Cortistatin Is Induced in Brain Tissue and Exerts Neuroprotection in a Rat Model of Bacterial Meningoencephalitis

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There are fewer reports of brain infection by Klebsiella pneumoniae than there are in other organs, but an increase incidence and morbidity has been noted. We have previously developed a rat model of K. pneumoniae meningoencephalitis. Cortistatin (CST) is a recently discovered neuropeptide with endocrine activities in humans. In this study, we found that brain infection by K. pneumoniae increased endogenous prepro-CST messenger RNA expression, which occurred earlier than did leukocyte infiltration in vivo and also occurred in cultured neuron-glia. Postinfection treatment with CST (either intracerebroventricularly or intraperitoneally), but not somatostatin, reduced leukocyte recruitment and clinical illness as revealed by fever and clinical score in vivo. Postinfection increases of proinflammatory cytokine messenger RNA levels were attenuated by CST in neuron-glia cultures, further confirming a direct effect on neuroinflammation. Administration of CST resulted in less postinfection neuronal loss in vitro, suggesting a direct neuroprotective effect and potential as an adjuvant for treating bacterial meningoencephalitis.

Acute bacterial infection of the central nervous system has remained a critical care problem associated with high mortality and morbidity worldwide despite advances in antibiotic therapy [1–3]. Meningitis-associated brain injury and neuronal death is not mediated simply by the presence of viable bacteria but by a consequence of host response to pathogens [4]. We have previously demonstrated that glial cells are important cellular sources of proinflammatory cytokines in cerebrospinal fluid (CSF) in a rat model of Klebsiella pneumoniae meningoencephalitis [5]. A striking increase in incidence and morbidity of K. pneumoniae meningitis has been noted in Taiwan [6–9]. The induction and amplification of host inflammatory responses during bacterial meningitis may be a potential target for adjunctive therapy.

Cortistatin (CST) is a cyclic neuropeptide with predominantly cortical expression and the ability to depress cortical activity [10]. CST originates from prepro-CST, a 27-residue apparent secretion signal sequence; it may be cleaved to human CST, which shares 13 of the last 14 residues with rat and mouse CST-14 with similar biological activities (reviewed by de Lecea [11]). CST shares some functions with somatostatin (SST), including depression of neuronal activity through binding to SST receptors [12]. However, CST, but not SST, has been detected in various human immune cells, including lymphocytes, monocytes, macrophages, and dendritic cells. CST has immunomodulatory effects, and the levels of CST correlate with the degree of inflammatory cell differentiation and activation [13, 14]. Previous studies have demonstrated that CST has anti-inflammatory effects in different experimental models, including ulcerative colitis, arthritis, and endotoxemia [15–18]. In human studies, CST has been shown to have endocrine activities [19, 20].

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The present study was aimed to investigate the brain expression of CST after *K. pneumoniae* infection in rats and evaluate the effects of CST administration on *K. pneumoniae* meningoencephalitis. We provide evidence that endogenous CST could be induced after *K. pneumoniae* meningoencephalitis in brain tissue, and that exogenous CST administration has anti-inflammatory and neuroprotective effects.

**MATERIALS AND METHODS**

**Animal Model of Meningoencephalitis**

The pathogen *K. pneumoniae* was isolated from the CSF culture of a patient with meningitis. The animal model for meningoencephalitis was established using adult male Sprague-Dawley rats weighing 300–400 g by stereotactic inoculation into the right lateral ventricle with *K. pneumoniae* (1 × 10³ colony-forming units [CFUs] in 10 μL) after the rat was anesthetized with pentobarbital (65 mg/kg; intraperitoneal injection) [5]. Primary cultures of neuron-glia were prepared from 1-day-old neonatal Sprague-Dawley rats as described elsewhere [21, 22]. All animal protocols were approved by the Institutional Committee for Care and Use of Animals at the National Defense Medical Center and Taipei Medical University, Taiwan.

**Drug Administration**

CST (Peptides International, Louisville, KY) was dissolved in phosphate-buffered saline (PBS; pH, 7.4) for intracerebroventricular (ICV) administration or in sterile saline for intraperitoneal administration. Effects of CST were also compared with those of SST (Sigma-Aldrich Chemical) or dexamethasone (Merck, Darmstadt, Germany) in animal and culture studies. The primer sequences for detection of prepro-CST (forward) and 3′ primer sequences used for detection of prepro-CST (reverse).

**Functional Assessment With Body Temperature and Clinical Score**

Core temperature was measured by a thermocouple inserted into the rectum at 24 hours after infection. Clinical scores were evaluated according to a method described elsewhere [23] at 24 hours after infection. Scoring criteria were as follows: normal activity, 5 points; inactivity but normal locomotion after stimulation, 4 points; ataxic gait, 3 points; delayed righting, 2 points; unable to right, 1 point; dead, 0 points.

**Sampling and Analysis of CSF**

Approximately 50–100 μL of CSF was collected from animals anesthetized with pentobarbital (65 mg/kg; intraperitoneal injection) by use of a 23G needle of winged infusion set inserted through the midline of the atlanto-occipital membrane. Leukocytes in 10 μL of CSF were counted in a Bürker chamber. After CSF collection at various time points, rats were killed and brain tissue samples were collected for histological and immunohistochemical staining (with transcardial perfusion; tissue fixed with paraformaldehyde) for reverse-transcription polymerase chain reaction (RT-PCR; without transcardial perfusion; tissue quickly frozen in liquid nitrogen).

**Quantitative Measurement of Messenger RNA Encoding for Prepro-CST or Proinflammatory Cytokines**

Approximately 50 mg of tissue was homogenized with an ultrasonic cell disruptor (Microson, Farmingdale, NY). Total RNA was extracted from cells by use of TRIzol reagent (Invitrogen Life Technologies, Paisley, Scotland) treated with DNase I and then subjected to reverse transcription by use of SuperScript II RNase H reverse transcriptase (Invitrogen, Carlsbad, CA). The primer sequences used for detection of prepro-CST messenger RNA (mRNA) were 5′-GGGGATGAGTGGCTTCC-3′ (forward) and 5′-GGGGATGAGTGGCTTCC-3′ (reverse). The primer sequences for β-actin, which was used as an internal control, were 5′-GACCAGATCTAGTTTGAGACCCT-3′ (sense) and 5′-GGGGATGAGTGGCTTCC-3′ (antisense). Quantitative (real-time) PCR was conducted using SYBR Green Master mix (Applied Biosystems, Foster City, CA) with primers in an ABI PRISM 7500 (Applied Biosystems, Foster City, CA). Reaction conditions were 2 minutes at 50°C and 10 minutes at 95°C, followed by 45–50 cycles of 15 seconds at 95°C and 30 seconds at 60°C, followed by 35 seconds at 75°C. Melting curve and sequencing data were used to confirm the specificity of the PCR products. The comparative cycle threshold (Ct) method was used as described elsewhere [24]. Levels of prepro-CST mRNA were normalized to those of β-actin and were expressed as values relative to the control. Measurement of mRNA levels of proinflammatory cytokines tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6) was performed using TaqMan primers and probes as described elsewhere [5].

**Detection of Apoptosis by Immunohistochemical Staining of Caspase-3**

Paraffin sections (thickness, 3 μm) of brain were deparaffinized, submitted to antigen retrieval, and quenched with 0.3% hydrogen peroxide in methanol. The sections were incubated with rabbit anti-caspase-3 antibody (1:200 dilution; Cell Signaling Technology, Danvers, MA) overnight and visualized by the avidin-biotin peroxidase complex (ABC Elite kit; Vector Laboratories, Burlingame, CA) and diaminobenzidine-based peroxidase substrate. Quantification of caspase-3–positive cells was performed on 5–8 images per slide by an independent observer blinded to the experiment.

**Measurement of Cytokine Concentrations in CSF**

A multiplex bead array assay (Bio-Rad Laboratories, Hercules, CA) was used to measure concentrations of cytokines in CSF as...
described elsewhere [5]. The standard curves were generated using a 5-parameter logistic method.

Primary Cultures of Neuron-Glia Co-culture
Primary cultures of neuro-glia co-culture were prepared from the cerebral cortex of 1-day-old neonatal Sprague-Dawley rats as described elsewhere [21, 22] with slight modifications. Cells were dissociated, suspended in 10% fetal bovine serum in Dulbecco minimum essential medium (Gibco BRL, Grand Island, NY), plated at a density of 5 × 10⁵ cells/mL, and then incubated at 37°C in a humidified 5% carbon dioxide and 95% air atmosphere. We routinely examined the cell morphology and performed immunocytochemical staining (see below). Our neuron-glia co-cultures consisted of 43.1% ± 0.8% neurons, 48% ± 1.5% astrocytes, and 9.6% ± 1.4% microglia (mean ± SD). The cell cultures were used 10–14 days after the plating.

Assessment of Cell Injury
Cell injury was detected by staining with propidium iodide (PI; 5 μg/mL; Sigma-Aldrich Chemical) for 15 minutes, washing with PBS, and fixation in 4% paraformaldehyde (PFA) for 15 minutes. Cultures were then counterstained with 4,6-diamino-2-phenyl-indol dihydrochloride (DAPI; 0.01 mg/mL in PBS; Sigma, St Louis, MO). Images of PI-labeled cells were captured with a digital camera attached to a fluorescent microscope (model BX-51; Olympus, Tokyo, Japan) and quantified.

Cell Death Measured by Lactate Dehydrogenase Activity
The cytotoxicity was assessed by measuring the activity of lactate dehydrogenase (LDH) released from the cells into the medium of neuron-glia co-culture according to methods described elsewhere [22, 25].

Western Blotting for Cleaved Caspase-3
Western blot analysis of cleaved caspase-3 was performed as described elsewhere [26, 27]. Protein levels were quantified from gel images by use of the UVP BioImaging system (Biospectrum AC Imaging System, CA).

Immunocytochemistry
Immunocytochemical staining of cultured cells was performed as described elsewhere [21, 22]. Briefly, cultures were incubated with the appropriate primary antibodies mouse anti-Neuronal Nuclei [NeuN; 1:500 dilution; Chemicon, Temecula, CA] for neurons, mouse anti-glial fibrillary acidic protein [GFAP; 1:1000 dilution; Chemicon] for astrocytes, mouse anti-ED1 [1:500 dilution; Serotec, Bicester, UK] for microglia, and rabbit anti–caspase-3 antibody [1:200 dilution; Cell Signaling Technology, Danvers, MA], followed by incubation with appropriate biotinylated secondary antibodies, and visualized using the avidin-biotin peroxidase complex method (ABC Elite kit; Vector Laboratories, Burlingame, CA). The numbers of NeuN-, GFAP-, ED1-, and caspase-3–positive cells were counted in 5 randomly selected fields by means of Spot image analysis software (Diagnostic Instruments, Sterling Heights, MI).

Statistical Analysis
Differences among groups were evaluated with 1-way analysis of variance. A post hoc test (Bonferroni t test) was used to determine individual group differences. Results are expressed as means (± SD). All statistical analyses were performed using Sigma Stat (version 2.0; Jandel Scientific, San Diego, CA).
RESULTS

Time-Dependent Induction of Endogenous CST mRNA in Brain Cells in K. pneumoniae Meningoencephalitis
To examine whether endogenous CST would be regulated by K. pneumoniae infection, we compared prepro-CST mRNA levels in brain tissue in sham animals and K. pneumoniae–infected animals by reverse transcription followed by real-time PCR (quantitative RT-PCR). Our results indicated that levels of prepro-CST mRNA increased up to 11.13-fold as early as 2 h after infection and reached 46.65-fold at 8 hours after infection (Figure 1). Because it is known that CST can be expressed by neurons and also by leukocytes, we further compared the time course of leukocyte infiltration into CSF and prepro-CST mRNA expression in brain tissue. We found that leukocyte counts in CSF increased in a time-dependent manner following K. pneumoniae infection, with a significant increase as early as 6 hours after infection (Figure 2). Because the expression of prepro-CST mRNA occurred earlier than did leukocyte infiltration, we further examined the time course of prepro-CST mRNA expression in primary cultures of neuron-glia (resident brain cells) after K. pneumoniae infection. Our results revealed the prepro-CST mRNA expression could be induced at 4 and 8 hours after infection in cultured brain cells (Figure 1).

Intraperitoneal Administration of CST Was as Effective as ICV Administration in Reducing Leukocytosis in Brain and Clinical Illness
Leukocyte counts in CSF increased in a time-dependent manner following K. pneumoniae infection (Figure 2). ICV administration of CST at the dose of 1 nmol/10 μL, but not 0.1 nmol/10 μL, reduced white blood cell (WBC) infiltration in cerebrospinal fluid (CSF) and diminishing clinical symptoms after intracerebroventricular (ICV) and intraperitoneal (IP) treatment with cortistatin (CST) in Klebsiella pneumoniae–infected rats (inf). A, Time course of WBC counts in CSF after K. pneumoniae infection. **P < .01 and ***P < .001 versus sham rats; †P < .05 versus 4 hours after infection; ††P < .05 versus 6 hours after infection; †††P < .01 versus 8 hours after infection. B, Significant increase in the number of WBCs in CSF in infected animals treated with vehicle (veh; phosphate-buffered saline for ICV treatment and saline for IP treatment) at 8 hours after infection. **P < .01 versus sham rats. Because data from ICV vehicle treatment were not statistically different from those from IP vehicle treatment, data were pooled and expressed as vehicle data. After treatment at 1 hour after K. pneumoniae inoculation with CST (ICV; 1 nmol/10 μL), there was significant attenuation in the recruitment of WBCs into cerebrospinal fluid (CSF). Treatment with CST (IP; 250 μg/kg) also attenuated the leukocytosis in CSF from rats at 8 hours after infection. †P < .05 and ††P < .01 versus vehicle-treated infected rats. C, D, Comparison of core temperatures and clinical scores, respectively, among animals in the sham group, vehicle-treated infected group, ICV CST-treated infected group, and IP CST-treated infected group at 24 hours after infection. Data are expressed as means (± SD; n = 5 in each group). *P < .05, **P < .01, and ***P < .001 versus sham rats; †P < .05 and ††P < .01 versus vehicle-treated infected rats.
cell infiltration into CSF at 8 hours after infection (Figure 2). A higher ICV dose at 10 nmol/10 μL of CST had a similar effect to that of a dose of 1 nmol/10 μL (data not shown); we therefore chose the dose of 1 nmol/10 μL for subsequent experiments. Interestingly, postinfection (1 hour) treatment via intraperitoneal injection at a dose of 250 μg/kg—the dose previously demonstrated to produce protective effects against lethal endotoxemia [17]—also produced similar effects on leukocyte recruitment (Figure 2). The specificity of CST was examined by parallel comparison with the effects of SST (1 and 10 nmol/10 μL; ICV injection). In contrast to the effects of CST, the leukocyte counts in CSF at 8 hours after K. pneumoniae infection (12 217 ± 2146 cells/μL; n = 5) were not affected by SST (9000 ± 605 cells/μL for 1 nmol/10 μL and 12 400 ± 1213 cells/μL for 10 nmol/10 μL, respectively). In additional experiments, we also examined the effects of CST (250 μg/kg; intraperitoneal injection) in combination with dexamethasone (0.7 mg/kg; subcutaneous injection). The leukocyte counts in CSF at 8 hours after K. pneumoniae infection were significantly different from those in control group (6680 ± 585 cells/μL vs 12 217 ± 2146 cells/μL, respectively; n = 5; P < .05), but they were not significantly different from those in ICV and intraperitoneal CST groups. The core temperature increased up to 38.85°C ± 0.11°C at 24 hours after K. pneumoniae infection (Figure 2). Postinfection treatment with CST (1 nmol/10 μL; ICV injection) significantly attenuated the elevation of core temperature and improved clinical symptoms (Figure 2). The systemic route of CST administration (250 μg/kg; intraperitoneal injection) could achieve similar effects to those of the central route (ICV injection) on core temperature and clinical score (Figure 2). In contrast to the effects of CST, the core temperature and clinical score at 24 hours after K. pneumoniae infection...
infection were not affected by SST (data not shown). Similarly, the effects of CST (250 μg/kg; intraperitoneal injection) in combination with dexamethasone (0.7 mg/kg; twice daily; subcutaneous injection) on core temperature and clinical scores were not significantly different from those in the ICV or intraperitoneal CST groups (data not shown).

CST Treatment Attenuated the Apoptosis of Cortex and Hippocampus After Infection

More caspase-3 positively labeled cells, indicating apoptotic cells, were noted in the cortex (346 ± 23.12 cells/mm²) and hippocampus (53.11 ± 8.94 cells/mm²) of infected animals at 24 hours after infection than in sham animals (cortex, 4.03...
levels after infection that were significantly attenuated by CST treatment (ICV and intraperitoneally) (Figure 4). Similarly, postinfection treatment with CST by either ICV or intraperitoneal injection reduced the elevated concentrations of these cytokines in CSF at 8 hours after infection (Figure 4).

Postinfection Treatment With CST Reduced De Novo Synthesis of Prolinflammatory Cytokines in Primary Culture of Neuron-Glia

The expression of mRNA of proinflammatory cytokines in cultured neuron-glia was upregulated at 4 hours after infection. Post-treatment (1 hour) with CST (30 or 100 nM) attenuated the elevation of mRNA levels of proinflammatory cytokines.
Cortistatin Was Neuroprotective Against Cell Injury Caused by *K. pneumoniae* Infection In Vitro

PI-DAPI staining, which stains injured cells, indicated that exposure to *K. pneumoniae* (10³ CFUs) for 24 hours resulted in injury to 27% ± 4% of cells (with a mean plating density of 1133 cells/mm²), which was attenuated by CST (30 and 100 nmol/L) to 21% ± 2% and 7% ± 1%, respectively (Figure 6). Quantification of cytotoxicity by measurement of LDH activity in culture medium also indicated that the significant cell injury in cultured cells exposed to *K. pneumoniae* was attenuated in a concentration-dependent manner by CST (30 and 100 nmol/L), most prominently at 100 nmol/L (Figure 6). The ratio of cleaved caspase-3 to total caspase-3 (procaspase-3 and cleaved caspase-3), indicating apoptotic cell death, significantly increased after infection and was reduced by CST treatment (100 nmol/L) (Figure 6). Immunocytochemical staining of caspase-3–positive (apoptotic) cells indicated that *K. pneumoniae* infection significantly increased the percentage of apoptotic cells as compared with the control (from 0.59% to 23.56%). This phenomenon could be reversed by CST (data not shown). CST treatment (100 nmol/L) given at 1 hour after infection attenuated the reduction of neurons (NeuN-positive cells) at 24 hours after infection (Figure 7). The numbers of astrocytes (GFAP-positive) and microglia (ED1-positive) were not significantly changed with or without CST treatment in infected cultures (data not shown).

**DISCUSSION**

In this study, we first demonstrated a time-dependent induction of endogenous prepro-CST mRNA in brain tissue from animals with *K. pneumoniae* meningoencephalitis. The prepro-CST mRNA could be expressed resident brain cells or infiltrating immune cells, because CST is known to be expressed in immune cells. Interestingly, we found that the expression of endogenous prepro-CST mRNA happened earlier than did leukocyte infiltration. We further demonstrated that prepro-CST mRNA expression could be induced at 4 and 8 hours after infection in cultured brain cells. These findings indicate that de novo synthesis of endogenous CST can be produced in cells of central nervous system origin.

To examine the biological effects of exogenous CST, we first demonstrated that ICV administration of CST (1 nmol/10 µL), but not SST (1 or 10 nmol/10 µL), effectively attenuated leukocyte...
recruitment at 8 hours after K. pneumonia infection as compared with infected animals treated with vehicle, indicating that the effect of CST on leukocyte recruitment is specific. We further proved that systemically administered CST at a previously reported dose (250 μg/kg; intraperitoneal injection) [17] also significantly attenuated leukocyte recruitment to an extent similar to that produced by ICV administered CST and comparable to that of dexamethasone (0.7 mg/kg; subcutaneous injection). Postinfection treatment with CST (either ICV or intraperitoneal), but not SST (ICV), reduced the clinical signs of illness (such as fever) and improved the clinical score (Figure 2). Combined treatment with CST and dexamethasone did not augment the effects of CST. Apoptotic cell injury, which is commonly observed after bacterial meningitis [28], was also noted at the cortex and hippocampus after K. pneumonia infection. Postinfection treatment with CST (either ICV or intraperitoneal) significantly attenuated apoptosis in the cortex and hippocampus. The anti-inflammatory effect of CST was further proved by the results that intraperitoneal as well as ICV administration of CST suppressed mRNA expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in infected animals. Our results are consistent with those of previous studies demonstrating that CST has anti-inflammatory effects in different experimental models, including ulcerative colitis, arthritis, and endotoxemia [15–18].

Cytokines in CSF might be derived from recruited leukocytes or from glial cells [5]. The reduced cytokine production by CST was likely due to reduced leukocyte recruitment into the central nervous system. The reduction of mRNA expression of proinflammatory cytokines in cultured cells by CST indicated that CST also suppressed the production of cytokines from glial cells. Therefore, CST could reduce cytokine levels in CSF by both mechanisms—that is, reduced leukocyte recruitment and reduced production from glial cells.

Multiple adjunctive therapeutic approaches for central nervous system infection have been reported (reviewed by Nau and Bruck [28]). However, only a few approaches show beneficial effects in clinical trials, such as adjunctive dexamethasone or oral glycerol [29, 30]. Dexamethasone provides an anti-inflammatory effect, but it increases hippocampal neuronal injury and aggravates learning deficits in meningitis animal experiments [31, 32]. In this study we found that, unlike dexamethasone, postinfection treatment with CST did not have the adverse effect of increased apoptosis in the hippocampus.

Not only did we not detect any adverse effect of CST in our animal model, but our results in primary neuron-glia co-cultures also indicated that CST alone did not cause cytotoxicity as revealed by the biochemical indices of cell death (LDH) and apoptosis (cleaved caspase-3). This is consistent with the results of previous studies in humans showing that CST administration elicits no adverse effects [19, 20]. In meningitis, the mode of cell death (neuronal injury) consists of apoptosis, necrosis, or hybrid forms of both [28]. Our results from PI-DAPI staining, LDH measurements, caspase-3 Western blot analysis, and immunocytochemical staining in cell culture experiments indicate that the cellular death after K. pneumonia infection included apoptosis and necrosis. Further identification of cell types indicated that K. pneumonia infection for 24 hours significantly reduced the number of neurons but not the numbers of astrocytes or microglia. The neuronal loss induced by K. pneumonia infection was attenuated by CST. Our results suggest that CST might be used as an adjuvant therapeutic agent in bacterial meningitis, but further clinical studies are warranted.

In conclusion, our study demonstrates that endogenous CST is induced after K. pneumoniae meningoencephalitis. Exogenous CST reduces clinical illness not only by a reduction in leukocyte infiltration into the brain and in meningitis-associated inflammation but also by a direct neuroprotective effect. These results suggest a potential use of CST as an adjuvant to antibiotics in bacterial meningoencephalitis.

Notes

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References


