Vasoactive Intestinal Peptide (VIP) and Pituitary Adenylate Cyclase-Activation Polypeptide (PACAP) Protect Mice from Lethal Endotoxemia Through the Inhibition of TNF-α and IL-6

Mario Delgado, Carmen Martinez, David Pozo, Juan R. Calvo, Javier Leceta, Doina Ganea, and Rosa P. Gomariz

The neuropeptides vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) down-regulate cytokine production. Because human septic shock involves excessive cytokine production, the effect of VIP/PACAP was investigated in a high endotoxemia murine model. Both peptides protect against endotoxin-induced lethality and prevent septic shock-associated histopathological alterations. VIP/PACAP reduce serum and peritoneal TNF-α and IL-6, suggesting that the protective effect is exerted by inhibiting the production of endogenous TNF-α/IL-6. Consistent with this mechanism, VIP does not protect against septic shock induced by exogenous TNF-α. The immunomodulatory role of VIP in vivo is supported by the appearance of high levels of VIP in serum and peritoneal fluid following LPS administration. Thus, the neuropeptides VIP/PACAP protect from the lethal effect of high endotoxia, presumably by down-regulating TNF-α and IL-6 production, and may offer an alternative in the treatment of human septic shock syndrome.

Materials and Methods

Experimentally induced endotoxic shock

Eight-week-old male BALB/c mice (Iffa Credo, L’Arbresle, France) were injected i.p. with different concentrations (25–600 μg) of LPS (from Salmonella enteridis; Sigma, St. Louis, MO), and survival was monitored over the next 4–7 days. Various doses of VIP/PACAP (Novabiochem, Laufelfingen, Switzerland) were administered i.p. 6 h before LPS to serve as a positive control for survival. Lethal shock was induced in some animals by injecting TNF-α (50 and 25 μg) (PharMingen) i.p. All survival studies were conducted in a blind and
random fashion. All endotoxin-treated mice appeared acutely ill, displaying lethargy, ruffled fur, and diarrhea. The animals that succumbed to the effect of LPS treatment were necropsied immediately, and all VIP/PACAP-treated survivors were killed for necropsy at the end of the experimental period. Histopathological examination was performed on lung, small and large intestine, and kidney, and spleen-fixed with Bouin solution; sections were stained with hematoxylin-eosin or Masson’s haemalum and picroindigocarmine using standard techniques.

Mice receiving LPS (400 μg) concurrently with either medium or VIP/PACAP-38 (5 nmol) were sacrificed after various time points. Blood was removed through cardiac puncture, and peritoneal exudate was obtained as described previously (43). The blood samples were allowed to clot for 1 h at room temperature; serum was obtained and kept frozen until TNF-α, IL-6, and VIP ELISA analysis. The peritoneal suspension was centrifuged for 5 min at 1800 × g, and cell free supernatant was harvested and assayed for cytokine and VIP ELISA. The peritoneal cells were subjected to Northern blot analysis as described below.

Cytokine and neuropeptide quantitation: TNF-α, IL-6, and VIP

TNF-α and IL-6 levels in serum and peritoneal fluid were determined using commercially available murine-specific sandwich ELISAs (PharMingen). Serum and peritoneal fluid VIP levels were measured using a competitive ELISA. The mAb CURE.55 (44) (2.5 μg/ml; 100 μl/well in 96-well plates), kindly provided by Dr. H. C. Wong (University of California, Los Angeles, School of Medicine), was used for detection of TNF-α, IL-6, and VIP ELISA analysis. The peritoneal suspension was centrifuged for 5 min at 1800 × g, and cell free supernatant was harvested and assayed for cytokine and VIP ELISA. The peritoneal cells were subjected to Northern blot analysis as described below.

Quantitation of TNF-α and IL-6 mRNA

Northern blot analyses were performed according to standard methods. Total RNA was isolated from peritoneal cells using the Ultraspec RNA Isolation System (Biotex Laboratories, Houston, TX) according to the manufacturer’s instructions. Twenty micrograms of total RNA from each sample were electrophoresed on 1.2% agarose-formaldehyde gel, transferred to nylon membranes, and cross-linked using UV light. Membranes were hybridized with specific probes for TNF-α (5′-TTGACCTGCGGTCTGAGTTGGTCCTGGAGGACACTCATG-3′) and IL-6 (5′-CCAAGAGGCAAACCTGGAATGCGCTCTCTGCAAGAAGGAATTCCAT-3′) that were designed from murine TNF-α and IL-6 cDNA published sequences (46, 47). The probe for the murine 18S RNA, as a quantity control for RNA, was an oligonucleotide (5′-CCAAATACGGGCTGAAAGAGTCTTCATA-3′) derived from the published sequence. Oligonucleotides were 3′-labeled with digoxigenin-dUTP/dATP mix using terminal transferase, and hybridization and detection of chemoluminscent signal were performed using a commercially available kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s instructions.

Statistical analysis

All values are given as means ± SD. Survival curves were analyzed by the Kaplan-Meier method, and the log rank test was generated to test the homogeneity between treatment groups. Serum and peritoneal cytokines and VIP levels in different experimental groups were analyzed for statistical significance using the nonparametric Wilcoxon rank sum test. A value of p < 0.05 was considered to represent a significant difference.

Results and Discussion

VIP/PACAP protect from LPS-induced lethality

BALB/c mice were injected simultaneously with a lethal dose of LPS and VIP or PACAP-38, and survival was monitored. Both VIP and PACAP-38 protect against the lethal effect of LPS as analyzed by the Kaplan-Meier method, and the protective effect is similar to that of anti-TNF-α Abs used as control (Fig. 1A). The effect of VIP/PACAP was dose-dependent, with doses as low as 1 nmol being partially protective (Fig. 1B). The protective effect of VIP occurred over a large range of LPS concentrations, and VIP shifted the LD50 from 100 to 327 μg LPS (Fig. 1C). Animals injected with VIP had a survival rate of ~60%. However, even for the nonsurvivors, VIP almost dou-
**FIGURE 1.** A–D, VIP and PACAP-38 protect mice from LPS-induced lethality. A, BALB/c mice (12 per group) were injected i.p. with 400 μg LPS (control), LPS and VIP or PACAP-38 (5 nmol), or with anti-TNF-α Abs (1 mg) followed by LPS 6 h later. Survival was monitored over the next 96 h, and the survival curves were analyzed by the Kaplan-Meier method. Similar results were obtained in three identical independent experiments. The log-rank test was used for homogeneity between treatment groups (\( p, p, p, 0.001 \)). B, BALB/c mice (six per group) were injected i.p. with 400 μg LPS and various concentrations of VIP or PACAP-38 (from 0 to 10 nmol/animal). Survival was monitored over a 96-h period. Similar results were obtained in four identical independent experiments. C, BALB/c mice (8–12 per group) were injected i.p. with various concentrations of LPS in the absence or presence of 5 nmol VIP. Survival curves were used to calculate LD₅₀. Horizontal bars indicate the 95% confidence limits of LD₅₀ determinations. Inset, At the end of 96 h, the average survival time was calculated for nonsurvivors in both the LPS (600 and 400 μg/animal) and LPS plus VIP (5 nmol/animal) treated groups. Similar results were obtained in three identical independent experiments. *, \( p < 0.001 \) with respect to control mice without VIP treatment as determined.
Based on these considerations, we investigated the effect of VIP and PACAP on TNF-α and IL-6 production. Both VIP and PACAP reduced by ~50% the levels of secreted TNF-α in serum and peritoneal fluid (Fig. 3A). Similar reductions were observed for serum and peritoneal IL-6 (56–57%) (Fig. 3A). Northern blots confirmed that VIP/PACAP significantly reduce the steady-state mRNA levels for both TNF-α and IL-6 in peritoneal exudate cells (Fig. 3B). If VIP/PACAP protect against the lethal effect of endotoxin through down-regulating the expression of proinflammatory cytokines such as TNF-α and IL-6, the neuropeptides should not protect against death caused by direct cytokine administration. Indeed, VIP/PACAP did not affect the survival of mice injected with lethal doses of TNF-α (Fig. 3C).

The findings presented in this study indicate that the neuropeptides VIP and PACAP protect from high-dose endotoxin shock. This protective effect appears to be associated with decreased expression of two proinflammatory cytokines, TNF-α and IL-6, which were previously shown to mediate the deleterious effects of endotoxin. The effect of VIP/PACAP on the expression of TNF-α and IL-6 is in agreement with previous studies regarding the inhibitory effect of VIP/PACAP on cytokine production. High levels of VIP in both serum and peritoneal fluid are induced following the

**FIGURE 2.** Histopathological analysis of tissue sections from LPS-injected mice treated with and without VIP. Mice were injected i.p. with 400 μg LPS in the absence (a and d) or presence of 5 nmol VIP (b and e). Control animals were injected with RPMI 1640 medium (c and f). Lung and large intestine sections were obtained and stained with hematoxylin-eosin (a–c) or with Masson’s haemalum and picroindigocarmine (d–f), respectively. Magnification factor of sections is approximately ×150.

**FIGURE 1.** A–D, Continued by the Wilcoxon rank sum test. D, BALB/c mice (six per group) were injected i.p. with LPS (400 μg) and VIP or PACAP-38 (5 nmol) at times 0, 30 min, 90 min and 4 h after LPS administration. Survival was monitored over the next 7 days. **E**, LPS induces VIP secretion in vivo. BALB/c mice were injected i.p. with LPS (25 and 400 μg) or with RPMI 1640 medium (control). At times 0, 4, 8, 12 and 24 h peritoneal fluid and serum were obtained. Three animals were used for each time point. VIP concentration was determined through a competitive ELISA assay using the monoclonal anti-VIP Ab (clone CURE.V55) (44) as a capture Ab. Similar results were obtained in four identical independent experiments. **E**, p < 0.001 with respect to control mice without LPS treatment as determined by the Wilcoxon rank sum test.
lethal endotoxic challenge and were previously reported in patients with septic shock. However, LPS-induced endogenous VIP levels are two to three orders of magnitude lower than the concentrations of protective exogenous VIP. We propose that during a normal immune response, the timely production and/or release of VIP and possibly PACAP within the lymphoid organs following antigenic stimulation serves to down-regulate the ongoing immune response, mostly through modulation of cytokine expression. During septic shock, however, due to severe septicemia leading to an overstimulation of the immune system, the effect of checkpoint molecules such as IL-10, IL-13, VIP, and PACAP is overwhelmed by the proinflammatory cytokine network. However, based on the protective effect of VIP/PACAP in the high-dose endotoxic animal model, the exogenous administration of these anti-inflammatory neuropeptides could offer an alternative to existing treatments for septic shock syndrome.

References