 100% lysed by 1% Triton X-100. For the MTT assay, data are presented as a percentage of the value obtained from cells incubated in fresh medium only.

2.4. Reverse-transcriptase polymerase chain reaction (RT-PCR) and real-time quantitative PCR

Total RNA was isolated from human microglia, astrocytes, THP-1, U373, SK-N-MC, NT-2 and SH-SY5Y neurons using TRIzol (GIBCO-BRL, Gathersburg, MD). Cells (10^6 cells) were lysed with TRIzol solution and incubated at room temperature for 1 h. The lysates were centrifuged at 10,000 rpm for 10 min and supernatants transferred to new tubes. The purity and amount of the RNA was measured spectrophotometrically. Total RNA (20 μg) was used to synthesize the first strand complementary DNA (cDNA) using Moloney murine leukemia virus (M-MLV) reverse transcriptase (GIBCO-BRL). The cDNA products were then amplified by PCR using a GeneAmp thermal cycler (Applied Biosystems, Foster City, CA). Specific sense and antisense primers for LL-37/hCAP18 and GAPDH are LL-37/hCAP18: Forward 5′-ATCATTGCCCAGGTCCTCAG-3′ and Reverse 5′-GTCCCCATACACCGCTTCAC-3′ (251 bp) [22], and GAPDH: Forward 5′-CCATGTTCGTCATGGGTGTGAACCA-3′ and Reverse 5′-GCCAGTAGAGGCAGGGATGATGTTC-3′ (251 bp) [21]. PCR conditions were as follows: initial denaturation at 95 °C for 6 min followed by a 30-cycle amplification program consisting of denaturation at 95 °C for 45 s, annealing at 55–60 °C for 1 min and extension at 72 °C for 1 min. A final extension was carried out at 72 °C for 10 min. The amplified PCR products were identified using 1.5% agarose gels containing ethidium bromide (final concentration 0.5 μg/ml) and visualized under ultraviolet light.

Quantitative real-time PCR (qPCR) was also performed. Total RNA (5 μg) and the primers listed above were mixed with Fast SYBR® Green Master Mix (Life Technologies, Carlsbad, CA). PCR was performed using a Quantstudio 6 Flex (Applied Biosystems, Foster City, CA). Comparative C_T (Delta delta C_T, $\Delta\Delta C_T$) values were used to determine the relative expression of LL-37/hCAP18 [23].

2.5. Measurement of released IL-1β, IL-6, IL-8 and CCL-2

Cytokine and chemokine levels were measured in cell-free supernatants following 6 h or 48 h incubation of THP-1 cells, U373 cells, microglial cells and astrocytes. The cell stimulation protocols were the same as described above for measuring cell viability. Quantitation was performed with ELISA detection kits (Peprotech, NJ) following protocols described by the manufacturer.

2.6. Western blotting

Western blotting on cell lysates was performed as described by Lee et al. [24]. Briefly, after exposure to stimulants, human microglia and astrocytes were treated with a lysis buffer (150 mM NaCl, 12 mM deoxycholic acid, 0.1% Nonidet P-40, 0.1% Triton X-100 and 5 mM Tris–EDTA, pH 7.4). The protein concentration of the cell lysates was then determined using a BCA protein assay reagent kit (Pierce, Rockford, IL). Human organs were similarly treated with the lysis buffer and homogenized. Homogenates were centrifuged and the supernatants collected for western blotting.

The loading quantities of lysate proteins were 100 μg. Proteins in each sample were loaded onto gels and separated by 10% SDS-PAGE (150 V, 1.5 h). Following SDS-PAGE, proteins were transferred to a PVDF membrane (Bio-Rad, CA) at 30 mA for 2 h. The membranes were blocked with 5% milk in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween 20, pH 7.4) for 1 h and incubated overnight at 4 °C with a polyclonal anti-phospho-P38 MAP kinase antibody (9211, Cell Signaling, Beverly, MA, 1/2000), anti-phospho-P65 NFκB antibody (3031, Cell Signaling, Beverly, MA, 1/1000). The membranes were then treated with a horseradish peroxidase-conjugated anti-IgG (P0448, DAKO, Mississauga, Ontario, CA, 1/2000) for 3 h at room temperature and the bands visualized with an enhanced chemiluminescence system and exposure to photographic film (Hyperfilm ECL™, Amersham Pharmacia Biotech, Little Chalfont, UK). Equalization of protein loading was assessed independently using α-tubulin as the housekeeping protein. The primary antibody was anti α-tubulin (T6074, Sigma, St. Louis, MO, 1/2000) and the secondary antibody anti-mouse IgG (A3682, Sigma, 1/3000). Primary antibody incubation was overnight at 4 °C and secondary antibody incubation 3 h at room temperature. For expression of LL-37, the monoclonal anti LL-37 antibody (Hycult Biotech, Plymouth Meeting, PA, 1/2000) was used. For the secondary antibody, anti-mouse IgG antibody (A3682, Sigma, 1/3000) was utilized.

2.7. Immunostaining

Three elderly control cases without known neurological symptoms (age 71–78 years) and five cases with Alzheimer disease (age 71–87 years, Braak stage IV and V) were selected for study from our brain bank at the University of British Columbia. Immunohistochemistry was carried out as previously described in detail [16]. Briefly, brain tissues were fixed in 4% paraformaldehyde, and, after 3–4 days, transferred to a 15% buffered sucrose maintenance solution. Other tissues were fixed in formalin and embedded in paraffin. Cryostat cut sections of 30-μm thickness were pretreated with 0.5% H_2O_2, blocked with 5% skim milk, and incubated in a primary monoclonal anti-LL-37 antibody (Hycult, Biotech, Plymouth Meeting, PA, 1C-12, 1/500) in PBS-T for 72 h at 4 °C or overnight at room temperature. The sections were next treated with the appropriate biotinylated secondary antibody (DAKO, 1/2000) for 2 h at room temperature, followed by incubation in an avidin-biotinylated horseradish peroxidase complex (DAKO) for 1 h at room temperature. Peroxidase labeling was visualized by incubation in 0.01% 3,3′-diaminobenzidine containing 1% nickel ammonium sulfate, 5 mm imidazole, and 0.001% H_2O_2 in 0.05 m Tris–HCl buffer, pH 7.6. When a dark purple color developed, sections were washed and then mounted on glass slides.

2.8. Data analysis

The significance of differences in data sets was analyzed by one-way or two-way ANOVA tests. Multiple group comparisons were followed where appropriate by a *post hoc* Bonferroni test.

3. Results

Firstly we examined the expression of hCAP 18 and LL-37 in post mortem human tissues by western blot analysis. All tissues

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Quantitative results. Values are mean ± SEM, $n = 3$. Significance of differences was tested by t-test. $^{**}P < 0.01$ for pneumonia patients compared with controls. Note that protein expression was higher in lungs of pneumonia patients (2.5 fold increase) than controls. (D) Expression of hCAP18 and LL-37 in brain in normal and AD patients. Right panel: Quantitative results. Values are mean ± SEM, $n = 3$. Significance of differences was tested by t-test. $^{**}P < 0.01$ for substantia nigra (SN) and sensory cortex (SC) of AD patients compared with those of controls. Note that protein expression was higher in substantia nigra (SN) and sensory cortex (SC) of AD patients (2.5 fold for SN and 3.7 fold for SC) than those of controls.
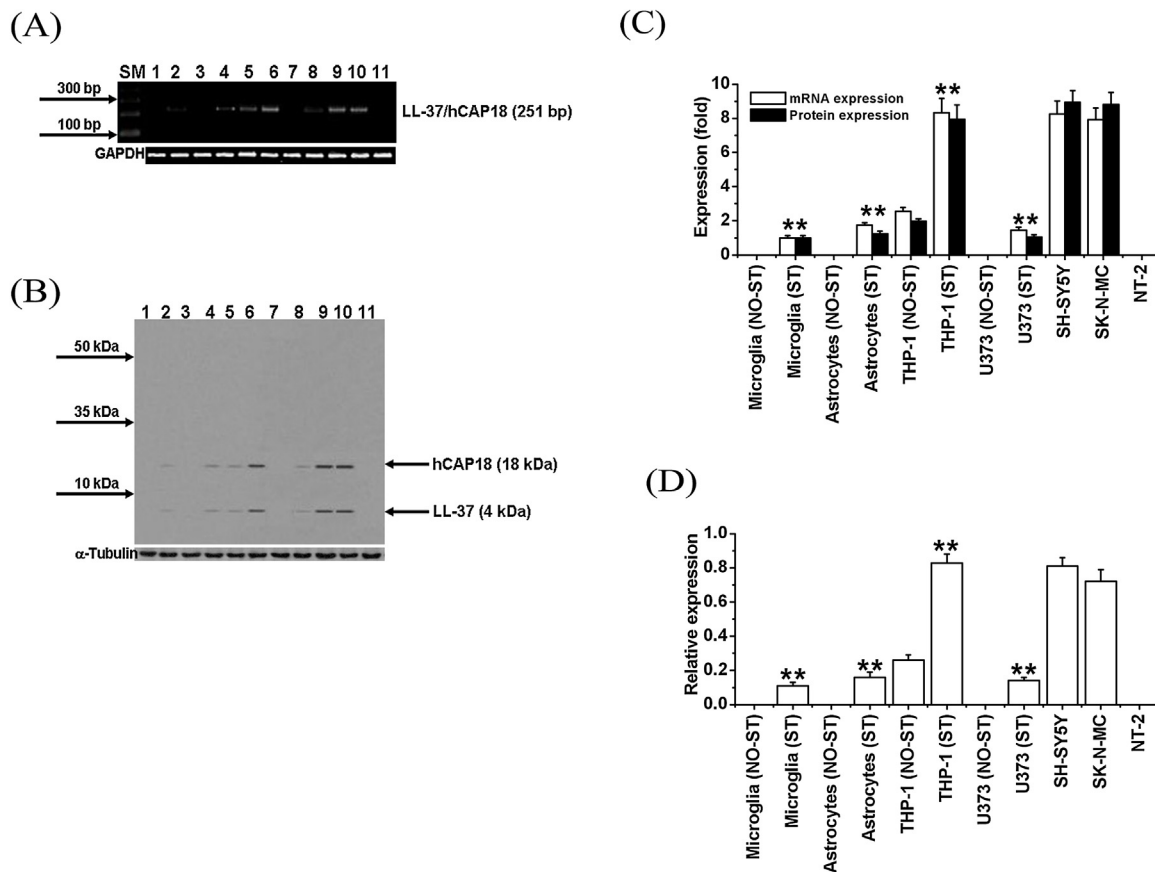
Fig. 2. Expression of LL-37/hCAP18 in stimulated or unstimulated cells. (A) mRNAs by RT-PCR and (B) proteins by Western blot analyses. (A and B) SM: 100 bp ladder; 1: unstimulated microglia; 2: LPS/IFNγ-stimulated microglia; 3: unstimulated astrocytes; 4: IFNγ-stimulated astrocytes; 5: unstimulated THP-1 cells; 6: LPS/IFNγ-stimulated THP-1 cells; 7: unstimulated U373 cells; 8: IFNγ-stimulated U373 cells; 9: SH-SY5Y cells; 10: SK-N-MC cells; and 11: NT-2 cells. GAPDH loading controls for mRNA, and α-tubulin loading controls for protein are shown in the lower panels. Unstimulated THP-1 cells, but not microglia, astrocytes and U373 cells, expressed LL-37. When stimulated microglia, astrocytes and U373 cells induced expression of LL-37. THP-1 cells potentiated expression of this protein by 4-fold after stimulation for 2 days. SH-SY5Y cells and SK-N-MC cells, but not NT-2 cells, expressed this protein. (C) Quantitative results. The densitometry values of each band were normalized to GAPDH or α-tubulin, respectively. For mRNA and protein data, the densities of bands were compared with those of stimulated microglial cells. Values are mean ± SEM, $n = 3$. Significance of differences was tested by *t*-test. **$P < 0.01$ for stimulated microglia, astrocytes, THP-1 cells and U373 cells compared with their unstimulated (NO-ST) cells. (D) Expression of LL-37/hCAP18 mRNAs in stimulated or unstimulated cells by quantitative real time PCR (qPCR). Real time PCR was performed with Fast SYBR® Green Master Mix using a Quantstudio 6 Flex amplification cycler. ΔΔC$_T$ values were used to express relative expression of LL-37/hCAP18 mRNAs. Values are mean ± SEM, $n = 3$. Significance of differences was tested by *t*-test. **$P < 0.01$ for stimulated microglia, astrocytes, THP-1 cells and U373 cells compared with their unstimulated (NO-ST) cells.

examined demonstrated significant expression of both compounds. The results are shown in Fig. 1. Fig. 1A shows that LL-37 and hCAP18 were found to be expressed in the GI tract, brain sensory cortex and substantia nigra, pancreas, lung, heart, liver, spleen and kidney. Fig. 1B shows the relative expression in organs compared with the GI tract which had the highest levels. Brain

areas showed the next highest levels followed by lung and liver. Fig. 1C shows the relative levels in the case with chronic lung disease. It shows upregulation of hCAP and LL-37 in the lung compared with other organs. Fig. 1D shows the relative levels of the case dying of Alzheimer disease. It shows relative upregulation of hCAP and LL-37 in the brain compared with other organs. These
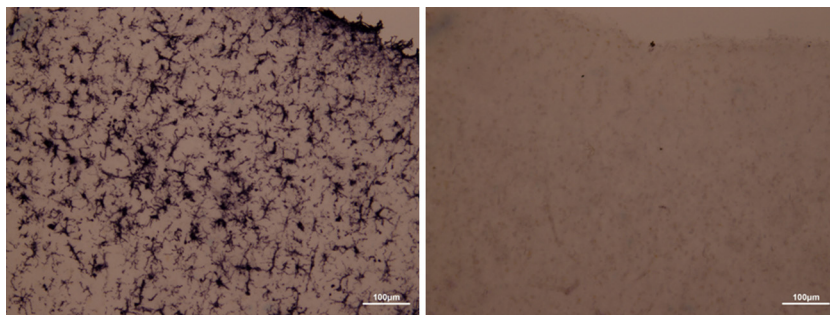

Fig. 3. Expression of LL-37 in AD and control post-mortem temporal cortex. LL-37 is strongly expressed in microglia and astrocytes in AD temporal cortex (left panel) but is not detectably expressed in control brain (right panel).
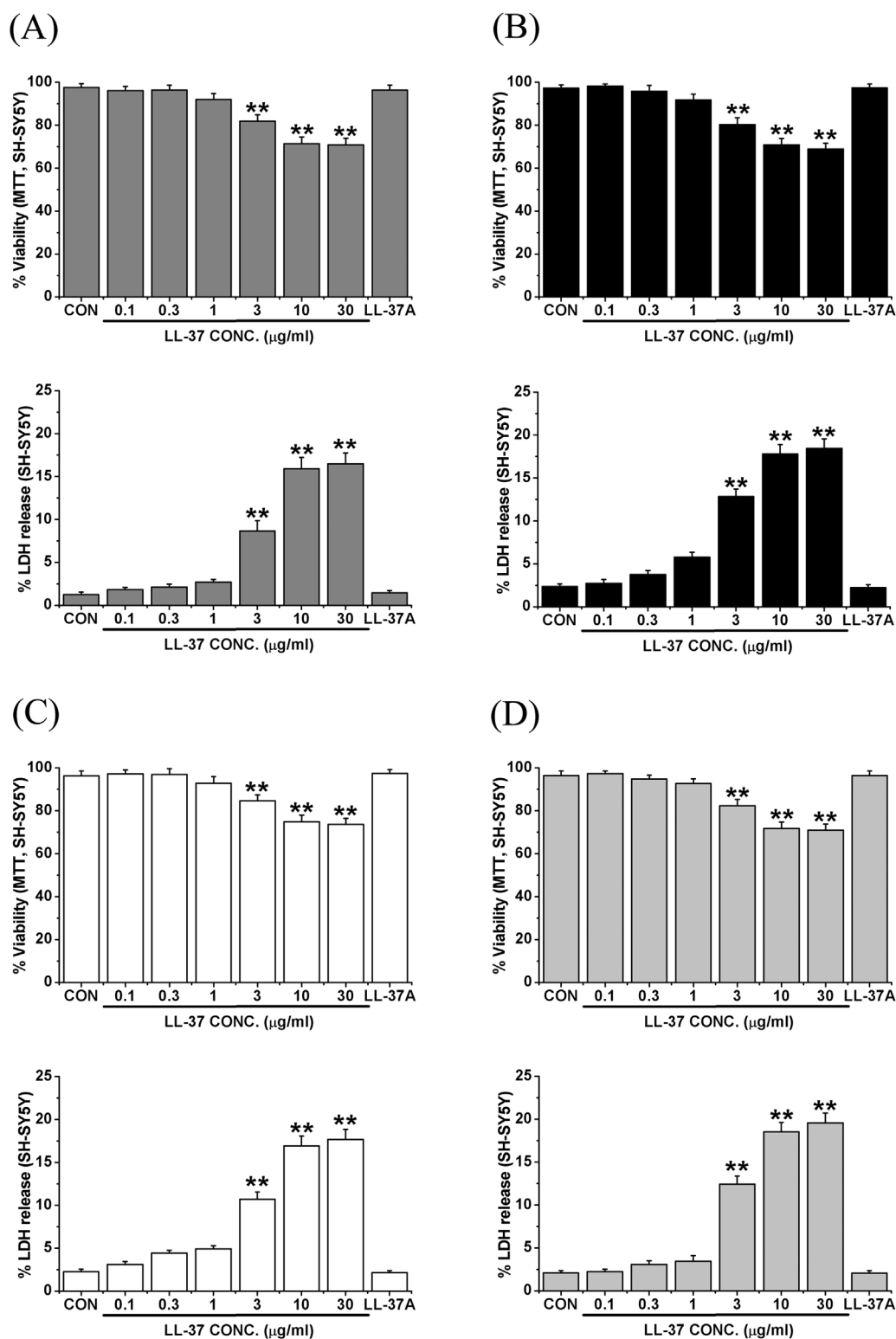
Fig. 4. SH-SY5Y viability changes induced by supernatants from (A) stimulated human microglia, (B) stimulated human astrocytes, (C) stimulated THP-1 cells and (D) stimulated U373 cells by LL-37 (0.1–30 μg/ml). After glial cells were treated with various concentrations of LL-37 for 2 days, their cell-free supernatants were transferred to SH-SY5Y cells. MTT and LDH release assays were performed in 3 days. Reductions in numbers of live cells are indicated by the MTT assay (upper panels), and increases in the numbers of dead cells by LDH release (lower panels). Notice that LL-37 at the concentrations of 3–30 μg/ml increased the toxicity of glial supernatants toward SH-SY5Y cells, but LL-37 was not toxic to SH-SY5Y cells when directly added (LL-37A in *X*-axis). Values are mean ± SEM, *n* = 4. One-way ANOVA was carried out to test significance. Multiple comparisons were followed with *post hoc* Bonferroni tests where appropriate. **P < 0.01 for LL-37 stimulated cells (3–30 μg/ml group) compared with unstimulated cells (CON group).

data demonstrate that there are base levels of hCAP and LL-37 expressed in all organs and that these levels are upregulated in chronically diseased areas.

We next examined by RT-PCR and western blot analyses LL-37/hCAP18 levels in all of the cultured cell types with and without stimulation. As stimulants for expression of LL-37 in microglia and THP-1 cells, we utilized LPS/IFNγ (LPS: 1 μg/ml and IFNγ: 333 U/ml) [25]. For astrocytes and U373 cells, we utilized IFNγ alone (IFNγ: 150 U/ml) since addition of LPS did not potentiate astrocytic neuroinflammation [26], which is closely associated with LL-37 expression. Fig. 2A and B demonstrates the mRNA and protein expression of LL-37/hCAP18 in unstimulated, compared with stimulated cells. The cells included astrocytes, U373 cells, microglia and THP-1 cells, as well as the three neuronally committed cell lines SH-SY5Y, NT-2, and SK-N-MC. Only THP-1

cells expressed detectable levels of LL-37/hCAP18 mRNA and proteins.

LL-37 expression was induced by treatment for 2 days with LPS/IFNγ for microglia and THP-1 cells, and IFNγ for astrocytes and U373 cells. In the case of THP-1 cells, LL-37 expression was increased approximately 4-fold ($P < 0.01$, lanes 1–8 in Fig. 2A for mRNA and 2B for protein). SK-N-MC cells and SH-SY5Y cells, but not NT-2 cells, also expressed LL-37, the amount of which was comparable to that in LPS/IFNγ-treated THP-1 cells (lanes 9–11 in Fig. 2A and B). The α-tubulin loading control for protein expression is shown at the bottom of each lane (Fig. 2B). The blot is a representative of three independent experiments. These data are shown in Fig. 2C. The relative expression of LL-37/hCAP18 in each cell line was further confirmed by quantitative real time PCR (qPCR). The results are shown in Fig. 2D.
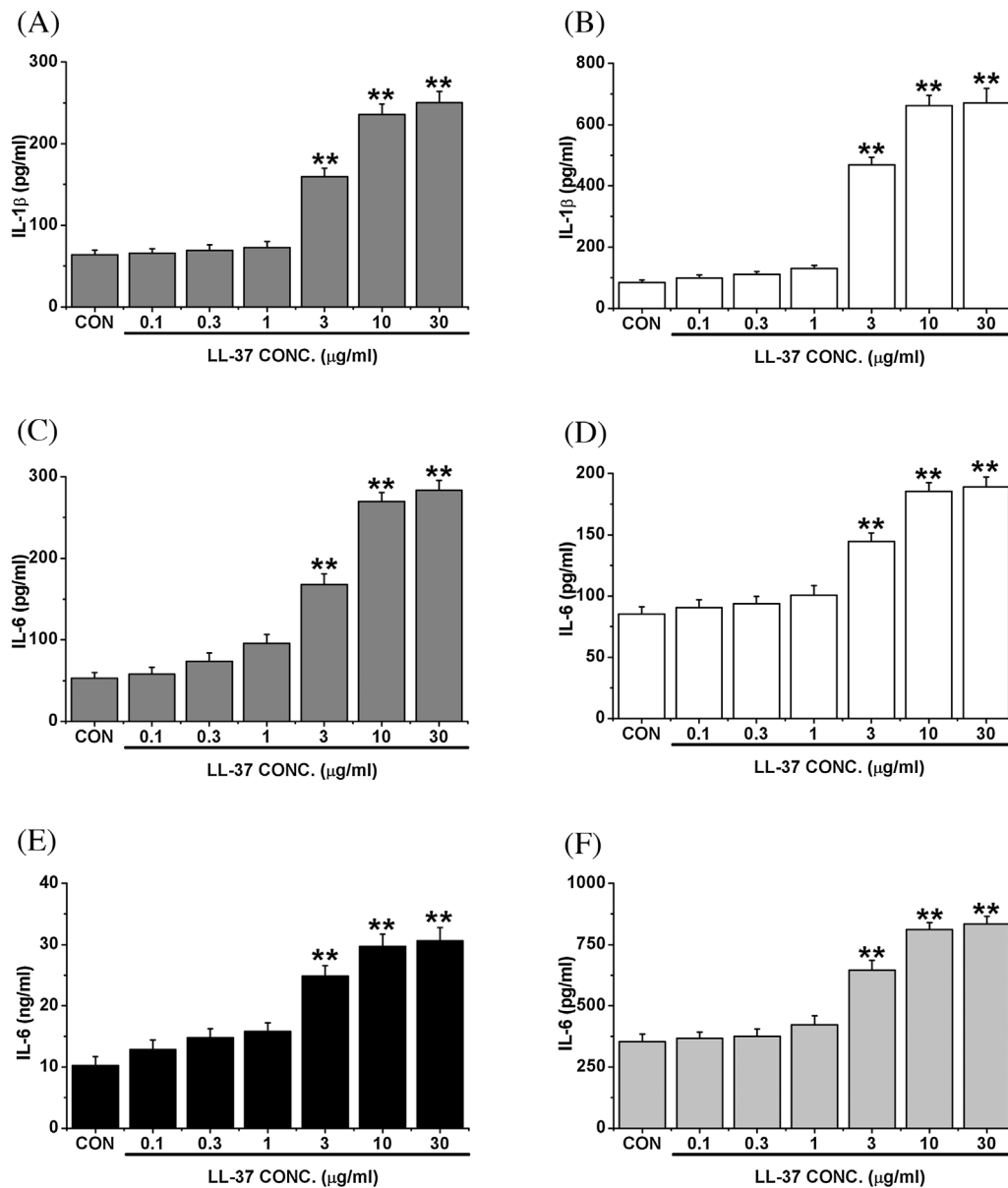

Fig. 5. Release of the inflammatory cytokines IL-1β from microglia (A), THP-1 cells (B), as well as IL-6 from microglia (C), THP-1 cells (D), astrocytes (E), and U373 cells (F) by LL-37. After microglia, THP-1 cells, astrocytes and U373 cells were treated with LL-37 (0.1–30 μg/ml) for 2 days, the released cytokine levels were measured using ELISA kits. Values are mean ± SEM, $n = 4$. Notice that LL-37 at concentrations of 3–30 μg/ml induced release of both IL-1β and IL-6 in all cells. One-way ANOVA was carried out to test the significance of differences. Multiple comparisons were followed with *post hoc* Bonferroni tests where appropriate. **$P < 0.01$ for LL-37 stimulated cells (3–30 μg/ml group) compared with unstimulated cells (CON group).

Typical results of immunostaining are shown in Fig. 3. In control brains there was no immunostaining of LL-37 (Fig. 3, right panel). In AD brain there was extensive LL-37 immunostaining of microglia and astrocytes (Fig. 3, left panel).

We next investigated whether treatment with LL-37 stimulated glial cells to release inflammatory factors toxic to neuroblastoma SH-SY5Y cells. For these experiments, microglia, and astrocytes, and their surrogate THP-1 and U373 cells, were incubated with LL-37 (0.1–30 μg/ml) for 2 days. Their cell free supernatants were then incubated with SH-SY5Y cells for 72 h. The resultant viability of the SH-SY5Y cells was measured by the MTT and lactate dehydrogenase (LDH) release assays. Fig. 4 demonstrates the results. MTT data are shown in the upper panel and LDH release in the lower panel. Exposure of microglia and astrocytes to 0.1–1 μg/ml LL-37 did not induce SH-SY5Y cell viability loss, but LL-37 at concentrations of 3–30 μg/ml decreased SH-SY5Y cell viability in a concentration-dependent manner ($P < 0.01$, Fig. 4A for microglia and B for astrocytes). This was confirmed by LDH release data. LDH release was 16–18% at 30 μg/ml LL-37 ($P < 0.01$). The effects of LL-37 on stimulated THP-1 or U373 toxicity toward SH-SY5Y cells replicated the microglial and astrocytic data (Fig. 4C for THP-1 cells and D for U373 cells). There was no direct effect of LL-37 treatment of SH-SY5Y cells at a concentration of 30 μg/ml for 3 days. Control experiments indicate that LL-37 (0.1–30 μg/ml) did not reduce the viability of the four types of glial cells (data not shown).

Further experiments were performed to investigate whether there were differences in viability change between differentiated and undifferentiated SH-SY5Y cells. For this investigation, SH-SY5Y cells were first differentiated with retinoic acid at 5 μM for 4 days [17]. THP-1 cells, U373 cells, astrocytes and microglia were exposed to LL-37 for 48 h. Differentiated or undifferentiated SH-SY5Y cells were then exposed to their conditioned media for 3 days. The MTT assay was employed to examine viability changes. The data indicate that viability loss of differentiated and undifferentiated SH-SY5Y cells induced by the conditioned medium were similar in the presence of LL-37 (data not shown). Therefore we performed further experiments with undifferentiated SH-SY5Y cells.

To determine whether the LL-37 effects were correlated with the release of inflammatory factors, the levels of IL-1β and IL-6 were measured in supernatants from LL-37 treated microglia, astrocytes, THP-1 cells and U373 cells that had been treated for 2 days. The results are shown in Fig. 5. At low levels there was no effect, but there was an increase in levels of IL-1β and IL-6 in microglia and THP-1 cells in a concentration-dependent fashion between 3 and 30 μg/ml. For IL-1β at 30 μg/ml, there was a 3.9-fold increase in microglia and an 8.1-fold increase in THP-1 cells ($P < 0.01$, Fig. 5A and B). For IL-6 at this concentration there was a 5.5-fold increase in microglia and a 2.3-fold increase in THP-1 cells ($P < 0.01$, Fig. 5C and D).

As far as astrocytes are concerned, IL-6 is the main inflammatory mediator that is generated [27]. There was an increase in the supernatant concentration of IL-6 from astrocytes and U373 cells in a concentration-dependent manner in the same 3–30 μg/ml range. At 30 μg/ml there was a 3-fold increase in astrocytes and a 2.5-fold increase in U373 cells ($P < 0.01$, Fig. 5E and F). Treatment with LL-37 after the culture medium was separated from cells did not change the levels of released IL-1β and IL-6 indicating that LL-37 had no direct effect (data not shown).
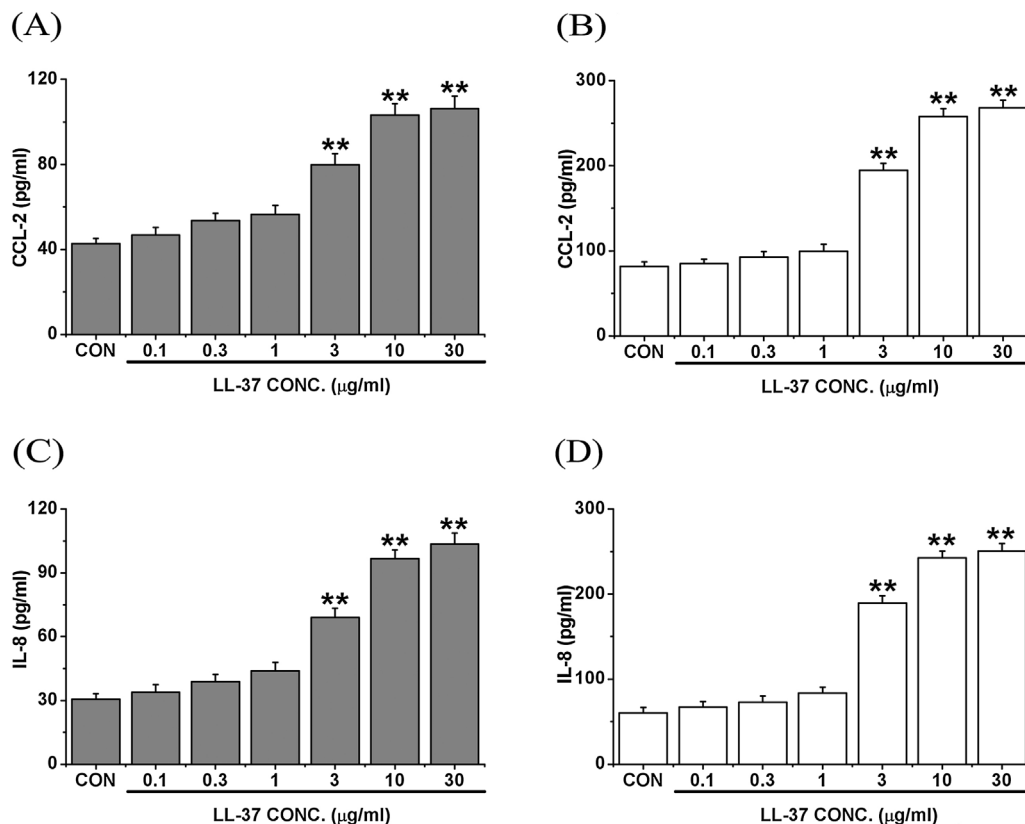

Fig. 6. Release of the inflammatory chemokines CCL-2 and IL-8 from microglia (A and C) and THP-1 cells (B and D) by LL-37. After microglia and THP-1 cells were treated with LL-37 (0.1–30 μg/ml) for 2 days the released cytokine levels were measured using ELISA kits. Values are mean ± SEM, $n = 4$. Notice that LL-37 at concentrations of 3–30 μg/ml induced release of both CCL-2 and IL-8 in both cells. One-way ANOVA was carried out to test the significance of differences. Multiple comparisons were followed with *post hoc* Bonferroni tests where appropriate. **$P < 0.01$ for LL-37 stimulated cells (3–30 μg/ml group) compared with unstimulated cells (CON group).

We also measured released levels of chemokines such as CCL-2 and IL-8 from microglia and their surrogate THP-1 cells after 48 h incubation with LL-37. Fig. 6 shows the results. The levels of IL-8 and CCL-2 were also increased in both microglia and THP-1 cells in a concentration-dependent fashion between 3 and 30 μg/ml (IL-8: 2.5-fold increase in microglia and 3.2-fold increase in THP-1 cells at 30 μg/ml, $P < 0.01$, Fig. 6A and C, and CCL-2: 3.3-fold increase in microglia and 4.1-fold increase in THP-1 cells at 30 μg/ml, $P < 0.01$, Fig. 6B and D). Treatment with LL-37 after culture medium was separated from cells did not change the levels of released IL-8 and CCL-2 (data not shown).

To investigate the mechanisms by which LL-37 influences the neuroinflammatory response, we examined extracts of microglial and astrocytic cells for evidence of activation of known intracellular inflammatory pathways. Cells were treated with LL-37 (0.1, 1 and 10 μg/ml) for 6 h at 37 °C. Western blotting results are detailed in Fig. 7A (left panel for microglial extracts, and right panel for astrocytic extracts). To ensure equal loading, the densitometric value of each band was normalized to the corresponding band for α-tubulin. Fig. 7B summarizes the quantitative results. Treatment with LL-37 at concentrations of 0.1 and 1 μg/ml did not activate intracellular inflammatory pathways such as phospho-P38 MAPK and phospho-NFκB proteins. Treatment with 10 μg/ml LL-37 produced an induction of phospho-NFκB (8-fold for microglia and 9-fold for astrocytes) and phospho-P38 MAPK (7.5-fold for microglia and 8.1-fold for astrocytes).
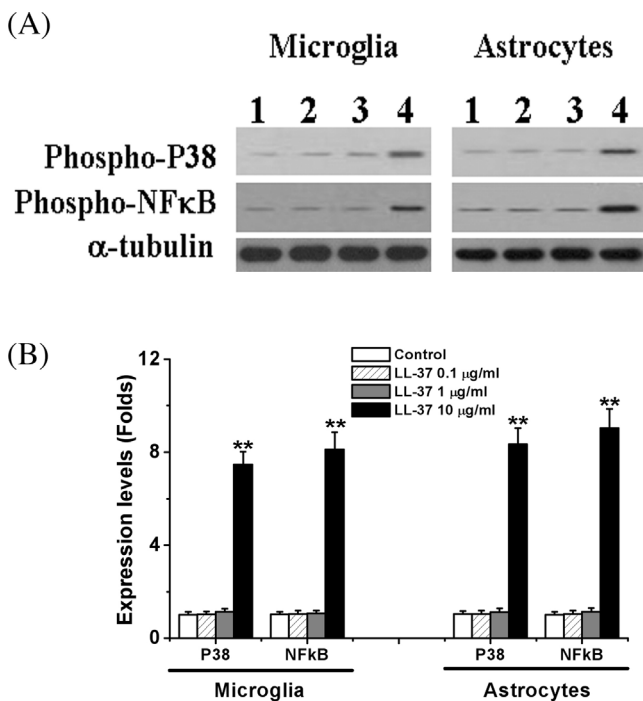
To confirm the effects of LL-37 on toxicity of conditioned medium from microglia or astrocytes toward SH-SY5Y cells, the microglia and astrocytes were treated with LL-37 at 30 μg/ml and anti LL-37 antibody (200 μg/ml) for 2 days. Their cell-free supernatants were then transferred to SH-SY5Y cells. After 3 days the MTT assay was employed to measure the SH-SY5Y cell viability. Levels of cytokines such as IL-1β and IL-6 in the presence of the antibody in conditioned medium were also measured. Fig. 8 shows the results. Addition of the anti LL-37 antibody completely blocked the effects of LL-37 on toxicity of the stimulated glial conditioned medium (Fig. 8A and B). It also reduced the levels of released cytokines from the stimulated microglia and astrocytes (Fig. 8C–E). The antibody itself did not affect SH-SY5Y cell viability when applied directly (Fig. 8A and B: X-axis: [LL-37 + Ab]A).

A further set of experiments was conducted to investigate the possible involvement of an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and intracellular phosphinositide 3-kinase (PI3K), protein kinase C (PKC) and MEK-1/2 kinase since previous studies indicate that PI3K, PKC and MEK kinase could be possible links between LL-37 binding proteins on the cell surface and P38 MAP kinase/NFκB induction [28–32]. We incubated astrocytes and microglia with inhibitors of these proteins (bisindolylmaleimide for PKC, 1 μM; wortmanin for PI3K, 1 μM and U0126 for MEK-1/2, 10 μM) for 6 h. We then measured levels of cytokines and examined their effects on glial toxicity toward SH-SY5Y cells. We also used a Ca^{2+} chelator, BAPTA-AM (300 μM) for 6 h.

These inhibitors and the Ca^{2+} chelator reduced levels of IL-1β (Fig. 9C) and IL-6 (Fig. 9D) in microglia by 70–90%, with a similar reduction of IL-6 in astrocytes (Fig. 9E). They also completely blocked LL-37-mediated glial toxicity toward SH-SY5Y cells by the MTT assay (Fig. 9A for microglia and B for astrocytes). These results indicate the involvement of PKC, PI3K and MEK in LL-37 stimulation of inflammatory pathways in both astrocytes and microglia. However, treatment with these inhibitors and the Ca^{2+}-chelator for 6 h did not change viability of SH-SY5Y cells (Fig. 9A and B) and microglia and astrocytes (data not shown).

4. Discussion

In the present study we demonstrated that hCAP18 and its active metabolite LL-37 were expressed in all the human organs that were studied. Its production is evidently much more widespread than previously reported. Such widespread production is consistent with a fundamental role in innate immune responses. The low levels detected in apparently normal tissues would suggest the possibility of standby expression and turnover of LL-37 to provide an instant response to immune challenges. The upregulated levels in lung of a respiratory case, and in brain of an Alzheimer disease case show that chronic inflammation in diseased areas will lead to upregulation of LL-37 expression, consistent with an innate immune response.

We also demonstrated by RT-PCR and qPCR that the mRNA of LL-37/hCP18 is expressed in LPS/IFNγ-stimulated microglia, IFNγ-stimulated astrocytes, and especially the SH-SY5Y and SK-N-MC neuronal cell lines. This was confirmed by LL-37 immunostaining results in post-mortem brains (Fig. 3).

We further found that LL-37 in a concentration of more than 3 μg/ml acted as an inflammatory stimulant to astrocytes and microglia. It induced phosphorylation of P38 MAPK and NFκB. This resulted in an increased release of the proinflammatory cytokines IL-1β and IL-6, the chemokines IL-8 and CCL-2, and other materials toxic to SH-SY5Y cells. The mechanism was confirmed by removal of the effect with an anti-LL-37 antibody. We also found that inhibitors of PKC, PI3K, MEK-1/2 or an intracellular Ca^{2+}-chelator,

(A)

Microglia   Astrocytes
1 2 3 4   1 2 3 4

Phospho-P38
Phospho-NFκB
α-tubulin

(B)

Fig. 7. Induction of intracellular inflammatory pathway phospho-P38 MAPK and phospho-P65-NFκB proteins in human microglia (A, left panel) and astrocytes (A, right panel). After microglia and astrocytes were treated with LL-37 (0.1, 1 and 10 μg/ml) for 6 h, cell extracts were prepared and the proteins separated by SDS-PAGE. Representative blots are shown in (A) and quantitative results in (B). (A) Lane 1: control, lane 2: 0.1 μg/ml, lane 3: 1 μg/ml and lane 4: 10 μg/ml: to ensure equal loading, the densitometric value of each band was normalized to the corresponding band for α-tubulin. Notice the sharp increase in the inflammatory markers phospho-P38 MAPK and phospho-P65-NFκB at 10 μg/ml LL-37 (approximately 8-fold increase). Values are mean ± SEM, n = 3. One-way ANOVA was carried out to examine the significance of differences. **$P < 0.01$ for stimulated cells (10 μg/ml LL-37 group) compared with unstimulated cells (CON group).
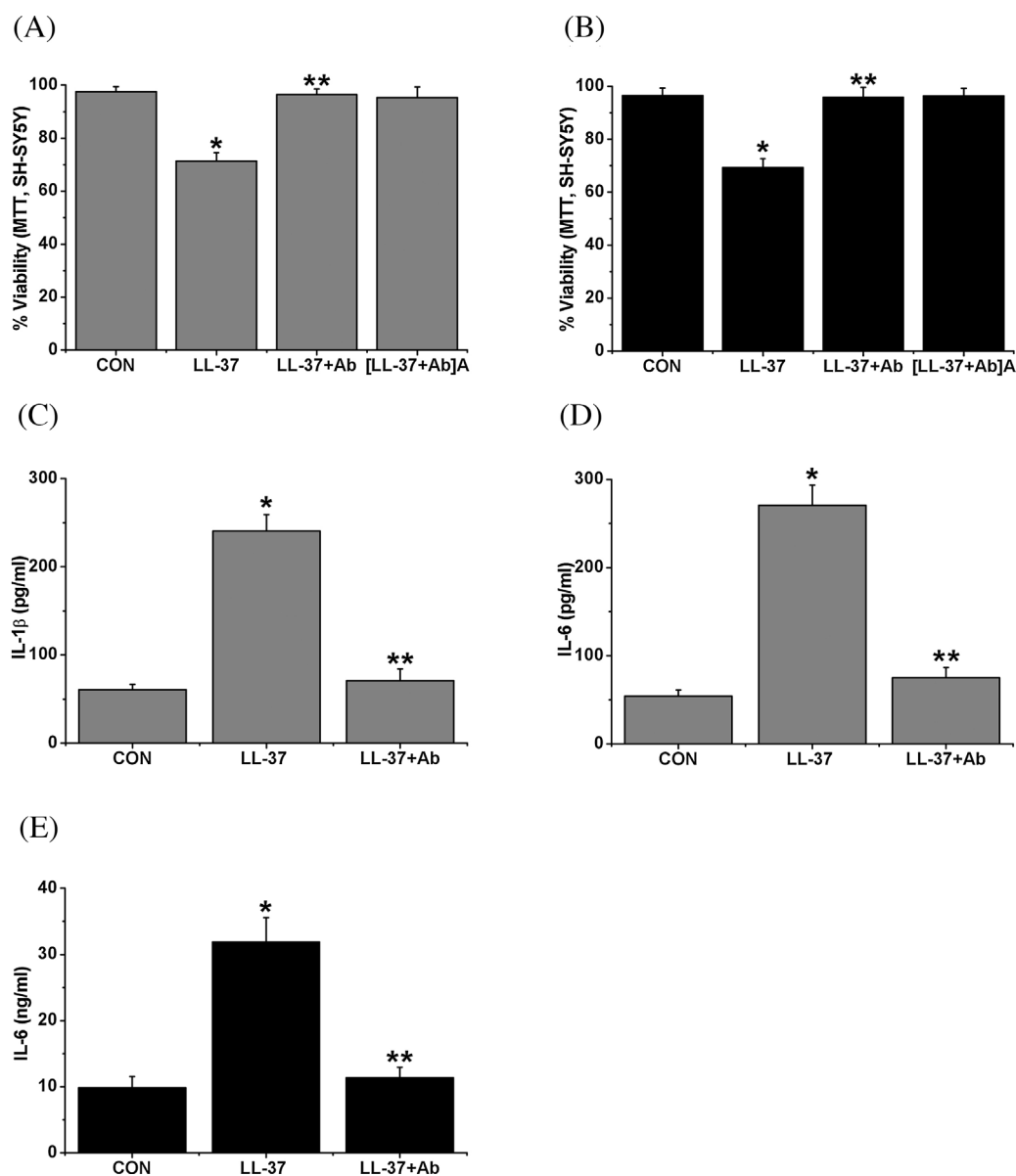
(A)

(B)

(C)

(D)

(E)

Fig. 8. Effects of treatment with anti LL-37 antibody (200 µg/ml) on LL-37-mediated neurotoxicity and cytokine release. Microglia (A) and astrocytes (B) were activated in the presence of LL-37 30 µg/ml plus anti LL-37 antibody (200 µg/ml) for 2 days and their conditioned media transferred to SH-SY5Y cells. The MTT assay was performed on SH-SY5Y after 3 days showing that the LL-37-mediated microglial and astrocytic toxicity was blocked by the LL-37 antibody. The bar designated [LL-37 + Ab]A refers to the direct application of the LL-37 antibody to SH-SY5Y cells. A similar blockade was shown for release of IL-1β (C) and IL-6 (D) from microglia, and IL-6 (E) from astrocytes following stimulation of the cells for 48 h. Values are mean ± SEM, $n = 4$. Significance by one-way ANOVA. Notice that in each case, treatment with the anti LL-37 antibody eliminated the effect of treatment with LL-37. *$P < 0.01$ for stimulated cells (LL-37 group) compared with unstimulated cells (CON group) and **$P < 0.01$ for LL-37 + Ab group compared with LL-37 group.

BAPTA-AM, also attenuated the effect, indicating the involvement of these proteins in the intracellular signaling cascade.

The mechanism by which LL-37 activates microglia and astrocytes is totally unclear. Previous studies suggest that LL-37 binding proteins might include the N-formylpeptide receptor like-1 (FPRL-1), a G-protein-coupled receptor [33], P2X7 receptors [34] and P2Y11 receptors [35]. Stimulation of these receptors increases [Ca^{2+}]$_i$, and activates PI3K, PKC and MAP kinases. This results in expression and release of inflammatory cytokines, chemokines and other materials toxic to nearby neurons.

Therefore it is possible to postulate a mechanism underlying LL-37-mediated neuroinflammation and neurotoxicity. When neurons are injured in chronic neurodegenerative disorders such as Alzheimer disease, they may release LL-37 which activates microglia and astrocytes. These stimulated microglia and astrocytes release LL-37 which can then bind to receptors such as FPRL-1, P2X7 and P2Y11. The result can be translocation of NFκB proteins to the nucleus. This, in turn, can lead to expression and release of inflammatory proteins such as TNFα, IL-1 and IL-6 providing a positive feedback mechanism leading to further destruction of neurons.

Our data indicate that LL-37 expression may play a role in neurological diseases where neuroinflammation is one of the main components of disease progression. It is known that Aβ plaques activate microglia in early stage of the AD. The possibilities could be explored, for example, in transgenic mouse models of Alzheimer disease where prominent astrocytic and microglial reactions occur surrounding Aβ deposits.
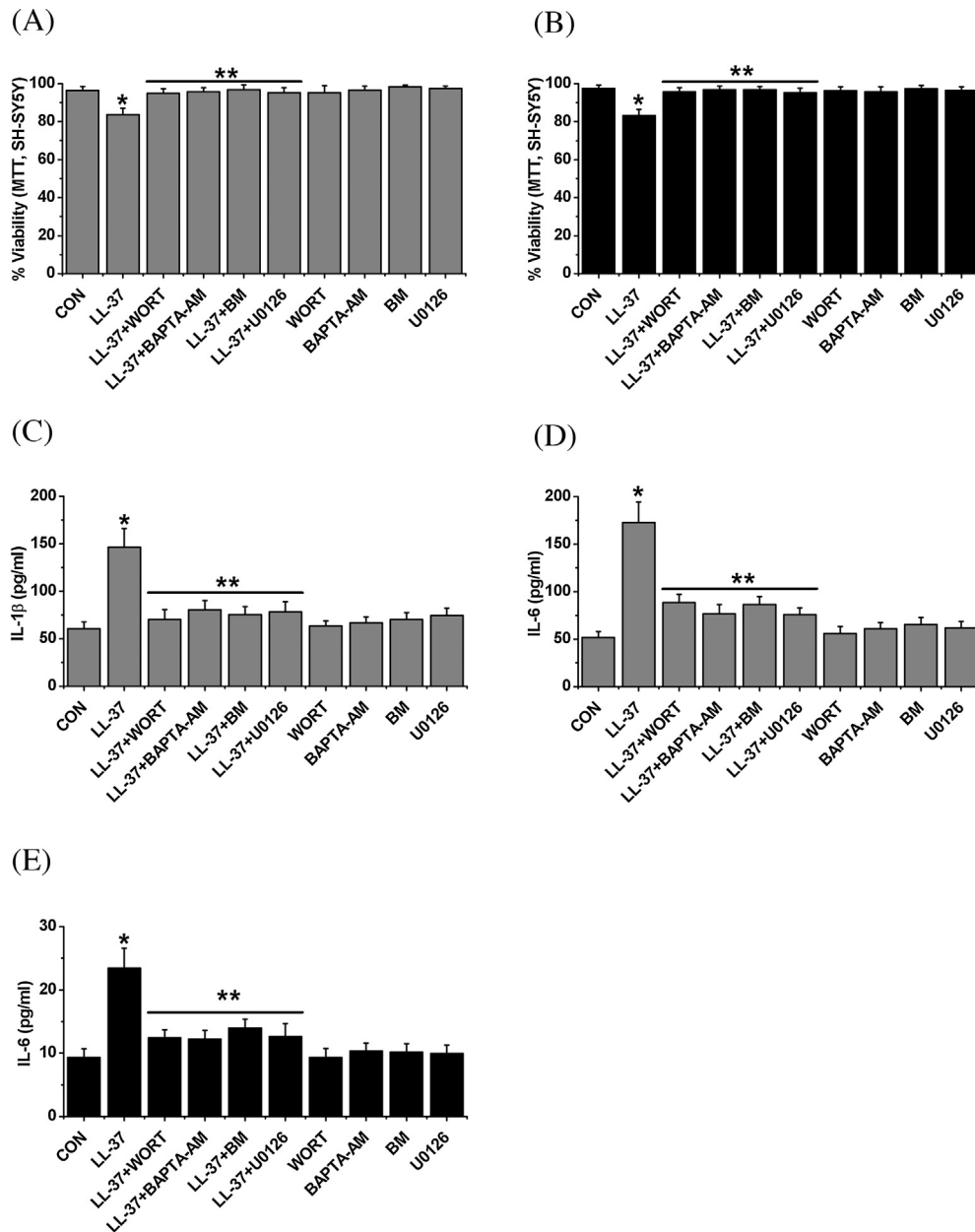
(A)

(B)

(C)

(D)

(E)

Fig. 9. Effect of treatment with wortmanin (WORT, a PI3K inhibitor, 1 μM,), BAPTA-AM (A Ca^{2+} chelator, 300 μM), bisindolylmaleimide (BM, a PKC inhibitor, 1 μM), or U0126 (a MEK-1/2 kinase inhibitor, 10 μM) on LL-37 (10 μg/ml)-mediated cytokine release and glial neurotoxicity. Released levels of IL-1β (C) and IL-6 (D) from microglia, and IL-6 (E) from astrocytes for 6 h. MTT results on SH-SY5Y cell viability changes induced by stimulated microglia (A) and astrocytes (B). Values are mean ± SEM, n = 4. Significance by one-way ANOVA. Notice that each inhibitor significantly reduced the effect of LL-37 stimulation. *$P < 0.01$ for stimulated cells (LL-37 group) compared with unstimulated cells (CON group) and **$P < 0.01$ for LL-37 + WORT, LL-39 + BAPTA-AM, LL-37 + BM and LL-37 + U0126 groups compared with LL-37 group.

Conflicts of interest

None of the authors have any conflicts of interest.

Acknowledgements

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